Research article

Exposure Impacts of Diquat dibromide herbicide formulation on amphibian larval development

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ABSTRACT

Many anthropogenic chemicals in general, and specifically aquatic herbicide formulations have the potential to modulate the thyroid pathways of the endocrine system of aquatic organisms, because they are normally applied directly into the aquatic system, to manage aquatic weeds. These thyroidal effects have been widely linked with disruption in developmental and reproductive processes. In fact, the exposure impacts of many of these substances on metamorphic organisms could produce a precocious metamorphosis. Using Xenopus Metamorphosis Assay (XEMA) protocol, this study assessed the thyroidal effects of environmentally relevant concentrations of Diquat dibromide at 0.05, 0.11, and 0.14 mg/L on Xenopus laevis metamorphosis. The formulation significantly reduced both the fore and hind limb lengths, and disrupted the developmental stage at concentrations of 0.11 and 0.14 mg/L, with a median at NF-stage 57, while median of NF-stage 60 was recorded in the control. Histopathologically, although there was no significant difference in thyroid gland area, the thyroid colloidal area was significantly reduced at 0.14 mg/L, while the mean height of the thyroid follicle increased at 0.05 mg/L. The result indicates an extra-thyroidal pathway, due to the dissociation between stage developmental effects and thyroid histopathology. The role of stress pathway occasioned by oxidative mode of action, involving lipid peroxidation and cell damage observed in this study need further investigation, in order to further characterize the physiological and ecological effects on wildlife.

1. Introduction

1.1. Chemical factors in amphibian declines

Numerous anthropogenic substances originating from industrial, pharmaceutical, and agricultural activities (herbicides, fungicides, and insecticides etc) are found in the environment. These substances majorly end up in the aquatic ecosystem, which becomes the warehouse of their residues (Ortiz-Delgado et al., 2019). Many of these substances have the potential to modulate the thyroid functions through different pathways including hormone synthesis and release, hormone transportation through the blood, hormone metabolism as well as the final clearance (Diamanti-Kandarakis et al., 2009). Hence, the exposure impact of these anthropogenic substances often results in growth and physiological modulation, including disruption in metamorphosis of metamorphic organisms (Nugegoda and Kibria, 2017) and particularly amphibians (Kloas et al., 1999; Hayes et al., 2010). In these metamorphic organisms, the metamorphosis process is a developmental phase where numerous important physiological changes occurs, which are sensitive to sub-lethal effects of chemicals (Denver, 1997). The processes obligatorily depends on thyroid hormone (Organisation for Economic Co-operation and Development (OECD), 2007a, b; Awkerman and Raimondo, 2018), and this is best exemplified by the fact that surgical removal of the thyroid gland or chemical blockage of thyroid hormone synthesis often leads to the complete blockage of metamorphosis (Optiz et al., 2005). The pre-metamorphic amphibian larvae for example, are highly sensitive to exogenous thyroid hormone, producing precocious metamorphosis when exposed (Helbing et al., 2010).

This means that thyroid hormone receptors mediate both early and late developmental programs of metamorphosis, and that growing organisms, particularly during early development, are at risk from these substances (Kloas et al., 1999; van Wyk, 2013; Denver, 2013). Already, there is growing concern about the safety of larval stages of aquatic organisms (Nugegoda and Kibria, 2017) and amphibians in particular (Hayes et al., 2010), given the current global amphibians’ population decline (Wagner et al., 2013; Munoz et al., 2013).
2015). This suggests that a better understanding of thyroid modulating activities of substances acting as agonists or antagonists is of great importance. In this regards, there is a dearth of information on the health and ecological impacts of several globally used herbicides (particularly the aquatic herbicides) on amphibian metamorphosis. Whereas the exposure impacts of a few herbicides and their formulations (e.g., glyphosate) are relatively well studied, the exposure impacts of most herbicides (e.g., Diquat dibromide, Paraquat, Glufosinate ammonium, Imazapyr etc.) are much less well known. This ecological gap makes the study of thyroidal activities of these herbicides very important, as part of their endocrine disruption indicators. These herbicides include glyphosate, diquat dibromide, Paraquat, glufosinate ammonium etc.

Diquat dibromide (9, 10-dihydro-8a, 10a-diazonia phenanthrene ion) is a post emergent herbicide. It is a non-selective contact herbicide and crop desiccant that is also used for aquatic weeds control (Emmett, 2002). Diquat has been registered with United States Environmental Protection Agency (USEPA) for terrestrial and aquatic ecosystem (Chung et al., 2009). Diquat is manufactured as bromide salt (C12H12N2Br2) (dibromide salt), and it can also exist as cation (C12H12N2+)(Howard 1991; USEPA 1995a, b). The chemical name for the dibromide salt is 6, 7-dihydrodipyrido(1,2-a:2,1-c)pyrazinedi-ium dibromide (USEPA 1995a, b). This herbicide formulation is widely used in the United States, North America, Europe, Australia, Japan and Africa (WHO, 2004). The expected environmental concentration of diquat dibromide is 0.073 mg/L (Peterson et al., 1994). This study was carried out in Canada, where they used an Expected Environmental Concentration (EEC) in evaluating the hazard of pesticides to non-target aquatic organisms. Test organisms were selected based on ecological relevance and present use in the test protocols. They calculated the EEC by assuming an overspray of a 15 cm deep fresh water body at the label application rate. The EEC of pesticides is then related to the EC50 for a given aquatic test organism. The phytotoxicity of the EEC of 23 different pesticides to ten algae (24 h inhibition of 14C uptake) and one vascular plant (7-day growth inhibition) was determined in an effort to examine the question of interspecific sensitivity and its relation to the development of pesticide registration guidelines. Diquat is not readily degraded by microbes, and exhibit low susceptibility to photodegradation (Howard, 1991; Paul et al., 1994; Chung et al., 2008; Siemering et al., 2008). It has high affinity for organic matter and water sediments, which normally decreases the concentration of free diquat in the water column (Ghalwa et al., 2012). In aquatic environment, diquat sorbs to suspended solid sediment and also to aquatic vegetation (Simsiman and Chesters, 1976; HSDB, 2003). Unfortunately, this adsorption to the organic matters and sediment is another important route of entry of toxicant in some amphibian species because of their feeding habit, whereby they nibble on organic matters in the water sediment. This study therefore assessed the exposure impacts of Diquat dibromide herbicide formulation on the thyroid system of developing Xenopus laevis.

The Xenopus metamorphosis assay (XEMA) is an animal-based model designed to identify substances that may interfere with the normal functioning of the hypothalamic-pituitary-thyroid (HPT) axis using X. laevis (Organisation for Economic Co-operation and Development (OECD), 2007a, b; Grim et al., 2009). The primary endpoints, including hind-limb length, developmental stage and thyroid gland histology, while snout vent length, wet weight, and mortality also help to distinguish between thyroid specific effect and generalized toxicity (Grim et al., 2009). The assay has several advantages over other animal-based in-vivo assays. It displays a temporal coupling of TH exposure to subsequent observable, and measurable morphological outcome to a degree unmatched by any other animal processes (Helbing et al., 2010). According to Helbing et al. (2010), the fact that this assay occurs entirely in aquatic medium, the ecological niche of the anuran larvae, also makes the protocol unique.

2. Materials and methods

2.1. The herbicide formulation

Midstream (Syngenta Ltd., South Africa) containing 373 g/L Diquat dibromide.

2.2. Xenopus laevis breeding and tadpole culture

Adult males and females X. laevis obtained from our in-house breeding stock were maintained separately in 15 L glass tanks containing buffered (2.5 g sea salt/10 L) reverse osmosis water (Kloas et al. 1999). The frogs were fed three times per week with fish pellets (Aqua-Nutro, South Africa). After each feeding, the tanks were cleaned and refilled with clean water. Gutter down-piping sections were placed in the holding tanks to create hide-outs for the frogs. The Breeding procedure was performed following protocol. Four days prior to the breeding, males and females were first primed with 100 IU human chorionic gonadotropin (hCG) (Merck Ltd Germany), injected into their dorsal lymph sac. This is followed by a second treatment just prior to the mating of 100 IU and 300 IU hCG to the males and females respectively. Single male and female were paired together in a 15L breeding tank, lined with plastic netting (to separate the eggs from the adults during oviposition), and placed in a well-ventilated dark place. The eggs were collected and spread out into several aerated 15 L tanks. Feeding of the tadpoles commenced at NF-stage 48, and they were fed with Sera Micron (Sera Heinsberg, Germany) twice daily until NF-stage 51. The tadpoles started on food ration of 30 mg/animal/day, which was later increased to 50 mg/animal/day to compensate for growth increase (OECD, 2007a, b). Staging of the tadpoles was done using a normal developmental atlas described by Nieuwkoop and Faber (1994). Xenopus laevis used for this study were collected, cultured, cared for, and treated under strict compliance with all ethical practises and law and approved by the Animal Research Ethical Committee of the Stellenbosch University (Approval no- SU-ACUM 12-00015).

2.3. Test procedure

2.3.1. Exposure set-up

At the NF-stage 51, twenty (n = 20) premetamorphic tadpoles were carefully picked from the holding tanks and transferred to new 15 L tanks. Individual exposure tanks were replicated twice at each exposure concentration and the control. Even though we did several pre exposure tests with four (4) replicates, our results didn’t show much effects of these replicates. Secondly, because we worked on six herbicide formulations, requiring very large numbers of tadpoles, therefore, we did not consider replicate tanks as the experimental unit but individual tadpoles. We tested for a ‘tank effect’. The exposures were done under controlled physical conditions following XEMA protocol: water temperature at 23 ± 1 °C, pH of 7.5–8.5, dissolved oxygen of >6.5 mg/L, 12 h of light and dark photoperiod (L12:D12) regime (OECD, 2008).

2.3.2. Exposure concentrations

The selected exposure concentrations were centred on 96-h LC50 at NF-stage 48 of the X. laevis tadpoles (Babalola & van Wyk, 2018) (Table 1). The Mortality incidence was monitored daily while the exposure medium in the tanks was completely replaced at interval of three days. The three days was chosen as diquat has been noted to have 8 days half-life photo-degradation in distilled water with 210–260 nm light
(Mackay et al., 1997), and is known to be stable to hydrolysis at pH 5-9
(USEPA, 1995a, b), with no hydrolysis measured after 30 days at pH 5 or 7
(Ritter et al., 2000). Only mortality incidence less than 10% in the
control group was accepted for the experiment (OECD, 2008).

2.3.3. Nominal concentration test
To confirm the exposure concentrations in the study, 100 ml samples
from the exposure water was taken from each of the exposure tanks, one
hour after the introduction of the herbicide formulation. For the replications, 100 ml from each of the tanks was taken and then mixed together,
from where a single 100 ml was pooled to represent that concentration.
The collected water samples from the exposure were immediately stored
in icebox pack at a temperature range of -5 to -10 °C before being sent to
Envirotech Laboratory, Lagos, Nigeria, for the determination of herbicide concentration, which was conducted within 10 h of the collection. The
analysis of the water sample was done using the gas chromatography mass spectrometric (GC-MS) method with a detection limit of 0.05 μg/L
(De Almeida and Yonamine, 2007). The result of the detected concentra

tions showed low variations relative to the predicted nominal concentra
tions (Table 2).

2.3.4. Autopsy procedure and morphometric measurements
At the 21-day termination of the exposure, the tadpoles were
collected and euthanized in 0.1 % benzocaine. They were blotted dry and
individually weighed (to nearest 0.01 g), and measured snout–vent
length (to nearest 0.1 mm). They were then fixed in Davidson’s solution
for 72 h prior to being transferred to, and preserved in 4 % neutral
buffered formalin (Shi et al., 2012). The fore and hind-limbs lengths were
measured using Leica EZ4D stereo microscope (Leica Microscope Ltd,
Germany). Limb lengths were determined on digital photographs of the
tadpoles using the metric trace ruler software that has the capacity to
measure both straight and curved lines using traced lines. The heads of
tadpoles enclosing the thyroid glands were carefully severed transversely
using a sharp blade, just posterior to the eye, and subjected to routine
paraffin wax imbedding histological procedures (Bancroft and Stevens,
1977). Sectioning, mounting and staining then followed.

2.3.5. Developmental stage (NF-stage) determination
To measure the impacts on histology, histopathological variation in
the thyroid gland was used. For this, five tadpoles per tank (at same
stage) were randomly selected per concentration and compared to the
matched five median developmental stage of the control group using the
thyroid gland histopathological features. All tadpoles were first staged as
described by Nieuwkoop and Faber (1994). This was then followed by
matching five median developmental stage of the control with that of the
exposure tanks at each of the concentrations, as recommended by the
OECD, 2008 protocol.

Table 2. The selected exposure concentrations (centred on the 15, 30 and 45% of
96-hour LC50 for NF-stage 48 of X. laevis tadpoles) of the Midstream formulation.

| Formulation | Exposure concentrations |
|-------------|-------------------------|
| Midstream   | 0, 0.05, 0.11 and 0.14   |

2.3.6. Histological procedures
The lower jaw samples containing the thyroid glands were removed
from formalin, washed in running tap water, and processed for routine
paraffin wax-based histology (Bancroft and Stevens, 1977). The jaws
were dehydrated in series of graded alcohol and embedded (in frontal
plane to facilitate the caudal surface of the tissue first) in histowax
(Histolab, Sweden). The tissues were sectioned at 7-8 μm using
Reichert-Jung microtome (Cambridge Instrument, Germany), then the
sections were mounted on clean albumin coated slides, and oven-dried
(40 °C) overnight. The sections were then dewaxed, stained with hae
matoxylin and eosin (H & E) (Bancroft and Stevens, 1977), cleared in
xylene before mounting with glass cover slips using a resin-based me
edium (DPX, Sigma Ltd).

2.3.7. Histological measurement of the thyroid gland
Using the right-side thyroid, the thyroid image, taken with Leica DMLB
microscope equipped with digital camera (Leica Microscope Ltd, Ger
many) was used to measure the epithelia cell heights by taking measu
rement from the base to the apical edge of the cell. For each tadpole
specimen, 15 epithelial cell height measurements were taken for four
thyroid gland follicles, resulting in 60 epithelial cell height measurements
per individual. A mean value was then calculated per individual and used
with other individual group members to compute a group mean for follicle
cell height. Follicular cross-sectional area (follicle lumen area), as well as
thyroid gland cross-sectional area were also measured and calculated
(using image analysis software (Sigmascan, Systat Software Inc.) using the
right thyroid gland side for each individual specimen, 10 thyroid follicles
in each section were measured, making 10 thyroid follicles per tadpole.
The data was then combined for all the tadpoles in each exposure group.
The average sum total of the thyroid gland cross-sectional areas gives the
gland area for each exposure group.

2.3.8. Data analysis
The non-parametric Kruskal-Wallis test was used to assess variation in
median NF-stage among exposure groups because stage is a categorical
variable. The Dunn’s multiple comparison test was subsequently used to
identify significant pairwise differences in stages among treatments (Shi
et al., 2012). Front limb length (FLL) and hind limb length (HLL) were
normalized to snout vent length, to correct for the influence of devel
opmental stage on limb size. Normality and homogeneity of variance in
FLL, HLL, wet body mass (WBM), whole body length (WBL) and snout to
vent length (SVL) data were assessed using Shapiro-Wilk’s and Levene’s
tests respectively. One-Way ANOVA and Kruskal-Wallis ANOVA for
parametric and non-parametric data respectively were used to analyse
variation in morphometric endpoints (WBM, WBL and SVL) among
exposure groups. The effect of treatment (i.e. specific pesticide concen
tration), developmental stage and the treatment-stage interaction on FLL
and HLL was tested using mixed model ANOVA, with individual tadpole
as a random factor. Pairwise differences in WBM, WBL, SVL and
normalized FLL and HLL between treatments and the control groups
were assessed using the Tukey HSD test with Sjostoflin/Stoline correction
for parametric data or the Dunn’s test for non-parametric data. Significant
differences between treatments were taken at P < 0.05. All statistical
analyses were performed using Statistica V12 (Statsoft Inc., USA).

3. Results

3.1. Mortality
No incidence of mortality (0 %) was observed in any of the exposure
and the control tanks throughout the 21-day exposure period.

3.2. Variation in developmental stages

The exposure altered the frequency distribution pattern of the
developmental stages. The median of the frequency distribution shifted

Table 2. The gas chromatography analytical result for Midstream (Diguat
dibromide) with very low variations compared to the predicted nominal concen
trations). The limit of detection was 0.05 μg/L.

| Nominal | Detected |
|---------|----------|
| 0       | 0        |
| 0.05    | 0.048    |
| 0.11    | 0.10     |
| 0.14    | 0.13     |
to NF-stage 57 at the two highest exposure concentrations of 0.11 and 0.14 mg/L compared to NF-stage 60 at the control (Figure 1). Midstream treatment was a significant source of variation in developmental stage \((H_{3,120} = 25.15, P < 0.001, \text{Kruskal-Wallis ANOVA})\), followed by a significant variation (delay) in NF-stage development between the control and concentrations of 0.11 and 0.14 mg/L was confirmed \((P = 0.0001, z = 4.25\) and \(P = 0.03, z = 2.82\) respectively, Dunn's test) (Figure 2).

### 3.3. Morphometric analyses

The Midstream formulation resulted in slight reduction in mean whole body length (WBL) alongside slight increase in mean snout-vent length (SVL) (Table 2). The variation was, however not significantly different to that of the control group \((\text{WBL: } 0.05 \text{ mg/L, } P = 1, z = 0.23; 0.11 \text{ mg/L, } P = 0.45, z = 1.78; 0.14 \text{ mg/L, } P = 0.13, z = 2.29; \text{SVL: } 0.05 \text{ mg/L, } P = 1, z = 1.18; 0.11 \text{ mg/L, } P = 1, z = 0.34; 0.14 \text{ mg/L, } P = 1, z = 0.42\), Dunn's test). Similarly, variation in mean whole body mass (WBM) of the treated tadpoles (Table 3) also showed no significant difference when compared to the control \((0.05 \text{ mg/L, } P = 0.45, z = 1.78; 0.11 \text{ mg/L, } P = 1, z = 1.13; 0.14 \text{ mg/L, } P = 0.13, z = 2.29\), Dunn's test). However, Midstream treatment was a significant source of variance in both normalised mean hind limb length (HLL) \((F_{3,74} = 35.72, P < 0.0001, \text{Mixed Model ANOVA})\) and front limb length (FLL) \((F_{3,74} = 20.49, P < 0.0001, \text{Mixed Model ANOVA})\) (Table 2). In particular, HLL and FLL was found to be significantly reduced in the 0.11 mg/L and 0.14 mg/L treatments relative to the control \((\text{HLL: } P = 0.0001 \text{ and } P = 0.0003 \text{ respectively, Tukey HSD test}; \text{FLL: } P = 0.0001, z = 4.87 \text{ and } P = 0.0005, z = 3.96 \text{ respectively, Dunn's test}).

### 3.4. Histopathological endpoints

The thyroid follicular lumen area in the treated tadpoles showed evidence of atrophy, as it significantly reduced at the highest exposure concentration of 0.14 mg/L compared to the control \((P < 0.0001, z = 5.05\), Dunn's test) (Table 4). The mean height of the thyroid follicle epithelium also showed hypertrophy with a significant increase at only the lowest exposure concentration of 0.05 mg/L compared to the control \((P < 0.0001, z = 5.57\), Dunn's test). The mean of thyroid gland area (Figure 3), on the other hand, showed no significant difference at all the exposure concentrations compared to the control \((P = 0.58, z = 1.66; P = 1, z = 0.48; P = 0.193, 2.14 \text{ respectively, Dunn's test}).

### 4. Discussion

The global widespread malformation and decline in amphibian population is already established, but what remain unknown are some specific causes driving this decline, sometimes in relation to some species. The increasing use of pesticides (including aquatic herbicides) has been variously suggested as one of the possible declines (Grain and Guillette, 1997; Stuart et al., 2004; Khan and Law, 2005; Egea-Serrano et al., 2012). There is increasing concern about the health impacts of these pesticides on human and wildlife, particularly on thyroid system (Miyata and Ose, 2012). The significant function of these thyroid hormones makes it important to identify toxicants that could disrupt their functions. This study therefore assessed the thyroidal impacts of Diquat dibromide herbicide (Midstream formulation), using X. laevis as sentinel organism.

The control tadpoles from this study were healthy and went through the processes and stages of metamorphosis at the expected rate. At test termination, the control tadpoles were between NF-stage 57-61 (NF 60 median stages), which is consistent with OECD pre-validation guidelines, according to which control tadpoles should minimally be at median developmental stage of NF 57 at the test termination (Organisation for Economic Co-operation and Development (OECD), 2007a, b).

The exposure concentrations in this study were environmentally relevant and showed no toxicity on the survival of the X. laevis, as none of the exposed tadpoles died in the course of the exposure. Compared with the OECD's phase 1 pre-validation studies at stage 51 tadpoles, which has 0.944 g and 19.5 mm as the mean weight and mean snout-vent length respectively (Organisation for Economic Co-operation and Development (OECD), 2007a, b; Coady et al., 2010), the overall mean wet weight and snout-vent length \((\pm \text{SD})\) of the control tadpoles in this study after 21 days were 0.84 \pm 0.11 g and 21.12 \pm 1.39 mm for this formulation. These values compared favourably with the OECD pre-validation tadpoles, as the tadpoles in this study were even longer. As noted by Coady et al. (2010), the difference in length could be due to the current Sera micron food administered compared to combination of Tetrafin, spirulina algae, Silver Cup Trout Starter and live brine shrimp that was administered in the OECD test studies.

Exposure to the Diquat formulation resulted in a significant reduction in mean developmental stage at concentrations of 0.11 and 0.14 mg/L, which suggests potential inhibitory tendency in this formulation. However, histological observation showed that the gland area was not...
significantly different when compared to the control tadpoles, although there was occurrence of atrophy in follicular lumen area (at 0.14 mg/L) alongside a slight hypertrophy in follicular epithelium (significant at 0.05 mg/L). The dissociation of developmental effects and thyroid histopathology could imply a target tissue effect (extra-thyroidal pathways) (OECD 2008; Shi et al., 2012) or the inhibition of T₄ to T₃ transformation (Miyata and Ose, 2012), rather than HPT regulatory effects. Saka et al. (2013) reported a similar result for Simetryn herbicide, and concluded that the delay in development may be due to non-thyroidal effects. As also pointed out by Fort et al. (2011), when chemical affect the whole

Table 3. Morphometric of tadpoles exposed to Midstream formulation (±SD) including whole body mass (WBM), whole body length (WBL), hind limb length (HLL), front limb length (FLL) and snout vent length (SVL) after 21-day exposure. Asterisks indicate significant different (P < 0.05).

| Herbicide | Conc. (mg/L) | WBM (g) | WBL (mm) | HLL (mm) | FLL (mm) | SVL (mm) |
|-----------|-------------|---------|----------|----------|----------|----------|
| Midstream | 0           | 0.84 ± 0.11 | 61.23 ± 2.81 | 6.67 ± 1.22 | 2.54 ± 0.34 | 21.12 ± 1.39 |
|           | 0.05        | 0.89 ± 0.11 | 61.23 ± 2.98 | 6.54 ± 1.28 | 2.39 ± 0.40 | 21.42 ± 1.12 |
|           | 0.11        | 0.78 ± 0.15 | 59.57 ± 3.88 | 4.89 ± 1.48* | 2.0 ± 0.40* | 20.95 ± 1.27 |
|           | 0.14        | 0.74 ± 0.13 | 58.83 ± 4.58 | 5.12 ± 1.59* | 2.11 ± 0.48* | 21.02 ± 1.64 |

Table 4. Histo-morphometric data following a 21-day XEMA exposure to graded concentrations of Midstream formulation. Values represent the mean ± SD. Asterisks represent significant difference relative to the control.

| Treatment | Mean Follicle Epithelium Area (μm ± SD) | Mean Follicular lumen Area (μm ± SD) | Mean Gland Area (μm ± SD) |
|-----------|-----------------------------------------|--------------------------------------|----------------------------|
| 0         | 7.82 (1.65)                             | 3410.64 (2299.2)                     | 53903.80 (27630.7)        |
| 0.05      | 8.92 (1.68)*                            | 3146.04 (1793.6)                     | 69585.69 (12328.7)        |
| 0.11      | 8.27 (1.22)                             | 2413.78 (1195.1)                     | 59964.97 (15335.3)        |
| 0.14      | 8.28 (1.33)                             | 1832.86 (999.8)*                     | 39340.26 (10961.2)        |

Figure 2. Tadpoles stage differentiation after 21-day treatment with Midstream formulation relative to control (Ctrl). Asterisks indicate significant difference (P < 0.05) from control.

Figure 3. The gland size area of tadpoles exposed to Midstream formulation at 0.14 mg/L compared to the control (Mag X 100).
body length, snout vent length, and whole body mass, but not hind limb length and thyroid gland, (it indicates non-endocrine effects, but extra-thyroidal pathways.

Several studies have linked diquat to the formation of toxic radicals in the cell, which leads to oxidative stress (Sewalk et al., 2001; Hook et al., 2006; Higuchi et al., 2011; Bouetard et al., 2013), and severe stress condition (Yadav et al., 2013). The radicals from the diquat and the hydroxyl, which form from the oxygen radicals, absorbs electrons from cell membrane lipids, leading to lipid peroxidation, cell damage and cell death (Jones and Vale, 2000; Chung et al., 2008). According to Lushchak (2014), oxidative stress is usually the product of imbalance between reactive oxygen species (ROS) generation and the elimination, leading to certain consequences for cell physiology. Paraquat, a similarly quaternary herbicide, has been noted to exert its toxicity by production of the superoxides anion, which leads to the formation of more toxic reactive oxygen species (ROS), such as hydrogen peroxide and hydroxyl radicals.

The observed growth inhibition of the tadpoles in this study could be a short term physiological response to the toxicity of the herbicide formulation. The stress response physiology can also cause negative impacts on growth and reproduction in the chronically stressed organisms, as the body respond to protecting the organism (Cribb, 2018). When organisms are under mild or intermediate oxidative stress, their system usually block the general programs of their life cycle such as immune system, reproduction or extensive biosynthesis (which could cause growth inhibition) to develop responses towards preventing or neutralizing the negative effects of reactive oxygen species (ROS) (Lushchak, 2014). It could also be a long term response due to severity of the toxic exposure.

For this test formulation to have severely impacted the developmental stages of the treated tadpoles, the observed toxicity could be directed against the antioxidant defence systems, that operate to provide optimum reactive oxygen species homeostasis, or by increasing the production of ROS, leading to severe or high intensity oxidative stress in the tadpoles (Hurd and Murphy, 2009; Gomez-Mestre et al., 2013; Lushchak, 2014).

Another possible pathway is that the disruption of ROS homeostasis could suppress their roles as secondary messengers in cell signalling and developmental functions, leading to growth inhibition as witnessed in this study (Mittler, 2002; Hurd and Murphy, 2009; Gomez-Mestre et al., 2013). Although the function of normalized reactive oxygen species as secondary messenger in the cell signalling and developmental function is not yet full characterized, studies have shown their essential contributions in diverse physiological processes and many normal functions of living organisms (Gomez-Mestre et al., 2013; Lushchak, 2014).

In this redox homeostasis disruption, several pathways are possible. The introduction of certain oxidant(s) that could be beyond the capacity of the tadpoles antioxidants; the increasing production of reactive oxygen species (ROS) beyond the normal; depletion of reserves of low molecular mass antioxidants (ascorbic acids, tocophenol, carotenoids, polyphenols etc); decreased production of antioxidant enzymes and low molecular mass antioxidants as well as certain combination of two or more of all these pathways could lead to serious impacts on cellular homeostasis and cause severe oxidative damage in both short and long term (Costantini, 2008; Metcalfe and Alonso-Alvarez, 2010; Lushchak, 2014).

As noted by Cribb (2018), while the short term effect of the increased oxidative stress may include general metabolic processes. The long term effects may result in numerous serious physiological effects including the immune system, the reproductive system and even reduced longevity of the organisms in general that may not be reversible for the tadpoles (Hurd and Murphy, 2009; Gomez-Mestre et al., 2013; Lushchak, 2014).

In all, the inhibition of developmental stages and other thyroid controlled endpoint such as hind limb length by the Diquat formulation at concentration below 0.733 mg/L, which is the expected environmental concentration of this formulation (Peterson et al., 1994) is a serious ecological highpoint that deserves further attention.

5. Conclusion

An assessment of the chronic exposure impact of Diquat dibromide formulation (Midstream) based on both morphometric and histopathological endpoints, following the XEMA protocol revealed that this formulation disrupted the developmental stage of the treated tadpoles, and reduced the mean hind limb length, but without significant changes in thyroid gland histology. This disconnection between the morphometric endpoints and the thyroid gland histology showed that the exposure impacts of this Diquat formulation is through extra-thyroidal pathway and not thyroidal effects. Therefore, further study on this formulation will be necessary to characterise how it affect some critical thyroid dependent endpoints like hind limb length and mean colloidal area in thyroid gland, even without the thyroid gland effects. The role of oxidative stress due to reactive oxygen species from the exposure impacts of the Diquat formulation should also be further investigated. The result of this study also showed that many salient properties of the test formulation that could cause critical ecological impacts on aquatic organisms, particularly through the stress pathway, still exist. Therefore, exposure impacts of other similar aquatic herbicides need further attention, particularly on amphibian populations.

Declarations

Author contribution statement

Babalola, O. Oluwaseun: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Van Wyk, Hannes Johannes: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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