EFFECTS OF TRICHLORFON USED IN THE TREATMENT OF PARASITOSIS ON BIOLOGICAL METRICS OF FARMED *Ctenopharingodon idella* (Valenciennes, 1844)

Efeitos da aplicação de trichlorfon utilizado no tratamento de parasitoses sobre métricas biológicas de Ctenopharingodon idella (Valenciennes, 1844)

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ABSTRACT: This study evaluated the effect of Trichlorfon treatment to control fish's parasites on the growth, histological damage and bioaccumulation in grass carp tissues, under control conditions in laboratory. Two assays with the same experimental design were conducted with C. idella distributed in two tanks with 40L (control and treatment), each with n=10, under controlled abiotic conditions. The Trichlorfon active ingredient was added to the tank water at a concentration of 0.25 mg/L, once a week for five consecutive weeks. At the first assay, during the Trichlorfon treatment, we took biometric measurements, and at the second assay we performed histological analysis of gills and chromatography of the fish muscle. The results indicate that Trichlorfon promoted physiological changes in the analyzed fish, with inhibition of appetite and growth decrease in the treated group. There were no significant histopathological differences between control and treated groups. The muscle analysis of treated fish, collected five days after the last application of Trichlorfon, indicates no evidence of bioaccumulation for Trichlorfon or its metabolic residues. Thus, it can be preliminarily said that this type of treatment had a low effect on the morphological structures of the vital organs and was not accumulated in fish muscle. However, the treatment acts directly on zootechnic parameter, one of the most important factors in the context of fish farming.
Keywords: bioaccumulation; carp; fish farming; histomorphology; organophosphates.

RESUMO: Este estudo avaliou o efeito do organofosforado Trichlorfon, utilizado para controle de parasitas de peixes, sobre o crescimento, danos histológicos e bioacumulação em tecidos de carpa-carpim, em ambiente laboratorial com condições controladas. Dois ensaios com o mesmo desenho experimental foram conduzidos com espécimes de *C. idella* distribuídos em dois tanques de 40 L (controle e tratamento), cada qual com 10 indivíduos, sob condições abióticas controladas. O ingrediente ativo de Triclorfon foi adicionado à água do tanque a uma concentração de 0,25 mg/L, uma vez por semana durante cinco semanas consecutivas. Durante esse período, no primeiro ensaio foram tomadas medidas biométricas e no segundo ensaio, no mesmo período, realizamos análises histológicas de brânquias e análises cromatográficas da musculatura dos peixes para avaliação de bioacumulação. Os resultados indicam que Triclorfon promoveu alterações fisiológicas nos peixes analisados, sendo que no grupo tratado com o

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organofosforado houve inibição do apetite e redução na taxa de crescimento. Não houve diferenças histopatológicas significativas entre as amostras dos grupos controle e de tratamento. As análises do músculo dos peixes tratados, coletado cinco dias após a última aplicação de Triclorfon, não indicam evidências de bioacumulação de Triclorfon ou seus resíduos metabólicos. Assim, pode-se dizer preliminarmente que esse tipo de tratamento teve um baixo efeito sobre as estruturas morfológicas dos órgãos vitais e não foi acumulado na musculatura dos animais. No entanto, o tratamento atua diretamente sobre os parâmetros zootécnicos, sendo estes os fatores mais importantes no contexto da piscicultura.

Palavras-chave: bioacumulação; carpa; piscicultura; histomorfologia; organofosforados.

INTRODUCTION

Production in aquaculture systems may be affected by pathogens and parasites, causing economic and environmental problems (Kubitza, 2010; Schalch, 2011). For the treatment against parasites, farmers often use chemical-based products, such as sodium chloride (NaCl) against fungi (Silva et al., 2009), albendazole and praziquantel against nematode and cestodes (Fujimoto et al., 2006), and organophosphorus pesticides against copepods and other ectoparasites (Schalch and Moraes, 2005). However, there are few drugs registered for use in aquaculture (Campos, 2005; Jaafar and Buchmann, 2011; Tavechio et al., 2009), which often leads to the indiscriminate use of various chemicals, for which studies on the administration, processing, and period of consumption are still scarce.

Organophosphates are largely used in agriculture as insecticides (Barbieri and Ferreira, 2011; Jokanovi, 2001; Oliveira and Machado, 2004). In the metabolism of acetylcholine by cholinesterases, acetic acid (metabolic product) may be detected, which is an excellent parameter for the quantification of enzyme activity and hence markers of organophosphorus pesticides in the environment (Oliveira et al., 2007; Rodrigues et al., 2011; Santos et al., 2007; Sturm et al., 1999).

Among the widely used organophosphates against fish parasites, the most efficient are those whose active ingredient is Trichlorfon (2,2,2-trichloro-1-hydroxyethylphosphonate) (Tojo and Santamarina, 1998). Trichlorfon is mainly used in the treatment of several parasite taxa (Venturini et al. 2015), but especially *Argulus* sp., *Ergasilus* sp., *Lernea* sp., *Dactylogyrus* sp. and *Trichodina* sp. (Burridge et al., 2010).

Despite the problems associated with the use of Trichlorfon, products that incorporate this organophosphate as an active ingredient are often used in Brazilian aquaculture indiscriminately, repeatedly, at high concentrations, and without specialized technical guidance (personal observation). In this context, this paper postulates that organophosphates act negatively on biological parameters, with consequent damage to the growth and development of the organism. Thus, we evaluate, under laboratory conditions, the influence of using 2,2,2-trichloro-1hydroxyethyl phosphonate (Trichlorfon) at the recommended levels in the literature on growth, gill histology and tissue bioaccumulation in grass carp (*Ctenopharyngodon idella*), after the treatment period.

MATERIALS AND METHODS

Experimental Design: Two similar experiment were conducted in a completely randomized design of the type undereplicate (Tincani et al. 2017). The animals were considered experimental units and distributed in two 40 L tanks (Control and Experiment), in each assay, containing ten specimens of C. idella each, from the fish farm in Pescobrás (Rodovia Alexandra/Matinhos, Paraná, Brazil). Experiments in ecotoxicology that do not present true replicates (ie, several animals per tank) present equivalent results and with the same error value of type I and II as experiments with replication (ie one animal per tank) (Tinciani et al. 2017). Water filtration, oxygenation, and temperature (25°C) remained constant, and the photoperiod was maintained at 12-h light/12-h dark. The animals were fed once a day (4% average biomass per tank) with extruded feed. The maintenance of tanks was made weekly; deposited food remains were removed by siphoning, they were dried and weighed in a digital scale and 50% of total volume of water was

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replaced with clean water. It is noteworthy that the maintenance of the tanks during the treatment with Trichlorfon was always carried out before the weekly application of the doses of the active principle.

Before the beginning of the assays, fish were acclimated in their respective tanks for ten days. Trichlorfon was added to the water of the treatment tank to reach the concentration of 0.25 mg/L, once a week, for five consecutive weeks, reaching a final concentration of 1.25 mg/L, as recommended by Nordmo (1993) for the control of dactylogyrosis and lerneosis. The first assay of this study evaluated the growth pattern and the second assessed the histomorphological modifications and bioaccumulation.

Growth Analysis: For 14 weeks, all fish were measured for total length (mm), weight (g) and biometric measurements. The first five measurements were performed during the five weeks of treatment. Subsequently, measurements were performed every two weeks. Throughout the measurements, inspections were performed and changes were verified in the body of each animal, such as coloration and deformities.

The feed conversion rate (FCR) is calculated, to demonstrate the mathematical relationship between the input of the feed that has been fed and the weight gain of fish sample. The formula used was as follows: FCR. = Feed given / Animal weight gain. The FCR measurement units used was g of food.g of weight gain-1.

Histological Analysis: In the second assay, after the completion of treatment with Trichlorfon in the five week, three animals from each tank - control and treatment - were subjected to spinal cord section. The 2nd gill arches were collected from each fish and fixed in Alfac (Beçak and Paulete, 1976). Subsequently, the samples were

transferred to 70% alcohol. The collected material was subjected to routine processing. The sections were stained with Alcian Blue pH 2.5 to show cells secreting acidic carboxylated and sulfated glycosaminoglycans (Beçak and Paulete, 1976). After analysis, permanent slides were selected and photographed with the help of an Olympus PM 10 AD microscope.

In order to compare the number of mucous cells of the gills between the control and treatment, we randomly chose ten regions of gill lamellae of each individual. Each region was digitized and analyzed using the Image Tool for Windows 28.1 (The University of Texas Health Science Center in San Antonio).

Bioaccumulation Analysis: To detect traces of the product and determine the period of accumulation in fish muscle, three samples were collected consisting of a pool of muscle material from three fish. The first sample was taken after five days of the last application of the product (33 days after the start of the experiment); the second, after ten days (38 days); and the last, after twenty days (48 days). The three samples were subjected to Soxhlet extraction to extract the chemicals (Andrews et al., 1993; Makarewicz et al., 1993), with dichloromethane as a solvent.

After extraction of the product, the sample was evaporated in gaseous nitrogen and subsequently solubilized in methanol. After chromatograph calibration, the sample was injected and analyzed for 20 minutes. The chromatograph was HPLC type, which had a reversed-phase ODS silica column. The mobile phase consisted of 15% acetonitrile and 85% of a 0.01 M phosphoric acid solution at pH 3.0 dissolved in MilliQ water (Samuelsen, 1987). The UV detector coupled to the chromatograph was calibrated for a length of 205 nm. The solvent flow rate

was 1 mL/min at a pressure close to 270 atm.

Statistical analysis: Morphometric data were analyzed subjected to mist model of Repeated Measures Factorial Anova. The number of mucous cells of the control and treated animals were compared using the t-test for independent samples, after checking the assumptions of normality (Shapiro-Wilk test) and homoscedasticity (F-test). All analysis was made using the software Statistica 7.0 (StatSoft, Inc.).

RESULTADOS

Growth Analysis: The control animals showed a weight and length increase equal to 53.26% and 8.3%, respectively (tab.1). The treatment animals presented values equal to 17.81% and 4%, respectively (tab.1).

There was a significant difference in weight ($F10, 130 = 6.74$; $p<0.0001$), with lower mean values in treated animals from the 10th week, i.e., five weeks after the end of treatment with Trichlorfon (Fig. 1A). There was no significant difference between the groups in total length ($F10, 130 = 1.635$; $p = 0.104$). The significant increase in total length growth ($p<0.05$) occurred in both groups from the 3rd experimental week (Fig. 1B).

Analysis of the weekly increase of these two variables suggests higher growth for the control fish compared to treated individuals. The rates of weight increase remained lower in the treated fish during the period of exposure to Trichlorfon ($F_{9,117} = 6.626$; $p<0.0001$) and tended to further decrease, reaching a zero rate of increase in weight in the seventh week after the end of treatment (Fig. 2A). The total length growth rate of the treated fish immediately reduced at the beginning of treatment, but showed the same growth performance as the control group ($F_{9,117} = 1.536$; $p = 0.143$) (Fig. 2B).

The relative rates of weekly growth suggest that there is a reduction in growth rates during and after the treatment with Trichlorfon, especially for the weight of *C. idella*. These growth rates do not return to similar levels to those of the control during the study period. It is also worth noting that 30% of the treated fish presented lordosis in the vertebral column, whereas no control animal presented any type of alteration during the inspections.

The reduction of the growth rate in weight and length observed in the treated group can be explained by the increase in feed requirement (Fig. 3). Throughout the experiment, it was possible to observe that control group required, on average, 0.33 ± 1.0 g of food to convert in one gram of weight. On the other hand, the treated group required, on average, 4.9 ± 8.1 g of food, and in the fourth and fifth weeks of treatment with Trichlorfon the feed conversion rate increased to 18 g of food.g of weight gain^{-1}. After treatment completion, feed conversion rates return to values similar to those in the control group.

Table 1 – Mean values of weight (± SE) and total length (± SE), coefficient of variation (CV%) of individuals of *Ctenopharyngodon idella* before and after the bioassay. SD= standard deviation.

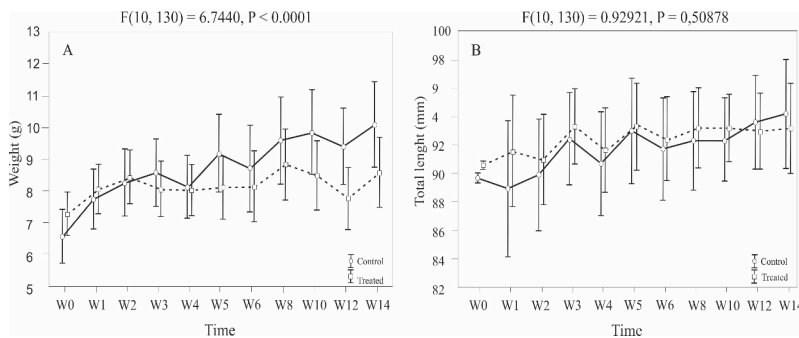|	Stage	Weight (g) (± SE)	CV% Weight	Length (mm)(±SE)	CV% Length
Control	Initial	6.59 + 0.43	18%	87.01 + 0.85	2%
	End	10.10 + 0.80	22%	94.20 + 1.38	4%
Treatment	Initial	7.30 + 0.30	19%	89.65 + 1.24	4%
	End	8.60 + 0.38	13%	93.22 + 1.65	5%

Figure 1 – Means and confidence intervals (95% CI) of biometric measures in Control and Treated groups over the experimental period. A) Weight (g). B) Total length (mm).
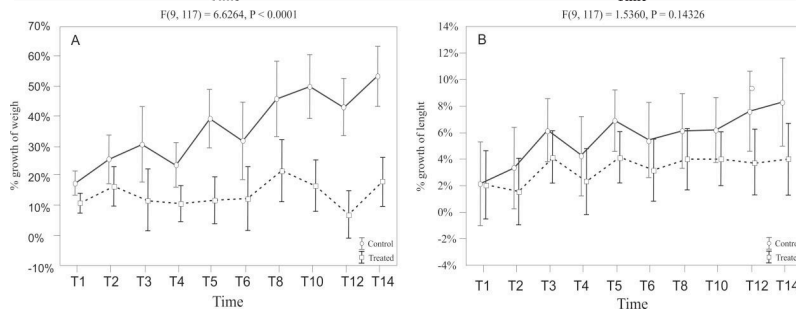

Figure 2 – Means and confidence intervals (95% CI) of % growth of biometric measures in Control and Treated groups over the experimental period. A) % Weight Growth. B) % Total length Growth.
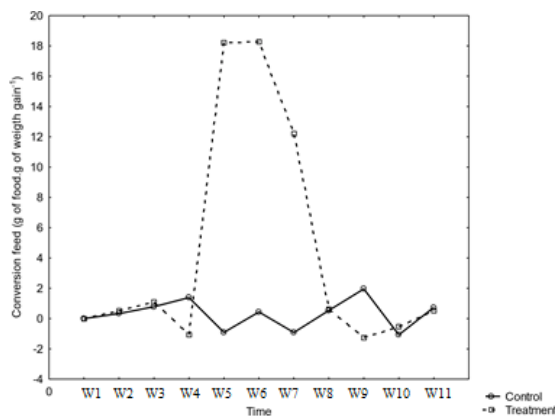
Trichlorfon at a retention time of 8.802 (equivalent to 8.48 min).

Figure 3 - Conversion feed in Control and Treated groups over the experimental period.

Histopathological Analysis: There was no evidence of histological change in animals exposed to Trichlorfon. There was no significant difference in the number of mucous cells of the gills between fish of the treatment (5.9 ± 2.4 cells/field) and control (5.2 ± 2.6 cells/field) (t = 0.83; p = 0.414) (Figs. 4).

Chromatographic Analysis: A standard aqueous solution of Trichlorfon (97.02%) at 50 mg/L was prepared, diluted in acetonitrile and applied to chromatographs. The resulting chromatogram (Fig. 5A) showed two peaks: one representing acetonitrile at the time of 3.165 (equivalent to 3.09 min), and another representing
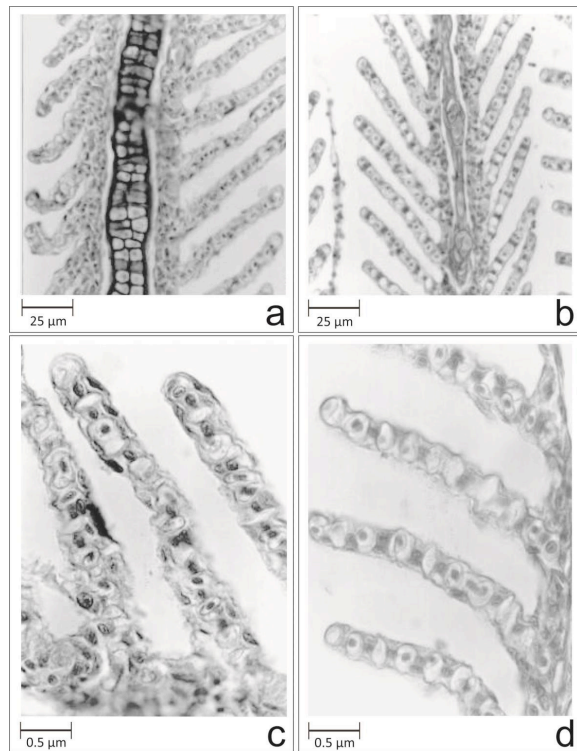

Figure 4 – Photomicrography of gills of *C. idella*, of 10 µm scale; A, B - control and treated respectively stained with HE. C, D - control and treated respectively stained with Alcian Blue pH 2.5.

This standard was used for comparison with other experimental samples.

The chromatogram of the fish muscle samples collected at five days (33 experimental days) after the last treatment indicated a no-retention peak of Trichlorfon (Fig. 5B). The same result was observed for fish muscle samples collected ten and twenty days after the last application of Trichlorfon. This result provides no evidence of bioaccumulation of the product or its metabolic residues in the muscles of treated fish.
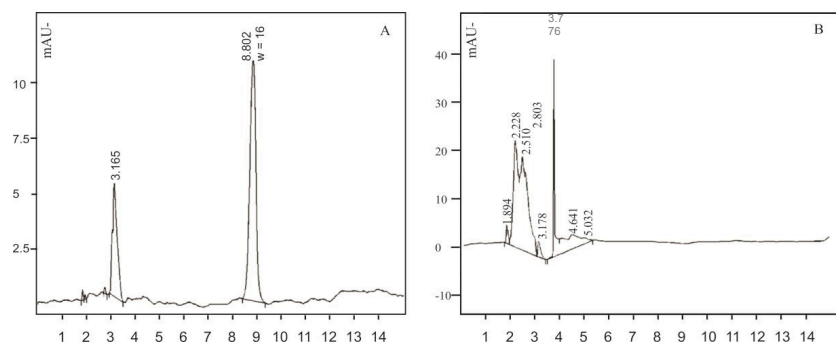

Figure 5 – A) Chromatogram of the liquid phase of the Trichlorfon standard. Retention time of acetonitrile = 3.165. Retention time of Trichlorfon = 8.802. B) Chromatogram of the liquid phase of the sample 5 days after the last application of 0.25 mg/L Trichlorfon. Retention time of acetonitrile = 3.776. Retention time of methanol = 2.228.

DISCUSSÃO

Trichlorfon has been used as an effective antiparasitic chemical by fishes for several decades. Brandal and Egidius (1979) used Neguvon for the treatment of salmon lice in farmed salmon. Hispano et al. (2013) suggested the bath treatment within the range of 0.3–0.5 ppm of Trichorfon to eliminating *Gnathia maxillaries* of fishes. Thing et al. (2016) showed that Trichlorfon killed all the larval parasites, even at a concentration of 0.2 ppm. Although there are many studies that show the efficacy of Trichlorfon in the treatment against fish parasites, it is worth analyzing the zootechnic parameters of the fish and the health of fish cultured.

On the present study, the accumulation of feed remains on the bottom of the treatment tank is strong evidence of the negative effects of Trichlorfon to the zootechnic parameters of grass-carp. Appetite inhibition is a side effect of the action of organophosphates on cholinesterase (Barbieri and Ferreira, 2011; Eto, 1975; Groh et al., 2015; Jokanovi, 2001; Larini, 1979; Salte et al., 1987), which promotes alterations in the development of fish, with a consequent decrease in weight growth. The comparison of the weight-growth curves between the control and treated animals corroborates the hypothesis that the energy stored by the fish treated with Trichlorfon is used only for the growth in length, without any increase in weight.

The requirement for essential nutrients varies depending on various factors such as the metabolic demand (Tavares-Dias, 2009). A nutrient-rich diet provides an increased hippocampal-cholinergic neurotransmission, which in turn influences cognitive effects (Cansev et al., 2015). On the other hand, fish species reduce their growth under dietary deficiency of essential amino acids (e.g. tryptophan), lipids, mineral salts (e.g. magnesium, phosphorus) and, especially, vitamin C and D (Tacon, 1992). This is directly related to the lack of food intake, derived from the appetite inhibition, resulting from possible contamination by organophosphate, which can result in spinal lesions (Arbuatti et al., 2013; Tacon, 1992).

Additionally, spinal lesions were identified in 30% of fish of the treatment and were probably associated with the lack of essential elements coming from the feed. Vitamin C deficiency in fish can cause growth reduction, increased mortality and bone deformities such as lordosis, kyphosis and scoliosis

(Chagas et al., 2003; Chagas and Val, 2006; Koshio, 2007; Salaro et al., 2013). Besides these deleterious effects, changes may occur in the production of hydroxyproline, an essential component of the collagen matrix, and where calcium is deposited to form the vertebrae. With vitamin deficiency, these structures become weakened, even with normal calcium deposition (Darias et al., 2011; Sandnes, 1991; Tavares-Dias, 2009). This syndrome can occur due to the oxidizing depletion dependent on Vitamin C. Vitamin D, on the other hand, stimulates calcium absorption in the intestine, acting directly on bone maintenance and on osteoblasts. As the fish are unable to synthesize both vitamins (Darias et al., 2011), a restrictive diet or the lack of appetite can lead to a deficiency of these vitamins, with consequent damage to growth and bone formation.

Moreover, another possible cause of spinal lesions is the direct inhibition caused by organophosphate. Skeletal deformities have been observed in wild fish subject to deficient diets and exposed to pesticides and heavy metals (Kessabi et al., 2013). Organophosphate can cause twisting of the body due to a muscle hyper-contraction (Johnson, 1994). Furthermore, pesticides can affect the absorption of vitamin C and, consequently, the development of skeletal collagen (Arbuatti et al., 2013; Silverstone and Hammell, 2002). Bone deformities have also been reported during developmental stages in environments contaminated with heavy metals (Sfakianakis et al., 2015).

Independent of how the application of Trichlorfon acts negatively on the rates of growth in weight and length of the grass carp, the deleterious effect seems to remain even after the end of treatment. Growth rates of the treated fish, in general, did not return to similar levels to those observed for control fish in the 14 experimental weeks. This may be related to the exposure of fish to the organophosphate and its ability to bioaccumulate in aquatic animal tissues (Amaraneni and Pillala, 2001; Lopes et al., 2006; Luvizotto-Santos et al., 2009). Meanwhile, our findings indicate that the accumulation of Trichlorfon and its metabolites is below of the detection limit or virtually non-existent in our experimental model, corroborating with Essa and Korni (2018) that demonstrated that residues were not found neither in water nor in fish larvae treated with Trichlorfon; and Mackinnon (1997) that showed that dichlorvos residues were not detected in fish, even a week after treatment.

Regarding the gills, no morphological difference was detected. According to Mallatt (1985), the presence of a toxic substance in the medium, such as e.g. organophosphates, can cause the body to react, releasing a greater amount of mucus, which is a defense mechanism. A thick mucus layer acts as a filter to minimize damage to the lamellae (Rezende et al., 2013). Nevertheless, as no signs of organophosphate bioaccumulation were observed, the treatment possibly did not change the number of mucous cells of the gills compared to control animals.

CONCLUSION

The results presented herein were obtained from a laboratory bioassay that can be extrapolated to a real culture situation. Thus, we suggest that treatment with Trichlorfon against parasites has little effect on the morphological structures of the vital organs, and the compound and its metabolites do not accumulate in the fish muscle.

However, it directly inhibits the fish growth, one of the most important factors in fish production. In this case, growth of treated animals is impaired

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precisely by the acute effects of Trichlorfon.

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