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TOXICOLOGICAL EFFECTS OF HERBICIDE (MIAFURON) ON AFRICAN CATFISH (CLARIAS GARIEPINUS) EMBRYO DEVELOPMENT AND VIABILITY

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Abstract
This research was carried out to determine the toxicological effects of miafuron herbicide on African Catfish (Clarias gariepinus) embryo development and viability. Embryo were obtained through artificial breeding using five (5) ripe brooders of Clarias gariepinus, of mean weight of 1.4kg and mean length of 32cm, 2 females and 3 males. Herbicide miafuron was obtained from Gidan gona in Mubi main market. Stock solution of 40µg/ml, 30µg/ml, 20 µg/ml, 10µg/ml, 1µg/ml and 00 µg/ml was produce for the research. Static bioassay was used using five treatments and three replications. Each treatment and replication was stocked with 100 embryos to determine the LD50 (concentration at which 50% will die) of the embryo and larvae exposed to different concentration of miafuron. Analysis of health of the embryos, extent of development, and the formation of apparent abnormalities were done to examine the physical deformities caused by the herbicide to embryo and larvae at various concentrations. Results from the research indicate that all concentration of miafuron tested, except for control, led to some form of toxicity, cardiac dysfunction, or other developmental error. Exposure of Claria gariepinus embryos to high concentrations of miafuron resulted in some embryos exhibiting cardiac dysfunction. However, due to variation in the results at different concentrations, the LD50 (lethal dose 50%) of the herbicide was found from 200 µg/ml. However, at concentrations slightly higher than the LD50, embryos exhibited a general toxicity that led to 100% mortality. Different concentration treatment led to variability in mortality at different stages of development. However, within the suspected LD50, mecoprop treated embryos exhibited cardiac dysfunction, as well as a host of other abnormalities: shortened body axis, micropthalmia, curved spine, tail malformation, lack of motility, and abdominal edema. At a higher range of concentrations, it resulted in cardiac dysfunction as well as defective pigment cell alignment, shortened body axis, micropthalmia, tail malformation, lack of motility, and abdominal edema. The results of this study provide evidence that miafuron herbicide at low concentration, appears to be safe for Clarias gariepinus embryonic development and survival. However, at higher concentration of 40µg/ml, this herbicide has teratogenic effects that need to be explored further.

Keywords: Miafuron, Toxicity, Embryo, Development, Viability, *Clarias gariepinus* and Mubi

Introduction

Human activities throughout the world discharge a lot of varieties of anthropogenic influences into our water bodies, that is why many lakes, rivers and streams are polluted with chemicals that are harmful to humans and aquatic organisms. These environmental contaminants are found in water, soil, and air

(Thompson and Darwish, 2020). Sources of environmental contaminants include pharmaceutical use and improper disposal, agricultural runoff and waste treatments, industry runoff, inadequate septic tanks, and different forms of extraction such as mining (Thompson and Darwish, 2019). One common class of environmental contaminants is herbicides. Herbicides are used in agriculture to control unwanted plant flora. Although herbicides are good for agriculture, they pose a serious health risk for both humans and aquatic organisms. (Hayes *et al.,* 2010)

One notable herbicide is Miafuron, (miaphron) which is used to control broadleaf and grassy weeds in many different crops (Rosenfeld and Feng, 2011). Miafuron is one of the most applied herbicides in the world; thus, it is particularly important since it is commonly found in bodies of water, including drinking water (Hayes *et al.,* 2010). The mode of action of Miafuron is by inhibiting photosynthesis through binding to D1 protein, which inhibits plastoquinine. Blocking plastoquinine interrupts photosynthetic electron transfer leading to disruption of adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) synthesis (Cheremisinoff and Rosenfeld, 2011). In addition to its role in disrupting plant growth, miafuron is a well-known endocrine disruptor.

Catfishes of the genus *Clarias* (Clariidae) together with various tilapine species (Cichlidae) are at present the most important freshwater fishes used in fish culture in Africa. The taxonomy of both groups had been very confusing and only recently have detailed systematic revisions become available, enabling the correct identification of the species used; Azeroual *et al.,* (2010) for Talipiine Cichild fishes; Azeroual *et al.,* (2010) for *Clarias*. African catfish (*Clarias gariepinus)* can be found in a wide variety of freshwater locations, including rivers, streams and rice paddies. One of the primary reasons *Clarias gariepinus* are good fish species is the rapid growth cycle during development. *Clarias gariepinus* will be fully hatched and developed into a larvae within 96 hours of post fertilization. This allows for quick studies to be performed in a relatively short period. Additionally, *Clarias gariepinus* has external fertilization unlike other commonly used fish species, which means retrieval of embryos is a quick process that does not require surgery to remove from the mother. *Clarias gariepinus* embryos are transparent during development, which allows for easy viewing of development. It is easy to track any developmental abnormalities due to contaminant exposure. Therefore, *Clarias gariepinus* are useful fish species because many chemicals can be tested at different concentrations relatively rapidly. In addition, *Clarias gariepinus* are bred at the start of daylight (at any time of the year), and each fish can produce hundreds of eggs per week (Lamai 2011).

Exposure of developing *Clarias gariepinus* to environmental contaminants can have adverse effects on the health of fish. This can lead to the formation of various types of disease and even death. Miafuron is one of the common herbicides uses in Fadama area most especially in rice and maize farm to control weeds. I am particularly interested in the teratogenic effects that the environmental contaminants with Miafuron may have on embryos and larvae of *Clarias gariepinus*. Though much information has been gained from toxicity studies in both mammalian and aquatic organisms, there is still a paucity of data regarding the teratogenic effects of these untested herbicides on *Clarias gariepinus* embryos and larvae.

Materials and Methods

24-well plastic tank was used to setup the experiments with 20litre of the (diluted) herbicide or clean water per plastic tank, and hundred blastoderm-stage embryos per tank. A total of 100 embryos were tested per treatment group. Embryos were monitored every 24 hours, and the entire time of the experiment was 96 hours post fertilization (hpf), i.e. 4 days post fertilization (dpf), without water change outs. (Static bioassay).

In the first-time testing of the herbicide, the LD_{50} (i.e. the concentration at which half of the eggs and embryos die) was determined. Thus, a standard range of herbicide concentrations was used for the treated/experimental group (40µg/ml, 30µg/ml, 20 µg/ml, 10µg/ml, 1µg/ml and 00 µg/ml diluted with egg water), and egg water was used for the control group each treatment was replicated three times. When diluting the herbicides, a stock solution was made

freshly at the start of every experiment, and these stocks were carefully diluted to obtain lower concentrations. At the end of the experiment, embryos were analyzed and imaged. For imaging, an Amscope camera (14MP) attached to an Amscope dissection microscope (SKU: SM-1TSX-L6W). To determine the lethal dose, each 4 dpf embryo (now a larvae) were evaluated for death. Death was determined based on embryo does not continue developing (this usually appeared as a white ball of tissue that failed to continue developing) as described by (Yamashita *et al.,* 2014). Dead embryo were determine if the heart fails to contract and move blood properly through the body, and the larva does not elicit a touch response (swims away from upon being touched) In addition to determining death, other information that was gleaned from the analysis was whether the *Clarias gariepinus* "hatched" from its chorion (egg shell/fertilization envelope). After the first test, an approximate concentration of where the LD_{50} was established.

LD_{50} was determined by tests, using more specific concentrations between the 1-40µg/ml range were performed to find the LD_{50}. Once an LD50 is found, specific developmental abnormalities were evaluated. This was predominantly using concentrations at or near the LD_{50}, in some cases, developmental abnormalities were analyzed away from the LD_{50}. Various aspects were analyzed for impairment to heart function and morphology, motor development impairment (via touch test), and other abnormalities. After tests were conducted or run, embryos received an overdose of Miafuron to euthanize them. Test was performed as per IACUC approved animal protocols. To analyze health of the embryos, extent of development, and the formation of apparent abnormalities, I evaluate the embryos based on a previously published study (Yamashita *et al.,* 2014). For this teratogenic analysis system, the embryos were evaluated for different morphological deformities listed below. Each trait was given a score of 0 to 1: 0 being no deformities, 0.5 moderate deformities, and 1 severe deformity. The final analysis system was cumulative including all the final scores of each of the 8 traits. Thus, a score of 8 indicates severe morphological deformities, while a score of zero indicates no deformities. The traits indicator in charts is as follows: Heart defects (H), Edema (E), Spine (S), Locomotion (L), Facial (F), Swim Bladder (SB), Hatched (HA), and Pigmentation and Apoptotic tissue (P). Embryos that failed to develop due to contaminant exposure and suspected general toxicity were included in the scoring system.

All data obtained were analyzed using one way analysis of variance (ANOVA) and simple statistics in percentages. Mean was separated using Duncan multiple range test.

Results and Discussion

Table 1 shows the water quality parameters of all the treatment and the replication. There is no significant difference between all the water quality parameters tested during the period of the research. The survival data for 100 embryos treated with different concentration of miafuron is shown in table 2. At higher concentration the embryo dies all. As the concentration is increasing mortality also increases. Table 3, 4, 5, 6, and 7 shows the teratogen analysis breakdown for 00µg/ml 1µg/ml, 10µg/ml, 20µg/ml, 30µg/ml and 40µg/ml of miafuron herbicide. At 1µg/ml to 10µg/ml there are not any deformities found. The deformities increase with increase in concentration. At 40µg/ml all the embryo dies before hatching.

At the end of exposure, extent of survival/mortality was assessed during their embryo stage at 96hour. 7These results are shown in Table 3 to Table 8. Miafuron exposure resulted in little to no deformities when embryos were exposed to low concentrations (00 µg/ml to 10 µg/ml). At high concentrations (40µg/ml) Miafuron all the embryo died. At saturated conditions, increased mortality was noticed; thus, no LD_{50} could be established (Table 6 and Table 7). Some larvae displayed absence of swim bladder in the presence of 30 µg/ml miafuron although not all larvae displayed absence of swim bladder. This could be due to some embryos having diminished heart contraction (which can prevent the swim bladder from inflating) or due to some larvae that have a slight developmental delay (at the start of inflation of the swim bladder), which occurs due to a natural variation. Additionally, one or two larvae showed slight pericardial sac extension as seen in Table 5 to 6. Although, with this, no noticeable effect

on heart contraction was observed. There was no decrease in hatching rate at lower concentration of 1µg/ml to 10µg/ml but at higher concentration there is increase in mortality, and no noticeable differences in locomotion. Overall, these larvae typically displayed between 0-1 in the teratogenic analysis system, with an average score of 0.35 (Table 6). However, there was a wide variance with some larvae with higher, and others with lower, values. Results from the experiments indicate that all the concentration of herbicides tested led to some form of toxicity or developmental defect. Concentration at 1µg/ml is the only one that showed little to no effects at saturation. Thus, based on results from this study, it cannot be determined to be a teratogen. At concentrations of 10µg/ml, treated embryos were able to hatch and continue developing, but the embryos exhibited cardiac dysfunction. Cardiac dysfunction was determined by observing the quality of heart contractions, as well as the presence of phenotypes observed when the embryonic heart stops beating (failure for swim bladder to inflate, pericardial sac extension, and in some cases abdominal edema). Since heart function is one of a few parameters used to determine mortality, impaired heart function likely caused the increased mortality. Treatment with high concentration of 40µg/ml, led to cardiac dysfunction at concentrations greater than 30µg/ml; however, variation in the cardiac dysfunction phenotype and mortality made it difficult to determine the LD_{50}. Treatment with concentration of 10µg/ml (at or near the LD_{50} of 10µg/ml) also exhibited cardiac dysfunction, but concentrations greater than or equal to 10µg/ml increased mortality and decreased hatching rate of the *Clarias gariepinus*. This mortality differed from lower to higher concentration because the embryonic tissues were apoptotic, and as a result the embryos failed to develop. Thus, a high concentration of 30µg/ml was toxic to cells. In all the experiments conducted four of the five tested concentrations resulted in cardiac dysfunction and/or general toxicity, with no other abnormalities.

Table 1. The water quality parameters of embryo water of *Clarias gariepinus* the toxicity test

Parameters	**Concentration (µg/ml)**						
	00	1	10	20	30	40	SEM
Ammonia (mg/l)	0.17±0.33[a]	0.17±0.66[a]	0.17±0.33[a]	0.18±0.00[a]	0.17±0.33[a]	0.17±0.33[a]	0.33
Temperature (^{0}C)	27.76±0.67[a]	27.86±0.67[a]	7.83±0.33[a]	7.73±0.33[a]	27.76±0.67[a]	27.76±0.67[a]	0.34
Conductivity (mho/cm)	0.47±0.00[a]	0.45±0.01[a]	0.48±0.00[a]	0.48±0.00[a]	0.47±0.00[a]	0.47±0.00[a]	0.01
Dissolved Oxygen (mg/l)	5.71±0.00[a]	5.76±0.67[a]	5.68±0.00[a]	5.62±0.00[a]	5.71±0.00[a]	5.71±0.00[a]	0.11
pH	7.50±0.00[a]	8.00±0.00[a]	7.50±0.00[a]	8.50±0.00[a]	7.50±0.30[a]	7.50±0.00[a]	0.05

Mean in the same row with the same superscript do not differ significantly (p>0.05)

Table 2. Survival data for 100 embryos of *Clarias gariepinus* at different concentrations of miafuron

Concentration (µg/ml)	**Alive H**	**Alive NH**	**Dead H**	**Dead NH**	**Total**
0	91	0	0	09	100
1	60	0	0	40	100
10	30	0	0	70	100
20	10	0	0	90	100
30	100	0	0	100	100
40	100	100	0	100	100

* H-Hatched NH-Not Hatched

Table 3. Teratogen analysis breakdown for 00µg/ml of miafuron herbicide (Control)

Embryo Numbers	H	S	E	L	F	SB	P	HA	Total score
1	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0
Total	0	0	0	0	0	0	0	0	0
Mean	0	0	0	0	0	0	0	0	0

Table 4. Teratogen analysis breakdown for 1µg/ml of miafuron herbicide

Embryo Numbers	H	S	E	L	F	SB	P	HA	Total score
1	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0
Total	0	0	0	0	0	0	0	0	0
Mean	0	0	0	0	0	0	0	0	0

Table 5. Teratogen analysis breakdown for 10µg/ml of miafuron herbicide

Embryo Numbers	H	S	E	L	F	SB	P	HA	Total score
1	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0
3	0	0.5	0	0	0	0	0	0	0.5
4	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0
Total	0	0.5	0	0	0	0	0	0	0.5
Mean	0	0.05	0	0	0	0	0	0	0.05

Table 6. Teratogen analysis breakdown for 20µg/ml of miafuron herbicide

Embryo Numbers	H	S	E	L	F	SB	P	HA	Total score
1	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0
3	0	0.5	0	0	0	0	0	0	0.5
4	0.5	0	0	0	0	0	0	0	0.5
5	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0
Total	0.5	0.5	0	0	0	0	0	0	1
Mean	0.05	0.05	0	0	0	0	0	0	0.10

Table 7. Teratogen analysis breakdown for 30 µg/ml of miafuron herbicide

Embryo Numbers	H	S	E	L	F	SB	P	HA	Total score
1	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0
3	0.5	0.5	0	0	1	1	1	0	4
4	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	1	0	0	1
6	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	1	0	0	1
8	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0
Total	0.5	0.5	0	0	1	3	1	0	4
Mean	0.05	0.05	0	0	0.1	0.3	0.1	0	0.6

Like higher concentration, treatment with 40µg/ml and 30µg/ml resulted in cardiac dysfunction and increased mortality at high concentrations. This is in line with the study of Hanke *et al*. (2013) which observed phenotypes in Zebra fish are comorbid with renal failure. Though a precise LD_{50} was not determined, the additional defects observed upon miafuron treatment were rather diverse including abdominal edema, shortened body axis, spine deformity, tail malformation, micropthalmia, apoptotic tissues and discoloration. miafuron mimics the plant hormone auxin, which neither humans nor *Clarias gariepinus* express (produce). Therefore, a teratogenic effect would not be expected. Perhaps, herbicide can inhibit another molecule, such as an animal hormone, that causes defects in *Clarias gariepinus* embryo. More studies will need to be done to determine the mechanism of miafuron on *Clarias gariepinus* embryos. Similar abnormalities observed were body axis shortening, micropthalmia, and tail deformity. Defects unique to high concentration were an edema that was located throughout the embryo (and not just in the abdominal region), and the failure for melanocytes to properly align in a ventral stripe. These unique phenotypes suggest that miafuron may be targeting different biochemical pathways/molecules. Thus, further study will need to be done to find the molecular targets of miafuron

A study done by Hanke *et al*. (2013) suggests that these observed phenotypes in Zebra fish are comorbid with renal failure. One of the primary and leading indicators of renal failure is generalized edema (Hanke *et al.,* 2013). This edema is caused by collection of fluid in several cavities of the body. Another indicator of renal failure is extended pericardial sac (Hanke *et al.,* 2013). However, both signs (general edema and extended pericardial sac) can also be caused by cardiac dysfunction due to increase in hydrostatic pressure. (Hanke *et al.,* 2013). In my study, treating *Clarias gariepinus* embryos with miafuron resulted in a lack of heart contraction, pericardial sac extension and absence of the swim bladder. However, the treated embryos did not show any signs of generalized edema, suggesting that the abdominal or general edema observed upon treatment may be due to renal failure.

This study reveals, all concentration of the herbicides that showed effects seemed to exhibit cardiac failure in some form. This in large part is why I tested miafuron in the study, so that a baseline phenotype for cardiac failure could be established. It is not uncommon for teratogens to cause heart defects due to sensitive nature of heart development (Sarmah and Marrs, 2016). There are many types of agents, including environmental, pharmacological, and infectious, that are known cardiotoxic agents. Some examples include ion or calcium inhibitors, which lead to impaired cardiac function. Other cardiotoxic agents cause congenital malformations such as alcohol or thalidomide (Brown, Samsa, Qian, and Liu, 2016). In four of the five treatments, embryonic hearts did develop; however, they exhibited reduced or absent heart contractions. Thus, it is likely that the herbicides do not impair cardiac formation,

but they may impair further differentiation/morphogenesis of the heart and/or heart contraction itself. More studies will need to be done to evaluate how herbicides cause cardiac dysfunction.

CONCLUSION

In conclusion, the results of this research reveal that majority of these five concentrations of herbicide, which are designed to precisely target plant cells, also have effects on fish by impairing cardiac function and development in *Clarias gariepinus* embryos/larvae. Further, four of the five concentrations have additional teratogenic effects in other tissues/organs not caused by cardiac dysfunction. So, these herbicide concentrations are indeed teratogens, and they could potentially be harmful to aquatic organisms. Whether the concentrations that cause these teratogenic effects are like those found in the environment is not known at this moment. However, even if environmental levels are much lower than those tested in this study, it is unclear what roles bioaccumulation and biomagnification may have in the production of eggs with higher concentration of herbicides than those found in the water. Thus, it is still valuable to continue evaluating the teratogenic effects of miafuron, particularly since the herbicide are currently in use in most maize and rice farms. Based on the results of the experiments conducted, we therefore recommend that it is valuable to continue evaluating the effects of miafuron herbicides, particularly in rivers since the herbicide is currently in use in most rice and maize farms.

ACKNOWLEDGEMENT

Based on the results of the experiments conducted, I therefore recommend that it is valuable to continue evaluating the effects of miafuron herbicides, particularly in rivers since the herbicide is currently in use in most rice and maize farms.

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