International Journal of Mosquito Research

ISSN: **2348-5906**
CODEN: **IJMRK2**
IJMR 2015; 2(3): 05-09

Received: 05-06-2015
Accepted: 07-07-2015

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Efficacy and persistence of two *Bacillus thuringiensis israelensis* formulations for the control of *Aedes aegypti* (Linnaeus, 1762) under simulated field conditions

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Abstract
The objective of this work was to evaluate the efficacy and persistence of *Bacillus thuringiensis israelensis*, when used against immatures of *Ae. aegypti* and to monitor water quality with respect to pH, conductivity (mS), dissolved (mg/L) and saturated (%) oxygen. The microbial behavior and persistence of bacteria were determined. The experiment was carried out in an unshaded area, utilizing five barrels with 80 liters of water for each product together with 50 immatures. Vectobac T at $0.9x10^{-3}$ g/L produced 100% mortality of larvae up to the eighth day and 98.8% up to the fifteenth day. Vectobac WDG, at $2.4x10^{-4}$ g/L, controlled 100% of immatures up to the first 15 days. Conductivity and pH increased gradually with time in the experiment. Oxygen concentration was always about 7 mg/L. Vectobac WDG disperses rapidly in the environment, while Vectobac T is slowly released, with significant reduction in Bti during 22 days.

Keywords: water quality, dengue fever, biological control, Culicidae.

1. Introduction
Aedes (*Stegomyia*) *aegypti* (Linnaeus 1762) is the principal vector of the viruses of dengue and urban yellow fever in Brazil. Dengue fever is the most important arboviruses in the world, with a 30-fold increase in the number of cases recorded annually in the last 50 years, where *Ae. aegypti* exposes almost half the global population to the risk of this virosis. Fifty to 100 million new cases are estimated per year in more than 100 endemic countries [30]. In South America, more than 1.5 million cases of dengue were recorded in 2013 [20], and this situation is aggravated with the reintroduction of serotype DENV IV and entrance of Chikungunya virus in this region. *Aedes aegypti* and *Ae. albopictus* reached alarming transmission rates for three genotypes of Chikungunya in Brazil in tests performed in the laboratory [26].
This substantial number of dengue cases is linked to, among other factors, the rapid and disorganized growth of cities, industrialization, population migration and climate changes [8]. The occurrence of artificial breeding sites coming from anthropic activities, principally recyclables and water storage tanks for various purposes, favor the proliferation of mosquito larvae [16]. The availability and diversity of breeding sites, high reproductive capacity, strong domestication and anthropophily, and selection of populations of mosquitoes resistant to synthetic insecticides, are also factors that contribute to the marked increase in dengue annually. In Brazil, the number of cases of the disease in 2013 was 190% more than in 2012, with 50% of cases caused by DENV-4 [20].
Various strategies have been adopted for the control of mosquitoes, where the main ones are elimination of breeding sites and the use of synthetic chemical insecticides. However, the difficulty in the control of the vector has led to the need for new alternatives. Bioinsecticides, with the active principle being crystals of *Bacillus thuringiensis israelensis* (Bti), have been used in various countries, through large scale programs for controlling mosquito larvae (Culicidae) and black flies (Simullidae) [27]. Toxicity to these insects is due to the production of protein crystals composed of the proteins Cry and Cyt [7]. These proteins show highly specific entomopathogenic activity, causing no damage to non-target insects, vertebrates or even the environment [11].
Under field condition, this formulated bioinsecticide is affected by various abiotic factors. Some authors, using simulated field conditions for the control of *Ae. Aegypti,* have demonstrated the influence of sunlight on the persistence of the bioinsecticide [17, 37, 32] or have

reported the possible effects of water quality on spores and formulations of *B. thuringiensis israelensis* [23]. However, there is divergence in our understanding when considering the behavior of these commercial products in relation to their persistence in breeding sites, which is an important factor in the possible interference of water quality with product performance, since they can be used in homes as well as various sectors of production.

To contribute to our knowledge about the action of *B. thuringiensis*-based products, the aim of this study was to evaluate the efficacy and persistence of two commercial formulations based on *B. thuringiensis israelensis* against immatures of *Ae. aegypti*. Water quality was monitored in relation to pH, conductivity, and dissolved and saturated oxygen during the action of the products, as well as quantifying the presence of spores and vegetative cells of the bacteria at the breeding site during the experiment in the field.

2. Material and Methods

2.1 Experiment setup and larval mortality

Two formulations of *B. thuringiensis israelensis*, Vectobac® WDG 3000 ITU/mg, lot 72-72-578-PG, and Vectobac® T 2200 ITU/mg, lot PK009-2, were tested for efficacy as well as persistence of bioinsecticide activity against larvae of *Ae. aegypti*, in breeding sites set up in the field. The experiment was performed in a grassy area without shade (23°19'28.61"S and 51°12'04.00"W; altitude of 598 meters), on the campus of the State University of Londrina, Londrina, Paraná, Brazil, during December 12-31, 2004.

We used 16 plastic barrels with a capacity of 100 liters, filled with 80 liters of water from the public supply, arranged at a distance of 1.5 meters one from the other (Fig. 1A). The containers were covered using lids cut in the middle, to simulate conditions similar to those found with half-open water tanks or barrels not completely closed for storing water at homes (Fig. 1B, C).

Five barrels were utilized to evaluate Vectobac T, where 0.072 g product was added to each barrel ($0.9x10^{-3}$ g/L). The other five barrels were used in the tests with Vectobac WDG, adding 0.0193 g product per barrel ($2.4x10^{-4}$ g/L). Afterwards, 50 4th instar larvae of *Ae. aegypti* were added to each container. The larvae were obtained from the insectary maintained in the General and Medical Entomology Laboratory of the State University of Londrina, which originated from samplings of eggs in the field, utilizing ovitraps. Three barrels were used as controls for each bioinsecticide tested: two had bioinsecticide added but no larvae and one had larvae added but no product.

The analyses of larval mortality were done 24 hours and 8, 15 and 22 days after the start of the experiment. In each analysis, there was no removal of dead larvae from the containers, and another 50 1st instar immatures were introduced to the barrels, simulating natural reinfestations at artificial breeding sites. Each barrel also received 0.040 g cat food that had been autoclaved and triturated into particles of approximately 1 mm. At the same time points as analysis, live larvae present in the control barrels were quantified and replaced with 1st instar immatures. Pupae that eventually emerged were counted and removed before the emergence of adults. The experiment was monitored daily.

2.2 Physical and chemical monitoring of breeding site

Physical and chemical parameters of the water were evaluated, including pH, conductivity, dissolved and saturated oxygen and temperature, using a Hanna HI 9828 multiparameter meter. Ambient temperature and relative humidity were measured with the help of Gehaka thermohygrometer and thermometers installed on site (Fig. 1B, E). There parameters were measured before and after the application of the products at five minutes and one, eight, 15 and 22 days.

2.3 Sampling of microbiological material

The presence of spores and vegetative cells of *B. thuringiensis israelensis* was evaluated in all containers. A small opening was established, closed with a rubber stopper, in the lower portion of each barrel, close to the bottom, for drawing samples of water to be analyzed for the presence or absence of spores. During each evaluation of mortality, 10-mL samples of water were taken from each breeding site, using a sterile disposable syringe, for later inoculation into culture medium to count colony forming units (CFUs) (Fig. 1D). These samples were kept at –20 ºC until the time of processing. One milliliter of each sample was seeded in plates containing Bacto-peptone medium (BP) with 100 μg mL^{-1} ampicillin. After five days of incubation at 30 ºC, colonies obtained were evaluated with respect to morphology and examined with a light microscope (Zeiss 1000 x) to confirm the formation of protein crystals typical of *B. thuringiensis israelensis*.

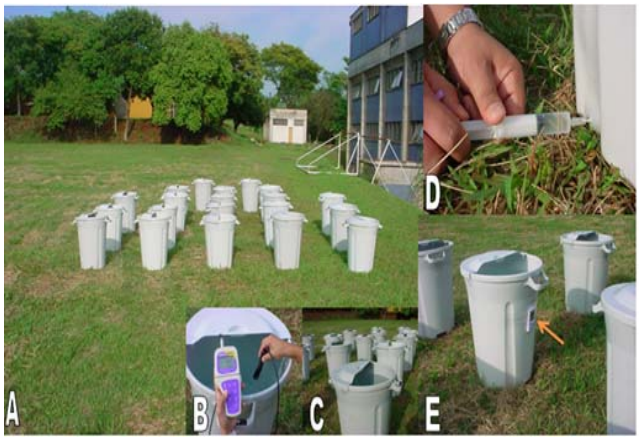

Fig 1: A. Location and layout of the experiment in semi-field conditions. B. Measurements of abiotic parameters and details of the lid simulating a semi-open water tank. D. Sampling of water with a disposable syringe. E. Instrument for measuring ambient temperature (arrow).

2.4 Analysis of data

The data for the two products tested in 5 replicates and controls were subjected to analysis of variance. The means of each replicate were compared by the Tukey test at the 5% level of significance using the program SPSS® 14.0 package for Windows [22].

3. Results

Vectobac T applied at a concentration of 0.0009 g/L controlled 100% of immatures of *Ae. aegypti* during a period of eight days. At 15 days after application, larval mortality was 98.8% (Table 1). Vectobac WDG, also applied under the same conditions and at a concentration of 0.00024 g/L, killed 100% of immatures up to the fifteenth day (Table 1).

At 22 days of the experiment, the mortality rate was less than 95% in tests with the two products, which determined the end of the experiment, considering this percentage limit for successful mosquito control under non-experimental conditions (Table 1).

Table 1: Percentage of mortality of *Aedes aegypti* larvae exposed to Vectobac T (VT) at 0.0009 g/L and Vectobac WDG (VWDG) at 0.00024 g/L with monitoring of water quality and microbial behavior, for period of December 9-31, 2004, Londrina, Paraná.

Application	(n) Total[≠]		Dead larvae %		Mean pH		Conductivity (µS)	
(Days)	VT	VWDG	VT	VWDG	VT	VWDG	VT	VWDG
0	250	250	0.0	0.0	6.28[*] c[+]	6.84[*] c[+]	83.00 d	83.00 c
1	250	250	100	100	7.08 c	6.82 c	91.30 c	91.40 b
8	250	250	100	100	7.53 c	7.56 b	91.93 c	90.70 b
15	250	250	98.8	100	8.15 a	8.14 a	96.27 b	94.00 b
22	0.0	0.0	<95	<95	8.16 a	8.21a	102.73 a	102.07 a
CV´%	-	-	-	-	2.0	1.9	1.5	1.6

[*] Original data
[+]Means followed by same letter in the column do not differ according to the Tukey test at 5% level of significance.
´ Coefficient of variation of Tukey test
[≠]Number of larvae introduced to breeding site

In the controls, five immatures died on the eighth day of the experiment, accumulating a mean of 98.75% of survivors in the whole experiment (Table 2).

Table 2: Mortality of larvae in the control (without bioinsecticide) and means of pH and conductivity in control barrels without larvae but containing Vectobac WDG (0.00024 g/L) and Vectobac T (0.0009 g/L)

Time of experiment						
Controls	Application	1 day	8 days	15 days	22 days	CV%
Control[≠] (larvae)	100	100	100	100	0.0	-
Mortality (%)	0.0	0.0	5.0	0.0	0.0	-
Mean pH	6.85[*] d[+]	7.03 c	7.60 a	7.29 b	7.07c	0.8
Conductivity (µS)	83.03 d	91.73 c	90.97 c	96.67 b	104.73 a	0.9
Control WDG Mean pH	6.82 e	7.08 d	7.52 c	7.7 b	8.60 a	0.6
Conductivity (µS)	83.03 d	91.50 c	91.30 c	96.30 b	104.30 a	0.1
Control T Mean pH	6.84 e	7.11 d	7.41 c	8.06 b	8.20 a	0.6
Conductivity (µS)	83.03 d	91.70 c	91.40 c	96.00 b	102.20 a	0.7

[*] Original data
[+]Means followed by same letter in the row do not differ according to the Tukey test at 5% level of significance.
[≠]Number of larvae introduced to breeding site

The experiment was conducted at a mean ambient temperature of 26.51 ºC (16.7 – 37.4 ºC) and water temperature of 26.15 ºC (18.4 – 32.9 ºC), with mean relative humidity of 68.94% (46.0 – 99.0%). Significant alterations occurred in pH and conductivity, according to the Tukey test, with increase in the two different treatments (Tables 1 and 2). Dissolved and saturated oxygen remained about constant (6.5 to 7.0 mg/L or 92.8 to 100%) in the whole sampling period.

With regard to the release of Bti in the water of breeding sites, the water samples taken immediately before the application of the bioinsectisides showed no presence of the products. In the water samples 5 minutes after application of Vectobac WDG, the Bti spore levels were in the range of 1555 to 10,220 CFU mL^{-1} (Table 3). The samples 5 minutes after application of Vectobac T showed spores levels between 110 and 1785 CFU mL^{-1}**.** One day after application, bacteria spore levels in water samples decreased to 197-2270 CFU mL^{-1} for Vectobac WDG and 87-1520 CFU mL^{-1} for Vectobac T. At 8, 15 and 22 days, for both Vectobac WDG and Vectobac T, Bti spore counts varied between 200 and 1100 CFU mL^{-1} (Table 3). All samples analyzed in the control breeding sites, with or without larvae, showed no presence of Bti spores (Table 3).

Table 3: Number of spores of *B. thuringiensis israelensis* in samples of water, after the application of Vectobac WDG and Vectobac T at 0.00024 and 0.0009 g/L, respectively, and in the control, under semi-field conditions in the period of December 9-31, 2004.

	UFC mL^{-1}		
Sampling	Vectobac WDG (min. –max.)	Vectobac T (min. –max.)	Control (min. –max.)
5 minutes	1555-10,220[*]	110-1785	0
1 day	197-2270	87-1520	0
8 days	450-790	200-580	0
15 days	640-1100	330-950	0
22 days	350-660	200-760	0

[*] Ranges for three replicates for each sampling

4. Discussion

The biological activity of the bioinsecticides Vectobac T and Vectobac WDG was maintained up to 22 days, where these results were similar to those obtained by [1] who tested Vectobac T at a concentration of 0.375 and 0.75 g per breeding site, finding a mortality rate of up to 100% of *Ae. aegypti* for 2 and 3 weeks, respectively [14] in a semi-field assay utilizing Vectobac WDG against *Ae. Aegypti* and obtained more than 70% mortality between 30 and 36 days in plastic, concrete or asbestos containers. In general, the commercially formulated or experimental products with *B. thuringiensis israelensis* did not exceed four weeks of persistence [17, 4], and often the increase in concentration of product did not result in greater mortality [18]. Jacups *et al.* [12] tested Vectobac WDG at concentrations of 400 and 800 g/ha, and found mortality rates greater than 80% for up to nine weeks using semi-field assay

with *Aedes* in cryptic breeding sites in Australia.

The results indicate that the formulations tested are suitable for the control of *Ae. aegypti*, where it is recommended that reapplications be carried out every three weeks. The World Health Organization recommends its use as an alternative in potable water reservoirs [29] or other types of breeding sites that cannot be treated with nonspecific chemical products such as organophosphates. Therefore, this type of formulation represents a complementary alternative in control programs in regions where the populations of larvae show resistance to conventional insecticides, which has occurred in Brazil [6, 3, 5, 15].

The ranges of temperature and relative humidity recorded in this study are typical summer conditions in northern Paraná State, favorable conditions for the proliferation of *Ae. aegypti.* Temperature and light intensity can indirectly affect the action of Bti-based products, since at low temperatures, immatures filter less food, and therefore, greater concentrations of product are necessary to obtain satisfactory results in mosquito control [1]. Nayar *et al.* [19] tested, under laboratory conditions, the action of Bti at different temperatures for *Culex nigripalpus* Theobald, 1901 and *Ae. taeniorhynchus* (Wiedemann, 1821), and found that a higher concentration of product was needed at 25 °C compared to 15 °C to reach the same LC_{50}. Santos *et al.* [21] also observed a decrease in the LC_{50} and LC_{95} of the product with increase in temperature, testing 15, 25 and 35°C and utilizing various isolates of Bti against 4th instar larvae of *Ae. aegypti.*

We found a gradual and statistically significant change in pH of water, which went from slightly acid to basic after 22 days of exposure, including the control containers with no application of bioinsecticide (Tables 1 and 2), demonstrating that the alteration observed was not a result of the application of the bioinsecticides tested, but a natural consequence of organic enrichment. Since it is known that larvae of *Ae. aegypti* develop at alkaline pH [21], the increase in pH of water can be one of the factors that contribute to the decrease in mortality of immatures of *Ae. aegypti.* In an earlier study, Santos *et al.* [21] demonstrated that bioassays carried out at pH below 7.0 showed LC_{50} values lower than those at alkaline pH. These results can be correlated with the fact of larvae being more phagoactive at acidic pH. Bem-Dov *et al.* [2] pointed out that alkaline pH probably activates the crystals in the medium, before being ingested by the larvae, and also, the nutritive particles at alkaline pH can last longer in the intestine of the larvae, thereby decreasing the toxicity of the crystals.

Conductivity increased in all treatments (Table 1), but without statistical significance (Tables 1 and 2), indicating that it increases in a natural manner in this type of environment. This increase can be related to the organic and mineral enrichment of the medium. The effectiveness of *B. thuringiensis israelensis* is inversely proportional to organic matter content [25]. Bem-Dov *et al.* [2] found that the efficacy of Bti is substantially reduced in the presence of food for *Ae. aegypti,* since the particles ingested protect the intestinal wall against the effect of endotoxins, by covering its surface and impeding the binding of the bacteria to membrane receptors.

All experiments were carried out on site with incidence of direct sunlight, in half-covered barrels simulating semi-open water tanks. Direct sunlight can interfere with the persistence of crystals, diminishing their toxicity [1, 13]. Melo-Santos *et al.* [17] tested Vectobac T under simulated field conditions for control of *Ae. aegypti*, and found persistence with larval mortality of 13-35 days and 40-54 days, respectively, for breeding sites exposed to sun and shade. Vilarinhos and Monnerat [28] utilizing Vectobac T, found persistence of two weeks with control of 100% of larvae of *Ae. aegypti*, even in water tanks exposed to direct sunlight. Under the same conditions, Vectobac WDG provided three weeks of 100% control of larvae and maintained the level of control very close to 100% for 12 weeks, in water tanks where the water was not exposed to direct sunlight. Very often, people use semi-open water tanks for storing water, which can favor the development of the vector due to shading. This mixed environment (sun/shade) can have implications on the action of Bti, because ultraviolet rays affect the persistence of protein crystals in the environment and consequently their action on the larvae [13], but according to [24], ultraviolet rays increase the sensitivity of mosquito larvae to Bti, thereby enhancing its efficacy in a short space of time.

Several studies have analyzed the presence of spores of *B. thuringiensis* in water samples after applications of Bti products [10, 9]. In the present study, Bti spores were detected in similar quantities, during the whole experiment, except for Vectobac WDG in the first sampling, carried out five minutes after its application, where the values were higher. The formulation Vectobac WDG dissolved more rapidly than Vectobac T (Table 3), which is due to the nature of its formulation, i.e., water dispersible granules (WDG). On the other hand, Vectobac T is a compacted granulated formulation for slow release. This more rapid or slow action of formulation, as well as their persistence, should be taken into consideration when choosing a product for mosquito control measures. Both products showed a decrease in the number of spores over time, which was more apparent for Vectobac WDG.

5. Conclusions

We conclude that Vectobac WDG and Vectobac T are effective for the control of *Ae. aegypti* and do not alter the physical conditions of the water. The low persistence of toxicity observed for the two formulations is probably related to organic enrichment of the breeding site**,** alkaline pH, sunlight intensity and low quantity applied. The bacteria did not show recycling in the environment, indicating the need for fortnightly applications of bioinsecticide at the breeding sites of *Aedes Ae. aegypti,* under these conditions.

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