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COPPER AND CADMIUM ACCUMULATION IN GILLS AND MUSCULAR TISSUE OF TILAPIA (*Oreochromis niloticus*) UNDER EXPERIMENTAL CONDITIONS*

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ABSTRACT

The world wide use of tilapia for different approaches in fish bioassay was exploited to assess the accumulation of copper and cadmium, in isolated forms and in combination in gills and muscular tissue in the specie *Oreochromis niloticus*, which is of economical relevance in fish consumption in the Sao Paulo State, Brazil. To reach for these goals, semi-static chronic toxicity tests were carried out during 21 days by using two dissolved concentrations of each trace element as follow: $LC_{50}/10$ and the average of $LC_{50}/10$ and $LC_{50}/100$. Fish samplings to assess for the kinetic trace element absorptions with time were performed at 24, 96 hours, 7, 14 and 21 days. After 14 days of exposure gills had higher concentrations for both elements (5.20 mg Kg^{-1} Cu and 4.89 mg kg^{-1} Cd), than the muscular tissue (0.79 mg Kg^{-1} Cu e 0.32 mg Kg^{-1} Cd). A competition for absorption was established when both elements were in combination, being the maximum absorbed concentrations, 1.81 mg Kg^{-1} Cu and 1.54 mg Kg^{-1} Cd for the gills and 0.63 mg Kg^{-1} Cu and 0.12 mg Kg^{-1} Cd for the muscular tissue. The Tukey test used for the statistical evaluation of the exposure period times dissolved metal concentration interactions revealed the interference of the basal Cd and Cu contents of the fish on the results. Despite the verified bioaccumulation, in which BCF for Cd were lower than the BCF for Cu, the fractions of the LC_{s50} were not lethal to the fish. Tilapia did not concentrate Cu and Cd in the edible tissue at concentrations to bring restrictions for human consumption.

Key words: bioaccumulation; copper; cadmium; chronic toxicity; *Oreochromis niloticus*.

ACUMULAÇÃO DE COBRE E CÁDMIO EM BRÂNQUIAS E TECIDO MUSCULAR DE TILÁPIA (*Oreochromis niloticus*) SOB CONDIÇÕES EXPERIMENTAIS

RESUMO

O largo uso mundial da tilápia para diferentes finalidades em bioensaios foi explorado para se verificar a acumulação de cobre e cádmium, nas formas isoladas e em combinação, em brânquias e tecido muscular da espécie *Oreochromis niloticus*, cujo consumo é de relevância econômica no Estado de São Paulo, Brasil. Para tanto, ensaios semi-estáticos de toxicidade crônica foram conduzidos por 21 dias em duas concentrações para cada elemento traço, baseadas nos valores $CL_{50}/10$ e nas médias dos logaritmos das $CL_{50}/10$ e $CL_{50}/100$. Amostragens para avaliar as cinéticas das absorções dos elementos traços com o tempo foram realizadas após 24, 96 horas, 7, 14 e 21 dias do início do experimento. Decorridos 14 dias, as brânquias apresentaram maiores concentrações que o tecido muscular para ambos os metais, sendo os respectivos valores máximos iguais a 5,20 mg Kg^{-1} Cu e 4,89 mg kg^{-1} Cd, e 0,79 mg Kg^{-1} Cu e 0,32 mg Kg^{-1} Cd. Uma competição foi estabelecida quando os elementos traços estavam em combinação, sendo os valores máximos encontrados para as brânquias de 1,81 mg Kg^{-1} Cu e 1,54 mg Kg^{-1} Cd e 0,63 mg Kg^{-1} Cu e 0,12 mg Kg^{-1} Cd para o tecido muscular. O teste de Tukey utilizado para a avaliação estatística das interações período de exposição e concentração dos metais dissolvidos, revelou a interferência do conteúdo basal de Cd e Cu dos peixes quando da análise dos resultados. Apesar da bioacumulação verificada, onde o BCF do Cd foi inferior ao BCF do Cu, as frações das LC_{s50} não foram letais aos organismos. A tilápia não concentrou suficientemente Cu e Cd no tecido comestível para representar restrições ao consumo humano.

Palavras-chave: bioacumulação; cobre; cádmio; toxicidade crônica; *Oreochromis niloticus*.

INTRODUCTION

Population growth, intensification of agricultural activities and indiscriminate use of natural resources, coupled with fast industrialization, has led to increased contamination of the environment, being the aquatic systems an important end member in this process (ÇOĞUN and KARGIN, 2004; ATLI and CANLI, 2008). In these ecosystems, the ichthyofauna occupies an important role as bioindicator of the occurrence of organic and inorganic chemical species dissolved in the abiotic environment, since they occur in higher concentrations in their tissues due to bioconcentration and biomagnification as well, depending on its position level in the web food chain (SARAVI and SHOKRZADEH, 2013; BARBIERI *et al*., 2017).

Another alternative to assess for the interactions of a xenobiotic with the ichthyofauna can be through the use of bioassays under controlled laboratory conditions, which allow to estimate the lethal and chronic concentrations of chemicals on the studied species (EPA, 2002; ABNT, 2011, 2016). The great advantage of the study in laboratory is to enable the most varied conditions of the abiotic environment, to provide stress to the organisms, allowing extrapolation of biota responses to the actual environment (MAGALHÃES and FILHO, 2008). Therefore, the fish bioassay is a powerful tool to predict the anthropic impacts to which the environment is exposed (MAGALHÃES and FILHO, 2008; DAMATO and BARBIERI, 2012).

The major difference in the observation of chemicals in fish in nature and in the experimental conditions is the absence of a food chain in this latter, thus not occurring in this situation the process of biomagnification. Food webs occur in nature at different levels of complexity, and may, for example, result in different bioconcentration factors of a chemical species, and these should be some orders of magnitude smaller for experimental laboratory conditions. Another important difference to point out when considering the exposure of a fish in the nature and in a bioassay is related to the fate of chemicals due to the composition of the abiotic medium, bring as result different degrees of chemicals bioavailability (BUFFLE and DE VITRE, 1994). Many factors, which control the fate of chemicals in the environment, need to be considered when studying the bioaccumulation in aquatic organisms. Some of them are solubility, stability and molecule stoichiometry. Abiotic medium parameters such as dissolved organic carbon, total hardness (FERREIRA *et al*., 1997) and salinity (SANTOS *et al*., 2014) should also be considered.

Usually the dissolved analyte concentration is much lower in the nature than in the bioassays, which employes the LC_{50} or fractions of it in chronic dosage. However, the two studies have their specific characteristics, being both of them useful in their respective purposes.

Through the web food chain some organic and inorganic chemical species accumulate in organisms, inducing chronic or acute toxicity effects which can affect human health (TUNDISI and TUNDISI, 2008). The knowledge of xenobiotic concentrations in tissues and organs can be used to aware for the excessive human consumption of aquatic organisms, contributing not only to reduce the impact of these pollutants on the aquatic systems, but also improving consequently the human health conditions, as fish is an important source of protein in the human diet (SILVA FILHO *et al*., 2000). Due to their differential sensitivity to pollution, fish has been increasingly used to detect potential environmental problems (BOMBAIL *et al*., 2001).

The bioaccumulation of metals is a complex process. This is a result of metals toxicokinetic, which includes, metals absorption, distribution, metabolism and excreption (ERCAL *et al*., 2001). Among metals, there are the essential ones, like Cu, involved in the metabolism and growth of plants and animals and non-essential ones, such as Cd, without any recognized biological function. Thus the Brazilian National Council for the Environment – CONAMA establish levels of 1.0 mg L^{-1} Cu and 0.2 mg L^{-1} Cd for effluents to be discharged in the environment. (AZEVEDO and CHASIN, 2003; BRASIL, 2011).

Once chemicals are inside the body, physiological and biochemical processes essential to the organism metabolism can suffer alterations (ATLI and CANLI, 2008). The degree of organism response to them is upon on the chemical toxicity, properties and characteristics of the organism itself such as lipid content, diet and metabolic rate. The metallothionein formation in the body should also be taken into account (AZEVEDO and CHASIN, 2003; HUANG *et al*., 2007; VIRGA *et al*., 2007; AGAH *et al*., 2009; NAKAYAMA *et al*., 2010).

In this situation, tilapia is a fish widespread in Brazil, both in commercial crops and in reservoirs and dams. Among their several species, the Nile (*Oreochromis niloticus*), has been the most cultivated, thus becoming the most popular species in the country. The high quality of its meat makes tilapia a product of great interest for industrial processing with good acceptance by the consumer market (DIAS *et al*., 2007). In addition, this specie is widely used in fish bioassay (ALMEIDA *et al*., 2001; GARCIA-SANTOS *et al*., 2006; DIAS *et al*., 2007; GARCIA-SANTOS *et al*., 2007; GIRÓN-PÉREZ *et al*., 2007; ATLI and CANLI, 2008).

The aim of the present work was to document the accumulation of copper ($CuCl_2$), cadmium ($CdCl_2$) and copper + cadmium ($CuCl_2$ + $CdCl_2$) in gills and muscle tissue of tilapia, a fish that is not only largely used under experimental conditions, but also a fish of commercial importance in the State of São Paulo, Brazil, in order to assess for the uptake of an essential element in comparison to a non essential one. In both cases to check if the observed kinetics can imply in a bioaccumulation which represents risk for the human consumption.

METHODS

All research protocols in this work followed the guidelines of the United States Environmental Protection Agency (EPA, 2002) and the Brazilian Association of Technical Standards (ABNT, 2011) for fish toxicity tests, manipulating animals gently and carefully to minimize stress. All organisms, including those of the control treatment, were humanely destroyed and all effluents were adequately purified prior to disposal. Following the recommendations of the Brazilian National Institute for the

Environment – IBAMA, there is no need of a Government special license for scientific manipulation of tilapia, an exotic species, supplied by a fish farm.

Juvenile specimens of *Oreochromis niloticus*, male-reverted, with an average weight of 10.0±0.8 grams were kept in the laboratory under acclimatization conditions for a period of fifteen to twenty days. During this time the organisms remained under controlled conditions of light and temperature, alternating photoperiod of 12 hours of light and 12 hours of darkness; the temperature was established as 25.0±1.0 °C according to the procedures described in ABNT Standard 15088 (ABNT, 2011).

Chronic assays using two concentrations of each trace element in isolation and in combination, which were approximately $LC_{50\text{-}96h}/10$ and the mean logarithms of $LC_{50\text{-}96h}/10 + LC_{50\text{-}96h}/100$ were used. These concentrations being 0.35 mg L^{-1} Cu and 0.12 mg L^{-1} Cu; 2.0 mg L^{-1} Cd and 0.65 mg L^{-1} Cd; and 0.14 mg L^{-1} (Cu + Cd) and 0.05 mg L^{-1} (Cu + Cd). These concentrations were obtained in a previous assay (SILVA *et al*., 2017).

Organisms were submitted to acclimatization in experimental vats for a period of 48 hours prior to the beginning of the assays. In this period, they were kept with only the same water used in the chronic tests and fed daily during the experimental period of 21 days.

The assays were performed semi-statically, in triplicate, containing 15 organisms in each of the 40.0 L of experimental solution. Four units were used as control treatment, and two of these aquaria were not altered during the duration of the experiment, in order to verify the sanity of the organisms and the validity of the assay.

Exchanges of approximately ¼ of the water volume were performed every 96 hours in order to maintain the initial concentrations, with a tolerance level of ± 10.0%. Three individuals from each of the replicates were collected at 0, 24 and 96 hours and every 7 days for the determination of the total Cu and Cd concentrations in the gills and muscle.

During the collection, the physico-chemical variables of the test solutions, such as pH, temperature, dissolved oxygen (Table 1), total hardness, ammonia and metals concentrations (Table 2) were determined.

Temperature, pH and dissolved oxygen were measured in the vats with the YSI 556 MPS probe (Ohio, USA), while the total hardness, determined by using the EDTA titrator method, based on ABNT Standard 5761 (ABNT, 1984).

Dissolved trace element concentrations were determined after filtration on 0.45 µm cellulose acetate filters by using a Thermo Scientific ICP 6000 series (Waltham, USA) spectrometer with induced argon plasma (ICP-AES). The standard solutions used

Table 1. Temperature, pH and Dissolved Oxygen (DO) for the treatments, monitored at 0, 24 and 96 hours, 7, 14 and 21 days of the chronic test (n = 3) with solutions of $CuCl_2$, $CdCl_2.H_2O$ and ($CuCl_2$ + $CdCl_2.H_2O$).

Concentrations	Temperature (°C)	pH	DO (mg L^{-1})
0.0 mg L^{-1}	24.5±0.08-25.7±0.13	7.0±0.01-7.6±0.00	5.9±0.12-8.2±0.30
0.12 mg L^{-1} Cu	24.8±0.06-25.9±0.12	6.7±0.10-7.7±0.10	5.8±0.04-8.4±0.23
0.35 mg L^{-1} Cu	25.0±0.05-26.0±0.04	7.2±0.04-7.7±0.05	5.7±0.01-8.1±0.98
0.65 mg L^{-1} Cd	25.1±0.04-26.1±0.00	6.6±0.06-7.6±0.11	6.2±0.03-7.6±0.12
2.0 mg L^{-1} Cd	25.0±0.09-26.2±0.07	7.3±0.08-7.6±0.08	6.5±0.03-8.1±0.03
0.05 mg L^{-1} (Cu+Cd)	25.1±0.01-26.2±0.04	6.4±0.15-7.7±0.03	6.5±0.25-7.9±0.81
0.14 mg L^{-1} (Cu+Cd)	25.0±0.04-26.1±0.06	6.5±0.02-7.6±0.03	6.4±0.08-7.6±0.12

Table 2. Dissolved trace element concentrations (n=3) of 0.12 and 0.35 mg L^{-1} Cu; 0.65 and 2.0 mg L^{-1} Cd; 0.05 and 0.14 mg L^{-1} (Cu+Cd) which were established based on the $LC_{50\text{-}96h}$ for the species. Measurements were performed at 0, 24, 96 hours, 7, 14 and 21 days.

Trace elemento	Hours/Days					
	0h	*24h*	*96h*	*7d*	*14d*	*21d*
0.12 mg L^{-1} Cu	0.12±0.00	0.12±0.00	0.09±0.01	0.12±0.00	0.10±0.001	0.11±0.00
0.35 mg L^{-1} Cu	0.33±0.00	0.41±0.05	0.30±0.01	0.33±0.03	0.28±0.01	0.29±0.02
0.65 mg L^{-1} Cd	0.58±0.00	0.60±0.01	0.54±0.04	0.67±0.06	0.53±0.02	0.60±0.01
2.0 mg L^{-1} Cd	1.81±0.03	2.03±0.01	1.92±0.26	2.43±0.23	1.72±0.12	1.91±0.02
0.05 mg L^{-1} Cu+Cd						
Cu	0.06±0.00	0.06±0.00	0.05±0.00	0.05±0.00	0.05±0.00	0.05±0.00
Cd	0.05±0.00	0.06±0.02	0.05±0.01	0.05±0.00	0.04±0.01	0.06±0.01
0.14 mg L^{-1} Cu+Cd						
Cu	0.14±0.01	0.14±0.00	0.12±0.00	0.14±0.00	0.12±0.01	0.13±0.02
Cd	0.13±0.01	0.13±0.00	0.10±0.01	0.12±0.00	0.10±0.00	0.12±0.00

in these determinations were 0.0 mg L^{-1}, 0.1 mg L^{-1}, 0.5 mg L^{-1}, 1.0 mg L^{-1} and 2.0 mg L^{-1}. For Cu, the 324.7 nm line was used and for Cd, the 228.8 nm one, being the detection limits (LODs) 1.51 µg L^{-1} and 1.02 µg L^{-1} respectively.

Ammonium ion determinations were performed by flow injection (REIS *et al*., 1997) and the quantification of the non-ionized species was calculated as a function of the pH of the medium.

To assess the bioaccumulations, gills and muscular tissue of the individuals were removed. Samples were stored frozen until analyses. The low mass of liver samples was insufficient for chemical analysis of trace elements and therefore not taken into account. The other tissues had enough weight to be analysed by the analytical method described below.

It is important to note that samples were thoroughly washed with deionized water prior to packaging. This protocol proved to be essential for the gills due to their high surface area of exposure.

Samples were submitted to an acid digestion in a microwave pressurized system, Berghof, Speed Wave model (Eningen, Germany). For solubilization, 100.0 to 500.0 mg of samples were introduced into digestion flasks, followed by the addition of 4.0 mL HNO_3 50.0% (v/v) and 1.0 mL H_2O_2 solutions. The programming used for sample solubilization is presented in Table 3.

After solubilization, samples were transferred to a 15.0 mL Falcon tubes, being the final volume filled up to 10.0 mL. Prior the determination, the extracts were kept at 4 °C.

Analytical curves for both elements (Cu, Cd) were built as follows: 0.0 mg L^{-1}, 0.10 mg L^{-1}, 0.25 mg L^{-1}, 0.5 mg L^{-1} and 1.0 mg L^{-1}. Detection limits were 1.33 µg L^{-1} Cu and 0.60 µg L^{-1} Cd.

Results are in µg g^{-1} of trace element on wet weight basis. The analytical accuracy was evaluated with DORM-2 Certified Reference Material provided by the National Research Council of Canada - NRCC, with recoveries of 82% for Cu and slightly higher than 100% for Cd. Results were expressed as mean and standard deviation of the mean.

Statistical analysis was carried out taking into account the subdivided plot design, the splitplot, with primary factor being concentration of the dissolved metal and the second one period, which is the time of exposure of the fish in each of the concentrations used. For Cu, it were established the Control level (0.0 mg L^{-1} Cu), 0.12 mg L^{-1} Cu and 0.35 mg L^{-1} Cu and for Cd, Control (0.0 mg L^{-1} Cd), 0.65 mg L^{-1} Cd and 2.0 mg L^{-1} Cd in isolated situations. When the two trace elements were mixed together, Control 0.0 mg L^{-1} (Cu + Cd), 0.05 mg L^{-1} (Cu + Cd), and 0.14 mg L^{-1} (Cu + Cd).

There was a need for data transformation, seeking for the residues normality and homocedasticity. In order to identify the main treatments to explain the response variable, a Tukey test was performed at a significance level of $P<0.05$.

RESULTS

Temperature, pH and dissolved oxygen of experimental solutions at 0, 24 and 96 hours, 07, 14 and 21 days are presented in Table 1.

Total hardness average, which was monitored only in the control at the beginning and at the end of the experiments, was 65.2±1.03 mg L^{-1} $CaCO_3$. The concentrations obtained for ammonia during the chronic assays, varied from 0.001 to 0.1 mg L^{-1} NH_3.

The dissolved trace element concentrations (mg L^{-1}) obtained for all sampling periods are presented in Table 2, denoting good stability for sample solutions concentrations.

Tables 4 and 5 are indicative of copper and cadmium average concentrations (mg kg^{-1}) in gills and muscular tissues of the organisms. Data were obtained in triplicate for metals in isolated and combination mode along 21 days. The selected concentrations in water were based on the respective LC_{s50} for the species.

At the 96-hour period, for most treatments, there were increases in metal bioaccumulations, both in isolated and in combination mode. Afterwards bioaccumulation follows increasing, while dissolved trace element concentrations keep more or less stable. It was found that bioaccumulation occurs on a larger scale in the gills for both trace elements, being more pronounced as the concentrations increase in the water. In muscle tissue this differentiation was not significant.

In Table 6 it is present the maximum, minimum and average concentrations with standard deviations of Cu and Cd basal levels obtained in muscle tissue and gills for the fish population utilized in the assays. Along the entire period of the experiment they constituted the Control treatment for every single sampling time interval.

An overview of metals interactions with fish can be seen in Figure 1, which illustrates the kinetic of absorption in gills and muscular tissue along the 21 days exposure in a combined experimental conditions (Cu+Cd).

For the Tukey calculations, of the eight possible combinations, among the experiments conducted individually and with the two trace elements together, only 3 of them had their interactions calculated directly, the others being obtained through reverse transformation. These were, for the exposure of both metals, Cu in muscle, and Cd in gills. In the single-assayed trials, only Cd in gills was obtained directly.

Table 3. Programming of time, temperature and pressure used in the microwave oven for the acid solubilization of samples.

STEP	°C	RAMP (min)	PRESSURE (bar)	TIME (min)	% MAGNETRON
STEP 01	170.0	10.0	35.0	10.0	80.0
STEP 02	200.0	1.0	35.0	20.0	80.0
STEP 03	50.0	2.0	35.0	1.0	0.0

Table 4. Copper, cadmium and copper+cadmium average concentrations (mg kg^{-1}), (n=3), in gill samples of *Oreochromis niloticus* along the chronic toxicity test at 0.12 and 0.35 mg L^{-1} Cu; 0.65 and 2.0 mg L^{-1} Cd; 0.05 and 0.14 mg L^{-1} (Cu+Cd). Measurements were performed at 24, 96 hours, 7, 14 and 21 days.

Gill							
Trace element		**Concentrations (mg L^{-1})**	**Time**				
			24h	***96h***	***7d***	***14d***	***21d***
Cu		0.12	1.35±0.30	1.54±0.03	1.50±0.87	2.22±0.61	2.53±0.00
		0.35	2.26±1.37	3.78±1.75	2.99±1.76	5.20±0.37	4.50±2.10
Cd		0.65	0.59±0.21	0.96±0.28	0.87±0.49	2.42±1.44	2.20±0.56
		2.0	1.64±0.49	2.17±0.63	3.16±0.85	4.89±0.80	4.07±0.55
Cu+Cd	Cu	0.05	0.53±0.10	0.72±0.08	0.60±0.04	0.86±0.03	1.38±0.26
		0.14	0.80±0.21	1.29±0.07	1.05±0.12	1.81±0.44	1.85±0.09
	Cd	0.05	0.26±0.13	0.89±0.14	1.14±0.60	1.54±0.78	3.43±0.36
		0.14	0.32±0.18	0.65±0.04	1.24±0.45	1.13±0.01	2.87±1.49

Table 5. Copper, cadmium and copper+cadmium concentrations (mg kg^{-1}), (n=3), in muscle samples of *Oreochromis niloticus* along the chronic toxicity test at 0.12 and 0.35 mg L^{-1} Cu; 0.65 and 2.0 mg L^{-1} Cd; 0.05 and 0.14 mg L^{-1} Cu+Cd. Measurements were performed at 0, 24, 96 hours, 7, 14 and 21 days.

Muscle							
Trace element		**Concentrations (mg L^{-1})**	**Time**				
			24	***96***	***7d***	***14d***	***21d***
Cu		0.12	0.39±0.15	0.45±0.16	0.46±0.08	0.79±0.57	0.49±0.00
		0.35	0.39±0.05	0.92±0.01	0.56±0.24	0.67±0.04	0.68±0.15
Cd		0.65	0.16±0.03	0.05±0.02	0.08±0.01	0.32±0.29	0.19±0.07
		2.0	0.10±0.07	0.48±0.17	0.14±0.05	0.22±0.01	0.17±0.02
Cu+Cd	Cu	0.05	0.43±0.15	0.38±0.05	0.49±0.12	0.39±0.07	0.44±0.01
		0.14	0.58±0.02	0.49±0.11	0.48±0.17	0.63±0.03	0.43±0.12
	Cd	0.05	0.03±0.01	0.10±0.09	0.06±0.00	0.09±0.03	0.09±0.03
		0.14	0.06±0.01	0.05±0.01	0.05±0.00	0.12±0.02	0.11±0.00

Table 6. Copper, cadmium basal concentrations (mg kg^{-1}), (n=3), in muscle and gills samples of *Oreochromis niloticus* along the chronic toxicity test. Measurements were performed at 0, 24, 96 hours, 7, 14 and 21 days.

Fish tissue	Average±rsd mg L^{-1}	% rsd	Maximum	Minimum
		Muscle		
Cu	0.537±0.357	66.562	0.909	0.403
Cd	0.029±0.006	19.589	0.047	0.011
		Gills		
Cu	1.301±1.049	80.677	4.394	0.518
Cd	0.171±0.147	86.552	0.471	0.029

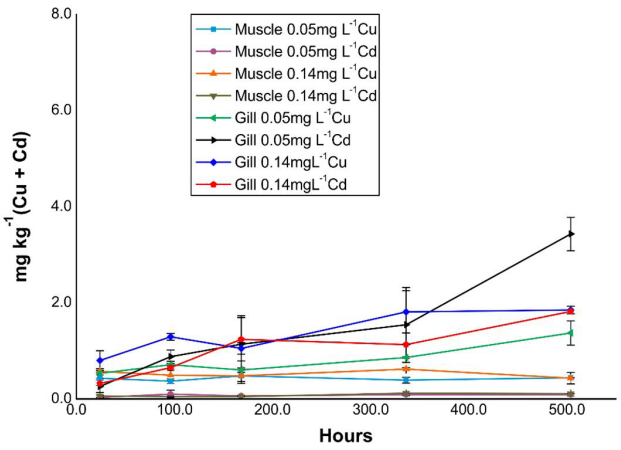

Figure 1. Copper and cadmium concentrations (mg kg^{-1}) in muscle tissue and gill samples (n=3) of *Oreochromis niloticus* for the chronic cadmium plus copper chloride test at 0.05 mg L^{-1} (Cu+Cd) and 0.14 mg L^{-1} (Cu+Cd) and sampling periods of 24, 96 hours, 7, 14 and 21 days.

DISCUSSION

Experimental abiotic medium and dissolved trace elements concentrations

During the whole experimental period, temperature, pH and dissolved oxygen remained nearly stable for the different treatments, thus reducing the possibility of effects on biota due to variations in the abiotic conditions (Table 1).

The observed concentration range for ammonia is indicative of alterations in the physiology of the specimens present in the experimental vats and upon the semi-static system operation, with changes of ¼ of the solution volumes every 96 hours of experimentation. However, the range obtained in the chronic trial, even when considering the highest concentration, was still significantly lower than the maximum limits allowed for *Oreochromis niloticus*. According to EVANS *et al*. (2006), the $CL_{50\text{-}96h}$ NH_3 found for this species was 0.98 mg L^{-1} NH_3 and for BENLI and KÖKSAL (2005), $CL_{50\text{-}48h}$ values for *Oreochromis niloticus* larvae and fry were 1.01±0.02 mg L^{-1} NH_3 and 7.40±0.01 mg L^{-1} NH_3 respectively.

Although the total hardness was slightly above values recommended for bioassays with aquatic organisms (ABNT, 2011), its influence over the biota would be minimized by its stability and pH values observed in all treatments of this experiment (SILVA *et al*., 2017). The preparation of synthetic water, as indicated in the literature (ABNT, 2011), would be difficult from the operational and practical point of view, due to the high volumes of water required.

In general, small positive and negative variations were observed for Cd and Cu concentrations in the different treatments during the entire 21 days experimental period. This can be due to solution preparations used to carry out the substitutions of ¼ of the solution volumes every 96 hours to restore the nominal concentrations. Values below the expected concentrations may have occurred by trace element adsorption to the experimental vessel walls and also by the bioaccumulation of trace element by the organisms.

Metals bioaccumulation an overview

It can be seen that for gills the bioconcentration increases for both elements with metal dissolved concentration, either in isolated or in combination mode (Table 4). For the muscular tissue this is not so clear (Table 5). Different kinetic absorptions for the dissolved trace elements in their isolated forms were achieved by the organisms. In combination these dynamics were modified. It was found that the trace element concentrations rise in the gills for both trace element with time, being more pronounced as the metal concentration in the medium is higher (Tables 4 and 5). In muscle tissue, this differentiation was not well noticeable and it was not possible to evaluate the variation of the concentrations of both trace elements with time, either in their isolated forms or in combination. This can be explained by the fact that gills present high capacity to absorb and store dissolved trace element, due to their surface of exposure for the gas exchanges, being able in this way, to reduce the amount of trace element to be transferred to the blood and subsequently distributed to other organs (MCGEER *et al*., 2000a, 2000b).

Tables 4 and 5 show also that the mechanism of absorption for the dissolved chemical species seems to differ in the situation in combination, possibly by trace element competition for the active sites of the complexing proteins (KALAY and ERDEM, 2003).

It were observed a correspondence of results of the present study with works done by ÇOĞUN *et al*. (2003) and NOGAMI *et al*. (2000). The first study verified a bioaccumulation of copper ($CuSO_4.5H_2O$) and cadmium ($CdSO_4.8H_2O$) in *Oreochromis niloticus*, higher in the gills (16.1-30.9 mg Kg^{-1} Cu; 22.3-31.4 mg Kg^{-1} Cd) than in muscle tissue (4.2-6.0 mg Kg^{-1} Cu; 3.6-4.2 mg Kg^{-1} Cd). The second found a greater accumulation of Cd in the viscera (1.8-40.0 mg Kg^{-1}) when compared to muscle tissue (0.6 – 1.8 mg Kg^{-1}) for the same species.

In their study VISNJIC-JEFTIC *et al*. (2010) observed an accumulation of trace element in *Alosa immaculata* muscle, liver and gills; Al, Sr, Ba, Mg and Li presented high concentration in gills, while Cd, Cu, Zn, Fe and B showed a higher concentration in the liver. Authors verified, as in the present study, that the muscle tissue had the lowest concentrations of the elements analyzed.

The role of Metallothioneins (Mts) should also be taken into account in the bioaccumulation process. A study related to the bioaccumulation of Cd ($CdCl_2.2.5H_2O$) in the kidneys, liver and muscle in *Cyprinus carpio*, revealed that the concentration of the trace element in the kidneys and liver increases up to saturation levels, with a positive correlation between the presence of Mts and increased fish tolerance to the trace element. The authors also concluded that after three months of exposure of the organisms to Cd, trace element concentration increased in the muscle, indicating that after reaching a saturation limit in the liver and kidneys, trace element accumulation in muscle tissue was increased (CONTO CINIER *et al*., 1997).

One aspect to be emphasized in this work is the biological essentiality of Cu and the non-identified function for Cd, which distinguish biologically these two trace elements. The chemical analyses of organisms in the control treatments showed concentrations for Cu in the muscle tissue between 0.403 mg kg^{-1} Cu and 0.909 mg kg^{-1} Cu, with a mean concentration of 0.537 mg kg^{-1} Cu. Cd occurred at concentration range between 0.011 and 0.047 mg kg^{-1} Cd, with a mean concentration of 0.029 mg kg^{-1} Cd. So, on average, the Cu concentration was 18.5 times higher than Cd, in the muscle tissue of tilapia. With rare exceptions, this would lead to obtain negative values in the determination of copper in the muscular tissue if they were subtracted by the average content found in the organs belonging to the control treatments. For Cd this was not verified. The low Cd concentrations present in the muscle tissue of the control treatments resulted in a percentual standard deviation around 19.59% for the individuals analyzed.

This should be taken into account in the analysis of metals in gills, where the population used in the control treatments had a maximum value of 4.394 mg kg^{-1} Cu and a minimum of 0.518 mg kg^{-1} Cu, with an average value of 1.301 mg kg^{-1} Cu, which leads to a percentual standard deviation of 80.68%.

In the case of Cd, maximum values of 0.471 mg kg^{-1} Cd and a minimum of 0.029 mg kg^{-1} Cd, with an average value of 0.171 mg kg^{-1} Cd, can lead to variations in concentrations of 86.55%.

The above statments denote that organism absorbs Cu preferably to Cd. Quantifying these differences for the highest concentrations adopted in the chronic toxicity tests for Cu and Cd alone, it was found that Cd was used in a concentration 5.71 times higher than that of Cu. This difference between the concentrations of dissolved chemical species is coherent and may explain the higher toxicity of Cu, an essential element to organisms.

It can also be verified that the log-base concentrations of LCs_{50} (SILVA *et al*., 2017) were established in a safe manner and no deaths were recorded during the entire period of experiment, even at the respective highest concentrations for each trace element, either in isolation or in combination.

It is the presence of trace element in fish muscular tissues that is of greatest interest, yet it is there that concentrations are lowest - this both from the feed point of view (RASHED, 2001; TAO *et al*., 2012) and environmental aspects (REPULA *et al*., 2012). For feed, reasons are self-explanatory. Regarding the environment, fish are organisms known as bioindicators because usually they have higher analyte concentrations than are in the abiotic medium (LINS *et al*., 2010; ABDEL-BAKI *et al*., 2011). This is verified by their ability to concentrate the dissolved species.

Concentrations resulting from trace element in muscle tissue, both in isolation and in combination, indicate that there is no compromise regarding the consumption of Nile Tilapia, since Brazilian legislation has limits of 30.0 mg kg^{-1} Cu (BRASIL, 1998) and 1.0 mg kg^{-1} Cd (BRASIL, 1965 - Decree No. 55871/65) for food. This information is of utmost importance from the point of view of human health, contributing to the incentive to trade in tilapia, fish of economic expression in the inland fisheries of the State of São Paulo.

Metals bioaccumulation, a statistical approach

From the statistical point of view, the general tendency of Cu and Cd bioaccumulation in muscle tissue and gills observed to tilapia for the period and concentrations of dissolved metals considered indicates situations of high significance and others with absence of these proximities when are confronted period of exposure times dissolved concentration. Thus, due to the wide variability of results obtained, each interaction should be considered separately.

Starting by analyzing the bioaccumulation of Cu in the muscular tissue, which was significant for the interactions, focusing on concentrations for the same period of exposure, the highest average concentration at 24h was obtained for the Control (b) > 0.35 mg L^{-1} Cu (a) > 0.12 mg L^{-1} Cu (a). For the 96 h period this trend was modified, obtaining higher averages for 0.35 mg L^{-1} Cu (b) > 0.12 mg L^{-1} Cu (a) > Control (a). For the sequential times of 7, 14 and 21 d, the Tukey's test at $p<0.05$, indicated that there were no significant differences for Cu accumulation in muscle tissue with time. When considering the period within the concentration, the following decreasing sequence were obtained, for 0.12 mg L^{-1} Cu, 14 d (b) > 21 d (ab) > 7 d (ab) > 96 h (ab) > 24 h (a). The lower average concentration for the 21 d period in comparison to the 14 d one is linked to a defense of the organism by the Mt formation (CONTO CINIER *et al*., 1997).

For 0.35 mg L^{-1} Cu, with the exception of the 96 h period of exposure, an expected decreasing sequence with time was obtained, 96 h (c) > 21 d (bc) > 14 d (bc) > 7 d (ab) and 24 h (a). This can be either by a basal Cu concentration in the analysed organisms or by 96 h be the selected time for most of the establishment of the acute toxicity in fish bioassay (BRASIL, 2011). No statistical differences were observed for the Control with time, being all average Cu concentrations considered similar.

The interaction for Cd with muscular tissue indicated that always the Control organisms had the lowest trace metal accumulation, with alternating rankings for 2.0 mg L^{-1} Cd and 0.65 mg L^{-1} Cd. It should be pointed out that the observed alternances were not statistically significative, unless for the 96 h period were the accumulation sequence was 2.0 mg L^{-1} Cd (c) > 0.65 mg L^{-1} Cd (b) > Control. Thus, the Cd accumulation in the muscular tissue was proportional to the dissolved concentration of the element. When comparing the periods for the same concentration, three different responses were obtained. For 0.65 mg L^{-1} Cd, 14 d (b) > 21 d (b) > 24 h (bc) > 7 d (ab) > 96 h (a) was obtained. For 2.0 mg L^{-1} Cd the concentration sequence versus time observed was 96 h (c) > 14 d (bc) > 21 d (ab) > 7 d (ab) > 24h (a), constituting in both situations unpredictable sequences, not demonstrating a tendency to be higher in tissue concentrations, the longer the exposure time of organisms. Observing the results obtained for the Control, a statistical significance over time was verified, which can only be explained by the basal concentrations of Cd present in the tissues of the analyzed organisms, 14 d (b) > 21 d (b) > 24 h (ab) > 96 h (a) > 7 d (a). This finding explains the statistical analyzes obtained in relation to the Cd in the muscle for the studied concentrations.

Related to the experiment of bioaccumulation of Cu in the gills, similar to what was verified for muscle tissue, a significant

interaction was found. In the unfolding of the combinations, for the different periods greater bioaccumulations, the higher the concentration of the dissolved metal. However, different meanings were obtained as a function of the exposure time considered. A single exception of the increasing sequence of concentrations occurring at the 14 day exposure time, where the following sequence was obtained, 0.35 mg L^{-1} Cu (b) > Control (ab) > 0.12 mg L^{-1} Cu (a).

When it were considered the variation of the dissolved concentration with the exposure time, an expected but not significant sequence was obtained for 0.12 mg L^{-1} Cu, 21 d (a) > 14 d (a) > 7 d (a) > 96 h (a) 24 h (a), with a no logical sequence for 0.35 mg Cu L^{-1}, 14 d (b) > 21 d (ab) > 96 h (ab) > 7 d (ab) > 24 h (a), and significant variation being verified in Control with time, which should not happen, 14 d (d) > 21 d (cd) > 24 h (bc) > 96 h (ab) > 7 d (a).

The behavior of Cd bioaccumulation in the gills, which was analyzed directly, did not present a significant interaction as occurred for Cu. It was verified that the highest concentration of this chemical species in the gills occurred at the concentration 2.0 mg L^{-1} Cd (a), followed by 0.65 mg L^{-1} Cd and Control Treatment (c). As a function of the exposure time, the following significance was obtained: 14d (a) > 21d (a) > 7 d (b) > 96 h (b) > 24 h (b), indicating what would normally be expected for the interaction, where the longer the exposure time, the greater the accumulation of metal in the tissues, followed by the tendency of organisms to excrete the metal after 14 days.

The bioaccumulation responses for the mixture of both metals indicated for Cu in the muscle non-significant interaction in the presence of Cd and that the treatments differed from each other, with the sequence 0.14 mg L^{-1} Cu and Control (a) and 0.05 mg L^{-1} Cu (b). For the different periods, the following significance was obtained 24 h (a) > 14 d (ab) > 7 d (ab) > 21d (ab) > 96h (b), an unexpected sequence, indicating similarities between the longer exposure (7, 14, and 21 d) times used . This arrangement is probably also due to the baseline values of the organisms analyzed. When the interaction of Cu with the gills was considered, a highly significant result was verified, the combination of which showed the same sequence of significance for all periods, 0.14 mg L -1 Cu (b) > 0.05 mg L -1 Cu (b) > Control (a). The treatment schedule with the period indicated no differences for the averages obtained for 0.14 and 0.05 mg L-1 Cu. However, where one might least expect, statistical significance was found for the means of the Control, namely: 21 d (c) > 14 d (c) > 96 h (b) > 7 d (ab) > 24 h (a). It should be noted that the temporal sequence observed showed no trends for the period-to-concentration interactions for the three concentrations of the dissolved species, corroborating with the strong influence of the basal Cu concentration in this tissue.

Concluding by the assays observed for Cd in the muscle and the gill in the presence of Cu, a significant interaction was obtained with the sequence 0.14 mg L^{-1} Cd (c) > 0.05 mg L^{-1} Cd (b) > Control (a) for the 24-hour period. For 96 h and 7 d the following sequence was observed, 0.05 mg L^{-1} Cd (b) > 0.14 mg L^{-1} (b) > Control (a). With a different sequence of this, for the longer periods of exposure, 14 and 21 days, it were observed 0.14 mg L^{-1} Cd (b) > 0.05 mg L^{-1} Cd (b) > Control (a). That is, it is significant evidence that the higher the concentrations of dissolved Cd, the greater bioaccumulation is verified for the muscle, even in the presence of Cu. In other words, Cd behaves in a situation similar to its condition of isolated presence.

In the case of gills, the concentration versus time interactions for Cd, analyzed directly by the Tukey's test, were not significant, although the concentration and period factors were highly significant when considered in isolation. The simple concentration effect grouped the treatments in the sequence 0.14 mg L^{-1} Cd (a) > 0.05 mg L^{-1} Cd (b) > Control (c), in a theoretically expected sequence, with the highest concentrations being found in the environments with higher concentrations of dissolved metal. As a function of the period, two groups were statistically formed, 14 d (a) > 21 d (a) > 7 d (b) > 96 h (b) and 24 h (b), as also expected, with the highest concentrations being obtained according to the highest time of exposure of the organism. It is also evidenced the tendency of the organism to initiate a process of excreting the metal after 14 days, as a defense mechanism, probably triggered by the formation of Mts (CONTO CINIER *et al*., 1997).

The discussion of this item was initiated referring to the wide variability of the obtained results, indicating a randomness of the interactions observed, depending to a greater or lesser degree of the considered element and the bioaccumulated tissue. Within this reality there is no way to establish conclusive results from the statistical point of view of the analyzed data.

The qualitative evaluation of the data, as presented in detail in the previous item, can establish a tendency of the kinetic aspects of absorption of the metals by the biota, which presented increasing concentrations as a function of time and higher bioaccumulation the higher the concentration of the dissolved chemical species. As an example, in the case of the presence of metals in combination, where a competition was verified, the approach is presented on Figure 1.

Basal concentrations for both Cu and Cd are uncontrolled variables in the chronic toxicity tests with tilapia, leading to significant interferences in the interactions of these dissolved species with organisms, making it difficult to obtain expected results. This influence is likely to be lessened in acute toxicity trials, where high concentrations of dissolved chemical species are employed, inducing a significantly higher bioaccumulation, close to lethality, rendering more despicable those that the organism presents naturally in its tissues.

Bioconcentration factors (bcfs) for copper and cadmium

At the 14 day experiment, different bio-concentration factors (BCFs) of the trace elements isolated and in combination were found. Organisms present in Cu treatments at concentrations of 0.12 and 0.35 mg L^{-1} Cu presented BCFs for muscular tissue equal to 6.58 and 1.91 L Kg^{-1} respectively. For the same tissue, treatments with Cd at concentrations of 0.65 and 2.0 mg L^{-1} Cd, BCFs smaller than the unit were found, being 0.49 and 0.11 L Kg^{-1}, respectively (Table 4 and 5). The same phenomenon was observed for both trace elements in the gills, where BCFs of 18.54 and 3.71 L Kg^{-1} were found respectively for Cu in the concentration 0.12 mg L^{-1} Cu and Cd in the concentration 0.65 mg L^{-1} Cd.

Increasing concentrations at 0.35 mgL^{-1} Cu and 2.0 mg L^{-1} Cd, BCFs of 14.86 L Kg^{-1} and 2.45 L Kg^{-1} were obtained for the same elements.

Experimental conditions show in both situations that BCFs varies with trace element water concentration, increasing when it decreases.

Changes were observed for the treatments in combination where, for the concentrations of 0.05 and 0.14 mg L^{-1} (Cu + Cd), BCFs for muscle of 7.8 and 4.46 L Kg^{-1}and 1.76 and 0.87 L Kg^{-1} for Cu and Cd were respectively obtained (Table 4 and 5). However, if we observe the BCFs obtained for Cu in the muscular tissue in isolated mode at the concentration 0.12 mg L^{-1} Cu (BCF = 6.58 L Kg^{-1}) and Cu in combination at concentration 0.14 mg L^{-1} (Cu + Cd) (BCF = 4.46 L Kg^{-1}), which are close to each other, we found that the BCF in combination was lower, due to the presence of the Cd, as discussed above.

Values obtained for BCFs in the experimental conditions differ from those found in natural ones for the studied metals, where metals are found in the abiotic medium in significantly lower concentrations (ZAGATTO and BERTOLETTI, 2008; CHOWDHURY *et al*., 2016; NOLI and TSAMOS, 2016; BARBIERI *et al*., 2016). In the laboratory, the mechanism of metal absorption is directly verified by the respiration of the organisms through the gills. In nature, metal absorption is dependent on the food chain, trophic level of the organism, and on biomagnification of the chemical specie (KEHRIG *et al*., 2011). In addition, it was evidenced that the available metal to the biota is lower than the dissolved nominal concentrations presented (SILVA *et al*., 2017). Therefore, the BCFs for both Cu and Cd in the nature should be higher than the calculated values above. In addition, it should be emphasized that although BCFs were low in this assay, probably by the low residence time of fish exposure and physiology, fish use to have elevated BCFs according to the abiotic medium conditions surrounding (SARI *et al*., 2016), being used as bioindicators. The BCF for *O.niloticus* in the nature must be low due its omnivorous feed bahaviour and certainly much lower to not reach concentrations of Cu and Cd above to those limits established by ANVISA for food.

As observed in this paper, Cu and Cd varies in a wide range of concentrations in tilapias' muscle. For this reason, the BCFs for both elements were determined without subtracting the average metals concentrations of the controls, just considering the concentrations of metals quantified in the biological material for each treatment. Thus, the error in considering the wide variety of concentrations in which these metals may be present in a fish population is minimized.

CONCLUSIONS

Physico-chemical data and ammonia concentration along the whole experiment indicates the good operational conditions at the laboratory. Moreover, no deaths were observed during the entire period of the experiment. These aspects denote the importance of the previous assays to establish the metals LC_{50}, in isolate and in combination forms. Small positive and negative variations in the dissolved metal concentrations were observed during the entire 21 days experimental period. Metal bio-concentrations were higher in the gills than in the muscle tissue, with a maximum value achieved after 14 days of the beginning of the experiments. Then there is a decrease in concentration towards the 21 days, probably by metals excretion and metalothioneins formation. The kinetic of absorptions are modified when both metals are in combination due to the competition established for available sites of the proteins. Although the metals absorption by fish are higher for higher metals dissolved concentration, the BCFs in the experimental conditions for both metals were much lower than those observed in nature. Beside the occurrence of metals in a natural environment being much lower than in the experimental conditions, there are interactions with the colloids of the medium, and competition among dissolved chemical species, which diminish availability. In addition, the biomagnification process, which does not occur in the experimental conditions, increases significantly the metal concentrations in the whole fish. In all situations, it was observed that BCFs of Cu, an essential element, were higher than those observed for Cd. For both elements a reduction of the BCFs are observed in the presence of Cu + Cd in the medium. The evaluation of the different interactions of Cu and Cd with the species *Oreochromis niloticus*, in isolated situations and in combination presented wide variability measured on the response variable for all the treatments (combination of the levels of the period and levels of the concentration), being a function of the chemical element and the analyzed tissue. The wide variations in the Cu and Cd concentrations presented by the organisms used in the tests represented a great interference in the application of the statistical analyzes of the obtained results, leading to that in many situations, random and unexpected results were obtained. These effects should be reduced in the case of acute toxicity tests where organisms bioaccumulate higher amounts of metals, which minimize their background content influence in the results. The low BCFs obtained for Cu and Cd in the experimental chronic conditions can predicted that tilapia should be considered, from Cu and Cd point of view, a safe source of protein, moreover considering its omnivorous trophic level in the web food chain.

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