# REVERSIBLE HEMATOLOGICAL AND BIOCHEMICAL TOXIC EFFECTS OF AZOXYSTROBIN AND DIFENCONAZOLE MIXTURE EXPOSURES IN *OREOCHROMIS NILOTICUS* UNDER INCREASED HEAT STRESS.

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## ABSTRACT

As one of the fastest growing sectors, aquaculture contributes to global food security. While pesticides play an important role in improving land productivity and food quality for a growing world population, especially in developing countries, their presence in agricultural drainage is a serious risk to all components of aquatic ecosystems. Additionally, there is a high risk that environmental contaminants will enter the human food chain and threaten public health. Therefore, there is an increasing need to find reliable approaches to rapidly determine the conditions of aquaculture ecosystems exposed to agricultural runoff and assess fish health and welfare. In addition to increases in pollutant inputs, increasing surface water temperatures due to climate change are affecting the physical condition of the aquatic system and coastal systems worldwide. Difenoconazole is a typical triazole fungicide used for the control of fungal disease in vegetables, grains and other field crops. Azoxystrobin is the most widely used fungicide worldwide. Although temperature is an important factor in toxicity, there are not enough studies to elucidate the metabolism related to the effects of temperature on pesticide toxicity. Therefore, the effects of pesticide exposures to azoxystrobin and difenconazole mixture (0.5ppb, 5 ppb, 10 ppb) under heat stress (22ºC and 30ºC) on defense mechanisms in *Oreochromis niloticus* blood tissue and the reversibility of these effects was examined in order to evaluate the health status of aquatic creatures and determine water quality. In this study, it was determined that hematological, enzymatic and metabolic responses occurred in *O. niloticus* against exposure to the pesticide mixture azoxystrobin and difenconazole.

**Keywords:** P*esticides, Fish, Enzyme, Blood, Heat stress*

## INTRODUCTION

Climate change is a global concern in response to anthropogenic activities. Due to climate change, extreme weather events are experienced with increasing frequency and

intensity around the world (Shahjahann et al., 2022). While global warming may have horizontal negative perturbations by expanding temperatures (synergistic effect) of other anthropogenic stressors (Verberk et al. 2016), warming is allowed to track antagonistically with other stress characteristics, which appears to be a pattern of pressure in freshwater ecosystems (Jackson et al. 2016). In addition to global warming, other anthropogenic stressors that threaten freshwater biodiversity include pesticides. Environmental factors such as temperature caused by natural (seasonal changes) or anthropogenic (thermal pollution) factors affect pesticide toxicity in aquatic organisms, and temperature changes cause changes in homeostasis and physiological parameters in fish (Gandar et al., 2017). Although temperature is an important factor in toxicity, there are not enough studies to elucidate the metabolism regarding the effects of temperature on pesticide toxicity. It has been reported that there is an increase in the toxicity of pesticides at high temperatures. It has been reported that fish are sensitive to environmental pollutants and respond quickly to contamination (Fiorino et al., 2018). Therefore, fish are considered a good model to evaluate the toxicity of water-based chemicals (Sehonova et al., 2017).

Fish are sensitive organisms to environmental changes (Rudneva et al., 2012; Shahjahan et al., 2017). Therefore, it can be measured the physiological responses of fish to the environmental stressors. Using more comprehensive testing methods to understand the effects of environmental conditions has the potential to improve our ability to unravel the possible causes and dynamics of perturbations and even detrimental confounding factors, allowing the use of more appropriate diagnostic biomarkers for fish health (Shahjahan et al., 2022). Because they have biochemical responses similar to those found in mammals, teleost fish, including tilapia, can be good indicators of contamination by a wide range of pollutants. Tilapia (*Oreochromis niloticus*) is a species of fish widely grown and consumed worldwide.

Difenoconazole provides protection against fungi in vegetables, grains and other field crops. Azoxystrobin is a typical triazole fungicide used to control the disease. Its concentration in agricultural runoff and surface water was found to be 1-50 μg/L.

However, except for a few studies on species and pesticides, the data collected on the effects of global warming on the toxicity of pesticides are not comprehensive enough for environmental risk assessments. Therefore, more studies are needed to better understand the interactions of global warming and pesticides.

In this present study, we wanted to determine the potential harmful effects of environmentally realistic concentrations of the Azoxystrobin/Difenconazole mixture, which is

widely used all over the world, and heat stress on biochemical and hematological biomarkers in *Oreochromis niloticus* under different temperature conditions.

## MATERIALS AND METHODS

Fish (*O. niloticus*) were obtained from the Cukurova University Fish Culture Pools. They were acclimated to the new conditions in glass aquariums containing 100 L of tap water (40x 40x100 cm) at least two weeks before the experiments.

Prior to the pesticide exposure, fish were gradually acclimatized during 15 days to the experimental temperatures of 22 and 30◦C. For that, the temperature was increased by 1◦C every 24 h until reaching the experimental temperature. Water was heated to the respective temperature with external heating units. Fish were kept 15 d to acclimate to each temperature regime. In the final 96 h (4 d) of the temperature exposure, fish were also simultaneously coexposed to azoxystrobin/Difenconazole mixture in concentrations of 5, and 50 µg/L. Pesticide concentrations were chosen from a range of reported measured concentrations during runoff events. Semi-static toxicity test was performed.

Following the adaptation period, fish were individually exposed to different temperatures alone ($22^0$C and $30^0$C) and combination of temperature and azoxystrobin/difenoconazole mixture commercial formulation (200g/L Azoxystrobin+125 g/L Difenoconazole, Quadris Maxx, Sygenta) at environmentally relevant concentrations of 5 µg/L and 50 µg/L at 22ºC and at the elevated water temperatures (30ºC) for acute eposure (96 hours). Each exposure concentration and control contained 6 fish which the length (40.38±2.20 cm) and weight (17.18±1.39 cm) were similar ($P>0.05$) among exposure groups. Aquaria media were changed every day to keep pesticide concentrations close to the nominal values and clean the exposure media. During adaptation and experiments, animals received fish feed purchased from Pinar Company (Izmir, Turkey). Feeding was done just 1 h before water renewal.

At the end of experiments, six fish per each experimental group (n=6) were sampled. The blood samples were collected via severing the caudal peduncle of fish. To avoid coagulation, EDTA (1.26 mg/0,6 ml) was used. Blood sample were centrifuged at 3000 rpm at 4ºC for 10 min and the plasma was removed and stored at -80ºC the biochemical analysis. The hematological parameters including hemoglobin (Hb), heamatocrit (Hct) and hematological indices like mean cell hemoglobin (MCH), mean cell volume (MCV) and mean cell hemoglobin concentration (MCHC) were determined by Sysmexpoc H100i. Anticoagulant was not added to the blood samples for serum biochemical analysis. The blood sample was kept on ice for an hour, followed by centrifugation (3000 rpm, for 10 min) for serum isolation. The samples were stored (-80 ºC) for further analysis of different biochemical

parameters. Biochemical parameters including glucose and trigliseride amount were determined by Roche Cobas Integra 800.

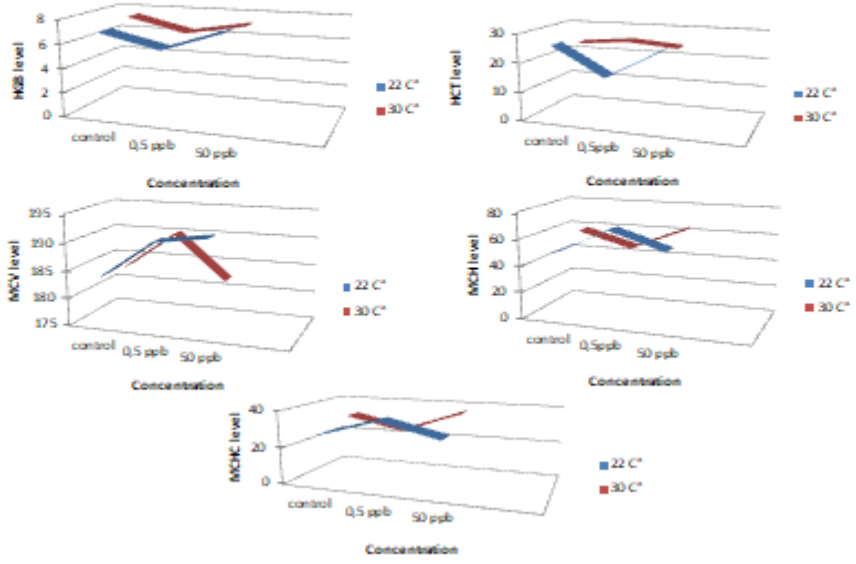
All data were analyzed using analysis of variance (ANOVA) with SPSS 22.0 software (version 22, SPSS Inc., Armonk, NY, USA) and are shown as means ± standard deviation (SD) at $p < 0.05$. If the variance homogeneity threshold could be met, the data were analyzed with SNK's test.

## RESULTS AND DISCUSSION

Blood assessment is an important technique to determine the physiological health of fish. Hematological indices such as hemoglobin (Hb), mean cell hemoglobin (MCH), mean cell volume (MCV) and mean cell hemoglobin concentration (MCHC), as well as biochemical parameters such as plasma glucose and triglyceride are widely used in the evaluation of toxic stress (Ismail et al., 2017). Enzyme activities, another category of sensitive indicators, have also been used to determine tissue damage in fish exposed to various groups of water pollutants (Saravanan et al., 2011).

Effects of various concentrations of Azoxystrobin/Difenconazole mixture and heat stress on hematological parameters of fish are shown in Figures 1-2.

**Figure 1.** Changes in hematological parameters of freshwater fish *O. niloticus* after exposure to sublethal concentrations of azoxystrobin/difenconazole mixtures and heat stress; HGB (Hemoglobin), HCT (Hematocrit) and hematological indices like mean cell hemoglobin (MCH), mean cell volume (MCV) and mean cell hemoglobin concentration (MCHC).




Pesticide exposure caused significant changes in Hct and Hb in *O. niloticus*. Jenkins et al. stated that the decrease in hematological parameters may be related to a decrease in the rate of erythrocyte production, destruction and/or anemia resulting from pesticide toxicity in fish (Jenkins et al., 2003). The pesticide caused a significant decrease in Hct, Hb, HCM and MCHC, indicating an anemic state. At the other hand, at low dose exposure, an increase

which may be attributed to the release of new red blood cells into the circulation in response to stress (Pereira et al., 2013).

**Figure 2.** Changes in hematological parameters of freshwater fish *O. niloticus* exposed to sublethal concentrations of azoxystrobin/difenconazole mixtures and heat stress after depuration period for 7 days; HGB (Hemoglobin), HCT (Hematocrit) and hematological indices like mean cell hemoglobin (MCH), mean cell volume (MCV) and mean cell hemoglobin concentration (MCHC).

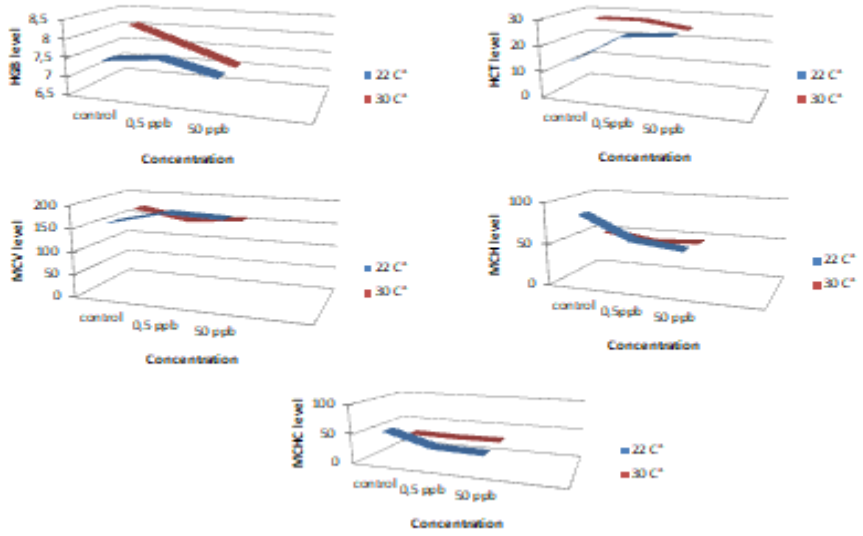
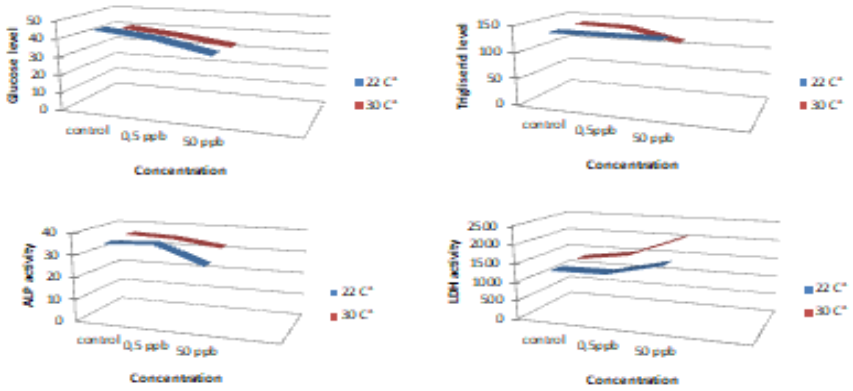
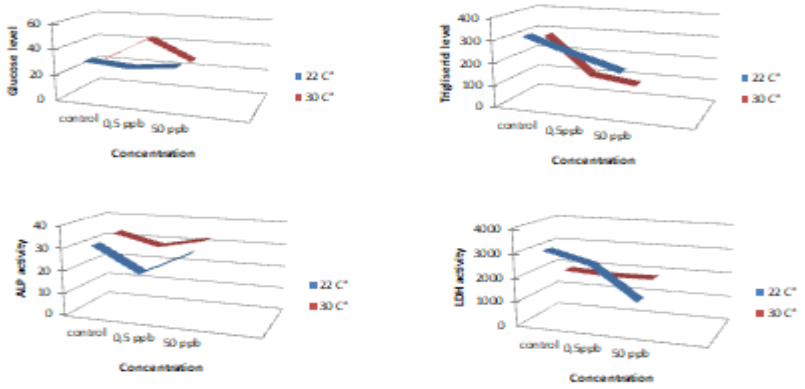



**Figure 3.** Changes in biochemical parameters of freshwater fish *O. niloticus* after exposure to sublethal concentrations of azoxystrobin/difenconazole mixtures and heat stress




Temperature also affects glucose and triglyceride levels in fish (Figure 3-4). It was found that glucose and triglyceride content increased during high temperature exposure, while serum glucose and triglyceride tended to decrease at some exposure concentrations (Islam et al., 2020). Alterations in metabolites in fish exposed to extreme temperatures can be used as an indicator of increasing energy demands to metabolically adapte.

**Figure 4.** Changes in biochemical parameters of freshwater fish *O. niloticus* exposed to sublethal concentrations of azoxystrobin/difenconazole mixtures and heat stress after depuration period for 7 days.




Differences in hematobiochemical parameters serve as indicators of physiological changes associated with environmental changes in response to environmental pollution. Hemoglobin (Hb), hematocrit (Hct), red blood cell (RBC) and some hematological parameters such as mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) contribute significantly to the environmental risk assesment's studies. Hematological changes are also used to evaluate the health of fish (Ismail et al., 2017). The results of hematological assessments in fish are affected by several factors, such as stress levels and duration. Moreover, blood biochemical parameters related to metabolism such as glucose (Glu), triglyceride (TG) can be viewed as important indicators of fish health and physiology. These parameters can be used to monitor fish physiology in response to varying diets, disease states, and environmental environments to monitor changes in fish health (Ashaf-Ud-Doulah et al., 2019). Plasma glucose is one of the stress indicators in fish; Low glucose levels indicate a state of high stress (Hossain et al., 2015). Hyperglycemia has been stated in various fish species exposed to a stressor through stimulation of glycogenolysis and gluconeogenesis in response to changes in temperature (Islam et al., 2020).

The induction of alkaline phosphatase and lactate dehydrogenase activities is a result of the anaerobic activity of tissues at the effect of stress conditions and, appear to be as reliable biomarker by which tissue damage caused by toxic substances can be recognized (Nemcsok and Benedeczky, 1990). Biochemical biomarkers are often evaluated as primary diagnostic tools when an organism is exposed to pollutants (Gholami-Seyedkolaei et al., 2013). Moreover, because biomarkers represent early warning of environmental contamination and thermal stress, they are useful for the development of public policies focused on finding

alternatives that can minimize the effects of pollutants on a specific population (Freitas et al., 2017).

## CONCLUSIONS

We showed that temperature has an imporant effect on the hematological and biochemical parameters of *O. niloticus* exposed to pesticides. Because the activities of ALP and LDH enzymes were significantly changed in the blood of pesticide exposed tilapia, the alterations of these parameters can be successfully used as a valuable biomarkers to determine health of fish and aquatic environment.

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