Effects of the Aquatic Herbicide, Reward®, on the Fathead Minnow and Northwestern Salamander

by

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Abstract

This study assessed the toxicity of the aquatic herbicide Reward® (active ingredient diquat dibromide [DB]) in its commercial formulation. For larval and adult Fathead minnows (*Pimephales promelas*; FHM), after two 24 h pulse exposures spaced two weeks apart, LC_{50}'s of 4.19 mg/L and 6.71 mg/L were obtained, respectively. A lowest observed effect concentration of 1.18 mg/L for larval growth in FHMs during a 7 d continuous exposure was also derived. An acute 96 h LC_{50} of 71.5 mg/L DB was obtained for Northwestern salamander (*Ambystoma gracile*) larvae, while a 21 d continuous exposure LC_{50} was dramatically lower (1.56 mg/L DB) for these larvae. Few reports of environmental concentrations of this pesticide could be found for Canadian waters. Whether aquatic and/or terrestrial applications and/or DB's persistent nature in sediments translate into continuous toxicity exposure scenarios in Canadian aquatic systems is unknown and should be the focus of future studies.

Keywords: toxicology; aquatic herbicide; Reward®, diquat dibromide; fathead minnow; northwestern salamander

To those who have gone before and those who will come after.

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I, along with SFU, acknowledge our three campuses stand upon the Squamish, Musqueam, Tsleil-Waututh, Katzie and Kwikwetlem peoples territory, who lived here before us and continue living here now.

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Thank you to my wonderful family, whose strong spirit of resiliency and loyalty will endure within me forever. Every success is ours.

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List of Acronyms

BCL2	Biological Containment Level 2
F_1	First Generation of Offspring
FHM	Fathead minnow
H50	Time to reach 50 % hatch success
H90	Time to reach 90 % hatch success
Kow	Octanol/Water Partition Coefficient
LC_{50}	Lethal Concentration 50%
LOEC	Lowest Observed Effect Concentration
MRL	Maximum Residue Limit
NCAG	National Contaminants Advisory Group
NOEC	No Observed Effect Concentration
NWS	Northwestern Salamander
OECD	Organisation for Economic Cooperation & Development
PMRA	Pest Management Regulatory Agency
ROS	Reactive Oxygen Species
TRED	Tolerance Reassessment Programs and Risk Management Decision
USEPA	United States Environmental Protection Agency
VHS	Viral Hemorrhagic Septicemia
WHO	World Health Organization

Chapter 1. General Introduction

1.1. Pesticides

Pesticides are substances that are used to limit or destroy pest populations in domestic or commercial settings (Health 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 (USEPA, 2012). Since the early 1980s, pesticide use has increased in agriculture with the main output being in herbicide application. 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). While herbicides are generally formulated to target biochemical pathways that are specific to plants, they can also potentially cause harm to non-plant wildlife. Since most herbicides can be persistent and mobile in the environment, they can accumulate in areas distant from the site of application (Figure 1) potentially inducing 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 United States, 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 traditional apical endpoints and sub-lethal endocrine disrupting effects (USEPA, 2012). It is therefore important that new data are collected through rigorous scientific method so that regulatory bodies around the world can enact evidence-based policies that protect non-target wildlife, including humans.

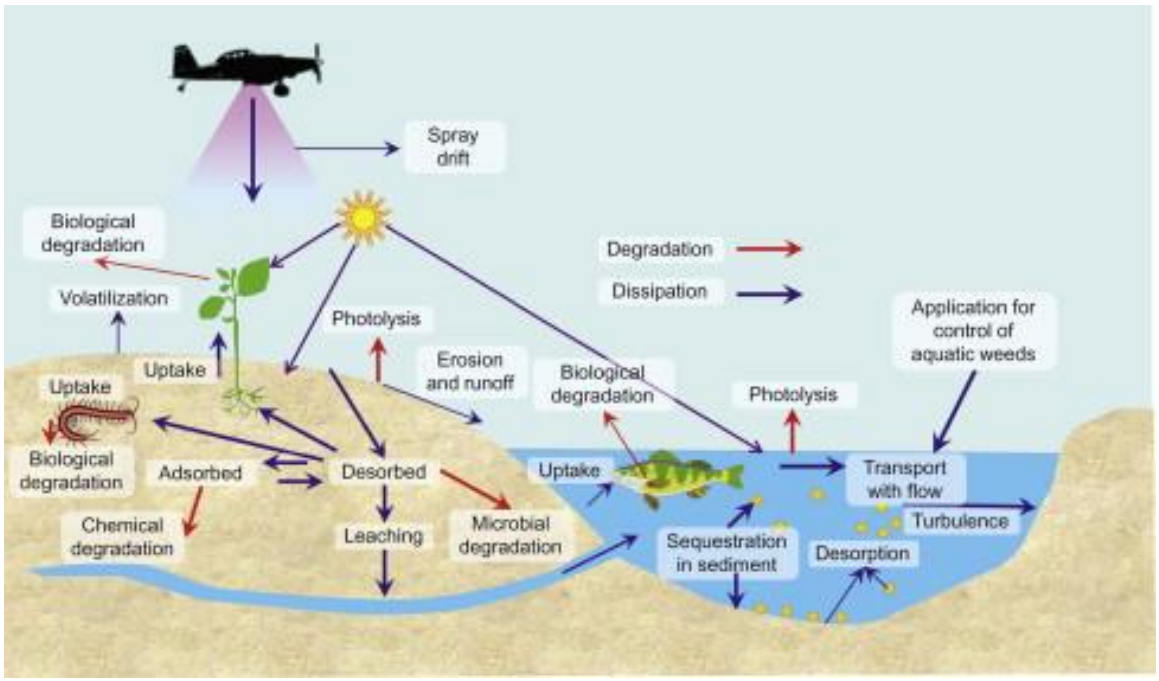

Figure 1. Pesticides that are broadly applied often have a variable fate in the environment, capable of traveling long distances via air or water to affect organisms that were not the intended target of the pest control (Solomon *et al*., 2013).

Pesticides in Canada are regulated under the Pest Control Products Act, registered after initial evidence-based risk assessment, and are subject to re-evaluation on a 15-year renewal basis to align with the latest scientific assessment (Health Canada, 2009). The Pest Management Regulatory Agency (PMRA) is made up of over 420 employees who represent an interdisciplinary team of scientists and administrators (PMRA, 2018). The PMRA is responsible for assessing new products as they become available to market and regulating health and safety standards regarding the product's spatial and temporal limits of application. The responsibility to adhere to these regulations falls solely on the applicator and consumers are warned to critically assess whether pesticide use is necessary, considering the wider environmental impact that their use may have (PMRA, 2017).

1.2. Diquat Dibromide

Commercial and recreational agriculturists alike have long faced the task of battling unintended growth of pest species of plants alongside the cultivation of crops or gardens and keeping water drainage ways or irrigation ditches clear. Mechanical removal can be

extremely laborious and expensive, consuming huge sums of personnel and economic resource especially as agricultural treatment area increases (Rejmánek & Pitcairn, 2002). Several categories of herbicides exist and target specific types of plant pest species by a variety of molecular mechanisms. Regulating the pest-management industry and informing policy with up-to-date sources of toxicological information about pesticides is important, especially in agriculture-rich economies (Carriquiriborde *et al.,* 2014). The herbicide examined in this research is diquat dibromide (DB) and is known by several trade names some which include Reward®, Reglone®, Aquacide®, Dextrone® and Reglox® (EPA, 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 coefficient (K_{OW}) is -4.60, which is more than 9 orders of magnitude smaller than the generally accepted minimum K_{OW} value of 5, indicating it is unlikely to accumulate in tissues and biomagnify up trophic levels (Mackay *et al.,* 2017). This low 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 with limited terrestrial applications, thus the bulk of its use is likely to be applied in aquatic settings. It is important to consider its fate once applied to bodies of water, given water's mobility, persistence of dissolved chemicals, and the broad scope of wildlife that might utilize treated water.

Currently, DB is not part of the National Pesticides Monitoring and Surveillance Network program and consequently there is no data on environmental concentrations in Canada (NCAG, 2018). When Reward® is applied according to the manufacturer's instructions (0.454 kg/1233 m^3, or ~0.37 mg/L), the maximum concentration is 0.37 mg/L DB. Due to its indiscriminate herbicidal action it is effective against common targets including Duckweed, Coontail, Canadian Water Weed, Water Chestnut and Flowering Rush (Syngenta, 2015). DB is a bipyridyl herbicide compound, similar in structure to its predecessor paraquat (Figure 2), which was first registered in 1964. Unlike paraquat, DB is registered as a restricted use pesticide in Canada (Health Canada, 2010). While many DB-based products are designed for aquatic application, it is also used directly as a pre-harvest desiccant on crops destined for consumption such as potatoes, peas, beans, canola, and control of pest weeds surrounding vegetables and apple trees (Health Canada, 2008).

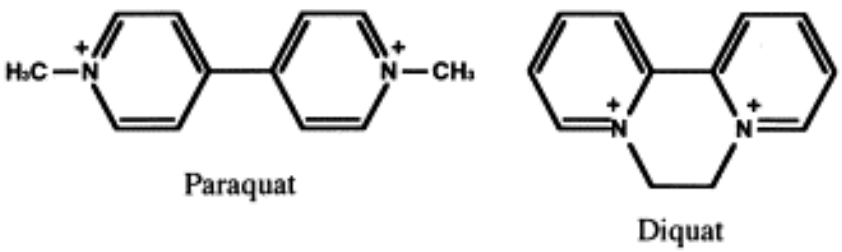

Figure 2. Comparative chemical structure of paraquat and diquat ions as would be considered in their biologically active forms (Fuke *et al*., 2002). The nomenclature of DB according to the International Union of Pure and Applied Chemistry is 6,7-dihydrodipyrido[1,2-b:1',2'-e]pyrazine-5,8-diium; dibromide (National Centre for Biotechnology Information, 2018).

According to Syngenta's Reward® Landscape and Aquatic herbicide (Reward®) factsheet, the product's active ingredient DB (present at 373 g/L in the commercial formulation; 240 g/L diquat cation) is a non-selective herbicide that is rapidly taken up by the parts of the plant to which it adheres causing diffuse desiccation (Syngenta, 2003; Miller, 2002). DB is classified as a group 22 herbicide, which represents compounds with a toxic mode of action that causes plant death through cell membrane disruption (Alberta's Ministry of Agriculture and Forestry, 2016). Closer analysis of its mode of action indicates that DB causes plant cell death by inhibiting the reduction of NADP+ in photosystem I (Harris & Dodge, 1970) by cycling electrons (Figure 3). Diquat is subsequently oxidized during the production of hydrogen peroxide which, though naturally occurring, is a highly reactive molecule that can subsequently reduce and damage other macromolecules or produce other reactive oxygen species (ROS; e.g. superoxides, hydroxyl radicals etc.; Choudhury *et al.*, 2017; Demidchik, 2015; Scandalios, 1993). ROS can perform irreversible damage to cellular components that ultimately lead to cell death (Dixon & Stockwell, 2014). Typically, plant cells contain specialized enzymes (antioxidants) to remove or neutralize ROS, though in the case of DB exposure the ratio of substrate to antioxidant enzymes overwhelms the cells and ROS can initiate a chain reaction of oxidative cellular damage (Vale & Jones, 2000).

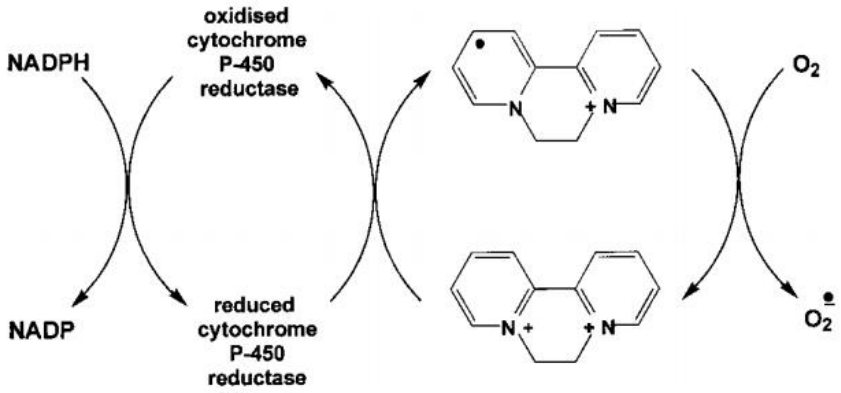

Figure 3. Schematic representation of electron cycling and generation of free radicals within cells (Jones & Vale, 2000).

Currently, herbicide products containing DB are approved for use in Canada, Europe and the United States. It is a currently restricted-use pesticide according to Canada's Pest Management Regulatory Agency in their most recent assessment document (Health Canada, 2010), and approved for restricted use in the United States since their last Tolerance Reassessment Programs and Risk Management Decision (TRED) for DB (USEPA, 2002). The current European Union approval expires June 30th, 2018 and will enter review at that time (European Commission, 2017; European Food Safety Authority, 2015). Federal regulators in Canada have most recently ordered a maximum residue limit (MRL) on crops of up to 0.5 mg/L (Health Canada, 2010). The maximum acceptable concentration of DB in drinking water is 0.07 mg/L, based on an allowable exposure limit of 0.008 mg/kg bodyweight per day as established by the World Health Organisation (Government of Canada, 2009).

1.3. Environmental Fate of Diquat Dibromide

The World Health Organization (WHO) reported the half-life of DB in natural waters to be <48 h (WHO, 1995). A WHO assessment of groundwater at two different sites in Japan where DB had been commercially applied for more than 5 years showed no evidence of persisting diquat (limit detection = 0.1 mg/L) (WHO, 1998). It is proposed that concentrations rapidly decrease to 0.1 mg/L after 24 h and 0.01 mg/L after 4 d (Syngenta, 2003). Simsiman & Chesters (1976) reported slow to negligible microbial degradation of radio-labelled DB in a lake sediment experiment. Their results suggest diquat does not readily desorb from sediment to which it is adhered, and that microbes do not play a major role in removing DB from the water column. Cabridenc & Petit (1995) reported DB to be

susceptible to photodegradation (75% ^{14}C radio-labelled DB degraded after 96 hours). Despite this, it is purported that DB's primary environmental fate is adsorption to soil substrates, rendering it biologically inert (Howard, 1991). Owing to its dual positive charge, the cation is strongly attracted to negatively charged clay particles, where it remains tightly bound (Ketterings, *et al.,* 2002). In aerobic soil, DB is reported to have a half-life of 3450 d (Pateiro-Moure *et al.,* 2010). Despite its strong affinity for organic matter, it is unclear whether it will be re-released when mechanically disturbed or if soil/sediment becomes saturated and therefore presents further threat to aquatic life. Current international assessments have rendered DB largely safe when applied according to manufacturer's directions (Environmental Protection Agency, 1995; European Food Safety Authority, 2015; Health Canada, 2010).

1.4. Toxicity of Diquat Dibromide in Animals

1.4.1. Fish

Many studies testing the toxicity of DB in fish demonstrate lethal concentrations in the low mg/L range. For example, Paul *et al.*, (1994) determined LC_{50} concentrations on embryo, juvenile and adult Walleye (*Stizostedion vitreum*) after 24-, 48-, 72-, and 96-hr. The resulting LC_{50} 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. A study with Rainbow trout of unreported life stage reported a 96 h LC_{50} of 11.2 mg/L (Gilderhus, 1965). However, the highest values were reported for Grass carp (*Ctenopharyngodon idella*; 96 h LC_{50} was 53.0 mg/L) (El-Deen & Roger, 1993) and Mosquitofish (*Gambusia affins*; 96 h LC_{50} was 289 mg/L) (Leung *et al*., 1983).

The necessity to understand adverse sub-lethal effects of chemicals on an organism are vital to understanding the extent of damage that may be caused at levels below the established lethal limits and under more realistic environmental exposure scenarios. Emmett (2002) reports the lowest observed effect concentration (LOEC) in early life stage (egg to fry) exposed for 34 d Fathead Minnow (*Pimephales promelas*) to be 0.32 mg/L. This chronic exposure used bodyweight to measure significant effects at this concentration. Toxicity data tend to show a relationship between life stage and sensitivity, such that the younger the organism, the more sensitive they are to DB. de

Peyster & Long, (1993) identified a decrease in swim speed and mobility in FHM adults under exposure to at least 9.2 mg/L DB. Bimber *et al*. (1976) reported respiratory distress in two-year old yellow perch that were exposed to sub-lethal levels of DB (1 and 5 mg/L), represented by a dramatic increase of "coughs" per hour compared to the control. The rate of coughing increased steadily as time progressed from the initial addition of DB to 48 h post-exposure. Post-exposure assessment of physiological damage from these respiratory fits were not conducted. Dodson & Mayfield (1979) reported significantly decreased swim speed in rainbow trout after a 24 h exposure to all concentrations of DB (0.5, 1.5, and 5.0 mg/L). These sub-lethal endpoints may point to an adverse ecological consequence facing susceptible aquatic organisms, with decreased predator evasion or prey capture. Not all the effects of herbicidal treatment may be exclusively harmful, however. One interesting publication in a diquat-treated Nigerian creek saw an increase in fish species numbers after herbicidal treatment (Olaleye & Akintunde, 1993). This is likely owing to the herbicidal removal of water hyacinth (*Eichhornia crassipes*) and its compromise of water quality and physical clogging of water ways. This stands as one anecdotal case where sub-lethal exposure provided fish with a net benefit after the removal of a more harmful factor and should not brand DB as helpful for organismal flourishing.

Molecular changes at the cellular level that precede whole organism level changes are useful clues that can help elucidate a chemical's mode(s) of action and assist in predicting adverse effects on the whole organism. Sub-lethal work performed on rainbow trout (*Oncorhynchus mykiss*) indicated a significant increase in hepatic proteome effects at 0.37 mg/L DB in a Reward® exposure (McCuaig, 2018). Protein analysis showed 315 protein changes in exposed fish compared with controls at the pre-feeding swim-up fry stage but only 84 proteins changed in the feeding swim-up fry stage. This is indicative of increased sensitivity at earlier life stages, but also how both life stages responded to exposure; despite the difference in protein changes, both early and later life stage fish had changes in oxidative stress proteins along with those participating in immune pathways, and an increase in Atk/mTOR and caspase proteins which indicates possible hepatoxicity. Hook *et al*. (2006) highlighted the importance of using changes in gene expression and other genetic markers to determine the effects of exposure to toxicants in whole animals. In their study of *O. mykiss*, exposure to DB (via intraperitoneal injection) produced dramatic changes in liver gene expression. Among the upregulated genes (upwards of 20

times the control) were acyl carrier protein, phosphoinositide-3-kinase and nucleic acid binding proteins which participate in fatty acid synthesis, cellular function, and gene expression, respectively (Byers & Gong, 2007; Cheng *et al.,* 2009; Travers, 2001). Other genes, like those involved in glycolysis, showed a decrease in expression. A slightly longer exposure of young grass carp (*Ctenopharyngodon idella*; 168 h, life stage not reported) noted that fish exposed to 53.0 mg/L showed significantly lower total muscle and blood plasma levels of protein compared to the control fish, though none of the 53.0 mg/L fish survived to 168 h. There was no difference reported between control fish and those exposed to 2.0 mg/L DB (El-Deen *et al*. 1993). These studies then help to illustrate a more nuanced picture of the effects DB exposure has on an organism, even if it does not seem to harm them on a macro level.

Applying laboratory-based toxicity data to the field can introduce many complicating factors, which can include commercial formulations versus technical active ingredients, multiple exposures versus continuous, and modifying factors like water hardness. Pulse exposures of some chemicals have been shown to have an additive effect, that is previous exposures affect subsequent exposures, albeit this is dependent on the length of interval between exposures (Hoang *et al*., 2007). This study also raises the potential for organismal recovery in a multi-phasic pulse scenario, but few studies exist that mimic this intermittent exposure scenario that is practiced in the field. Ali *et al*., (2017) also show a compensatory response in FHM larvae exposed to a collection of pesticides present in agricultural run-off. This post-exposure recovery period after the run-off event was represented in significantly increased body weight, length, and condition factor in FHM larvae compared with the control. Evidence of over-compensation was present not just in these apical end points, but in the relative gene expression of the androgen receptor, which increased after recovery. Pulse exposures are not often performed and currently are not represented for DB in the literature. In addition, because most of the existing DB data were collected from exposure to DB alone as an active ingredient, there remains a gap in the literature of DB exposures performed with commercial formulation. While DB data exists, it does not fully reflect a real-world exposure scenario since other chemicals are added to the commercial formula, in fact 62.7 % of Reward® formulation is undisclosed “inerts” (Syngenta, 2003). Secondly, there is a lack of data on pulsed application (i.e. with a recovery period between applications), as is regulated in some products. DB applied intermittently has not previously been reported in the literature and

the 14 d waiting period between applications, as mandated by the label, should be investigated. Finally, modifiers of toxicity may also exist for certain pesticides as an important observation by Surber & Pickering (1962) noted the LC_{50} for FHM to DB is 7.6 mg/L in softwater (19-27 mg/L Ca2+) and 70 mg/L in hardwater (320-382 mg/L Ca2+). Unfortunately, reports of water hardness along with DB concentrations in fish toxicity studies are not often reported, but trends showing that water hardness can dictate fish sensitivity to a toxicant should not be ignored when considering risk assessment (Everall *et al*., 1989; Horne & Dunson, 1995; Oliveira-Filho *et al*., 2014) especially when water quality varies with geography.

1.4.2. Amphibians

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 favour of the well-characterized frog species, *Xenopus laevis* (OECD, 2012). Amphibians have largely exhibited sensitivity to environmental pollutants as evidenced by decreases in survival and growth, along with large increases in the incidence of developmental abnormalities (Ega-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 traditional frog species and investigating relative sensitivities of related taxa.

Although no studies testing a commercial formulation of diquat dibromide have been performed on an amphibian species, some studies in frogs testing the toxicity of the pure active ingredient DB have been reported. For example, a NOEC of 2 mg/L for the early gastrula life stage of Northern leopard frogs after 16 day exposures to 2, 5 and 10 mg/L was reported by Dial *et al.* (1987). Interestingly, in this study 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 d old larvae) at 10 mg/L (the only concentration tested; Dial *et al*., 1987). Together the results of the study by

Dial *et al*. (1987) suggest that the 16 d LC_{50} for gastrula stage larvae is between 5 and 10 mg/L.

Currently, little work has been conducted on the Northwestern salamander (NWS) with respect to sensitivity to contaminants, which is native to the North America's Pacific west coast (Government of British Columbia, 2017). The species is currently listed as of least concern to the International Union for Conservation of Nature (IUCN, 2015) and is therefore of interest as a common non-target organism that may exhibit sensitivity to pesticides used near high pesticide use areas. The adaptation of established toxicity assays for this understudied amphibian are needed to begin to fill in the gap in knowledge surrounding this representative salamander species' sensitivity to contaminants prevalent in the Canadian environment.

1.4.3. Mammals

The mode of action of DB alone or within commercial formulations of herbicides in animals is not known. Because of the innate contrasts with plant physiology (e.g. there is no photosystem I to disrupt), the mechanism of toxicity is not conserved between plants and animals (Zheng *et al.,*2017; Fussell *et al.,* 2011; Osburn *et al.,* 2006; Yumino *et al.,* 2002). The most sensitive mammal appears to be the cow with a LD_{50} reported to be 30-56 mg/kg (American Conference of Governmental Industrial Hygienists, 2007). Davis & Golly (1963) determined an LD_{50} for oral dosing in rats to be 116 mg/kg body weight. In the USEPA re-registration decision (1995), a 2-generation rat reproduction study showed the lowest observed effect level equivalent to 12 mg/kg/d DB, which was administered to the rats through DB-treated food. Rodent testing indicates poor bioavailability when administered orally (<37%), but this route of exposure decreases reproductive success in rats and is associated with an increased incidence of cataracts (World Health Organization, 2004; Zhang *et al.,* 2016; Government of Canada, 2009). Research in Wistar rats and dogs (World Health Organization, 1970 & 1977) support Health Canada's assessment that when applied as directed by the manufacturer, DB does not present a significant health concern to humans outside of direct, prolonged dermal exposure; this despite reported cases of DB poisoning that have been generally deemed as rare (Health Canada, 2010; California Environmental Protection Agency, 1994; Jović-Stosić *et al.,* 2009; Saeed *et al.,* 2001; Rudež *et al.,* 1999).

The consequences of large single-time dose exposures (concentrations DB >38.6 g/L) in humans is rare but can be severe, leading to gastrointestinal distress, respiratory and renal dysfunction, ataxia, and localized redness and paresthesia (Fortenberry *et al.,* 2016). The primary targets for adverse effects in humans include organ failure of the heart (Jović-Stosić *et al.,* 2009), kidneys, central nervous system, and depending on the route of administration, can produce extremely painful burns to the skin or mucosa (Rudež, *et al.,* 1999; Manoguerra, 1990; Ronnen *et al.,* 1995). Rohanish (2012) notes many acutely toxic effects in humans stem from direct exposure of concentrated DB, which is typically limited to incidents occurring with those who apply the herbicide. There has been question regarding a connection between DB exposure and the development of Parkinsonism which is compounded by its chemical similarity to paraquat, a suspected contributor to developing Parkinson's disease (PD) (McCormack *et al.* 2002). Nisar *et al.*, (2015) investigated possible targeted neurotoxicity by DB and reported caspase activation (via RIP1 kinase) in the same neural tissue that is subject to necrosis in patients with PD. Similarly, mice exposed to paraquat and maneb fungicide in an early life stage showed dysregulation of the nigrostriatal dopamine system and subsequent neurotoxic effects, reducing motor activity and producing permanent brain lesions (Thiruchelvam *et al.,* 2002). A meta-analysis of over 100 studies also introduces question about a direct connection between PD and paraquat, noting that self-reported rates of PD were higher among those exposed to any kind of pesticide (Pezzoli & Cereda, 2013). Hence while a causal relationship is far from being established, prophylactic measures against paraquat have already been implemented in the European Union, having prohibited its use since 2007 (Díaz *et al.,* 2016). For these reasons, while there seems to be less concern about DB and its neurological effects on humans than other pesticides, future consideration would not be unwarranted.

The potential of a toxicant to cause irreversible damage to DNA is also an important consideration in risk assessment. Dimitrov *et al.* 2006 reported no mutagenic effects of DB from Reglone® (an analogous herbicide with active ingredient DB present in 240 g/L) by Syngenta Canada (2016) in either plant or mouse models with continuous exposure reaching 5 d. However, all concentrations in these assays produced an increase in micronucleus induction, and indication of genotoxicity or chromosome instability. This assay is one of the standard Organisation for Economic Development and Cooperation (OECD) tests for detecting genotoxicity where chemicals cause chromosomes to be

inequitably distributed to daughter cells during mitosis (Norppa & Falck, 2003). While some studies report genetic alterations in bacteria and human cell *in vitro* tests (Siebert & Lemperle, 1974; Benigni *et al.,* 1979), the concentrations producing these results are substantially higher than what is required for plant desiccation (Syngenta Canada, 2015). An *in vivo* murine dominant lethal test performed by Pasi *et al.* (1974) detected no mutagenic effects in the germ line of exposed rats, though it did decrease pregnancy rates. Zhang *et al.* (2012) note that the dual positively-charged heterocyclic moieties of DB pose a special potential to bind the negatively charged backbone of DNA helices. Although these interactions do not directly cause mutagenesis, questioning the biological risk of these *in vivo* interactions detected by Zhang *et al*. (2012) is warranted because these electrostatic potentials serve common functional roles in cells like initiating apoptosis (Ye *et al.,* 2002). In fact, as outlined by Jones *et al.* (2003), positive electrostatic potential is one means by which proteins successfully interact with DNA-binding domains in regular cellular function. These data considered, the USEPA (1995) reported that DB is non-carcinogenic, citing only a statistically insignificant increase in the incidence of several benign and rare tumours in male rats dosed up to 44.88 mg/kg/d over 104 weeks. To date DB has not been assessed by the International Agency for Research on Cancer (IARC, 2018).

1.5. Research Objectives

Although DB strongly adsorbs to organic matter and plants in surface water, it is highly water soluble and is resistant to microbial degradation. Therefore, due to its tendency to remain intact in the environment, there is potential for chronic exposure scenarios for non-target aquatic organisms. The specific objectives of the present study were: 1) to examine the acute toxicity of the commercial formulation of the broad spectrum herbicide Reward® in larval and adult fathead minnow (*Pimphales promales*) and larval Northwestern salamanders (*Ambystoma gracile*); 2) to determine the toxicity of Reward® in FHM after 'pulse' exposures that mimic direct aquatic applications of this herbicide; and 3) to determine acute and sub-chronic toxicity for an amphibian species native to North America's west coast, the Northwestern Salamander (NWS; *Ambystoma gracile*).

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Chapter 2.

Toxicity of Reward® to Fathead Minnow Larval and Adult Life Stages

2.1. Abstract

The objectives of this study were to assess the lethal and sub-lethal effects of the herbicidal commercial formulation, Reward® (373 g/L DB), using application scenarios prescribed by the manufacturer. Specifically, a 14 d period between applications of Reward® in a water body undergoing treatment is required, yet the effects of these 'pulse' exposure scenarios on aquatic wildlife such as fish are unknown. In the first experiment early life stage FHM were exposed to continuous DB concentrations from 0.102-12.6 mg/L which yielded a larval 7 d LC_{50} of 2.04 mg/L as well as a significant decrease in body mass (25.0 ± 11.6 %) at the 1.18 mg/L Reward® concentration. In a second experiment, FHM larvae were exposed for 24 h and then reared in clean water for 14 d followed by a second 24 h exposure to Reward® which produced a 16 d LC_{50} value of 4.19 mg/L. In a third experiment, adult FHM were exposed in a pulse/discontinuous manner to Reward® with a calculated 21 d LC_{50} value of 6.71 mg/L. No significant changes were detected in gonadosomatic index or fecundity with respect to the cumulative number of eggs laid in each treatment. A fourth experiment assessed the F_1 generation's hatch success by incubating eggs laid by treated adults. No differences were shown in any of the treatment groups compared to the control. These findings suggest that concentrations causing adverse effects occur above the maximum concentration predicted by the manufacturer when applied according to the label (i.e. >0.37 mg/L).

2.2. Introduction

Diquat dibromide (DB) is a herbicide widely used in North America for industrial and recreational control of terrestrial and aquatic weeds, and is the active ingredient in the herbicide Reward® (Syngenta, 2003). It is used in aquatic and terrestrial applications in Canada, the United States and Europe (Health Canada, 2010; USEPA, 2002; European Commission, 2000). DB is highly water soluble (>700 g/L; Shiu *et al.,* 1990) and binds tightly to organic matter, particularly clay and sediment, causing its rapid evacuation from

the water column (Ketterings *et al.,* 2002). To what degree soil can become saturated with DB, therefore preventing clearance from the water column in subsequent applications, is not currently known (Birmingham & Colman, 1983). The primary use of DB as an aquatic pesticide draws attention to the sensitivity of aquatic organisms. Though life stages are often unpublished, there is some indication that earlier life stages show lower survival tolerance to DB than adults as seen in the walleye (*Stizostedion vitreum*) which indicates a larval 24 h LC_{50} of 2.9 mg/L versus adult 24 h LC_{50} of 7.8 mg/L (Paul *et al.,* 1994). Gilderhus (1965) reported the 96 h LC_{50} for rainbow trout to be 11.2 mg/L. Hardier 96 h LC_{50} values were seen in grass carp (*Ctenopharyngodon idella*; 53.0 mg/L; El-Deen & Rogers, 1993) and mosquitofish (*Gambubsia affins*; 289 mg/L; Leung *et al.,* 1983). According to application methods and rates published by the manufacturer, the maximum concentration of DB that should accumulate in a water body is 0.37 mg/L (Syngenta, 2003). As mandated by Canada's Pest Management Regulatory Agency (PMRA), there is a mandatory two week waiting period between multiple applications of Reward® (Health Canada, 2010). This recovery period exists to protect non-target aquatic organisms from the peripheral effects of pesticide use. In plants, its primary mode of action is to disrupt photosystem I by cycling electrons between NADPH and reactive oxygen species (Jones & Vale, 2000). In vertebrates, several studies have demonstrated that adverse effects in non-target animals are due to increasing intracellular quantities of reactive oxygen species (*Sus scrofa*, Zheng *et al.,* 2017). There are currently no studies published that examine DB's potential as an endocrine disruptor.

Fathead Minnows (FHM; *Pimephales promelas*) are small (0.81-3.37 g) teleosts that school in lakes, rivers and ponds across North America (Crane & Ferrari, 2015). The FHM has also been adopted as a hardy, convenient model species that has been extensively characterized for toxicological research throughout each stage of its lifecycle (Ankley *et al.,* 2001). Routine endpoints using the FHM model in toxicological studies include body morphometrics, development, deformity rates, behaviour, and survival (Kolok & L'Etoile-Lopes, 2005). FHM have been well established as model organisms for evaluating the effects of endocrine-disrupting toxicants, particularly through a reproductive assay using sexually mature adults (Jensen *et al.,* 2001; OECD, 2012). Changes to sex hormone-related pathways (eg. phenotypic feminization of males, vitellogenin levels etc.) are indicators that a toxicant interferes with the reproductive endocrine axis (Fort *et al.,* 2015; Zhang *et al.,* 2015; Yonkos *et al.,* 2010). Utilizing the FHM model species to evaluate

the adverse effects of pesticides used in Canada will aid in more accurately assessing the risks of pesticides on non-target teleosts native to this geographic region.

The objectives of this study were to explore the adverse effects of Reward® using the FHM model by examining established whole-organism level effects (survival, body length and weight, gonadosomatic index, egg production, and egg hatch success) while preserving tissues for future molecular analysis (e.g. gene expression and proteomics). The novelty of these experiments lay in using the commercially available formulation of the herbicide Reward® instead of DB alone, and mimicking the real-world application method, with the two week recovery period as mandated by the PMRA, on two life stages of FHM. In addition to overt toxicity data, the effects of DB on reproductive capacity and sexual dimorphism were assessed in FHM adults to determine potential for endocrine disruption.

2.3. Methods

2.3.1. Culture Methods

Adult FHM were imported from Aquatic Biosystems, Fort Collins, Colorado, USA under approval from the Canadian Food Inspected Agency (import number: Q-2016-00463-4). Upon arrival the fish, which were separated by sex, were acclimated in breeding pairs at 25 ± 2 ˚C for 14 d in 20.8 L glass aquaria. Aquaria were first washed with Alconox detergent, followed by rinsing with 100% acetone, a triple deionized water rinse, followed by a 10% HNO_3 rinse followed by a triple deionized water rinse and a dechlorinated water rinse (Sigma-Aldrich, 2018). Aquarium water temperature was maintained at 23-27 ˚C and dissolved oxygen levels >9.0 mg/L (>100%). FHM were cultured in 14 L of aerated, dechlorinated water in aquaria containing one PVC breeding tile as described in OECD Test 229 (2012). Daily egg production was monitored and recorded. Eggs for the larval study were removed from breeding tiles by gently scraping off the tile using a metal spatula and placed in aerated moderately hard water [96 mg/L $NaHCO_3$, 60 mg/L $CaSO_4$, 60 mg/L $MgSO_4$, 4 mg/L KCl dissolved in deionized water] in 1.5 L glass aquaria (loading density 100-300 eggs/L). Daily water renewals (80%) were performed during embryo and larval rearing. Once hatched, larvae were randomly transferred to 400 mL low form glass beakers containing 250 mL of moderately hard water (5 larvae/vessel in 7 d exposure; 10 larvae/vessel in 16 d exposure) and reared to 24 h post-hatch, at which point the exposure

experiment began. Larval rearing procedures were performed according to the Test of Larval Growth and Survival Using Fathead Minnows (Environment Canada, 2011).

Larvae were fed live brine shrimp *ad libitum* (*Artemia salina;* Canadian Aqua Farm, Maple Ridge, BC) twice per day. Two brine shrimp cultures were prepared each day in separatory funnels containing 500 mL dechlorinated water and ~10 g brine shrimp (*Artemia salina)* cyst/salt mixture. The funnels were kept under direct light exposure (100 W bulb) and at least 30˚C temperatures with vigorous aeration. After removing the airline and allowing the shrimp to settle, live, hatched shrimp were separated from the hatched cyst shells by gravity and washed three times with dechlorinated water followed by suspending the live hatched shrimp in 20-50 mL moderately hard water. Each beaker of larvae was fed brine shrimp *ad libitum* which entailed administering 50-150 µL aliquots depending on the brine shrimp concentration from daily batches of brine shrimp 1-2 times per day, such that larvae had brine shrimp available throughout the day light hours and each beaker was fed the same amount based on volume dispensed per feeding. Visual confirmation of feeding was evident by the larval stomachs turning orange with shrimp.

Due to the risk of Viral Hemorrhagic Septicemia (VHS) associated with *Pimephales promelas,* all the operations adhered to quarantine procedures for a BCL2 (Biological safety containment level 2) pathogen. In summary, all BCL2 fish, disposable materials and supplies, and water used in these studies were autoclaved or disposed of as per existing policy. Reusable equipment and supplies were sterilized by either autoclaving wherever possible or soaking in a 70% isopropanol or 1% sodium hypochlorite solution for at least 5 minutes. No adults or larvae exhibited symptoms of VHS throughout these experiments.

2.3.2. 7 d Continuous Larval FHM Exposure to Reward®

The 7 d continuous exposure experiment was initiated when larvae were <24 h post-hatch using the following treatment groups: moderately hard water control, and Reward® concentrations of 0.12, 0.37, 1.18, 3.79 and 12.12 mg/L based on the concentration of the active ingredient diquat ion. Each treatment included four biological replicates of 5 larvae each. Unless otherwise stated, experimental methods adhered to the Biological Test Method (Environment Canada, 2011). Water changes during this sub-chronic larval exposure were performed every 24 h, renewing 80% of the volume with freshly prepared solutions. For each treatment group, a concentrated stock solution (10

000x the nominal concentration) was prepared by serial dilution (Appendix A) and held in 125 mL Erlenmeyer flasks stored in the dark at 4 ˚C. Daily solution renewal was conducted by diluting these stocks by 10 000x in moderately hard water and distributing to each vessel. A split sample of each test concentration was prepared on d 1; half was used for the exposure and the other submitted for analysis of DB (ALS Environmental, Burnaby, BC). During the daily water changes and feeding, survival was monitored and excess food and feces were removed.

After the 7 d exposure period, feeding was suspended 12 h prior to euthanization. Larvae were collected by gently pouring the test solution for a single test vessel into a net; larvae were immediately euthanized in buffered MS222 (0.4 g/L). Larvae were removed and carefully placed onto pre-weighed aluminum weigh boats, placed into a 60 ˚C incubator for 24 h, then re-weighed at random. The final measurement of dry biomass was calculated for each replicate as an average per individual larva.

2.3.3. 16 d Pulse Larval FHM Exposure to Reward®

FHM larvae were exposed to varying concentrations of Reward® for two 24 h period pulses that were 14 d apart, modified from the Test of Larval Growth and Survival Using Fathead Minnows (Environment Canada, 2011). All larvae used in this study were ≤24 h post-hatch and were collected from adult FHM (Aquatic Biosystems, Fort Collins, CO) reared and bred in the Alcan Research Centre, Simon Fraser University (Burnaby, BC) as described above. In this pulse exposure experiment, the following treatments were included: moderately hard water control and Reward® concentrations of 0.12, 0.37, 1.18, 3.79 and 12.12 mg/L based on the active ingredient diquat ion. Ten larvae aged ≤ 24 h post-hatch were placed into 250 mL of the control or DB concentrations in 400 mL low form glass beakers, with four biological replicates per treatment. Feeding began immediately with freshly hatched, live brine shrimp as described above. After 24 h of exposure, all test beakers underwent an 80% water renewal with clean moderately hard water. This renewal with fresh moderately hard water was repeated every 24 h for 14 d, at which point the water was removed to 20% volume and refilled with a 120% concentration of test solution to yield the desired nominal concentration for a second 24 h exposure. Feeding was suspended at least 12 h prior to termination. Larvae from each vessel were removed and released into a secondary holding tank of deionized water for euthanization with MS222. Once euthanized, excess water was carefully blotted and the

wet body weight and length were measured before snap freezing the whole body on dry ice and storing at -80 ºC for future molecular analysis.

2.3.4. 21 d Pulse Adult FHM Exposure to Reward®

The adult FHM exposure adhered to the standardized Organisation for Economic Co-operation and Development Test: 229 Fish Short Term Reproduction Assay (OECD, 2012), but was modified to reflect a pulse exposure design. Specifically, adult FHMs were exposed to two 24 h exposure periods to the formulation 14 d apart as above for larvae. Adult FHM were distributed per vessel with a male: female ratio of 2:4 and acclimated for two weeks in municipal dechlorinated tap water in aquaria (four per treatment) containing 7.5 cm diameter PVC pipe sectioned longitudinally and cut into ~9 cm long segments as an egg-laying substrate. The underside of the PVC pipe was scored with a razor blade to increase adhesion of eggs after spawning events, creating a relatively uniform grid of 2-5mm^2. The sides of the glass tanks housing the FHM were covered externally with black plastic to minimize visual disturbance. The fish were subject to an automatically timed 16 h light: 8 h dark photoperiod. Fish were fed 2.76% bodyweight of Finfish starter #1 fish food (Ziegler Bros, Inc., PA) twice a day at least 3 h apart. Aquarium water was renewed every 48 h remove uneaten food and feces, replacing 80% of the tank volume.

For the first 24 h Reward® pulse, 80% of water in each tank was removed and refilled with 120% the desired nominal concentrations. Water samples were collected as a 1 L composite from all four replicate tanks at each concentration immediately following the addition of first of Reward® on d 1; samples were analyzed by ALS Environmental (Burnaby, BC) and yielded measured concentrations of 0, 0.10, 0.33, 1.17, 3.57, and 12.6 mg/L (based on the active ingredient diquat ion concentration). Following the first 24 h pulse, an 80% water renewal was performed and then every 48 h with clean water. On d 15, a second 24 h Reward® pulse exposure occurred as described above. Daily measures of water quality were collected along with ammonia measurements which were taken 3 times per week. Daily records of egg production throughout both the acclimation and exposure period were taken. Eggs that were not collected for subsequent hatching experiments were discarded. To measure hatch success in the F_1 generation after Reward® pulse exposures, eggs from the control, 0.10, 0.33 and 0.17 mg/L DB test concentrations (from all 4 replicate tanks) were collected on d 20 of the test and distributed into clean 400 mL low form glass beakers (10 eggs/beaker; 3 beakers per treatment).

Eggs were incubated at 25 ± 2˚C in clean and aerated moderately hard water for 5 d with daily hatch assessment, water renewal, and water quality monitoring.

Termination of the adult FHM exposure occurred over d 20/21, from two of the four replicate tanks for each test concentration per day. After euthanasia in buffered MS222, body weight and snout-fork length were recorded, and the liver rapidly dissected and snap frozen on dry ice and transferred to long-term storage at -80 ˚C. The gonads were then removed, weighed and along with dissected fish heads (decapitated slightly anterior to the operculum), preserved in Davidson's for 24 h, followed by rinsing in tap water and immersion in 10% Neutral Buffered Formalin at room temperature for long term storage. Subsequent quantification and classification of nuptial tubercles on the snout of the preserved head was performed and adhered to the OECD Fish Short Term Reproduction Assay test guideline (Test 229; OECD, 2012).

2.3.5. Data Analysis

Statistical analysis was performed using SPSS v. 24 (IBM Corporation, Armonk, New York, USA). Survival and body morphometric data were analysed using one-way analysis of variance (ANOVA) followed by a Tukey's post hoc (P<0.05). These data passed the criteria for normality and homogeneity of variance based on evaluating these data via the Shapiro-Wilk's test for normality and Levene's homogeneity of variance test. LC_{50}s were calculated using the binomial method if survival dropped from 100% to 0% between two test concentrations (i.e. 2 data points) (Environment Canada, 2007). In cases where survival displayed a more gradual dose response (i.e. 3 or greater data points), the probit method or the trimmed Spearman-Karber method was employed (Environment Canada, 2007).

2.4. Results

2.4.1. Water Chemistry

Water chemistry analysis was performed only on the first pulse of adult exposures and therefore all larval FHM figures present nominal concentrations and adult FHM figures present measured concentrations. Measured concentrations were similar to nominal values (mean deviation = 5.6 ± 2.0 %; Table 1).

2.4.2. 7 d Continuous Larval FHM Exposure to Reward®

Survival decreased to 0 % in the two highest concentrations (3.79 and 12.1 mg/L; (Figure 4). No significant differences were seen in the two lowest treatment groups compared to controls, however at the highest concentration where fish survived (1.18 mg/L), significant decreases in total body weight (p=0.027) were seen. After 96 h, the larval LC_{50} was 3.82 mg/L and 7 d larval LC_{50} was 2.11 mg/L (Table A2). The lowest observed effect concentration (LOEC) on biomass (Figure 4) was 1.18 mg/L and was significantly decreased by 25.0 % to 188.5 ± 14.9 µg (n=4) compared to the average control larvae body weights (251.5 ± 21.8 µg; p=0.027). The mean daily dissolved oxygen was 7.80 mg/L, the conductivity 346.4 µs/cm, the temperature 24.6 ˚C and pH 7.52.

2.4.3. 16 d Pulse Larval FHM Exposure to Reward®

Survival of FHM larvae decreased in the two highest concentration compared with the control (Figure 5). The 3.79 mg/L concentration decreased survival to 72.2 ± 6.0% (p=0.00038) and the 12.1 mg/L concentration caused 100 % mortality after the 2nd 24 h pulse. No significant differences were seen in length or body weight compared to the controls. Calculated 16 d LC_{50} value was 4.19 mg/L. The mean daily dissolved oxygen was 7.61 mg/L, the conductivity 347.5 µs/cm, the temperature 25.4˚C and pH 8.36.

2.4.4. 21 d Pulse Adult FHM Exposure to Reward®

Survival of the adult FHM exposed to two 24 h exposures to Reward® was 100% in all treatment groups from 0.10-3.57 mg/L (Figure 6). All fish died in the 12.6 mg/L after the second exposure period. Morphometric data including weight, length, and gonadosomatic index (GSI = 100 * gonad weight / bodyweight) showed no significant changes in any of the treatment groups compared to control, whether analyzed together or by sex. The calculated 21 d LC_{50} value was 6.71 mg/L. The mean daily dissolved oxygen was 7.22 mg/L, the conductivity 36.7 µs/cm, the temperature 24.9 ˚C and pH 7.40.

2.4.5. Adult Reproduction

Average daily egg counts as well as total amounts over the course of the 21 d are shown in Figure 7 (A and B, respectively). No significant changes in daily average or total

egg production were seen between treatment groups during the exposure. Figure 7 (C) illustrates the male tubercle score averages in the surviving treatment groups compared to controls; no significant differences were seen between treatment groups. There was no evidence of tubercles developing on females.

The final experiment assessed hatch success on eggs (F_1) that were laid by adults exposed to DB (Figure 8). There was a significant decrease in final hatch success in the 0.33 mg/L group compared to the 0.10 mg/L, as the total lowered to 86.7 ± 3.3 % (p=0.046) from the 100.0 % success in the 0.10 mg/L. However, there was no difference between the controls and any of the treatment groups that laid eggs near the end of the test (ie. 0.10-3.57 mg/L). The number of days for embryos in each treatment to reach 50% (H50) or 90% (H90) hatch was not significantly different between a treatment group (p = 0.808 and p = 0.0553 respectively; one-way analysis of variance; data not shown). The mean time-to-hatch values (± SE) were H50 = 2.16-2.5 ± 0.08 d and H90 = 3.8125-4.5 ± 0.15 d (Table A2).

Table 1. Nominal values of expected diquat ion present in treatment solutions based on serial dilution of concentrated Reward® herbicide compared with values measured by ALS Environmental (Burnaby, BC).

Nominal Concentration (mg/L)	Measured Concentration (mg/L)
0	<0.001
0.1156	0.102
0.37	0.33
1.184	1.17
3.788	3.57
12.124	12.6

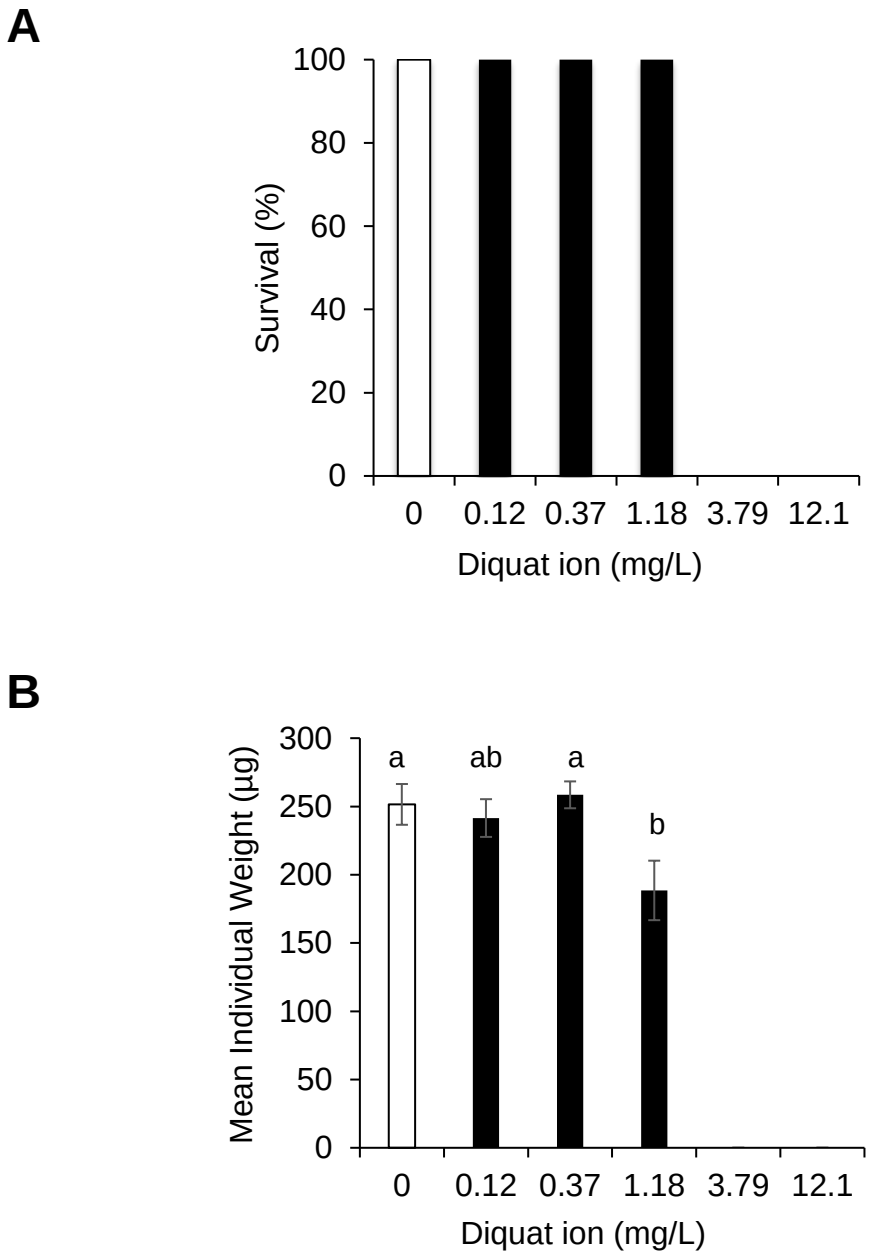
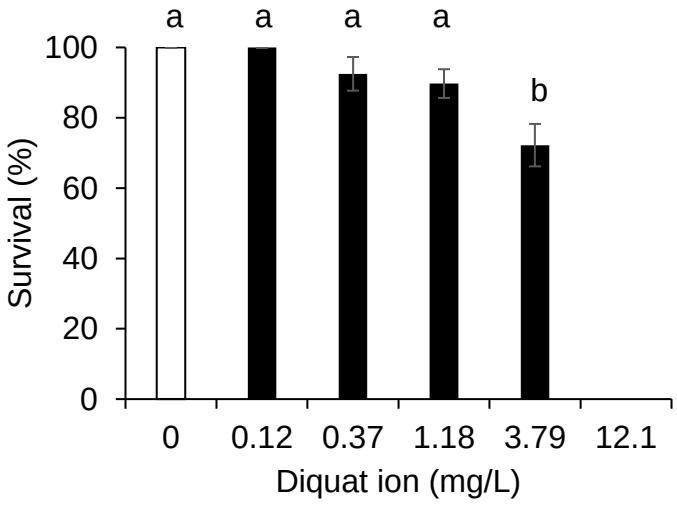
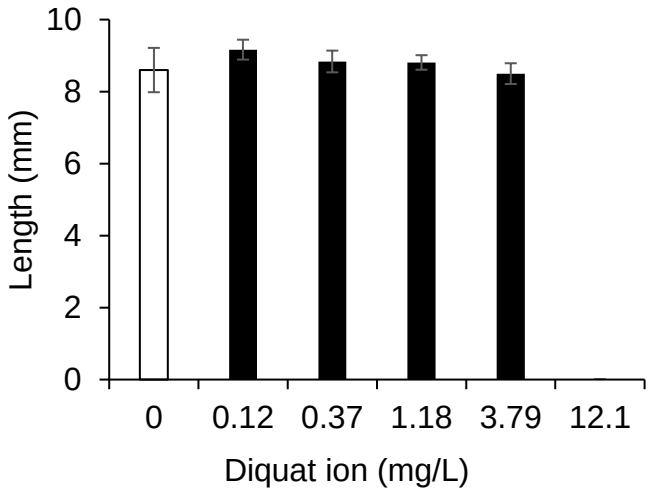


Figure 4. **The effects of continuous 7 d Reward® exposures on FHM larval A) survival and B) mean individual larval weight (total dry weight of all individuals in a replicate divided by number of individuals). Values presented are means of 4 replicates ± standard error (5 larvae/replicate and 4 replicates per test concentration). Different superscripts indicate significant differences between treatments (one-way analysis of variance followed by a Tukey's post-hoc, P<0.05).**

A


a
a
a
a
b
100
80
60
40
20
0
Survival (%)
0
0.12
0.37
1.18
3.79
12.1
Diquat ion (mg/L)

B


10
8
6
4
2
0
Length (mm)
0
0.12
0.37
1.18
3.79
12.1
Diquat ion (mg/L)

C

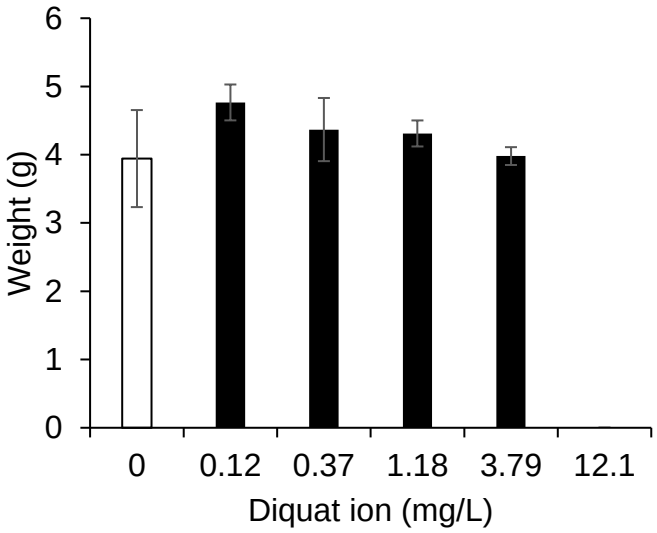
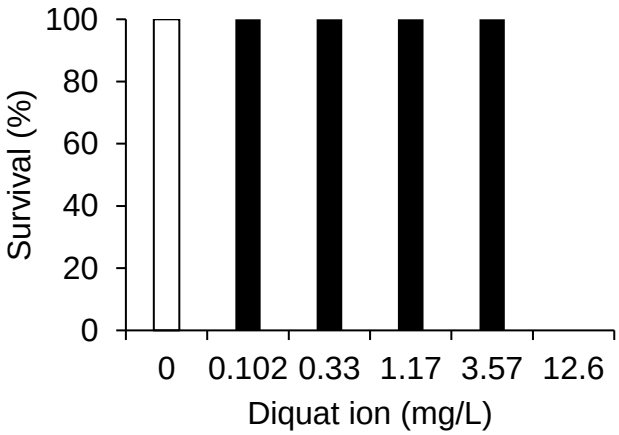
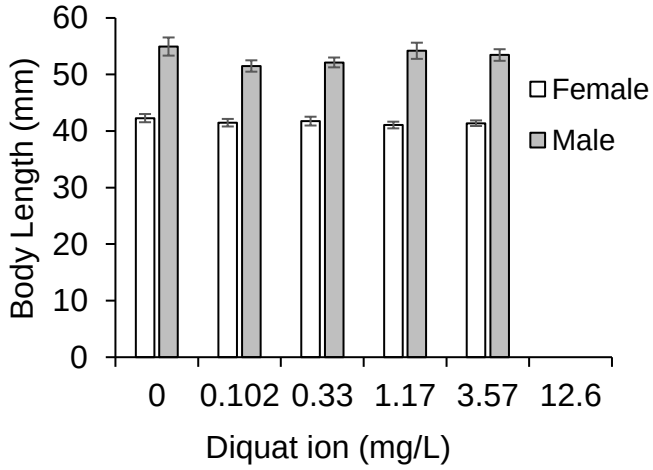


Figure 5. **The effects of two 24 h pulses of Reward® on FHM larvae after 16 d on A) survival, B) fin-fork to snout length and C) wet weight of individuals. Values presented are the means ± standard error (10 larvae/replicate, 4 replicates per treatment). Superscript letters indicate significant differences between treatments (one-way analysis of variance followed by Tukey's post-hoc, P<0.05).**

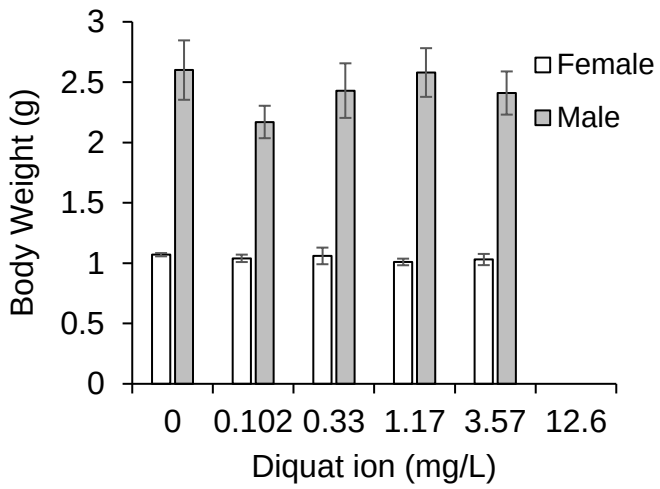
A


100
80
60
40
20
0
Survival (%)
0
0.102
0.33
1.17
3.57
12.6
Diquat ion (mg/L)

B


60
50
40
30
20
10
0
Body Length (mm)
Female
Male
0
0.102
0.33
1.17
3.57
12.6
Diquat ion (mg/L)

C



D

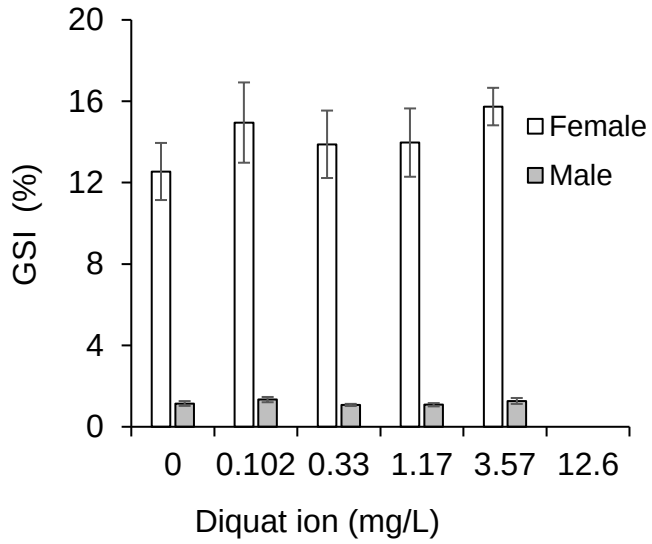
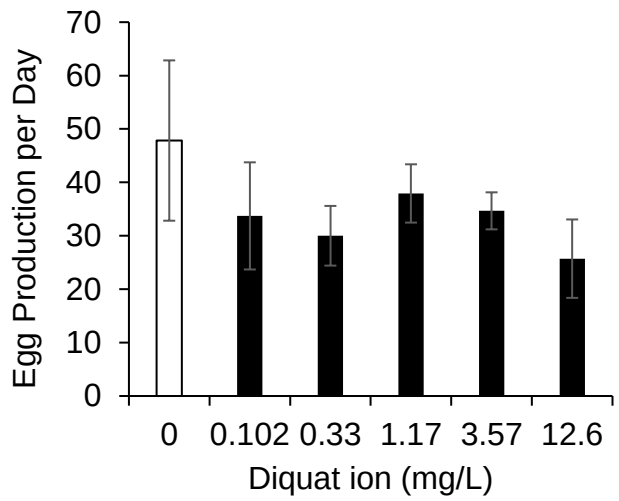
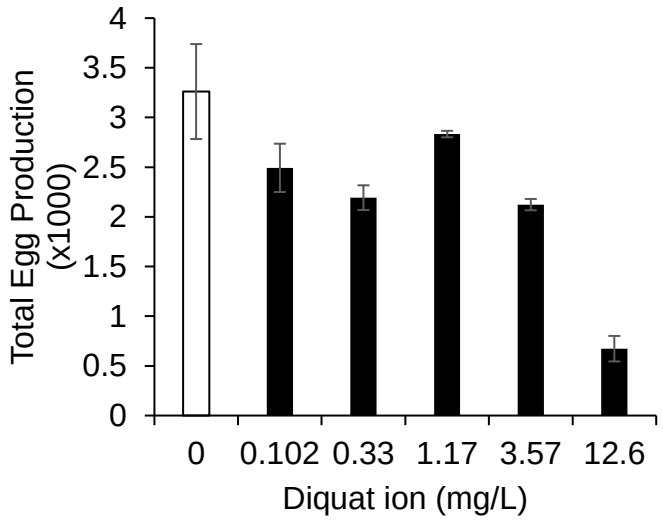


Figure 6. **Effects of two 24 h Reward® exposures over 22 d separated by 14 d of recovery in clean water on adult FHM A) survival, B) body length by sex, C) body weight by sex, and D) gonadosomatic index (GSI= 100*average gonad weight ÷ body weight). Clear bars represent females and shaded bars represent males. Values represent means ± standard error (2 males, 4 females per replicate and 4 replicates per treatment). No significance was calculated in one-way analysis of variance followed by Tukey's post-hoc, P<0.05.**

A


70
60
50
40
30
20
10
0
Egg Production per Day
0
0.102
0.33
1.17
3.57
12.6
Diquat ion (mg/L)

B


4
3.5
3
2.5
2
1.5
1
0.5
0
Total Egg Production (x1000)
0
0.102
0.33
1.17
3.57
12.6
Diquat ion (mg/L)

C

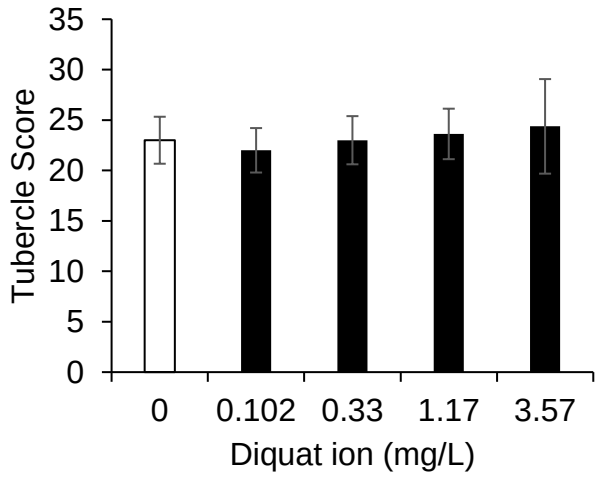


Figure 7. **Effects of two 24 h Reward® exposures over 22 d separated by 14 d of recovery in clean water on adult FHM A) average egg production per d for each treatment of the four replicates, B) total egg production sum of all replicates over the course of the exposure, and C) average male tubercle score from all four replicates in each treatment. Females showed no evidence of tubercle formation. No significant differences between treatments were observed (one-way analysis of variance followed by Tukey's post-hoc, $P<0.05$).**

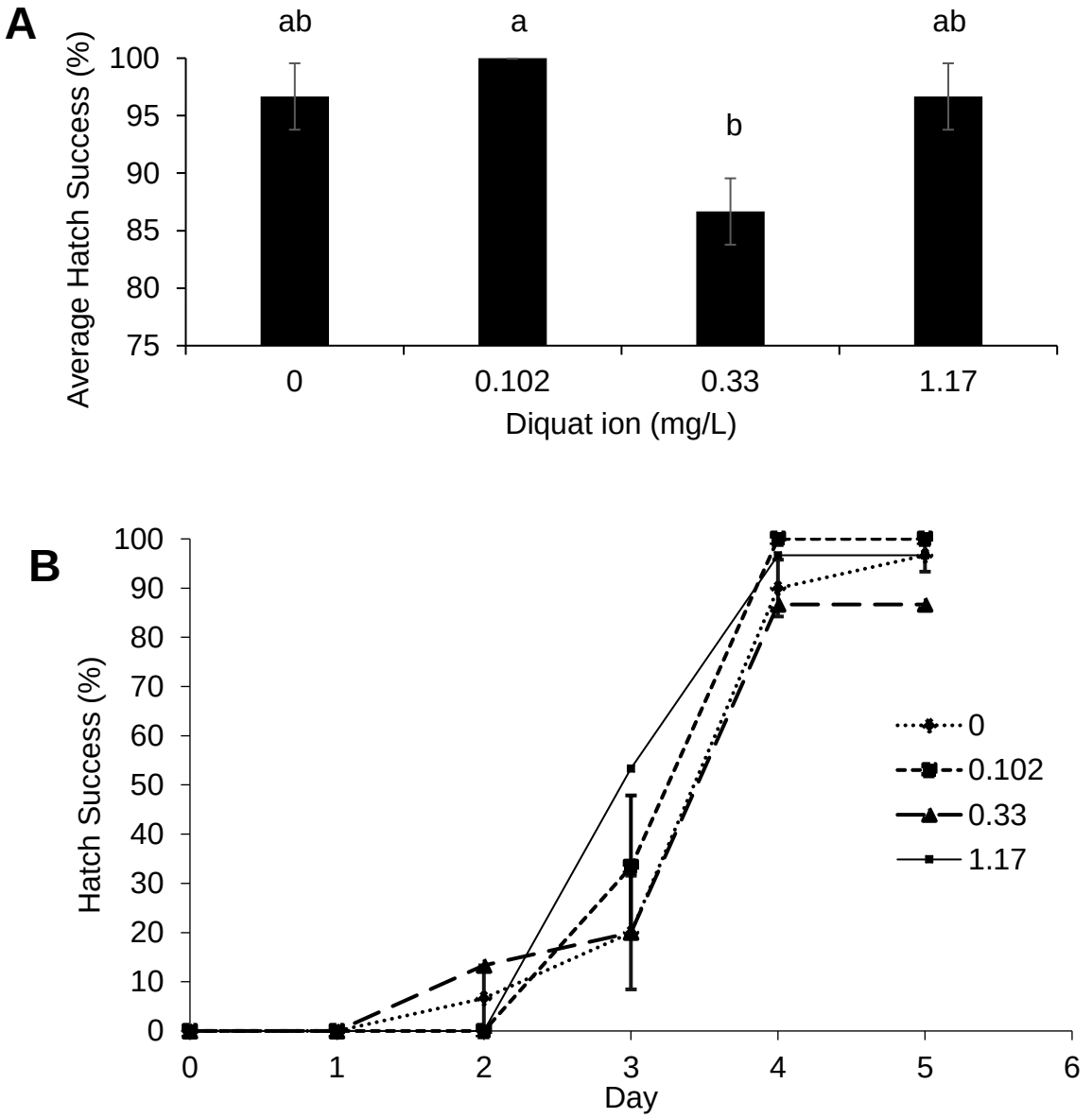


Figure 8. **Hatch success of eggs collected from adult FHM exposed to two 24 h periods in Reward® for treatments that laid eggs on termination day. Eggs were collected, incubated in clean moderately hard water and monitored daily for hatching. The results are displayed for A) overall hatch success and B) progress of hatch success from d 1-6. Relative length of dashes represents increasing concentration from the shortest dash (control) to the solid line (highest). Values represent averages and standard error (10 eggs per treatment and 3 replicates per treatment). Superscripts indicate significant differences between treatments (one-way analysis of variance followed by Tukey's post-hoc $P<0.05$).**

2.5. Discussion

The objective of this study was to examine the acute and sub-chronic toxicity of the commercial formulation (Reward®) of the aquatic herbicide DB, on multiple life developmental stages of the fathead minnow. The continuous exposure experiments indicated a 96 h LC_{50} of 3.82 mg/L and a 7 d LC_{50} of 2.11 mg/L DB on 8 d post-hatch FHM larvae. Exposure also resulted in a significant decrease in body weight at 1.18 mg/L in this experiment, the highest concentration with surviving larvae. The acute toxicity value is lower than reported for the pure active ingredient in rainbow trout of unknown ages (96 h LC_{50} 11.2 mg/L DB; Gilderhus, 1965). Although no effects on growth of FHM larvae or adults were evident after two 24 h pulse exposures ranging from 0.11 to 12.12 mg/L DB, lethality was observed and the LC_{50} values of 4.19 mg/L and 6.71 mg/L were obtained in these two life stages, respectively. These data show that larval fathead minnow are more sensitive than adult life stages to this commercial formulation and, as expected, sub-chronic continuous exposure elicits greater mortality and sub-lethal effects (ie. decreased weight) compared to a pulsed application with clean water renewal. Although environmental levels of DB have not been reported in Canada, the manufacturer claims that the highest water concentration of DB is 0.37 mg/L after aquatic applications complying with the label of Reward® to treat aquatic pest plants. They also claim that concentrations are expected to dissipate to 0.01 mg/L within 24 h, though the conditions (e.g. water temperature, pH, turbidity, etc.) for this dissipation rate are unpublished. However, based on these purported concentrations of Reward® in the field, direct applications to water bodies adhering to the label instructions are unlikely to result in mortality even during sensitive early life stages of FHMs, since significant mortality was observed at concentrations ten times this reported maximum water concentration of 0.37 mg/L.

Pulse exposure scenarios of FHM larvae to DB in a commercial formulation (Reward®) are less toxic than the cumulative effects associated with continuous exposures at the same concentrations. While this phenomenon was expected, it has not been previously tested. And, in the case of Reward® as an aquatic herbicide with directions for use indicating applications are to be administered to a water body 14 days apart, this was an important line of investigation to capture a realistic environmental exposure scenario. Indeed, whether organisms recover, become resilient or more sensitive to these

discontinuous exposures to DB and other pesticides that are likely input into water in pulses is poorly studied. Nonetheless, this phenomenon of continuous exposure translating into lower concentrations being more toxic was evident based on the LC_{50} value for pulse exposures separated by 14 d of non-exposure being twice that observed for the 7 d continuous exposure scenario (4.19 mg/L vs. 2.11 mg/L, respectively). Additionally, in the present study no effects on larval weight were observed during the pulse exposure scenario up to and including 3.79 mg/L, while the LOEC for larval growth in the 7 d continuous exposure was 1.18 mg/L. This suggests that the prescribed 14 d period between applications of this commercial aquatic herbicide is unlikely to impact traditional whole organism (or apical) endpoints in the FHM up to 3.79 mg/L. Based on these results, it is clear that this waiting period increases safety to fish compared to if the herbicide was applied in continuous succession, since exposure to the same concentrations in the 7 d continuous exposure had greater effects on FHM survival and growth. This is further supported by the lack of effects observed on FHM adults with respect to fecundity and growth during the adult pulse exposure experiments in the present study up to 12.6 mg/L, with the only effect of 100% mortality occurring at this concentration. Therefore, at the highest expected concentration of Reward®, it appears no lethal effects are seen in FHM larvae and the fish show higher survival after exposure when they are allowed to recover between pesticide applications.

The results of the present 21 d pulse experiment parallel those observed in a seasonal pulse-style agrichemical exposure to FHM by Zhang *et al.* (2015), where at the end of the exposure period, there were no morphometric (body weight, gonadosomatic index) differences between adult FHM control and treatment groups. The FHM were exposed in mesocosms fed by circulating river water during which at least 9 pesticides were measured in separate pulses comparing single-week low discharge and high discharge events, separated by 7 d. During the river's high discharge pulse, the highest concentrations measured included atrazine (6.27 µg/L), acetochlor (4.77 µg/L), DEA (1.12 µg/L), and metolachlor (1.43 µg/L). Breaking up the exposure to these pesticide residues may have mimicked what was seen in the present study, where the clean water recovery period provides the FHM time to compensate for deleterious effects and survive the exposure period without significant variation compared to control animals. It should be noted that because Zhang *et al.* (2015) measured a mixture of pesticides from agricultural run-off events, the comparison to a controlled single-chemical exposure is not fully

comparable considering possible synergistic or antagonistic interactions of the pesticides. However, this pulse-related compensatory pattern is duplicated in a similar agrichemical pulse work by Ali *et al.* (2017) in run-off waters from which 8 pesticides were detected by passive collection over a 6 week period and concurrent FHM larval exposures were conducted. Polar organic chemical integrative samplers were deployed three times for 2 week intervals and the quantity of each chemical was analysed to be many fold higher in the middle interval than the preceding or following two weeks (Ali *et al.,* 2017). The authors exposed FHM <5 d post-hatch to a suite of agricultural pesticides in run-off water for two weeks and then allowed for recovery in clean river water for two weeks once the agricultural run-off had subsided. Mid-point and end sampling (at d 14 and 28) revealed that directly after the exposure, there was significant suppression of body mass and androgenic gene expression (Ali *et al*., 2017). However, after two weeks of recovery, every measured endpoint had not only recovered to control animal levels, but significantly increased past the control (Ali *et al*., 2017). Though none of the measured run-off pesticides included DB, it is interesting to note that a hyper-compensatory response was observed during the recovery period. In balance to this observation, it should be considered that the potential for recovery is only available provided the initial pulse concentration does not induce lethal or irreversible damage to the organism. Nonetheless, the results of the present study follow the notion that the longer the duration of exposure to a toxicant, the more pronounced the toxic effects will be and that within the confines of the maximum predicted concentration of DB, the 14 d period in between Reward® applications provides a less toxic exposure scenario in this model teleost.

The LC_{50} values for the FHM continuous and pulse exposures to DB in a commercial formulation in this study are similar, and in some instances lower, than the values reported in other teleosts during exposure to pure DB. For example, the acute toxicity of the continuous 96 h exposure for larval FHM (LC_{50} of 3.82 mg/L) in the present study is lower than presented previously for exposures conducted using the pure active ingredient, DB, on rainbow trout of unknown ages (96 h LC_{50} of 11.2 mg/L DB; Gilderhus, 1965). A sub-chronic toxicity study conducted by Tapp and Caunter (1989) on juvenile rainbow trout for 21 d (48.6 mm fingerling) reported an LC_{50} of 2.9 mg/L DB (European Commission, 2000), which is similar to the 7 d continuous LC_{50} of 2.11 mg/L for larval FHM (initiated in larvae aged <24 hours post-hatch) in the present study, which suggests the older rainbow trout are able to survive in higher concentrations of DB compared to FHM

larvae. The adult FHM LC_{50} of 6.71 mg/L in the pulse Reward® exposures (i.e. two 24 h pulses separated by 2 weeks in clean water) in the present study is slightly lower than the range of LC_{50}s reported for continuous acute exposures in other teleosts. For example, several studies included in the United States Environmental Protection Agency (USEPA) *Registration Eligibility Decision for Diquat Dibromide* (USEPA, 1995) assessment to evaluate DB toxicity to fish reported adult rainbow trout 96 h LC_{50} values between 14.8 mg/L and <18.7 mg/L. The adult FHM also appears to be more sensitive than adult Chinook salmon that exhibited a 48 h LC_{50} of 28.5 mg/L and the northern pike a 96 h LC_{50} of 16 mg/L after continuous exposure to pure DB (USEPA, 1995). Because of a lack of published adult FHM tests, it is difficult to conclude whether FHM are more sensitive to DB than other species, or if the commercial formulation induces greater toxicity. Further testing comparing the commercial formulation toxicity to existing DB data in different teleosts is warranted since pesticide products are what organisms will be exposed to in the environment.

The sensitivity of the larval life stage of the FHM to Reward® exposures in the present study appears to be higher compared to other early life stage teleosts during pure DB exposures, though it is difficult to discern whether the commercial formulation causes greater toxic effects or if FHM are more susceptible to DB. In the present study the 7 d larval FHM LOEC was 1.18 mg/L, which reflects the LOEL reported by the USEPA for early life stage FHM of 1.5 mg/L to DB alone (USEPA, 1995). These similar values may suggest similarity in toxicity between DB and Reward®. The FHM are slightly more sensitive than the embryo largemouth bass (*Micropterus dolomieu*), with a 96 h LC_{50} of 4.8 mg/L to DB. The FHM LC_{50} value reflects the embryo smallmouth bass (*Micropterus* salmoides) 96 h LC_{50} of 3.9 mg/L (Paul *et al.,* 1994). In contrast, walleye embryos exposed to DB yielded a 96 h LC_{50} of 0.75 mg/L, being over 5 times more sensitive than the FHM larvae exposed to Reward® (de Peyster & Long, 1993). Further testing is required to ascertain if the larval walleye and FHM are more sensitive to DB, or if this difference in sensitivity compared to other teleosts is due to varying experimental conditions (e.g. water hardness, temperature, etc.) or the use of the pure active ingredient instead of a commercial formulation of DB.

In the current study, there were no effects of DB ranging from 0.11 to 12.12 mg/L on the reproductive endocrine axis of the adult FHM after Reward® exposures administered as two 24 h pulses separated by two weeks in clean water. This

interpretation is based on the lack of effects on gonadosomatic index, egg production and male nuptial tubercle numbers between control and Reward®-treated adult FHMs. Although the estrogenic biomarker vitellogenin was not measured in the present study, previous work investigating DB's potential for estrogenic activity suggests no estrogenic effects of this herbicide. Specifically, Xie *et al*. (2005) reported no changes in vitellogenin protein in rainbow trout exposed to 2.07 mg/L DB and Kojima *et al*. (2004) reported no transactivation of estrogen receptors-*α* or -*β* in Chinese hamster ovary cells tested *in vitro* (Kojima *et al.,* 2004). During this FHM adult exposure experiment, eggs were collected and reared in clean water to examine effects on the F_1 offspring, and although there was significantly lowered success in eggs between the 1.17 and 0.37 mg/L DB treatment groups, there was no significant effects on hatch success compared with the control group in the lowest (0.102 mg/L) or highest concentrations (1.17 mg/L). No previous studies have examined the effects of DB on hatching success in fish nor on transgenerational effects after parental exposures. These data indicate that transient exposure of adult FHM during typical application of DB in the commercial formulation, Reward®, is not likely to affect reproduction or F_1 early development.

One of the novel aspects of this study was testing an environmentally-realistic exposure scenario (i.e. two pulses) of a commercial formulation of the herbicide Reward®. The importance of adhering to Reward®'s label instructions when applying this herbicide directly to bodies of water is evident based on the FHM toxicity studies presented here. According to the manufacturer's instructions, the expected concentrations of DB in the water column is 0.37 mg/L immediately after single applications of Reward® separated by two weeks, thus, this herbicide does not appear to be toxic to adult or larval FHMs when used as directed. However, a LOEC of 1.18 mg/L for larval growth in FHM during a 7 d continuous exposure experiment was observed in the present study, which is 3.2 times higher than the maximum expected concentration after the aforementioned aquatic applications. It is noted that that these expected concentrations are based on appropriately calculated application after the herbicide dissolves into solution represented by the entire volume of water. Thus, prudence in aquatic application methodology, including accurate calculations of water body volumes to ensure application rates do not exceed the expected maximum concentration of 0.37 mg/L is critical. Furthermore, no monitoring studies reporting environmental concentrations of DB have been performed in Canada. Therefore, whether this maximum expected environmental concentration of 0.37 mg/L is exceeded

during aquatic pest plant treatments, run-off events from terrestrial applications, or if this herbicide persists in natural aquatic systems in concentrations that translate into acute or sub-chronic toxicity exposure scenarios is unknown and should be the focus of future studies.

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Chapter 3.

Toxicity of Reward® Case Study: Northwestern Salamander

3.1. 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. The objectives of this study were to assess the lethality of the commercial formulation of the aquatic herbicide, Reward®, on the Pacific Northwest amphibian species, the Northwestern Salamander (NWS; *Ambystoma gracile*). This research provides data on the sensitivity of this species to Reward®, a commercial DB formulation. NWS egg clutches were collected from the wild and reared until hatching. A 96 h acute exposure (0.37-151.7 mg/L DB) and a continuous 21 d exposure (0.37-94.7 mg/L DB) in the Reward® formulation were conducted. The 96 h LC_{50} value in larvae was 71.5 mg/L and the 21 d LC_{50} value was 1.56 mg/L. Collectively, the results of this study demonstrate that early life stage NWS larvae appear largely insensitive to acute Reward® exposures compared to early life stage fish. However, NWS larvae are considerably more sensitive during sub-chronic exposure 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.

3.2. Introduction

Risk to amphibian populations at large is proposed to be vastly underestimated globally (Wake & Vredenburg, 2016). However, a literature review of ecotoxicology papers published between 1972 and 1998 revealed that a mere 2.7 % of vertebrate studies concerned amphibians (Sparling *et al.,* 2000). Current standard toxicity tests for amphibians are largely limited to the tropical species *Xenopus laevis* and are routinely used by governments in Europe, the US and Japan (Johnson *et al.,* 2017). The Organisation for Economic Cooperation and Development (OECD) mandates adherence to Test No. 231: Amphibian Metamorphosis Assay using this test species. Though *X. laevis* has been thoroughly characterized (Karpinka *et al.,* 2015), one limitation in its use

as a model organism is how its native ecology of Sub-Saharan Africa does not reflect the climate in which many North American species find themselves, especially in Canada (Rödder *et al.,* 2017). Little work has been done on the Pacific Northwestern amphibians the field of ecotoxicology which presents a significant opportunity to expand knowledge of common species. By better understanding the sensitivity of common species, the field would gain invaluable prophylactic knowledge to mitigate harm to a population entrenched in the ecological web of the North West.

The Northwestern Salamander (NWS; *Ambystoma gracile)* is a carnivorous amphibian native to the west coast of Canada and the United States ranging from Alaska to Northern California (Government of British Columbia, 2017). Based on the most recent Canadian government population size assessment available by the Committee on the Status of Endangered Wildlife, this species is not currently at risk (Government of Canada, 1999). Like other amphibians, the NWS 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 the NWS from embryo to adult, like other amphibians, is influenced by various factors such as clutch size, food availability, temperature and photoperiod (Morrison & Hero, 2003). Between embryogenesis and sexual maturity, however, temperature appears to be the primary factor dictating the developmental timeline. The NWS is one of 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 & 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 favourable 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 the development, growth and ratio of aquatic: terrestrial adults in nature and what adverse effects, if any, exist to a NWS population.

Generally, in the Pacific Northwest, the NWS emerges from hibernation within rotten wood structures to lay eggs between late January and mid-March (Snyder, 1956), although it is expected that local population breeding seasons likely vary depending on temperature. For example, local populations in Abbotsford in the Lower Fraser Valley of BC breed well into April based on egg mass enumerations between 2014 and 2018

(personal communication, Dr. Vicki Marlatt, 2018). Adult habitats are typically rich in organic soils, woody brush and lower vegetation density relative to *Ambystoma macrodactylum* (Long-toed salamander), another common salamander species in the Pacific Northwest (Hoffman *et al*., 2003). Breeding grounds, usually in the form of permanent ponds appear to be favoured regardless of whether they occur naturally have been artificially constructed (Holzer, 2014). As noted by Snyder (1956), non-neotenic NWS reach their first terrestrial-permitting metamorphic phase nearly a year after hatching. Prior to this emergence on land the larvae live, move, feed and develop in water (Licht, 1991).

The question of whether urbanization causes ecological disruption is not debated, but the effects are complicated. Though habitat fragmentation leads to instability of biodiversity, many aquatic and semi-aquatic species are versatile enough to find alternative dwelling during habitat disturbance (Holtmann *et al.,* 2017). Urban ponds have been shown to exhibit statistically similar levels of macro-invertebrate diversity when broadly compared with non-urban ponds across the United Kingdom (Hill *et al.,* 2016; Turrini & Knop, 2015). This could pose a risk of pesticide exposure to carnivorous vertebrates through direct exposure in the water column, further exacerbating the potential harm as Reward® is often used in recreational pond applications. Overflow storm water ponds, designed to temporarily divert water from urban areas, are unintentionally becoming alternative habitats for some species. Hassall & Anderson (2015) noted significant differences in water chemistry between many such storm water ponds from unmanaged wetlands, while observing similar levels of biodiversity. This finding speaks to the likelihood of residual toxicants building up in such storm water ponds, some of which could include pesticide runoff from treated green spaces in urban environments. As these ponds are designed to only temporarily hold diverted water, there is incentive to quickly release their contents, which may be inhibited by rapid-growth weeds. Such problematic drain-clogging weeds may be controlled through the application of herbicides like Reward®, one of its primary purposes. Because these spaces are not designed to accommodate wildlife, there may be little forethought surrounding the risk of pesticide use in these areas or for pesticide application further upstream in urban green spaces. In particular, if repeated applications cause the sediment to approach a DB-saturation point (Birmingham & Colman, 1983), this could result in DB persisting in the water column and thus chronic exposure scenarios for aquatic wildlife.

Another aspect of potential chemical sensitivity in the NWS is the presence of algal cultures that adhere to the embryonic capsule during embryonic development before hatching (developmental stage 25-40, the interval immediately preceding hatch as described by Harrison, 1969) (Bishop & Miller, 2013). Marco & Blaustein (2000) describe the relationship between NWS embryo and green algae as symbiotic. Thus as the reported mechanism of DB in plants is to disrupt photosynthesis (Jones & Vale, 2000), compromising the integrity of this bilateral relationship could pose a threat to the successful development of NWS embryos. Primary benefits to the developing embryo are speculated to be related to water quality, boosting the partial pressure of O_2, fixing CO_2 and removing ammonia wastes (Figure 9; Tattersal & Spiegelaar, 2008; Graham *et al*., 2008; Bianchini *et al*., 2012). These interdependent relationships between organisms further complicate an understanding of the adverse effects of xenobiotic exposure in aquatic species, like that of DB in Reward®.

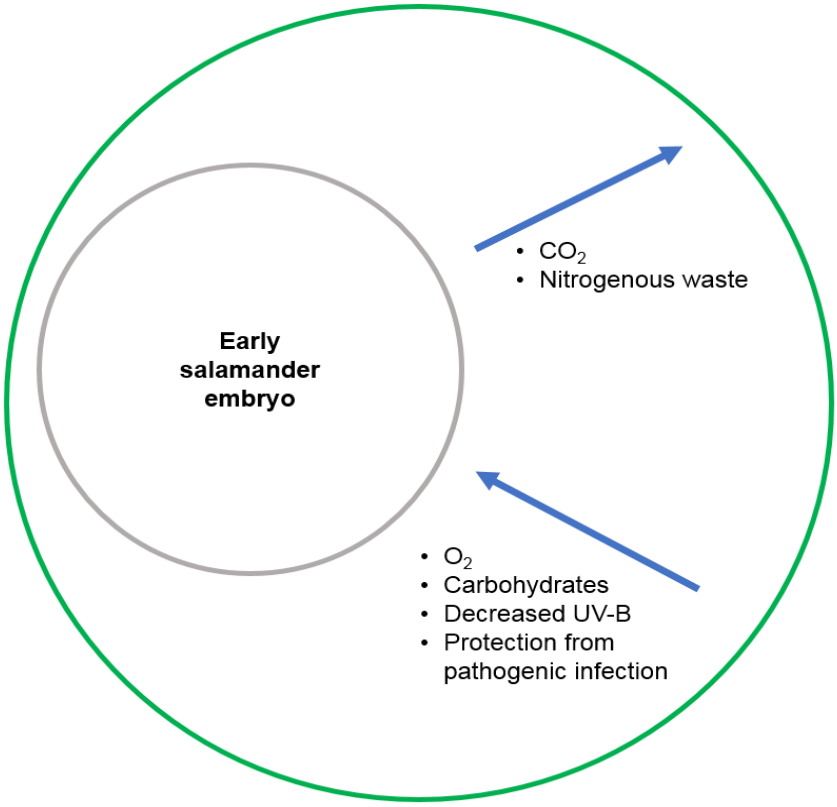


Figure 9. **Representation of the symbiotic relationship between salamander embryos and intramembrane green algae. The developing embryo is housed within a gelatinous outer membrane that becomes lined with green algae that reduces toxic metabolic waste products and provides the embryo with oxygen, energy source and protection from biotic and abiotic threats (Adapted from Kerney, 2011).**

The goal 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 work employs two phases of the tier 3 OECD Test 231: Amphibian Metamorphosis Assay (OECD, 2009) to assess first the short-term toxicity of Reward® (96 h acute exposure), and second, to the potential effects on their development during the full metamorphic assay (21 d sub-chronic exposure). Concentrations for the 96 h bioassay were chosen based on the reported maximum environmental accumulation of DB in the water column immediately after aquatic applications of this herbicide (0.37 mg/L; Syngenta, 2003). NWS were exposed continuously for 96 h and effects on survival, body weight, and length were determined. In the second experiment, employing a continuous exposure to Reward® for 21 d allowed for a better understanding of sub-lethal effects (body weight, length) to be determined in addition to the effects of sub-chronic exposure to Reward® on NWS larval survival.

3.3. Methods

3.3.1. 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 Northwestern Salamander (NWS) 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). 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. Water quality (dissolved oxygen, pH, ammonia) was monitored daily until hatching and larvae were then randomly distributed to glass test vessels. The embryos hatched 8-10 d after collection. Larvae less than 5 d old (stages 41-45) were used in the 96 h acute study and less than 14 d (stages 45-46; Harrison, 1969) were used in the 21 d sub-chronic exposure.

3.3.2. 96 h Acute Larval NWS Exposure to Reward®

Stage 43 NWS were continuously exposed to 5 concentrations (0, 0.37, 1.67, 7.49, 33.71, 151.72 mg/L) of active diquat ion in Reward® dissolved in dechlorinated municipal

tap water for 96 h in four replicates per treatment. The concentrations of DB were increased by a factor of 4.5 and were selected to test concentrations 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, 2015). Nine larvae were added to each glass aquaria, which were arranged randomly, containing 7 L of Reward® solution or dechlorinated water. Water temperature was maintained at 18.4 ± 0.1˚C: range 17.3-20.4 ˚C) with gentle aeration throughout the 96 h exposure period. 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.

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 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.

3.3.3. 21 d Sub-Chronic Larval NWS Exposure to Reward®

This 21 d continuous exposure followed the parameters of the Organisation 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 at stage 46 (Harrison, 1969). The test concentrations were adjusted from the 96 h acute study to reflect a 4-fold increase in each group, starting from the maximum reported concentration when applied according to the manufacturer's instructions (Syngenta, 2015). Organisms were exposed to concentrations of: 0, 0.37, 1.48, 5.92, 23.7, 94.7 mg/L DB in Reward®. The animals were maintained under a 12 h light: 12 h dark photoperiod and tanks were covered with dark plastic. The loading density was <2 larvae/L and 80 % test chemical/control water renewals were performed every 72

h. 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*.

3.3.4. Data Analysis

Statistical analysis was performed using SPSS v. 24 (IBM Corporation, Armonk, New York, USA). Survival and morphometric data were analysed using a one-way analysis of variance (ANOVA) followed by a Tukey's post hoc to determine significance ($P<0.05$). 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 or trimmed Spearman-Karber methods were used to calculate LC_{50} or effect concentration (Environment Canada, 2007).

3.4. Results

3.4.1. 96 h Acute Larval NWS Exposure to Reward®

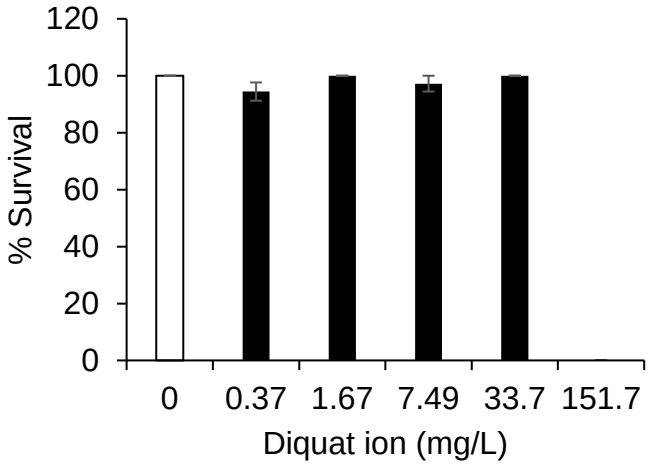
No mortality was observed after 96 hours of exposure to 0.37, 1.67, 7.49, 33.72 mg/L DB on 1-3 d old NWS larvae, however there was 100 % mortality at the highest concentration of 151.72 mg/L DB at d 3 (Figure 10A). The average body weight of larvae was significantly decreased after 96 h of exposure to 33.72 mg/L DB (the highest test concentration with 100% survival), compared to the control (P=0.003; Figure 10B). There was no significant difference in the snout-vent length after 96 h of exposure. The total body length (snout-tip of tail) showed significant increase in body length in the 7.49 mg/L test concentration (P=0.002; Figure 10C). The 96 h LC_{50} value was 71.5 mg/L. The daily dissolved oxygen ranged from 8.38-9.70 mg/L, the conductivity ranged from 27.2-261 μs/cm and pH ranged from 7.01-8.31.

3.4.2. 21 d Sub-Chronic Larval NWS Exposure to Reward®

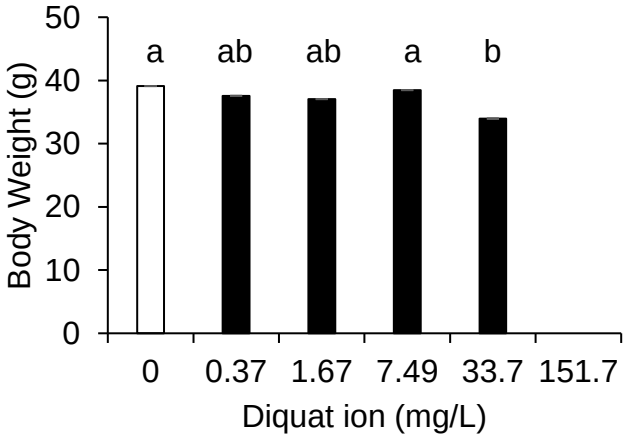
The 21 d sub-chronic NWS larval exposure showed 100% mortality at concentrations of 5.92, 23.7, and 94.7 mg/L, active diquat in Reward®. Survival during the

exposure period decreased to 0% for concentrations ≥23.7 mg/L after 11 d and in the 5.92 mg/L after 18 d (Figure 12). The 7 d LC_{50} for NWS larvae was 1.72 mg/L. A significant decrease in survival after 21 d was observed in the larvae exposed to 1.48 mg/L compared to controls (P=0.015; Figure 11A). The 21 d LC_{50} value was 1.56 mg/L. A similar concentration-response is shown in Figure 11B as body weight decreased with increasing concentration of Reward®. NWS in the highest surviving concentration of DB (1.49 mg/L) exhibited a 74% decrease in body weight compared to controls (P=0.003; Figure 11B). 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. The average water temperature was 18.9 ± 1.5 ˚C (Range 16.1-21.8 ˚C) throughout the 21 d exposure. The dissolved oxygen ranged from 7.80-9.67 mg/L, the conductivity ranged from 33.3-178.8 μs/cm and pH ranged from 7.22-7.54.

A


120
100
80
60
40
20
0
% Survival
0
0.37
1.67
7.49
33.7
151.7
Diquat ion (mg/L)

B


50
40
30
20
10
0
Body Weight (g)
a
ab
ab
a
b
0
0.37
1.67
7.49
33.7
151.7
Diquat ion (mg/L)

C

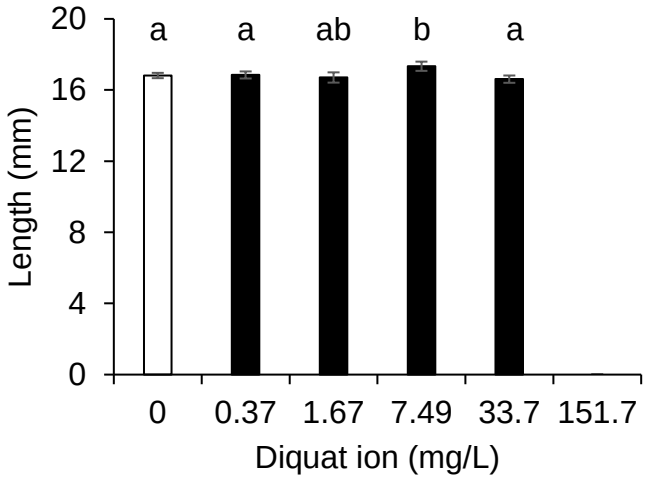
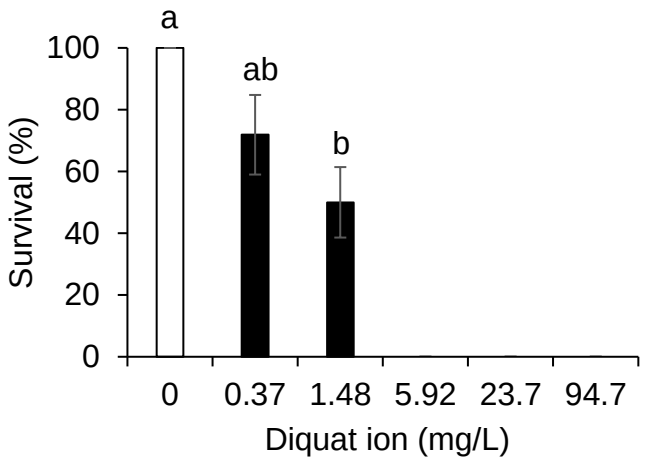
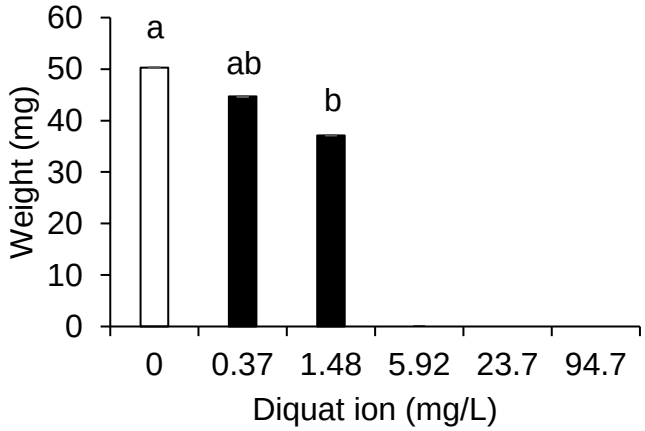


Figure 10. **Effects of 96 h continuous exposure to Reward® on NWS larval (aged <5 d post-hatch) A) survival, B) body weight, and C) length from snout to tip of tail. Values represent means plus standard error (9 larvae per replicate and four replicates per treatment). Differing superscripts indicate significant difference (one-way analysis of variance followed by Tukey's post-hoc, P<0.05).**

A


a
ab
b
100
80
60
40
20
0
Survival (%)
0
0.37
1.48
5.92
23.7
94.7
Diquat ion (mg/L)

B


a
ab
b
60
50
40
30
20
10
0
Weight (mg)
0
0.37
1.48
5.92
23.7
94.7
Diquat ion (mg/L)

C

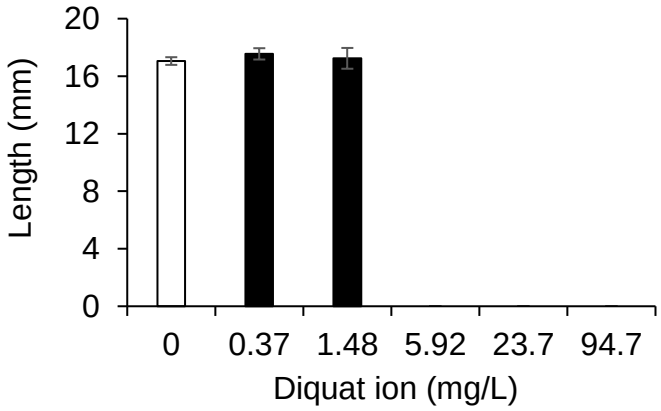


Figure 11. **The effects of 21 d sub-chronic exposure to Reward® NWS larvae (<5 d post-hatch) on A) survival, B) bodyweight, and C). Values represent means and standard error (8 larvae per replicate and 4 replicates per treatment). Significant differences between treatments are denoted by different superscript letters (one-way analysis of variance followed by Tukey's post-hoc, P<0.05).**

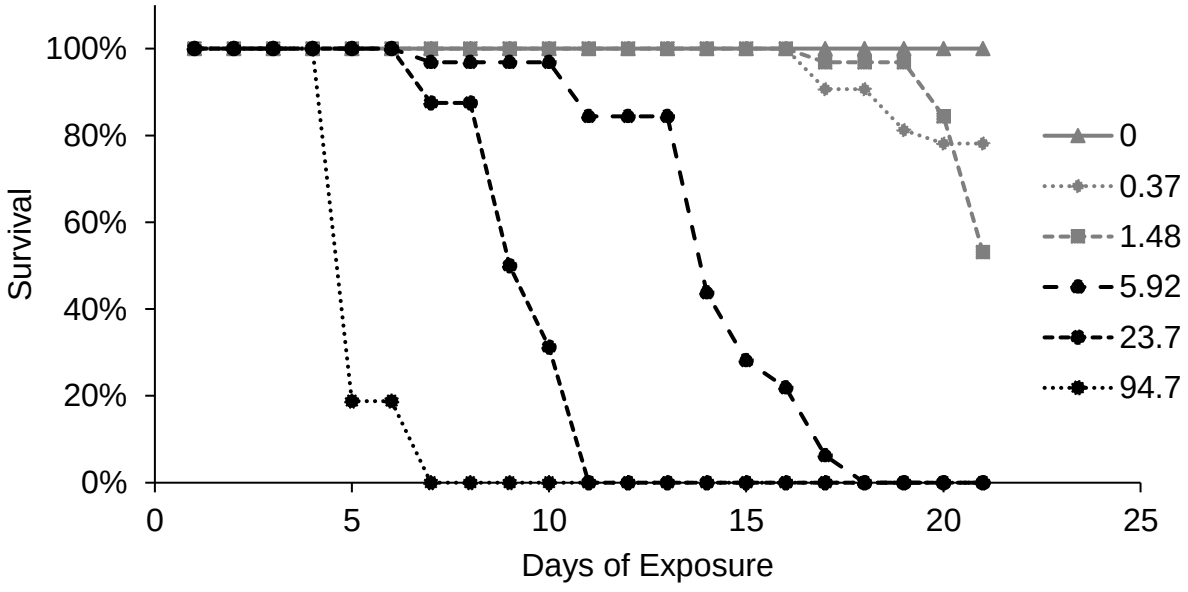


Figure 12. **Effects of Reward® on survival of NWS larvae over the 21 d continuous exposure. Values represent mean survival of all treatment replicates per day (8 larvae/replicate, n=4). Treatment groups represent concentration of DB in the commercial formulation of Reward® as dictated by the legend (mg/L).**

3.5. Discussion

This is the first study examining the toxicity of the aquatic herbicide formulation Reward® on the Northwestern salamander (NWS; *Ambystoma gracile*) under acute and sub-chronic exposure scenarios. The acute 96 h LC_{50} value obtained in this study for NWS larvae was 71.5 mg/L DB, suggesting the NWS are generally less sensitive after acute exposure than early life-stage and adult FHM and other fish reported in previous studies with pure DB (Chapter 2; USEPA 1995, Syngenta, 2004). Interestingly, in the present study after a 21 d continuous exposure of NWS 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 this study for the NWS. 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 the NWS is ~3 times more sensitive using lethality as an endpoint after similar exposure duration to pure DB (Dial *et al.*, 1987). Overall, the results of the present study demonstrate that early life stage NWS larvae appear relatively insensitive under acute exposure scenarios to Reward® containing the active ingredient DB compared to the studies reported for fish. However, NWS larvae are considerably more sensitive during sub-chronic exposures with lethal and sub-lethal effects occurring in the 1 to 2 mg/L range, which more closely resembles the previously reported larval fish sensitivities to this active ingredient.

The present study used the 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. With respect to acute studies, the 96 h LC_{50} obtained in this study for NWS larvae was 71.5 mg/L, which is >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 FHM larvae (96 h LC_{50} = 3.82 mg/L; Chapter 2). Sub-chronic or chronic continuous studies of DB toxicity comparable to the present NWS experiment are fewer, however, in addition to the FHM results presented in Chapter 2 of this thesis one other study indicates higher sensitivity of FHM to DB than the NWS. Specifically, FHM larvae were exposed to waterborne DB during the egg to fry stage for 34 d 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; Surpernant, 1987; Emmett (2002). In NWS, larvae were unaffected at 0.37 mg/L DB based on survival and growth endpoints during 21 d exposures, and a higher LOEC of 1.48 mg/L was observed for both endpoints indicating lower sensitivity compared to larval FHM. 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 the NWS, as well as identify any toxicity modifying factor of DB during aquatic exposures.

The question of water hardness affecting the toxicity to amphibians should also be considered in light of DB exposure (Horne & Dunson, 1995). One study reports effects on survival after experiments exposing Northern leopard frogs (*Rana pipiens*) to pure DB for 16 d (water hardness was 374 mg/L $CaCO_3$) during the following 2 life stages: embryos pre-hatching during the early gastrula stage initiated 1 d post-hatch; and, larvae 15 d old (Dial *et al.,* 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 *et al.,* 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 d old larvae) at 10 mg/L which was the only concentration tested by Dial *et al*. (1987). Together the results of the study by Dial *et al*. (1987) suggest that the 16 d 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 NWS larvae of similar age after 21 d 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 *et al*. 1987), while in the present study, the water was considerably softer (10 mg/L $CaCO_3$) and Reward® formulation was used. The extent of these factors affecting these species-specific sensitivities to DB and/or Reward® is unknown, and additional studies in multiple amphibians under similar experimental conditions would help complete the picture of water hardness as a toxicity modifier in amphibians.

In contrast to the reduced weight observed by 1.48 mg/L in NWS in the present 21 d Reward® exposure study, 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 d 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 (Government of British Columbia, 2017) evidently found useful for gaining weight. The increase in body weight was confirmed to be correlated with an increase in intestinal content (i.e. algae, diatoms, etc.) versus swelling or water retention, compared with the controls. Since NWS are mainly carnivorous, the opposing effect after Reward® exposure observed in the present study suggests adverse effects on feeding behaviour or metabolism of food that has been consumed. Furthermore, there was an unexpectedly slight, but significant increase in average total body length of NWS 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. Additional studies examining natural and toxicant-induced changes in growth and development in the NWS are required to further characterize the sensitivity of these apical endpoints in this amphibian species.

Other studies testing the sensitivity of several amphibians, including the NWS, 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 Reylea & 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 NWS 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, the Folmar *et al.* (1979) study 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 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 NWS larvae from an acute 96 h exposure to the 21 d 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 NWS.

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 NWS. 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, 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 present threats to larval growth and survival.

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Chapter 4. General Discussion

As pesticide use in Canada continues to rise, the bulk of chemical output into the environment remains in the application of agricultural herbicides (Agriculture and Agri-Food Canada, 2016). Herbicides are often specifically designed to target nuisance plant growth, but many are multi-modal, capable of causing harm in much of the biota that lives in the areas of application. A crucial step in mitigating harm to non-target organisms is limiting the quantity and timing of herbicide application. As noted by Folmar *et al.* (1979), it is also important to appreciate that testing the toxicity solely of an active ingredient is not necessarily reflective of the potential harm faced by organisms in the field where commercial formulae are applied. The present study was designed to address several such issues surrounding the herbicidal compound, diquat dibromide (DB). DB is the active ingredient in Reward® Landscape and Aquatic Herbicide product by Syngenta and is registered in Canada.

The first objective of this research was to examine the acute toxicity of the commercial formulation of the broad-spectrum herbicide Reward® in larval and adult FHM and larval NWS. With respect to the continuous acute Reward® experiments, the 96 h LC_{50} obtained for NWS larvae was 71.5 mg/L DB, which was over 18 times less sensitive than FHM larvae (96 h LC_{50} = 3.82 mg/L DB). Although the low mg/L LC_{50} obtained for FHM larvae falls within previous reports of DB acute toxicity in fish, the high 96 h LC_{50} value derived from the NWS exposures is higher than any LC_{50} value reported for amphibians and fish. This finding agrees with a meta-analytic trend determined by Weltje *et al*. (2013), who found fish to be more acutely sensitive to almost 90% of the chemicals surveyed in their analysis. In the present study after a 21 d sub-chronic exposure of NWS larvae to Reward® a 21 d LC_{50} value of 1.56 mg/L was determined, which is ~46 fold lower than the determined acute LC_{50} value. In the present study, the higher lethality at lower concentrations of Reward® in the sub-chronic compared to concentrations in the acute study were also associated with sub-lethal effects on growth, with decreased growth occurring at 1.48 mg/L. Future investigations using NWS to verify the large difference in sensitivity of acute compared to sub-chronic exposures are necessary to evaluate if factors such as parentage, more sensitive larval life stages included in longer duration studies or aspects related to Reward® contributed to this phenomenon. While the larval developmental progress is obviously different between the acute and sub-chronic

exposures, it cannot be deduced whether this played a part in the large discrepancy between the two tests. This contrast between acute and sub-chronic toxicity tests also reveals that relying on acute toxicity data alone can underestimate the toxicity of a chemical toxicant exposure. For example, Kerby *et al.,* (2010) who conducted a meta-analysis of 23 942 toxicity tests on 1075 different invertebrate and vertebrate species found that 44 amphibian studies reported lower xenobiotic sensitivity than the mean estimates of all other species. However, all studies included in this meta-analysis were 24-96 h acute toxicity studies and therefore the species sensitivity distributions for chronic toxicity responses were not considered. In the case of the NWS, the conclusions based on acute toxicity data does not fully capture the sensitivity of this species to Reward®, and whether this phenomenon is prevalent in other poorly studied vertebrates merits further investigation.

The second objective of this study was to test the toxicity of Reward® on the FHM model after 'pulse' exposures that mimic direct aquatic applications of this herbicide. Although no effects on growth of FHM larvae or adults were evident after two 24 h pulse exposures ranging from 0.11 to 12.12 mg/L of DB during Reward® exposures separated by a two week non-exposure period, lethality was observed and the LC_{50} values of 4.19 mg/L (16 d) and 6.71 mg/L (21 d) were obtained in these two life stages, respectively. These data show that larval fathead minnow are more sensitive than adult life stages to this commercial formulation containing DB and, as expected, sub-chronic continuous exposure causes more harm in terms of lethal and sub-lethal effects (i.e. growth) as seen in the larvae. This is the first study to examine the effects of DB on adult FHM reproduction, and no effects on gonadosomatic index, egg production and male nuptial tubercle numbers were observed after these Reward® experiments. Although environmental levels of DB are not measured in Canada, Syngenta claims (2003) that the highest water concentration of DB is 0.37 mg/L after aquatic applications of Reward® to treat pest aquatic plants, and that concentrations are expected to dissipate to 0.01 mg/L within 24 h. Thus, direct applications to water bodies adhering to the label instructions are unlikely to result in mortality even during sensitive early life stages of FHMs. In conclusion, the 24 h pulse exposure scenarios using Reward® separated by 14 d are considerably less toxic than the cumulative effects associated with continuous exposures where effects and growth are evident in the 1 to 2 mg/L DB range.

Currently, Canadian fish toxicity data is heavily relied upon to estimate the risks of anthropogenic chemicals to amphibians (Johnson *et al.,* 2016). Indeed, there is a lack of standardized protocols for amphibians relative to other aquatic taxa, and there are no standardized test methods developed for salamander species in Canada. Therefore, the third objective of this research was to determine sensitivity data on the poorly studied amphibian species, the Northwestern Salamander (NWS; *Ambystoma gracile*). The NWS is primarily confined to the west coast (ie. south eastern Alaska to the Gualala River, California; IUCN, 2018) and represents a niche amphibian species that may prove to be a valuable organism in regulatory toxicity testing in North America because of its ecological relevance to this region. Expanding this work on amphibian species in ecological risk assessments of anthropogenic chemicals is necessary because amphibians experience dramatically different exposure conditions at different life stages compared to fish, and little is known about this phenomenon save for a handful of amphibian species. For example, the way NWS eggs reside in the water contrasts the environment in which FHM eggs are laid and reared from the location to the type and number of egg membranes. In short, the NWS embryos are enclosed within an exterior membrane and combined with dozens of other embryos within a jelly-like substance and symbiotic algae that forms around the cluster of eggs. Several questions remain unanswered regarding these basic life cycle features of the NWS, for example: what are the impacts of toxicants on female gametogenesis, reproductive courting behavior and ovulation; and, how would the jelly/algal matrix surrounding the eggs affect exposure of embryos to toxicants? In the case of DB, reports indicate it has low persistence in the water column because it sorbs to organic matter, but whether it would sorb to the jelly/algal matrix coating the eggs is not known. These types of data would be useful for informing potential risks not only to NWS, but also to other amphibians, since the jelly matrix serves crucial functions like fertilization in these taxa (Peavy *et al.,* 2003). The clusters of embryos were not treated in this study in order to supply the OECD 21 d amphibian metamorphic assay with unexposed larvae (OECD, 2009) which provides a benchmark for future work with NWS. Lastly, unlike the thoroughly characterized *Xenopus laevis,* a developmental stage atlas for salamanders, including the NWS, is only partially completed (Harrison, 1969) thus the effects of Reward® on development could not be assessed. Further studies codifying the natural physiological and morphological changes of NWS, both preceding and following metamorphosis, are needed to fully establish the utility of the NWS for assessing the risk of environmental contaminants.

This research provides novel toxicity data for two North American species to a commercial formulation of the herbicide Reward®, registered for use on aquatic plants. The importance of adhering to Reward®'s label instructions when applying this herbicide directly to bodies of water is evident based on the FHM and NWS toxicity studies presented here. In particular, accurate calculations of water body volumes to ensure application rates do not exceed the expected maximum concentration of 0.37 mg/L and prudence in waiting the mandatory two week recovery period before reapplying. Nonetheless, since no monitoring studies reporting environmental concentrations of DB have been performed in Canada, future work should focus on establishing environmental concentrations of DB after aquatic and/or terrestrial applications of this pesticide to provide a more thorough exposure characterization for aquatic wildlife. Indeed, whether this maximum expected environmental concentration of 0.37 mg/L is exceeded during aquatic pest plant treatments, run-off events from terrestrial applications, or if this herbicide persists in natural aquatic systems in concentrations that translate to acute or chronic toxicity exposure scenarios is unknown and should be the focus of future studies.

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Appendix.

Serial Dilution

Table A1. Calculations for serial dilution used to create each stock solution for the respective treatment groups in both larval and adult experiments.

Level	Final Tank Diquat Ion (mg/L)	Required Stock Concentration (mg/L)	20% Greater Concentration to account for 80% water change	Concentration of stock used (mg/L)	Dilution FACTOR from original stock	Volume of Each Stock Diquat Ion (mL)	Volume of Moderately Hard Water (mL)
Very High	12.1241	121241	145489.2	240000	1.6496	0.1	999.9
High	3.7888	37888	45465.6	145489.92	3.2	0.1	999.9
Medium	1.184	11840	14208	45465.6	3.2	0.1	999.9
Low	0.37	3700	4440	14208	3.2	0.1	999.9
Very Low	0.115625	1156.25	1387.5	4440	3.2	0.1	999.9
Control	0	0	0	0	0	0.1	999.9

Table A2. Duration and values of LC_{50}s within the 7 d continuous FHM larvae exposure and the progress of hatch success of F_1 generation eggs laid by treated adult FHM.

Experiment	Duration	LC_{50} Value (mg/L)	Hatch Success
7 d Continuous FHM	96 h	3.82	-
7 d Continuous FHM	168 h	2.11	-
21 d Pulse Adult FHM	2.16 – 2.5 d	-	H50
21 d Pulse Adult FHM	3.81 – 4.5 d	-	H90