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Toxicological effects of four chemicals used for prophylaxis of Amazonian ornamental fish

Henrique Malta Dias[1]
Ruda Fernandes Brandão Santos[2]
Nicolas Levy Pereira[3]
Natalino da Costa Sousa[4]
Marcia Valeria Silva do Couto[4]
Higo Andrade Abe[4]
Rodrigo Yudi Fujimoto[4*]

1. University Nilton Lins, Avenida Tefé, 700, Japiim, cep: 69078-000, Manaus-AM, Brasil. Department of Biochemistry.
2. Department of Biochemistry, University Federal of Pernambuco (UFPE). Avenida Prof. Moraes Rego, s/n, cidade Universitária, 50670-420, Recife-PE, Brasil.
3. Centro of Aquaculture-CAUNESP–UNESP. Prof. Paulo Donato Castellane, s/n, 14884-900, Jaboticabal-SP, Brasil.
4. Laboratory of aquaculture of Embrapa tabuleiro costeiro. Avenida Beira Mar, 3250, jardins, 49025-040, Aracaju, SE, Brasil.
**Autor para correspondência: ryfujim@hotmail.com*

ABSTRACT

The objective of this study was to determine the acute toxicity of chemotherapeutic drugs used to diseases control in ornamental fish cardinal tetra (*Paracheirodon axelrodi*). The fish were exposed to four chemicals: formalin, oxytetracycline, copper sulfate and malachite green. Four experiments were performed, distributed in a completely randomized design with seven concentrations of product test (formalin, oxytetracycline, copper sulfate and malachite green), a control and three repetitions. A static system with five fish per recipient containing 2 L of water for 96 hours was used. The estimated lethal concentration 50% (LC initial (I) $_{50\text{-}96h}$) were 67.94 mg.L^{-1}, 3.83 mg.L^{-1}, 1.65 mg.L^{-1} and 0.85 mg.L^{-1} to formalin, oxytetracycline, copper sulfate and malachite green, respectively. Thus the present study considered the formalin low toxicity for the cardinal tetra and the copper sulfate, oxytetracycline and malachite green are moderately toxic.

Keywords: ornamental fish; fish diseases; chemotherapeutic; lethal concentration.

Efeito toxicológico de quatro produtos químicos utilizados na profilaxia de peixe ornamental amazônico

RESUMO

O objetivo deste estudo foi determinar a toxicidade aguda de quimioterápicos utilizados para o controle de doenças em peixes cardinais ornamentais (*Paracheirodon axelrodi*). Os peixes foram expostos a quatro produtos químicos: formalina, oxitetraciclina, sulfato de cobre e verde malaquita. Foram realizados quatro experimentos, distribuídos em delineamento inteiramente casualizado com sete concentrações do produto teste (formalina, oxitetraciclina, sulfato de cobre e verde malaquita), um controle e três repetições. Utilizou-se um sistema estático com cinco peixes por recipiente contendo 2 L de água durante 96 horas. Foram estimadas as concentrações letais 50% (LC inicial (I) $_{50\text{-}96h}$) de 67,94 mg.L^{-1}, 3,83 mg.L^{-1}, 1,65 mg.L^{-1} e 0,85 mg.L^{-1} para formalina, oxitetraciclina, sulfato de cobre e verde de malaquita, respectivamente. Assim, no presente estudo a formalina foi considerada de baixa toxicidade para o tetra cardinal e o sulfato de cobre, oxitetraciclina e verde malaquita são moderadamente tóxicos.

Palavras-chave: Peixe ornamental, doenças de peixes, quimioterápicos, concentração letal.

Introduction

The state of Amazonas is recognized by its ornamental fish industry. This activity generates an incoming of more than US$ 6 million every year to the State of Amazonas (TAVARES-DIAS et al., 2009). The cardinal tetra *(Paracheirodon axelrodi*, Schultz 1956) is the main specie commercialized, very attractive for its strong glowing blue, represents almost 80% of all the fish caught in this region (CHAO et al., 2001).

However all the fishery processes occur under serious technical restrictions, mainly related to the unorganized capture of the fish, inadequate conditions of transport (SANTOS; SANTOS, 2005) and maintenance, resulting in mortalities rates reaching 50% from the capture to the destiny as America, Europe or Asia (TLUSTY et al., 2005). But in front of this considerable decrease of profits, some substances are used in the attempt to control the diseases outbreaks.

Among the most used chemicals are the malachite green, the formalin, the copper sulfate and the oxytetracycline. The malachite green is a water soluble triphenylmethane dye, presenting a high effectiveness against protozoa and fungal infections (CARNEIRO et al., 2005), however known by its carcinogenic, teratogenic and mutagenic potential (FERNANDES et al., 1991; RAO, 1995; CARNEIRO et al., 2005). The formalin is an aqueous solution of 37-49% formaldehyde gas, usually used as parasiticide, mainly for ectoparasites as protozoan and monogeneans, but can be used also as bactericide and algaecide (NOGA, 2010), leading the cells to death by cross-linking proteins (SANCHES et al., 2007). The copper sulfate (CuSO4) is an efficient algaecide and can be used as a therapeutic for protozoan parasites (STRAUS et al., 2009). The oxytetracycline, an antibiotic of the tetracyclines group with wide range of action, is frequently used in some human and animal disease treatments (DOLLERY, 1999; CARRASCHI et al., 2012) and acts inhibiting the bacterial protein synthesis.

Although these chemicals constitute part of the routine in this fish production, there are not studies validating their use for cardinal tetra, what obligates the fishermen to follow the prescription for other fish. Moreover, the application of incorrect doses of therapeutics is the most harmful problem in this activity, resulting in substantial financial and environmental losses as reported by Torres et al. (2008). Hence, the realization of aquatic toxicity assays can measure the degree of fish responses exposure to level of the chemical concentration and it is the first step to characterize optimal doses for disease treatments in this specie.

The objective of the present work was to evaluate the acute toxicity of the chemicals formalin, oxytetracycline, malaquite green and copper sulfate for cardinal tetra.

Material and Methods

Prior to the present study, 1000 cardinal tetra fish (*Paracheirodon axelrodi*) was acquired from a commercial store (Manaus, Amazonas, Brazil) and placed in 310 L glass fiber tanks, in order to acclimate to the laboratory conditions, all under constant aeration and water recirculation. Afterwards, the animals were submitted to 24 hours of feed restriction and then placed five fish per aquarium (mean weight of 1.73g ± 0.08), each one containing 700 mL of water. After this, another 300 mL, containing the test

chemicals were added to complete one liter per aquarium. All the recipients were provided with constant artificial aeration. All the concentrations of the tested chemicals are given in table 1. The fish mortality was monitored each hour, during the first 12 hours and each six hours until the final of the experiment (96 h).

Table 1. Chemotherapeutics concentrations used in tests with neon (*Paracheirodon axelrodi*). / **Tabela 1.** Concentrações de quimioterápicos utilizadas nos testes com neon (*Paracheirodon axelrodi*).

Chemotherapeutic	Concentration mg.L^{-1}							
Formalin	0	20	40	60	80	100	120	140
Copper sulfate	0	0.5	1	1.5	2.0	2.5	3.0	3.5
Oxytetracycline	0	1	2	3	4	5	6	7
Malachite green	0	0.2	0.5	0.8	1.1	1.4	1.7	2.0

Four substances (copper sulfate, oxytetracycline, malachite green and formalin) commonly used in the disease control of ornamental fish were tested. For each chemical was prepared a main solution adding one g of the product to one L of water (1 mg.mL^{-1}). To the formalin concentrations, the volume was pipeted directly to the recipients. Previously to the definitive assays, a potassium chloride assay (KCl) was performed using nine concentrations (0; 0.5; 1.0; 1.5; 2.0; 2.5; 3.0; 3.5 and 4.0 g L^{-1}) and three replicates.

An adaptation of Zuker classification to determinate the degree of toxicity of each product was used (ZUCKER, 1985). The compounds were classified into: highly toxic (when the Lethal concentration- LC_{50} is lesser then 0.1 mg.L^{-1}), very toxic (0.1 mg.L^{-1}> LC_{50}> 1 mg.L^{-1}), moderately toxic (1 mg.L^{-1}> LC_{50}> 10 mg.L^{-1}), hardly toxic (10 mg.L^{-1}> LC_{50}> 100 mg.L^{-1}) and barely nontoxic (LC_{50}> 100 mg.L^{-1}).

The water parameters as dissolved oxygen, temperature, pH and conductivity were measured daily using specific probes (LUTRON probes models PH-221 and DO-5519, and HANNA probe model HI-8733), and the total ammonium was monitored before and after the experiment with a photocolorimeter (HANNA). The dead fish were captured with aid of net to prevent the water deterioration.

All data were submitted to Trimmed Spearman Karber method (HAMILTON et al., 1977) for determination of the LC_{50} for each chemical.

Results

During the toxicity test the water parameters were stable: 5.6 ± 0.21 of pH, 6.02 ± 0.19 mg.L^{-1} of dissolved oxygen, temperature of 27.1 ± 0.11ºC, electric conductivity of 322.4 ± 12.2 µS.cm^{-1} and 0.01 mg.L^{-1} of total ammonia.

The lethal concentration $LC(I)_{50\text{-}96h}$ to potassium chloride was 1.92 g.L^{-1}. The mortality rates of the toxicity assays to the different chemicals are shown in table 2. The mortality of formalin started at the 40 mg.L^{-1}. However, the copper sulfate presented toxicity starting from one mg.L^{-1}, and malachite green at 0.4 mg.L^{-1}. The oxytetracycline, at four mg.L^{-1}, presented 100% of mortality.

Table 2. Cardinal tetra (*P. axelrodi*) mortality (n=15 fish per treatment) submitted at different concentrations of formalin, copper sulphate, oxytetracycline and malachite green after 96 hours of exposure. / **Tabela 2.** Mortalidade (n=15 peixes por tratamento) do Cardinal tetra (*P. axelrodi*) submetido em diferentes concentrações de formalina, sulfato de cobre, oxitetraciclina e verde da malaquita após 96 horas de exposição.

Formalin								
Concentration mg.L^{-1}	0	20	40	60	80	100	120	140
Mortality (%)	0	0	0	40	53.3	100	100	100
Copper sulfate								
Concentration mg.L^{-1}	0	0.5	1	1.5	2	2.5	3	3.5
Mortality (%)	0	0	6.7	26.67	66.67	100	100	100
Oxytetracycline								
Concentration mg.L^{-1}	0	1	2	3	4	5	6	7
Mortality (%)	0	0	0	46.67	100	100	100	100
Malachite green								
Concentration mg.L^{-1}	0	0.2	0.5	0.8	1.1	1.4	1.7	2
Mortality (%)	0	0	0	46.67	66.67	100	100	100

The $LC(I)_{50\text{-}96h}$ found to formalin was 67.94 mg.L^{-1}, with an inferior threshold of 60.89 mg.L^{-1} and a superior of 75.82 mg.L^{-1}. In relation to the oxytetracycline, the $LC(I)_{50\text{-}96h}$ was 3.83 mg.L^{-1} with an inferior threshold of 3.5 mg.L^{-1} and o superior of 4.25 mg.L^{-1}. For copper sulfate the $LC(I)_{50\text{-}96h}$ was 1.65 mg.L^{-1} with an inferior threshold of 1.47 mg.L^{-1} and a superior of 1.87 mg.L^{-1}. The malachite green presented a $LC(I)_{50\text{-}96h}$ of 0.85 mg.L^{-1}, with an inferior threshold of 0.76 mg.L^{-1} and a superior of 0.97 mg.L^{-1}.

The shorter period that cardinal tetra fish was exposed to each substance without any mortality, is presented in table 3. The formalin presented the first mortality after 24 h of exposure, at the concentration of 60 mg.L^{-1}. The copper sulfate showed a lethal toxicity after 48 h exposed to a concentration of one mg.L^{-1}, and the oxytetracycline after 36 h, at 2 mg.L^{-1}. The malachite green group had its first mortality after two h of exposure at a concentration of 0.8 mg.L^{-1}.

Table 3. Cardinal tetra exposure (n=15 fish per treatment) time to occur the first mortality in different concentrations of chemotherapeutics. / **Tabela 3**. Tempo de exposição (n=15 peixes por tratamento) do tetra-cardinal para ocorrer a primeira mortalidade em diferentes concentrações de quimioterapêuticos.

Formalin								
Concentration mg.L^{-1}	0	20	40	60	80	100	120	140
Exposure time (h)	96	96	96	24	24	6	4	2
Oxytetracycline								
Concentration mg.L^{-1}	0	1	2	3	4	5	6	7
Exposure time	96	96	36	8	2	2	2	1
Copper sulfate								
Concentration mg.L^{-1}	0	05	1	1.5	2	2.5	3	3.5
Exposure time	96	96	48	10	4	2	2	2
Malachite green								
Concentration mg.L^{-1}	0	0.2	0.5	0.8	1.1	1.4	1.7	2
Exposure time	96	96	96	2	1	1	1	1

Discussion

In the present study toxicity of four chemicals commonly used to disease treatment in fish was tested on cardinal tetra fish, in different periods of exposure.

None of the water parameters were restrictive to the maintenance of fish to the experimental conditions, being within the range recommended by IBAMA (1987). There was no correlation between the concentrations of the different chemicals used the water parameters from the conduced tests.

Macniven and Little (2001) found that the formalin was less toxic to Nile tilapia (*Oreochromisniloticus*), where the $LC(I)_{50\text{-}96h}$ was 429.68 mg.L^{-1} but, Tister and Koncan (SANTOS et al., 2012) observed a higher sensibility in rainbow trout (*Oncorhynchu smykiss*), with a $LC(I)_{50\text{-}48h}$ of 6.1 mg.L^{-1}. The *Coridora melanistius*, an ornamental fish from Amazon rivers, presented a $LC(I)_{50\text{-}96h}$ of 50.75 mg.L^{-1} to formalin, similarly to the LC found in the present experiment, but morphological alterations as hyperplasia of the gills cells were also found, reaching at lamellar fusion (SANTOS et al., 2012).

This indicates that, despite the formaldehyde to be considered, according to Zucker (1985) classification, just "slightly toxic", the exposure of the cardinal tetra to this chemical may cause pathophysiological problems.

Paixão et al. (2013) submitted the *Hemigrammus* sp, Amazonian species inhabiting the same environment as the Cardinal tetra at three concentrations of formalin: 250mg.L^{-1} and 100mg.L^{-1} submitted to one hour bath and 25mg.L^{-1} at 24 hours baths for three days

The cardinal tetra show mortality at concentration of 140 mg.L^{-1} after 2 hours of exposure to formaldehyde, indicating that for this species, the recommendation of short baths (up to 60 min, 150 mg.L^{-1}) can be performed without resulting in mortality (Martins, 2004). But analyzing the results obtained by Paixão et al. (2013) and the present work is possible to identify a large variability in the physiological response of the species, even inhabiting the same environment, highlighting the need for such tests.

Regarding recommended by Martins (2004) for long duration bath (up to 24 h, 10 to 15 mg.L^{-1}) became totally suitable to the cardinal tetra since the dose and duration of treatment are compatible the LC_{50} presented here. Considering the results obtained by Santos et al. (2012) from the toxicological and histological results and Paixão et al. (2013) from the assessment of mortality and changes in hematological parameters, this concentration can be used to control parasites

The copper sulfate toxicity is also influenced by some water parameter (MAZON et al., 2002), being highly dependent of alkalinity and pH, which, when is low, turns the heavy metals more available in the environment. Because of this, the treatment with this substance must be carefully monitored with kits that identify and quantify total, chelated or free copper.

In the present study, the toxicity of the copper sulfate to cardinal tetra can be classified as moderate (1 $mg.L^{-1}$> LC_{50}> 10 $mg.L^{-1}$) but, since the LC_{50} was found very close to the threshold of "very toxic" interval (1.65 $mg.L^{-1}$), this chemical must considered very carefully to use in this specie.

The toxicity in channel catfish (*Ictalurus punctatus*) presenting a $LC(I)_{50\text{-}96h}$ of 1.5 to 1.9 $mg.L^{-1}$ (STRAUS; TUCKER, 1993). This fluctuation values are due to alkalinity (76 to 240 $mg.L^{-1}$), however in the present study this variable was not analyzed.

In the present study was observed that the concentration of 0.5 $mg.L^{-1}$ of copper sulfate can be used to therapeutic baths of long duration without any mortality to cardinal tetra (table 3), agreeing to Bassler (2009), which recommends a concentration of 0.1 to 0.2 $mg.L^{-1}$ of the chemical.

However Paixão et al. (2013) exposed the Hemigramus sp. concentration of 0.3 $mg.L^{-1}$ for 24 hours showed increased mortality and decreased lymphocytes and neutrophils, monocytes and eosinophils, behavior significantly similar for all variables in the control group but significantly different for the treatment that caused the lowest mortality rate during the experiment (25 $mg.L^{-1}$ formalin). Indicating that the treatment itself can be as stressful as the parasitic infestation. Thus, the concentration of 0.5 $mg.L^{-1}$ used in this trial with cardinal tetra probably resulted in hematologic changes and the applicability of this concentration on the realization of the control of parasites may not be applicable due to the stress associated with parasitic infestation, may result in higher mortalities.

In the present study, the oxytetracycline was classified as moderately toxic for the cardinal tetra with a $LC(I)_{50\text{-}96h}$ of 3.83 $mg.L^{-1}$. Similar sensibility was found by Cruz et al. (2006) in guaru (*Phallocerus caudimaculatus*), with a $LC(I)_{50\text{-}96h}$ of 3.4 $mg.L^{-1}$ of oxytetracycline. According to Bassler (2009), the best dose of oxytetracycline in the control of bacteriosis is 150 to 200 $mg.L^{-1}$ for short duration baths, and 2 to 20 $mg.L^{-1}$ for long duration bath.

Yanog (2010) recommend a concentration of 10 $mg.L^{-1}$ of oxytetracycline in baths of 6 to 12 h, during a period of 10 days. According to our data, the cardinal tetra can be submitted to the treatments of Bassler (2009) and Yanog (2010) without any risk of mortality, however it is important the study of antibiotic efficacy for each of the pathogenic bacteria.

In the commercial chain of cardinal tetra in Brazil, the use of antibiotics as a prophylactic strategy is a common practice adopted by Amazonic exporters (WAICHMAN et al., 2001), being already reported by Mendonça and Camargo (2008) to cardinal tetra.

On the other hand, the appearance of bacterial strains resistant to one or more drugs, including the oxytetracycline, have already been reported a severe menace to public health (LEVY; MARSHALL, 2004) and the World Health Organization, since 2001, tries to forbid the nontherapeutic use of these substances (WHO, 2001). This highlights the importance to determine, for each fish species, its sensibility to this class of substances.

Bacterial strains isolated from tilapia (*Enterococcus sp., V. harveyi, V. alginolyticus and V. anguilarum*) were resistant to oxytetracycline demonstrating the infeasibility of using this antibiotic (JATOBÁ et al., 2008). According to Jatobá et al. (2012) several hemolytic strains isolated from red discus *Symphysodon* spp and rainbow fish *Melanotaenia* spp had a largest resistance for some antibiotics administered during to the culture in fish farming.

Corroborating these results Declerq et al. (2013) found that strains of *Flavobacterium columnare* isolates from cardinal tetra and neon tetra in 2000 showed bacterial resistance to tetracycline. Comparing the data obtained by Declerq et al. (2013) and those obtained in this work, the highest concentration of tetracycline (2 $mg.L^{-1}$) causing no mortality in cardinal tetra is not effective in inhibiting bacterial infection at least one strain of *Flavobacterium columnaris* isolated from neon tetra or cardinal tetra .

Thus, oxytetracycline cannot be used as stress reducer for transport and handling of ornamental fish as reported by Waichman et al. (2001); Fossa et al. (2007); Jatobá et al. (2008); Mendonça and Camargo (2008); Bassler (2009) and Yanog (2010).

Regarding the determination of the acute toxicity of malachite green was observed that in the present study was determined a $LC(I)_{50\text{-}96h}$ of 0.85 $mg.L^{-1}$ being classified as very toxic to the specie. The fish *Palaemonetes kadiakensis* and the *Heteropneustes fossilis* (SRIVASTAVA et al., 2004) presented a $LC(I)_{50\text{-}96h}$ of 1.9 and 1 $mg.L^{-1}$, respectively, demonstrating that both species are more resistant than the cardinal tetra. However, these species are cool water fish and no toxicological studies has been found for species of acidic water and low hardness in tropical regions, not knowing the toxicity of this substance in water with these characteristics.

The importance of carrying out the test with malachite green is due to the fact this substance to be is a biocide widely used in the aquaculture industry for its high efficiency against protozoa and fungi (FERNANDES et al., 1991; BIDARI et al., 2011). However, this substance is carcinogen and teratogenic (MARTINS, 2004). But its use in the ornamental fish industry is still recommended (FOSSA et al., 2007; BASSLEER, 2009) which reiterates conducting toxicity tests with this substance, even to demonstrate its negative effect on fish survival.

According to Harms (1996) 2 $mg.L^{-1}$ of malachite green during 5 hours to the control of external parasite was recommend. Nonetheless, for the present study was observed 93.24% of mortality with the same concentration during one hour preventing the recommendation above. Also was observed in literature the use of FMC (3.5 mg of malachite green; 3.5 mg of methylene blue and 1L of formalin) for treatment of parasite as *Cryptobia, Ichthyobodo, Chilodonela, Trichodina, Trichodinella* and some fungi infection (BASSLEER, 2009). Although the efficacy of this mixture should be evaluated in further experiments, since for the cardinal tetra the concentrations of formalin and green malachite are inside the range considered safe.

Conclusion

The present study determined the lethal dose and exposure time of formalin, malachite green, copper sulfate and oxytetracycline to the cardinal tetra. For the formalin, it's had a low toxicity over captivity conditions. Other chemicals applied in the present study showed a moderately toxic effect. These chemotherapeutics tested when applied as a treatment to the fish culture should be administered correctly to avoid mortality to organisms.

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