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Hematological, biochemical and physiological evaluation of tilapia juveniles treated with organophosphate trichlorfon

Avaliação hematológica, bioquímica e fisiológica de juvenis de tilápia tratados com organofosforado triclorfon

Evaluación hematológica, bioquímica y fisiológica de juveniles de tilapia tratados con el organofosforado triclorfón

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ABSTRACT

Trichlorfon (TCF) is an organophosphate parasiticide commonly used in aquaculture to treat ectoparasites. However, studies across various fish species have reported adverse effects even at sublethal doses. This study assessed the hematological and biochemical responses of Nile tilapia juveniles (*Oreochromis niloticus*) following a one-hour exposure to a 0.5 mg L^{-1} bath of a commercial TCF-based product. It also evaluated the fish's physiological recovery 28 hours after exposure. Results showed elevated plasma cortisol levels and a downregulation of glutathione-S-transferase (GST) activity in liver tissue among TCF-exposed fish compared to controls. Alterations in the leukogram of treated fish indicate an increased vulnerability to disease. These findings suggest that a 28-hour recovery period is insufficient to restore the physiological health of Nile tilapia juveniles after TCF exposure. The study highlights the importance of adopting best management practices (BMPs) when applying TCF treatments in aquaculture and underscores the need for further research on the short- and long-term physiological impacts of chemical treatments on fish.

Keywords: Blood Glucose. Plasma Cortisol. Oxidative Stress. Ectoparasites Treatment. Physiological Response. *Oreochromis niloticus*.

RESUMO

O triclorfon (TCF) é um parasiticida organofosforado comumente usado na aquicultura para tratar ectoparasitas. No entanto, estudos em várias espécies de peixes relataram efeitos adversos mesmo em doses subletais. Este estudo avaliou as respostas hematológicas e bioquímicas de juvenis de tilápia do Nilo (Oreochromis niloticus) após uma hora de exposição a um banho de 0,5 mg L^{-1} de um produto comercial à base de TCF. O estudo também avaliou a recuperação fisiológica dos peixes 28 horas após a exposição. Os resultados mostraram níveis elevados de cortisol no plasma e uma regulação negativa da atividade da glutationa-S-transferase (GST) no tecido hepático entre os peixes expostos ao TCF em comparação com os controles. As alterações no leucograma dos peixes tratados indicam uma maior vulnerabilidade à doença. Essas descobertas sugerem que um período de recuperação de 28 horas é insuficiente para restaurar a saúde fisiológica dos juvenis de tilápia do Nilo após a exposição ao TCF. O estudo destaca a importância de adotar as melhores práticas de gerenciamento (BMPs) ao aplicar tratamentos com TCF na aquicultura e ressalta a necessidade de mais pesquisas sobre os impactos fisiológicos de curto e longo prazo dos tratamentos químicos em peixes.

Palavras-chave: Glicose no Sangue. Cortisol Plasmático. Estresse Oxidativo. Tratamento de Ectoparasitas. Resposta Fisiológica. *Oreochromis niloticus*.

RESUMEN

El triclorfón (TCF) es un parasiticida organofosforado utilizado habitualmente en acuicultura para tratar ectoparásitos. Sin embargo, los estudios realizados en varias especies de peces han informado de efectos adversos incluso a dosis subletales. Este estudio evaluó las respuestas hematológicas y bioquímicas de juveniles de tilapia del Nilo (Oreochromis niloticus) tras una exposición de una hora a un baño de 0,5 mg L^{-1} de un producto comercial a base de TCF. También se evaluó la recuperación fisiológica de los peces 28 horas después de la exposición. Los resultados mostraron niveles elevados de cortisol en plasma y una regulación a la baja de la actividad de la glutatión-S-transferasa (GST) en el tejido hepático entre los peces expuestos al TCF en comparación con los controles. Las alteraciones en el leucograma de los peces tratados indican una mayor vulnerabilidad a la enfermedad. Estos hallazgos sugieren que un periodo de recuperación de 28 horas es insuficiente para restaurar la salud fisiológica de los juveniles de tilapia del Nilo tras la exposición a TCF. El estudio pone de relieve la importancia de adoptar las mejores prácticas de gestión (BMP) al aplicar tratamientos con TCF en acuicultura y subraya la necesidad de seguir investigando sobre los impactos fisiológicos a corto y largo plazo de los tratamientos químicos en los peces.

Palabras clave: Glucosa en Sangre. Cortisol Plasmático. Estrés Oxidativo. Tratamiento Ectoparasitario. Respuesta Fisiológica. *Oreochromis niloticus*.

1 INTRODUCTION

Fish culture production in Brazil reached 968,745 tons in 2024, representing an increase of 9.21% over the previous year. Nile tilapia *(Oreochromis niloticus)* is the most cultivated species in Brazil contributing with 68.36% of the total with 662,230 tons. Over the last ten years, tilapia production in Brazil jumped from 285.000 tons to 579.000 tons, an increase of 103%. Tilapia culture is carried out in all regions of the national territory with exception of Amazonas, Rondônia and Roraima States (Brazilian Fish Farming Association - Peixe BR, 2025). Brazil stands out as the world's fourth largest tilapia producer, only behind China (1.93 million tons), Indonesia (1.35 million tons) and Egypt (900 thousand tons) (FAO, 2020).

Trichlorfon (TCF; dimethyl 2, 2,2 – trichloro – 1 – hydroxyethyl phosphate) is an insecticide of organophosphates class present as active principle in some products used by agricultural sector (IPCS, 1992; ANVISA, 2009). In aquaculture, TCF has stood out for its effectiveness in eliminating undesirable ectoparasites in fish culture units, representing an alternative to reduce the economic losses

associated with epidemics caused by these organisms (Heo and Shin, 2009; Burridge *et al*., 2010; Coelho *et al*., 2011).

Often, the chemical treatment with TCF of infected fish is done by the fish farmer himself and according to the technical data sheets of commercial products, which normally recommend applying an immersion bath for one hour. The number of baths can vary between two and four in the interval of 48h depending on the degree of parasites infestation and fish species. The dosage of application of TCF can vary from 0.1 to 1.0 mg L^{-1} (Xu *et al*., 2012). The main parasites controlled by this pesticide are *Lernae sp., Argulus, Ergasilus sp., Lernea sp., Dactylogyrus sp. and Trichodina sp.* (Pavanelli *et al*., 2002; Heo and Shin, 2009; Burridge *et al*., 2010; Coelho *et al*., 2011).

Despite the efficiency of using TCF to control parasites, some studies have already demonstrated its toxic effects in sublethal concentrations for several freshwater fish species commercially produced. Some of the side effects already recorded for fish exposed TCF were, for example, leukopenia, lymphopenia and reduced acetylcholinesterase (AChE) activity for common carp (*Cyprinus carpio*) exposed to concentrations of 0.25 and 0.5 mg L^{-1} (Chandrasekara and Pathiratne, 2005); induction of oxidative stress in Nile tilapia (*Oreochromis niloticus*) exposed to a concentration of 0.5 mg L^{-1} (Thomaz *et al*., 2009); apoptosis, cell membrane rupture, collapse of the cytoskeleton and loss of cytoplasm in hepatocyte culture cells in cross carp (*Carassius auratus gibelio*) when exposed to concentrations of 0.01, 0.1 and 1.0 mg L^{-1}; reduced AChE activity and altered metabolism in alkaline phosphatase (ALP) and acid phosphatase (ACP) after exposure of Pacu (*Piaractus mesopotamicus*) for 96h at 8.4 µg L^{-1}.

TCF is classified by ANVISA (National Health Surveillance Agency) as a chemical with a high degree of toxicity, what means that animals that survive its chemical intoxication may still have several sequels. Thus, due to the toxic potential of TCF it is essential to monitor the health status of fish after chemical exposure to TCF in order to assess, whether the animal is sufficiently recovered.

The monitoring and evaluation of hematological and biochemical parameters are used as a diagnostic tool for toxicology and aquatic biomonitoring (Satake *et al*., 2009; Tavares-Dias and Mariano, 2015). Consequently, after any toxicological impacts caused by a chemical stressor the blood parameters are

often measured to evaluate fish physiology and to estimate the potential of disease occurrence (Cardoso *et al*., 2020; Ferri *et al*., 2020, Sampaio *et al*., 2012). In addition, the antioxidant defense system under controlled environmental conditions can provide a baseline for assessing toxic effects of pesticides on fish, as these enzymes play an important role in neutralizing xenobiotics and free radicals (ROS), resulting from chemical biotransformation processes in the liver tissue. However, there is still a great lack of information available about the use of biological parameters or biomarkers in assessing the clinical status of fish during the recovery period after exposure to TCF.

In this sense, the aim of this study was to assess the effects of an immersion bath of TCF for one hour at a concentration of 0.5 mg L^{-1} on Nile tilapia juveniles health , and also if the 28h period after exposure is enough for the physiological recovery of fish.

2 MATERIAL AND METHODS

2.1 FISH MAINTENANCE

The experiments were carried out with 90 healthy juveniles of Nile tilapia with approximately 30 grams of average weight obtained from a commercial fish farm located at Mogi Mirim, SP.

Fish acclimatization was done during a period of three weeks in a modular experimental aquaculture system composed of four 300 L aquariums with water recirculation (4 L min^{-1} per aquarium) through a 2,000 L biological filter consisting of expanded clay and gravel substrate. Water temperature of all aquariums was maintained by a heater with a digital thermostat connected to an electrical panel, and aeration was done with a 2.0 hp radial blower for air distribution in each aquarium.

During acclimatization a commercial feed for tilapia from Guabi® with 4-5 mm, 10% humidity, 32% crude protein (PB), 6.5% fat was used to fed the fish *ad libitum* two times a day (8 a.m., 4.30 p.m.).

During the experimental period the temperature (° C), dissolved oxygen (mg L^{-1}) and pH (pH units) were measured daily with a HORIBA multiparameter probe,

model U-50, Minami-ku, Kyoto, Japan (Table 1). The concentration of total ammonia nitrogen (mg L^{-1}) was determinate with a digital spectrophotometer HACH, model DR 2200, Loveland, CO, USA.

2.2 EXPERIMENTAL PROTOCOL

After the three-week acclimatization period, 72 fish were anesthetized with benzocaine (65 mg L^{-1}) for biometrics check, and then randomly distributed in 6 aquariums of 150 L with controlled temperature and continuous aeration, but without water recirculation. The experimental design was completely randomized in 2 x 3 factorial scheme: (TC) control with fish not exposed to TCF, and (TCF) with fish exposed to TCF, and recovery times of 3, 6 and 28 h after exposure. For blood sampling 12 fish were collected from each treatment.

Fish exposed to TCF were submitted to an immersion bath of 0.5 mg L^{-1} of trichlorfon (Masoten®) for 1 h. The dosage of TCF used in this study was the same recommended for conventional treatments for parasitic control (Pavanelli *et al*., 2002; Chandrasekara and Pathiratne, 2005). After 1 h period of exposure to TCF immersion bath the animals were transferred to 150 L aquariums filled with clean water, and monitored for 28 hours, called recovery period. During the recovery period, biological material from all the fish were collected for both treatments after 3h, 6h and 28h to evaluate the physiological and enzymatic responses.

2.3 HEMATOLOGICAL ANALYSIS, BLOOD GLUCOSE AND CORTISOL

Determinations of hemoglobin (Hb), erythrocyte (RBC), and blood glucose concentrations were made with blood samples collected from 4 fish per aquarium by a caudal puncture using a syringe bathed with EDTA (3%).

Hemoglobin was determined according to Collier (1944) with a dilution of 10 µl of blood in 2.5 ml of Drabkin's reagent. After homogenization the samples remained at rest for 20 minutes. The absorbance readings were done with a spectrophotometer (Model Nova 1600 UV) with a wavelength of 540 nm. The erythrocyte count was carried on by diluting 10 µl of whole blood in 2 ml solution using the method described by Natt and Herrick (1952). The erythrocyte count was

done in a Neubauer camera using an optical microscope (Leica DM750 w) with a 40 x magnification.

Differential and total leukocyte and thrombocyte counts were done according to Hrubec and Smith (1998). A Leica microscope, model DM 2500, was used with a 100 times magnification oil immersion objective. The blood extension slides were made and stained with the May-Grunwald-Giemsa-Wright dye.

Blood glucose measurements were done using whole blood with a reactive tape and glucometer (Roche, AccuChek® Performa). Cortisol concentrations were done using blood plasma by immunoenzymatic assay with a commercial kit (DRG International, Inc, USA, Cortisol ELISA - EIA - 1887).

2.4 ENZYMATIC ANALYZES OF HEPATIC OXIDATIVE STRESS

All the fish were euthanized after blood collection by exposure to an overdose of benzocaine 100 mg L^{-1}. Their livers were collected and washed with saline solution (0.9% NaCl), dried with filter paper, identified and stored at -80 ° C for further biochemical analysis. To evaluate the lipid peroxidation (LPO) and enzymes activity catalase (CAT) and glutathione S-transferase (GST) the fish tissues sampled were weighed and centrifuged at 12,000 rpm in phosphate buffer solution for 30 minutes. For centrifugation a refrigerated Hermle-Z323K centrifuge (Hermle Labortechnik, Germany) was used and the supernatant was used as an enzyme source.

The liver protein was determined using the method described by Bradford (1976) and adapted by Kruger (1994) for a Dynex MRXTC 250 micro plate reader. LPO was quantified using the FOX method (ferrous oxidation-xylenol orange) described by Jiang *et al*., 1992. CAT activity was determined according to Aebi *et al*. (1974) for the continuous evaluation of hydrogen peroxide ($H2O_2$) concentration decrease at λ = 240 nm. GST activity was measured according to Habig *et al*. (1974), using 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate. Microplate readings were done using a Tecan-SNR reader (Sunrise-Basic Tecan, NS 1105003419 Groding, Austria).

2.5 STATISTICAL ANALYSIS

All data were submitted to Shapiro-Wilk normality. To assess possible interactions between factors the means were subjected to an analysis of variance (two-way ANOVA). The means were compared using the Tukey test with a p-value <0.05 to estimate the level of significance. For unbalanced data ANOVA was used with Generalized Linear Model (GLM, GLIMMIX), and means were compared using Tukey test with a p-value <0.05 to estimate the level of significance. The SAS® program was used to do all the statistical analyzes (University edition, Cary, North Carolina).

3 ETHICAL STATEMENT

All procedures involving the use of animals during this study followed the Ethical Principles for Animal Research approved by the Ethics Committee for Animal Experimentation (CEUA) of Embrapa Environment (protocol nº 0082017) - Jaguariúna, SP, Brazil.

4 RESULTS

The exposure of Nile tilapia juveniles to 0.5 mg L^{-1} TCF immersion bath did not cause fish mortality, regardless of recovery time. It was also not observed any differences between the hemoglobin concentration and the number of erythrocytes between treatments (Fig. 1 A,B).

There was no interaction between exposure factors and recovery time for cortisol ($p = 0.144$) and glucose ($p = 0.05$) determined by blood plasma. Both treatments showed the same response pattern during the recovery period (Fig. 2 A, B). However, it was found that fish exposed to TCF showed higher cortisol values ($p < 0.05$) between recovery times when compared to control. For glycemia, a significant difference ($p < 0.05$) between treatments was observed only at 3h.

The number of leukocyte cells for control and TCF treatments were similar for 3h ($p = 0.293$) recovery period. But, there was a significant difference between 6h ($p < 0.0148$) and 28 h ($p < 0.007$). This indicates that the response pattern of

these cells is influenced by the interaction of exposure condition and fish recovery time. Regarding thrombocytes, it was observed that there is an effect of time over fish exposure conditions for the 3h period ($p < 0.001$), however, this effect was not verified for the periods of 6h ($p = 0.148$) and 28h ($p = 0.346$) (Fig. 3B). In addition, there was an interaction between the exposure condition and recovery time for 3h ($p < 0.02$) and 28h ($p < 0.042$) related to monocytes, and for the 6h period the results of control and TCF treatments were similar. For neutrophils, it is unlikely that there is an interaction between exposure and time factors, because the number of these cells were similar for control and TCF treatment (Fig. 3C).

Catalase activity and LPO marker were not influenced by interaction between the exposure condition and the recovery time for both treatments ($p = 0.05$) (Fig. 4 A, B). The GST activity showed a significant difference between the periods of 3h ($p < 0.03$) and 28h ($p < 0.014$) due to the exposure condition and fish recovery time (Fig. 4 C). The GST activity was lower for fish exposed to TCF for 3h and 28h period when compared to control, and for the 6h period there were no significant difference ($p = 0.369$) between treatments.

5 DISCUSSION

The exposure of Nile tilapia juveniles to 0.5 mg L^{-1} TCF to an immersion bath for 1h did not cause mortality during the exposure period or 28h after recovery in water free of TCF. Therefore, based on the results obtained it is possible to affirm that the concentration of TCF used is safe to eliminate ectoparasites of Nile tilapia juveniles, and with the advantage of not causing any increases of mortality. Although, was not observed any increases in fish mortality, it does not mean that fish exposed to TCF have not suffered stress and physiological changes that might compromised their welfare and health status.

In this sense, this study has contributed to demonstrate that fish exposed to TCF presented alterations in the leukogram despite the absence of any alterations in the erythrogram. However, according to Banaee (2013), the effects of different pesticides in the erythropoietic tissue reduces the hematological response and may indicate an aggravation of fish health, predisposing them to develop anemia and even leading to death.

Previous studies that evaluated hematological parameters during the recovery period of fish after exposure to TCF have demonstrated the need to use a recovery period longer than 28h. For example, Venturini *et al*., (2015) found that the exposure of pacu *(Piaractus mesopotamicus*) to TCF at the concentration of 8.4 µg L^{-1} for 96 h did not alter the hematological profile of fish after 7 days of recovery. Chandrasekara & Asoka Pathiratne (2005) observed that the hematocrit of Common Carp (*Cyprinus carpio L.)* exposed for 1h at 0.25 and 0.5 mg L^{-1} of TCF does not change after 7 days of recovery.

In addition, it was possible to verify that cortisol concentrations for all recovery times have showed higher values for fish exposed to TCF when compared to control (Fig.2 A). The measurement of plasma cortisol has been an excellent tool used to assess the acute effects of a particular stressor on fish (Aerts *et al*., 2015; Sadoul and Geffroy, 2019). One of the functions of cortisol is to act as glucocorticoids, stimulating glycogenolysis and hepatic gluconeogenesis what may increase blood glucose levels (Mommsen *et al*., 1999). This fact may explain why blood glucose values were higher for fish exposed to TCF at 3h and 6h period compared to control.

According to Balasch and Tort (2019) in fish, the cortisol regulates neuroimmunoendocrine circuitries elicits stress-induced immunosuppression and contributes to allostatic imbalances.

The same effect was observed in the amount of leukocytes for the treatment with TCF (Fig. 3A). There was a reduction of thrombocytes (Fig. 3B) and monocytes values (Fig. 3D) during recovery period for fish exposed to TCF. According to Svoboda *et al*. (2001), it can be expected that acute exposure to organophosphate pesticides predisposes fish to diseases in reason of less leukocytes. The reduced number of these cells in fish exposed to TCF results in lower resistance to pathogens (Kaattari & Piganelli, 1996). Thus, it is necessary to be careful in relation to TCF use in animals with a high rate of parasitic infestation, since the significant reduction of monocytes and thrombocytes in treated fish in relation to untreated suggests that fish are more susceptible to develop diseases, because their capacity of defense against pathogens is smaller.

In addition, a healthy organism has a balance between the production of reactive oxygen species (ROS) and the antioxidant system to protect cells

(Slaninova *et al*., 2009). Therefore, oxidative stress occurs when this balance is broken by the depletion of antioxidants or excessive accumulation of ROS, or both (Sampaio *et al*., 2016). However, the formation of ROSs such as superoxide anion (O_2^-) hydrogen peroxide (H_2O_2) and hydroxyl radical (OH) can be accelerated as a consequence of stress caused by xenobiotics (Scandalios, 2005), producing enzymatic inactivation, lipid peroxidation and damage to DNA, resulting in oxidative stress (Giordano *et al*., 2009; Kurutas, 2015; Nita and Grzybowski, 2016).

Catalase enzymes (CAT) and glutathione-S-transferase (GST) are important constituents of antioxidant enzymes group involved in the protection process against harmful effects of xenobiotics and free radicals in the animals' organisms. CAT belongs to the first line of defense against oxidative stress and occurs in peroxisomes in an oxy-reduction reaction where H_2O_2 is decomposed into O_2 and H_2O (Coelho *et al*., 2011). Glutathione-S-transferase (GST) belongs to a family of multifunctional and, mainly, cytotoxic enzymes that act in the biotransformation and detoxification of xenobiotics, turning the products less toxic and more soluble in water and thus facilitating excretion (Wheatley *et al*., 1994; Rao, 2006; Huber *et al*., 2008).

CAT activity was not affected by fish exposure to TCF. Nevertheless, the short period of fish exposure to pesticide was enough to suppress GST activity during the recovery period. According to Xu *et al* (2012), under TCF stress the critical balance between free radicals and antioxidant substances is interrupted, and the defense mechanism of antioxidants can be reduced in fish. In this case, the reduction of GST can compromise the antioxidant defense system of the animals, because this enzyme catalyzes the conjugation of xenobiotics (or phase I metabolites) turning them into an easily excreted form in function of their high solubility in water (Huber *et al*., 2008; Clasen *et al*., 2018).

6 CONCLUSION

An immersion bath with TCF at a concentration of 0.5 mg L^{-1} for 1 hour did not result in fish mortality or significantly impact the erythrocyte response. However, some physiological alterations were observed, including changes in the leukogram, elevated plasma cortisol levels, and increased activity of the

antioxidant enzyme GST in the liver tissue of Nile tilapia juveniles. The 28-hour observation period was insufficient for the tilapia to fully recover physiologically. These findings underscore the importance of implementing best management practices (BMPs) when using TCF baths in fish treatment. Additionally, they highlight the need for further research into the physiological effects of chemical treatments on fish, both during and after exposure.

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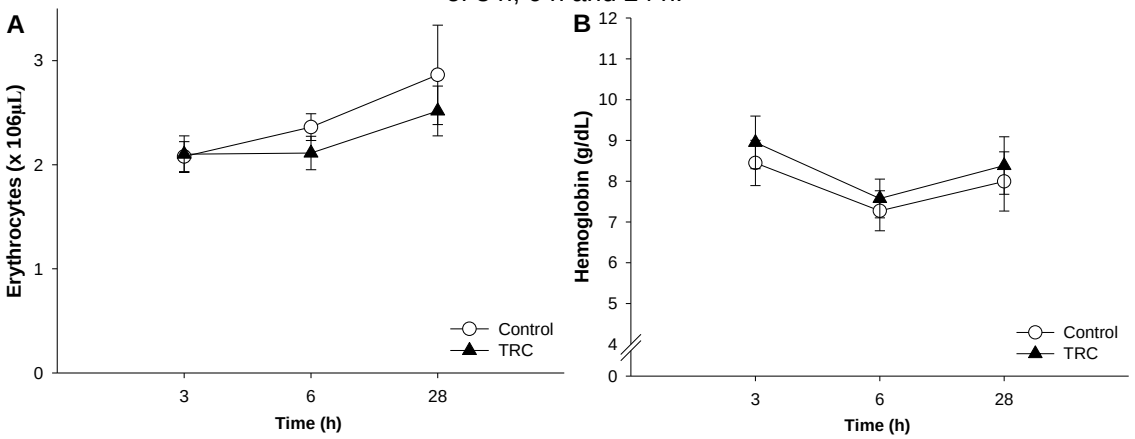
ANNEXES

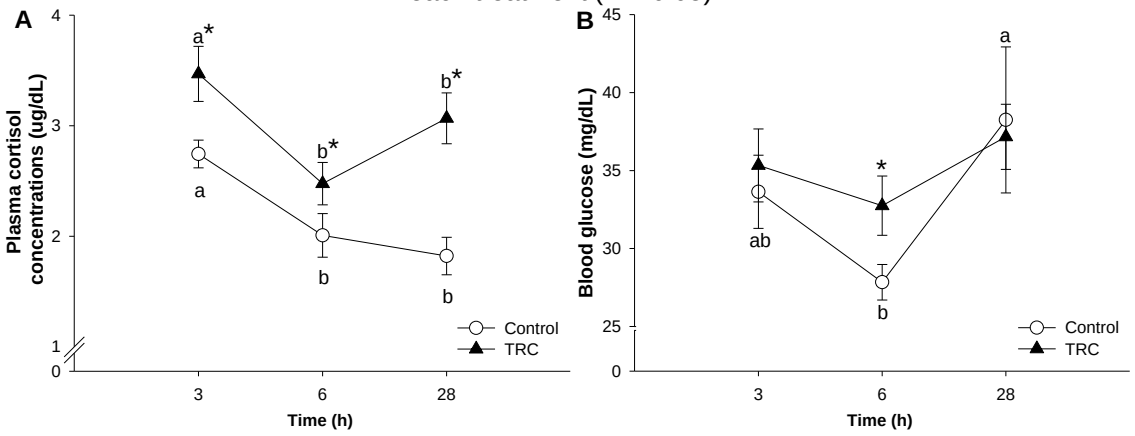
FIGURES

Figure 1. Erythrocyte number (A), hemoglobin concentrations (B) in Nile tilapia juveniles (*Oreochromis niloticus*) for control and Trichlorfon (TCF) treatments in function of recovery times of 3 h, 6 h and 24 h.



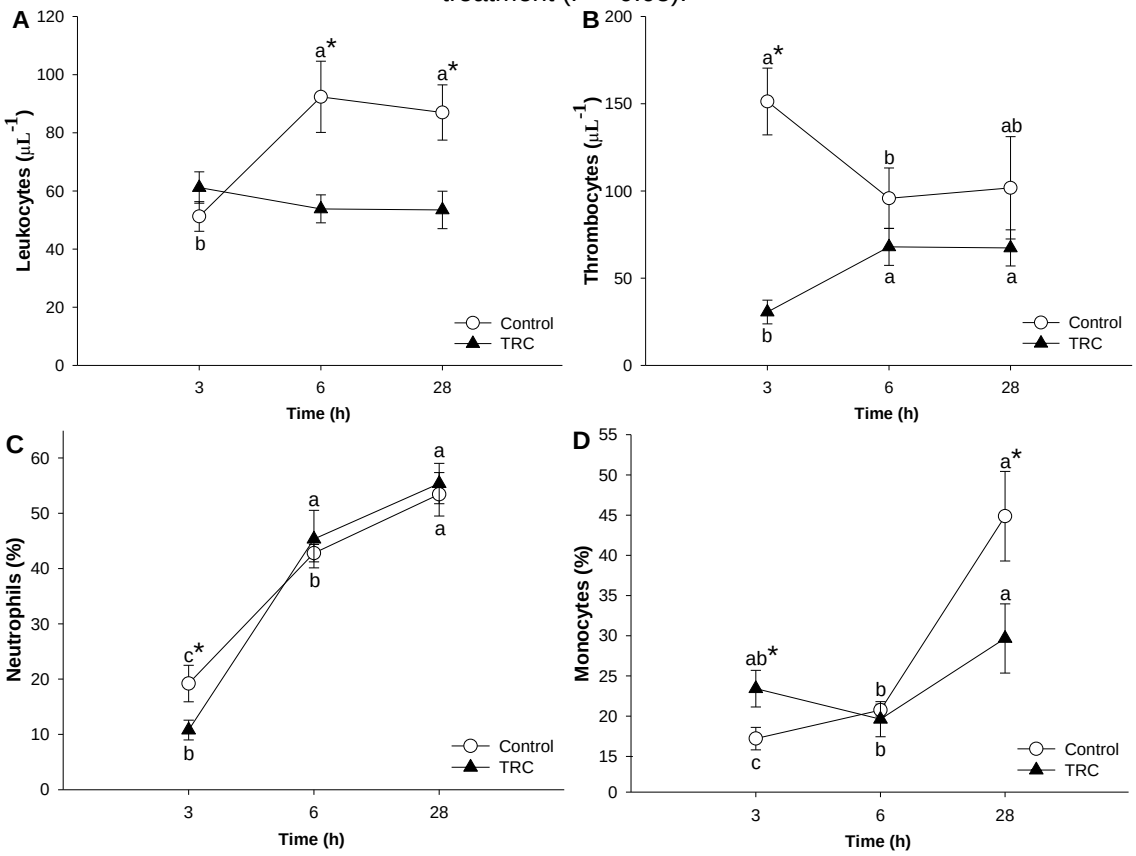
Sources: Authors.

Figure 2. Plasma cortisol (A) and Blood glucose (B) in Nile tilapia juveniles (*Oreochromis niloticus*) for control and Trichlorfon (TCF) treatments in function of recovery times of 3 h, 6 h and 24 h. Small letters compare the treatments in each phase and symbol (*) compare the phases in each treatment ($P < 0.05$).



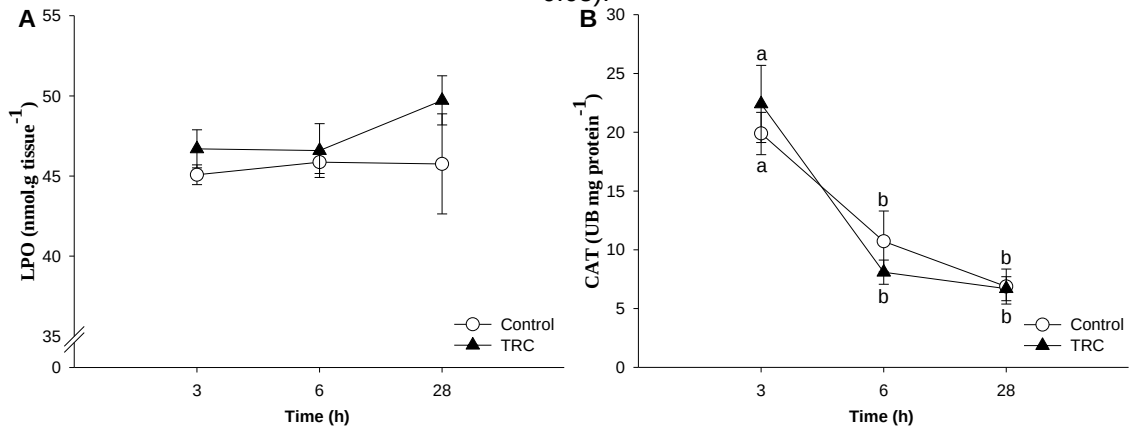
Sources: Authors.

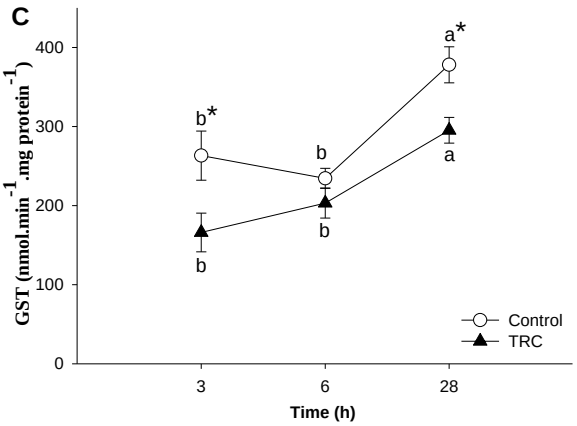
Figure 3. Leukocytes (A), thrombocytes (B), neutrophils (C) and monocytes (D) counting for Nile tilapia juveniles (*Oreochromis niloticus*) for control and Trichlorfon (TCF) treatments in function of recovering times of 3h, 6h and 24h. Mean values of leukocytes and thrombocytes x 10^3. Small letters compare the treatments in each phase and symbol (*) compare the phases in each treatment ($P < 0.05$).



Sources: Authors.

Figure 4. Levels of lipid peroxidation - LPO (A), catalase activity - CAT (B) and glutathione S-transferase activity - GST (C) in Nile tilapia juveniles (*Oreochromis niloticus*) for control and Trichlorfon (TCF) treatments in function of recovering times of 3h, 6h and 24h. Small letters compare the treatments in each phase and symbol (*) compare the phases in each treatment ($P < 0.05$).





Sources: Authors.