Embryotoxic, Developmental and Genotoxic Evaluations of a Endosulfan and Deltamethrin Mixture on the African Sharptooth Catfish (*Clarias gariepinus*)

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Abstract

The evaluation of pesticides using early life stages of fish yields high throughput results that can inform one about the developmental effects of these toxicants. The effects of sublethal concentrations of a commercial mixture of endosulfan and deltamethrin (ratio 280:2) were evaluated in the embryos and juveniles of *Clarias gariepinus*. Embryotoxic and developmental evaluations were conducted in the embryos from 0 - 26 hours post fertilization. Genotoxic effects were assessed in the erythrocytes of the juveniles using the micronucleus assay over a period of 28 days. The acute toxicity studies revealed that the 24 hLC_{50} and 26 hEC_{50} (non-hatching) values for embryos were 25.25 µg/L and 12.96 µg/L respectively while the 96 hLC_{50} value for fingerlings was 12.67 µg/L. Hatching success was lower in the exposed embryos compared to the control. The percentage developmental abnormalities and number of heartbeats per minute were statistically higher (p<0.05) in the exposed embryos compared to the control. Developmental abnormalities observed included pericardial and yolk sac oedema, scoliosis and tail curvature. The genotoxicity studies showed that there was a significant increase (p<0.05) in micronuclei in the erythrocytes of the exposed juveniles by days 14 and 28 compared to the control animals. These results imply that the pesticide mixture can cause a decline in the population of non-target organisms such as fish at sub-lethal or environmentally realistic concentrations. A review of the use of this pesticide formulation and development of environmentally friendly methods for pest management are recommended to safeguard non-target organisms such as the African sharptooth catfish.

Introduction

Endosulfan (6, 7, 8, 9, 10, 10-Hexachloro-1, 5, 5a, 6, 9, 9a-hexahydro- 6, 9-methano-2, 4, 3-benzodioxathiepine-3-oxide, CAS No. 115-29- 7) is an organochlorine insecticide that is ubiquitous, persistent and toxic to animals (Quinete, 2013). It is widely used to protect food crops in agriculture and integrated aquaculture farms (Piazza et al., 2015). Pyrethroids such as deltamethrin ([(*S*)-Cyano-(3-phenoxyphenyl)-methyl] (1R, 3R)-3-(2, 2-dibromoethenyl)-2,2-dimethyl-cyclopropane-1-carboxylate, CAS No. 52918-63-5) are regarded as safer alternatives to other insecticides due to their efficacy and biodegradability (Hamed, 2016). These insecticides contaminate soil as well as surface and ground water causing deleterious effects in non-target organisms like aquatic animals and soil microorganisms (Liang *et al.,* 2013; Meffe and de Bustamante, 2014). The biological effects of these insecticides when acting singly on non-target organisms have been demonstrated. For example, delayed hatching, reduced hatching success, altered mobility, were observed in embryos of the Japanese rice fish, *Oryzias latipes* (Family Adrianichthyidae) exposed to endosulfan concentrations of 0.01 – 1.0 µg/L (Gormley *et al.*, 2003). Deltamethrin has also been shown to alter development and decrease hatching success (that is, the level of hatching) in the common Carp, *Cyprinus carpio* embryos (Family Cyprinidae) (Koprucu and Aydin, 2004); induce neuro-behavioural effects (spastic movements), developmental abnormalities such as pericardial oedema and craniofacial abnormalities in embryos of the Zebrafish, *Danio rerio* (Family Cyprinidae)

(DeMicco *et al.*, 2010); and cause fry lethality and loss of equilibrium in the Rainbow trout, *Oncorhyncus mykiss* (Family Salmonidae) (Ural and Saglam, 2005). Beyond biological effects at the individual and population levels of organization, macromolecular effects on the genetic material in exposed organisms is essential in order to provide early warning signals before observable effects at higher levels of biological organization. Consequently, induction of genetic damage in the DNA following acute and/or chronic exposure to xenobiotics can be evaluated using the micronucleus and the single cell gel electrophoresis (SCGE) assays (Ruiz de Arcaute *et al.*, 2016; Sogbanmu *et al.*, 2016).

The issues of insecticide resistance and efficacy of insecticides when acting singly have led to the development and usage of insecticide mixtures. This is based on the theory that binary mixtures of insecticides will ensure that insects that survive exposure to one insecticide will be killed by the other ultimately delaying the development of resistance to the insecticide (Shi *et al.*, 2012). Furthermore, insecticide mixtures may be synergistic leading to reduced costs and increased efficacy against insect pests. However, the biological effects of such mixtures in non-target organisms such as fishes, earthworms, snails and benthos which are inadvertently exposed to these pesticide mixtures via different routes (atmospheric deposition and surface run-off from farmlands) are mostly unknown. Due to the widespread use of pesticides in agriculture, home pest control, protection of foodstuff and disease vector control, humans may be exposed through ingestion of food contaminated with low levels of the pesticide residues or through inhalation and/or dermal exposure in or around the home and other indoor environments (Orlu, 2013).

The African sharptooth catfish (*Clarias gariepinus*; Burchell 1822) (Family Clariidae) is a species of air-breathing fish due to the presence of accessory respiratory organs beside the gills which enable it to survive for a long time in debilitating anoxic environments or outside water (Olson *et al.*, 1995). It is an ecologically and commercially important freshwater fish that is found in the wild and one of the most cultured fish species in several African countries especially Nigeria (Esenowo and Ugwumba, 2010). It is also a model organism that has been utilized extensively for various eco-toxicological studies, particularly as a non-target aquatic organism that is impacted by pesticide run-offs from agricultural farms into surface waters (Datta and Kaviraj, 2003; Jenyo-Oni *et al.* 2011). Thus, the aim of this study was to evaluate the acute toxicity, developmental and genotoxic effects of environmentally-realistic (sublethal) concentrations of a commercial mixture of endosulfan and deltamethrin on the embryos and juveniles of *Clarias gariepinus*.

Materials and methods

Test compound

The pesticide used in this study was Cracker 282 EC (Endosulfan - 280 g/L + Deltamethrin - 2 g/L). It was obtained from the Department of Entomology, Cocoa Research Institute of Nigeria (CRIN), Ibadan, Nigeria. It is manufactured by Hoechst Schering Agrevo SA. It is a light yellow coloured liquid which was stored at room temperature.

Test animal: collection and acclimatization

Fingerlings (weight: 6 – 10 g; length: 4.8 – 6.0 cm) and Juveniles (weight: 17 – 25 g and length: 14.5 - 17.1 cm) of *Clarias gariepinus*

were obtained from a fish farm in Gbagada, Lagos, Nigeria. Two male broodstocks of *C. gariepinus* (weight: 1 ± 0.2 kg; length: 49.2–49.9 cm) were obtained from a fish farm in Surulere, Lagos, Nigeria, while two female broodstocks of *C. gariepinus* (weight: 1 ± 0.2 kg; length: 46.8–50.4 cm) were obtained from the Department of Marine Science, University of Lagos, Lagos, Nigeria. During acclimatization, the fish were fed twice daily with a commercial fish feed (Coppens) and the water in the holding tanks was renewed daily (this was done by replenishing 50% of the water in the stock tank to avoid stress to the fish). Feeding was stopped 24 h prior to commencement of the bioassays (Qayoom *et al.*, 2016).

Procedure for spawning Clarias gariepinus embryos

Embryos were spawned as described in Sogbanmu (2015). Briefly, a female broodstock *C. gariepinus* was injected with OVAPRIM hormone (Salmon Gonadotropin Releasing Hormone Analogue and Domperidone injection manufactured by Syndel Laboratories Ltd, Canada) at 0.5 mL per kg of fish. After 10 hours latency period, slight pressure (by hand) was applied to the abdomen of the females to induce the release of eggs which were collected in a plastic bowl. A male broodstock *C. gariepinus* was euthanized (by a single sharp blow to the head with the aid of a hammer followed by pithing (AVMA, 2013)) and the testes carefully removed with the aid of a new razor blade within 5 min. The testes were kept on white paper prior to dissection. These were slightly dissected to let out the milt used for fertilizing the eggs. Fertilization was aided with the addition of saline water to the mixture and the bowl was gently swirled to ensure adequate mixing of the milt with the eggs within 3 min at 27 ± 2 °C. Fertilized eggs were identified and confirmed with the aid of a dissecting microscope (Ceti Star – 13 ED Stereomicroscope, Medline Scientific, United Kingdom). Fertilization was considered to have occurred when the egg-yolk was transparent greenish-orange and cell division was clearly visible in the blastodisc (Oellermann, 1995).

Acute toxicity studies with Clarias gariepinus embryos and fingerlings

This was conducted with embryos (age: 3 min post-fertilization) and fingerlings of *C. gariepinus* in petri dishes (diameter: 90 mm) and 5 L glass tanks respectively. Working solutions were prepared from a stock solution of 1 mL/L (that is, 282 mg/L) of the test compound. Initial range finding tests in which test animals were exposed to the test compound in duplicates (that is, test concentrations were replicated) were conducted to establish the range of concentrations for the test compound before the definitive tests. For the definitive tests, four (4) fingerlings of similar sizes in duplicates and fifty (50) fertilized embryos in triplicates were exposed to varying concentrations of 5, 10, 20, 40, 80 µg/L of Cracker 282 and untreated control (water alone). A static non-renewable bioassay protocol was employed in which test media was not renewed throughout the duration of the experiment (24 h for embryos, 96 h for fingerlings) following the standard methods of APHA (1998) and OECD (2013). Mortality was assessed every 6 h over a period of 24 h for embryos, with the aid of a dissecting microscope and every 24 h over a period of 96 h for fingerlings. The percentage of embryos that did not hatch (non-hatching) after 26 h

was also assessed (OECD, 2013).

Sublethal toxicity studies with Clarias gariepinus embryos and juveniles

Embryotoxicity and developmental toxicity studies with *Clarias gariepinus* embryos

Clarias gariepinus embryos were exposed to sublethal concentrations (1/10th and 1/100th of 96 hLC50 value) of the test compound. Fifty (50) fertilized embryos were exposed to test media in triplicates including control from 0 to 26 hours post-fertilization (hpf). Hatching success, developmental abnormalities and number of heart beats per minute (NHBpM) were assessed at 26 hpf according to Sogbanmu et al. (2016). Hatching success was measured as the percentage of embryos that hatched (fully emerge from the chorion) at 26 hpf. Developmental abnormalities were calculated as the percentage of embryos observed under the dissecting microscope with one or more developmental abnormalities such as pericardial oedema, yolk-sac oedema, curved and/or stunted tail and scoliosis. The number of heart beats per minute in the fish embryos were evaluated as the number of heartbeats recorded as viewed with the aid of a dissecting microscope for 30 secs. This number was extrapolated to cover the number of heart beats per minute (60 secs).

Genotoxicity studies (micronucleus assay) with *Clarias gariepinus* juveniles

Clarias gariepinus juveniles (weight: 17 – 25 g and length: 14.5 - 17.1 cm) were also exposed to sublethal concentrations (1/10th and 1/100th of 96 hLC_{50} value) of the test compound in triplicates for 28 days. At post-treatment periods of 14 and 28 days, blood samples were collected from fishes exposed to the test compound and the control set up for micronucleus assay (Obiakor et al., 2014). The peripheral blood was collected from the caudal vein of the fishes. A smear was prepared on a clean glass slide and fixed with methanol for 5 mins, air dried and stained with 2% Giemsa. The slides were staged and analysed for 1000 cells/individual with micronuclei.

Statistical analysis

The relative acute toxicity data for embryos and fingerlings were analyzed by probit using SPSS 20.0 version. One way analysis of variance (ANOVA), Fisher's LSD (least significance difference) test and multiple comparison analysis were employed to test for significant difference between treatment means and control at $p<0.05$. Results are presented as mean ± standard error. Figures were prepared using Microsoft Office Excel 2010 version.

Results

Acute toxicity studies

The results of the relative acute toxicity studies with *C. gariepinus* (Table 1) embryos and fingerlings showed that the fingerlings (96 hLC_{50} – 12.67 µg/L) were more susceptible to the pesticide compared to the embryos (24 hLC_{50} – 25.25 µg/L). The median concentration (26 hEC_{50} – 12.96 µg/L) for embryos that did not hatch also revealed that the endpoint (non-hatching) was a precursor for lethality to the embryos.

Embryotoxicity and developmental toxicity studies with Clarias gariepinus embryos

For the embryotoxicity and developmental toxicity studies, environmentally realistic concentrations (1/10th and 1/100th of 96 hLC_{50} – 1.27 µg/L and 0.13 µg/L respectively) of the test compound showed that there were

TABLE 1
Acute toxicity of Cracker 282 (Endosulfan:Deltamethrin ratio 280:2) against *Clarias gariepinus* Embryos and Fingerlings

Fish Life stage	Endpoint (µg/L)	Slope ± SE	Probit Line Equation
Embryo	24 hLC_{50} - 25.25	2.14 ± 0.18	Y= 2.78 + 2.14x
Embryo	26 hEC_{50} -12.96	1.75 ± 0.22	Y= 2.25 + 1.75x
Fingerling	96 hLC_{50} - 12.67	2.50 ± 1.04	Y= -3.00 + 2.50x

KEY: SE - Standard Error, LC - Lethal concentration, EC – Effective Concentration (Non-Hatching)

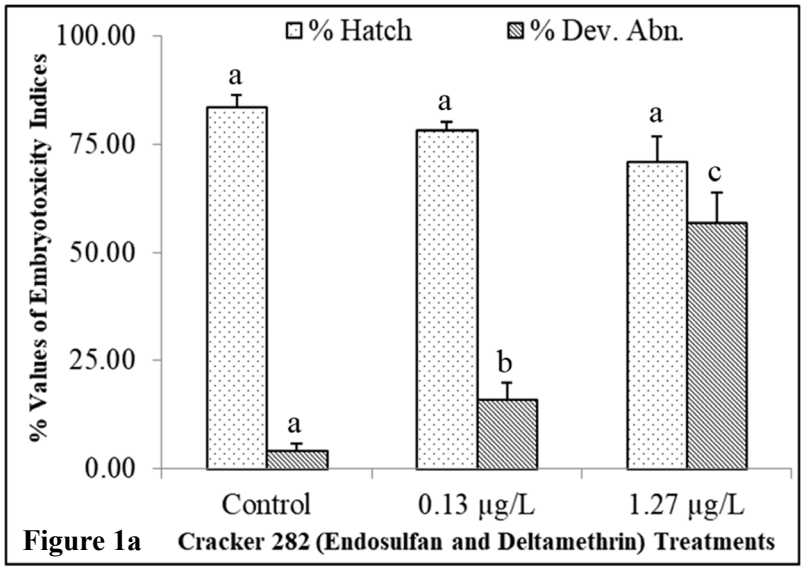

Figure 1a

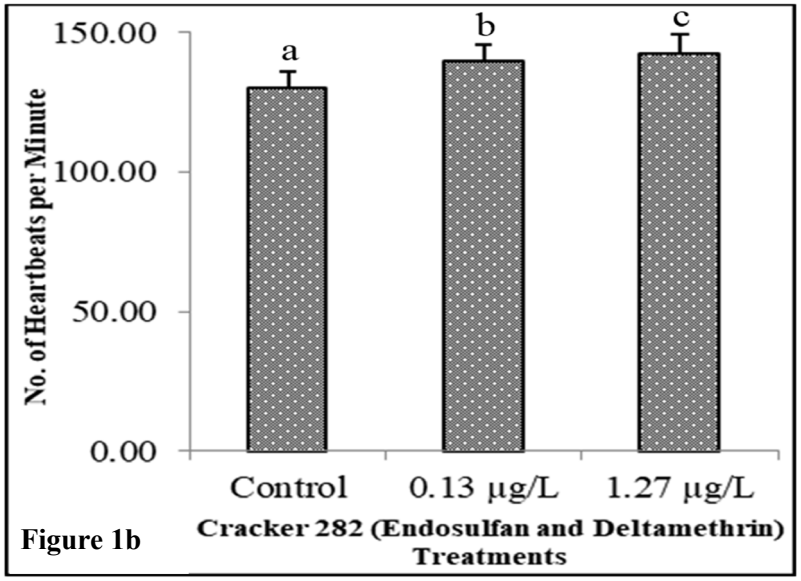

Figure 1b

Figure 1a & b: Percentage Hatching Success, Developmental Abnormalities (Figure 1a) and Number of Heartbeats per Minute (Figure 1b) in *Clarias gariepinus* Embryos exposed to Cracker 282 (Endosulfan:Deltamethrin ratio 280:2) for 26 hours.

Key: % Hatch – Percentage Hatching Success, % Dev. Abn. – Percentage Developmental Abnormality, Dissimilar letters (in superscripts) represent significant difference between treatment means and control at $p<0.05$. For hatching success (hatch) and developmental abnormality (dev. abnor.), n=150 embryos, number of heartbeats per minute (NHBpM), n = 15 embryos.

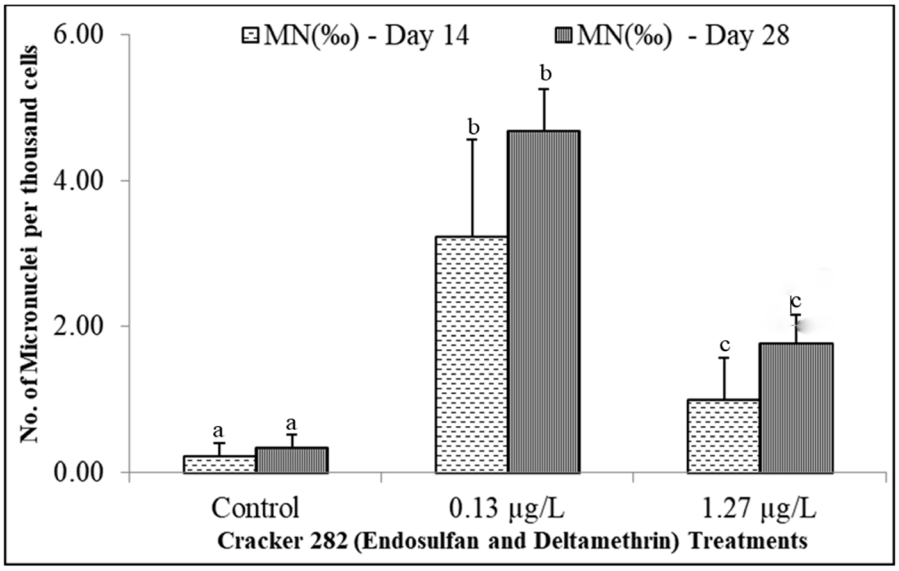

Figure 2: Genotoxicity of Cracker 282 (Endosulfan:Deltamethrin ratio 280:2) on *Clarias gariepinus* over a period of 28 days.

Note: Dissimilar letters (in superscripts) represent significant difference between treatment means and control at p<0.05. MN (‰) – micronuclei per thousand cells, n=9000 cells.

no statistically significant (p>0.05) differences in the hatching success of exposed embryos and control (Figure 1a). Percentage hatching success was highest for embryos in the control (83.33 % ± 3.05) followed by embryos in the lowest treatment of 0.13 µg/L (78.00 % ± 2.00) and the highest treatment of 1.27 µg/L (70.66 % ± 6.11).

Developmental abnormalities and number of heartbeats per minute were however statistically higher (p<0.05) in the exposed embryos compared to the control. Developmental abnormalities observed were pericardial and yolk sac oedema, scoliosis and tail curvature. These abnormalities were highest in the 1.27 µg/L treatment (56.67 % ± 7.02), followed by the 0.13 µg/L treatment (16.00 % ± 4.00), and control was 4.00 % ± 2.00 (Figure 1a). The number of heartbeats per minute was highest in the 1.27 µg/L treatment (142.67 ± 6.62) followed by the 0.13 µg/L treatment (139.60 ± 5.87) and control was 130.13 ± 5.68 (Figure 1b).

Genotoxicity studies (micronucleus assay) with Clarias gariepinus juveniles

The assessment of genotoxicity using the micronucleus assay showed that the frequency of micronuclei increased statistically from day w0 to 28 in the exposed fish compared to control (Figure 2). Although, micronuclei were higher in fish exposed at the lowest concentration of 0.13 µg/L (Day 14 – 3.22 ± 1.34 ‰; Day 28 – 4.67 ± 0.58 ‰) compared to the highest concentration of 1.27 µg/L (Day 14 – 1.00 ± 0.57 ‰; Day 28 – 1.77 ± 0.39 ‰) of the pesticide. Micronuclei in the control fish blood were 0.22 ± 0.19 ‰ and 0.33 ± 0.19 ‰ on days 14 and 28 respectively.

Discussion

Data from acute toxicity studies based on mortality are of biological and ecological importance as they can be used to determine application factors for derivation of water quality guidelines (Kumar *et al.,* 2016). In this study, the mortality of embryos and fingerlings

of *Clarias gariepinus* were dose-dependent (Table 1). There is hardly any report on the 96 h LC_{50} values of endosulfan and deltamethrin mixtures though various authors have reported similar values as obtained in this study for endosulfan and deltamethrin acting singly against fishes. Boateng *et al.* (2006) reported a 96 hLC_{50} value of 15.47 µg/L of deltamethrin against *Oreochromis niloticus.* Conversely, the 96 hL_{50} value (12.67 µg/L) of the pesticide (endosulfan :deltamethrin ratio 280:2) to *C. gariepinus* in this study is far lower (more toxic) than that obtained for *Chanos chanos* (Milk fish) exposed to endosulfan alone (21.50 µg/L). Jenyo-Oni *et al.* (2011) reported a 96 hLC_{50} value of 2.09 µg/L for endosulfan on fingerlings of *C. gariepinus*. According to Datta and Kaviraj (2003), the 96 hLC_{50} of deltamethrin on *C. gariepinus* was 0.004 µg/L. A probable reason for the variations in toxicity of the chemicals could be as a result of differences in testing protocol used, susceptibility and tolerance of the animals related to their accumulation, biotransformation, and excretion (Omitoyin *et al.,* 2006).

Furthermore, *C. gariepinus* embryos were more resistant to the test compound (Cracker 282 EC (Endosulfan - 280 g/L + Deltamethrin - 2 g/L)) than the fingerlings (24 hLC_{50} embryo – 25.25 µg/L; 96 hLC_{50} fingerlings – 12.67 µg/L). This could be attributed to the presence of the embryonic chorion which serves as a protective layer preventing the test compound from penetrating the embryo. Embryonic stages of fish have been recognized as a more resistant life stage than those immediately following hatching (eleutheroembryo) and continuing through the juvenile stages (Léonard *et al.*, 2005). The evaluation on non-lethal end points in acute toxicity studies provides an indication of subtle effects of exposure to toxic substances within a short time which usually precedes mortality. In this study, based on the 26 hEC_{50} value (12.96 µg/L) for non-hatching of exposed *C. gariepinus* embryos, mortality of the embryos at higher concentrations may be preceded by no hatching success at lower concentrations of the test compound. In other words, if the concentration of the test compound in the environment is increased beyond the EC_{50} level, non-hatching would precede death eventually.

The developmental effects and morphological abnormalities observed in this study were also dose-dependent (Figures 1a and 1b). Similar results were observed in Oryzias latipes embryos exposed to endosulfan concentrations of 0.01 – 1.0 µg/L (Gormley *et al.,* 2003), *Cyprinus carpio* embryos exposed to deltamethrin (Koprucu and Aydin 2004) and *Danio rerio* embryos exposed to deltamethrin (DeMicco *et al.*, 2010). These physiological, behavioural and population level end points are driven by genetic and cellular effects or mechanisms which result in oxidative stress in a time and concentration-dependent manner (Paskova *et al.*, 2011; Safari *et al.*, 2016). These are phenotypically expressed as observed in this study and will ultimately result in the death or decline in population of such exposed organisms.

The micronucleus test was employed to assess the potential genotoxic effect of the test compound on juveniles of *Clarias gariepinus.* The induction of micronuclei in peripheral erythrocytes of *Clarias gariepinus* juveniles exposed to sublethal concentrations of the test chemical is consistent with the results of Grisciola *et al*. (2002) and Ansari *et al.* (2009), who reported that deltamethrin increased

micronuclei frequency in *Tilapia rendalli* and the fresh water fish, *Channa puncata* respectively. It is also in agreement with the results obtained by Dar *et al.* (2015) who reported that endosulfan induced genotoxicity in freshwater cyprinid fish (*Carassius carassius*) by micronucleus formation in all treated groups. Genetic damage in fish cells could play a major role in decreasing the fitness of fish populations with short and long-term consequences on their survival (Thomas *et al*., 2014).

Conclusion

This study has shown the potential developmental and genotoxic effects of sublethal concentrations of Cracker 282 (Endosulfan /Deltamethrin ratio 280:2) on *Clarias gariepinus* which can serve as biomarkers of effects of this insecticide mixture. The results also imply that the insecticide mixture may cause a decline in the population of non-target organisms such as fish at sub-lethal or environmentally realistic concentrations based on the embryotoxic and developmental effects observed. In view of the results, we recommend further studies in the field to ascertain the levels of the pesticides that run-off into freshwater ecosystems and evaluation of other biological responses at higher levels of organization. A review of the use of this insecticide formulation and development of more environmentally friendly methods for pest management are also recommended to safeguard non-target organisms such as the African sharptooth catfish.

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