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EVALUATION OF ZEBRA FISH EMBRYOS EXPOSED TO DIFFERENT CONCENTRATIONS OF ABAMECTIN

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Abstract: Despite the benefits brought to public health by the application of pesticides to control disease vectors, their damage to the environment, such as loss of biodiversity and effects on non-target organisms, need to be further evaluated. Abamectin (ABA) is an active ingredient belonging to the group of avermectins (AVMs), widely used in insecticides, acaricides and nematicides. In Brazil, it is the main pesticide used in the cultivation of strawberries (Fragaria spp.), coffee (Coffea spp.) and other vegetables. The objective of this study was to evaluate the lethal and sublethal effects of abamectin, using zebrafish (Danio rerio) embryos at different stages of development as an experimental model. For this, fish embryo toxicity tests (FET) were carried out, exposing 2-hour post-fertilization (hpf) zebrafish embryos for 96 hours to different concentrations of abamectin (0.5 - 11.7 mg.L-1). The main parameters evaluated were mortality outcomes, developmental delays, malformations and occurrence of edema. Significant effects were observed in relation to lack of larvae hatching, egg coagulation and pericardial edema. Considering the seriousness of the results obtained, it is suggested that new studies using different living organisms be carried out, especially in countries where abamectin is registered.
Keywords: zebrafish embryo; Danio rerio embryology; acute toxicity; sublethal toxicity; pesticides

INTRODUCTION

Abamectin (ABA) belongs to the group of avermectins (AVMs) which are macrocyclic lactones derived from the fermentation of the soil bacteria *Streptomyces avermitilis* (The Pesticide Manual, 2015). They are products used worldwide in veterinary medicine as antiparasitic agents, and in agriculture as insecticides and acaricides (Siddique et al., 2014; Mesa et al. 2017). "The commercial product ABA contains two active compounds of approximately 80% avermectin B1a and 20% avermectin B1b and both have similar biological properties" (Santiago et al., 2023; The Pesticide Manual, 2015).

The primary mechanism of action of ABA in living organisms acts mainly on the chlorine channels, controlled by glutamic acid, and secondarily on the chlorine channels, thus, inducing an increase in the flow of ions to the nerve synapses in helminths and in the neuromuscular plaque in arthropods. This hyperpolarization of nerve membranes and muscle cells results in the paralysis and death of organisms exposed to ABA. Abamectin is a highly lipophilic compound that can accumulate in fat cells. "The mechanisms of action are not clear in human" (Zortéa et al. 2017; Novelli et al. 2012).

As for toxicity, ABA can demonstrate different results according to the trophic level of the studied species, affecting non-target organisms and causing deleterious effects on zooplankton (Mesa *et al*. 2017), and extremely toxic effects on microcrustaceans such as *Daphnia magna* (Novelli 2012) as well as vertebrates such as amphibians (Amaral 2018; Montalvão and Malafaia 2017) and birds (Weldemariam and Getachew 2016). In mammals, studies with rats detected the presence of ABA in their milk (Mossa et al. 2017). It was demonstrated, in fish ABA can cross the blood-brain barrier (Høy et al. 1990; Novelli et al. 2016).

The introduction of AVMs in aquatic ecosystems can occur through many indirect routes, such as run off in areas of agricultural production, rainfall, and infiltration, or direct routes like their accidental use for pest and disease vectors in seasons of fish farming (GHais et.al. 2019).

Despite toxicity tests routinely used to assess the contamination of aquatic ecosystems, *in*

vivo studies assessing the toxicity and effects of agrochemicals, such as AVMs, have been carried out using zebrafish embryos (*Danio rerio*) as an experimental model (Sanches et al. 2017). In addition to the high degree of similarity with mammals in terms of molecular mechanisms of development and cell physiology, studies using zebrafish embryos have many advantages. The manipulation of zebrafish embryos is relatively easy when compared to other experimental models, such as rats and mice, and the high number of progenies per individual allows for statistically significant sampling in a single experiment. Moreover, their embryonic development is rapid, and due to the optical transparency of embryos (Chang et al. 2020) direct real-time observation of each stage of development is perfectly possible making the zebrafish a particular and unique experimental model (Hollert et al. 2003; Braunbeck et al. 2005; Rodeck and Dicker 2007). It is currently one of the most used models worldwide for the toxicological assessment of water samples and studies on how toxins can harm the environment (Bambino and Chu 2017).

In our study the Fish Embryo Toxicity Test (FET), (OECD 2013) was used to expose zebrafish embryos to an ABA formulation at different concentrations. The main parameters analyzed were mortality end points (coagulation of eggs, lack of somite formation, lack of detachment of the yolk sac tail, and absence of heartbeat), delay in embryo development, occurrence of edema, and malformations. As a result, the lack of larvae hatching, egg coagulation, and pericardial edema were observed.

Considering the results obtained, further studies are proposed regarding the effects of ABA on living organisms at different trophic levels, in countries where this compound is reported for different uses.

MATERIAL AND METHODS

TEST ORGANISMS

The zebrafish embryos were donated by the "Zebrafish Unit" of the Central Animal Facility of the USP School of Medicine.

FET TEST

Fertilized eggs were selected by an inverted Zeiss Axio microscope at 50x and used up to the 16-cell stage (figure 1) in the tests carried out at the Biological Institute. The Fish Embryo Toxicity (FET) Test of the Organization for Economic Cooperation and Development (2013) was followed.

Essays with zebrafish embryos (*Danio rerio*) were carried out at the Toxicology Laboratory of the Environmental Protection Center of the Biological Institute and at the Interinstitutional Aquaculture Laboratory of the Biological and Fisheries Institute, São Paulo, Brazil.

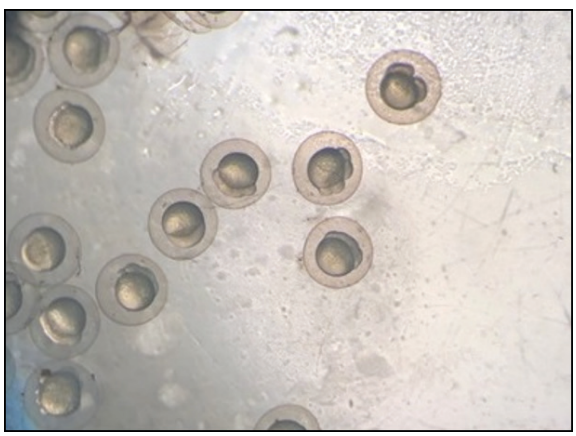

Fig. 1 2 cell to 16 cell stage zebrafish embryos. Source: Scientific researcher Cíntia Badaró Pedroso

CONCENTRATIONS USED, ANALYSIS OF QUANTITY OF ACTIVE INGREDIENT, AND PREPARATION OF DILUTED EMBRYONIC MEDIUM

In these tests, five concentrations of active ingredient were used, prepared with the commercial product in dilution water: 0.5 $mg.L^{-1}$; 1.1 $mg.L^{-1}$; 2.4 $mg.L^{-1}$; 5.3 $mg.L^{-1}$; 11.7 $mg.L^{-1}$. Three Essays were performed with these concentrations. These concentrations were based on Sanches et al. (2018) study who examined another commercial brand of content twice as high as the one used in herein to assess toxicity in embryos as well.

The contents of the active ingredient in the stock solution and in the lowest concentration were measured by an outsourced laboratory (Mérieux NutriSciences) using the EPA 8321B method (USEPA, 2007) and were within the recovery limits acceptable, that is, the variation in relation to the nominal concentration was less than 10% for the stock solution and less than 15% for the concentration of 0.5 $mg.L^{-1}$.

The diluted embryonic medium was prepared in 2000 mL volumetric flasks and consisted of ultrapure water and salts that allow the embryo to survive, as occurs in rivers and streams. The Zebrafish Unit of the Central Animal Facility of the USP School of Medicine supplied the concentrated salt suspensions (consisting of ultrapure water, NaCl, KCl, $CaCl_2$, $2H_2O$, and $MgSO_4.7H_2O$), also called concentrated embryonic medium. This concentrated embryonic medium was transported in a thermal bag and kept at a low temperature in a refrigerator at the Toxicology Laboratory until used. To prepare the dilution water, a volume of 20mL of embryonic medium and 50 mL of sea salt suspension (1.2 $g.L^{-1}$) were used, simulating the culture water, and ultrapure water were added to provide one liter of dilution water. The hardness of the diluted water prepared was around 83 mg/L in five measurements on different days of preparation.

Suspensions were serially diluted starting from the highest concentration. After preparation, the dilutions were transferred with automatic pipettes with a capacity of 1000 µL, pipetting twice into each well of sterile 24-well polystyrene cell culture plates in the afternoon prior to the Essay. These plates were kept refrigerated at 8ºC and removed from the refrigerator the following morning, waiting for them to reach room temperature, while the fertilized eggs brought from the School of Medicine were selected and after transferred one egg per cell. The test method used was static.

EMBRYO EXPOSURE, INCUBATION, AND OBSERVATION OF EMBRYOS

Before and after exposure to pesticides, pH and dissolved oxygen were measured in the Toxicology lab and were within acceptable parameters for the FET test. Dilution water samples were sent to the Fisheries Institute's Water Quality Laboratory, and before exposure the dilution water hardness and conductivity were measured.

The plates containing the embryos were placed randomly in an incubator with a photoperiod of 12 h light/12 h darkness, with a constant temperature of 26.5°C and were removed only for readings under the microscope and then returned.

Plate readings were carried out in the laboratories of the Biological Institute in São Paulo, using an inverted Zeiss® Axio model microscope at 100x magnification at 24, 48, 72, and 96 hours postfertilization (hpf), mainly observing 4 outcomes or end points: egg clotting, lack of somite formation, lack of heartbeat, and lack of yolk sac tail detachment. Additionally, the occurrence of developmental delay, yolk sac and cardiac edema, spinal deformation, malformations, and lack of

larvae hatching were also observed.

STATISTICAL ANALYSIS

The response variables were categorical (positive/negative) and imported into an Excel spreadsheet from Word. Test replicates were considered independent samples.

For descriptive statistics, data are presented in absolute numbers of occurrence when positive and their respective proportion in relation to negative responses, thus, generating the rates of occurrence of the observed phenomena.

For inferential statistics, the Chi-squared test and, when appropriate, Fisher's Exact test were performed to compare the different concentration doses in relation to the respective control groups. The significance level used was 0.05.

RESULTS AND DISCUSSION

EFFECT OF ABAMECTIN ON ZEBRAFISH EMBRYO MORTALITY

In the set of three Essays, mortality in the negative abamectin control (dilution water) was less than or equal to 11.7%. When analyzed together, the data from the three Essays show that for embryos exposed to abamectin for up to 96 hpf, there was an increase in mortality as a function of concentration and exposure time. Mortality rates above 60% were recorded at an exposure concentration of 11.7 $mg.L^{-1}$ at 72 hpf and up to 96 hpf.

From the data of each concentration of abamectin, the movement of the embryos was observed at concentrations much lower than those that affected the survival of all tested individuals. It was specifically verified that there was a lack of spontaneous movements of the embryos in the tested concentrations. The main end point found was the clotting of embryos at all concentrations.

Over time, from 24 to 96 hpf, a significant and increasing mortality rate was observed at the abamectin treatment of 11.7 $mg.L^{-1}$ ($p < 0.001$) when the three Essays were analysed together (Table 1).

When analyzed separately, significant mortality rates for different concentrations of abamectin in the three Essays performed were observed (Tables 2 to 7).

In Essay 1, 40% of the embryos died with the treatment at 5.3 $mg.L^{-1}$, and 100% with a 11.7 $mg.L^{-1}$ treatment, both mortality values were statistically significant when compared to the control ($p \leq 0.05$) (Tables 2 and 3). Moreover, the difference between the concentrations 5.3 $mg.L^{-1}$ and 11.7 $mg.L^{-1}$ ($p \leq 0.05$) was also significant, as shown in Tables 2 and 3.

In Essay 3, the effects on mortality of concentrations 0.5 $mg.L^{-1}$ (3 dead embryos) and 1.1 $mg.L^{-1}$ (4 dead embryos) did not differ from each other or from the negative control (1 dead embryo) and this differed significantly from the other concentrations. The 0.5 $mg.L^{-1}$ concentration did not differ from the 2.4 $mg.L^{-1}$ concentration (6 dead), but significantly differed from the 5.3 $mg.L^{-1}$ (9 dead) and 11.7 $mg.L^{-1}$ (16 dead) concentrations. Moreover, the 2.4 $mg.L^{-1}$ concentration did not differ significantly from the 5.3 $mg.L^{-1}$ concentration ($p = 0.32$). The effects attributed to abamectin at the highest concentration 11.7 $mg.L^{-1}$ (16 dead) were statistically greater than at all other concentrations (Tables 6 and 7).

The repeatability analysis between the 3 Essays detected that the treatments with 0.5 $mg.L^{-1}$, 1.1 $mg.L^{-1}$, and 5.3 $mg.L^{-1}$ were not statistically different, showing similar results between the three tests ($p>0.05$). There was no significant repeatability at the other two concentrations (Table 8).

LC_{50} DEFINITION

In the analysis of abamectin, only at 96 hpf, the regression model was statistically significant, (p = 0.0015) and considering the three Essays analyzed together the LC_{50} value was 4.11 mg. L^{-1}.

SUBLETHAL EFFECTS OBSERVED UPON EXPOSURE TO FORMULATED ABAMECTIN

EFFECT OF ABAMECTIN ON THE HATCHING OF ZEBRAFISH LARVAE

In Essay 1 there was a significant delay in the hatching of zebrafish embryos at all concentrations compared to the negative control ($p<0.001$). At the highest concentration, 11.7 $mg.L^{-1}$, the delay was not statistically determined due to the mortality of all embryos (Tables 9 and 10).

In Essay 2, all concentrations caused a significant delay in hatching compared to the control ($p \leq 0.005$) (Tables 11 and 12). The 0.5 mg.L -1 concentration had the greatest number of delays in hatching, differing significantly from the treatments at 2.4 mg.L-1, 5.3 mg.L-1, and 11.7 mg.L-1 ($p \leq 0.05$) (Tables 11 and 12). However, this result does not mean that this concentration was more toxic, as the number of deaths was higher at higher concentrations.

In Essay 3, the negative control had an embryo with delayed hatching, which was within the acceptable range. In this Essay only the highest concentration of 11.7 $mg.L^{-1}$ did not detect significant hatching delays (p = 0.148) (Table 13). This fact is likely associated with the greater number of deaths. Among the concentrations for which significant delays were detected, those of 0.5 $mg.L^{-1}$ and 1.1 $mg.L^{-1}$ had significantly greater delays than those at a concentration of 5.3 $mg.L^{-1}$ (p = 0.013 and p = 0.038, respectively) (Table 14).

The repeatability analysis between the 3 trials detected that treatments with 0.5 mg. L^{-1} and 5.3 $mg.L^{-1}$ were not statistically different, nor the control, showing similar results between the three Essays ($p> 0.05$) (Table 15). Because the repetitions were performed on different days, as directed by the "Zebrafish Unit" of the USP School of Medicine, and the controlled conditions, the significant p-values were probably due to phenotypic conditions of the embryos.

Regarding developmental damage, it may be inferred that these effects are related to mortality, since effects such as developmental delay, malformations, and edema occurred with a relative frequency in % of the total exposed for each concentration in a smaller number of than the respective mortality rates.

Out of the total exposed embryos (60), the abamectin-exposed group of embryos with developmental delay reached 5% at 72 hpf. Delays persisted in the 1.1 $mg.L^{-1}$ and 11.7$mg.l^{-1}$ concentration groups reaching less than 2% at 96 hpf.

Malformations were mainly found in the two lower concentrations, persisting throughout the test until 96 hpf, reaching a rate of 25% for the concentration of 0.5 $mg.L^{-1}$. At concentrations of 5.3 and 11.7 $mg.L^{-1}$ they were, respectively, 20% and less than 15% at 96 hpf; the same occurred with observed edema in embryos and larvae.

It was observed that 25% to 30% of the embryos exposed to abamectin at the highest concentrations of the pesticide (5.3 $mg.L^{-1}$ and 11.7 $mg.L^{-1}$), had an intensified blood supply. This effect triggered a hemorrhage-like process and, consequently, a pericardial clot, leading to the embryo's death.

In the FET test, the tested organisms are exposed in a laboratory under controlled conditions to different concentrations of the samples under analysis and the pathogenic effects generated were studied and quantified.

This study used the active ingredient concentrations predetermined by Sanches *et*

al. (2018), although using another trademark, and arrived at an LC_{50} value (4.11 mg abamectin.L^{-1}) within the range reported in the study by the aforementioned author.

Egg coagulation was observed as the main mortality end point compared to the negative control and that embryos exposed to the five concentrations of abamectin showed a reduction in movement within the chorion. This fact is relevant considering that embryos had already developed the somites which must allow them to move within the chorion, thus, demonstrating neurotoxic damage. Although abamectin is not of a high molecular weight (Sanches *et al*., 2018), it is a highly lipophilic substance which hinders its entry through the chorion. However, a partial lack of hatching of exposed larvae was also detected, confirming the entry of the substance. In fish, abamectin acts as an agonist of gamma aminobutyric acid (GABA), mimicking GABA, and competes for its receptors. This binding to the receptor increases the permeability of muscle and nerve cells to chloride ions, impairing the passage of nerve impulses, since it can cross the blood-brain barrier (Hoy et al. 1990; Katharios et al. 2004; Sanches et al. 2018).

We also found high hatching delay rates, even at the lowest concentration, 0.5 mg.L^{-1} and higher mortality at the highest concentration. Similar to the studies by Moura et al. (2017) and Sanches et al. (2018), it was observed herein that the formulation applied contained 18g.L^{-1}, corroborating what was mentioned previously that there are several reasons for a reduced hatching rate, such as a denaturation or abnormality in the chorionase enzyme that acts on the inner layers of the egg. Moreover, abamectin altered blood circulation as well. All of these observations lead to the interpretation of a possible neurotoxic effect of the pesticide, reducing the movement of embryos, and affecting the success of hatching.

According to Rahman 2023, experiments carried out with embryos of the short-horned grasshopper *Heteracris littoralis* exposed to sublethal concentrations of ABA, provided a significant reduction in the hatchability of the eggs tested. Furthermore, many embryos stopped developing at certain stages of development.

Although some researchers report that in laboratory tests an absolute and conclusive answer to the harmful effects to humans is not obtained, given the difficulty of extrapolating the toxicity results between species, other scientists corroborate that neurotoxicity tests in zebrafish provide useful results for human health, due to the conservation of the main response mechanisms (Ogungbemi et al. 2020).

Formulated products may have different toxicity from the active ingredients. Emerging contaminants like pesticides are not monitored in the environment (Santiago et al., 2023; Sposito et al. 2018; Richardson and Ternes 2018; Masiá et al. 2015). Nevertheless, they can cause ecological damage such as declining biodiversity of aquatic species (Hayasaka et al. 2012) and for Public Health (Sposito et al. 2018).

The present work demonstrated that sublethal effects such as delayed hatching of larvae, malformations and edema were observed at the lowest tested concentrations of 0.5mg.L-1 and 1.1mg.L-1, which differed significantly from the control with mode of action probably related to a neurotoxic effect.

CONCLUSIONS

The main effects observed in embryos exposed to sublethal doses of abamectin in different dilutions of the formulation were: inhibition of embryo movement within the chorion intensification of blood circulation with hemorrhage and pericardial edema.

We advise that these abamectin-based products be further researched from the point

of view of environmental and human health.

DECLARATIONS

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The zebrafish embryos used in this study were donated by the "Zebrafish Unit" of the Central Animal Facility of the USP School of Medicine. This study was approved by the Ethics Committee on the use of animals (CEUA) of ``Faculdade de Medicina da Universidade de São Paulo`` (Brazil) and was registered under number 1123/2018. The first FET Test stage was approved by the CEUA of the Biological Institute (São Paulo, Brazil) under number 161/2019.

AVAILABILITY OF DATA AND MATERIALS

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

MRS conducted the experiments, analyzed and interpreted the data, and wrote the manuscript. LMS suggested experimental modifications, wrote the abstract, and contributed with writing results and discussion. CBP was a major contributor in revising the manuscript. EMFC designed the research project and was a major contributor in revising the manuscript. All authors read and approved the final manuscript.

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TABLES

Treatment	24 hpf		48 hpf	72 hpf	96 hpf
	N	Dead n (%)	Dead n (%)	Dead n (%)	Dead n (%)
Negative Control	60	3 (5.0 %)	4 (6.6%)	4 (6.6%)	7 (11.7%)
0.5 mg.L^{-1}	60	2 (3.3%)	7 (11.7%)	9 (15%)	10 (16.7%)
1.1 mg.L^{-1}	60	11 (18.3%)*	13 (21.7%)	13(21.7%)*	16 (26.7%)*
2.4 mg.L^{-1}	60	8 (13.3%)	8 (13.3%)	8 (13,3%)	23 (38.3%)*
5.3 mg.L^{-1}	60	7 (11.7%)	7 (11.7%)	9 (15%)	25 (41.7%)*
11.7 mg.L^{-1}	60	20 (33.3%)*	22 (36.7%)*	39 (65%)*	47 (78.3%)*

Table 1 Embryo mortality at 24-, 48-, 72-, and 96-hours postfertilization (hpf) in treatments of five concentrations of abamectin

Source: MR Santiago Thesis Project

N: Total number of organisms

n: number of dead embryos

*: Statistically significant p-values (p<0.001) in Fisher's Exact test.

Treatment	Essay 1		
	n	Dead	% Dead
Negative Control	24	2	8.30
0.5 mg.L^{-1}	20	3	15.00
1.1 mg.L^{-1}	20	3	15.00
2.4 mg.L^{-1}	20	4	20.00
5.3 mg.L^{-1}	20	8	40.00*
11.7 mg.L^{-1}	20	20	100.00*

Table 2 Effects of exposure to treatments with different concentrations of abamectin on zebrafish embryo mortality in Essay 1 at 96 hpf.

Source: MR Santiago Thesis Project

n: number of embryos per treatment

*: statistically significant values compared to the negative control, $p \leq 0.05$.

Treatment	Essay 1 (p-value)					
	Negative Control	0.5 mg.L^{-1}	1.1 mg.L^{-1}	2.4 mg.L^{-1}	5.3 mg.L^{-1}	11.7 mg.L^{-1}
Negative Control	-	0.646	0.646	0.387	0.013*	0
0.5 mg.L^{-1}	-	-	1	1	0.077	0.231
1.1 mg.L^{-1}	-	-	-	1	0.077	0.231
2.4 mg.L^{-1}	-	-	-	-	0.168	0.106
5.3 mg.L^{-1}	-	-	-	-	-	0.003

Table 3 Essay 1 p-values assuming mortality of zebrafish embryos due to abamectin concentrations.

Source: MR Santiago Thesis Project

*: statistically significant values, $p \leq 0.05$.

Treatment	Essay 2		
	n	Dead	%_Dead
Negative Control	24	2	8.3
0.5 mg.L^{-1}	20	5	25.0
1.1 mg.L^{-1}	20	9	45.0*
2.4 mg.L^{-1}	20	14	70.0*
5.3 mg.L^{-1}	20	12	60.0*
11.7 mg.L^{-1}	20	13	65.0*

Table 4 Effects of exposure to treatments with different concentrations of abamectin on zebrafish embryo mortality in Essay 2 at 96 hpf.

Source: MR Santiago Thesis Project

n: number of embryos per treatment

*: statistically significant values compared to the negative control, $p \leq 0.05$.

Treatment	Essay 2 (p-value)					
	Negative Control	0.5 mg.L^{-1}	1.1 mg.L^{-1}	2.4 mg.L^{-1}	5.3 mg.L^{-1}	11.7 mg.L^{-1}
Negative Control	-	0.217	0.012	0	0	0
0.5 mg.L^{-1}		-	0.185	0.004	0.025	0.011
1.1 mg.L^{-1}			-	0.2	0.342	0.204
2.4 mg.L^{-1}				-	0.507	0.736
5.3 mg.L^{-1}					-	0.744

Table 5 Essay 2 P-values assuming mortality of zebrafish embryos due to abamectin concentrations.

Source: MR Santiago Thesis Project

statistically significant values, $p \leq 0.05$.

Treatment	Essay 3		
	n	Dead	%_Dead
Negative Control	27	1	3.7
0.5 mg.L^{-1}	21	3	14.3
1.1 mg.L^{-1}	21	4	19.0
2.4 mg.L^{-1}	20	6	30.0*
5.3 mg.L^{-1}	20	9	45.0*
11.7 mg.L^{-1}	20	16	80.0*

Table 6 Effects of exposure to treatments with different concentrations of abamectin on zebrafish embryo mortality in Essay 3 at 96 hpf.

Source: MR Santiago Thesis Project

n: number of embryos per treatment

*: statistically significant values compared to the negative control, $p \leq 0.05$.

Treatment	Essay 3 (p-value)					
	Negative Control	0.5 mg.L^{-1}	1.1 mg.L^{-1}	2.4 mg.L^{-1}	5.3 mg.L^{-1}	11.7 mg.L^{-1}
Negative Control	-	0.306	0.153	0.000	0.001	0.000
0.5 mg.L^{-1}		-	1.000	0.224	0.031	0.000
1.1 mg.L^{-1}			-	0.484	0.074	0.000
2.4 mg.L^{-1}				-	0.327	0.001
5.3 mg.L^{-1}					-	0.022

Table 7 Essay 3 p-values assuming assuming mortality of zebrafish embryos due to abamectin abamectin concentrations.

Source: MR Santiago Thesis Project

statistically significant values, $p \leq 0.05$.

Chi-squared Test of Essays 1 vs 2 vs 3	
Treatment	P-value
Negative Control	0.743
0.5 mg.L^{-1}	0.612
1.1 mg.L^{-1}	0.064
2.4 mg.L^{-1}	0.003*
5.3 mg.L^{-1}	0.420
11.7 mg.L^{-1}	0.016*

Table 8 Comparison between the three Essays in relation to mortality of zebrafish embryos exposed to treatments with different concentrations of abamectin.

Source: MR Santiago Thesis Project

*: statistically significant values, $p \leq 0.05$

Treatment	Trial 1		
	n	Hatching Delay	% Hatching Delay
Negative Control	24	0	0
0.5 mg.L^{-1}	20	17	85.0*
1.1 mg.L^{-1}	20	17	85.0*
2.4 mg.L^{-1}	20	16	80.0*
5.3 mg.L^{-1}	20	12	60.0*
11.7 mg.L^{-1}	20	NA	NA

Table 9 Effects of exposure to treatments with different concentrations of abamectin on zebrafish embryo hatching in Essay 1 at 96 hpf.

Source: MR Santiago Thesis Project

n: number of embryos per treatment

NA: Not analyzed due to 100% death of embryos.

*: statistically significant values compared to the negative control, $p \leq 0.05$

Treatment	Essay 1 (P-value)					
	Negative Control	0.5 mg.L^{-1}	1.1 mg.L^{-1}	2.4 mg.L^{-1}	5.3 mg.L^{-1}	11.7 mg.L^{-1}
Negative Control	-	0	0	0	0	NA
0.5 mg.L^{-1}	-	-	1	1	0.077	NA
1.1 mg.L^{-1}	-	-	-	1	0.077	NA
2.4 mg.L^{-1}	-	-	-	-	0.168	NA
5.3 mg.L^{-1}	-	-	-	-	-	NA

Table 10 Essay 1 p-values1, assuming hatch delays of zebrafish due to abamectin concentrations at 96 hpf.

Source: MR Santiago Thesis Project

1The p-value tables are interpreted by initially associating the same concentration in the row with the column, continuing the comparisons of the other concentrations in the same row.

Treatment	Essay 2		
	n	Hatching Delay	% Hatching Delay
Negative Control	24	0	0
0.5 mg.L^{-1}	20	15	75.0*
1.1 mg.L^{-1}	20	11	55.0*
2.4 mg.L^{-1}	20	6	30.0*
5.3 mg.L^{-1}	20	8	40.0*
11.7 mg.L^{-1}	20	7	35.0*

Table 11: Effects of exposure to treatments with different concentrations of abamectin on zebrafish embryo hatching in Essay 2 at 96 hpf.

Source: MR Santiago Thesis Project

n: number of embryos per treatment

*: statistically significant values compared to the negative control, $p \leq 0.05$

Treatment	Essay 2 (p-value)					
	Negative Control	0.5 mg.L^{-1}	1.1 mg.L^{-1}	2.4 mg.L^{-1}	5.3 mg.L^{-1}	11.7 mg.L^{-1}
Negative Control	-	0	0	0.005	0.001	0.002
0.5 mg.L^{-1}		-	0.32	0.01	0.025	0.011
1.1 mg.L^{-1}			-	0.20	0.342	0.204
2.4 mg.L^{-1}				-	0.507	0.736
5.3 mg.L^{-1}					-	0.744

Table 12 P-values in Essay 2, assuming hatching delay of zebrafish due to formulation.

Source: MR Santiago Thesis Project

Statistically significant values p≤ 0.05

Treatment	Essay 3		
	n	Hatching Delay	% Hatching Delay
Negative Control	27	1	3.7
0.5 mg.L^{-1}	21	18	86.0*
1.1 mg.L^{-1}	21	17	81.0*
2.4 mg.L^{-1}	20	14	70.0*
5.3 mg.L^{-1}	20	11	55.0*
11.7 mg.L^{-1}	20	4	20.0

Table 13: Effects of exposure to treatments with different concentrations of abamectin on zebrafish embryo hatching in Essay 3 at 96 hpf.

Source: MR Santiago Thesis Project

n: number of embryos per treatment

*: statistically significant values compared to the negative control, p ≤ 0.05.

Treatment	Essay 3 (p-value)					
	Negative Control	0.5 mg.L^{-1}	1.1 mg.L^{-1}	2.4 mg.L^{-1}	5.3 mg.L^{-1}	11.7 mg.L^{-1}
Negative Control	-	0*	0*	0*	0*	0.148
0.5 mg.L^{-1}		-	1	0.235	0.013	0
1.1 mg.L^{-1}			-	0.451	0.038	0
2.4 mg.L^{-1}				-	0.327	0.001
5.3 mg.L^{-1}					-	0.022

Table 14 Essay 3 p-values, attributing hatch delays of zebrafish to the formulation.

Source: MR Santiago Thesis Project

*: statistically significant values compared to the negative control, p ≤ 0.05.

Pearson's Chi-squared Test of Essays 1 vs 2 vs 3	
Treatment	**P-value**
Negative Control	0.406
0.5 $mg.L^{-1}$	0.432
1.1 $mg.L^{-1}$	0.041*
2.4 $mg.L^{-1}$	0.003*
5.3 $mg.L^{-1}$	0.420
11.7 $mg.L^{-1}$	0.016*

Table 15 Comparison between the three Essays in relation to the delay in hatching of zebrafish embryos exposed to treatments with different concentrations of abamectin.

Source: MR Santiago Thesis Project

*: statistically significant values, $p \leq 0.05$