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RESEARCH ARTICLE

Characteristics of VectoBac® WG spray, a *Bacillus thuringiensis israelensis* (Bti) strain AM65-62 water-dispersible granule formulation, from a backpack sprayer Stihl® SR420 to achieve effective dengue vector control in operational programs

Teoh, G.N.[2*], Nazni, W.A.[1*], Noor-Aisyah, A.[1], Tanusshni, M.[1], Seleena, B.[2]

[1]Medical Entomology Unit, Institute for Medical Research, Ministry of Health, Jalan Pahang, Kuala Lumpur 50588, Wilayah Persekutuan Kuala Lumpur, Malaysia
[2]Valent Biosciences LLC, Kuala Lumpur, Malaysia
*Corresponding authors: guatney.teoh@valentbiosciences.com; nazni@moh.gov.my

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ABSTRACT

The productive outdoor larval habitats of dengue vectors are widespread, cryptic and hard-to-reach. It is a challenge to larvicide such habitats using traditional manual sprayers. A wide-area treatment with *Bacillus thuringiensis israelensis* (Bti) microdroplets dispersed from a motorized backpack sprayer into such habitats is a proven method to suppress the vector population and interrupt disease transmission. This paper provides the answers to the constant inquiries by program operators on the characteristics of a Bti wide-area spray from a motorized backpack sprayer. The Bti spray mix can be either 125g or 250g in 10 liters water with a walking speed of 1m in 6 secs or 1m in 3 secs, respectively, to achieve the recommended dose of 400g/ha–500g/ha. The spray dispersed at a flowrate of 567mL-650mL through the No. 2 sprayer orifice, reached a 16m swath. A 90%-100% larval mortality was obtained in containers that were left outdoors for 7 to 14 days post spray under a shade, exposed to ambient temperature and sunlight. Higher Bti doses, at 2x and 3x to the recommended dose, achieved 90%-100% mortality in containers left outdoors for more than 14 days, but higher doses are not recommended in areas with persistent rainfall.

Keywords: Dengue vector control; Bti microdroplets; wide-area spray treatment; motorized backpack mist blower.

INTRODUCTION

Dengue is a global disease burden, with an estimate of 390 million dengue infections per year, and about 500,000 cases develop into severe dengue. The disease results up to 25,000 deaths annually worldwide (WHO, 2021). In the last two decades, the number of dengue cases reported to WHO has increased over 8 folds, from 505,430 cases in 2000, to over 2.4 million in 2010, and 5.2 million in 2019 (WHO, 2021). Asia is the most severely affected region with 70% of the global disease burden in the tropical and subtropical regions (Nuraini *et al*., 2021; WHO, 2021). In Malaysia, a total of 90,304 cases with 145 deaths were reported in 2020 (MOH, 2022). In 2022, there were 66,102 reported cases, resulting in 56 fatalities (MOH, 2022). However, in 2023, the number of dengue cases skyrocketed to 123,133, with 100 recorded fatalities (MOH, 2023). In 2024, as of epidemiology week 49, the country experienced 116,224 dengue cases with 248 deaths (MOH, 2024).

The process of urbanization and globalization has played a significant role in the geographic expansion of dengue transmission (Gubler, 2011). Urbanization has increased the breeding habitats of the vector mosquitoes (Ong, 2016). In Malaysia, *Aedes aegypti* (L.) and *Ae. albopictus* Skuse are the primary vectors for dengue. The habitat surveillance studies on dengue vectors have been conducted extensively over the years in Malaysia, as well as in Singapore and Thailand, which share similar climates. It was found that *Ae. aegypti* was found equally breeding in indoor and outdoor artificial containers. However, *Ae. albopictus* exhibited as the dominant breeder in outdoor artificial containers, 2.34 fold higher than *Ae. aegypti* (Oreenaiza *et al*., 2017). These vectors show great adaptation with human population (Oreenaiza *et al*., 2017).

The current national dengue control strategy in Malaysia focuses on targeting the mosquito vectors, conducting adulticiding, larviciding and source reduction. The MOH Vector Control Unit conducts larviciding more frequently than adulticiding. Larviciding with temephos and *Bacillus thuringiensis israelensis* (Bti) are implemented as a preventive dengue control measure and during the outbreak (MOH, 2019). The larvicides are applied as direct application into the water storage containers or sprayed into the widespread *Aedes* spp. larval habitats.

This paper focuses on the spray application of VectoBac WG, Bti strain AM65-52, a biological larvicide that is used in the Malaysian National Dengue Control Program since 2014. VectoBac WG, a product of 3000 International Toxin Units (ITU) per milligram against *Ae. aegypti*, is manufactured by Valent BioSciences LLC, USA and it has the pre-qualification status with WHO (WHO, 2012). It is used as direct application or spray application depending on the

type of mosquito larval habitat. The Malaysian VectoBac WG label describes both the direct and spray application methods. For the spray application, there are recommended spray mix rates for the different types of spray equipment. The spray mix can be dispersed using a compression hand pump sprayer, motorized backpack sprayer or a truck mounted ultra-low volume (ULV) generator. The spray is targeted towards the widespread *Aedes* spp. larval habitats. Spray applications from backpack sprayers is more common than compression hand-pump sprayers or the truck mounted ULV generators.

On the local VectoBac WG label, the recommended mix rate for the motorized backpack sprayer is 125g Bti in 12 liters of water. The 12 liters spray mix is sufficient to cover the varied *Aedes* spp. larval habitats which are found within an area of 2500m^2 (0.25ha). The recommended walking speed for the operator is 1m in 6 secs, to ensure a complete coverage of the widespread larval habitats which are many and varied, small and cryptic. At this spray mix rate and walking speed, a dosage of about 500g/ha is achieved and has been shown to effectively reduce dengue vector population and helped to curb dengue outbreak in Malaysia (Bohari *et al.*, 2020).

In the recent years, due to dengue outbreaks in multiple sites and due to limitation of manpower during the COVID-19 pandemic, the dengue vector control operators preferred to use 250g in 10L with a faster walking speed of 1m in 3 secs to cover wider areas within their working hours. Interruption of dengue transmission was achieved with the above spray regime. But the program managers at the MOH questioned whether the successful interruption in the disease transmission was due to a double dose rate or a higher dose rate than what was supposed to be implemented at 400g/ha-500g/ha. Thus, MOH requested the Institute for Medical Research (IMR) to conduct trials to confirm the dose achieved by spraying a 250g in 10 liters with a walking speed of 1m in 3 secs in comparison to 125g in 10 liters with a walking speed of 1m in 6 secs. IMR conducted the requested study by MOH and included additional evaluations: Bti spray mix dispersed through different nozzle orifices from the backpack sprayer; droplet profile of the Bti sprays; efficacy of the Bti sprayed droplets over time (7 days to 42 days post spray) and over distance (2m-16m from the spray path); and the impact of the outdoor environmental factors on the efficacy of the Bti sprayed droplets. This comprehensive study supported to determine the Bti spray characteristics from a backpack sprayer.

In the efforts of improving the dengue vector control strategies in Malaysia and in other countries with similar *Aedes* spp. larval habitats, this paper serves as a fine-tuned guideline for the application of Bti via backpack sprayers.

MATERIALS AND METHODS

Overall study design

A total of 11 studies were conducted between October 2020 and May 2022. This set of studies was denoted by alphabet K, followed with the study number, K1-K11. The studies covered the following 8 test arms as described in Table 1. The 2 different Bti spray mixes sprayed through orifice No. 2 with a lower flow rate or through orifice No. 4 with a higher flow rate by an operator who either walks in a slow walking speed of 1m in 6 secs or in a faster walking speed of 1m in 3 secs. All test arms were evaluated by measuring the larval mortality in cups exposed to the spray on Day-1, Day-21 and Day-42 post spray, except for the final 2 spray studies where the larval mortality was measured in cups exposed to the spray on Day-1, Day-7 and Day-14 post spray. The final 2 studies were conducted to determine the larval mortality in Days-7 and 14 post spray after confirming the optimum spray characteristics for the 125g in 10 liters and 250g in 10 liters from the previous studies. The Days-7 and 14 post spray evaluation were conducted because of the ongoing operational programs in Malaysia and Singapore with routine Bti spray intervals once in 7 days or in 14 days (Lee *et al*., 2010; Bohari *et al*., 2020).

The Day-1 treated cups were kept indoors, while Day-7, Day-14, Day-21 and Day-42 treated cups were left outdoors for the stipulated time for the Bti toxins to be exposed to the outdoor environmental conditions.

Study site

This study was conducted in an open field at IMR, Jalan Pahang, Kuala Lumpur (3°10’6.12”N; 101°41’57.12”E). The open field measured 42m x 20m (840m^2) with no vegetation taller than short grass. The test area was within the field site, measuring 41m x 16m (656m^2).

Test product

The Bti product tested was VectoBac WG, Lot 310-971-PG30, with the manufacturing date as February 2020.

Table 1. Summary of 8 test arms for VectoBac WG (Bti) spray dispersed from a motorized backpack sprayer, Stihl SR420. Eleven K studies covered the 8 test arms

Test Arm	Bti Mix Rate	Flow Rate	Walking Speed	Post spray larval mortality evaluation in treated cups	Study Label
1	125g in 10 Liters	Orifice No. 2 with lower flowrate	Slower walking speed (1m per 6 secs)	Day-1, Day-21 and Day-42	K1
2	125g in 10 Liters	Orifice No. 2 with lower flowrate	Slower walking speed (1m per 6 secs)	Day-1, Day-7 and Day-14	K10
3	125g in 10 Liters	Orifice No. 2 with lower flowrate	Faster walking speed (1m per 3 secs)	Day-1, Day-21 and Day-42	K8
4	125g in 10 Liters	Orifice No. 4 with higher flowrate	Faster walking speed (1m per 3 secs)	Day-1, Day-21 and Day-42	K3 & K6
5	250g in 10 Liters	Orifice No. 2 with lower flowrate	Slower walking speed (1m per 6 secs)	Day-1, Day-21 and Day-42	K2
6	250g in 10 Liters	Orifice No. 2 with lower flowrate	Faster walking speed (1m per 3 secs)	Day-1, Day-21 and Day-42	K9
7	250g in 10 Liters	Orifice No. 2 with lower flowrate	Faster walking speed (1m per 3 secs)	Day-1, Day-7 and Day-14	K11
8	250g in 10 Liters	Orifice No. 4 with higher flowrate	Faster walking speed (1m per 3 secs)	Day-1, Day-21 and Day-42	K4, K5 & K7

Test equipment

A motorized backpack sprayer, Stihl SR420, was used in this study. This model is manufactured in Brazil by STIHL Ferramentas Motorizadas Ltda, located in Industria Brasileira, Brazil. Stihl SR420 is a commonly used model by the MOH Malaysia vector control program. The unit was sent for routine maintenance before the studies were conducted. It was warmed for 15 minutes before each operation. The sprayer nozzle has 6 orifices from No. 1 to 6, with increasing flowrate. The studies were conducted either through orifice No. 2 or orifice No. 4, under maximum throttle. The flowrates were determined just before each study. After each spray, the sprayer was well cleaned with detergent and water to prevent accumulation of product residue at the nozzle and prevent microbial growth in the spray tubes.

Test mosquito

The test *Ae. aegypti* larvae were colonized in the insectarium of the Medical Entomology Unit, IMR. The temephos-resistant *Ae. aegypti* larvae were collected via ovitrap surveillance in Melaka State and was colonized to F7-F9 generation for the bio-efficacy testing. Freshly hatched larvae were transferred to rearing trays (30cm x 23cm x 7.5cm) containing 2 L of dechlorinated water, with an estimated population of 500 - 700 larvae per tray. The average room temperature was 26 ± 3°C, with an average relative humidity of 72 ± 3%. The larvae were fed with fish base protein flakes at regular intervals.

The temephos resistance status was determined using the WHO guidelines (WHO, 2016). Four replicates of the third instar larvae were exposed to discriminating concentration (DC) of 0.012 mg/L. Mortality was recorded at 24 hours post exposure. The larvae were categorized as temephos insecticide resistant as the mortality was ≤ 96% (Nazni & Lee, 2000).

Bti spray study protocol

For each study, there were 3 transects of test containers and magnesium oxide (MgO) coated slides. The 3 transects were 6m apart. The test containers and MgO slides were placed at 2m, 4m, 8m, 12m, and 16m from the spray path. At each test distance per transect 9 dry test containers of size 8.5cm diameter by 7.5cm height (per container) and one MgO slide were placed. A total of 45 containers were placed in each transect. The test containers and the MgO slides were removed 30 mins post spray.

The spray studies were conducted in the early morning hours, 0600H–0700H or in the evening hours after the sundown, 1900H–2100H. The spray operator walked along the spray path, pointing the spray pipe towards the transects. The wind speed was measured before and during the spray, using the handheld NK Kestrel® 1000 Pocket Wind Meter.

The two tested Bti spray mix rates were either 125g or 250g per 10 liters water and was prepared fresh just before the study. The two walking speeds of the operator were either 1m in 6 secs (slow speed) or 1m in 3 secs (fast speed).

Two spray applicators operated the sprayer. The first operator, JF, was only able to do the first two K studies. The remaining studies were conducted by the second operator, AM. We had the same operator to standardize the height of the operator, which directly affects the height of the spray pipe from the ground, which in turn affects the deposition of the droplets into the test containers and MgO slides.

The same Stihl SR420 sprayer was used for all the 11 studies, to ensure that the operational efficiency of the backpack sprayer was standardized for all the studies. The flowrate check was made before each study. At orifice No. 2 the flowrate was between 567 mL–650 mL per minute, while at orifice No. 4 the flowrate was at 2000 mL per minute. The amount of Bti dispersed in the study site, grams per ha, was determined from the time taken by the operator to cover the spray path of 41m at 1m in 6 secs or 1m in 3 secs for the specific flowrate.

Larval bio-efficacy

For K1-K9 studies, the 9 treated containers from each test distance were divided into 3 groups as Day-1, Day-21, and Day-42. As for K10 and K11 studies, the containers were grouped as Day-1, Day-7, and Day-14. The larval bio-efficacy was evaluated in the lab which has an average room temperature of 26 ± 2°C, with an average relative humidity of 80 ± 10%.

100 mL seasoned water was introduced into Day-1 test containers with 10 *Ae. aegypti* larvae, of third to early fourth instar, on the spray day itself. The Day-1 containers were kept indoors throughout the 4 weeks of testing.

The Day-7, Day-14, Day-21, and Day-42 containers were kept outdoors in the verandah, not under direct exposure to the sunlight and the rain. The verandah was in the first floor of a double -storey house, with a roof but did not have exterior walls. Each container had a lid, but the lid was placed loosely on the container. On respective days, the containers were brought to the lab and were processed in the same manner as the Day-1 containers.

For studies K1, K2, K6, K7, K8 and K9, the efficacy of the Bti sprayed toxins was measured in Day-21 and Day-42 containers which were left outdoors in the verandah for 21 days and 42 days post spray, respectively. As for K10 and K11 studies, which were sprayed with the same spray parameters as K1 and K9 studies, respectively, the efficacy of the Bti sprayed toxins was measured in Day-7 and Day-14 containers, which were left outdoors for 7 days and 14 days after treatment, respectively.

Larval mortality (LM) was recorded 3-hr and 24-hr post-introduction. In actual field operations, the objective is always to have the larvae killed as rapidly as possible. Thus, for this reason, we measured the LM at 3-hr post exposure of the larvae to the deposited Bti toxins in the containers.

Any surviving larvae on 24-hr post introduction was allowed to remain in the test container until the next introduction of larvae. If any one of the containers had one surviving larvae or pupae within the 7 days of exposure, the container was not subjected to further evaluations and was considered as zero mortality for the remaining evaluations.

After the initial evaluation, all the test containers were evaluated for residual efficacy (RE) of the sprayed Bti toxins on weekly intervals until week 4. In each week, 10 fresh *Ae. aegypti* larvae were introduced, and mortality was recorded as previous evaluations. The dead larvae from previous evaluations were not removed before introducing the 10 fresh larvae, to avoid removing any sprayed Bti toxins that could still be present in the container. Control cups were also set up accordingly.

Droplet analysis

Magnesium oxide (MgO) slides were used to collect the Bti spray droplets during a spray. Clear glass slides (25.4mm x 76.2mm) were coated with a uniform layer of magnesium ash covering ¾ of the slide length. MgO slides were placed at each test distance and were collected 30 minutes post spray and were stored in a closed slide box until droplet measurement.

Droplet analysis included both droplet numbers and droplet size. The numbers were determined by counting the droplet numbers under 40x magnification using a Rax Vision Y103 compound microscope in 9 fields per slide. The mean droplet numbers ± standard error (SE) was then determined for each test distance. The overall mean droplet numbers were also determined for each study.

The size of the droplets was determined using 100x magnification under the same microscope with the aid of JVC color video camera to visualize the droplets. A total of 60 droplets per

slide was measured using Dimas Ver 5.0 Professional Edition. The volume median diameter (Vmd) was determined using ULV Droplet Analysis Program (Sofield & Kent, 1984).

Data analysis

Larval mortality (LM) was scored for each test container. The mean mortality ± standard error (SE) was determined for each test distance by combining the mortality from all the 9 test containers at that specific distance from the 3 transects.

The statistical analysis for the LM was conducted using IBM Statistical Package for Social Science Software (SPSS) ver. 25. The Kolmogorov-Smirnov test was selected to assess normality due to the large sample size ($p<0.05$). Since the dataset was not normally distributed, the non-parametric Kruskal-Wallis H test at a 95% confidence level was applied to compare parameters between the spray studies of the 2 different Bti spray mix rates and 2 different walking speeds in relation to the distances from the spray path for the LM of the first evaluations in Day-1, Day-7, Day-14, Day-21 and Day-42 test containers ($p<0.05$). The Friedman non-parametric test was used to compare larval mortalities between 3 hours and 24 hours in containers placed outdoors for 7-, 14-, 21- and 42-days post spray ($p<0.05$).

The non-parametric Kruskal-Wallis H test at a 95% confidence level was used to compare the droplet numbers of K1 and K10 studies with 125g in 10 liters water with a walking speed of 1m in 6 secs versus K9 and K11 studies with 250g in 10 liters water with a walking speed of 1m in 3 secs ($p<0.05$).

The WHO guidelines for the evaluation of common larvicides and bacterial larvicide products, consider at least 90% LM at practical dosages as a good potential for mosquito control (WHO, 1996). This is reiterated under the guidelines for the testing of mosquito larvicides where 80% or 90% reduction can be used to determine the residual effect and optimum application dosage (WHO, 2005). Based on these WHO guidelines, we determined the effective Bti treatment for the tested parameters will be 90%–100% LM for the varied distances (2m–16m) from the spray path for Day-1 post spray containers and also for the treated containers which were exposed to outdoors for 7 days, 14 days, 21 days, and 42 days post spray. Thus, test distances which gave 90%-100% LM was denoted in this study as effective larvicidal treatment (ELT).

Graphs were also plotted using the Microsoft Excel to visualize the LM data at varied test distances.

RESULTS

General observation

We conducted 11 studies, but in this report, we present 8 studies which were not affected by wind during the time of spray. Among the 8 studies, K1 study is the only study that experienced a very momentary gust at transect No. 2 when the operator was just passing by the transect with the sprayer.

Table 2 describes the 8 studies that were conducted from a backpack sprayer, Stihl SR420, with the spray mix of 125g or 250g VectoBac WG in 10 liters water, dispersed from orifice No. 2 or orifice No. 4 of the sprayer nozzle, with the operator walking at a speed of 1m per 3 secs or 1m per 6 secs.

The target Bti dose for dengue control, following the manufacturer's recommended standard operating procedure (SOP) is between 400g/ha-500g/ha. The target dose was achieved when the spray mix was sprayed through orifice No. 2 with 125g per 10 liters water at a walking speed of 1m in 6 secs (K1 and K10 studies), or when the spray mix was 250g per 10 liters water with a faster walking speed of 1m in 3 secs (K9 and K11 studies). When the spray mix was sprayed at a higher flow rate, through orifice No. 4, higher dose rates were achieved as in K6 and K7 studies.

Table 2. Summary of eight studies with VectoBac WG (Bti) spray dispersed from a motorized backpack sprayer, Stihl SR420. The table details the flow rate and the final Bti dose achieved for each study, together with the wind speed at each transect of the study

Date and study time	Study	Orifice number on spray nozzle	Flow rate (ml/min)	Bti in 10 liters water	Time taken to walk 1 meter	Bti dose (g/ha)	Wind speed at each transect (T)(m/sec)
31 October 2020 0500H – 0600H	K1	2	633.33	125 g	6 seconds	422.4	T1 = 0 T2 = 0.28 T3 = 0
31 October 2020 0600H – 0700H	K2	2	633.33	250 g	6 seconds	965.4	T1 = 0 T2 = 0 T3 = 0
5 December 2020 0500H – 0600H	K6	4	2000.00	125 g	3 seconds	844.8	T1 = 0 T2 = 0 T3 = 0
5 December 2020 0600H – 0700H	K7	4	2000.00	250 g	3 seconds	1676.8	T1 = 0 T2 = 0 T3 = 0
13 March 2021 1900H – 2000H	K8	2	566.67	125 g	3 seconds	232.4	T1 = 0 T2 = 0 T3 = 0
13 March 2021 2000H – 2100H	K9	2	566.67	250 g	3 seconds	472.2	T1 = 0 T2 = 0 T3 = 0
13 May 2022 1900H – 2000H	K10	2	650.00	125 g	6 seconds	468.7	T1 = 0 T2 = 0 T3 = 0
13 May 2022 2000H – 2100H	K11	2	650.00	250 g	3 seconds	476.4	T1 = 0 T2 = 0 T3 = 0

g: grams; m/sec: meter per second; ml/min: milliliters per minute; g/ha: grams per hectare; WG: water-dispersible granules.

The containers that were left outdoors were not exposed to direct sunlight but were exposed to the sunrays and sprinkles from the rain. All containers were covered with a lid that was placed loosely on the container. The containers did not get flooded from the heavy rains. The containers were placed randomly under the verandah, not in a sequence to the spray distance, so each container had different exposures to the intensity of the sunrays.

Larval mortality (LM)

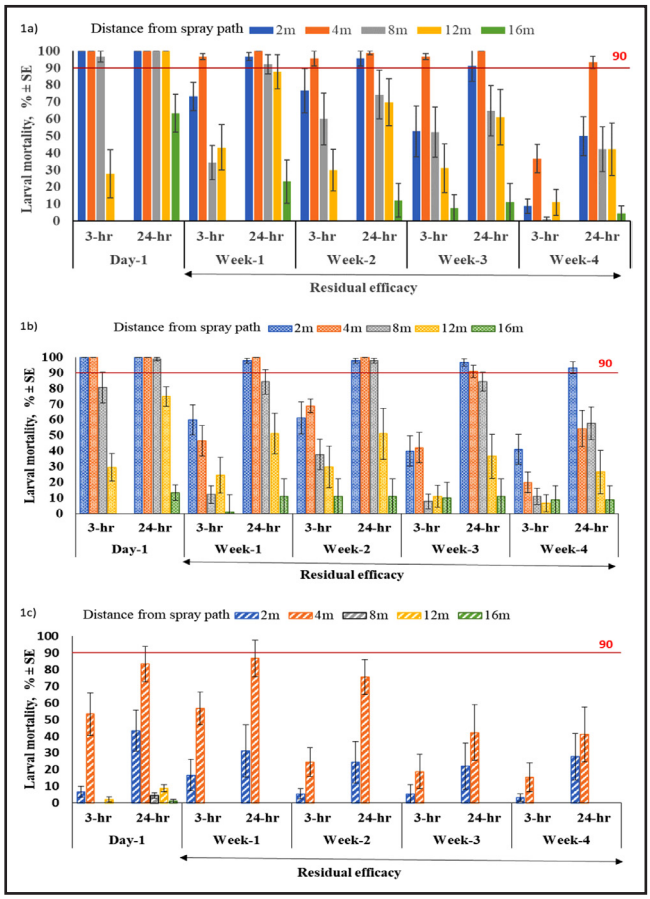
The LM for the controls and treated containers was measured at 3-hr and 24-hr post larval exposure. The LM in the control containers ranged between 0%–2%. As the control LM was less than 5%, it did not require any correction in the LM of the treated containers.

Figure 1–8 show the percentage larval mortality (%LM) ± standard error (SE) for each study.

The Day-1 containers for all studies, generally as per observation from the Figure 1a-8a, the LM decreases with distance from the spray path, with the containers at 16m having a lower mortality than containers placed at 2m–12m (Figure 1a-8a).

The LM was usually higher in 24-hr than in 3-hr post larval exposure for all distances (Figure 1a-8a).

Overall, for the 24-hr post larval exposure, in the Day-1 containers placed at 2m–12m from the spray path, there was no significant difference in the LM between all studies, from the lowest dose at 232g/ha (K8) to the highest dose at 1677g/ha (K7) (Kruskal-Wallis H test, $\chi^2 = 6.811$, d.f. = 7, p = 0.450). In the Day-1 containers

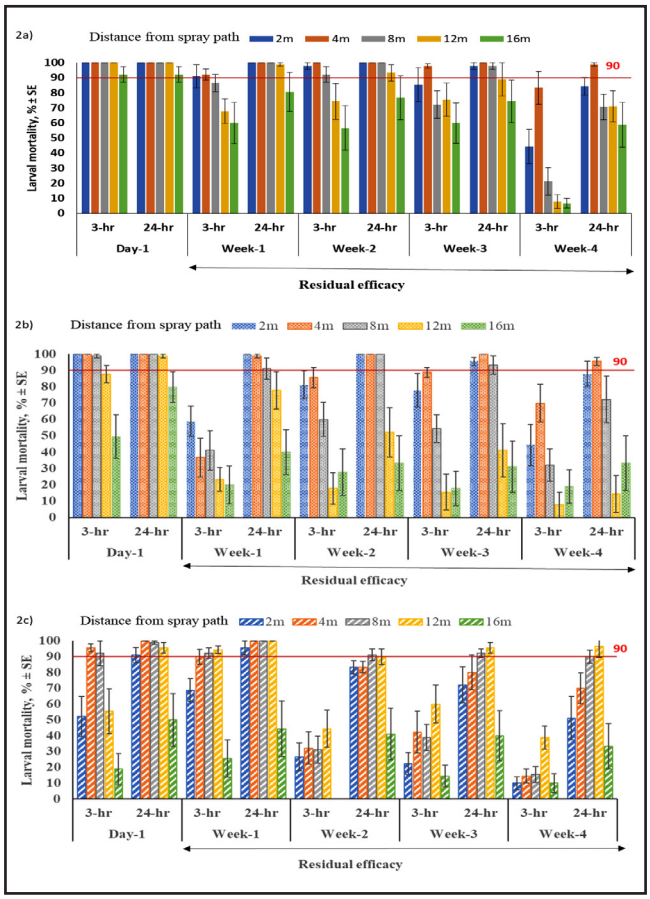


g/ha: grams per hectare; hr: hour; m: meters; SE: standard error.

Figure 1a. Larval mortality (LM) for Day-1 containers that were left indoors from K1 study (422.4 g/ha), that were placed at 2m – 16m from the spray path. The LM was measured on Day-1 post spray, and for the next 4 weeks residual efficacy evaluation in the same containers. The % LM±SE was measured for 3-hr and 24-hr post larval exposure.

Figure 1b. Larval mortality (LM) for Day-21 containers from K1 study (422.4 g/ha), which were exposed outdoors for 21 days, that were placed at 2m – 16m from the spray path. The LM was measured on Day-21 post spray and for the next 4 weeks residual efficacy evaluation in the same containers. The % LM±SE was measured for 3-hr and 24-hr post larval exposure.

Figure 1c. Larval mortality (LM) for Day-42 containers from K1 study (422.4 g/ha), which were exposed outdoors for 42 days, that were placed at 2m – 16m from the spray path. The LM was measured on Day-42 post spray and for the next 4 weeks residual efficacy evaluation in the same containers. The % LM±SE was measured for 3-hr and 24-hr post larval exposure.

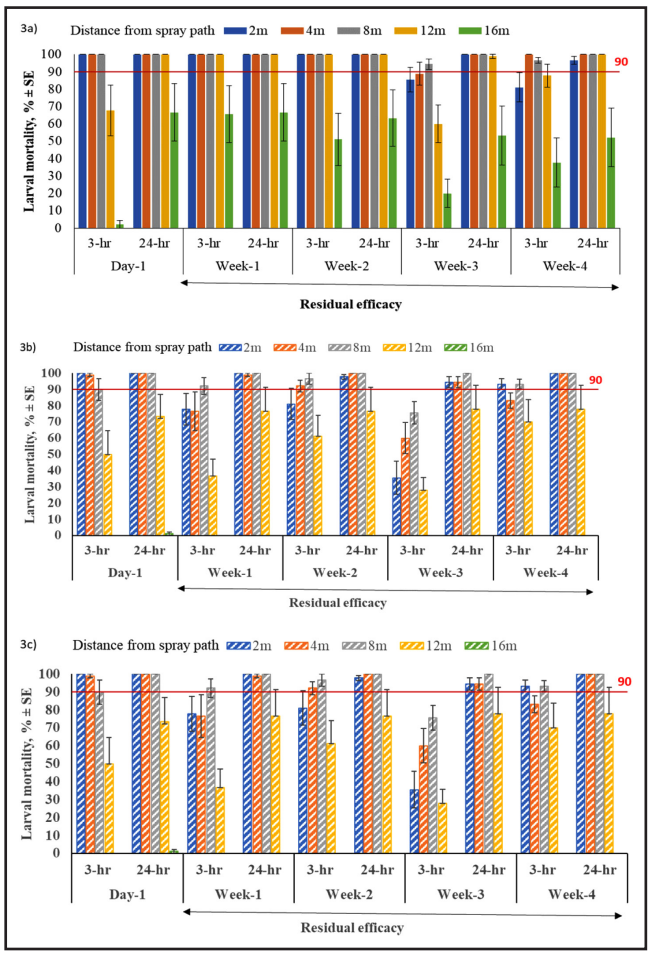


g/ha: grams per hectare; hr: hour; m: meters; SE: standard error.

Figure 2a. Larval mortality (LM) for Day-1 containers that were left indoors from K2 study (965.4 g/ha), that were placed at 2m – 16m from the spray path. The LM was measured on Day-1 post spray and for the next 4 weeks residual efficacy evaluation in the same containers. The % LM±SE was measured for 3-hr and 24-hr post larval exposure.

Figure 2b. Larval mortality (LM) for Day-21 containers from K2 study (965.4 g/ha), which were exposed outdoors for 21 days, that were placed at 2m – 16m from the spray path. The LM was measured on Day-21 post spray and for the next 4 weeks residual efficacy evaluation in the same containers. The % LM±SE was measured for 3-hr and 24-hr post larval exposure.

Figure 2c. Larval mortality (LM) for Day-42 containers from K2 study (965.4 g/ha), which were exposed outdoors for 42 days, that were placed at 2m – 16m from the spray path. The LM was measured on Day-42 post spray and for the next 4 weeks residual efficacy evaluation in the same containers. The % LM±SE was measured for 3-hr and 24-hr post larval exposure.

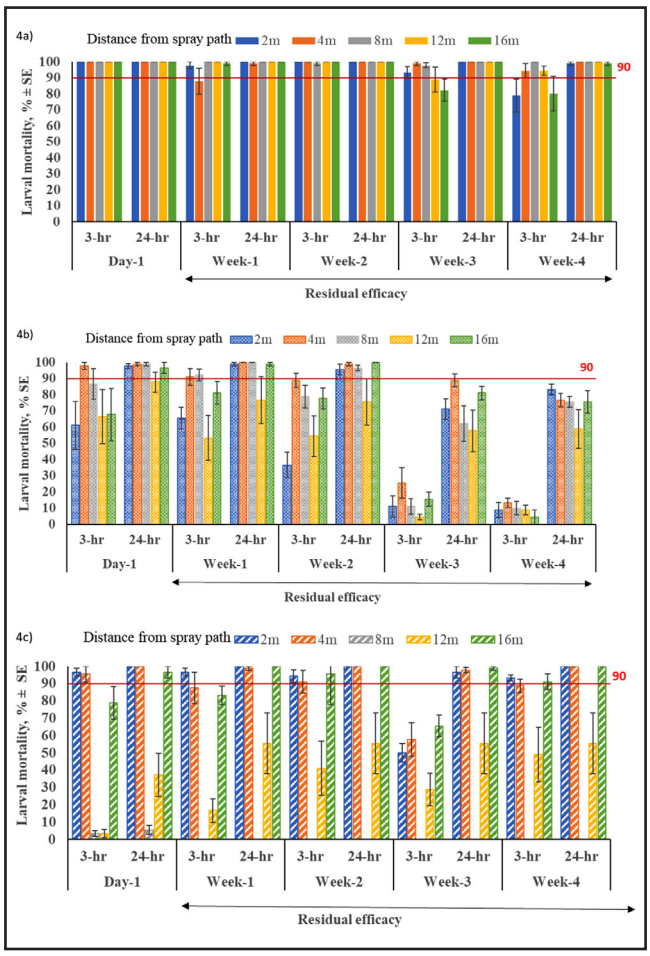


g/ha: grams per hectare; hr: hour; m: meters; SE: standard error.

Figure 3a. Larval mortality (LM) for Day-1 containers that were left indoors from K6 study (844.8 g/ha), that were placed at 2m – 16m from the spray path. The LM was measured on Day-1 post spray and for the next 4 weeks residual efficacy evaluation in the same containers. The % LM±SE was measured for 3-hr and 24-hr post larval exposure.

Figure 3b. Larval mortality (LM) for Day-21 containers from K6 study (844.8 g/ha), which were exposed outdoors for 21 days, that were placed at 2m – 16m from the spray path. The LM was measured on Day-21 post spray and for the next 4 weeks residual efficacy evaluation in the same containers. The % LM±SE was measured for 3-hr and 24-hr post larval exposure.

Figure 3c. Larval mortality (LM) for Day-42 containers from K6 study (844.8 g/ha), which were exposed outdoors for 42 days, that were placed at 2m – 16m from the spray path. The LM was measured on Day-42 post spray and for the next 4 weeks residual efficacy evaluation in the same containers. The % LM±SE was measured for 3-hr and 24-hr post larval exposure.



g/ha: grams per hectare; hr: hour; m: meters; SE: standard error.

Figure 4a. Larval mortality (LM) for Day-1 containers that were left indoors from K7 study (1676.8 g/ha), that were placed at 2m – 16m from the spray path. The LM was measured on Day-1 post spray and for the next 4 weeks residual efficacy evaluation in the same containers. The % LM±SE was measured for 3-hr and 24-hr post larval exposure.

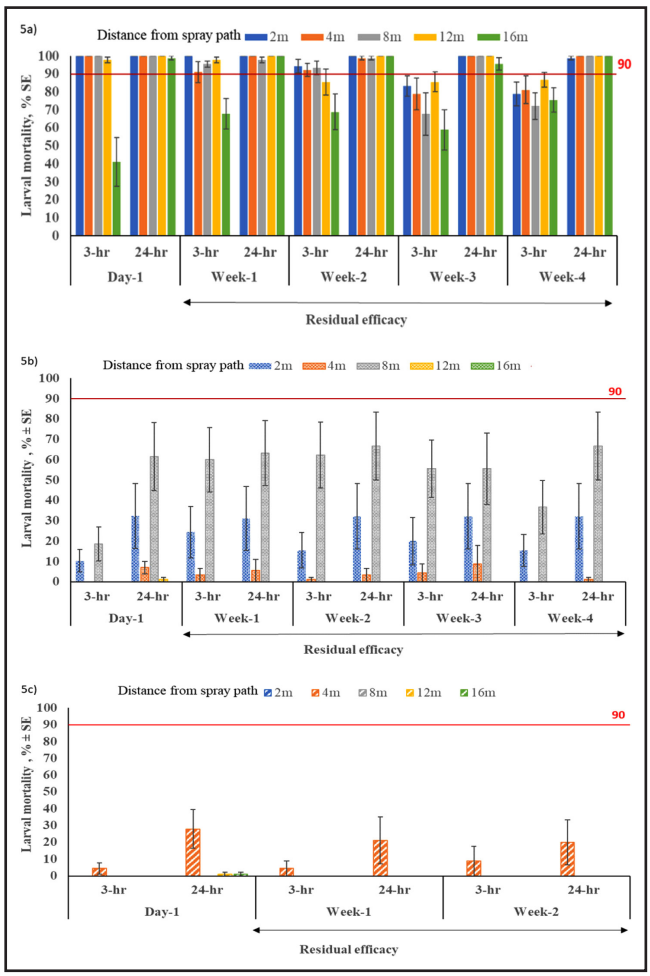
Figure 4b. Larval mortality (LM) for Day-21 containers from K7 study (1676.8 g/ha), which were exposed outdoors for 21 days, that were placed at 2m – 16m from the spray path. The LM was measured on Day-21 post spray and for the next 4 weeks residual efficacy evaluation in the same containers. The % LM±SE was measured for 3-hr and 24-hr post larval exposure.

Figure 4c. Larval mortality (LM) for Day-42 containers from K7 study (1676.8 g/ha), which were exposed outdoors for 42 days, that were placed at 2m – 16m from the spray path. The LM was measured on Day-42 post spray and for the next 4 weeks residual efficacy evaluation in the same containers. The % LM±SE was measured for 3-hr and 24-hr post larval exposure.

at 16m, there was also no significant difference between all studies, except for the K1 study (422g/ha) with a significant difference to the LM of K7, K8, K9, and K11 (Kruskal-Wallis H test, χ^2 = 25.472, d.f. = 7, p = 0.001). There was also no significant difference in the mortality achieved in the same Day-1 containers for all 8 studies at 2m and 4m distances for the following 4 weeks of RE evaluation (Kruskal-Wallis H test, χ^2 = 0.000, d.f. = 7, p = 1.000). However, a significantly lower mortality in the Day-1 containers of the K1 study at 8m and 12m from week 2 (Kruskal-Wallis H test, χ^2 = 49.173, d.f.=7, p=0.000) and significant lower mortality in the Day-1 containers of K1 at 16m from week 1 (Kruskal-Wallis H test, χ^2 = 40.793, d.f.=7, p=0.000). The lower mortality could be due to the sudden wind gust which happened during the K1 spray, which might have drifted the sprayed Bti toxins from the target containers (Table 2).

All containers exposed to outdoors 7-, 14-, 21- and 42-days post spray had a lower mortality than Day-1 containers (Figure 1b–8b and Figure 1c–8c). The containers that were exposed outdoors for a longer period of time had a lower LM compared to containers that were kept outdoors for a shorter period of time.

In the 3-hr larval exposure, the LM in containers placed outdoors for the different time periods had lower mortality (Figure 1b–8b and Figure 1c–8c), than the 3-hr larval exposure in the Day-1

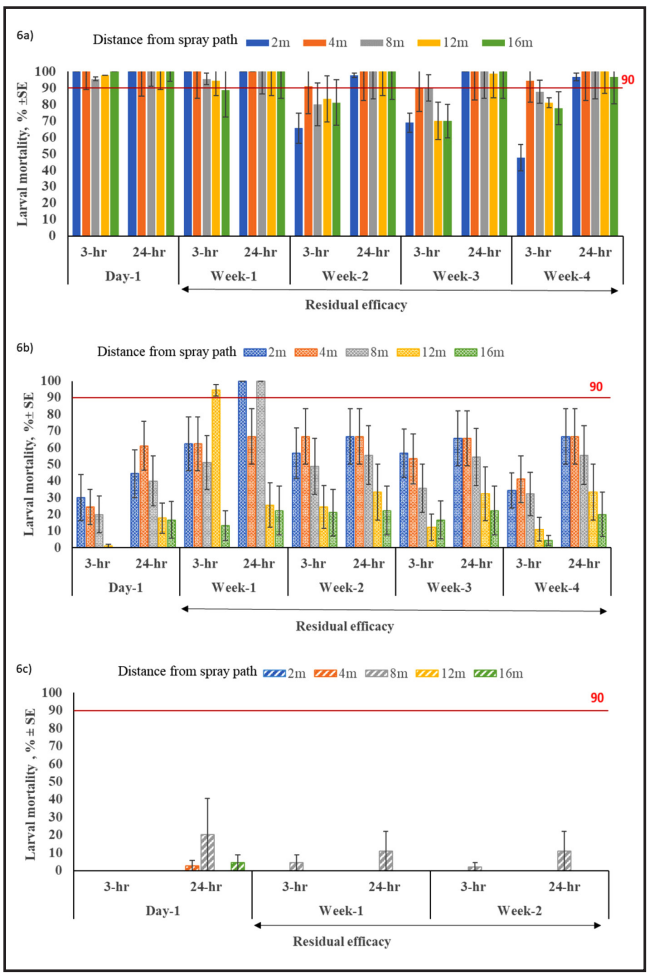


g/ha: grams per hectare; hr: hour; m: meters; SE: standard error.

Figure 5a. Larval mortality (LM) for Day-1 containers that were left indoors from K8 study (232.4 g/ha), that were placed at 2m – 16m from the spray path. The LM was measured on Day-1 post spray and for the next 4 weeks residual efficacy evaluation in the same containers. The % LM±SE was measured for 3-hr and 24-hr post larval exposure.

Figure 5b. Larval mortality (LM) for Day-21 containers from K8 study (232.4 g/ha), which were exposed outdoors for 21 days, that were placed at 2m – 16m from the spray path. The LM was measured on Day-21 post spray and for the next 4 weeks residual efficacy evaluation in the same containers. The % LM±SE was measured for 3-hr and 24-hr post larval exposure.

Figure 5c. Larval mortality (LM) for Day-42 containers from K8 study (232.4 g/ha), which were exposed outdoors for 42 days, that were placed at 2m – 16m from the spray path. The LM was measured on Day-42 post spray and for the next 4 weeks residual efficacy evaluation in the same containers. The % LM±SE was measured for 3-hr and 24-hr post larval exposure.



g/ha: grams per hectare; hr: hour; m: meters; SE: standard error.

Figure 6a. Larval mortality (LM) for Day-1 containers that were left indoors from K9 study (472.2 g/ha), that were placed at 2m – 16m from the spray path. The LM was measured on Day-1 post spray and for the next 4 weeks residual efficacy evaluation in the same containers. The % LM±SE was measured for 3-hr and 24-hr post larval exposure.

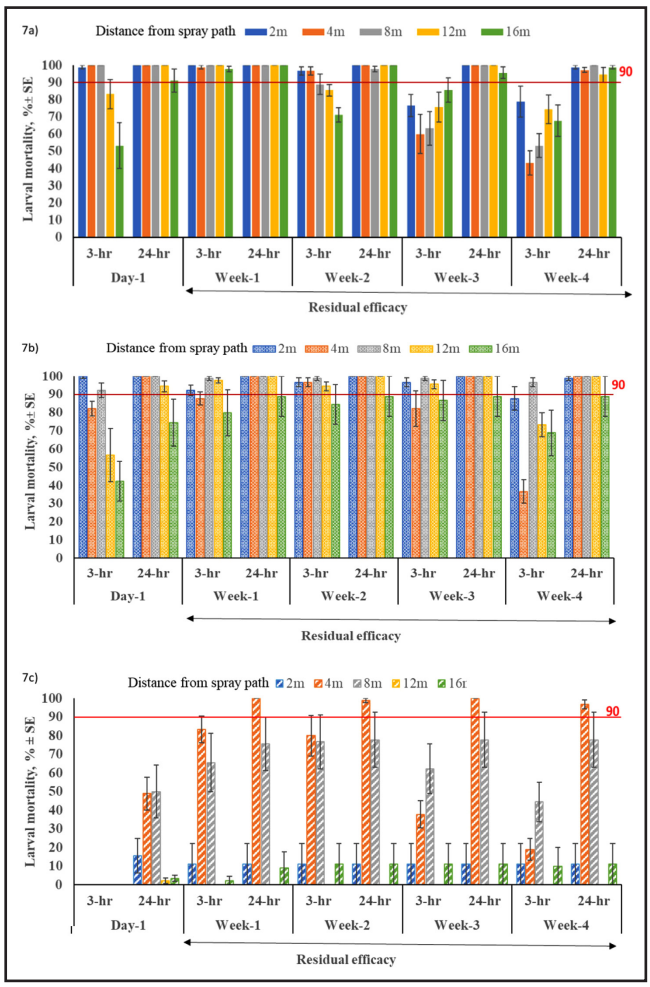
Figure 6b. Larval mortality (LM) for Day-21 containers from K9 study (472.2 g/ha), which were exposed outdoors for 21 days, that were placed at 2m – 16m from the spray path. The LM was measured on Day-21 post spray and for the next 4 weeks residual efficacy evaluation in the same containers. The % LM±SE was measured for 3-hr and 24-hr post larval exposure.

Figure 6c. Larval mortality (LM) for Day-42 containers from K9 study (472.2 g/ha), which were exposed outdoors for 42 days, that were placed at 2m – 16m from the spray path. The LM was measured on Day-42 post spray and for the next 4 weeks residual efficacy evaluation in the same containers. The % LM±SE was measured for 3-hr and 24-hr post larval exposure.

containers (Figure 1a–8a), and this is most probably due to lesser viable Bti toxins in the treated containers that were placed outdoors. Nevertheless, the amount of Bti toxins was sufficient to produce a significantly higher larval mortality (LM) at 24-hour larval exposure compared to 3-hour exposure on Day-7 (Friedman test, $\chi^2 = 66.000$, $p = 0.000$), Day-14 ($\chi^2 = 70.000$, $p = 0.000$), Day-21 ($\chi^2 = 56.333$, $p = 0.000$), and Day-42 ($\chi^2 = 102.038$, $p = 0.000$).

Containers exposed to a higher dose of Bti in the K2, K6 and K7 studies had significantly higher mortality when left outdoors for 21 days (Kruskal-Wallis H test, $\chi^2 = 91.595$, d.f.=1, p=0.000) (Figure 2b, 3b, 4b) and 42 days (Kruskal-Wallis H test, $\chi^2 = 120.540$, d.f.=1, p=0.000) (Figure 2c, 3c, 4c) in comparison to containers exposed to lower dose in K1, K8 and K9. Containers exposed to the lowest dose of Bti at 232g/ha in the K8 study had very low mortality when placed outdoors for 21 days (Figure 5b) and near- zero mortality when placed outdoors for 42 days (Figure 5c).

Residual efficacy (RE) of the Bti toxins was measured in all containers after the first LM evaluation for all 8 studies. The containers were left indoors in the laboratory and 10 fresh larvae were introduced weekly for the next 4 weeks. The LM decreased as the week progressed (Figure 1-8). Thus, indicating that the Bti toxins which were present in the water progressively decreased with weeks

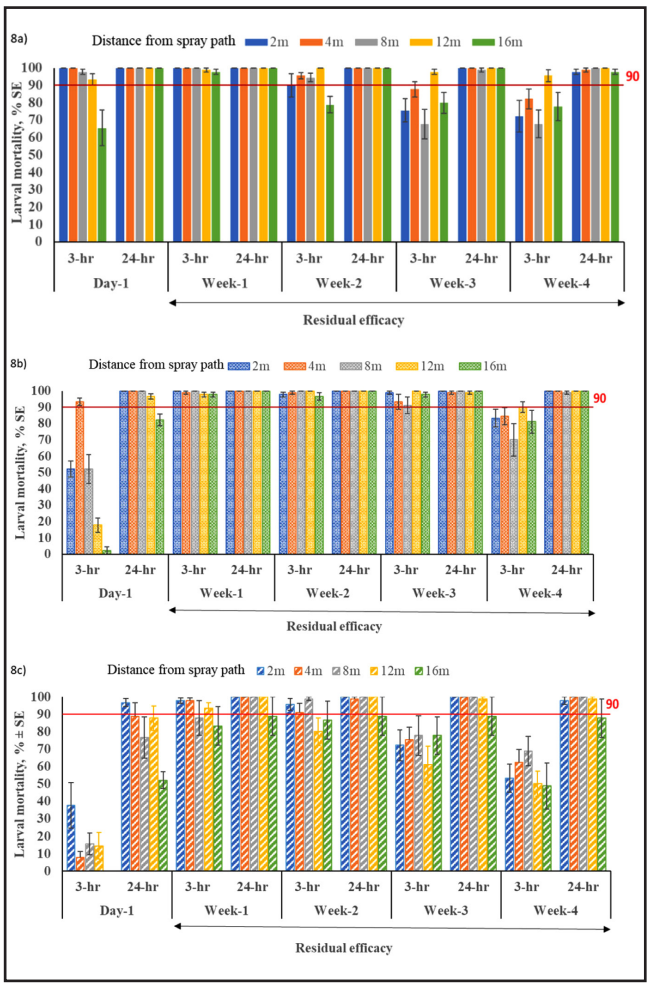


g/ha: grams per hectare; hr: hour; m: meters; SE: standard error.

Figure 7a. Larval mortality (LM) for Day-1 containers that were left indoors from K10 study (468.7 g/ha), that were placed at 2m – 16m from the spray path. The LM was measured on Day-1 post spray and for the next 4 weeks residual efficacy evaluation in the same containers. The % LM±SE was measured for 3-hr and 24-hr post larval exposure.

Figure 7b. Larval mortality (LM) for Day-7 containers from K10 study (468.7 g/ha), which were exposed outdoors for 7 days, that were placed at 2m – 16m from the spray path. The LM was measured on Day-7 post spray and for the next 4 weeks residual efficacy evaluation in the same containers. The % LM±SE was measured for 3-hr and 24-hr post larval exposure.

Figure 7c. Larval mortality (LM) for Day-14 containers from K10 study (468.7 g/ha), which were exposed outdoors for 14 days, that were placed at 2m – 16m from the spray path. The LM was measured on Day-14 post spray and for the next 4 weeks residual efficacy evaluation in the same containers. The % LM±SE was measured for 3-hr and 24-hr post larval exposure.



g/ha: grams per hectare; hr: hour; m: meters; SE: standard error.

Figure 8a. Larval mortality (LM) for Day-1 containers that were left indoors from K11 study (476.4 g/ha), that were placed at 2m – 16m from the spray path. The LM was measured on Day-1 post spray and for the next 4 weeks residual efficacy evaluation in the same containers. The % LM±SE was measured for 3-hr and 24-hr post larval exposure.

Figure 8b. Larval mortality (LM) for Day-7 containers from K11 study (476.4 g/ha), which were exposed outdoors for 7 days, that were placed at 2m – 16m from the spray path. The LM was measured on Day-7 post spray and for the next 4 weeks residual efficacy evaluation in the same containers. The % LM±SE was measured for 3-hr and 24-hr post larval exposure.

Figure 8c. Larval mortality (LM) for Day-14 containers from K11 study (476.4 g/ha), which were exposed outdoors for 14 days, that were placed at 2m – 16m from the spray path. The LM was measured on Day-14 post spray and for the next 4 weeks residual efficacy evaluation in the same containers. The % LM±SE was measured for 3-hr and 24-hr post larval exposure.

as the freshly introduced larvae actively ingested the Bti toxins and also due to natural degradation of the toxins in the water.

Effective larvicidal treatment (ELT)

For this entire study, we determined the distance from the spray path with effective larvicidal treatment (ELT), that is distance with 90%–100% LM, for Day-1 post spray containers and for the treated containers which were exposed to outdoors for 7 days, 14 days, 21 days, and 42 days post spray (Tables 3-6).

Table 3 describes the ELT for Day-1 post spray containers, and for the 4 weeks evaluation of RE in the same containers that were exposed from the lowest dose to highest tested dose at 1677g/ha. The ELT was until the maximum distance of 16m from spray path for 6 of the 8 studies, while the K1 and K6 studies the ELT was until 12m from the spray path. The impact of the momentary gust in the K1 study at 0.28m/sec did affect the spray, whereby Bti droplets were only sufficient to give 90%–100% LM for one week post spray until 12m from the spray path. Beyond the first week, the ELT for the K1

Table 3. Effective larvicidal treatment (ELT) of VectoBac WG (Bti) in Day-1 containers that were kept indoors, and for the next 4 weeks residual efficacy evaluation in the same containers which were kept indoors

Study	Bti dose (g/ha)	Distance from the spray path with 90-100% larval mortality in Day-1 containers					
		Day-1 containers		Residual efficacy			
		Larval exp 3-hr	Larval exp 24-hr	Week 1 larval exp 24-hr	Week 2 larval exp 24-hr	Week 3 larval exp 24-hr	Week 4 larval exp 24-hr
K8	232.4	2m – 12m	2m – 16m	2m – 16m	2m – 16m	2m – 16m	2m – 16m
K1	422.4	2m – 8m	2m – 12m	2m – 12m	2m – 4m	2m – 4m	4m
K10	468.7	2m – 8m	2m – 16m	2m – 16m	2m – 16m	2m – 16m	2m – 16m
K9	472.2	2m – 16m	2m - 16m	2m – 16m	2m – 16m	2m – 16m	2m – 16m
K11	476.4	2m – 12m	2m – 16m	2m – 16m	2m – 16m	2m – 16m	2m – 16m
K6	844.8	2m – 8m	2m – 12m	2m – 12m	2m – 12m	2m – 12m	2m – 12m
K2	965.4	2m – 16m	2m – 16m	2m – 12m	2m – 12m	2m – 12m	4m
K7	1676.8	2m – 16m	2m – 16 m	2m – 16m	2m – 16m	2m – 16m	2m – 16m

exp: exposure; g/ha: grams per hectare; hr: hour; m: meters; WG: water-dispersible granules.

Table 4. Effective larvicidal treatment (ELT) of VectoBac WG (Bti) in Day-21 post spray containers that were left outdoors for 21 days, and for the next 4 weeks residual efficacy evaluation in the same containers which were kept indoors

Study	Bti dose (g/ha)	Distance from the spray path with 90-100% larval mortality in Day-21 containers which were exposed outdoors for 21 days					
		Day-21 containers		Residual efficacy			
		Larval exp 3-hr	Larval exp 24-hr	Week 1 larval exp 24-hr	Week 2 larval exp 24-hr	Week 3 larval exp 24-hr	Week 4 larval exp 24-hr
K8	232.4	NIL	NIL	NIL	NIL	NIL	NIL
K1	422.4	2m – 4m	2m – 8m	2m – 4m	2m – 8m	2m – 4m	2m
K9	472.2	NIL	NIL	2m & 8m	NIL	NIL	NIL
K6	844.8	2m – 8m	2m – 8m	2m – 8m	2m – 8m	2m – 8m	4m
K2	965.4	2m – 12m	2m – 12m	2m – 8m	2m – 8m	2m – 8m	2m – 4m
K7	1676.8	4m – 8m	2m – 16m	2m – 8m & 16m	2m – 8m & 16m	4m	NIL

exp: exposure; g/ha: grams per hectare; hr: hour; m: meters; NIL: nothing; WG: water-dispersible granules.

Table 5. Effective larvicidal treatment (ELT) of VectoBac WG (Bti) in Day-42 post spray containers that were left outdoors for 42 days, and for the next 4 weeks residual efficacy evaluation in the same containers which were kept indoors

Study	Bti dose (g/ha)	Distance from the spray path with 90-100% larval mortality in Day-42 containers which were exposed outdoors for 42 days					
		Day-42 containers		Residual efficacy			
		Larval exp 3-hr	Larval exp 24-hr	Week 1 larval exp 24-hr	Week 2 larval exp 24-hr	Week 3 larval exp 24-hr	Week 4 larval exp 24-hr
K8	232.4	NIL	NIL	NIL	NIL	NIL	NIL
K1	422.4	NIL	NIL	4m	NIL	NIL	NIL
K9	472.2	NIL	NIL	NIL	NIL	NIL	NIL
K6	844.8	2m – 8m	2m – 8m	2m – 8m	2m – 8m	2m – 8m	2m – 8m
K2	965.4	4m – 8m	2m – 12m	2m – 12m	8m – 12m	8m – 12m	8m – 12m
K7	1676.8	2m – 4m	2m – 4m &16m	2m – 4m &16m	2m – 4m &16m	2m – 4m &16m	2m – 4m & 16m

exp: exposure; g/ha: grams per hectare; hr: hour; m: meters; NIL: nothing; WG: water-dispersible granules.

study was until 4m from the spray path (Table 3). As for the other 5 studies, even at the lowest dose of 232g/ha, the ELT was until 16m from the spray path for 4 weeks post spray.

Table 4 describes the ELT for Day-21 post spray containers, and for the next 4 weeks evaluation of RE in the same containers. The higher the Bti dose (K2, K6 and K7 studies) the ELT was at 8m from the spray path for 2 weeks RE evaluation. This ELT distance at 8m for the Day-21 post spray containers, was a 50% decrease in the ELT distance and a shorter RE duration of 2 weeks compared to Day-1 post spray containers at 16m for 4 weeks (Table 3). The lowest dose of 232g/ha (K8 study) had no ELT in the Day-21 post spray containers, indicating that there were not sufficient viable Bti toxins in the treated containers, after the indirect exposure to the sunlight, to affect a 90%–100% LM. The Day-21 containers from the K2 study with 422.4 g/ha had ELT within 2m-8m for 2 weeks RE evaluation, but from the K9 study with a slightly higher dose at 472g/ha had hardly any ELT, except at 2 test points (2m and 8 m) at 1 week RE evaluation.

Table 5 describes the ELT for Day-42 post spray containers, and for the next 4 weeks evaluation of RE in the same containers. The 232g/ha (K8) and the 400 plus g/ha (K1, K9) did not have sufficient viable Bti toxins to affect an ELT. The higher doses (K2, K6 and K7) had sufficient Bti toxins for an ELT until 4m or 8m or 12m or 16m for 4 weeks of RE evaluation.

Table 6. Effective larvicidal treatment (ELT) of VectoBac WG (Bti) in Day-7 and Day-14 post spray containers that were left outdoors for 7 and 14 days, respectively, and for the next 4 weeks residual efficacy evaluation in the same containers which were kept indoors

Study	Bti dose (g/ha)	Distance from the spray path with 90-100% larval mortality in Day-7 and Day-14 containers which were exposed outdoors 7 days and 14 days, respectively.					
		Day-7 containers		Residual efficacy			
		Larval exp 3-hr	Larval exp 24-hr	Week 1 larval exp 24-hr	Week 2 larval exp 24-hr	Week 3 larval exp 24-hr	Week 4 larval exp 24-hr
Day-7 containers							
K10	468.7	2m & 8m	2m – 12m	2m – 16m	2m – 16m	2m – 16m	2m – 16m
K11	476.4	4m	2m – 12m	2m – 16m	2m – 16m	2m – 16m	2m – 16m
Study	Bti dose (g/ha)	Day-14 containers		Residual efficacy			
		Larval exp 3-hr	Larval exp 24-hr	Week 1 larval exp 24-hr	Week 2 larval exp 24-hr	Week 3 larval exp 24-hr	Week 4 larval exp 24-hr
Day-14 containers							
K10	468.7	NIL	NIL	4m	4m	4m	4m
K11	476.4	NIL	2m – 4m	2m – 16m	2m – 16m	2m – 16m	2m – 16m

exp: exposure; g/ha: grams per hectare; hr: hour; m: meters; NIL: nothing; WG: water-dispersible granules.

Table 7. Average VectoBac WG (Bti) spray droplet size reported as volume median diameter (VMD), at each test distance for each study

Distance from the spray path	Mean volume median diameter (VMD), µm ± SE							
	K1	K2	K6	K7	K8	K9	K10	K11
2 m	48.30 ± 8.31	95.21 ± 19.00	127.07 ± 24.05	112.18 ± 10.73	59.25± 3.83	105.73 ± 23.91	84.75 ± 0.70	78.81 ± 4.44
4 m	27.94 ± 6.57	90.17 ± 4.56	92.35 ± 5.72	66.31 ± 11.88	25.24 ± 5.78	78.69 ± 7.86	58.86 ± 4.24	56.10 ± 7.02
8 m	23.18 ± 5.73	88.37 ± 0.88	52.10 ± 20.44	34.93 ± 5.48	25.07 ± 0.78	65.44 ± 17.16	60.27 ± 19.09	44.26 ± 6.81
12 m	29.60 ± 4.27	56.35 ± 13.00	54.40 ± 10.95	40.81 ± 8.51	38.25± 5.34	29.96 ± 5.52	53.21 ± 2.55	51.51 ± 3.97
16 m	30.34 ± 1.45	42.36 ± 6.80	45.97 ± 10.12	49.01 ± 4.04	29.38 ± 4.26	22.80 ± 6.73	40.05± 2.30	47.61 ± 3.78

m: meters; µm: micrometers; SE: standard error; VMD: volume median diameter.

Table 8. Average numbers of VectoBac WG (Bti) spray droplet for each test distance of each study and for the overall spray of each study

Distance from the spray path	Number of droplets, mean ± SE							
	K1	K2	K6	K7	K8	K9	K10	K11
2 m	63.96 ± 5.52	83.30 ± 8.02	58.22 ± 5.02	27.48 ± 1.79	22.81 ± 0.97	10.59 ± 0.82	20.78 ± 0.87	17.37 ± 1.10
4 m	161.59 ± 14.57	126.22 ± 10.32	105.44 ± 8.08	70.56 ± 5.15	110.59 ± 10.00	54.78 ± 4.30	94.74 ± 4.96	74.81 ± 3.35
8 m	119.41 ± 12.71	88.67 ± 6.50	95.11 ± 3.76	153.44 ± 12.95	76.04 ± 3.33	80.22 ± 6.00	130.59 ± 4.10	50.00 ± 3.44
12 m	17.00 ± 2.51	49.85 ± 5.61	61.94 ± 9.70	129.82 ± 5.49	24.81 ± 3.29	54.89 ± 3.78	95.00 ± 7.93	22.78 ± 1.18
16 m	3.04 ± 0.33	10.04 ± 1.26	11.44 ± 1.93	52.15 ± 4.06	5.52 ± 1.25	42.11 ± 2.23	37.89 ± 4.30	19.04 ± 1.25

SE: standard error.

Table 6 describes the ELT for Day-7 and Day 14 post spray containers, and for the next 4 weeks evaluation of RE in the same containers.

The K10 and K11 studies were specifically conducted to determine the ELT for 7 days and 14 days post spray. The ELT for Day-7 post spray and for the next 4 weeks evaluation of RE in the same containers was undoubtedly at the maximum 16m from the spray path.

The Day-14 post spray for K11 at 476g/ha, the ELT was at 16m for 4 weeks evaluation of RE, but for K10 at 469g/ha, the ELT was at 4m only for the same period.

Droplet profile

Bti spray droplets were observed in all slides from 2m-16m from the spray path (Table 7). Generally, the droplets were in the range of 50um–100um in size. But two studies (K1 and K8) had a near full spectrum of ultra-low-volume (ULV) droplets (<50um) through all test distances. The biggest droplets fell closest to the spray path at 2m. Two of the studies, K6 and K7, had huge droplets (127µm and 112µm) at 2m and these droplets were sprayed from the bigger orifice No. 4, which had a higher flow rate of 2000 mL/min.

The mean droplet numbers (± SE) was determined for each distance for each spray study (Table 8). The droplet numbers were not in a consistent range for all the studies within the specific distance. The trend was that the larger number of droplets was at 4m-8m.

The overall droplet numbers dispersed through orifice No. 2 with a walking speed of 1m per 3 secs in K9 and K11 studies with 250g in 10 liters water was significantly 1.7 folds less than K1 and K10 studies with 125g in 10 liters water at a walking speed of 1m per 6 secs (Kruskal-Wallis H test, d.f.=1, p=0.000).

DISCUSSION

The larval habitats of *Ae. aegypti* and *Ae. albopictus* comprise of both natural and artificial containers and these containers are abundant, and widespread in any human dwelling. The larval productivity is higher in outdoor containers than indoors (Oreenaiza *et al*., 2017; Ferede *et al*., 2018). Globally, the type of colonizing containers has transitioned from storage water pots of large volumes to natural and artificial containers of small volumes: tires, plastic containers, discarded trash, concrete drains with stagnant water, leaf axils, tree holes and as such (Oreenaiza *et al*., 2017; Murray *et al*., 2021; Harris *et al*., 2021). The dengue control operational programs find it to be an impossible task to search and destroy these small volume containers which are widespread larval habitats, and at times they are hard-to-reach and cryptic too.

Since the 1990's, IMR Malaysia has shown over years of research that the superfluous breeding of *Aedes* spp. larvae in these widespread, cryptic containers can be controlled by fine Bti droplets sprayed from motorized backpack sprayers or truck mounted ultra-low-volume generators (Seleena *et al*., 1996; Lee *et al*., 1996; Lee *et al*., 2008). The wide-area larvicide spray has shown to suppress the mosquito vector population and interrupt disease transmission (Tan *et al*., 2012; Bohari *et al*., 2020). The suppression of the dengue vectors was also observed by spraying Bti from a vehicle-mounted sprayer in a vegetated area (Lam *et al*., 2010) and in densely populated urban area in Puerto Rico (Harris *et al*., 2021).

Since 2014, the Ministry of Health (MOH), Malaysia, has included Bti as one of the larvicides in the national dengue control program. Initially, handheld sprayers were used to disperse the Bti spray mix, which was more towards a direct spot-treatment onto the larval habitats. The positive results from operational programs (Bohari *et al.*, 2020) spearheaded the use of wide-area larvicide spray using motorized backpack sprayers.

The manufacturer's recommended Bti dose for dengue control is between 400g/ha-500g/ha. The Bti dose, Bti spray mix rate, spray specs of the backpack sprayer, together with the walking speed of the operator were all recommended by the manufacturer based on data from several operational studies conducted in Malaysia and in Singapore (Lee *et al*., 2008; Lam *et al*., 2010; Lee *et al*., 2010; Tan *et al*., 2012).

The semi-field studies, K1 and K10, confirmed that the manufacturer's recommended SOP: the Bti spray mix of 125g in 10 liters water sprayed through orifice No. 2 of the Stihl SR420 sprayer, at a walking speed of 1m in 6 secs achieved the target Bti dose for dengue control (Table 2). The same target dose can be achieved when a spray mix of 250g per 10 liters water is sprayed through the same orifice but at a faster walking speed of 1m in 3 secs (K9 and K11 studies) (Table 2). However, if the applicator walked slower at 1m in 6 secs with the 250g per 10 liters spray mix, the dose achieved was double at 965g/ha (K2 study) to the recommended dose for dengue control. In contrast, a 125g in 10 liters of water sprayed through orifice No. 2, with a faster walking speed of 1m in 3 secs, the dose was one-half at 232g/ha (K8 study) (Table 2).

The WHO guidelines on the evaluation of a bacterial larvicide product consider a 90%-100% LM as good potential for mosquito control (WHO, 1996) and a minimum 90% reduction can be used to determine the residual effect and optimum application dosage (WHO, 2005). Thus, in this study it was set that the distances which gave the 90%-100% LM as the effective larvicidal treatment (ELT) for the Bti spray from a backpack sprayer, Stihl SR420.

The LM was measured in containers placed from 2m to a maximum of 16m from the spray path. The maximum distance of 16m was identified from previous studies at IMR with backpack sprayer and from a study conducted in Australia (Jacups *et al*., 2013).

In the Day-1 post spray containers, irrespective of the test doses between 232g/ha–1677g/ha, a 90%-100% LM was achieved within 3-hr of larval exposure, but the LM was more evident at 24-hr larval exposure until 12m-16m from the spray path (Table 3). For 4 weeks post spray evaluation in the Day-1 treated containers, the ELT remained at 16m for 5 of the 8 studies, and 12m for one study, the K6 study (Table 3). This data indicates that sufficient viable Bti toxins were present in the treated containers from the lowest dose (232g/ha) to the highest dose (1677g/ha) to achieve a 90%-100% LM for 12m-16m from the spray path, provided that these containers were placed indoors, and not exposed to the outdoor conditions.

But when the treated containers were placed outdoors for 14 days and beyond under a roof, exposed to ambient temperature fluctuations and the sun rays during the daylight hours, the ELT decreased in distance from the spray path and a shorter residual efficacy (RE) was obtained compared to the Day-1 post spray containers that were kept indoors (Tables 4–6). Anomalies were observed in the outdoor containers, where containers that were placed at closer distances to the spray path had lower LM than the containers placed at further distances. This could have been due to the containers being placed randomly outdoors, not following the sequence of the distance. The containers which were at the edge and facing more to outdoors were exposed more to the sunrays. We also observed that containers from different studies but with similar dose rates (K10 and K11) had varying degrees of LM and this could be due to the same reason of varying degree of exposure to the sunrays.

The containers that were treated with higher doses of 845g/ha–1677g/ha (K2, K6 and K7) and were exposed outdoors, had an evidently better ELT in distance and a better RE compared to the containers that were treated at lower doses of 232g/ha – 476g/ha (Tables 4-6). The lowest dose at 232g/ha (K8 study) had null ELT in the Day-21 and Day-42 post spray containers.

Similar results were observed in the Australian studies with the Bti spray from a backpack mist blower at 800g/ha as it provided slightly greater residual control than the 400g/ha spray (Jacups *et al*., 2013) and in direct Bti application the mega doses of 10x, 20x and 50x of the recommended rate at 8mg/l provided longer residual control of >90% control of 8, 8 and 23 weeks, respectively (Ritchie *et al*., 2010).

The larvicidal Bti toxins are endotoxins, and they are protein in nature (Valtierra-de-Luis *et al*., 2020). The amount of Bti toxins in the VectoBac WG formulation is expressed as 3000 ITU per mg of the finished product. Thus, the amount of Bti toxins in the treated environment is directly related to the dose applied. The higher dose had more Bti toxins then the lower dose. Despite natural degradation and exposure to the sunlight, there were sufficient viable Bti toxins in the containers in the higher doses (845g/ha–1677g/ha) to provide a better ELT in distance and RE compared to the containers that were treated with lower doses (232g/ha-476g/ha) (Tables 4-6).

The Bti treated containers that were left outdoors were exposed to the ambient temperature fluctuations of 24°C-33°C in Kuala Lumpur and sunlight in the daylight hours. The UV-A/B range (280-380 nm) of sunlight reaching Earth's surface has been considered responsible for considerable amount of photo-degradation and consequent loss of toxicity of the Bti toxins (Manasherob *et al*., 2002). The exposure of the Bti toxins to the sun's rays together with natural degradation could have contributed to shorter ELT (Tables 4-6) than in the Day-1 containers which were kept indoors (Table 3).

This K-series study concludes that 400g/ha-500g/ha of Bti, specifically for VectoBac WG formulation, is effective to control the dengue vectors via backpack sprayer for wide-area larvicide spray application. A similar efficacy was achieved when spray applications were made from a truck mounted sprayer, giving a good *Ae. aegypti*

larval kill of more than 90% in Florida Keys for a maximum tested distance of 91m from the spray path (Murray *et al*., 2021) and the suppression of adult female *Ae. aegypti* mosquitoes in Puerto Rico (Harris *et al*., 2021).

The ELT of K10 and K11 studies at 400g/ha-500g/ha for Day-7 post spray and for the next 4 weeks evaluation of RE in the same containers was undoubtedly at the maximum 16m from the spray path. The Day-14 post spray for K11 at 476g/ha, the ELT was at 16m for 4 weeks evaluation of RE, but for K10 at 469g/ha, the ELT was at 4m only, during the same study period. The dose dispersed at K10 was just less by 7g/ha and this could not be the cause for the lower ELT at 4m. The lower ELT is most likely due to the containers being randomly placed outdoors in the verandah and exposed to the varying degrees of the UV rays for 14 days. Previous studies in two housing estates in Selangor State, Malaysia, where Bti was sprayed from truck mounted ULV generators, cups with water were placed randomly in the porch (exterior) of the study houses. The cups were kept for 7 days and 14 days post spray in the porch to test the persistency of the sprayed Bti toxins under field conditions against *Ae aegypti* and *Ae albopictus* larvae. In both studies, a 100% mortality was achieved for 14 days post truck mounted ULV spray in 75% of the containers that were placed in the exterior of homes, indicating the persistency of the sprayed Bti toxins amid natural degradation and exposure to UV rays (Seleena & Lee, 1998).

The backpack sprayer delivered the spray at 567mL-650mL per min through the nozzle orifice No. 2 or at 2000mL per min through the nozzle orifice No. 4. There are no available guidelines for optimum droplet size and numbers for a larvicide space spray. Nevertheless, magnesium oxides (MgO) slides were placed for all K studies to determine the droplet spectrum. The presence of the Bti microdroplets on the slides confirmed that the larval mortality in the exposed containers was indeed due to the Bti droplets that were deposited in the containers. Numerous Bti studies have been conducted at IMR since the 2004 with the same model backpack sprayer, Stihl SR420, but we were not able to identify the optimum droplet numbers that will give a 100% larval mortality.

It is known that most equipment used to apply space spray will produce a range of droplet sizes and the droplet size increases as the flowrate increases (WHO, 2023). The droplet spectrum varied at the same distance among the eight K studies. The obvious trend for the K study was that the droplet size and droplet numbers reduced with distance (Table 7 and Table 8). The spray nozzle produces a range of droplet sizes. The bigger Bti droplets fell closer to the spray path. The droplet fall is determined by the droplet size, whereby a bigger droplet of 100µm only takes about 36 secs to fall compared to a 50µm which will take approximately 135 secs to fall (WHO, 2023).

The fan in the Stihl sprayer generates the wind to push the droplets forward with a maximum air throughput of 1260 m^3/hour under the maximum throttle (Stihl, 2006). In an optimal indoor setting with still air, the maximum deposition of droplets happens at 5m – 6m from the nozzle and after which the deposition of the droplets tapers with distance (KTS Trading Sdn. Bhd. & THK Powertools (M) Sdn. Bhd.; Stihl distributors in Malaysia personal communication). This is in line with our study, in five of the K studies (K1, K2, K6, K8, K11) we observed the highest number of droplets at 4m (Table 8). We did not have any sampling points between 5m–7m, otherwise we would have detected higher droplet numbers at these points. The highest droplet numbers at 4m could be a probable reason for observing a 90%–100% LM at 4m only in several of the residual efficacy evaluations (Tables 3-5), despite the natural degradation of some Bti toxins with time and exposure to UV rays.

For wide-area larvicide spray, higher Bti dose rates than the recommended dose of 400g/ha–500 g/ha, do give longer residual control of more than 14 days post spray as shown in this K-series study and in Australia (Jacups *et al*., 2013), but we do not recommend higher doses because of the frequent heavy rainfall in Malaysia, a tropical country with a year-round rainfall. The sprayed Bti toxins will be washed away by the rain. In western Kenya, the rainfalls did interfere with the residual effect of bacterial larvicides, and vector control programs had to implement weekly Bti applications during the rainy seasons (Fillinger & Lindsay, 2006).

The motorized backpack sprayer plays a key role to disperse the Bti microdroplets in a wide-area for dengue vector control. The Stihl SR 400 series have been tested and used effectively in several global mosquito control programs. The spray jet at maximum throttle can treat larval habitats, both horizontally and vertically with uniform flowrate, to a distance of 12m–16m. The presence of droplets on the MgO slides (Table 8) and LM in containers placed at 12m–16m (Table 3) did confirm that the Bti spray did reach 12m–16m in the open test field. But this spray swath can be curtailed by the presence of vegetation and building structures in the treatment site. The spray swath can also be reduced if the sprayer is not functioning to its maximum capacity and the spray nozzle is clogged. This situation can be avoided if the spray equipment is well maintained through regular services and the sprayer is washed with detergent and water after every Bti application to prevent the sedimentation of the Bti particles in the spray tubes and in the nozzle.

Doloi (2021) in the review of vectors and vector-borne diseases stated that Bti has proven to be a competent control agent for *Aedes.* spp mosquitoes. But this can only be true if VectoBac WG is sprayed at the specified dose of 400g/ha-500g/ha via an efficient motorized backpack sprayer that is able to disperse microdroplets of the Bti toxins uniformly over a wide-area of *Aedes* spp. larval habitats. The Bti spray mix rate to be either 125g in 10 liters of water or 250g in 10 liters of water, depending on the walking speed of the spray operator, 1m in 6 secs or 1m in 3 secs, respectively.

In conclusion, VectoBac WG spray mix dispersed as microdroplets from a motorized backpack sprayer can reach the widespread larval habitats to give an effective kill in the *Aedes* spp. larval population. The efficacy does decrease with distance and exposure to sunlight, nevertheless effective kill with residual efficacy can be achieved in the *Aedes* spp. larval mosquitoes within the optimal distance.

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Conflicts of Interest Disclosure

Two of the authors, Seleena Benjamin, Sr. Business Manager, and Teoh Guat Ney, Technical Development Specialist, are employees of Valent BioSciences LLC, the manufacturer of the bacterial larvicide, VectoBac WG. The authors were involved in designing this study; in the collection, analyses and interpretation of data; and in the writing of the manuscript. The commercial affiliation does not alter our adherence to the Tropical Biomedicine journal policies on sharing data and materials.

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