Archives of Environmental Contamination and Toxicology (2021) 80:708–716
https://doi.org/10.1007/s00244-020-00756-5

Lethal and Teratogenic Impacts of Imazapyr, Diquat Dibromide, and Glufosinate Ammonium Herbicide Formulations Using Frog Embryo Teratogenesis Assay-Xenopus (FETAX)

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Received: 2 May 2020 / Accepted: 21 August 2020 / Published online: 8 September 2020

Abstract

Globally, amphibians are experiencing widespread abnormalities and population declines. One potential contributor to these challenges is the use of pesticides, particularly aquatic herbicides applied to aquatic habitats inhabited by amphibians. Critical issues of concern are the potential toxicity and teratogenicity of these herbicides towards amphibians. Using the FETAX protocol, three globally used formulations, including diquat dibromide (Midstream), glufosinate ammonium (Basta), and imazapyr (Arsenal), were assessed for embryotoxicity, teratogenicity, and growth inhibition. Developing *Xenopus laevis* embryos were exposed for 96 h at concentrations of 0.5–3.0 mg/L, 1.6–3.0 mg/L, and 20–45 mg/L for Midstream, Basta, and Arsenal respectively. The 96-h LC_{50} estimates were 0.83 mg/L acid equivalent (a.e.), 36 mg/L a.e., and 2.2 mg/L a.e., whereas the EC_{50} estimates were 0.24 mg/L a.e., 28.13 mg/L a.e., and 2.01 mg/L a.e. for the Midstream, Arsenal, and Basta formulations, respectively. These two estimates produced Teratogenic Index of 3.5, 1.3, and 1.1 for Midstream, Arsenal, and Basta, respectively, indicating a high risk of malformation induction by Midstream and moderate risk for Arsenal. Regarding growth inhibition, lowest observable effect concentrations of 0.5 mg/L, 25 mg/L, and 2.0 mg/L were computed for Midstream, Arsenal, and Basta, respectively, producing the minimum concentration inhibiting growth (MCIG) ratios of 0.62, 0.69, and 0.89 for the three formulations. These MICG values are higher than the standard 0.30 growth inhibitors benchmark, suggesting that the formulations are not growth inhibitors at the evaluated concentrations. This study provides evidence of the embryotoxic and teratogenic status of Midstream and the embryotoxicity of Basta. There is a need to further characterise the physiological and ecological impacts of these formulations to ensure responsible use and the safety of amphibians and other wildlife.

Pesticides, including herbicides, insecticides, and fungicides, have been of immense benefits to mankind (European Environmental Agency (EEA) 2011; WHO 2013). These anthropogenic chemicals can increase farm yield, enhance pest control, environmental management, and improve health. But despite their positive contributions, the presence of these chemicals in the environment has continuously deteriorated the quality of water and soil resources therein (WFW 2010).

Currently, freshwater habitats are experiencing rapid biodiversity modifications, largely due to chemicals associated with agricultural practices (Downing et al. 2008). In addition, agrochemicals have become a major source of water pollution and consequent risk to the health of humans and wildlife (WFW 2010). Some estimates of freshwater biodiversity loss suggest that the current rate of extinction is the most profound in the past 100,000 years (Eldredge 1998). Amphibians' vulnerability to pesticides has been linked to their specific ecological requirements, which usually links them to permanent or temporary shallow waterbodies that are essential to their life cycle. However, these waterbodies are frequently contaminated with pesticides. The concern regarding the global incidence of amphibian morphological

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00244-020-00756-5) contains supplementary material, which is available to authorized users.

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abnormalities and population decline highlights the urgent necessity to characterise the potential impact of pesticides on these organisms (Gungordu 2013; Lajmanovich et al. 2013).

Pesticide ecotoxicology studies and acute toxicity LC_{50} estimates for aquatic organisms are mostly with reference to fish species, whereas information regarding toxicity to amphibians is limited (Mann et al. 2009; Wagner et al. 2013). Because it has been shown that amphibians may be just as sensitive to environmental contaminants as fish due to their permeable skin, speculation that exposure to pesticides could be linked to global amphibian decline exists (Brühl et al. 2013), and more amphibian related studies are needed (Blaustein et al. 1994; Wagner et al. 2013).

South Africa is the largest pesticides user in Africa, with about 180 pesticide active ingredients commercially available, and registered as approximately 400 trade names (Meinhardt 2008; Ansara-Ross et al. 2012). Several of these herbicides, including diquat dibromide, imazapyr, and glufosinate, are widely used in aquatic weeds management globally. However, the teratogenic potential and impact of the herbicide formulations applied directly to the aquatic environment, including diquat dibromide, glufosinate ammonium, imazapyr, and certain glyphosate formulations, on vertebrate growth and development are still not well characterized.

Diquat dibromide (9, 10-dihydro-8a, 10a-diazonia phenanthrene ion) is a post-emergent, nonselective contact herbicide, and crop desiccant that also is used in aquatic weeds control (Emmett 2002; WHO 2004). This herbicide is widely used in the United States, Canada, Europe, Australia, and Japan (Emmett 2002; WHO 2004). The Arsenal formulation for example contains nonylphenol ethoxylate as surfactant, which has been shown to exhibit estrogenicity (Othman et al. 2009). There have been some conflicting evidence regarding the impacts of diquat dibromide on amphibians and wildlife in general. Anderson and Prahlad (1976) showed that Diquat formulation inhibited body growth and pigmentation and resulted in distorted body shape at low concentrations 0.75–2 µg/L. In addition, Bimber and Mitchell (1978) reported increased rates of exogastrulation and mortality in *Rana pipiens* exposed to 0.1 mg/L diquat. In another study, Selypes et al. (1980) exposed nullipara mice to 11 mg/kg of Reglone formulation of diquat on the ninth day of gravidity and showed that the death of foetus is concentration-dependent and that the average embryonic weight decreased as the number of embryos retarded in weight increased. Selypes et al. (1980) further reported that diquat caused retardation in the embryos of females repeatedly treated with low doses, with changes occuring in the skull, vertebrae, sternum, and limbs. Conversely, Dial and Dial (1987) reported that eggs of *R. pipiens* to be resistant at 2.0–10 mg/L diquat, with both early and late gastrula producing no abnormalities.

Imazapyr herbicide belongs to the imidazolinone chemical family (Liu et al. 1992). These compounds are usually degraded through photolysis in water, with a half-life ranging between 2.5 and 5.3 days (WSDA 2009). According to Grisolia et al. (2004), Arsenal, one of the imazapyr formulations, consists of 25 g/L of imazapyr, 186 g/L of ammonium hydroxide, 18 g/L of nonylphenol ethoxylate (with 9 ethoxylated units), and water. There is scarcity of literature on the teratogenicity and developmental toxicity of this herbicide. However, various reviews, including USEPA, 2006 Registration Eligibility Decision (RED), and Washington State Department of Agriculture (WSDA 2003), rated the active ingredient of this herbicide as having no developmental toxicity and teratogenicity.

Glufosinate ammonium [(GA) (ammonium-D,L- homoalanin-4-yl methyl) phosphate] is a broad-spectrum herbicide. One of the prominent glufosinate ammonium formulations is Basta, which contains an anionic sodium polyoxyethylene alkyether sulphate (AES) that constitute 30% alkylether-sulphate as surfactant, solvent (propylene glycol ether), defoamer, and a blue dyestuff (Koyama et al. 1997). Even though the herbicide and its analogues are now registered and used in more than 40 countries (Qian et al. 2008), not much is known concerning its potential teratogenicity and thyroid disrupting activity. Watanabe and Iwase (1996) examined developmental and dysmorphogenic effects of GA on mouse embryos in culture and observed that 8-day-old embryos cultured for 48 h showed a significant overall growth retardation and increased embryolethality (37.5% at 10 mg/L). Accordingly, all embryos in the treated group exhibited specific morphological defects, including blisters in the lateral head (100%), hypoplasia of the prosencephalon (57.1%), and visceral arches (42.9%). Using the micromass cell culture method, GA also inhibited the differentiation of midbrain cells in Day 12 embryos, with 50% inhibition occurring at 0.55 µg/L. The ratios of the half maximal inhibitory concentration (IC_{50}) for cell proliferation to differentiation in limb bud cells were 0.76 and 1.52 in embryos (Days 11 and 12). Watanabe and Iwase (1996) concluded that GA was embryotoxic in vitro.

The Frog Embryo Teratogenesis Assay-Xenopus (FETAX) is a standardised 4-day flexible bioassay for assessment of potential developmental and teratogenic effects (Mann and Bidwell 2000; Yu et al. 2013). It is widely used in aquatic toxicity testing and is well-suited to environmental testing (Bantle et al. 1999). FETAX protocol assesses mortality, malformation, and growth inhibition during the embryonic developmental phase and has been applied to assess numerous environmental chemicals, including nonylphenol ethoxylate (Mann and Bidwell 2000), heptanol (Bernardini et al. 1994), soil extract (Fort et al. 1995), acidic mine water (Dawson et al. 1985; Oberholster et al. 2014), atrazine herbicides (Morgan et al. 1996), glyphosate

herbicides (Babalola and van Wyk 2019), organophosphate (Boga et al. 2009), and organochlorine insecticides (Schuytema et al. 1994), nicotine (Dawson et al. 1988).

In South Africa for example, the exposure impacts of several pesticides applied directly to aquatic ecosystems have not been well studied (Ansara-Ross et al. 2012), particularly the developmental toxicity of formulations, such as Midstream, Basta, and Arsenal, remain unknown. The aim of the present study was to assess the potential developmental toxicity (including teratogenicity, growth inhibition, and malformation) of diquat dibromide (Midstream), glufosinate ammonium (Basta), and imazapyr (Arsenal) herbicide formulations using *X. laevis* as model organism.

Materials and Methods

Test Chemicals

The herbicide formulations include: Midstream (373 g/L, diquat dibromide, Syngenta Ltd, South Africa), Basta (200 g/L, glufosinate ammonium, Bayer Crop Science AG Ltd, Germany), and Arsenal (250 g/L, Imazapyr, Base Chemical Ltd).

Exposure Concentrations

Following the initial pilot studies (Babalola and van Wyk 2017) and some acute toxicity results from the literature, between four and six concentrations were selected for each of the herbicide formulations (Table 1).

Nominal Concentration Test

Analytical Assessment of Experimental Concentrations To confirm the exposure concentrations, at about 2 h after the introduction of the herbicide formulation, water samples were randomly taken from 70% of the exposure tanks, including the controls. The water samples were stored in 150-mL glass bottles for each sample, frozen in an ice pack before being transported to the laboratory for analysis. The analysis was performed on the water sample around 8 h after collection. The analysis was done at Envirotech Laboratory, Lagos Nigeria, using gas chromatography (Anisuzzaman et al. 2000; Budde 2003; Shen and Lee 2003; Liu et al. 2004; de Almeida and Yoramine 2007). The detected concentrations (at detection limit of 0.05 µg/L) showed low variations relative to the predicted nominal concentrations (Supplementary Material Table 1).

Table 1 Exposure concentrations for diquat dibromide (Midstream), glufosinate ammonium (Basta), and imazapyr (Arsenal) herbicide formulations

Treatment	Exposure concentrations
Midstream (Diquat dibromide)	0, 0.5, 1.0, 2.0, 2.5, and 3.0
Arsenal (Imazapyr)	0, 20, 25, 30, 35, 40, and 45
Basta (Glufosinate ammonium)	0, 1.6, 2.0, 2.5, and 3.0

Care of *Xenopus laevis* and Breeding of Tadpoles

For the breeding, three sexually mature male and female *X. laevis* were selected from the established breeding colony of the laboratory and separately maintained in two 15-L glass tanks containing carbon filtered water. The frogs were fed with fish pellets (Aqua-Nutro, RSA) every 3 days. Following the American Society for testing and materials (ASTM) 2014 protocol, breeding induction was performed. In short, males were initially primed with 100 IU human chorionic gonadotropin (hCG) (Merck Ltd, Germany), which was injected into their dorsal lymph sac, 4 days before mating. The males and females were injected with another 100 IU and 300 IU hCG respectively to initiate mating three days after the first injection. Each breeding pair was housed in a separate 15-L exposure tanks lined with plastic netting (to separate the eggs from the adults) and positioned in a well-ventilated dark climate room.

FETAX Bioassay

Following the basic guidelines described by American Society for Testing and Materials (ASTM 1998), the FETAX bioassay exposure was performed. In brief, the fertilised eggs harvested from one of the breeding pairs were de-jellied by swirling in 2% L-cysteine (Sigma, Germany) (prepared in FETAX solution and adjusted to pH 8.1 with NaOH) for 3 min (Dawson and Bantle 1987). After de-jellying, normal cleaving embryos were first individually selected using the microscope. Second-level sorting was performed about an hour later to guarantee the quality of embryos used for the experiment (Fort and Mathis 2018). The embryos were staged using the NF developmental atlas of Nieuwkoop and Faber (1956), with NF stages 8-11 (mid-blastula to early gastrula embryos) selected for the exposure. All breeding and exposure procedures followed the ethical protocol approved by the Animal Ethical Committee of Stellenbosch University (SU-ACUM12-00014).

Exposure Set-Up

Twenty haphazardly selected NF-stage 8-11 embryos were introduced into each exposure vessel (500 mL). Each concentration was represented by two replicates (totaling 40 embryos), while four replicates were used for the positive and negative controls (80 embryos each). The whole

experiment was haphazardly arranged in a controlled-climate room under the following conditions: water temperature 24 ± 1 °C, pH of 6.5–7.4, dissolved oxygen of > 6.5 mg/L, and 12 h light:dark photoperiod ($L_{12}D_{12}$) (OECD 2008). All of the herbicide stocks were freshly prepared in distilled water daily, to avoid chemical degradation/breakdown by environmental factors. For the study, a semi-static exposure approach was adopted, where the exposure medium was changed every 24 h. The chemical 6-ammonicotinade (6-AN) at 99% purity at concentrations of 5.5 mg/L and 2500 mg/L was used as positive control, as part of FETAX requirement for the 96-h toxicity test without metabolic activation (ASTM 2014). The experiments were repeated twice.

Mortality Assessment

Mortality observations were recorded every 8 h throughout the exposure period. The dead embryos were counted and removed to reduce contamination. At 96 h, the exposure was terminated, and the cumulative mortality data were used to define the 96-h LC_5, LC_{50}, and LC_{95} for each of the herbicides, where the average of the replicates per concentration was used. All of the exposed embryos attained stage 46, including the control groups. The surviving tadpoles were euthanized using MS 222 (Tricaine methane sulfonate) (200 mg/L buffered with sodium bicarbonate at 0.42–1.05 g/L) (OECD 2007).

Growth Inhibition Assessment

The lowest observable effect concentration (LOEC) was calculated by statistically comparing the mean 96-h head-to-tail length larval at each concentration relative to the control. The minimum concentrations inhibiting growth (MCIG) was derived by dividing the LOEC with 96-h LC_{50} (Fort and Mathis 2018).

Malformations and Teratogenic Index

Developmental malformations, including facial and axial malformations, in the treated embryos/larva were accessed using the Atlas of Abnormalities (Bantle et al. 1999). In addition, characteristic methods described by Fort and Paul (2002) was adopted in the assessment of malformation. The percentage incidences of abnormalities were used to determine the 96-h malformation (EC_{50}) index. The LC_{50} and EC_{50} values were subsequently used to derive the teratogenic index (TI) using the equation TI = LC/EC) (NICEATM 2000; ASTM 2014; Fort and Mathis 2018).

Data Analysis

The mortality and malformation data were used to compute the LC_{50} and EC_{50} values using the probit analysis program (USEPA 1998). Variance in body length data between the treated embryos/larva and the control were used to derive the minimum concentration inhibiting growth (MCIG). Normality and heterogeneity of variance of the embryo length data was assessed using normal probability plots and Levene's test, respectively. For parametric data, variance among treatments were assessed using one-way ANOVA, and pairwise differences using Tukey's HSD test with the Spjotvoll-Stoline correction for unequal sample sizes. Nonparametric data were analysed using Kruskal–Wallis ANOVA in combination with Dunn's pairwise comparisons test. $\alpha = 0.05$ was deemed as significant. Statistica v 13.5 (Tibco Inc., USA) was used for all ANOVA and post hoc tests.

Results

Embryolethality/Mortality

In this study, the Midstream formulation was found to be the most embryolethal, followed by the Basta formulation, whereas the Arsenal formulation was the least toxic among the three formulations. The 96-h LC_{50} for Midstream, Basta and Arsenal were 0.83, 2.24, and 36 mg/L a.e., respectively (Table 2).

Growth Effects

Exposure to the Midstream formulation resulted in a significant decrease in total length across the concentrations tested (0.5–3 mg/L) relative to the control (Fig. 1a). In the Arsenal formulation exposure, there was a concentration dependent

Table 2 Exposure concentrations (mg/L), 96-h LC_5, LC_{50}, LC_{95}, and EC_{50}, Teratogenic Index (TI), Lowest Observable Effect Concentration (LOEC), and Minimum Concentration Inhibiting Growth (MCIG) for the herbicide formulations (95% confidence Intervals in brackets)

Treatment	Exposure concentration	LC_5 (95% CI)	LC_{50} (95% CI)	LC_{95} (95% CI)	EC_{50} (95% CI)	TI	LOEC	MCIG (LOEC/LC_{50})
Midstream	0, 0.5, 1.0, 2.0, 2.5, 3.0	0.11 (0.01–0.26)	0.83 (0.43–1.14)	6.34 (3.62–30.0)	0.24	3.5	0.5	0.60
Arsenal	0, 20, 25, 30, 35, 40, 45	22 (17.2–25.2)	36 (33.3–40)	58.5 (50–79)	28.1 (26.2–30.0)	1.3	25	0.69
Basta	0, 1.6, 2.0, 2.5, 3.0	0.92 (0.6–1.14)	2.24 (1.97–2.7)	5.46 (4–10.5)	2.01 (1.78–2.31)	1.1	2.0	0.89

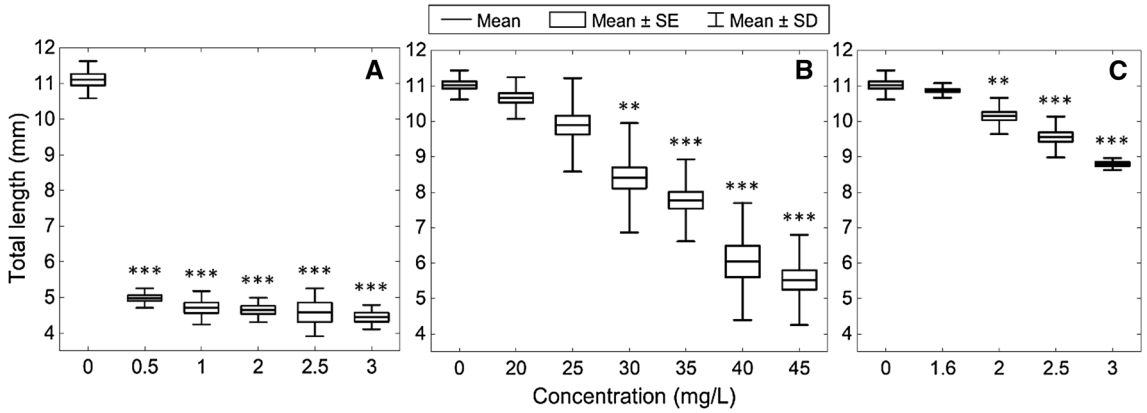

Fig. 1 Total length (mean ± SE ± SD) of *Xenopus laevis* tadpoles exposed to the Midstream (Diquat dibromide) (**a**), Arsenal (Imazapyr) (**b**), and Basta formulations (Glufosinate ammonium) (**c**) for 96-h relative to their negative control. Asterisks indicate significant differences relative to the negative control: $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$

decrease in length (Fig. 1b). The mean total lengths of tadpoles were only significantly reduced relative to the control at concentrations of 30–45 mg/L (Fig. 1b). Exposure to the Basta formulation resulted in a similar concentration dependent decrease in length in the treated tadpoles (Fig. 1c). The length reduction was only significant between 2 and 3 mg/L relative to the control (Fig. 1c). The growth inhibition potential ($MCIG/LC_{50}$) derived for the herbicides were 0.60, 0.69, and 0.89 for the Midstream, Arsenal, and Basta formulations, respectively (Table 2).

Malformation Index (MI) and Teratogenic Index (TI)

The 96-h EC_{50} values for malformation induction were 0.241 mg/L, 28.13 mg/L, and 2.01 mg/L for Midstream, Arsenal, and Basta formulations, respectively. TI produced was the highest for Midstream: 3.5, followed by 1.3, and 1.1 for the Arsenal and Basta formulations, respectively (Table 2).

Observed Malformations

Midstream Formulation

Following the characteristic malformation approach, the observed malformations following the Midstream treatments included generalised edema, cardiac and abdominal edema, blistering, improper gut formation/coiling abnormalities, wavy tails, and tail flexures that occurred in concentration dependent manner (Fig. 2). The most common malformation associated with Midstream exposure was generalised edema. But the most unique malformation was the occurrence of two-headed and multiple tails tadpole (Fig. 2h). The percentage incidences of malformations were as follows: edema (generalised, cardiac, and abdominal) (43%), gut abnormalities (11.4%), blistering (14%), axial malformation (wavy and curved tail) (10.04%), eye abnormalities (2.9%), and head (1.4%).

Arsenal Formulation

The observed characteristic malformations in the larva exposed to the Arsenal formulation include edema (generalised, cardiac, and abdominal), improper gut formation/coiling, and blistering (Fig. 2). The percentage incidences of malformations were as follows: edema (generalised, cardiac, and abdominal) (58.1%) (Fig. 2e), gut abnormalities (47.6%) (Fig. 2d), blistering (8.1%), head (2.3%), and eye (1.2%).

Basta Formulation

The observed characteristic malformations included edema (abdominal, generalised, and cardiac edema), gut abnormalities, and axial malformation (including wavy tails), which occurred with increasing concentration (Fig. 2). The percentage of various malformations occurred in the following order: edema (generalised, abdominal, and cardiac) (Fig. 2e) (50.4%), axial malformation (wavy and curved tail) (Fig. 2c) (38.8%), gut abnormalities(Fig. 2d) (15.4%), as well as eye and head abnormalities with (3.8%).

Discussion

There is increasing global concern regarding the health and ecological implications of pesticides in the environment (WHO 2013). Apart from numerous human health issues, including developmental, thyroid, and reproductive disruption (Lajmanovich et al. 2013; WHO 2013), the exposure impacts of pesticides to other nontarget organisms continues

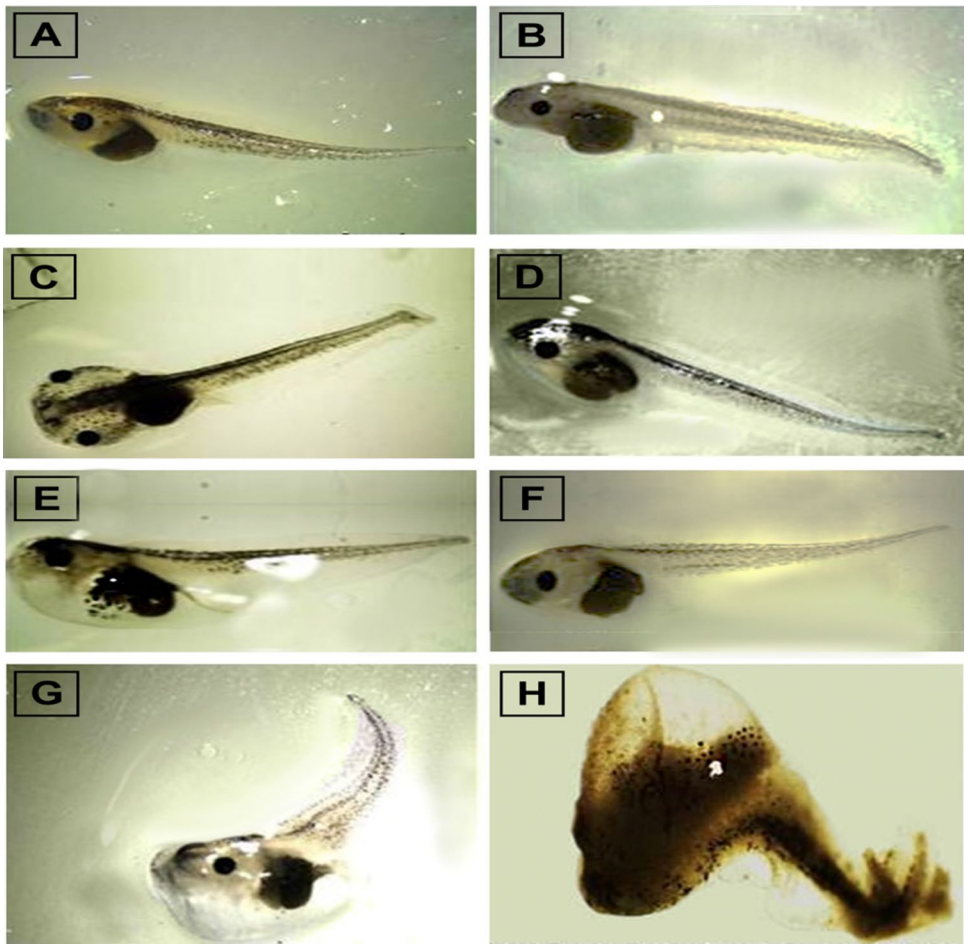

Fig. 2 Malformation types observed in the present investigation in *Xenopus laevis* larvae after 96-h exposure: (**a**) negative control; (**b**) oblong head; (**c**) wavy tail; (**d**) gut abnormality; (**e**) abdominal edema; (**f**) improper gut coiling; (**g**) cardiac and generalized edema; (**h**) multiple heads and tail

to incentivise research. This present study examined the toxicity, teratogenic, and malformation potential of three globally used herbicide formulations, including Midstream, Arsenal, and Basta, which are used extensively in commercial farming as well as to control alien plants in South Africa's water catchment areas (Working for Waters Programme) (Bold 2007; Mensah et al. 2013).

This study confirms that Midstream formulation has a high toxicity with an LC_{50} of 0.83 mg/L. The lethal concentration of this formulation is close to the expected environmental concentration (EEC), i.e., 0.73 mg/L, at the recommended application rate of 0.1–2.0 mg/L (Dial and Dial 1987; Peterson et al. 1994). This supports the findings of Anderson and Prahlad (1976), who reported that diquat at concentrations of 1 or 2 mg/L, was highly embryotoxic to *X. laevis*. Our results therefore suggest that more than 30% mortality will occur at the EEC concentration of 0.73 mg/L. Similarly, the related quaternary ammonium paraquat herbicide also has been reported to exhibit high toxicity in *X. laevis*, with an LC_{50} of 0.67 mg/L (Osano et al. 2002). The present results show that diquat dibromide is not appropriate for weed control in the aquatic environment, inhabited by amphibians and other equally sensitive organisms.

The Arsenal formulation was slightly toxic towards developing *X. laevis*, with an LC_{50} of 36 mg/L, which exceeds the expected environmental concentration of the herbicide, being 5.77 mg/L. The amphibian embryo therefore will not be at risk of acute toxicity at the normal application dosage of Arsenal. The Basta formulation was shown to be moderately toxic, with a 96-h LC_{50} of 2.24 mg/L. This result supports the findings of Ebert et al. (1990), who noted that glufosinate ammonium is slightly toxic following oral exposure in rats and dogs. However, with the Basta EEC of 1.0 mg/L in water, the safety of the tadpoles cannot be totally guaranteed because of the 96-h LC_{50} of 2.24 mg/L, as there will always be localised spots where concentrations higher than the EEC would be present, particularly immediately after application. The safety of the sensitive aquatic organisms is therefore in question.

Exposure to the Midstream formulation resulted in relatively high degree of growth inhibition, as the growth was inhibited even at the lowest exposure concentration of 0.5 mg/L (Fig. 1). The inhibition in the 0.5 mg/L treatment resulted in mean body length of 4.98 mm compared with 11.07 mm in the control. However, the minimum concentration inhibiting growth ratio (MCIG) was 0.6

and exceeded the 0.30 benchmark for a substance to be classified as growth inhibitor standard (NICEATM 2000). Nonetheless, the mere fact that even the lowest exposure concentration in this study inhibited the growth of the treated larva suggests that this formulation may be a high-risk growth inhibitor and requires further investigation. In particular, concentrations below 0.5 mg/L also could inhibit growth and lower MCIG due to the LOEC/LC_{50} ratio change. This current growth inhibition result supports the findings of Anderson and Prahlad (1976), who reported that diquat inhibited general body growth in *X. laevis* at a concentration of 1.5 mg/L. In the case of Arsenal and Basta, even though the two formulations caused significant concentration dependent growth reductions, particularly from 30–45 mg/L and 2.0–3.5 mg/L, respectively (Fig. 1), MCIG ratios were high at 0.69 and 0.89, respectively, compared with the 0.30 benchmark growth inhibitor level (NICEATM 2000). The data hence suggests that these two formulations are not growth inhibitors at the exposure concentrations tested. The occurrence of growth inhibition as observed in these two formulations therefore could be due to toxicity, which should be further investigated.

A high Teratogenic Index (TI) was observed for the Midstream formulation (Diquat). A TI value above 1.5 indicates teratogenic potential (ASTM 2014; Fort and Mathis 2018). The high TI observed for Midstream supports the findings of Osano et al. (2002), who reported a TI of 3.72 for paraquat, a related quaternary ammonium herbicide. High teratogenicity could therefore be a trait of members of the quaternary ammonium group. Severe generalised edema was one of the most common abnormalities observed in tadpoles exposed to Midstream. These edema abnormalities according to Osano et al. (2002) may be due to altered osmoregulation associated with disruption of cell membrane lipid layers. It is notable that the rare double headed and multiple tailed embryo-larva was observed in the Midstream exposed cohort. For Arsenal formulation (Imazapyr), the teratogenic index was 1.3, which is slightly below the 1.5 standard indicating teratogenic potential. According to Leconte and Mouche (2013), a TI > 1.2 should be regarded as positive dysmorphogenic, instead of 1.5 recommended by the ASTM. The TI of Arsenal therefore exceeds the 1.2 reference value and can be considered as teratogenic based on a study by Leconte and Mouche (2013). The leading abnormalities observed in individuals exposed to Arsenal were gut malformations that include the slightly improper gut coiling as well as complex improper gut formation. A high frequency of severe generalised edema was furthermore observed, as was the case with Midstream exposed tadpoles. The Basta formulation did not reach the teratogenic reference values of Leconte and Mouche (2013) or the ASTM, with a low TI of 1.1 suggesting the herbicide is not teratogenic towards *X. laevis*.

Conclusions

The results of this study indicate high toxicity and teratogenicity exhibited by Midstream (diquat dibromide) towards *X. laevis* embryo-larva, whereas Basta (glufosinate ammonium) was moderately toxic. The two formulations therefore have the potential to disrupt population dynamics of the embryo-larval stage of amphibians in a way that could lead to serious population decline if extensively applied. The current results furthermore indicate that the Midstream formulation has the potential to cause widespread malformations, given its high teratogenic index of 3.5. Further research regarding the ecological impacts of diquat dibromide and glufosinate ammonium-containing formulations are therefore imperative, not only related to amphibians, but other sensitive wildlife as well. Importantly, to protect all sensitive wildlife species, the continued use of diquat dibromide and glufosinate ammonium-containing formulations, particularly in aquatic ecosystems, should be revisited. In addition, the activity of these herbicides in terms of endocrine-disrupting potential in wildlife should be evaluated.

Acknowledgements The authors thank Dr. Olatunde Oladapo, formerly of Zoology and Environmental Biology Department, Lagos State University in Nigeria, for all his support. They wish him happy and good health in retirement.

Authors Contribution Babalola performed the laboratory work and manuscript writing. Truter performed all of the statistical and software analysis, and van Wyk performed the general supervision and conceptualization.

Funding This study was supported by the Water Research Commission, South Africa, Research grant (Grant Number K5/1952), as well as the Working for Water Department, Ministry of Water Affairs, South Africa, for the supply of all the herbicides used for this study. The authors declare that both the Water Research Commission and Working for Water Department, both in South Africa, did not in any way contribute to the design of the experiment, data analysis, as well as report writing and choice of publication.

Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest in financial, relationship, or otherwise.

Ethical Approval The authors declare that all experiments used in this study comply with the current laws in South Africa (Animal Ethics Permit No. SU-ACUM 12-00014).

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