A review of the effects of the biopesticides *Bacillus thuringiensis* serotypes *israelensis* (Bti) and *kurstaki* (Btk) in amphibians

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Abstract

Insecticides are important in agriculture, to reduce human disease, and to decrease the nuisance of biting insects. Despite this, many have the potential for environmental impacts and toxicity in non-target organisms. In this review, we review data on the effects of insecticides based on toxins from *Bacillus thuringiensis var. israelensis* (Bti) and *Bacillus thuringiensis var. kurstaki* (Btk) on amphibians. The few peer-reviewed publications that are available for Bti provide variable conclusions, ranging from few observable effects to evidence of acute toxicity at high concentrations. We briefly highlight the current controversies and identify key areas for future investigation.

Introduction

Insecticides to control mosquitos and crop pests were first introduced in the 1910s (Becker and Ludwig 1993; Stapleton 2004). The control of mosquitoes that are vectors of human diseases such as the West Nile virus, Dengue fever, and malaria (Succo et al. 2016; Calba et al. 2017) offers significant health benefits. In temperate regions, mosquito control may also be used to reduce the nuisance of mosquito bites (Becker et al. 2010; Halasa et al. 2014). With these economic, health, and potential lifestyle benefits, there are a series of risks from the use of insecticides that include environmental contamination, development of resistance, mortality, and sublethal impacts in non-target organisms (Hemingway and Ranson 2000; Coetzee and Koekemoer 2013; van den Berg et al. 2015). The potential harm that may result from the widespread use of mosquito control programs is exemplified by the insecticides Paris Green (copper (II) acetate triarsenite or copper (II) acetoarsenite) and dichlorodiphenyltrichloroethane (DDT) (National Pesticide Information Center 1999; Casida 2012). These products were used in agriculture against pests and to reduce mosquito-borne diseases. Despite their effectiveness, they are highly persistent in the environment and are toxic to non-target organisms, including humans. Paris Green was banned in the 1940s. While DDT is banned by some regions, countries in South America, Africa, and Asia it may use it in malaria vector control strategies as recommended with the World Health Organization.

The development of alternative insecticides with significantly less environmental and health impacts is of paramount importance. *Bacillus thuringiensis* (Bt) var. *israelensis* (Bti) is a Gram-positive bacterium that naturally occurs in the soil. It was discovered in 1976 and isolated

from a stagnant pond in the Negev Desert in Israel (Goldberg and Margalit 1977), then developed as a bioinsecticide to specifically target the order Diptera, predominantly mosquitoes and blackflies (Margalit 1990) and is used worldwide (Schäfer and Lundström 2014). The bacteria produce insecticidal proteins as crystal inclusions during growth, known as Cry and Cyt toxins, which have been proven effective for mosquito control programs (Goldberg and Margalit 1977; Bravo et al. 2011). The insecticide contains three Cry toxins (Cry4Aa, Cry4Ba, and Cry11Aa) and one Cyt toxin (Cyt1Aa) (Ben-Dov 2014), which after being ingested by a target insect, dissolves in the alkaline conditions of the Dipteran gut, releasing protoxins which are then activated by proteases (Rukmini et al. 2000; Vachon et al. 2012). The Cry toxins bind to specific protein receptors in the gut, oligomerize, and create pores in the gut membrane of the insects, leading to death. Bacterial spores then are released into the hemolymph where they germinate and can proliferate. Although mosquito resistance to individual Cry toxins has been reported, little resistance to the Bti insecticidal formulation has been found because it contains the mix of the four toxins (Goldberg and Margalit 1977; Soberon et al. 2013; Pardo-Lopez et al. 2013). High-resolution structural analysis (Tetreau et al. 2020) has recently revealed the key steps in the Cyt1Aa bioactivation cascade, from *in vivo* crystallization in Bti cells, to crystal dissolution, proteolytic activation, and membrane insertion and perforation through oligomerization. Thus, the mechanisms of Cyt protein toxicity in insects are emerging.

Another subspecies is *Bacillus thuringiensis* var. *kurstaki* (Btk). Commerical insecticide products containing Btk have been used for over 35 years in North America and is one of the most applied insecticides in Canada (Fuentealba et al. 2019), predominantly used in forestry and organic agriculture (Kreutzweiser et al. 1996). For example, over 10 million hectares of Canadian forests were sprayed with Btk-based insecticides between 1985 and 2012 to control defoliator pests such as the spruce budworm, gypsy moth, and hemlock looper (Fuentealba et al. 2019). It primarily targets over 200 Lepidopteran larvae species and contains five Cry toxins (Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa, and Cry2Ab) (Ben-Dov et al. 1999). The mechanism of action is similar to Bti where the toxin crystals ingested by target larvae dissolve in the alkaline gut conditions and bind to the midgut epithelial cells, which then produce pores in the gut membrane through cell lysis, resulting in the death of the insect larvae (Bravo et al. 2007; 2011).

In contrast to this mechanism of action in targeted pests, how commercial products containing Bti and Btk may affect amphibians is unknown. This is of concern because of the importance of amphibians in the food chain and for ecosystem services. Some species of frogs and salamanders inhabit wetlands or small ponds which are the typical sites for mosquito reproduction and may be treated with Bti (Becker and Lüthy 2017). On the other hand, there is the potential for Btk runoff, over-spraying, and deposition of sediment from agricultural sites that could infiltrate wetland ecosystems (Hoffman et al. 2000). Exposure to Bti or Btk through insecticide applications could potentially affect the health of amphibians during their larval (tadpole), juvenile (metamorphic), and adult phases, affecting their survival, growth, metamorphic success rate, physiological functions, and behaviour. Research on the effects of **Bti and Btk products** on amphibians is in the early stages. Current reports range from little to no observable effects to disruption of gut function. Here, we critically assess this scientific literature with the specific goal of identifying areas of future research on the effects of Bti and Btk on amphibians.

Effects on tadpole survival

Lajmanovich et al. (2015) assessed the toxicity of Introban® (Valent BioSciences Corporation, USA), an aqueous Bti suspension, in tadpoles of the South American frog, *Leptodactylus latrans.* No mortality was observed in the water control group. The calculated acute LC50 value (95% confidence limits) of Bti was 22.45 mg/L (19.59, 25.73) in the 48 h acute toxicity test. The exposure led to a mortality rate of 3.5% at 2.5 mg/L with a steady increase in mortality to about 20% at 20 mg/L. The highest concentration tested was 40 mg/L, and 100% of tadpoles died following the Introban® exposure (Lajmanovich et al. 2015). Allgeier et al. (2018) exposed *Rana temporaria* tadpoles to a nominal, two-fold, and ten-fold field rate (see Table 1) of VectoBac® WG ice and sand formulations (both containing 37.4% Bti) and VectoBac® 12AS liquid formulation (which contains 11.6% of Bti) (Valent BioSciences Corporation, Illinois, USA) and repeated these applications three times. In the nominal field rate of 3900 International Toxicity Units/L (ITU/L) of VectoBac® WG (ice), 3247 ITU/L of VectoBac® WG (sand), and 6494 ITU/L of VectoBac® 12AS (liquid) formulations, they reported 10% mortality. They also found a 10% mortality rate in the applied double field rate of 7800 ITU/L of VectoBac® WG (ice), 6494 ITU/L of VectoBac® WG (sand), and 12,988 ITU/L of VectoBac® 12AS (liquid) formulations. Results did not show a dose-response pattern as the 10-fold field rate yielded approximately a 5% mortality rate for the

ice and sand formulations and no mortality in the liquid formulation. There were no significant differences in survival from the various Bti formulations or application rates compared to the control group that had no exposure to Bti. Similarly, Schweizer et al. (2019) exposed *R. temporaria* tadpoles to VectoBacWG® (Valent BioSciences Corporation, Libertyville, IL, USA) at 1, 10, and 100 mg/L. The group of amphibians exposed to the lowest application rate of 1 mg/L exhibited 12% mortality, while animals exposed to the highest application rate of 100 mg/L exhibited 10% mortality. Schweizer et al. (2019) used a rice protein control and a negative control, and these groups had 2% and no recorded mortalities, respectively. There was no significant difference between the treatment groups. Schweizer et al. (2019) only exposed their tadpoles for 11 days, which may be short relative to likely exposure scenarios in a treated wetland throughout a season. There are many differences in the design of these studies. Firstly, Lajmanovich et al. (2015) used *L. latrans* while Allgeier et al. (2018), and Schweizer et al. (2019) used *R. temporaria*, so there are potential differential species sensitivities to Bti toxins. The oxygen dissipation, water hardness, temperature, feeding regiment, and pH may have varied between the studies. Different commercial formulations such as VectoBac® and Introban® could also produce differences in the results as Introban® contains 1200 ITU/L and 1.2% of Bti, while VectoBacWG® contains 3000 ITU/L and 37.4% of Bti. These commercial formulations also have additives that are known only to their respective manufacturers. It is unknown how these additives affect amphibians and other non-target organisms. Other differences can be found in the developmental stages exposed in these studies. Lajmanovich et al. (2015) used *L. latrans* tadpoles at Gosner Stage (GS) 26-30, Schweizer et al. (2019) used *R. temporaria* tadpoles at GS 23, while Allgeier et al. (2018) also used *R. temporaria* but at GS 21-23. Given the differences in the experimental design of the existing studies, that the composition of carriers and other formulation components are not reported, and that only a few anuran species have been tested, further acute toxicity testing in a wider range of amphibians is warranted.

Other studies found that higher application rates of Bti are used, it appears that the mortality rate increases. For example, Pauley et al. (2015) performed 300 L-mesocosm studies to test the effects of bioinsecticides in the presence and absence of dragonfly larvae as predators on the performance of GS 25 *Hyla versicolor* tadpoles. They treated commercial formulations of MosquitoBits® (containing 2.86% of Bti) and MosquitoDunks® (containing 10.31% of Bti) (Summit Chemical Co., Baltimore, MD), among other non-Bti insecticides. MosquitoBits® are

corn granules coated with Bti, whereas MosquitoDunks® are in the form of circular pucks, which can be applied to bodies of water where one puck is considered as one treatment (as described by the manufacturers). Pauley et al. (2015) applied one treatment of MosquitoDunks® every 30 days, one treatment of MosquitoBits®, consisting of 1.275 g, every 14 days, one treatment of Mosquito Torpedoes every 60 days (which does not contain Bti and therefore will not be discussed in this paper), and a control which received no insecticides, each allotted to three mesocosms. There were 24 mesocosms in total as each treatment was repeated with and without dragonfly larvae present. The total time of the mesocosm studies is not stated in the paper. The authors found no significant difference in tadpole survival between predators and insecticide treatments at $P < 0.05$. Despite this, mesocosms treated with MosquitoDunks® with predators present produced a mortality rate of 91% ($P = 0.06$) compared to the control mesocosm group that also had predators present which yielded a mortality rate of 64%. The authors suggested MosquitoDunks® may be the more toxic, as it contains a higher percentage of Bti than MosquitoBits®. In the control mesocosms, tadpole mortality rate without predators was approximately 20% and with predators present it was approximately 60%, suggesting that the stress response alone may have increased tadpole mortality by 20%. In comparison, MosquitoBits® led to approximately 40% mortality ($P = 0.26$) without the predators present and 70% mortality ($P = 0.66$) with the predators present (Pauley et al. 2015), suggesting there is an interaction between the natural predation stressor and the applied Bti bioinsecticide. Allgeier et al. (2019) reported similar results in their mesocosm study. They tested 3000 ITU/mg of VectoBacWG granules in 90 L mesocosms (equating to a high field rate) to test the development of *Lissotron vulgarus* and *Lissotron helveticus* newts and to compare food web communities. They found that with Bti treatments, the newts were more susceptible to intraguild predation from dragonfly nymphs (27% increase compared to the control), indicating that there was more competition over food sources. A trophic niche expansion was also found where newts consumed fewer chironomids in the Bti-treated mesocosms, especially with predators present. This may represent a suboptimal environment due to contaminants and limited or poor-quality food sources (Karlson et al. 2018). The studies by Pauley et al. (2015) and Allgeier et al. (2019) demonstrates an important contrast of how stressors could influence the effects of Bti, and how results may be vastly different in natural applications compared to a controlled laboratory setting. These environmentally relevant applications highlight what may occur in a natural setting. The Pauley et al. (2015) and Allgeier et al. (2019) mesocosm studies differ from Allgeier et al. (2018),

Lajmanovich et al. (2015), and Schweizer et al. (2019) as these were conducted in a laboratory. Mesocosm studies are advantageous because they can more closely replicate environmental factors (e.g., temperature, rain, UV, etc.); however, controlling variables in a mesocosm factorial design is also challenging. Well-controlled mesocosm studies, such as Pauley et al. (2015) and Allgeier et al. (2019) are useful for the study of interactions of Bti and predators; perhaps better reflecting conditions in the wild. These also highlight the indirect effects that may affect non-target organisms through food webs and predation. Brühl et al. (2020) also expressed concern regarding food web effects because mosquitos and chironomids are the main food sources for many species of amphibians (Becker and Ludwig 1983; Vinnersten et al. 2009; Gutierrez et al. 2017). In this regard, mesocosms would also be useful in investigating how Bti influences these effects.

Derua et al. (2018) examined the effects of Bti on the diversity, richness, and abundance of wild amphibians in three villages in the Western Kenya Highlands. One application of either FourStar® (Central Life Sciences, Sag Harbor, NY, USA) or LL3 (University of California, Irvine, CA, USA) briquets (both containing 1% Bti - potency of 70 ITU/mg - and 6% *Bacillus sphaericus* (Bs) - potency of 60 ITU/mg - the only difference being that LL3 briquets are formulated to float) were applied and monitored after the first 24 h, 3 days, then every week for 5 months. The study reported no significant differences in diversity, richness, or abundance of amphibians in sites treated with the insecticides compared to the control sites not treated with Bti. This study examined 289 sites consisting of abandoned gold mines, ponds, canals, rock pools, and swamps that were separated into three treatments of LL3, FourStar®, or the control and were monitored for 5 months from January to June 2016. It was not specified in the paper which habitats were allotted to each treatment. Because of the various types of sites in this study, there was likely considerable variation in turbidity, UV exposure, and the amount of vegetation present. This study also did not address the possible changes in biodiversity variables that could occur over several years and could influence the effectiveness of these insecticides. The briquets are designed to have a longer persistence in the environment as they slowly release active ingredients in the water column over time (Derua et al. 2018). Because environmental factors could affect the toxicological actions and persistence of Bti and Bs, the effects of these briquets on the health of amphibians should be examined more rigorously in the future in a controlled setting. It is difficult to compare this study to others because FourStar® and LL3 briquets not only contain Bti, but also Bs. However, all

commercial formulations also contain unknown additives, which may influence solubility, bioavailability, and thus, the ecotoxicological potential of the insecticide.

Junges et al. (2017) is the only study we could identify on the effects of Bti on amphibian behavior. They determined the effects of Introban® and two other non-Bti insecticides in *Rhinella arenarum, Rhinella fernandezae,* and *Physalaemus albonotatus* GS 33 tadpoles. The tested concentrations over 48 h of exposure were 1.5-40 mg/L and dechlorinated water was the negative control. It was found that Bti was less toxic than the other tested insecticides, and behavioral endpoints were altered by Introban® in *R. arenarum* where the tadpoles moved less compared to the controls. These results emphasize that there are likely species sensitivity differences to Bti-based insecticides.

There are currently only two studies about the effect of Btk on amphibians. This insecticide is important to research regarding how it may affect amphibians in the wetlands. These habitats can be exposed to Btk through run-off, over-spraying, and through the deposition of sediment from nearby agricultural operations (Hoffman et al. 2000). Weeks and Paris (2020) studied the effects of Monterrey® *B. thuringiensis* var. *kurstaki* (containing 98.35% of Btk) on Southern Leopard frog (*Lithobates sphenocephalus*) tadpole survival. In this laboratory experiment embryos were exposed to a control of no insecticide as well as a low, medium, and a high dose of Btk and pre-metamorphic tadpoles to a control of no insecticide, 0.0042, 0.42, and 2.73 mL/L of Btk. The lowest dose used was the expected environmental concentration (EEC) that would be seen in a shallow wetland sprayed with an application rate of 63 mL/100 m^2 (according to the Monterrey® product label) and that the highest dose is derived by using half of the maximum allowed concentration rate of 2.6 mL/L. They did not use the maximum concentration of 2.6 mL/L because it drastically reduced water quality and would not be expected to be applied in nature. After an exposure time of 7 days, they reported that the highest tested concentration of Monterrey® *B. thuringiensis* var. *kurstaki* (2.73 mL/L) significantly increased tadpole mortality to 52.5%, while the lower concentrations of 0.42 and 0.0042 mL/L Btk yielded 20.8% and 8.8% mortality, respectively, which were not significantly different compared to the no insecticide control group (4.2% mortality). Raimondo et al. (2003) examined the effects Btk could have on the abundance of salamander species in West Virginia. They set up nine 200-hectare plots in Monongahela National Forest where three blocks containing three plots were established. One of the three plots

in each block was treated through aerial application with fixed-wing aircraft with either 16 Billion International Units (BIU)/hectare of Forey48F® (consisting of 17.6% Btk), 8×10^{11} Polyhedral Inclusion Bodies (PIB)/hectare of Gypchek® (which does not contain Btk, but the nucleopolyhedrosis virus to kill gypsy moths), or a control that had no insecticide. These were observed from May to September in 1997 and from May to October in 1998. They analyzed the diet and abundance of the following five salamander species: *Desmognathus fuscus, Desmognathus ochrophaeus, Desmognathus monitocla, Plethodon cinereus,* and *Plethodon glutinosus*. When comparing the treated and control plots, the authors found no significant difference in species abundance. The authors did not sample the abundance of prey in the treated and control plots that would have strengthened their study design and results. With only two papers thus far identified, it is too early to draw firm conclusions regarding the potential toxicity of Btk. Further research on the effects of Btk in amphibians is therefore required.

Effects on Hatching Success, Growth, and Metamorphosis

Weeks and Paris (2020) investigated if 0.0042 and 2.73 mL/L of Monterrey® *B. thuringiensis* var. *kurstaki* affected the hatching success of *L. sphenocephalus* embryos (for details, refer to Table 1). There was no significant difference in the hatching success of *L. sphenocephalus* embryos between the no treatment control and the low dose (hatching success of 77% compared to 73%, respectively). There was, however, a significant difference between the highest Btk dose of 2.73 mL/L compared to the low and control groups where hatching success was reduced to only 16% (Weeks and Paris 2020). The hatching success for embryos exposed to 0.42 mL/L was not stated in the study. Survival to seven days was significantly reduced only in embryos that received a high dose. The species *L. sphenocephalus* is known to be sensitive to chemicals during development (Hanlon et al. 2015); therefore, this species could be less tolerant to pesticides than other amphibious species. Further studies on hatching success on both Bti and Btk formulations are required as this is the only study currently available.

Critical information is missing on the effects of Bti-containing pesticides on amphibian metamorphosis. Allgeier et al. (2018; 2019) found no significant differences in the time to complete metamorphosis between Bti-treated groups and control groups that had no treatment (see Table 1 for doses and formulations) for the frog species *R. temporaria*, and the newt *Lissotron helveticus* and *Lissotron vulgaris* species. Only their 2019 study included dragonfly nymphs as predators. The

presence of the nymphs somewhat affected the size of the newts, although the time to complete metamorphosis did not differ. The results of these studies also show that stressors could increase Bti effects that are likely seen in nature. Pauley et al. (2015) found that mesocosm groups with predators took longer to complete metamorphosis, regardless of if the tadpoles were treated with Bti formulations of MosquitoBits® or MosquitoDunks®, suggesting that stress induction was the variable affecting the time to complete metamorphosis. Without environmental stressors, insecticidal formulations of Bti do not seem to affect the growth of tadpoles when normal application rates (< 100 mg/L) are used (Pauley et al. 2015; Allgeier et al. 2018; Schweizer et al. 2019). Allgeier et al. (2018) reported no statistical differences in body mass of *R. temporaria* tadpoles exposed to ice, sand, and liquid Bti formulations (VectoBac® WG) (see Table 1). Schweizer et al. (2019) similarly found no significant difference in body mass of *R. temporaria* tadpoles exposed to Bti formulations of VectoBac® WG (for details, refer to Table 1) when compared to the negative control group. To account for potential nutritional influences of an increased supply of protein in the Bti formulation, Schweizer et al. (2019) also used a rice protein control. Amphibians in the rice protein control group had a smaller body mass compared to Bti-treated tadpoles.

Histopathology

Few studies have investigated the effects of Bti on gut morphology in amphibians. Lajmanovich et al. (2015) examined the intestinal tissues of *L. latrans* tadpoles treated with 2.5, 5, 10, 20, and 40 mg/L of Introban® (containing 1.2% Bti with 1200 ITU/mg potency; www.chemotecnica.com/introban). They found that Bti-exposed tadpoles exhibited signs of inflammation in the intestinal connective tissues and dilated blood vessels compared to the control. They also observed malformed erythrocytes (i.e., nuclear buds, pycnotic, kidney-shaped, and lobed nuclei) in the circulating blood and an increased frequency of micronuclei in the erythrocytes, where 2.5 mg/L and 10 mg/L of Introban® produced a micronuclei frequency of 2.21% and 2.74%, respectively compared to the control group (0.82%). Of note, the 20 mg/L Introban® group exhibited a frequency of 0.42%. While there may be effects on the incidence of micronuclear erythrocytes following exposure to the Bti formulations, there is no clear dose-response in this study, and little to compare it to. In contrast, Schweizer et al. (2019) tested the effects of 1, 10, and 100 mg/L of VectoBac® WG (containing 37.4% Bti with 3000 ITU/mg potency) in *R. temporaria*

tadpoles and following a histopathological assessment, found no impacts on the basal lamina or the muscular layers under the epithelium of the tadpole gut.

Effects on Biomarker Status

Several classic toxicological biomarkers have been studied regarding the potential effects of Bti formulations. Lajmanovich et al. (2015) reported that 48 h of exposure to Introban® significantly increased the antioxidant activity of GST (at 10 and 20 mg/L of Introban®) and catalase (CAT; at 20 mg/L of Introban®) in the intestinal tissues of GS 26-30 tadpoles of *L. latrans*. These results indicate that the tadpoles may have experienced phase II detoxification and antioxidant response to reduce reactive oxygen species (ROS). These data are in agreement with those of Allgeier et al. (2018) who also measured increases in detoxification and antioxidant enzymatic activity following Bti treatments. Allgeier et al. (2018) examined the effects of Bti-containing pesticides on the activity of the glutathione S-transferase (GST), glutathione reductase (GR), and acetylcholinesterase (AChE) as these are common indicators of toxicity. Tadpoles were exposed at GS 21-23 for the first application, GS 24-28 for the second application, and a third application at GS 36-40 to VectoBac® WG and VectoBac® 12AS (for details on doses, refer to Table 1). Data showed that both treatments induced significant increases of GST (37–550%), GR (5–140%), and AChE (38–137%), suggesting that detoxification, antioxidant activity, and alteration of neuronal activity are occurring in the Bti-treated animals. The authors found increases of both GR (140%) and AChE (38%) after the second round of Bti application, but no significant differences in enzymatic activity were noted for GR and AChE in the third application when compared to the control. In addition, no significant changes in the third application were noted.

In contrast, Schweizer et al. (2019) analyzed the activity of the heat shock protein 70 (Hsp70), AChE, and carboxylesterase in *R. temporaria* tadpoles at GS 23-25. The authors did not find any statistically significant changes among the Bti treatments and the control group. While the biomarkers used in the studies above are typically used in toxicology studies, they do not necessarily link mechanistically to the observed effects. For example, if inflammation is suspected, then the biomarkers chosen should be directly reflective of this process. There is a rich biomedical literature (Almradi et al. 2020; Eugene et al. 2020) on inflammatory bowel disease, interleukin responses, and other aspects that can be applied to amphibian ecotoxicology, and especially for the assessment of exposures causing gut inflammation. Modern approaches such as transcriptomic

profiling could also be used to identify novel biomarkers, as has been reported for numerous other environmental contaminants affecting amphibians (Gutierrez-Villagomez et al. 2019; Trudeau et al. 2020).

Conclusions

Data on the effects of Bti and Btk in amphibians are critically lacking. There are only a few studies that have assessed their effects on survival, growth, hatching success, metamorphosis, histopathology, and biomarkers. The diversity in formulations and exposure regimes, species, and developmental stages studied means that consensus views cannot yet be proposed. Nevertheless, under various regimes, these biopesticides may have both lethal and sublethal impacts. Chronic, environmentally relevant exposures that assess hatching success, development, and metamorphosis are of immediate importance. Mesocosm studies rigorously testing both direct and indirect (e.g., through reductions in insect prey items) effects of environmentally relevant levels of Bti and Btk have yet to be conducted. As with numerous other pesticide formulations, those with the described Bti and Btk toxins contain a host of other compounds, such as mixtures of proteins, spores and proprietary additives. It is thus challenging but necessary to develop appropriate controls that would help determine which effects on amphibians are due only to the Bti and Btk toxins compared to the potential effects of the additives in the commercial products. The establishment of physiologically relevant biomarkers and standardized analytical methods to quantify Cry and Cyt proteins are of paramount importance if we are to collectively make progress on risk assessment for Bti and Btk-based insecticides. This will contribute to the mitigation of potential effects on amphibians in wetland ecosystems.

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Table 1. Summary of studies of the effects of *Bacillus thuringiensis var. israelensis* (Bti) and *Bacillus thuringiensis var. kurstaki* (Btk) on amphibians

Species	Bti formulation	Concentrations (ITU/L)	Number of applications	Development stage	Exposure time	Studied variables	Effects	References
Common frog, *Rana temporaria*	VectoBac®WG (ice granules formulation), VectoBac®WG (sand granules formulation) VectoBac®12AS (liquid formulation)	[3900; 7800; 39000] [3237;6494; 32370] [6494; 12988; 64940]	1 application	G19 - G23	5 days	Medium GST activity	↗ 37% (GST)	Allgeier et al., 2018
						Medium GR activity	↗ 5% (GR)	
						Medium AChE activity (all mixed treatments)	↗ 38% (AChE)	
			2 applications	G23 - G25	11 days		↗ 150 %	
							↗ 140 %	
							↗ 137 %	
			3 applications	G25 - G39	43 days		↗ 550%	
							↗ 24%	
							No effect	
			Unique application	G19 - G25	11 days		↗ 48%	
							↗ 88%	
							No effect	
	VectoBac®WG (ice granule formulation)	[3900; 7800; 39000]	3 applications	G19 - G25	11 days	Mortality	No effect	
						Time to metamorphose	No effect	
						Size	No effect	
						Weight	No effect	
						Condition index	No effect	
	VectoBac®WG (sand granule formulation)	[3237;6494; 32370]	3 applications	G19 - G25	11 days	Mortality	No effect	
						Time to metamorphose	No effect	
						Size	No effect	
						Weight	No effect	
						Condition index	No effect	
	VectoBac®12AS (liquid formulation)	[6494; 12988; 64940]	3 applications	G19 - G25	11 days	Mortality	No effect	
						Time to metamorphose	No effect	
						Size	No effect	
						Weight	No effect	
						Condition index	No effect	
	VectoBac® WG	[3 000 ITU/L; 30 000 ITU/L; 300 000 ITU/L]	2 applications	G23 - G29	11 days	Mortality	No effect	Schweizer et al., 2019
						Weight		
						Intestine histology		
						Hsp70 activity		
						AChE activity		

						Carboxylesterase activity		
South American Spotted Grassfrog, *Leptodactylus latrans*	Introban®	[3000; 6000; 12 000; 24 000; 48 000]	Unique application	G26 - 30	48 hours	NOEC	3 000 ITU/L	Lajmanovich et al., 2015
						LOEC	6 000 ITU/L	
						LC50	26 940 ITU/L	
						LC100	48 000 ITU/L	
						Medium GST activity	↗ to 12 000 and 24 000 ITU/L	
						Medium CAT activity	↗ to 24 000 ITU/L	
						Micronuclei frequency	↗ 169 % to 3 000 ITU/L and ↗ 234 % to 12 000 ITU/L	
						Frequency of nuclei dividing	↗ 73 % to 000 ITU/L	
						Frequency of pyknosis	↗ 4 345 % to 3 000 ITU/L and ↗ 2 581 % to 6 000 ITU/L	
						Frequency of kidney shaped nuclei	↗ 74 % to 6 000 ITU/L and ↗ 100 % to 12 000 ITU/L	
						Frequency of lobed nuclei	↗ 180 % to 12 000 ITU/L	
						Intestine histology	Inflammatory infiltration of connective tissues under the epidermis and dilation of blood vessels (all treatments)	
Argitine toad, *Rhinella arenarum*	Introban®	[1 800 ITU/L - 48 000 ITU/L]	Unique application	GS33	24 h	LC50	24 612 ITU/L	Junges et al., 2017
						NOEC	22 656 ITU/L	
						LOEC	18 516 ITU/L	
		22 656 ITU/L				Distance travelled	↘ distance travelled	
						Time immobile	↗ time immobile	
						Global activity	↘ global activity	
		[1 800 ITU/L - 48 000 ITU/L]			48 h	LC50	23 100 ITU/L	
						NOEC	15 000 ITU/L	
						LOEC	22 656 ITU/L	
Bella Vista toad, *Rhinella fernandezae*		[1 800 ITU/L - 48 000 ITU/L]			24 h	LC50	12 876 ITU/L	
						NOEC	3 600 ITU/L	
						LOEC	6 000 ITU/L	

		3 600 ITU/L				Distance travelled	No effect	
						Time immobile	No effect	
						Global activity	No effect	
		[1 800 ITU/L - 48 000 ITU/L]			48 h	LC50	12 876 ITU/L	
						NOEC	3 600 ITU/L	
						LOEC	6 000 ITU/L	
Menwig frog, *Physalaemus albonotatus*		[1 800 ITU/L - 48 000 ITU/L]			24 h	LC50	14 244 ITU/L	
						NOEC	6 000 ITU/L	
						LOEC	9 960 UTI/L	
		6 000 ITU/L				Distance travelled	No effect	
						Time immobile	No effect	
						Global activity	No effect	
		[1 800 ITU/L - 48 000 ITU/L]			48 h	LC50	14 244 ITU/L	
						NOEC	6 000 ITU/L	
						LOEC	9 960 UTI/L	
Palmate newt, *Lissotriton helvecticus,* and smooth newt, *Lissotriton vulgaris* (in mesocosms)	VectoBac®WG	1 491 ITU/L	Unique application	NA	9 weeks	Predatory performance (1 predator present)	↘ 37% chironomid consumption	Allgeier et al., 2019
						Predatory performance (presence of another predator)	↘ 57 % chironomid consumption	
						Survival rate (presence of another predator)	↘ 27 %	
						Size of ecological niche (single)	↗ 30%	
						Size of ecological niche (presence of another predator)	↗ 70%	
						Size	No effect	
						Diet composition	No effect	
Gray treefrog, *Hyla versicolor*	Mosquito Dunks® Mosquito Bits®	11 156 ITU/L	1 application every 30 days	G25 - G46	ND	Survival (in presence of predator)	↘ for Mosquito Dunks®	Pauley et al., 2015
			1 application every 14 days			Survival (no predator present)	No effect	
						Size	No effect	
						Weight	No effect	
						Time to metamorphose	No effect	
Southern leopard frog, *Lithobates sphenocephala*	Monterrey® B.t. *kurstaki*	[0,0042 mL/L - 2,73 mL/L]	2 applications	G19	7 days	Hatching success	↘ to 2,73 mL/L	Weeks et al., 2020
						Survival		
				G25	7 days	Survival	↘ 37,5 % to 2,73 mL/L	
						LC50 (96h)	1,81 mL/L	

Northern dusky salamander (*Desmoghatnus fuscus),* seal salamander (*Desmognathus monticoloa),* Allegheny Mountain dusky salamander *(Desmognathus orchophaeus),* red-backed salamander *(Plethodon cinereus),* and Northern slimy salamander (*Plethodon glutinosus)*	Foray 48F® (Btk)	16 Billion International Units/hectare	1 application in May 1997 and 1998 (2 applications total)	N/A	May-September 1997 and May-October 1998	Abundance and diet analysis	No effect	Raimondo et al., 2003

AChE = Acetylcholinesterase, CAT = catalase, GR = glutathione reductase, GS = Gosner Stage, GST = glutathione S-transferase, ITU = International Toxicity Units. The papers chosen to be in this study were based on the criteria that they had to be peer-reviewed, published papers on the effects of Bti or Btk on amphibians. Given the rather limited number of publications assessment of the data quality of these papers was not conducted. Critical assessment of key publications is presented in the main body of the mini-review.