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Article

The transfer of heavy metals from the water "in the dish", through fish

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Abstract
The current work aimed to highlight the impact produced by copper intoxication on the freshwater fish species Cyprinus carpio L. Besides quantifying the degree of metal accumulation in the fish tissues, we also proposed a different approach, namely to see how much the thermal handling/preparation affects the heavy metal concentration in the final product. For this, boiling and frying techniques of intoxicate fish fillets were used. The results showed a considerable percentage decrease of copper in fish fillets subjected to thermal preparation compared to those not thermally treated (raw).

Keywords: *copper, Cyprinus carpio, fish fillets, intoxication, thermal preparation*

INTRODUCTION

Various types of food products are vital for human nutrition, as they are necessary to ensure a balanced diet. Fish is considered a significant part of a healthy and balanced diet due to its exceptional nutritional properties (high quality proteins, vitamins, omega-3 essential fatty acids). Thus, many public health authorities recommend the regular consumption of fish equivalent to at least 1or 2 portions per week to prevent chronic diseases due to food [1]. Some of these foods include fish, meat, vegetables or grains. Some are eaten raw, while others must be processed by cooking before consumption. Different cooking methods are available for processing food raw materials before consumption, so some of the commonly used methods include boiling, frying or grilling. Basically, cooking using one of these methods is done in such a way as to enhance the taste, aroma and to make it convenient to eat these foods. It has been reported that different cooking methods significantly affect the amounts of contaminants such as heavy metals present in the final product [2]. Often, fish and other seafood represent one of the main sources of exposure to metals among the general population [1]. The term heavy metal is commonly used to refer to those metallic elements that have a relatively high density, greater than 5g/cm^3. These groups of metals are worrisome because, unlike organic contaminants, they cannot be degraded by microorganisms, their transformation takes place only in terms of oxidation number changes. Although these metals are ubiquitous, therefore, they are found in all aspects of the environment, in such conditions they do not constitute damage, because they are less bioavailable. However, human activities such as industrialization, agriculture and modern technological explorations have greatly contributed to the higher content of these metals in the environment, thus causing them to have lethal consequences on humans and other organisms. It is known that food products such as meat and fish have remarkable nutritional values due to their

high protein content, despite their complex character that makes possible the bioaccumulation of toxic heavy metals [3].

In recent years, rapid industrial growth, agricultural development and urbanization have led to significant degradation of the aquatic environment. Heavy metals are the most common pollutants that affect the aquatic environment and include metals that are essential for organisms, but can also have toxic effects when concentrated in large quantities, such as copper, zinc and chromium, and small intakes of non-essential metals that are harmful to health, such as lead, cadmium and mercury. Lead, cadmium, chromium, arsenic and mercury are all highly carcinogenic substances that can affect the neurological, cardiovascular, respiratory and other vital human systems [4]. The increase in anthropogenic activities has caused global warming, and this constitutes a threat to organisms, especially ectotherms, whose body temperatures are practically identical to the temperature of the environment. However, ectotherms, such as fish, are able to respond to high temperatures through evolutionary thermal adaptation or acclimatization through phenotypic plasticity, resulting in improved superior thermal tolerance [5].

Among aquatic species, fish are widely used to assess the quality of the aquatic environment and can serve as excellent bioindicators of aquatic pollution [6]. The species *Cyprinus carpio* is one of the most important aquaculture species in the world due to its omnivorous feeding, disease tolerance, superior growth, prolific reproduction and ability to withstand large fluctuations in environmental conditions, and globally, the value of the fish industry, exceeds USD 3 billion [7]. Common carp (*Cyprinus carpio* L., 1758) is one of the most important freshwater aquaculture species worldwide, with an annual global production of 4.6 million metric tons [8]. In addition, this species is considered to be a good bioindicator for ecotoxicological studies and is recommended in the guidelines of the Organization for Economic Cooperation and Development (OECD), as one of the six fish species for regulatory testing [9]. As a model species, common carp is used to study the impact of metals both in the laboratory and in the field, for example on bioaccumulation, energy status, swimming capacity or oxidative stress [10]. It annually represents approximately 10% of the global freshwater aquaculture production. Due to its wide distribution and relatively simple anatomy, as well as its ability to tolerate environmental stress, *C. carpio* has proven to be an excellent indicator of toxic contaminants in natural freshwater bodies [11÷13].

Copper as an essential trace element plays an important role in the normal metabolic functioning of organisms. It acts in hemoglobin synthesis, hematopoiesis, cellular respiration, bone formation and as a cofactor for numerous enzymes such as super-oxide-dismutase, cytochrome-c-oxidase, ferroxidase, lysyl-oxidase, monooxygenases and ceruloplasmin. In aquaculture, Cu is used in the soluble forms of CuSO4 as an essential trace element in diets, as well as a therapeutic chemical to control algae blooms and bacterial infections. A dietary copper requirement of 3÷10 mg Cu/kg of dry diet has been reported for teleost fish [14].

Rainbow (2007), suggested that the concentration of copper in natural foods (invertebrates) could increase up to 3750 mg/kg in polluted areas [15].

Due to excessive exploitation and inefficient use of resources, heavy metal pollution is becoming a major environmental problem affecting ecosystems around the world [16]. Copper, as one of the heavy metals and an essential nutrient, can become toxic to living cells when its concentration reaches high levels [14]. It was identified as an environmental contaminant that was widely detected in soil and water. It has also been detected in various places, such as plants and seeds. Under such conditions, the heavy metal Cu could enter the animal and human body through different ways and accumulate in several organs, including lungs, liver, kidneys and reproductive organs. This excessive supply of Cu can cause oxidative damage to the organisms and can substantially affect the intestinal microbiome of the host animals. In addition, many studies have suggested that low concentrations of Cu have a toxic effect on the function of immune cells that can protect organisms from many diseases [11]. Thus, the study of the toxicological effects of Cu on organisms has received massive attention in recent years due to its toxicity [17].

Shaw and Handy showed that the level of Cu in the diet of about 1500 mg/kg food, decreased the growth of Nile Tilapia (*Oreochromis niloticus*) after feeding for 42 days [18]. Due to the importance

of fish as a food resource for humans and as a major component of the ecosystem, the assessment of the bioaccumulation pattern of metals in fish tissues is essential [19].
The aim of this work is to quantify the degree of accumulation of copper by the fish species *Cyprinus carpio* L. and to highlight the potential for bioaccumulation in aquatic organisms used as food sources, as well as the determination of copper in fish fillets that have been subjected to different thermal manipulation/preparation techniques.

EXPERIMENTAL PART

Acute intoxication test on freshwater fish (Cyprinus Carpio l. species)

The acute toxicity testing on *Cyprinus carpio* fish was carried out through a non-clinical study on the determination of acute toxicity on aquatic organisms (freshwater fish) in accordance with the methodology described in the OECD guide 203 [9]. The objective of the test was to intoxication the fish with a reference solution of Copper (Agilent, Copper AA Standard: 1000 µg/mL Cu in 5% HNO_3, MRC) in order to estimate the bioconcentration potential in fish tissue (muscle).
The biological material used in the toxicological experiments was 1-year-old carp juvenile (*Cyprinus carpio*), weighing 35÷45 g/individual, purchased in October 2021 from batches selected from the NUCET Aquaculture Research and Development Station from the experimental basins populated with juvenile, from healthy animals (according to Quality Certificate no. 738/21.10.2021). The fish species used is often used by the laboratory in acute lethal toxicity bioassays, showing ease of testing and relative sensitivity to potentially dangerous chemicals. After the acquisition, fish of similar length, weight and age were acclimatized in laboratory conditions, in the maintenance tanks of the aquatic biobase of the laboratory; the fish were healthy and showed no visible malformations.

Test conditions according to the methodology described by the OECD 203 guide

The toxicity experiment was carried out in glass aquariums, provided with an aeration system for the solutions and of adequate capacity (10 L), which allow the testing of at least 5 fish per experimental solution.
The theoretical concentration tested was 200 µg Cu/L. Throughout the experimental period, the specific concentration was supplemented to maintain it (on days 2, 4, 7, 10 and 14) depending on the analytical results. The determined values were: 85.7; 348; 200; 240; 214 µg Cu/L (the average analytical concentration during the test period was 217.54 µg Cu/L).
This initial concentration of copper (200 µg/L) in the test solution was chosen for several reasons. First of all, the maximum values allowed in the MMGA Order no. 161/2006 which stipulates that in order for a body of water to be included in the 5th class of surface water quality, the copper value must exceed 100 µg /L [20]. Another reason was that from the previous studies carried out on the surface waters of the Western area of Romania, it was found that the copper concentration determined in the waters did not exceed the limit value imposed by the order (higher than 100 µg/L).
The experimental duration was 14 days, during which was performed daily monitoring and recording of temperature, pH and dissolved oxygen in the test and blank solutions; daily recording of data on the fish used in the test, as well as mortality, every 24 hours and accumulated (after 14 days of experiment), for the test solution and control. Additionally, the periodic analytical determination of the tested compound (Cu) in the testing solution was carried out using standard ISO 11885:2007 [21].

Performing the acute lethal toxicity test

The test solution was prepared on the day of the test, by dissolving the corresponding amount of reference solution with copper directly in 5 liters of dilution water (dechlorinated tap water) so as to obtain the theoretical concentration of 200 µg Cu/L. In parallel with the test, a control test was also performed, in which dilution water was used, without the test substance.
In each test vessel, 5 liters of test solution were introduced and 5 fish selected according to weight and length were distributed, so as to comply with the test conditions imposed by the applied method. During the testing, the fish in the experimental vessels were not fed, and normal lighting conditions were ensured in the room intended for the acute lethal toxicity biotests (12÷16 hours of light daily).

The quality of the dilution water used in the preparation of the test solutions and in the control test was checked before the start of the experiments and the values determined for the physical-chemical indicators analyzed fell within the quality limits imposed by the applied OECD 203 method (table 1). The dilution water contains minimal amounts of metals, the value for Cu being 1.7 µg /L.
The conditions ensured in the test vessels were monitored daily, recording data on temperature, pH according to the standard ISO 10523:2008 [22] and dissolved oxygen content according to the standard ISO 5814:2012 [23]. In addition, during the execution of the acute lethal toxicity bioassays, the ambient conditions were monitored and recorded using a thermohydrometer, namely the temperature and humidity in the test room.

Table 1. Characteristics of dilution water used in testing

Analysed indicator	Experimentally determined values	Maximum allowed values
pH (pH units)	7.86	6.0 ÷8.5
Total hardness (mg/L $CaCO_3$)	123	40÷250 (preferably <180)
COD (mgO_2/L)	<30	max.10
Suspension (mg/L)	8.80	max. 5
Filterable residue (mg/L)	168	max. 500
Ammonia (mg/L)	<0.02	max. 0.001
Free residual chlorine (mg/L)	<0.03	max. 0.05
Dissolved oxygen (mg/L)	7.68	4 ÷7
Temperature (^{0}C)	22.5	20 ÷25^{0}C
Nitrate (mg/L)	3.00	< 9
Cu (µg/L)	1.70	-
Ni (µg/L)	1.40	-
Sr (µg/L)	86.5	-
Ca (mg/L)	45.2	-
Mg (mg/L)	5.25	-
Na (mg/L)	8.58	-
K (mg/L)	2.67	-
Al (µg/L)	14.0	-

Note: the values marked with "<" are below the limit of determination of the test method

Laboratory handling of intoxicated biological material
At the end of the 14 experimental days of fish intoxication in the laboratory, the biological material was subjected to thermal preparation and subsequently the copper in the tissues was determined. The next day, the fish were decapitated, gutted, cleaned of scales and washed well with distilled water to remove all impurities, thus obtaining fish fillets.
For the thermal preparation of the fish, the most qualitative individuals from the phenotypic point of view were chosen, consisting of three individuals for each experimental set. The experimental sets were the following: control, raw fish (not thermally treated) - fish intoxicated for 14 days with the Copper solution of 200 µg/L, but not subject to subsequent thermal preparation, boiled fish - intoxicated for 14 days and boiled for 5 minutes in distilled water and fried fish - intoxicated for 14 days and fried for 4 minutes in sunflower oil.
The next stage involved boiling the whole fish fillets for 5 minutes in a Berzelius glass containing 350 mL of distilled water. The heat source was an electric stove.
Another experimental stage involved frying the fish fillets for 2 minutes on each side, a total of 4 minutes, in a metal container with a diameter of 9.5 cm. A film of sunflower oil (approximately 10 mL) was added to avoid excessive sticking of the meat to its bottom.
After the heat treatments, the fish was left to dry outside in the air for 4 hours, after which it was cut into smaller pieces and placed in an oven to dry at 70ºC for about 2 hours, later raising the temperature to 90ºC for another 2 hours. After the 4 hours of drying in the oven, the animal tissue was transferred to a mortar and crushed until it disintegrated into powder (fig. 1) after which it was sieved.

Fig. 1. The fish powder obtained after the drying stages

The 0.50 g of powder was weighed and mineralized in a Milestone Ethos Easy microwave digestion system, following a predetermined digestion program. The biological samples were mixed with 10 mL of concentrated nitric acid and subjected to an increasing temperature up to 200ºC, after which they were filtered in 50 ml volumetric flasks. The characteristics of the digestion program are presented in figure 2.

After all these steps, the mineralized sample was obtained in which the amount of copper in the solution was determined by the technique of Atomic Absorption Spectrophotometry in Flame (AAS) using the Agilent 200 series AA device, model 280 FS AA in accordance with the standard ISO 8288: 1986 [24].

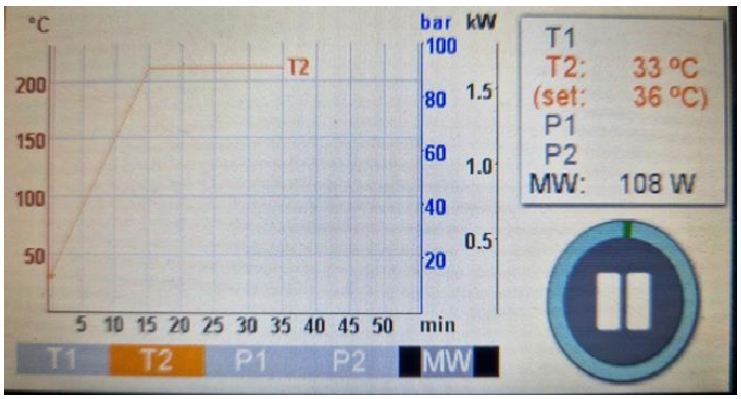

Fig. 2. The digestion program used in the mineralization of fish samples

Bioconcentration Factor (BCF)

In order to appreciate the amount of metal accumulated in the fish muscle in accordance with the amount of metal present in the test medium, the Bioconcentration Factor (BCF) was calculated. BCF is the ratio between the concentration of heavy metals in fish muscle (Cfm) and the concentration of heavy metals in water (Cw), calculated according to the formula: BCF=Cfm/Cw [25].

BCF is classified as follows: BCF < 1000 (low probability of accumulation), 1000 < BCF < 5000 (bioaccumulative) and BCF > 5000 (extremely bioaccumulative) [26].

RESULTS AND DISCUSSION

The sublethal effects or if mortality was recorded for the aquatic organisms (fish) used in the experiments. In table 2 are presented physical-chemical parameters monitored from blank and test solution.

Table 2. Physical-chemical parameters monitored in experimental tests (n=3)

Parameter / Measure unit	Initially 0 days	Final 14 days
Blank (dilution water)		
Dissolved oxygen concentration (mgO_2/L)	8.93-8.46	8.63-8.76
pH (pH units)	7.83-7.89	8.22-8.35
Conductivity (µS/cm)	316	323
Temperature (ºC)	20.0-22.2	20.1-22.4

Parameter / Measure unit	Initially 0 days	Final 14 days
Test solution 200 µg Cu/L		
Dissolved oxygen concentration (mgO_2/L)	7.52-8.06	8.41-8.51
pH (pH units)	8.25-8.46	8.22-8.35
Conductivity (µS/cm)	333	342
Temperature (^oC)	20.2 – 22.4	20.1 – 21.4
COD (mg/L)	<30	53

Note: the values marked with "<" are below the detection limit of the test method

In table 3 are presented the experimental data obtained during the acute toxicity test with freshwater fish.

Table 3. The toxic effects recorded in the experimental tests with fish juvenile (test solution 200 µg Cu/L)

Tested product (mg/L)	Fish no. used in the test	Mortality at 14 days %
Replicated 1	5	0
Replicated 2	5	0
Blank (dilution water without Cu)	5	0

No mortalities were evident at the tested concentration (200 µg/L). The test solutions showed no abnormal color. During the visual inspection of the fish in the first days of intoxication, a lack of response to stimuli was initially observed, which disappeared on the 4th day of exposure. At the end of the test, the fish did not show behavioral disorders or changes in the external organs.
Table 4 shows the final concentration of copper both in the control sample and in the samples subjected to thermal preparation. The data represent the average of three different samples and are expressed in mg/kg dry matter (d.m.).

Table 4. Copper concentration in different types of thermally treated tissue

Sample	Cupru, mg/kg d.m.
Control-Raw fish (not heat treated)	23.1
Boiled fish	10.6
Fried fish	4.55

Making a scale of copper concentrations in fish (mg/kg d.m.), was observed the following: Control-Raw Fish > Boiled Fish > Fried Fish.
Regarding the raw fish sample (not thermally treated), i.e. the sample that was intoxicated for 14 days with a solution of 200 µg Cu/L, but which was not subsequently subjected to thermal treatment, was founded 23.1 mg Cu/kg d.m in the fish tissues. The BCF equation results in a value of 116, which means that in the given test conditions, namely: the metal concentration in the intoxication solution, the exposure time, the subsequent handling of the biological material, etc., the *Cyprinus carpio* species presents a low probability of accumulation. Our results are similar to those reported by Noverita Dian Takarina et al., who reported BCF values of 160.57 in different species of copper-intoxicated fish from the Blanakan River estuary [25].
Regarding the samples of boiled and fried fish, was notice a considerable decrease of copper in the fish muscle, namely: for the sample of fish boiled for 5 minutes in distilled water, a copper amount of 10.6 mg/kg d.m. was reported, and for the fried fish sample a final amount of copper of 4.55 mg/kg d.m. was determinate. The percentage decrease of copper in the boiled fish sample, compared to the raw fish sample, is 54.1%, and in the fried fish sample it is 80.3%.
In our opinion, the decrease in the concentration of copper in fish muscle can be related to the contact with the thermal preparation condition (water or sunflower oil).

The obtained results are similar with other studies, which were conducted on several species of fish intoxicated with different toxics metals (lead, chromium, cadmium, nickel, arsenic and cobalt). These studies reported that the different thermal preparation methods (fried, boiled, fried fish, grilled, prepared in a microwave oven or baked in an electric oven), produced considerable decreases in the final concentration of toxic metals in fish fillets, compared to raw samples [27, 28].

CONCLUSIONS

By choosing appropriate methods of thermal preparation, it is possible that the concentration of heavy metals initially present in the fish meat will be reduced, as we demonstrated that from an initial concentration of 23.1 mg/kg d.m. in raw fish, a concentration of 10.6 mg/kg d.m. was reached in boiled fish fillet and 4.55 mg/kg d.m. in the fried fish fillet.

The present study strengthens the hypothesis that the species *Cyprinus carpio* L. intoxicated with copper at values of 200 µg/L and subsequently thermally prepared by boiling and frying, shows a decrease in the final metal concentration in the fillets.

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