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Survival analysis and biochemical response of the juvenile Nile tilapia (*Oreochromis niloticus*) exposed to metalaxyl fungicides

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ABSTRACT

The study examined the biochemical parameters and behavioral changes of *Oreochromis niloticus* exposed to different concentrations of metalaxyl fungicide using a survival analysis model. Batches of ten *O. niloticus* were randomly distributed into a set of 18 rectangular plastic tanks (75×45×45cm) each filled with 30L of unchlorinated water. The range-finding tests for metalaxyl (400, 500, 600, 700, and 800 ml) were carried out for 24h, and the definitive test (500, 600, 700, 800, and 900 ml) for 96h. At the end of 96h exposure of *O. niloticus* to metalaxyl, blood samples (5mL) were collected through the vertebral caudal blood vessel into sample bottles for biochemical analysis. The acute toxicity 96h LC_{50} was calculated to be 7.41 ml, while mortality rate and death prediction were determined using survival analysis. There was a significant increase ($p>0.05$) in the activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), as well as the levels of protein, cholesterol, and creatinine. Abnormal behavior such as fin deformation, air gulping, loss of reflex, molting and erratic swimming was observed in the experimental fish. Survival analysis results revealed that the average survival hour at 50% of events was 72h. The survival rate declines with an increase in the concentration and the chance of survival gets worse between 700 ml and 900 ml concentrations. The survival of fishes is significantly influenced by the concentrations at different levels at a time in point. However, alterations were seen in biochemical parameters and behavioral responses clearly indicate that the occurrence of metalaxyl in freshwater bodies, even in minute concentrations, could cause lethal effects on the biochemical parameters of fish and may threaten their survivability in the natural environment. Therefore, control measures should be taken to prevent the possible contamination of the aquatic environment by metalaxyl fungicide.

INTRODUCTION

Fish are an important resource for humans worldwide, especially as food. They are hunted by anglers or farmed in ponds and cages in inland water bodies and in the ocean. Fish have had a role in humanity through the ages, serving as deities, religious symbols, and as the subjects of art, books, and movies. They have become an inseparable part of the existence of man.

In tropical and subtropical freshwater, Nile tilapia (*Oreochromis niloticus*) is rated high as the most important fish species and this form the basis of commercial fisheries in many African countries **(Mohammed and Uraguchi, 2013)**. The Nile tilapia is mostly an herbivore, but with omnivorous tendencies, especially when young. It is of high economic value due to its ability to tolerate various environmental conditions and accept both formulated and natural feeds **(Adeyemi *et al.*, 2009)**.

Agrochemicals have been extensively used to increase productivity in agriculture by controlling pests and diseases. One of such agrochemicals is fungicides which invariably end up in our waterways. The presence of agrochemicals in the aquatic medium affects the tolerance limit of aquatic fauna and flora and also expose the ecosystem to dangers **(Desai and Parikh, 2012)**. **Ayoola (2008)** reported that water pollution by fungicide is a serious problem to all aquatic fauna and flora and to a considerable extent even man. This agrochemical adversely affects the non-target organisms, especially fish which are one of the most widely distributed organisms in an aquatic environment and being susceptible to environmental contamination may reflect the extent of the biological effects of environmental pollution in waters **(Desai and Parikh, 2012)**. Accounts of field application of fungicide in developed countries revealed that less than 0.1% of fungicide applied to crops reach target pest, thus over 99% moves into an environment to pollute the land, water and air **(Pimental, 2005)**. Exposure to the low level of fungicides has attested to cause profound effects on non-target organisms. Fungicides may also find their way into the food chain and cause functional damage and affect and alter the performance of the organism **(Silva and Gammon, 2009)**. Many times, the residues of these chemicals sink to the bottom of the water body where they often exert effects on aquatic lives especially fish. Their injuriousness persistency and propensity to gather in the organism was a menace generated by the agrochemical that sink into the bottom of the aquatic medium **(Joseph and Raj, 2010).** Any water contaminated by fungicides can directly or indirectly lead to fish kills, affect general productivity of fish or increase concentrations of undesirable toxicants in the tissue of fresh water edible fish which can affect the health of human beings that consume these fishes **(Adedeji *et al*., 2009).**

Metalaxyl is a systemic benzenoid fungicide of amide group. It is commonly used as foliar spray mixtures for tropical and subtropical crops, it is also used for soil treatment to control soil-borne pathogens, and to treat seed for prevention of downy mildews, fungal diseases on fruits, cotton, soybeans, peanuts, ornamentals, and grasses **(Ding *et al.*, 2012)**.

Contamination of natural waters by fungicides has become a matter of concern in recent years due to their large-scale use in home, farm, and industries. Many research works have been carried out on the effects of fungicide using *Oreochromis niloticus*. However, no much work has been done on the effect of metalaxyl fungicide on fish especially using *Oreochromis niloticus.* It is therefore important to determine the level at which metalaxyl fungicide will become lethal to *Oreochromis niloticus*, which is the first gap to be filled. Again, the second gap to be filled is to update the body of knowledge and address the dearth of information by employing survival analysis with emphasis to Kaplan-Meier technique and cox proportional hazards to achieve the objectives of this study. This will not only change the narrative from the old method of counting survival model, but statistically estimate the mortality rate and also predict the death of the subjects at the time in point. The advantages of survival analysis are: (i) it estimates the population survival curves from the sample obtained from the experiment, (ii) the curve is always estimated by computing the proportion of surviving at each time period if every subject is followed till death in the experiment, and (iii) the features of Kaplan-Meier curves provide wide applicability of the model as it presents a pictorial representation of the raw data, the failure (death) times and the censoring times with a mathematical estimate of the given survival model. Therefore, it is against this background that the study aimed at assessing changes in serum biochemical indices of *Oreochromis niloticus* juveniles exposed to different concentrations of metalaxyl fungicide.

MATERIALS AND METHODS

Experimental Site

The experiment was carried out in the Central Laboratory of Fisheries and Aquaculture Department, Faculty of Agriculture, Adekunle Ajasin University Akungba-Akoko, Ondo State; using Plastic tanks of 50 liters capacity (75 cm × 40 cm × 40 cm). All the 50 litters plastic tank was supplied with 30 liters of water that is unchlorinated.

Fish Collection and Experiment

Two hundred and fifty healthy *O. niloticus* juveniles were purchased from the Fisheries and Aquaculture Technology Research Farm at The Federal University of Technology Akure, Nigeria. The fish were later transported to the Central Laboratory of Fisheries and Aquaculture Department, Faculty of Agriculture, Adekunle Ajasin University Akungba-Akoko, Ondo State in a plastic container filled with pond water and oxygen. All the fish used for the research were confirmed to be healthy before the setting up of the experiment. Samples collected were acclimatized under laboratory conditions for two weeks prior to the commencement of the experiment.

Preparation of Toxicant

A stock solution of 50 g of metalaxyl was prepared by dissolving 3.3 g of metalaxyl in 1.0 liter of distilled water and carefully stirred with a glass rod for about 2-3 minutes. Treatments of different concentrations was taken out of the stock and added to each tank before introduction of the test animals.

Water Quality Parameter Measurements

The quality of the experimental water was measured before, during, and after the experiment. pH, conductivity, dissolved oxygen (DO), temperature, and total dissolved solids (TDS) was measured using HANNA Multi-Parameters Instrument (HI5521-02).

Toxicity Test

A range-finding test was carried out to determine the effective concentrations of metalaxyl to be used. Based on the results of the range finding test, a 96-hour defined test was carried out according to the static bioassay procedure described by **Parish (1985).** A batch of ten juvenile *O. niloticus* of 11.3-17.5 cm length and weighing 10.5-32.3g in a set of 18 rectangular plastic tanks (75×45×45 cm) each filled with 30 L of unchlorinated water. Six test solutions of metalaxyl 0.0, 500, 600, 700, 800, and 900 ml previously determined from the range finding test were introduced directly into the plastic tank in a single dose. The test fish were not fed during the 96h test. Change in behavior of the fish to the toxicant and mortality in each tank was monitored and recorded every 15 min for the first one hour, after which records were taken every hour for the next 4h, once every 4h for the next 24h and once every 24h for the rest 96h. Dead fish were immediately removed to avoid a consequent fouling of the test media.

Blood Collection

After 96h of exposure, fish were removed from each tank and blood of about 1-3 ml $fish^{-1}$ was collected for analysis. Collection of blood samples was through the vertebral caudal blood vessel, using disposable hypodermic syringe and needle to collect it. Collected blood samples were then emptied into 10 ml sample bottles.

Biochemical Parameters Analysis

Blood plasma, which was obtained from heparinized blood samples by centrifugation (4ºC, 800 ×*g*, 10 min) was used for the determination of selected biochemical indices. Biochemical indices analyzed in blood plasma includes; total protein, creatinine, cholesterol, alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Aspartate aminotransferase (AST) and alkaline phosphatase (ALT) activities were determined using UV test technique **(Bergmeyer *et al.*, 1986).** Measurement was based on the detection of the increase in absorbance due to the increase in the formation of p-nitrophenol in the reaction. Cholesterol was determined by Liebermann's method, an endpoint-coupled reaction in which cholesterol esters are hydrolyzed into free cholesterol and fatty acids by a microbial cholesterol esterase. The total plasma protein was determined by the method of **Cannon *et al.* (1972).**

Statistical Analysis

Data obtained for biochemical parameters of *O. niloticus* were subjected to Analysis of Variance (ANOVA) while the mean and standard error estimates were also generated. Mean generated was separated and compared by Duncan's New Multiple Range Test (DNMRT). The median lethal concentration, at selected period of exposure and an associated 95% confidence interval for each replicate toxicity test, was subjected to logit and probit analysis using minitab version 14. Again, the survival analysis also known as hazard model was employed for the study. The research defined "survival time" as the number of hours from the time the fish is put into the medium till one of the events "death" occurred. The study modeled treatment as the predictor variable while the time variable remains as hours. The Kaplan-Meier survival curves were used to determine the mortality rate while a cox proportional hazards model was used to determine the influence of the treatment on the mortality at 5% probability level.

The Kaplan-Meier plot depicts the survival probability which is the cumulative probability of a subject remaining alive at any time after the baseline. The equation for survival probability for hour, I is stated as:

$$S_{ti} = \frac{L_i - D_i}{L_i}$$

Where L_i is the number alive the period before i^{th}; D_i is the number died on period i^{th}. The cumulative survival probability to period (hour) i^{th}, signified $S_{(ti)}$.

We also perform the log-rank test for the differences in the survival function between the treatments as in the studies like **Allison (2010), Fagbamigbe *et al.* (2018), Falmata *et al*. (2019).**

The mathematical representation for the 5 treatments plus control is given as:

$$X^2_{log-rank} = \frac{(O_1 - E_1)}{E_1} + \cdots + \frac{(O_6 - E_6)}{E_6}$$

Where O is the observed frequency in each treatment. E is the expected frequency for the treatment and it is computed as:

$$E_i = \sum_{i=1}^{k} \frac{L_i D_i}{L_i}$$

Furthermore, a Cox regression was estimated to compare the hazards of the treatment groups as ratios. The model is stated as:

$$\gamma_{ti}(t) = \gamma_0(t) exp(\omega_1 x_1 + \omega_2 \omega_2 + \cdots + \omega_6 x_6)$$

Where, γ_{ti} is the hazard function at a time in point t for subject i; $\gamma_0(t)$ is the baseline of the hazard function (the hazard function at control when no treatment is applied); ω_i are the coefficient of treatments which is the hazard ratio interpreted as the predicted change in the hazard for a unit increase in the predictor.

RESULTS

The results of the biochemical parameters of *O. niloticus* exposed to metalaxyl fungicide was presented in Table "1". AST activities were found to be highest in T5 (232.50 ± 14.90), this was followed by T1 (218.70 ± 4.90), while T2 recorded the least value of AST (167.05 ± 22.25). T4 recorded the highest activity of ALT (116.20 ± 3.30), followed by T1 (97.55 ± 1.25), while T6 recorded the least value of ALT (78.30 ± 4.00). Total protein values were highest in T4 (152.70 ± 5.90), followed by T5 (133.05 ± 18.25), while the least value of total protein was observed in T1 (64.65 ± 0.85). Total protein values were significantly different ($P<0.05$) in T1 when compared to other treatments.

Also, T2 values were significantly different ($P<0.05$) when compared to T3, T4, and T5. Cholesterol values were highest in T4 (76.30 ± 11.60), followed by T3 (44.35 ± 3.95), while T1 recorded the least value of cholesterol (16.15 ± 6.65). A significant difference ($p<0.05$) was observed in cholesterol values when T4 was compared to other treatments. Creatinine values were found to be highest in T4 (155.60 ± 36.70), this was followed by T5 (76.35 ± 8.45), while T2 recorded the least value of creatinine (19.80 ± 2.80). Creatinine values were significantly different ($P<0.05$) in T4 when compared to T1, T2, T3, and T6. However, an insignificant difference ($p>0.05$) was observed between T1, T2, T3, T5, and T6 when compared to each other.

The water quality parameters of *Oreochromis niloticus* exposed to metalaxyl fungicide is shown in Table "2". Water quality parameters such as salinity, temperature and conductivity before introducing metalaxyl were not significantly different ($p>0.05$) when compared to the values obtained after introducing the toxicant. However, pH, total dissolved solids and dissolved oxygen values were significantly different ($p<0.05$) after introduction of the toxicant.

Log of concentration of metalaxyl fungicide and its probit value for juvenile Nile Tilapia *Oreochromis niloticus* is presented in Figure "1". Lethal concentration: The acute toxicity of metalaxyl to Nile Tilapia (*Oreochromis niloticus*) is 7.41 ml - 74.1 ml. This was calculated by multiplying the 96h LC_{50} by constant of 0.01-0.1. The LC_{50} represents the concentration at which 50% of a fish population is killed when exposed to toxins.

The behavioral responses of *O. niloticus* exposure to metalaxyl fungicide is presented in Table "3". In the control group (0.0), normal behavioral responses were observed. Fish exposed to different concentrations (500, 600, 700, 800, and 900 ml) of metalaxyl displayed behavioral responses such as loss of reflex, air gulping, erratic swimming, and fin deformation, and molting was noticed.

Table 1: Biochemical parameters of *O. niloticus* exposed to metalaxyl fungicide

Parameters	T1 (Control)	T2 (500ml)	T3 (600ml)	T4 (700ml)	T5 (800ml)	T6 (900ml)	P-value
AST(IU/L)	218.70 ±4.90	167.05±22.25	198.70 ± 13.70	191.75 ± 14.35	232.50 ± 14.90	168.80 ± 29.60	0.202
ALT(IU/L)	97.55 ± 1.25	95.25 ± 24.55	93.70 ± 8.60	116.20 ± 3.30	87.75 ± 6.55	78.30 ± 4.00	0.386
T protein (g/L)	64.65 ±0.85d	85.65±3.35cd	123.55 ±3.65ab	152.70 ± 5.90a	133.05±18.25ab	109.80 ±5.60bc	0.003
Cholesterol (mmol/L)	16.15 ±6.65b	23.05 ± 0.55b	44.35 ± 3.95b	76.30 ± 11.60a	34.25 ± 5.75b	37.90 ± 12.00b	0.016
Creatinine (µmol/l)	24.05±18.35b	19.80 ± 2.80b	52.35 ± 26.85b	155.60±36.70a	76.35 ± 8.45ab	45.25 ± 28.25b	0.045

Note: Means with the same row followed by the same letter are not significantly different (p>0.05) from each other.

Key: AST = Aspartate aminotransferase, ALT = Alanine aminotransferase

Table 2: Water quality parameters of *O. niloticus* exposed to metalaxyl fungicide

		Concentrations					
Times	**Parameter**	**0.0ml**	**500ml**	**600ml**	**700ml**	**800ml**	**900ml**
Before	**Temp**	25.33 ± 0.03^{a}	25.40 ± 0.00^{a}	25.57 ± 0.03^{b}	25.57 ± 0.03^{b}	25.80 ± 0.00^{c}	25.73 ± 0.03^{c}
	Cond	723.61 ± 0.01^{f}	144.57 ± 0.08^{a}	159.93 ± 0.07^{e}	155.03 ± 0.07^{c}	156.80 ± 0.06^{d}	150.33 ± 0.07^{b}
	Ph	7.72 ± 0.00^{d}	7.53 ± 0.00^{b}	7.75 ± 0.00^{e}	7.77 ± 0.00^{f}	7.63 ± 0.01^{c}	7.49 ± 0.00^{a}
	TDS	71.83 ± 0.07^{f}	72.18 ± 0.01^{a}	79.96 ± 0.01^{e}	77.46 ± 0.01^{c}	78.45 ± 0.01^{d}	75.06 ± 0.01^{b}
	DO	3.53 ± 0.00^{a}	3.53 ± 0.03^{d}	4.00 ± 0.00^{c}	3.53 ± 0.03^{e}	4.00 ± 0.00^{f}	$3.53\pm0.03^{b``}$
1hr	**Temp**	26.03 ± 0.03^{b}	25.80 ± 0.00^{a}	25.83 ± 0.03^{a}	26.03 ± 0.03^{b}	26.03 ± 0.03^{b}	26.37 ± 0.03^{c}
	Cond	154.77 ± 0.03^{c}	144.13 ± 0.03^{a}	156.13 ± 0.07^{d}	154.57 ± 0.03^{b}	160.47 ± 0.03^{f}	160.07 ± 0.03^{e}
	Ph	7.48 ± 0.00^{a}	7.48 ± 0.00^{b}	7.48 ± 0.00^{b}	7.51 ± 0.00^{d}	7.53 ± 0.00^{e}	7.51 ± 0.00^{c}
	TDS	77.06 ± 0.01^{b}	72.13 ± 0.01^{a}	78.02 ± 0.01^{d}	77.17 ± 0.01^{c}	80.07 ± 0.01^{f}	79.79 ± 0.00^{e}
	DO	3.70 ± 0.00^{e}	3.27 ± 0.03^{d}	2.73 ± 0.03^{b}	2.90 ± 0.00^{c}	2.60 ± 0.00^{a}	2.83 ± 0.03^{c}

2hrs	**Temp**	26.53±0.13^{a}	26.43±0.03^{a}	26.50±0.00^{a}	18.34±7.86^{a}	26.17±0.03^{a}	26.23±0.03^{a}
	Cond	147.83±0.07^{a}	351.73±19.9^{a}	150.67±0.07^{a}	146.83±0.07^{a}	156.83±0.03^{a}	158.27±0.03^{a}
	Ph	7.49±0.00^{d}	7.49±0.00^{d}	7.50±0.00^{e}	7.41±0.00^{b}	7.22±0.00^{a}	7.47±0.00^{c}
	TDS	73.73±0.07^{b}	75.17±0.03^{d}	74.80±0.06^{c}	73.27±0.03^{a}	78.63±0.03^{e}	79.23±0.07^{f}
	DO	2.80±0.00^{b}	2.60±0.10^{a}	2.60±0.00^{a}	2.77±0.03^{b}	2.83±0.03^{b}	2.77±0.03^{b}`
3hrs	**Temp**	26.27±0.03^{b}	26.10±0.00^{a}	26.10±0.00^{a}	26.57±0.03^{c}	26.03±0.03^{a}	26.33±0.03^{b}
	Cond	153.83±0.03^{e}	155.83±0.03^{f}	145.27±0.13^{c}	130.97±0.03^{a}	144.87±0.03^{b}	148.87±0.03^{d}
	Ph	7.59±0.00^{e}	7.49±0.00^{d}	7.65±0.00^{f}	7.40±0.00^{b}	7.01±0.00^{a}	7.47±0.00^{c}
	TDS	76.98±0.01^{e}	78.07±0.01^{f}	72.69±0.01^{b}	65.71±0.01^{a}	73.20±0.01^{c}	74.55±0.01^{d}
	DO	3.17±0.03^{cd}	2.33±0.17^{a}	3.27±0.27^{d}	3.03±0.03^{bcd}	2.77±0.07^{abc}	2.67±0.07^{ab}
4hrs	**Temp**	26.45±0.03^{bc}	27.17±0.03^{d}	26.13±0.07^{a}	26.40±0.00^{b}	26.47±0.03^{bc}	26.57±0.07^{c}
	Cond	164.60±0.10^{f}	158.33±0.07^{e}	150.50±0.10^{d}	149.07±0.03^{c}	147.67±0.07^{b}	145.47±0.13^{a}
	pH	7.19±0.00^{a}	7.28±0.00^{b}	7.49±0.00^{f}	7.43±0.00^{d}	7.46±0.00^{e}	7.36±0.00^{c}

	TDS	82.41±0.01^{f}	79.13±0.01^{e}	75.24±0.01^{d}	74.52±0.01^{c}	73.91±0.01^{b}	72.60±0.01^{a}
	DO	2.50±0.00^{a}	2.83±0.03^{c}	3.10±0.06^{d}	3.63±0.03^{e}	2.67±0.03^{b}	2.83±0.03^{c}
8hrs	**Temp**	27.70±0.65^{b}	26.67±0.03^{a}	26.37±0.03^{a}	26.23±0.03^{a}	26.40±0.03^{a}	26.43±0.03^{a}
	Cond	181.63±0.03^{f}	169.83±0.07^{e}	157.87±0.03^{d}	152.97±0.03^{b}	154.47±0.01^{c}	149.93±0.17^{a}
	pH	7.47±0.00^{c}	7.44±0.00^{a}	7.50±0.00^{d}	7.46±0.00^{b}	7.54±0.00^{e}	7.57±0.00^{f}
	TDS	7.47±0.00^{f}	7.44±0.00^{e}	7.50±0.00^{d}	7.46±0.00^{b}	7.54±0.00^{c}	7.57±0.00^{a}
	DO	3.77±0.07^{d}	2.93±0.03^{b}	3.00±0.00^{b}	3.33±0.03^{c}	3.30±0.00^{c}	2.63±0.03^{a}
12hrs	**Temp**	26.53±0.03^{ab}	26.67±0.07^{b}	26.60±0.00^{ab}	26.53±0.03^{ab}	26.47±0.07^{a}	26.47±0.07^{a}
	Cond	172.33±0.03^{f}	168.33±0.03^{e}	155.50±0.10^{a}	156.47±0.09^{b}	159.80±0.55^{d}	158.77±0.08^{c}
	pH	7.48±0.00^{b}	7.33±0.00^{a}	7.51±0.00^{c}	7.48±0.00^{b}	7.56±0.01^{d}	7.59±0.00^{e}
	TDS	86.00±0.00^{e}	83.25±0.04^{d}	77.73±0.04^{a}	79.20±0.17^{b}	79.53±0.04^{c}	79.46±0.01^{c}
	DO	3.00±0.00^{b}	3.23±0.07^{c}	3.23±0.03^{c}	2.47±0.07^{a}	2.50±0.07^{a}	2.43±0.03^{a}
16hrs	**Temp**	26.60±0.00^{e}	26.40±0.00^{bc}	26.23±0.03^{a}	26.33±0.03^{b}	26.43±0.03^{cd}	26.50±0.00^{d}

	Cond	147.83±0.07^{a}	164.30±0.00^{e}	157.83±0.07^{c}	157.33±0.07^{b}	164.30±0.10^{e}	162.27±0.18^{d}
	pH	7.45±0.00^{a}	7.49±0.00^{b}	7.56±0.01^{d}	7.58±0.00^{e}	7.50±0.00^{b}	7.54±0.00^{c}
	TDS	73.80±0.01^{a}	82.08±0.00^{e}	78.87±0.01^{c}	78.66±0.00^{b}	82.32±0.01^{f}	80.77±0.01^{d}
	DO	2.33±0.03^{a}	3.10±0.00^{c}	3.07±0.03^{b}	3.13±0.03^{c}	2.27±0.03^{a}	2.30±0.00^{a}
20hrs	**Temp**	26.57±0.03^{c}	26.43±0.03^{ab}	26.47±0.03^{ab}	26.40±0.00^{a}	26.50±0.00^{bc}	26.67±0.03^{d}
	Cond	157.27±0.13^{a}	175.33±0.07^{e}	168.50±0.10^{d}	165.80±0.06^{c}	157.70±0.10^{b}	175.27±0.13^{e}
	pH	7.08±0.00^{a}	7.41±0.00^{b}	7.47±0.00^{c}	7.54±0.00^{e}	7.55±0.00^{f}	7.49±0.00^{d}
	TDS	78.52±0.01^{a}	87.98±0.01^{f}	84.33±0.00^{d}	82.70±0.01^{c}	78.83±0.01^{b}	87.93±0.01^{e}
	DO	3.40±0.00^{c}	3.20±0.00^{e}	2.70±0.00^{a}	3.23±0.03^{b}	3.43±0.03^{c}	2.00±0.00^{d}
24hrs	**Temp**	26.40±0.00^{a}	26.43±0.03^{a}	26.43±0.03^{a}	26.40±0.00^{a}	26.40±0.00^{a}	26.63±0.03^{b}
	Cond	149.70±0.10^{a}	168.60±0.00^{c}	171.77±0.03^{d}	164.80±0.00^{b}	179.20±0.10^{e}	171.73±0.07^{d}
	pH	7.54±0.00^{c}	7.52±0.00^{a}	7.55±0..00^{d}	7.54±0.00^{b}	7.55±0.00^{d}	7.54±0.00^{b}
	TDS	74.87±0.02^{a}	84.33±0.00^{c}	85.94±0.01^{d}	82.04±0.00^{b}	89.66±0.01^{e}	85.95±0.01^{d}

	DO	3.00±0.00^{a}	3.43±0.03^{b}	2.13±0.03^{a}	3.27±0.07^{ab}	3.20±0.00^{ab}	2.20±0.020^{ab}
48hrs	**Temp**	26.50±0.00^{a}	26.47±0.03^{a}	26.40±0.00^{a}	26.63±003^{b}	26.77±0.03^{c}	26.80±0.06^{c}
	Cond	200.20±0.10^{f}	174.73±0.07^{b}	182.10±0.10^{e}	165.87±0.23^{a}	181.47±0.13^{d}	175.47±0.13^{c}
	pH	7.52±0.00^{a}	7.49±0.00^{a}	7.45±0.00^{a}	7.60±0.12^{ab}	7.52±0.00^{a}	7.69±0.00^{b}
	TDS	100.17±0.07^{f}	87.38±0.01^{b}	90.99±0.01^{e}	82.89±0.09^{a}	90.35±0.02^{d}	87.75±0.04^{c}
	DO	3.40±0.00^{b}	3.23±0.03^{a}	2.67±0.07^{c}	3.20±0.00^{a}	3.40±0.00^{b}	2.43±0.03^{b}
72hrs	**Temp**	26.63±0.09^{a}	26.50±0.00^{a}	26.60±0.10^{a}	26.50±0.00^{a}	26.50±0.00^{a}	26.67±0.03^{a}
	Cond	155.67±0.13^{a}	188.27±0.13^{f}	180.73±0.07^{d}	184.73±0.07^{e}	177.67±0.07^{b}	178.20±0.10^{c}
	pH	7.27±0.00^{a}	7.50±0.00^{b}	7.59±0.00^{d}	7.60±0.00^{f}	7.59±0.00^{c}	7.60±0.00^{e}
	TDS	77.77±0.10^{a}	94.78±0.10^{f}	90.32±0.10^{d}	92.45±0.01^{e}	88.91±0.10^{b}	88.98±0.01^{c}
	DO	3.60±0.00^{b}	3.50±0.03^{c}	3.23±0.03^{a}	3.50±0.00^{e}	3.43±0.03^{d}	2.60±0.00^{d}
96hrs	**Temp**	26.53±0.03^{b}	26.57±0.03^{bc}	26.50±0.00^{a} ^{b}	26.50±0.00^{ab}	26.43±0.03^{a}	26.63±0.03^{c}
	Cond	169.83±0.07^{a}	190.03±0.07^{c}	183.30±0.10^{b}	195.33±0.07^{d}	207.37±0.07^{f}	196.47±0.07^{e}

	pH	7.38±0.00^{c}	7.36±0.00^{b}	7.32±0.00^{a}	7.47±0.00^{d}	7.55±0.00^{e}	7.83±0.00^{f}
	TDS	84.88±0.01^{a}	94.98±0.01^{c}	91.65±0.01^{b}	97.64±0.01^{d}	103.67±0.07^{f}	98.42±0.01^{e}
	DO	3.55±0.00^{c}	3.45±0.00^{a}	3.20±0.00^{c}	3.40±0.00^{b}	3.34±0.03^{a}	2.65±0.00^{d}
After	**Temp**	26.57±0.03^{a}	26.50±0.06^{a}	26.57±0.03^{a}	26.60±0.00^{a}	26.57±0.03^{a}	26.57±0.03^{a}
	Cond	156.23±0.03^{a}	187.33±0.03^{bc}	184.97±3.71^{b}	194.97±2.67^{c}	196.17±4.43	190.40±3.00
	pH	7.28±0.00^{a}	7.25±0.00^{a}	7.30±0.07^{a}	7.50±0.06^{b}	7.69±0.07^{c}	7.83±0.00^{c}
	TDS	73.13±0.13^{a}	95.39±1.00^{bc}	93.24±2.03^{b}	98.52±0.97^{c}	98.53±1.77^{c}	95.74±1.33^{bc}
	DO	3.50±0.00^{a}	3.57±0.33^{a}	3.53±0.03^{a}	3.57±0.03^{a}	3.53±0.03^{a}	3.20±0.00^{a}

Note: Means with the same column followed by the same letter are not significantly different ($p>0.05$) from each other.

Table 3: Behavioral responses of *O. niloticus* exposure to metalaxyl fungicide

KEY: - = absent, + = present.

Behavior	24 hours exposure (ml)					
	.0	500	600	700	800	900
Loss of reflex		-	-	-	-	-
Air gulping		-	-	-	-	-
Erratic swimming		-	-	-	-	-
Fin deformation		-	-	-	-	-
Molting		-	-	-	-	-

Behavior	48 hours exposure (ml)					
	.0	500	600	700	800	900
Loss of reflex		-	-	-	-	-
Air gulping		-	-	-	-	-
Erratic swimming		-	-	-	-	-
Fin deformation		-	-	-	-	-
Molting		-	-	-	-	-

Behavior	72 hours exposure (ml)					
	.0	500	600	700	800	900
Loss of reflex		-	-	-	-	-
Air gulping		-	-	-	-	-
Erratic swimming		-	-	-	-	-
Fin deformation		-	+	+	-	-
Molting		-	-	-	-	-

Behavior	96 hours exposure (ml)					
	.0	500	600	700	800	900
Loss of reflex		-	-	-	-	-
Air gulping		-	-	-	-	-
Erratic swimming		-	-	-	-	-
Fin deformation		-	-	-	-	-
Molting		-	-	-	-	-

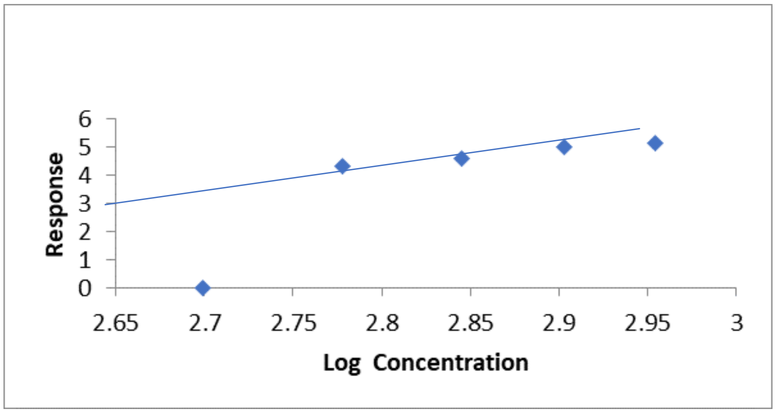

Figure 1: Log of concentration of metalaxyl fungicide and its probit value for juvenile Nile Tilapia *Oreochromis niloticus*

The results of the Kaplan-Meier technique of survival analysis were depicted in Figure "2" and Table "4". The results explain the survival rate of juvenile Nile Tilapia *Oreochromis niloticus* under different concentrations of metalaxyl fungicide. From the figure, it was revealed that the average survival at 50% of events was 72 h. The survival rate declines with an increase in the concentration used as the treatment. The chance of survival gets worse between the treatments of 700 ml and 900 ml. The control has not recorded any death as expected, while the events are minimal in the treatments 500 ml and 600 ml. In the Table "4", no event (death) was recorded in the control between 24 to 96h of the experiment. In treatment 500 ml, the chance of survival reduces as the time increases, at 24h it was about 90% chance of survival but gets worse by the time it reaches 96h (approximately 12% chance). About 85% of survival chance was recorded at 24h and nearly 11% by the time it reaches 96h under the 600 ml concentration. Again, it was noted that the chance of survival (85%) at a 24h period was the same for 600 ml and 700 ml concentrations but there is a clear difference for 700 ml at 96h where no survival was recorded. The chance of survival under 800 ml and 900 ml at 24h was approximately 80% and 75%, respectively and both recorded no survivor at 96h.

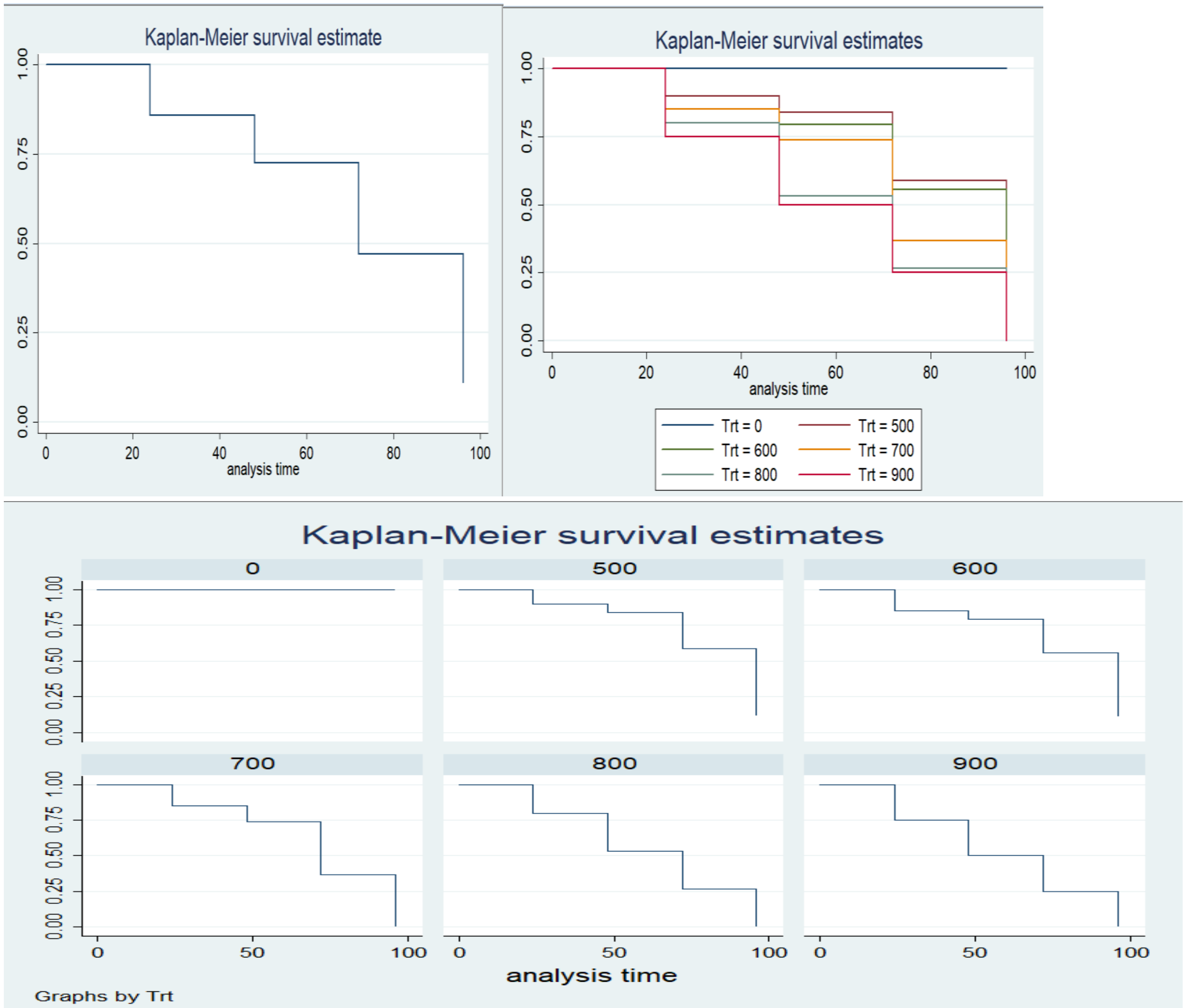

Figure 2: Kaplan-Meier Survival Curves Based on the Treatments

Table 4: The Results of Mean Time to Death of Juvenile Nile Tilapia *Oreochromis niloticus* under the Concentration of Metalaxyl Fungicide

```
        failure _d:  Event == 1
  analysis time _t:  Hours
```

Time	Beg. Total	Fail	Net Lost	Survivor Function	Std. Error	[95% Conf.	Int.]
Trt=0							
24	20	0	5	1.0000	.	.	.
48	15	0	5	1.0000	.	.	.
72	10	0	5	1.0000	.	.	.
96	5	0	5	1.0000	.	.	.
Trt=500							
24	20	2	3	0.9000	0.0671	0.6560	0.9740
48	15	1	4	0.8400	0.0853	0.5792	0.9459
72	10	3	2	0.5880	0.1356	0.2883	0.7971
96	5	4	1	0.1176	0.1086	0.0068	0.3990
Trt=600							
24	20	3	2	0.8500	0.0798	0.6038	0.9490
48	15	1	4	0.7933	0.0925	0.5374	0.9173
72	10	3	2	0.5553	0.1319	0.2730	0.7661
96	5	4	1	0.1111	0.1028	0.0066	0.3818
Trt=700							
24	20	3	2	0.8500	0.0798	0.6038	0.9490
48	15	2	3	0.7367	0.1018	0.4766	0.8816
72	10	5	0	0.3683	0.1271	0.1400	0.6020
96	5	5	0	0.0000	.	.	.
Trt=800							
24	20	4	1	0.8000	0.0894	0.5511	0.9198
48	15	5	0	0.5333	0.1142	0.2936	0.7244
72	10	5	0	0.2667	0.1018	0.0974	0.4722
96	5	5	0	0.0000	.	.	.
Trt=900							
24	20	5	0	0.7500	0.0968	0.4999	0.8875
48	15	5	0	0.5000	0.1118	0.2713	0.6919
72	10	5	0	0.2500	0.0968	0.0910	0.4485
96	5	5	0	0.0000	.	.	.

The incident rate at 95% confidence intervals was depicted in Table “5”. The incident rate’s trend increases as the concentration increases. The results showed that the average incident rate at 500 ml concentration was 0.8% with 95% confidence intervals (CI) of 0.4% to 1.5%. For 600 ml, the average incident rate at 95% CI was 0.9% (0.5 – 1.7%). About 1.3% (0.7 – 2.1%) incident rate at 95% CI was observed under 700 ml, while 1.6% (1.0 – 2.5%) and 1.7% (1.1 – 2.6%) for 800 ml and 900 ml, respectively, were recorded as the average incident rate at 95% CI.

Table 5: Incident Rate Estimates by Treatments (Trt)

Estimated rates and lower/upper bounds of 95% confidence intervals
(120 records included in the analysis)

Trt	D	Y	Rate	Lower	Upper
0	0	1.2e+03	0.0e+00	.	.
500	10	1.2e+03	0.0083333	0.0044838	0.0154879
600	11	1.2e+03	0.0091667	0.0050765	0.0165523
700	15	1.2e+03	0.0125000	0.0075358	0.0207343
800	19	1.2e+03	0.0158333	0.0100993	0.0248228
900	20	1.2e+03	0.0166667	0.0107526	0.0258335

The Log-rank test was used to test and confirm the null hypothesis that there is no significant difference in the survival times between the treatments at all time points in the experiment. The summary of the results was depicted in Table “6”. Using Chi-square test, it was showed that there is significant difference given the coefficient of 35.07 at 1% probability level.

Table 6: Test of Difference in Survival Times using Log-rank Test

Log-rank test for equality of survivor functions

Trt	Events observed	Events expected
0	0	12.50
500	10	12.50
600	11	12.50
700	15	12.50
800	19	12.50
900	20	12.50
Total	75	75.00
	chi2(5) =	35.07
	Pr>chi2 =	0.0000

Table "7" revealed the results of the hazard ratio estimated from the Cox Regression. The time at risk was 7200, while the failure was 75. The model has a goodness of fit given the correct negative signed of Log-likelihood of 292.14 with a statistical significance of Chi-square value of 32.91 at a 1% probability level. It is evident from the Table "7" that the survival of fishes is all influenced by the treatments at all concentrations in the experiment. The positive coefficients indicated a positive relationship between the treatment and hazard for the terminal event (death). This means that higher values on the treatment's coefficient are associated with less survival time (until the terminal event). The coefficient of 500 ml concentration was 1.24 times the risk of the event compared with the control. Also, 600 ml concentration was 1.36 times the risk of the event compared with the control while 700 ml concentration was 1.86 times the risk of the event compared with the control. The 800 ml concentration was 2.35 times the risk of the event compared with the control while 900 ml concentration was 2.47 times the risk of the event compared with the control in the experiment.

Table 7: Estimates of Treatments in Cox Regression

Cox regression -- Breslow method for ties

No. of subjects =	120	Number of obs =	120
No. of failures =	75		
Time at risk =	7200		
		LR chi2(4) =	32.91
Log likelihood =	-292.14068	Prob > chi2 =	0.0000

_t	Haz. Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
Trt						
500	1.24e+09	.	.	.	.	.
600	1.36e+09	5.95e+08	48.13	0.000	5.78e+08	3.20e+09
700	1.86e+09	7.58e+08	52.28	0.000	8.34e+08	4.13e+09
800	2.35e+09	9.18e+08	55.23	0.000	1.09e+09	5.05e+09
900	2.47e+09	9.58e+08	55.85	0.000	1.16e+09	5.29e+09

DISCUSSION

The mortality rates observed in the present study were clearly proportional to the increase in the dosage of metalaxyl. The LC_{50} of mtalaxyl to juvenile *O. niloticus*is was found to be 7.41 ml. This is similar to what was observed by Nimai *et al*., 2016 (11.68 mg) in the acute toxic effect of Mancozeb to *O. mossambicus*. The result of the 96h LC_{50} also corresponds with the findings on *punctatus ticto* (12.95 mg/l), *Clarias batracus* adult (14.36 mg/l), and fingerlings (14.04 mg/l) by **Srivastava and Singh (2013).**

The analysis of the biochemical parameters in aquatic organisms is mostly used to monitor the effect of pollutants on their health status. Moreover, biochemical parameters can serve as markers for toxicant exposure and its effects on fish **(Vutukuru, 2003)**. In this study, there is an increase in the activities of AST at 800 ml and ALT at 700 ml in the plasma of *O. niloticus* exposed to metalaxyl, which indicates that the exposure to metalaxyl caused tissue damage in fish. Transaminases play an important role in protein metabolism, especially in the liver. Large amounts of ALT and AST are released into the blood as a consequence of liver damage. The increase in ALT and AST activities might have been due to hepatocellular damage caused by exposure to metalaxyl. Increase in the plasma activities of AST and ALT has been reported in *O. mykiss* and *Channa punctatus* exposed to diazinon and monocrotophos, respectively, by **Banaee *et al.* (2011) and Agrahari *et al.* (2006).** This observation in this study is in line with the report by **Agrahari *et al.* (2007), Velisek *et al.* (2009), and Karmakar *et al.* (2016)** who noticed increased activities of AST and ALT in fish exposed to various concentrations of monocrotophos, deltamethrin and malathion for different time intervals. They all observed that ALT and AST activities might increase in the blood of fish exposed to pesticides due to induction of metabolic shifts in protein catabolism or lysis of hepatic cell membrane and leakage of these enzymes into the bloodstream of fish.

Hyperproteinemia was observed with increase in concentration of the toxicant. The increase in total protein observed in juvenile *O. niloticus* fish exposed to metalaxyl fungicide is in agreement with the work of **Dhunsi and Oranusi (2011)** on Biochemical profile of *Clarias gariepinus* exposed to chemical additives effluent. This is also similar to the work of **Onuc and Uner (1999)** who reported an increase in liver protein when *Cyprinus capio* was exposed to 2,4- Diamine. However, the observed increase in total protein observed in this study is contrary to the findings of **Li *et al.* (2010)** who reported decline in total protein after exposing *Oncorhynchus mykiss* to fungicides. **Farsani *et al.* (2015)** also observed decrease in total protein of *Cyprinus carpio* exposed to Carboxin-thiram.

Hepatobiliary disorder may also lead to decreased cholesterol clearance from the blood and its elevated concentration in plasma, as was revealed in this study. The increase in cholesterol level observed in this study corroborates the findings of **Sevcikova *et al.* (2016)** who documented an increase in cholesterol level of *Cyprinus carpio* after exposure to copper but contrary to what was reported by **Dahunsi and Oranusi (2011)** on biochemical profile of *Clarias gariepinus* exposed to sub-lethal concentration of chemical additives effluent. Since cholesterol and proteins are essential components of cell membranes, enzymes, and several stress marker hormones, determination of alteration in their concentrations in the fish blood could also provide critical information about liver and kidney tissue dysfunctions, impairment in metabolic pathways, and immune disorders **(Bharti and Rasool, 2021).**

An increase in creatinine level was observed after exposure of *O. niloticus* to metalaxyl fungicide. The increase in creatinine seen in this study could have resulted from glomerular dysfunction or increased renal tissue breakdown, or decreased urinary clearance by the kidney. The increase in creatinine observed in this study is in agreement with the observation of **Amin and Hashem (2012)** also reported abnormality in the increase of creatinine in *Clarias gariepinus* exposed to deltamethrin.

A significant difference was observed in water quality parameters such as pH, total dissolved solid and dissolved oxygen after the introduction of the toxicant. However, the temperature, conductivity and salinity were not significantly different after introducing the toxicant Water quality plays an important role in influencing fish survival, growth performance and reproduction. Unstable water quality affects aquatic animals including fish by causing mortality and change in behavior **(Akinsorotan, 2014).**

Behavioral alterations in fish are the most sensitive signs of potential lethal effects of pesticide exposure **(Rauf and Arain, 2013; Banaee *et al.*, 2011).** The behavioral responses observed during *O. niloticus* exposure to metalaxyl in the study are similar to observations made by **Alkahemal-Balawi *et al.* (2011)** on the responses of *Cyprinus carpio* exposed to different concentrations of fenthion. The abnormal behavior displayed by fish in the experimental groups include; loss of reflex, air gulping, erratic swimming, fin deformation and molting, which is due to inhibition of acetylcholinesterase activity leading to accumulation of acetylcholine in cholinergic synapses thus causing hyper stimulation of the toxicants and became exhausted owing to respiratory difficulty. Swimming performance is considered one of the measures which could serve as a possible sensitive sign of sub-lethal toxic exposure **(Adewumi *et al.*, 2018).** It has been reported that under stress conditions, fish become hyperactive, perhaps to get out of the stressful medium and would need an increased amount of oxygen to meet their energy demand **(Alkahemal-Balawi *et al.*, 2011).**

The Kaplan-Meier survival probability estimates and curves were used to compare the survival time of the juvenile Nile Tilapia (O. niloticus) under different concentrations of metalaxyl fungicide in the experiment. The probability of survival when the fish is exposed to the concentration at different level at a point in time recorded event (death). This indicates that the presence of metalaxyl fungicide in water cause a threat to the existence of fish and other aquatic organisms. This is because the control recorded no event and it was statistically different from the treated variables. From the results in Figure "2" and Table "4", the trend of survival time decreases as the hours of exposure and concentration increases. There is association between mortality rate and the high concentration of metalaxyl and it gets worse with longer hours of exposure. The average survival time was 72h above which mortality count is catastrophic and detrimental in any habitation. The survival time at 96h under the concentration of 700 ml and above has been zero, meaning that *O. niloticus* cannot survive in a medium of metalaxyl of 700 ml and above at 96h of exposure. Using different approach and toxicant (hydroelectric turbines), **Jahannes *et al*. (2021)** recorded average fish mortality of 22.3% (95% CI 17.5 – 26.7%), which is higher than the value gotten at 900 ml concentration in this study. Also, **Sahr *et al*. (2022)** reported that the pathogenicity of *Strptococcus agalactiae* was confirmed in *O. niloticus* at 90% cumulative mortality using counting technique. Furthermore, the incident rate increases with the increase in the concentration as depicted in the Table "5". The control has no incidence meaning that the water is safe for the fish when it is void of toxic substances. The concentration at 900 ml recorded the highest rate of about 1.7% and the duration of occurrence (D) in the Table "5" indicated about 20 times of terminal event compared to when no concentration of metalaxyl fungicide is added in a medium. This is a great economic loss if times 20 deaths are recorded within 96h when there is presence of any toxic substance such as the case of metalaxyl fungicide. This was also confirmed by **Wafaa *et al*. (2022)** who used nano-composites as a toxicant and reported that high concentrations alter the biochemical functions of organisms.

The difference in survival times was confirmed and established by the results of Chi-square test in the Table "6". The difference is evident that using any concentration of metalaxyl fungicide is detrimental to the fish. The survival times of the fish at different levels of the concentration also differs. It means that time is also a critical factor in explaining mortality rate when an organism is exposed to any toxic substance as also stated by **Falmata *et al*. (2019).** The Cox Regression approach is useful when modeling the time to a specific event based on the value of the treatment **(Fagbamigbe *et al*., 2018)**. The main statistical output is the hazard ratio which is the hazard in treatment experiment divided by the hazard in the control experiment. According to the Table 7, all the treatments significantly influenced the survival time. The positive coefficients indicate a worse prognosis effect of the treatment on the fish in the experiment. There is very high tendency of failure (death) at 900 ml concentration by 2.47 times for every addition of one hour's exposure. The significant trend was noticed in all the treatment. The results further buttress the graphical representation estimates of Kaplan-Meier curves above.

CONCLUSION

In conclusion, metalaxyl was toxic to *O. niloticus*. This study clearly indicates that the presence of metalaxyl in fresh water reservoirs, even in small concentration, causes deleterious effects on biochemical parameters of fish and will potentially disturb their survivability in the natural environment. On the average, in not more than 72 h, the probability of survival is 50% and the more the concentration of metalaxyl fungicide, the less the survival rate of the *O. niloticus*. The chance of survival at 96h at any concentration above 700 ml of metalaxyl fungicide will lead to terminal event (death). Therefore, the combination of longer hours of exposure and higher concentration of metalaxyl fungicide is highly associated with the mortality rate of fish and also have the potential to change the aquatic medium, affecting the tolerance limit of aquatic fauna and flora, as well as creating danger to the ecosystem.

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