Do Bio-Insecticides Affect Only Insect Species? Behavior, Regeneration and Sexual Reproduction of A Non-Target Freshwater Planarian

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Research Article

Abstract

Bio-insecticides have been increasingly used worldwide as ecofriendly alternatives to pesticides, but data on their effects in non-target freshwater organisms is still scarce and limited to insects. The aim of this study was to determine the lethal and sub-lethal effects of the bio-insecticides Bac Control (based on *Bacillus thuringiensis kurstaki* - Btk) and Boveril (based on *Beauveria bassiana* - Bb) on regeneration, behavioral and reproductive endpoints of the freshwater planarian *Girardia tigrina*. The estimated LC_{50-48h} were > 800 mg a.i./L for Btk and 60.74 mg a.i./L for Bb. In addition, exposure to Btk significantly decreased locomotion and feeding activities of planarians (lowest observed effect concentration (LOEC) of 12.5 mg a.i./L Btk) and fecundity rate (LOEC = 3.12 mg a.i./L Btk), whereas exposure to Bb significantly delayed regeneration (LOEC = 0.75 mg a.i./L Bb) and decreased fecundity rate (1.5 mg a.i./L Bb) of planarians. Thus, both bio-insecticides induced deleterious sub-lethal effects on a non-insect freshwater invertebrate species. However, only Bb-based formulation affected the survival, fecundity rate and regeneration at concentrations below the maximum predicted environmental concentration (PEC = 247 mg/L). Thus, care should be taken when using such formulations as alternatives to chemical insecticides near aquatic ecosystems.

1 Introduction

Bio-insecticides have been increasingly used since the 80's as alternatives to reduce the impacts on the environment and public health posed by application of chemical insecticides for the control of pests (Gupta and Dikshit 2010; Singh et al. 2018; Vivekanandhan et al. 2018). These biological compounds are based on microorganisms (bacteria, fungi, virus or protozoa) and have been considered ecofriendly due to their specificity, low toxicity, fast decomposition and efficacy when used in low concentrations (Gupta and Dikshit 2010; Kandpal 2014; EPA 2016). Therefore, they are considered safe to non-target organisms and humans (Mazid et al. 2011; Subbanna et al. 2019). Due to all their advantages and safety perception by consumers, the demand of microbiological products increased over the last 5 years showing a global market value of and US $ 5.2 billion in 2020 in opposition to US $ 2.3 billion in 2015 (Kumar 2015). According to the Environmental Protection Agency (EPA), 299 active ingredients have been registered and more than 1400 bio-based formulations were available in the market in 2016 (EPA 2016). Over the years, strains of *Bacillus thuringiensis* occupied prime position in biopesticide' market followed by entomopathogenic fungi, such as *Beauveria bassiana* (Thakore 2006; Olson 2015; Subbanna et al. 2019).

Bacillus thuringiensis kurstaki (Btk) is a facultative anaerobic gram-positive bacterium naturally occurring in soil, water, air and plants (Machado et al. 2017). It is known as an entomopathogenic bacterium that produces parasporal crystal proteins deathly toxic to insects after ingestion (Sanahuja et al. 2011; Castagnola and Stock 2014). The strains of *B. thuringiensis* (Bt) can produce different types of crystal proteins containing δ-endotoxins that specifically affect certain orders of insects after previous solubilization and activation in their midgut (OECD 2012). The predicted environmental concentration in surface water (PEC_{sw}) for different strains of *B. thuringiensis* kurstaki is lower than 100 μg a.i. Btk/ L (EFSA 2012).

Beauveria bassiana (Bb) is a filamentous fungus that belongs to the class of deuteromycetes (Sandhu et al. 2012; Berlitz et al. 2014). Briefly, it affects hosts through penetration of their cuticle by its conidia, followed by internal colonization of their tissues combining both mechanical pressure and enzymatic activities (Mascarin

and Jaronski 2016). The grown hyphae invade the hemocoel of hosts and use nutrients of hemolymph producing toxins that ultimately lead to death (Sayed and Behle 2017; Rustiguel et al. 2018). This species of fungus is commonly found worldwide as a saprophyte in soils, an endophyte in plants and acting as entomopathogen for arthropods (Rehner et al. 2011; Berlitz et al. 2014). The predicted environmental concentration in surface water (PEC_{sw}) for *B. bassiana* (different strain) range from 35,3 to 247,3 mg /L of commercial compound (EFSA 2015).

Despite all the advantages pinpointed to these bio-insecticides and the studies concerning their effects on target and non-target insects (Lajmanovich et al. 2015; Allgeier et al. 2019; Challa et al. 2019; Dornelas et al. 2020a), there is a lack of knowledge about their potential ecological effects to freshwater ecosystems (EFSA 2012; EFSA 2015). Moreover, some of these biological formulations of insecticides are also being used directly in aquatic ecosystems to control mosquito populations, that are vectors of human pathogens responsible for important diseases (Pelizza et al. 2010; Singh et al. 2018), which may increase their concentration in freshwater systems. Microbial insecticides-based Btk have been considered environmentally friendly, since they were considered harmless to non-target species due to its target-oriented mode of action (Álvarez and Biosca, 2017). In fact, 48h and 96h LC_{50} previously estimated (Table 1) for vertebrate (fish and amphibians) species were high and above 100 mg a.i. Bt/L (Becker and Margalit 1993; Karmrin 1997; WHO 1999), except for the frog *Leptodactylus latrans* with a LC_{50} of 22.45 mg/L (Lajmanovich et al. 2015). Moreover, studies performed with non-target insects showed that microbial insecticides were more acutely toxic for insects than other invertebrates, such as, the genus *Hydra* (Becker and Margalit 1993) (Table 1).

Thus, this study aims to determine the effects of the bio-insecticides Bac Control (based on *B. thuringiensis kurstaki)* and Boveril (based on *B. bassiana)* on the freshwater planarian *Girardia tigrina* Girard (Paludicola: Dugesiidae), not only to evaluate and compare their acute effects posed to planarians (mortality) with other studied species, but also to assess their sub-lethal effects using more sensitive endpoints, such as, locomotion, feeding, regeneration and sexual reproduction. In fact, freshwater planarians have been successfully used in environmental toxicology studies to assess the sub-lethal effects caused by different contaminants on such endpoints (Ofoegbu et al. 2016; Rodrigues et al. 2016; Saraiva et al. 2018; Wu and Li 2018; López et al. 2019; Ofoegbu et al. 2019a, b; Saraiva et al. 2020; Dornelas et al. 2020b; Simão et al. 2020; Simão et al. 2021).

Freshwater planarian's characteristics like their broadly geographic distribution, easy experimental manipulation and maintenance in laboratory (Guecheva et al. 2003; Knakievicz 2014; Knakievicz and Ferreira 2008), and regeneration capacity (Reddien and Alvarado 2004), make them ideal to evaluate sub-lethal effects that might be more relevant in terms for environmental risk assessment. Moreover, planarians are aquatic invertebrates found in a range of water systems (Knakievicz et al. 2006; McConnell 1965; Tyler 2000) and they prey on insect larvae that are common targets of microbial insecticides (Allgeier et al. 2019; Benzina et al. 2018; Bordalo et al. 2020; Hart and Merz 1998; Reddien and Alvarado 2004; Vila-Farré and Rink 2018).

2 Material And Methods

2.1 Bio-insecticides

Bac-Control® WP *is a formulation based in spores of B. thuringiensis, Berliner (25x10^9 spores per gram) as active ingredient. This bio-insecticide* was purchased from *Vectorcontrol Indústria e Comercio de Produtos Agropecuários Ltda.* Boveril® is a formulation based in the fungus strain ESALQ PL63 of *B. bassiana* (Bals.) Vuill., (1x10^8 conidia per gram) as active ingredient. *This bio-insecticide* was purchased from Koppert do Brasil Sistemas Biológicos Ltda.

2.2 Test organisms

Girardia tigrina was obtained from USP (University of São Paulo) and kept in the Laboratory of Ecotoxicology at the UFT (Universidade Federal do Tocantins – Functional and Applied Ecology Research Group) in ASTM (American Society for Testing and Materials) hard water medium (ASTM 1980) with constant aeration, controlled temperature (22 ± 1 ºC) and constant dark conditions. Planarians were fed with bovine liver (*ad libitum*) for periods of 2 hours once per week followed by renewal of ASTM medium. Seven days before bioassays, planarians were not fed and active organisms with no signs of injuries were used for experiments.

2.3 Acute tests

Planarians were exposed to a range of concentrations of the active ingredient (a.i.) present in the formulations of *B. thuringiensis* kurstaki (Btk) and *B. bassiana* (Bb). Btk nominal concentrations were: 50 mg a.i./L (39x10^9 spores/L), 125 mg a.i./L (97.6x10^9 spores/L), 200 mg a.i./L (156.2x10^9 spores/L), 275 mg a.i./L (214.8x10^9 spores/L), 350 mg a.i./L (273.4x10^9 spores/L), 425 mg a.i./L (332x10^9 spores/L), 500 mg a.i./L (390.6x10^9 spores/L), 575 mg a.i./L (449.2x10^9 spores/L), 650 mg a.i./L (507.8x10^9 spores/L), 725 mg a.i./L (566.44x10^9 spores/L) and 800 mg a.i./L (625x10^9 spores/L). *Beauveria bassiana* nominal concentrations were: 50 mg a.i./L (1x10^8 conidia/L), 55 mg a.i./L (1.1x10^8 conidia/L), 60 mg a.i./L (1.2x10^8 conidia/L), 65 mg a.i./L (1.3x10^8 conidia/L), 70 mg a.i./L (1.4x10^8 conidia/L), 75 mg a.i./L (1.5x10^8 conidia/L) and 80 mg a.i./L (1.6x10^8 conidia/L). A control treatment for each bioassay was performed using ASTM hard water only.

The length of test organisms used for the acute bioassays ranged from 8 to 10 mm and 25 organisms were used per experimental treatment (five planarians per replicate and 5 replicates). Exposure was carried out in Petri dishes (90x15 mm), containing 20 ml of each experimental solution and control treatment for each microbial insecticide. Planarians were exposed during 96 hours in statically system, with constant temperature (22 ± 1 ºC), in dark and without being fed. Mortality was checked at the end of 48 (to Bb and Btk) hours exposure. After the exposure period the number of dead organisms was registered for each replicate in order to allow estimation of Lethal Concentrations (LCs$_{50}$).

2.4 Behavior and regeneration of planarians

Planarians (8 to 10 mm, total length) were exposed during 8 days to nominal concentrations (the highest concentration of the chronic test did not exceed 10% of the LC$_{50}$) of *B. thuringiensis* [1.56 mg a.i./L (1.21x10^9 spores/*L*), 3.12 mg a.i./L (2.43x10^9 spores/*L*), *6.25 mg a.i./L (4.87*x10^9 spores/*L*), 12.5 mg a. i./L*

(9.75x10^9 spores/L) and 25 mg a.i./L (19.5x10^9 spores/L)] and *B. bassiana* [0.375 mg a.i./L (7.5x10^5 *conidia/L), 0.750 mg* a.i./L *(15x10^5 conidia/L), 1.5 mg* a.i./L *(30x*10^5 *conidia/L), 3 mg* a.i./L *(60x*10^5 *conidia/L) and 6 mg* a.i./L *(120x*10^5 *conidia*/L). *A control* treatment using ASTM hard water only was performed for each bioassay. Three replicates per condition (12 organisms per replicate) were used in a bottle glass containing 100 ml of each experimental solution. The tests were performed at standardized conditions of temperature 22 ± 1 ºC, in the dark and without food.

2.4.1 Locomotion

The post-exposure effects of both bio-insecticides on planarian locomotor velocity (pLMV) were evaluated individually (using 12 organisms per condition) by placing the organism into a recipient (Ø = 35 cm) containing ASTM hard water medium and covered with gridlines (spaced 0.5 cm per gridline). Locomotor velocity was measured by the number of crossed and re-crossed gridlines over a period of observation of 3 minutes. Results were expressed as the number of gridlines crossed per minute.

2.4.2 Feeding activity

Nine planarians per condition were individually placed into Petri dish containing 20 mL of ASTM hard water medium and 25 larvae of *Chironomus xanthus* (total length 0.6 ± 0.1cm, 2° instars). The post-exposure feeding rate of planarians was determined by counting the number of chironomids larvae totally digested and consumed over 3 hours. Results of feeding rate were expressed as number of consumed larvae per hour.

2.4.3 Regeneration

After exposure, twelve organisms per treatment were decapitated with a precise single cut behind the auricles. After decapitation, each organism was individually placed in a Petri dish containing 20 mL of ASTM hard water only. Blastema regeneration (i.e. length in mm) was observed after 48 hours decapitation and photoreceptors regeneration was monitored every 12 hours until complete regeneration, using a stereo microscopy (MIKROS®) with an eyepiece micrometer. Blastema length was expressed as mm after 48 hours regeneration, whereas photoreceptor formation was expressed as time in hours needed for formation of photoreceptors.

2.5 Reproduction

Sexual reproduction endpoints were determined on same conditions of Btk and Bb concentrations as described above, but the size of test organisms was 1.5 ± 0.1 cm, and the time of exposure 28 days. Four replicates per condition using ten organisms per replicate in a bottle glass containing 100 ml were used for each experimental condition including controls. Planarians were fed once a week followed by renewal of test solutions. The number of cocoons and newborn planarians were registered daily in order to determine fecundity and fertility rates (Knakievicz et al. 2006). Fecundity was expressed as the number of cocoons

produced divided by the number of planarians per treatment. Fertility was expressed as the number of offspring produced divided by total cocoons produced.

2.6 Statistical analyses

The 48h LCs$_{50}$ of Btk and Bb in *G. tigrina* were estimated using four parameter logistic curve: Y = Bottom + (Top-Bottom)/(1−10^((LogLC$_{50}$-x)*HillSlope)). For sub-lethal parameters, Kolmogorov-Smirnov and the Bartlett's tests were used to evaluate normality and homogeneity of variance of data, respectively. The effects of *B. thuringiensis* and *B. bassiana* on sub-lethal endpoints assessed in planarians were determined by one-way analysis of variance (ANOVA) followed by Dunnett's test. Locomotion data (*B. bassiana*) were transformed into rank (Y) for correction of non-normal data. Non-parametric test (Kruskal-Wallis test) followed by Dunn's test was performed for analysis of *G. tigrina* photoreceptor regeneration. Statistical analysis was performed using the software GraphPad Prism version 7.0 for Windows (GraphPad Software, La Jolla, California, USA).

3 Results

3.1 Lethal effects

The estimated LC$_{50-48h}$ for Btk was > 800 mg a.i./L (433.6×10^9 spores/L) and for Bb LC$_{50-48h}$ was 60.74 mg a.i./L (1.21×10^8 conidia/L) [95% CI: 59.61 - 61.89 mg a.i./L]. It was also observed that the highest concentrations of both formulations induced body deformations in planarians followed by their total body disintegration.

3.2 Sub-lethal effects

Locomotor velocity was significantly decreased in planarians exposed to Btk with a LOEC of 12.5 mg a.i/L ($F_{(5,66)}$ = 13.17, *p* < 0.0001, Figure 1a). Locomotor velocity was significantly altered by -43.22% and -54.23% in 12.5 and 25 mg a.i./L of *B. thuringiensis* respectively. In contrast, no significant differences were observed on locomotion of planarians exposed to Bb when compared to the control treatment ($F_{(5,66)}$ = 1.81, *p* = 0.31 Figure 1c).

Feeding activity was significantly decreased ($F_{(5,48)}$ = 16.89, *p* < 0.0001, Figure 1b) by Btk (LOEC= 12.5 mg a.i/L) showing altered feeding rates of -80% and -90% after exposure to 12.5 and 25 mg a.i./L, respectively. Again, feeding activity was not significantly affected by Bb concentrations compared to control treatment ($F_{(5,48)}$ = 2.32, *p* = 0.057, Figure 1d), despite the slight decrease observed.

The regeneration of blastema ($F_{(5,66)}$ = 1.39, *p* = 0.24, Figure 2a) and photoreceptors (H = 3.12, *p* = 0.68, Figure 2b) were not significantly affected on planarians exposed to Btk when compared to control. However, length of blastema was significantly decreased on planarians exposed to Bb (Figure 2c) with LOEC of 0.75 mg a.i./L. Moreover, decreased length at 48 hrs reached -16.2, -15.4, -17.9 and -27.3 % on planarians exposed to 0.75, 1.5,

3, and 6 mg a.i./L Bb, respectively ($F_{(5,66)}$ = 10.35, p < 0.0001, Figure 2c). In contrast, no significant differences were observed on the time needed for regeneration of photoreceptors (H = 4.34, p = 0.50, Figure 2d) on planarians exposed to Bb.

Fecundity rate was significantly decreased after exposure of planarians to Btk with a LOEC of 3.12 mg a.i/L ($F_{(5,18)}$ = 19.51, p < 0.0001; Figure 3a) and Bb with a LOEC of 1.5 mg a.i/L ($F_{(5,18)}$ = 11.54, p < 0.0001; Figure 3c). Fecundity rate reached a decrease of -64.2 on planarians exposed to the highest concentrations of Btk (Figure 3a) and -61.1% for Bb (Figure 3c). However, fertility rate was not significantly affected on planarians exposed to Btk ($F_{(5,18)}$ = 1.64, p = 0.2; Figure 3b) and Bb ($F_{(5,18)}$ = 2.40, p = 0.07, Figure 3d), compared to respective control.

4 Discussion

Exposure to Btk- and Bb-based insecticides decreased the survival of planarians. However, only the estimated LC_{50} value for Bb was below the PECsw and the concentrations recommended for its application in the field (EFSA 2015; Agboyi et al. 2020). This shows that adults of *G. tigrina*, a non-target and non-insect species, seem to be very sensitive to formulations based on Bb, but not so sensitive for Btk when compared to chironomids, *Daphnia magna*, the frog *Leptodactylus latrans*, and zebrafish (Table 1).

Survival tests performed with adults of *G. tigrina* showed that Bb-based formulations increased the mortality of planarians at estimated PECsw demonstrating that care should be taken when using this bio-insecticide near aquatic ecosystems. Data on other aquatic invertebrates, besides insects, is still very scarce in the literature and further toxicity studies should be performed, since such formulations have not been considered to represent a risk to aquatic environments (EFSA 2012; EFSA 2013). Furthermore, the soil fungus *B. bassiana* is not considered a potential risk to aquatic compartments, which can also be a valuable reason for the limited number of studies found (EFSA 2013).

Although, LC_{50} data are a simple approach to assess the toxicity of Btk and Bb, it represents the first line of evidence for environmental risk assessment (Lajmanovich et al. 2015). In fact, endpoints assessed on planarians at the organismal and population levels showed to be much more sensitive than its survival, as expected according to Sokolova and co-workers (2012) and other studies previously performed with planarians (Ofoegbu et al. 2016; Rodrigues et al. 2016; Saraiva et al. 2018; Wu and Li 2018; López et al. 2019; Ofoegbu et al. 2019a, b; Saraiva et al. 2020; Dornelas et al. 2020b).

The microbial formulation based on Btk decreased the locomotion, feeding activity and fecundity of adult planarians for concentrations as low as 12.5, 12.5 and 3.12 mg a.i./L, respectively. Nevertheless, these concentrations affecting behaviour and fecundity were still much higher than the PECsw value for Btk. In contrast, Bb-based formulation delayed the regeneration of blastema and decreased the fecundity of planarians at concentrations similar to the predicted environmental concentrations for surface water. Organisms exposed to moderate stress have high metabolic costs and high levels of energy consumption when trying to allocate energy to maintain defence mechanisms (Sokolova et al. 2012; Campos et al. 2017; Monteiro et al. 2019). No information of specific action mode of Btk or Bb regarding planarian's is available. However, exposure to bio-insecticides can interfere with the immune response of freshwater invertebrates

exacerbated by decreased feeding activity, and consequently the allocation of energy to other physiological processes, in an attempt to overcome an inflammatory response (Bordalo et al. 2020).

Planarians have exceptionally robust regenerative abilities and regulate neoblasts' proliferation (stem cells) in response to changes in metabolic status and wounding (Elliott and Alvarado 2012; Rink 2013). Is intriguing that the delay in blastema' regeneration of adults was not enough to affect the regeneration of photoreceptors. Some xenobiotics can target very specific pathways, thus not affecting the planarian regeneration system (Hagstrom et al. 2015). The dissimilar responses on behavioural (affected by Btk) and regeneration (affected by Bb, but not photoreceptor formation) endpoints are not surprising as they have distinct modes of action in insects (Sanahuja et al. 2011; Castagnola and Stock 2014; Mascarin and Jaronski 2016; Sayed and Behle 2017; Rustiguel et al. 2018; Bordalo et al. 2020). In this context, Bordalo et al. (2020) observed different responses between bio-insecticides-based *B. thuringiensis* and *B. bassiana* to life-history traits in *C. riparius* larvae. However, fecundity rate of planarians was also affected by both bio-insecticides showing that exposure to both formulations can induce adverse population level effects in planarians.

There is still scarce information on sub-lethtal effects of bio-insecticides on freshwater organisms and, to our knowledge, only a few studies were performed addressing development of larvae and emergence on *Chironomus xanthus* (Dornelas et al. 2020a, b) and *Chironomus riparius* (Charbonneau et al. 1994; Kästel et al. 2017; Bordalo et al. 2020). These previous studies with *C. xanthus* showed that Bt- and Bb-based formulations decreased growth rate of larvae and affected emergence, whereas Bt- did not affect time to emergence on study using *C. riparius*. Moreover, it was observed for insects that the toxicity of microbial insecticides may vary depending on the host species (Sanjayan et al. 1996; Beetz et al. 2008) and a few studies showed that this might be dependent on the mechanisms of response against bacterial and fungal and more specifically their immune response by recognition of microbial components and the triggering of a cascade of reactions leading to activation of phenoloxidase, that is crucial for the melanisation of pathogens and repair of damaged tissues (Cerenius et al. 2008; Grizanova et al. 2014). The host needs to cope also with reactive oxygen species and prevent oxidative damage as a consequence of immune response activation (González-Santoyo and Córdoba-Aguilar 2012; Saraiva et al. 2020).

Recent studies report the evaluation of the immune response of bio-insecticides based on *B. thuringiensis* and *B. bassiana* on *C. xanthus* (through total hemocyte count) and *C. riparius* (through Phenoloxidase activity) (Dornelas et al. 2020a; Bordalo et al. 2020). Phenoloxidase activity and oxidative damage has been previously assessed on planarians (Pang et al. 2010; Saraiva et al. 2020). Therefore, further research studies concerning the use of those biochemical endpoints would be desirable to further unravel the mechanisms of response of planarians to bacterial and fungal based insecticides, as well as, implications for consumption and allocation of energy. Interestingly, both microbial insecticides decreased the number of cocoons deposited by each planarian, but the number of newborns originated on each cocoon was not affected, showing that their development was not compromised by both formulations.

Overall, toxicity and sub-lethal effects of *B. bassiana* have been demonstrated at PECsw, that lead us to conclude that those formulations are not harmless to freshwater compartments and might not be safe to non-target freshwater invertebrate species. Concerning Btk-based formulation, further studies should be pursued, since its use is increasing considerably and our current study showed deleterious effects on planarians'

reproduction at much lower concentrations than the ones causing acute toxicity. Therefore, our study provides important information for the risk assessment of bio-insecticides in freshwater ecosystems. To our knowledge this is the first study concerning the reproductive effects of Btk - and Bb-based formulations in a non-target, non-insect freshwater invertebrate. Finally, the results obtained in this study are important to understand the possible direct effects of *B. thuringiensis* and *B. bassiana* on non-target aquatic organisms and validate the use of *Girardia tigrina* as a potential bioindicator species.

5 Conclusions

Our results showed evidence that microbial insecticides based on Bb might not be as ecologically friendly as previously suggested especially if used in the vicinity of aquatic ecosystems. This study also highlights the importance of using planarians as bioindicators of environmental contamination by bio-pesticides. Given the effects observed on fecundity rates of planarians exposed to environmentally relevant concentrations of Bb and Btk, it is critical to consider reproductive endpoints to better evaluate how microbial insecticides affect natural populations of non-target freshwater invertebrates.

Declarations

Ethics approval and consent to participate

'Not applicable'

Consent for publication

'Not applicable'

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors' contributions

LCRS conducted experiments, analyzed data and wrote the manuscript (writing - original draft, review and editing). ASPD conducted experiments. ASS, ASPD, CG, JLTP, AMVMS and RAS analyzed data, review and edit the manuscript; RAS and AMVMS conceived (funding acquisition) and designed research. All authors read, made corrections, and approved the manuscript.

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Tables

Table 1: Acute toxicity of *B. thuringiensis* and *B. bassiana* to aquatic organisms.

Microbial Insecticide	Species	Life stage	Test duration	LC_{50} or EC_{50}	Reference
Aquatic Invertebrates					
B. thuringiensis	Chironomus xanthus	Insect larvae (non-target) - 1st instar	48 hours	LC_{50} = 1534 µg a.i./L	Dornelas et al. (2020a)
B. thuringiensis	Chironomus riparius	Insect larvae (non-target) - 1st instar	48 hours	LC_{50} = 1.8 µg a.i./L	Bordalo et al. (2020)
B. thuringiensis	Chironomus riparius	Insect larvae (non-target) - 1st instar	48 hours	LC_{50} = 2.3 µg a.i./L	Kästel et al. (2017)
B. thuringiensis	Chironomus calligraphus	Insect larvae (non-target) - 3rd/4th instar	48 hours	LC_{50} = 11.2 µg a.i./L	Lavarías et al. (2017)
B. thuringiensis	Chironomus tepperi	Insect larvae (non-target) - 4th instar	48 hours	LC_{50} = 40 – 48 µg a.i./L	Hughes et al. (2005)
B. thuringiensis	Chironomus kiiensis	Insect larvae (non-target) - 4th instar	24 hours	LC_{50} = 0.3 – 1.6 mg a.i./L	Cao et al. (2012)
B. thuringiensis	Daphnia magna	Planktonic crustacean (non-target) ≤ 24h old	48 hours	EC_{50} = 0.15 µg a.i./L	Machado et al. (2017)
B. thuringiensis	Aedes aegypti	Insect larvae - 4th instar	24 hours	LC_{50} = 600 spores/ml	Khawaled et al. (1990)
B. bassiana	Chironomus xanthus	Insect larvae (non-target) - 1st instar	48 hours	LC_{50} = 6.3 µg a.i./L	Dornelas et al. (2020a)
B. bassiana	Chironomus riparius	Insect larvae (non-target) - 1st instar	48 hours	LC_{50} = 34.7 mg a.i./L	Bordalo et al. (2020)
B. bassiana	Anopheles stephensi	Insect larvae -	24 hours	LC_{50} =	Ragavendran

		4th instar		32.6 mg a.i./L	et al. (2017)
B. bassiana	*Culex quinquefasciatus*	Insect larvae - 4th instar	24 hours	LC$_{50}$ = 35.4 mg a.i./L	Ragavendran et al. (2017)
B. bassiana	*Culex quinquefasciatus*	Insect larvae – 1st instar	24 hours	LC$_{50}$ = 11.53 mg a.i./L	Vivekanandhan et al. (2018)
B. bassiana	*Aedes aegypti*	Insect larvae - 4th instar	24 hours	LC$_{50}$ = 47.1 mg a.i./L	Ragavendran et al. (2017)
B. bassiana	*Mysidopsis bahia*	shrimp/juveniles (non-target) ≤ 24h old	96 hours	LC$_{50}$ = 560 µg a.i./L	Genthner et al. (1994)
Aquatic Vertebrates					
B. thuringiensis	*Leptodactylus latrans*	amphibian (non-target) - larvae	48 hours	LC$_{50}$ = 22.45 mg a.i./L	Lajmanovich et al. (2015)
B. thuringiensis	*Danio rerio*	Zebrafish (non-target) - embryos and larvae	96 hours	LC$_{50}$ = 85.9 – 188.4 mg a.i./L	Grisolia et al. (2009)
B. thuringiensis	*Fundulus heteroclitus*	Fish (non-target) - Adult	96 hours	LC$_{50}$ = 980 mg a.i./L	Lee and Scott (1989)
B. thuringiensis	*Oncorhynchus mykiss*	Rainbow trout (non-target)	32 days	LC$_{50}$ > 143.5 MPCA/L*	EFSA (2012)
B. thuringiensis	*Lepomis macrochirus*	Bluegill Sunfish (non-target)	32 days	LC$_{50}$ > 143.5 MPCA/L*	EFSA (2012)
B. bassiana	*Oncorhynchus mykiss*	Rainbow trout (non-target) – embryos, larvae and adult	31 days	LC$_{50}$ = 7300 mg a.i./L	Zimmermann (2007)

* Microbial Pest Control Agent (MPCA) = Active Ingredient.

Figures

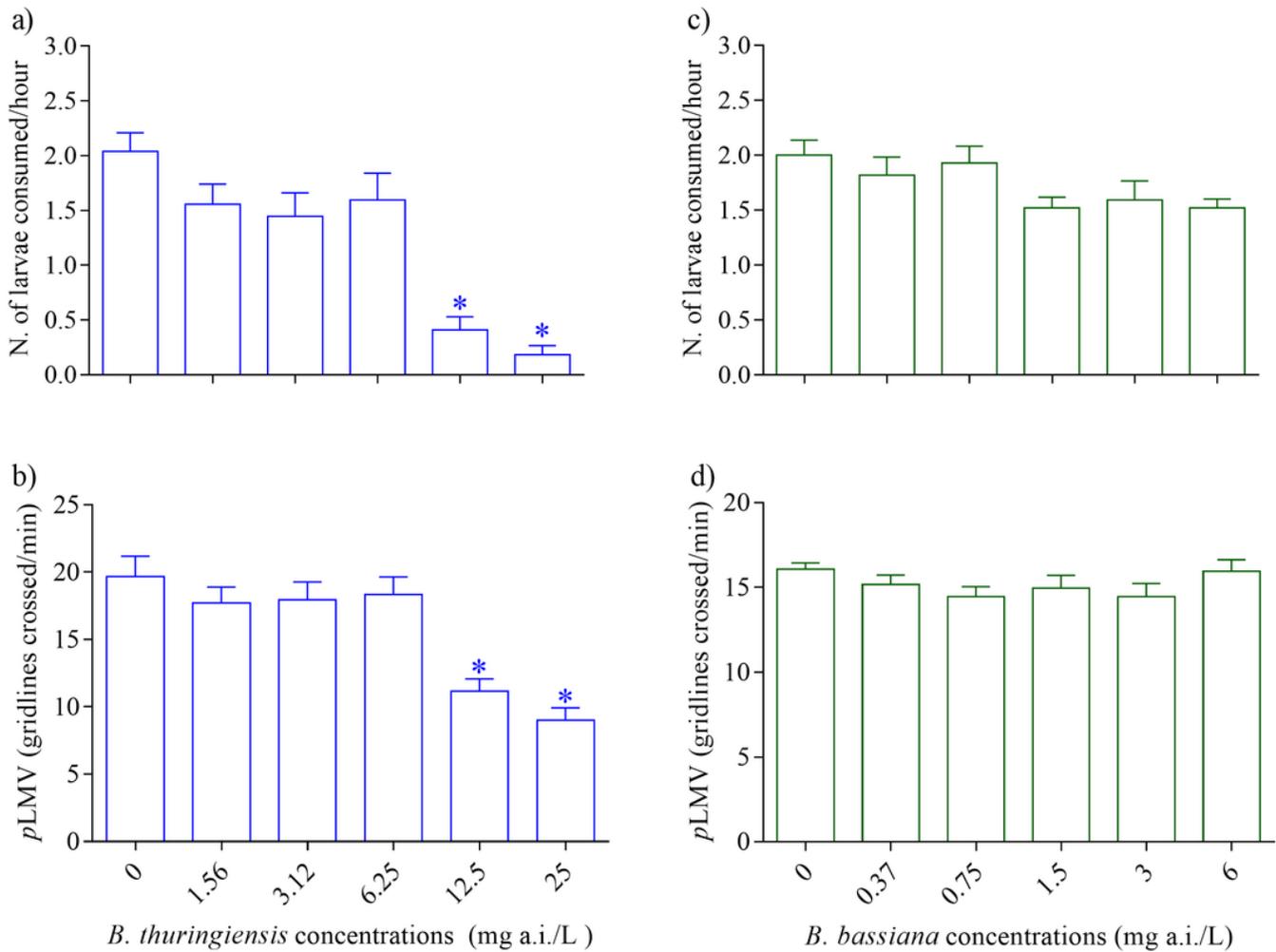## Figure 1

Feeding rate of G. tigrina (number of C. xanthus larvae totally consumed per hour; mean ± SEM) after exposure to sub lethal concentrations of B. thuringiensis [a)] and B. bassiana [c)]. Locomotor velocity of G. tigrina (gridlines crossed per minute; mean ± SEM) after exposure to sub lethal concentrations of B. thuringiensis [b)] and B. bassiana [d)]. *Denotes significant differences compared to the control treatment (Dunnett´s test)

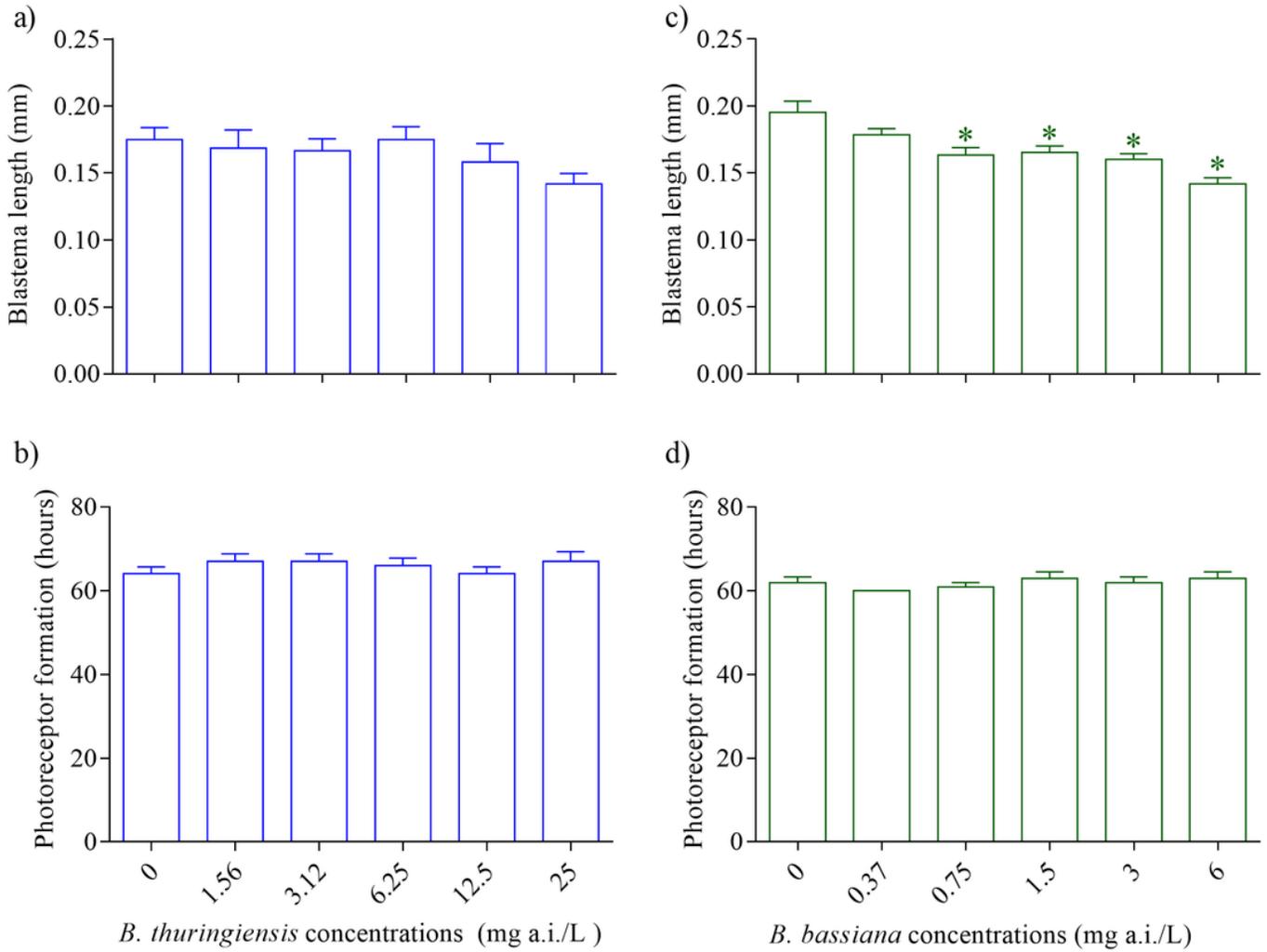## Figure 2

Blastema regeneration (mm - after 48 hours of decapitation; mean ± SEM) of G. tigrina after exposure to sub-lethal concentrations of B. thuringiensis [a)] and B. bassiana [c)]; Photoreceptor formation (hours; mean ± SEM) of G. tigrina after exposure to sub-lethal concentrations of B. thuringiensis [b)] and B. bassiana [d)]. *Denotes significant differences compared to the control treatment (Dunnett´s test)
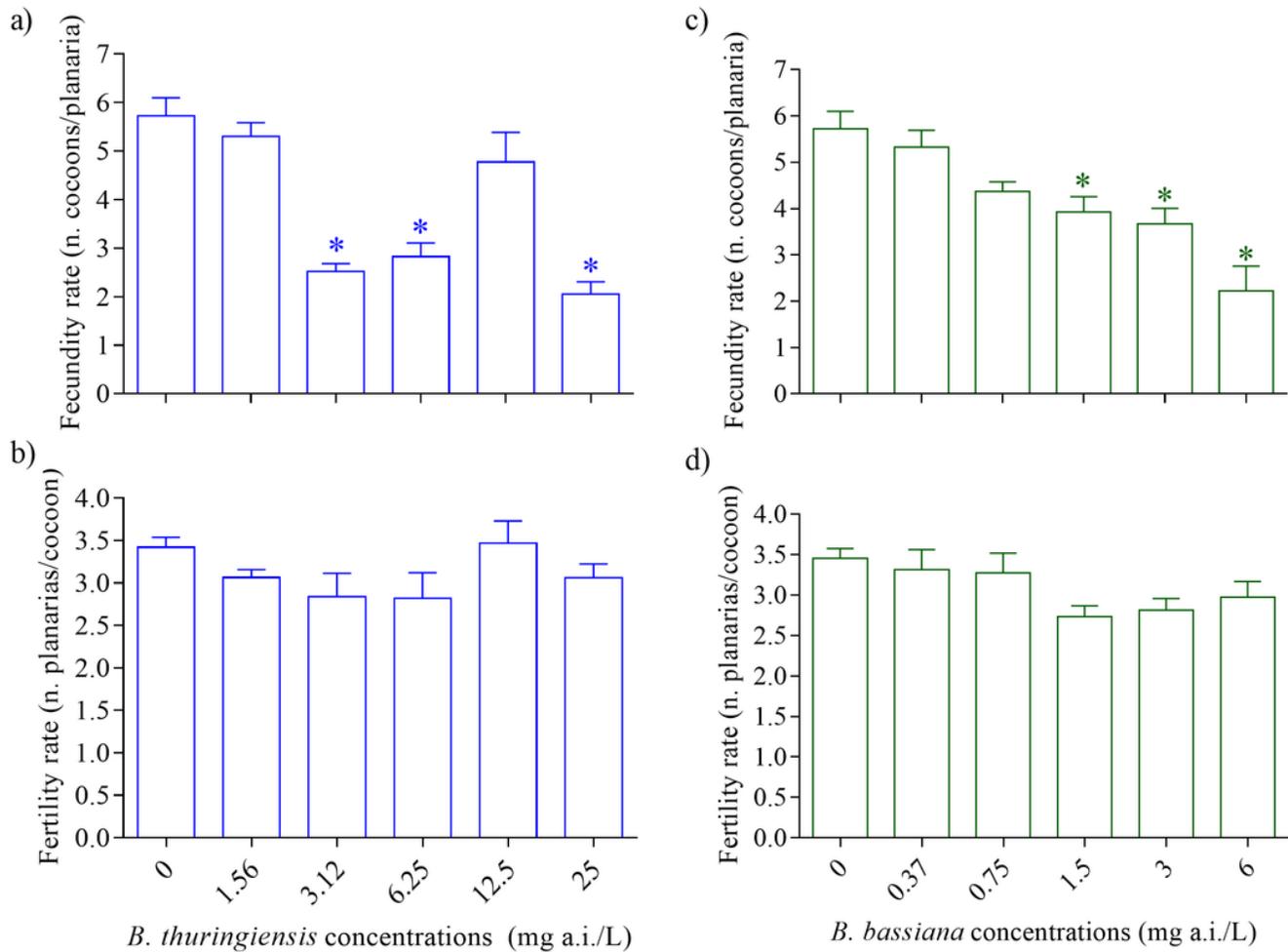
Figure 3

Fecundity rate (mean ± SEM) of G. tigrina exposed during four weeks to sub-lethal concentrations of B. thuringiensis [a)] and B. bassiana [c)]. Fertility rate (mean ± SEM) of G. tigrina exposed during four weeks to sub-lethal concentrations of B. thuringiensis [b)] and B. bassiana [d)]. *Denotes significant difference compared to the control treatment (Dunnett´s test)