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RESEARCH ARTICLE

Toxicity of the aquatic herbicide, reward®, to the northwestern salamander

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Abstract

Diquat dibromide (DB) is the active ingredient in several herbicide products used around the world for industrial and recreational control of terrestrial and aquatic pest plants. This study aimed to assess the adverse effects of the commercial formulation of the aquatic herbicide, Reward®, on the Pacific Northwest amphibian species, the northwestern salamander (*Ambystoma gracile*). Larvae were exposed to the Reward® herbicide in a 96-h acute bioassay (0.37–151.7 mg/L DB) and a continuous 21-day exposure (0.37–94.7 mg/L DB). The 96-h LC_{50} was 71.5 mg/L and the 21-day LC_{50} was 1.56 mg/L. Collectively, the results of this study demonstrate that early life stage *A. gracile* larvae appear largely insensitive to acute Reward® exposures compared to early life stage fish. However, *A. gracile* larvae are considerably more sensitive during sub-chronic exposure (21 days) with lethal and sub-lethal effects on growth occurring in the 1–2 mg/L range, which more closely resembles the larval fish lethal sensitivity to this active ingredient. This is the first study examining the toxicity of the aquatic herbicide formulation Reward® on *A. gracile* under acute and sub-chronic exposure scenarios.

Keywords Toxicology · Aquatic herbicide · Ecology · Diquat dibromide · Northwestern salamander · Amphibians

Introduction

The incorporation of amphibian toxicity data to existing evaluations of pesticides is currently lacking. Indeed, no current standardized amphibian toxicity tests exist for Canadian toxicity testing regimes, including within Environment and Climate Change Canada or British Columbia Ministry of Environment for an amphibian species. Furthermore, a meta-analysis of tens of thousands of published ecotoxicity tests showed a disproportionately low representation of amphibian species (Kerby et al. 2010). In particular, salamander species are often overlooked in favor of the well-characterized frog species, *Xenopus laevis* (OECD 2009). Amphibians have largely exhibited sensitivity to environmental pollutants as evidenced by decreased survival and growth, along with increases in the incidence of developmental abnormalities in polluted field and laboratory settings (Egea-Serrano et al. 2012). This reality of amphibian sensitivity combined with their underrepresentation in the literature presents a valuable opportunity to expand current knowledge by branching out from conventional frog species and investigating relative sensitivities for other amphibians, such as salamanders.

Currently, little work has been conducted on the sensitivity of contaminants to the northwestern salamander (*Ambystoma gracile*), a species native to North America's Pacific west coast (Government of British Columbia 2017). This species is currently listed as of least concern to the International Union for Conservation of Nature (International Union for Conservation of Nature 2015) and is a potential candidate model salamander to investigate the sensitivity of North American salamanders to environmental contaminants. *A. gracile* is a carnivorous amphibian ranging from Alaska to Northern California (Government of British Columbia 2017). Like other amphibians, *A. gracile* spends much of its life cycle in or near water sources, ultimately reproducing by laying its eggs underwater in a standing water body (Guderyahn et al. 2016). The development of *A. gracile* from embryo to adult is influenced by various factors such as clutch size, food availability, temperature, and photoperiod (Morrison and Hero 2003). Between embryogenesis and

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sexual maturity, however, temperature appears to be the primary factor dictating the developmental timeline (Smith-Gill and Berven 1979). *A. gracile is one o*f many amphibians that are polymorphic, where a subset of the population does not develop into a terrestrial adult form, but retains the larval characteristics including external gills until reaching sexual maturity in 1–2 years and is known as neotenic (Licht and Sever 1991). It is speculated that if metamorphosis is driven by environmental factors (resource limitation, pond desiccation, and temperature), then the "decision" to curtail metamorphosis may result in neotenic adults until more favorable environmental conditions arise (Eagleson 1976). The rate of neoteny may also increase with altitude (Hoffman et al. 2004). It is unknown how exposure to environmental contaminants might affect *A. gracile* survival, development, growth, and ratio of aquatic to terrestrial adults.

Pesticides are substances that are used to limit or destroy pest populations in domestic or commercial settings (Government of Canada 2017). The United States Environmental Protection Agency (USEPA) estimated that in 2007, the worldwide use of pesticides was 2.4 billion kg, with 40% representing herbicides (Grube et al. 2006). Since the early 1980s, pesticide use has increased in agriculture with herbicide application accounting for most of the increase. In 2011, upwards of 35% of agricultural land in the Canadian prairies and almost 21% in the province of Ontario were subject to herbicide treatment, with some of the highest relative risk of pesticide contamination being surface waters in agricultural areas surrounding Toronto and Winnipeg (Agriculture and Agri-Food Canada 2016). Contamination of waters presents risk to wildlife because many herbicides can be persistent and mobile in the environment, accumulating in areas distant from the site of application and could potentially induce adverse effects on non-target flora and fauna (Solomon et al. 2013). The need to discern the widespread effects of chemicals in the environment has increased as research has returned with warnings about the danger presented by using certain pesticides (Fent et al. 2006). In the USA, there are currently more pesticides in use that have unknown effects on non-target organisms than there are those with a robust toxicity profile, which may include conventional apical endpoints and sub-lethal endocrine disrupting effects (Grube et al. 2006). It is therefore important that new data are collected through the rigorous scientific method so that regulatory bodies around the world can enact evidence-based policies that protect non-target wildlife, including humans.

The herbicide examined in this research is diquat dibromide (DB) and is known by several trade names, such as, Reward®, Reglone®, Aquacide®, Dextrone®, and Reglox® (United States Environmental Protection Agency 1995). The molecular weight of DB is 344.05 g/mol, and it is soluble in water at 20 °C up to 708 g/L, nearly twice the solubility of sodium chloride (360 g/L at 25.0 °C). Its log octanol-water partition coefficient (K_{OW}) is − 4.60, which is more than 9 orders of magnitude smaller than the Canadian Environmental Protection Act (Environment and Climate Change Canada 2008) criteria for categorizing a substance as bioaccumulative (i.e., log $K_{OW} \geq 5$), indicating it is unlikely to accumulate in tissues and biomagnify (Mackay et al. 2018). This low log K_{OW} is likely due, at least in part, to its dual positive charge once dissociated from its anionic bromides. Due to the high water solubility of DB (708 g/L), Reward® is marketed in Canada (registration number 26271) primarily as an aquatic herbicide, but is also used in terrestrial applications in different commercial formulations to control various plant pests in the agricultural setting. Due to its indiscriminate herbicidal action, it is effective against common targets including Duckweed (*Lemna minor*), coontail (*Ceratophyllum demersum*), Canadian water weed (*Elodea canadensis*), water chestnut (*Trapa natans*), and flowering rush (*Butomus umbellatus*) (Syngenta Canada 2015). Currently, DB is not part of the National Pesticides Monitoring and Surveillance Network program in Canada, and consequently, there is no data on environmental concentrations in Canada (National Contaminants Advisory Group (NCAG) 2018). However, when Reward® is applied to water bodies directly to control aquatic plants at an application rate of 0.454 kg/1233 m^3 of water, the maximum instantaneous concentration according to the manufacturer would be 0.37 mg/L of DB and this dissipates to 0.1 mg/L after 24 h (Syngenta Canada 2015). Many studies testing the toxicity of the active ingredient, DB, in fish demonstrate lethal concentrations in the low mg/L range and at least twofold higher than the instantaneous aquatic application rate. For example, Paul et al. (1994) determined LC50 concentrations on embryo, juvenile and adult Walleye (*Stizostedion vitreum*) after 24, 48, 72, and 96 h. The resulting LC50 values ranged from the lowest concentrations corresponding to the longest period (96 h) to the highest concentrations (exposed for 24 h): embryo, 0.75–2.9 mg/L; juvenile, 1.5–3.1 mg/L; adult, 4.9–7.8 mg/L. Similarly, no studies testing a commercial formulation of DB have been performed on an amphibian species, but one study by Dial and Dial (1987) showed that the 16-day LC50 for Northern leopard frog gastrula stage larvae was between 5 and 10 mg/L DB. No acute or chronic toxicity studies examining pure DB or commercial formulations with this active ingredient have been reported for any salamander species.

The objective of the present study was to address the gap in knowledge surrounding the sensitivity of an amphibian species endemic to the west coast of North America to DB in the commercial formulation, Reward®. This present study assessed first the acute toxicity of Reward® (96-h acute exposure) and sub-chronic toxicity of Reward® during a 21-day exposure of larval *A. gracile*. Concentrations for these exposure experiments were selected based on the reported maximum environmental concentration of DB in the water

column immediately after aquatic applications of this herbicide according to the manufacturer's instructions (0.37 mg/L (Syngenta 2003)). *A. gracile* were exposed continuously during these experiments and adverse effects on survival, body weight, and length were determined.

Methods

Animal collection, hatching, and culture

Northwestern salamander eggs were collected from the wild under British Columbia Ministry of Environment permit: SU17-265445, and all protocols were adhered to under approval of Simon Fraser University Animal Care Protocol: 1240B-16. Clutches of *A. gracile* eggs in developmental stage 28 (Harrison 1969) were collected from a pond at the University of the Fraser Valley campus (Abbotsford, BC; 49° 01′ 41.4″ N 122° 17′ 05.9″ W). Upon collection of the clutches, there was no recorded use of Reward® herbicide or history of pesticide treatment. Individual egg masses were transported in pond water at 16 ± 1 °C and acclimated over 48 h to 20 ± 1 °C in separate 8 L aquaria with gentle aeration under a photoperiod of 12-h light:12-h dark to Simon Fraser University Alcan Aquatic Research Facility. Water quality was monitored daily until hatching in each 8-L aquarium with an HQd portable meter (Hach Company, Loveland, CO, USA; temperature 18.1 ± 0.38 °C, conductivity 93.1 ± 6.6 μs/cm, dissolved oxygen 8.99 ± 0.07 mg/L, and pH 7.73 ± 0.02) and larvae were then distributed to glass test vessels. The embryos hatched 8–10 days after collection. Larvae less than 5 days old (stages 41–45 (Harrison 1969)) were used in the 96-h acute study and less than 14 days (stages 45–46) were used in the 21-day sub-chronic exposure.

A previous study has shown that diquat does not adhere to glass vessels or volatilize during aeration after 24 h (McCuaig 2018). This present study follows previous fathead minnow (*Pimphales promelas*) exposure experiments that employed the same solution preparation methodology and included chemical analysis that verified nominal concentrations (Moreton 2018). For that reason, the concentrations displayed here represent nominal values. The dechlorinated municipal tap water used in this study is considered soft based (0–60 mg/L calcium carbonate), and although not measured, during these particular studies, it is monitored routinely multiple times per year at the Alcan Research Facility and has not exceeded 20 mg/L $CaCO_3$ over the last 5 years. The most recent hardness measurement was conducted approximately 1 month prior to this study and the water hardness was 10.7 mg/L $CaCO_3$ (determined via inductively coupled plasma-mass spectrometry, Maxxam Analytics, Burnaby, BC).

96-h acute larval *A. gracile* exposure to Reward®

The concentrations of DB were selected to test concentrations at and above the manufacturer's maximum reported environmental concentration of 0.37 mg/L DB in Reward® when applied according to the label's instructions (Syngenta Canada 2015), increasing thereafter by a factor of 4.5. Stage 43 *A. gracile* were continuously exposed to either a control or one of 5 nominal concentrations (0.37, 1.67, 7.49, 33.71, and 151.72 mg/L) of active diquat ion in Reward® dissolved in dechlorinated municipal tap water for 96 h in four replicates per treatment or control. In this static, non-renewal exposure, nine larvae were added to each of the four replicate glass aquaria (30 cm length × 20.5 cm height × 15 cm width) containing 7 L of Reward® solution or dechlorinated water. Larvae were fed ad libitum a mixture of freshly thawed *Mysis diluviana* (opossum shrimp; Piscine Energetics 2017) and *Chironomidae* larvae (bloodworms; Hikari USA 2017) once after 24 h. A photoperiod of 12-h light: 12-h dark was maintained throughout the experiment, and the exposed sides of the tanks were shielded from visual disturbances with black plastic. Daily checks on survival and removal of dead larvae were performed in the morning, and water quality was monitored daily using a HQd Portable Meter in one of the four replicate tanks (on a rotating basis so each replicate was monitored every 4 days) for each treatment and the water control.

After the 96-h Reward® exposure, larvae were removed by netting and euthanizing in 0.4 g/L buffered MS-222. Body metrics were recorded and included total wet body weight, snout-tail length, and snout-vent length under a dissecting microscope. Developmental stage was determined for each larvae based on visual inspection and the development stage system by Harrison (1969) using forelimb and hindlimb as markers. The whole body was then frozen on dry ice and transferred to storage at − 80 °C for future molecular work.

21-day sub-chronic larval *A. gracile* exposure to Reward®

This 21-day continuous exposure followed the parameters of the Organization for Economic Co-operation and Development Test 231: Amphibian Metamorphosis Assay (OECD 2009). Larvae hatched from 5 separate egg masses were individually assessed and separated into groups by developmental stage (stages 41–46 (Harrison 1969)) to standardize the average age between tanks used in this experiment. Each tank contained 5 larvae at stage 45 and 3 larvae at stage 46 (Harrison 1969). The test concentrations were adjusted based on the results observed in the 96-h acute study, and increased fourfold starting from the maximum reported concentration when applied to a water body to control aquatic plants according to the manufacturer's instructions (Syngenta Canada 2015). Specifically, larvae were exposed

to either a control or one of 5 nominal concentrations (0.37, 1.48, 5.92, 23.7, and 94.7 mg/L) of active diquat ion (DB) in Reward®. The animals were maintained under a 12-h light:12-h dark photoperiod, and the sides of the tanks were covered with dark plastic to minimize visual disturbance while technicians worked. The loading density was < 2 larvae/L and 80% test chemical/control water renewals were performed every 72 h. Water quality was monitored using a HQd portable meter before and after water renewals in one of the four replicate tanks (on a rotating basis so each replicate was monitored every 4 days) for each treatment and the water control. Larvae were fed ad libitum a mixture of freshly thawed *Mysis diluviana* (opossum shrimp; Piscine Energetics 2017) and *Chironomidae* larvae (bloodworms; Hikari USA 2017) once per day. This resulted in each tank receiving an average of 0.13 g of an approximately 1:1 ratio of *Chironomidae* larvae to *Mysis diluviana*. After the 21-day exposure, larvae were sampled in the same manner described in the 96-h test.

Data analysis

Statistical analysis was performed using SPSS v. 24 (IBM Corporation, Armonk, New York, USA). Survival, morphometric data, condition factor, and water conductivity were analyzed using a one-way analysis of variance (ANOVA) followed by a Tukey's post hoc to determine significance ($P < 0.05$; survival data log transformed prior to analysis). Condition factor (K) was calculated for each larvae as $K = 100 \times \left(\frac{\text{body weight}}{(\text{snout vent length})^3}\right)$. These data passed Shapiro-Wilk's test for normality and Levene's homogeneity of variance test. The LC_{50}s were calculated using the binomial method if mean survival dropped from 100 to 0% between two test concentrations (Environment Canada 2007). If a gradual dose response was displayed, the Probit method was used to calculate LC_{50} or effect concentration (Environment Canada 2007).

Results

96-h acute larval *A. gracile* exposure to reward®

No significant mortality was observed in the controls or after 96 h of exposure to 0.37, 1.67, 7.49, and 33.72 mg/L DB on 1–3-day-old *A. gracile* larvae; however, there was 100% mortality at the highest concentration of 151.72 mg/L DB at 72 h (Fig. 1a). The average body weight of larvae was significantly decreased by 13.2% after 96 h of exposure to 33.72 mg/L DB (the highest test concentration with 100% survival), compared to the control (P = 0.003; Fig. 1b). Although there was no significant difference in the snout-vent length in any of the concentrations after 96 h of exposure (P = 0.114; data not shown), total body length (snout-tip of tail) showed significant increase by 3.0% in the 7.49 mg/L test concentration (P = 0.002; Fig. 1c). The 96-h LC_{50} value was 71.5 mg/L (binomial method; Environment Canada 2007). Condition factor (K) was not significantly different between any treatments (P > 0.05; data not shown). Across all treatments, the average water temperature was 18.4 ± 0.78 °C (range 17.3–20.4 °C), daily dissolved oxygen ranged from 8.38 to 9.70 mg/L and pH ranged from 7.01 to 8.31. Ammonia levels were below detection (< 1 μg/L). The conductivity increased as DB concentration increased with the following ranges in μs/cm: control, 27.4–29.1; 0.37 mg/L DB, 28.0–30.1; 1.67 mg/L DB, 30.0–31.3; 7.49 mg/L DB, 38.7–40.8; 33.72 mg/L DB, 77.0–80.6; 151.72 mg/L DB, 247–261. Conductivity was significantly different between the control and all treatments except for 0.37 and 1.67 mg/L DB (P < 0.05; data not shown).

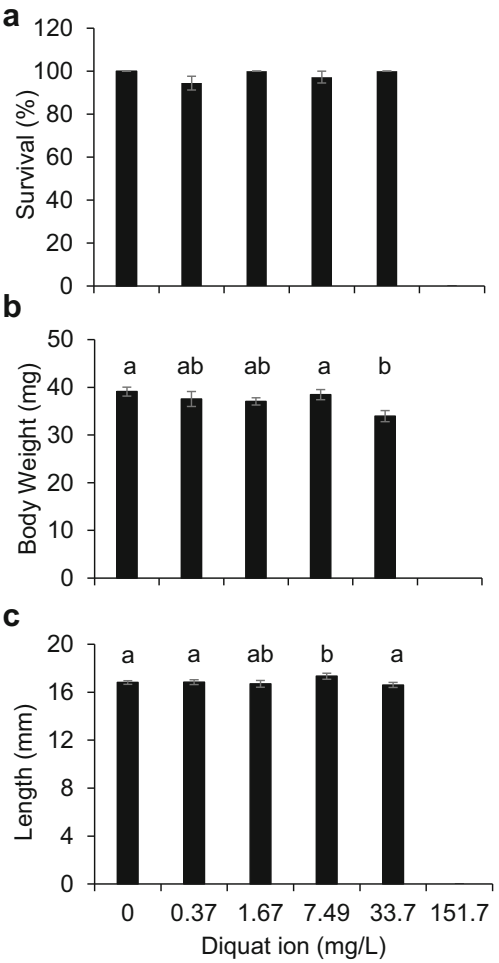

Fig. 1 Effects of 96 h continuous exposure to Reward® on *A. gracile* larval (aged < 5-day post-hatch). **a** Survival. **b** Body weight. **c** Length from snout to tip of tail. Values represent means ± standard error (9 larvae per replicate and four replicates per treatment). Differing superscripts indicate significant difference (one-way analysis of variance followed by Tukey's post-hoc, P < 0.05)

21-day sub-chronic larval *A. gracile* exposure to Reward®

The 21-day sub-chronic *A. gracile* larval exposure showed 100% mortality at concentrations of 5.92, 23.7, and 94.7 mg/L, DB in Reward®. Survival during the exposure period decreased to 0% for concentrations ≥ 23.7 mg/L after 11 days and in the 5.92 mg/L after 18 days. The 7-day LC_{50} for *A. gracile* larvae was 1.72 mg/L (probit method; Environment Canada 2007). A significant decrease in survival to 53.1% was observed after 21 days in the larvae exposed to 1.48 mg/L compared to controls ($P = 0.040$; Fig. 2a). The 21-day LC_{50} value was 1.56 mg/L (probit method; Environment Canada 2007). A similar concentration-response is shown in Fig. 2b as body weight decreased with increasing concentration of DB. *A. gracile* in the highest surviving concentration of DB (1.48 mg/L) exhibited a 74% decrease in body weight compared to controls ($P = 0.003$; Fig. 2b). The lowest observed effect concentration (LOEC) for body weight was 1.48 mg/L and the no-observed effect concentration (NOEC) for body weight was 0.37 mg/L. There was no significant difference in total body length (Fig. 2c). Condition factor (K) was significantly lower in the 0.37 and 1.48 mg/L treatments compared to the control ($P = 0.0030$; data not shown). Across all treatments, the average water temperature was 18.9 ± 1.5 °C (range 16.1–21.8 °C), dissolved oxygen across all treatments ranged from 7.80 to 9.67 mg/L and pH ranged from 7.22 to 7.54. Ammonia levels were below detection (< 1 μg/L). The conductivity increased as DB concentration increased with the following ranges in μs/cm: control, 31.6–35.3; 0.37 mg/L DB, 32.3–36.1; 1.48 mg/L DB, 33.8–37.3; 5.92 mg/L DB, 40.9–45.1; 23.68 mg/L DB, 67.8–71.9; and 94.72 mg/L DB, 173–186.3. Conductivity was significantly different between the control and all treatments except 0.37 mg/L DB ($P < 0.05$; data not shown).

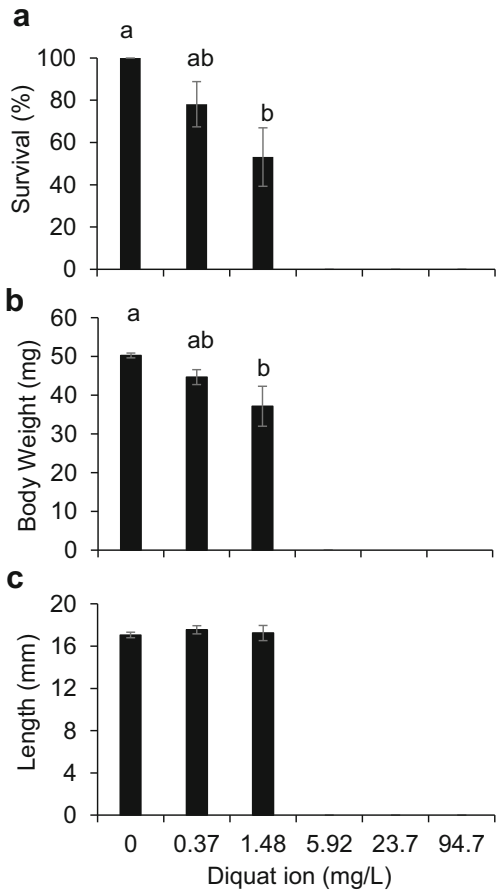

Fig. 2 The effects of 21-day sub-chronic exposure to Reward® on *A. gracile* larvae (< 5-day post-hatch). **a** Survival. **b** Body weight. **c** Length from snout to tail. Values represent means ± standard error (8 larvae per replicate and 4 replicates per treatment). Significant differences between treatments are denoted by different superscript letters (one-way analysis of variance followed by Tukey's post-hoc, $P < 0.05$)

Discussion

This is the first study examining the toxicity of the aquatic herbicide formulation Reward® on *A. gracile* under acute and sub-chronic exposure scenarios. The acute 96-h LC_{50} obtained in this study for larvae was 71.5 mg/L DB, suggesting *A. gracile* are generally less sensitive after acute exposure than early life-stage and adult *Pimephales promelas* and other fish reported in previous studies with pure DB (4.4–18.7 mg/L; United States Environmental Protection Agency 1995; Syngenta 2016). However, in the present study, after a 21-day continuous exposure of *A. gracile* larvae to Reward®, an LC_{50} of 1.56 mg/L DB was derived, which is dramatically (~ 46-fold) lower than the acute LC_{50} value observed in the present study for *A. gracile*. Furthermore, in the present study, the higher lethality at lower concentrations of Reward® observed during the sub-chronic exposure was also associated with sub-lethal effects on growth, with decreased growth occurring at 1.48 mg/L. Few toxicity studies of DB in amphibians are reported in the literature, but one study in Northern leopard frog (*Rana pipiens*) larvae suggests that *A. gracile* is ~ 3 times more sensitive using lethality as an endpoint after similar sub-chronic exposure duration to pure DB (Dial and Dial 1987). According to the Reward® label, the maximum concentration of DB that is expected to accumulate in the water column after aquatic applications of this herbicide is 0.37 mg/L, which is lower than the lethal and sub-lethal effects observed for *A. gracile larvae* in the present study during the chronic exposure experiment. Additionally, while DB has been shown to sorb to the organic components of soil and sediment (Ritter et al. 2000), there is no evidence that repeated applications will not saturate the soil and increase risk to aquatic organisms. Nevertheless, since the actual environmental concentrations of DB are not currently monitored in

Canada and it is utilized for both aquatic and terrestrial applications, if cumulative environmental concentrations reach low mg/L levels then larval growth and survival of *A. gracile* may be impeded.

The present study used the proprietary commercial formulation of DB, Reward®, which makes it difficult to compare these results directly to previous studies that were mainly conducted on fish using the pure active ingredient during waterborne toxicity bioassays. Furthermore, the present study reports nominal concentrations of DB only based on addition of Reward® volumes to water in test vessels. However, several previous fish experiments in our lab that employed the same Reward® original solution, stock dilution methodology, and water renewal regimes that did include DB water concentration measurements by a commercial laboratory showed a maximum variance of 30% between measured and nominal DB concentrations, with the majority of samples demonstrating 15% variance or less (McCuaig 2018; Moreton 2018). Furthermore, McCuaig (2018) demonstrated no volatilization or adherence to aquaria glass after 24 h of aeration without fish. This demonstrates DB does not likely evacuate from the water column without organic components present. Due to economic restrictions, chemical analysis was not possible, though the only substance present that may have sorbed DB was the food, present in small enough quantities (0.13 g/7 L) as to presume negligible effects. With respect to acute studies, the 96-h LC_{50} obtained in this study for *A. gracile* larvae was 71.5 mg/L, which is approximately > 4.5 times less sensitive than rainbow trout fingerlings (96-h LC_{50} = 15 mg/L; age not reported; water hardness < 52 mg/L (Emmett 2002)) and > 18 times less sensitive than *P. promelas* larvae (96-h LC_{50} = 3.82 mg/L (Moreton 2018)). Sub-chronic or chronic continuous studies of DB toxicity comparable to the present *A. gracile* experiment are rare. However in one sub-chronic test, *P. promelas* larvae were exposed to waterborne DB during the egg to fry stage for 34 days and resulted in a NOEC of 0.12 mg/L and a LOEC of 0.32 mg/L based on survival (water hardness not reported (European Commission 2001; Emmett 2002). In *A. gracile*, larvae were unaffected at 0.37 mg/L DB based on survival and growth endpoints during 21-day exposures, and a higher LOEC of 1.48 mg/L was observed for both endpoints indicating lower sensitivity compared to larval *P. promelas*. Future studies using the same experimental designs (duration, formulation, water hardness, etc.) to test Reward® on multiple species are necessary to determine if differences in sensitivity exist between fish and *A. gracile*, as well as identify any toxicity-modifying factor of DB during aquatic exposures.

The question of water hardness and conductivity and its influence on DB toxicity in NWS larvae in the present study is unclear, but in general, these factors have been known to impact xenobiotic toxicity in other amphibians. Previous work has demonstrated hard water's protective effects in fish to some xenobiotics (Charles et al. 2002; Horne and Dunson 1995; Perschbacher and Wurts 1999), which may be of importance as the Pacific North West generally contains low water hardness (US Geological Survey 1975). One study reports effects on survival after experiments exposing Northern leopard frogs (*Rana pipiens*) to pure DB for 16 days (water hardness was 374 mg/L $CaCO_3$) during the following 2 life stages: embryos pre-hatching during the early gastrula stage initiated 1-day post-hatch; and, larvae 15 days old (Dial and Dial 1987). Significant mortality was observed at 5 and 10 mg/L (~ 30% and 65%, respectively) and a NOEC of 2 mg/L for the early gastrula life stage was reported (Dial and Dial 1987). However, the older Northern leopard frog larvae appeared to be less sensitive than the younger larvae tested because no effects on survival were observed for the older life stage tested (i.e., 15-day-old larvae) at 10 mg/L which was the only concentration tested by Dial and Dial (1987). Together the results of the study by Dial and Dial (1987) suggest that the 16-day LC_{50} for gastrula stage larvae is between 5 and 10 mg/L, and this is ~ 4–10-fold higher than that observed in the present study with *A. gracile* larvae of similar age after 21-day exposure. This result also demonstrates that the older life stages of these frogs were less sensitive to DB. However, it is worthy of note that the water was harder (> 30-fold), and pure DB was used during the Northern leopard frog exposures (Dial and Dial 1987), while in the present study, the water was considerably softer (10.7 mg/L $CaCO_3$ determined via inductively coupled plasma-mass spectrometry, Maxxam Analytics, Burnaby, BC) and Reward® formulation was used. In addition, in the present study, the influence of increasing conductivity with increasing addition of Reward® to the test water on the health of the NWS larvae in this study is unknown. Whether DB and/or other proprietary components in this commercial herbicide formulation caused the increasing conductivity and the tolerance limits of the NWS with respect to conductivity was beyond the scope of this experimental design but warrants further investigation. Thus, water hardness and conductivity as toxicity-modifying factors as well as species-specific sensitivities to DB and/or Reward® remain to be discovered, and will require additional studies in multiple amphibians under similar experimental conditions.

In contrast to the reduced weight observed by 1.48 mg/L in *A. gracile* in the present 21-day Reward® exposure study and reduced condition factor in a non-concentration dependent manner, one early DB study reported increased weight in frog and toad tadpoles (*Rana temporaria* and *Bufo bufo*, respectively) exposed to 1.0 mg/L DB (Cooke 1977). At both 18- and 32-day post-exposure, a significant increase in body weight compared to tadpoles from untreated ponds was reported (Cooke 1977). This weight gain was attributed to algal blooms that developed after DB exposure and the death of macrophytes, which these normally carnivorous tadpoles evidently found useful for gaining weight (Government of

British Columbia 2017). The increase in body weight was confirmed to be correlated with an increase in intestinal content (i.e., algae, diatoms) versus swelling or water retention, compared with the controls. Since *A. gracile* are mainly carnivorous, the opposing effect after Reward® exposure observed in the present study suggests adverse effects on feeding behavior or metabolism of food that has been consumed. Furthermore, there was an unexpectedly slight, but significant increase in average total body length of *A. gracile* larvae in the third highest concentration tested during the 96-h acute study (7.49 mg/L DB), which may or may not have biological relevance to this species. Given the Harrison (1969) developmental staging guide only allows for morphological identification up to stage 46, our ability to differentiate development between exposure concentrations was limited and therefore may cause an unintended conflation of DB-induced weight reduction with normal developmental variability between individuals. Additional studies examining natural and toxicant-induced changes in growth and development in *A. gracile* are required to further characterize the sensitivity of these apical endpoints in this amphibian species.

Other studies testing the sensitivity of several amphibians, including *A. gracile*, to pesticides have demonstrated similar acute LC_{50} values across species that may help place the toxicity of Reward® among other products. In the study by Relyea and Jones (2009), a total of 13 different species of amphibians were exposed to increasing concentrations of Roundup® Original Max (1.12–5.26 mg/L glyphosate in Roundup® commercial formulation), and 100% mortality was observed in every species after 96 h in the highest concentration. The 96-h LC_{50} for the larval *A. gracile* was 2.8 mg/L and was comparable to the other salamander species (*Ambystoma maculatum*, *Ambystoma laterale*, and *Notophthalmus viridescens*; 2.8, 3.2, and 2.7 mg/L, respectively). The frog tadpoles were in a similar low mg/L range but were slightly more sensitive to glyphosate, with 96-h LC_{50}s ranging from 0.8 mg/L (*Rana catesbeiana*) to 2.0 mg/L (*Bufo boreas*). These data also suggest that early life stage amphibians are generally as sensitive as fish species exposed to the same chemical (i.e., glyphosate as Roundup®) (Folmar et al. 1979). Interestingly, a study (Folmar et al. 1979) conducted the same assay on multiple fish species (*Salmo gairdneri*, *Pimephales promelas*, *Ictalurus punctatus*, and *Lepomis macrochilas*) in separate Roundup®, a surfactant, and technical/pure glyphosate exposures, finding similar 96-h LC_{50}s for these fish for the surfactant alone compared to Roundup®, suggesting that glyphosate may not be the primary toxic agent of Roundup®. This phenomenon, also noted by Howe et al. (2004) may be a similar component to the toxicity of DB alone versus Reward®. The similar LC_{50}s derived from acute exposures across a variety of frog, amphibian, and fish species to Roundup® appear to suggest similar sensitivity of these taxa to Roundup® itself compared to Reward®. Thus it appears that some pesticides prove to have similar toxicities for all life stages while some are dissimilar; this could be explained by factors like variable windows of vulnerability during development, or the timeline of detoxification pathway emergence between different species, and must consider the mode of action of the toxicant (Herkovits et al. 1997). The present study showed a dramatic difference in the sensitivity of *A. gracile* larvae from an acute 96-h exposure to the 21-day exposure (71.5 and 1.56 mg/L DB in Reward®, respectively). Future studies testing the toxicity of Reward® under acute and chronic exposure scenarios in multiple amphibian species are required to understand the underlying causation of these dramatic differences in toxicity with respect to exposure length and general toxicity to *A. gracile*.

The novel aspect of this study was testing the toxicity of the commercial formulation of the widely used herbicide, Reward®, on an understudied amphibian native to the Pacific North West. When applied according the product's label, the maximum concentration of DB expected to accumulate in the water column is 0.37 mg/L, which does not appear to be an acute threat to larval *A. gracile*. However, in the 1 to 2 mg/L concentration range, decreased survival and body weight were observed during sub-chronic exposures. This illustrates the importance of adhering to the mandated temporal and rate restrictions during aquatic applications of Reward® to ensure the maximum predicted concentrations are not exceeded. While previous reports have indicated DB dissipates from the water column rapidly and sorbs to sediment and persists but remains inert (Ritter et al. 2000; Langeland and Warner 1986; Simsiman and Chesters 1976; Yeo 1967), the actual environmental concentrations of this herbicide are not currently monitored in Canada. Therefore, the cumulative risk of low-level chronic exposure to aquatic wildlife, such as amphibians, after terrestrial applications and the potential leaching or run-off into surface waters as well as aquatic applications of Reward® are unknown, but the results of this study suggest that continuous exposure at low mg/L levels presents threats to larval growth and survival.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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