METHOD FOR DETERMINING SETTLING RATES OF *BACILLUS THURINGIENSIS* SEROTYPE H-14 FORMULATIONS[1]

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ABSTRACT. A water-column apparatus is described in which settling rates of *Bacillus thuringiensis* serotype H-14 [*B.t.* (H-14)] formulations can be indirectly quantified using mortality of mosquito larvae at restricted depths as an index of *B.t.* (H-14) activity. To illustrate the type of data provided by this method, commercial *B.t.* (H-14) products (Bactimos®, Teknar®, Vectobac®) and experimental formulations were compared at the manufacturers' recommended rates for mosquito control. All evaluations utilized laboratory-reared, 4th-instar *Aedes aegypti* larvae. The procedure can be used to provide an index of suspension properties of different *B.t.* (H-14) formulations and to measure dispersion rates of granular formulations resting at the bottom of a water column. Standardization of the method provides a convenient and practical means of generating comparative data on the effectiveness of *B.t.* (H-14) and other mosquito larvicides against specific target species.

INTRODUCTION

Since the discovery of *Bacillus thuringiensis* serotype H-14 [*B.t.* (H-14)] as a potent mosquito larvicide (Goldberg and Margalit 1977), major attention has been directed at the development of formulations which will effectively deliver the material to target species. Various formulations, including aqueous suspensions, wettable powders, sustained-release floating briquets, floating and semi-floating corn-cob grit materials, and other granular products, are available commercially or are being developed.

Standardized procedures are needed in order to compare the numerous *B.t.* (H-14) isolates and formulations now available. McLaughlin et al. (1984) have proposed a U.S. Standard Bioassay to assess the potency of *B.t.* (H-14) using small wax-lined paper cups (depth ca 4.0 cm) in which mosquito larvae are treated. As in most such laboratory assays, test containers are shallow and do not take into account the effect of water depth on the efficacy of the materials being evaluated. This factor alone may have significant implications for field applications, accounting in part for disparities in laboratory performance of *B.t.* (H-14) products and their efficacies under field conditions.

Similar problems in trying to use laboratory data to predict field efficacies for *B. thuringiensis* isolates in controlling the cabbage looper, *Trichoplusia ni* (Hübner), are discussed by Dulmage et al. (1971) and Beegle et al. (1982). In the latter case, a significant factor is the amount of treated leaf tissue consumed. In mosquitoes it is the volume of treated water which the larvae filter and the amount of *B.t.*-contaminated particulate matter they ingest which largely determine efficacy (Aly 1983). Both factors are directly related to the dispersion of *B.t.* (H-14) in the water column and the rate at which it settles out.

In addition to retaining toxicity, formulations should remain suspended in the water long enough to ensure adequate ingestion by mosquito larvae. However, owing to significant differences in the larval feeding behavior of mosquito species, notably the depth at which they typically feed, many formulations do not effectively reach all target species. Floating formulations can be effective in controlling surface-feeding anopheline larvae, whereas sinking formulations are likely to be more effective in killing bottom feeders such as many *Aedes* spp. Larvae which feed at intermediate depths are likely to be more vulnerable to formulations which remain well suspended throughout the water column.

Because of the difficulty in directly quantifying the amount of *B.t.* (H-14) present at various depths of the water column following application, a method was devised to indirectly measure the amount of *B.t.* (H-14) present by assessing mortality of larvae confined to specific water depths. This simple technique permits determination of settling rates for individual *B.t.* (H-14) formulations as well as comparative evaluations among different products, formulations, and target species.

MATERIALS AND METHODS

The bioassay apparatus (Fig. 1) consists of paired, vertical glass cylinders, each with the inner cylinder just small enough in diameter to slide up and down within the outer cylinder (height = 40 cm, diam = 6 cm). The bottom end of the cylinder is covered with a taut, dacron organdy screen held in place with a rubber band.

[1] Supported in part by grant from Abbott Laboratories, North Chicago, IL 60064.

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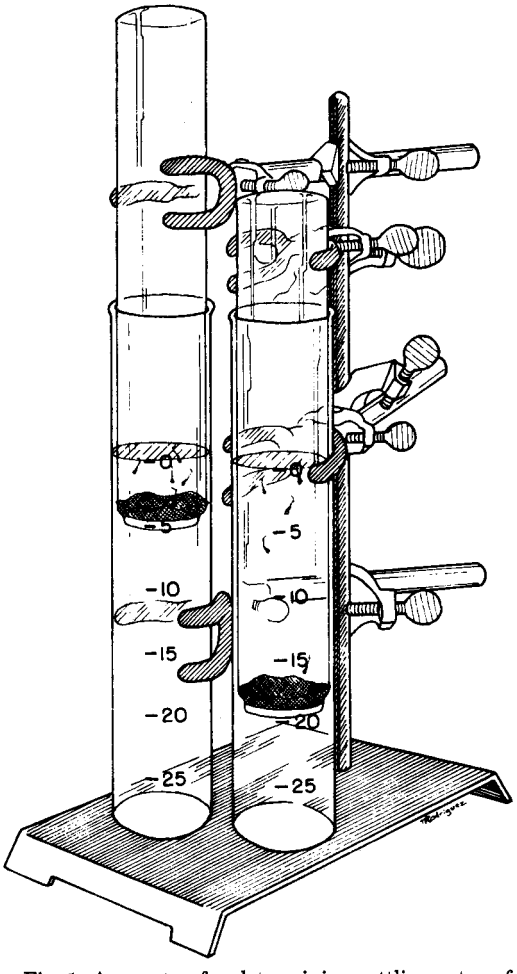

Fig. 1. Apparatus for determining settling rates of *Bacillus thuringiensis* (H-14) formulations in a 30-cm water column. Glass cylinders at left adjusted to restrict mosquito larvae to upper 5 cm of water column. Cylinders at right adjusted to allow mosquito larvae to move freely to depth of 20 cm.

The outer cylinder is closed at the bottom and secured in place with a utility clamp on a laboratory stand. The outer cylinder is marked from 0 to 30 cm in 5-cm increments beginning 10 cm from the top of the cylinder.

Prior to each assay, the outer chamber is filled with water to the 0-cm mark, providing a 30-cm water column. The inner cylinder is then carefully inserted, adjusted to the desired depth, and secured in place with a utility clamp at its upper end. By moving the inner cylinder up and down, the water column above the screened end can be adjusted to any depth from 0 to 30 cm. Mosquito larvae are then introduced, restricting them to prescribed depths from the water surface. Formulations of *B.t.* (H-14) are applied directly to the water surface either prior to or after the mosquito larvae are introduced, depending on the purpose of the assay.

Studies were conducted with commercial and experimental *B.t.* (H-14) formulations to illustrate the types of data which can be obtained using this technique. All assays were conducted at 25°C using laboratory-reared, early 4th-instar *Aedes aegypti* (Linn.) larvae and tap water that had been allowed to stand overnight.

Settling rates of commercial B.t. (H-14) products. Three *B.t.* (H-14) products were evaluated at the application rates recommended by their respective manufacturers: Bactimos®, wettable powder (0.25 and 0.5 kg/ha), Biochem Products, Inc; Teknar®, aqueous concentrate (0.5 and 1.0 pt/acre), Sandoz, Inc.; and Vectobac®, wettable powder (0.5 and 1.0 lb/a), Abbott Laboratories. The respective, equivalent International Toxic Units (ITU) for each of these formulations used in the described assays were: Bactimos, 247 and 495 ITU; Teknar, 244 and 489 ITU; and Vectobac, 318 and 637 ITU.

In a series of tests conducted with each product, *Ae. aegypti* larvae were confined to the upper 5, 10, 15, 20 or 25 cm of the water column. Following adjustment of the test cylinder to the appropriate depth, *B.t.* (H-14) was applied to the water surface at the desired rate. One hour after application, 20 larvae were introduced and left for 24 hours, at which time the number and percent mortality were recorded. The test was repeated for each product, application rate, and depth by introducing 20 larvae at 2, 3, 4, 5, 6, 7, 8, 12, 24 and 48 hr posttreatment and recording mortality in each case at 24 hr after larval introduction. Each test was replicated three times and compared with an untreated control. Percent larval mortality was used as an indicator of the amount of *B.t.* (H-14) present within a given portion of the water column, indirectly providing a measure of the rate at which the active component of the *B.t.* (H-14) material settled out.

Comparison of different Abbott Laboratory formulations. Five formulations of Vectobac were compared using the water-column apparatus to determine their relative settling rates. Three were experimental granules (ABG-6138, ABG-6140, ABG-6141), each applied at a rate of 5.0 lb/acre. The fourth formulation was an aqueous suspension (ABG-6145) applied at 1.0 pt/acre and 2.0 pt/acre, and the fifth formulation was the commercially available Vectobac wettable powder (WP) applied at 1.0 lb/acre.

The WP and aqueous suspensions were applied to the water surface after adjusting the test cylinders to the desired depths. Depths chosen for this series of tests were 5, 10 and 15 cm. Twenty *Ae. aegypti* larvae were introduced in successive tests at 1, 4, 8, 12 and 24 hr posttreat-

ment with mortality counts being made 24 hr later. These materials were compared with Teknar applied at 1.0 pt/acre, following the same procedure.

Because the granules were too large to pass through the screen at the lower end of the inner test cylinder, the protocol was modified for granular applications. Granules were applied to the water surface prior to insertion of the inner cylinder, allowing the material to settle in the water column unhindered by the screen. The test cylinder was then carefully inserted and adjusted to the desired depth just before introduction of mosquito larvae at 1, 4, 8, 12 and 24 hr posttreatment.

Larval feeding behavior. In order to compare the depths at which larvae of different mosquito species typically feed, an activity profile was determined for *Ae. aegypti* and *Culex pipiens* Linn.[3] Vertical movement of larvae was observed in a clear glass cylinder (height = 20 cm, diam = 3 cm) graduated in 3-cm increments and filled with water to a depth of 15 cm. In each test, ten 4th-instar larvae of a given species were placed in the cylinder and allowed to acclimate for 30 minutes. During the next hour the depth of each larva was recorded at one-minute intervals. Observations were made from a distance of 1 m to minimize any disturbance of the larvae as they freely ascended and descended in the water column. Tests were repeated 3 times for each species in the morning, afternoon and evening. From these data, the mean percent time spent at each depth was determined.

RESULTS AND DISCUSSION

Using *Ae. aegypti* larval mortality as a measure of the amount of *B.t.* (H-14) activity at a given depth, the mean percent mortality over time provides an index of the settling rate for each product (Fig. 2). Alternatively, the same data depict how well each product remains suspended in the water column during the indicated time periods.

In the case of larvae confined to the upper 5 cm of the water column, Vectobac remained suspended in sufficient quantity to kill larvae for only 3 to 4 hr posttreatment. The amount of suspended material decreased rapidly in the uppermost 5 cm of water after the first hour. Bactimos, in contrast, remained suspended in the upper 5 cm of the water column up to 5 hr following application, after which insufficient material was present to effect further mortality. Teknar settled out in the water column much more slowly than the other two products, with larvae being killed in the uppermost 5 cm of water up to 24 hr after treatment. These same trends are evident in the data for each of the other depths. The observation that mortality was still high at the lower levels of the water column for each product indicates that the reduction in larval mortality reflects a decreasing amount of *B.t.* (H-14) material at a given depth rather than simply its inactivation.

The settling rates of the aqueous suspensions were much slower than that of the wettable powder (Fig. 3). The WP remained suspended in the upper 5 cm of the water column less than 4 hr and in the upper 15 cm less than 8 hr. Both aqueous suspensions, on the other hand, effected complete kill of larvae at all depths during the 24-hr test. Performance of the aqueous suspensions of Vectobac was comparable to that of Teknar during the first 12 hr posttreatment and was better in the upper 5 cm at 24 hr (Fig. 3). Aqueous suspensions of *B.t.* (H-14) appear to be more effective than wettable powders in controlling mosquito larvae, due to their slower settling rates.

The three granular formulations also provided excellent larval control, resulting in 100% mortality of larvae at all depths throughout the 24-hr test period. The so-called "granular" formulations that were tested were in effect semi-floating. The particle size of the corn-cob carrier varied considerably, as did the rate at which the individual particles absorbed enough water to sink. Whereas ca 28% of the material sank to the bottom of the water column within minutes of application, 41% of the material remained floating at the surface 6 hr later. Seventy percent of the granules had sunk by 12 hr and 94% by 24 hr posttreatment.

The advantages of granular, semi-floating formulations are evident. Under field conditions they can effectively penetrate vegetation, reaching the water surface in situations where the use of liquid formulations is not practical. By settling at irregular rates, depending upon the nature of and consistency in particle size of the carrier, these materials can effectively disperse *B.t.* (H-14) throughout the water column for extended periods. Unlike the aqueous suspensions and wettable powders, granular and semi-floating formulations do not readily pass through the screen barrier of the water-column bioassay apparatus, necessitating some modifications of the assay procedure. Granular formulations, which sink quickly and uniformly, are simply allowed to sink before the inner cylinder is inserted and adjusted to the desired test depth. The rate and degree of dispersion of the active *B.t.* (H-14) material from the bottom of

[3] Hinkle, N. C. 1983. Factors affecting the efficacy of *Bacillus thuringiensis* var. *israelensis* as a mosquito larvicide. Auburn University, Auburn, AL. M.S. Thesis, 75 pp.

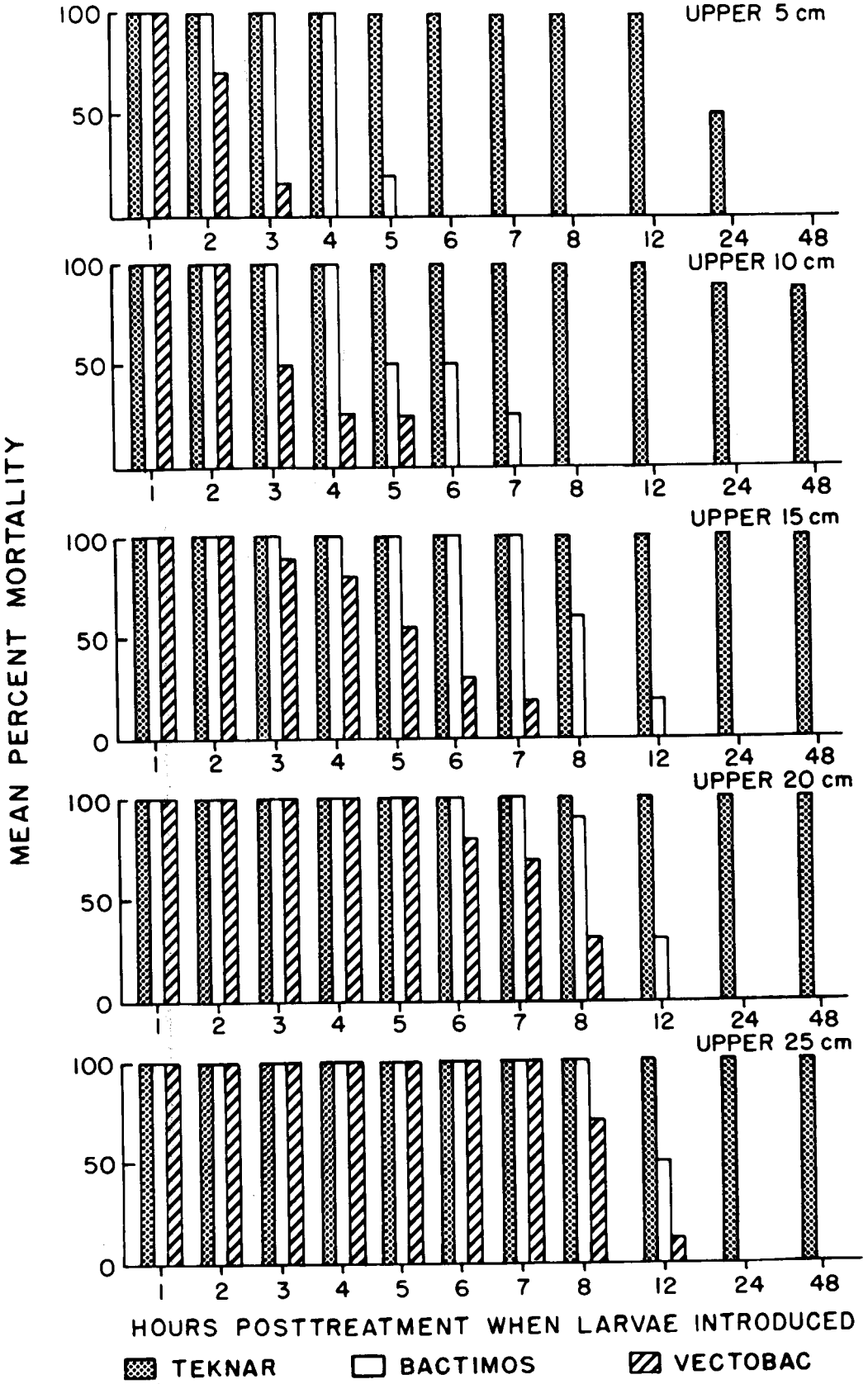

Fig. 2. Comparison of settling rates of 3 commercial *Bacillus thuringiensis* (H-14) products applied at manufacturers' recommended rates (Teknar 1.0 pt/acre, Bactimos 0.5 kg/ha and Vectobac 1.0 lb/acre) expressed as mean percent mortality of 4th-instar *Aedes aegypti* larvae retained at depths of 5–25 cm from surface in water-column apparatus. Larvae introduced at hourly intervals from 1 to 8 and at 12, 24 and 48 hr posttreatment. All mortality counts made 24 hr following introduction of larvae.

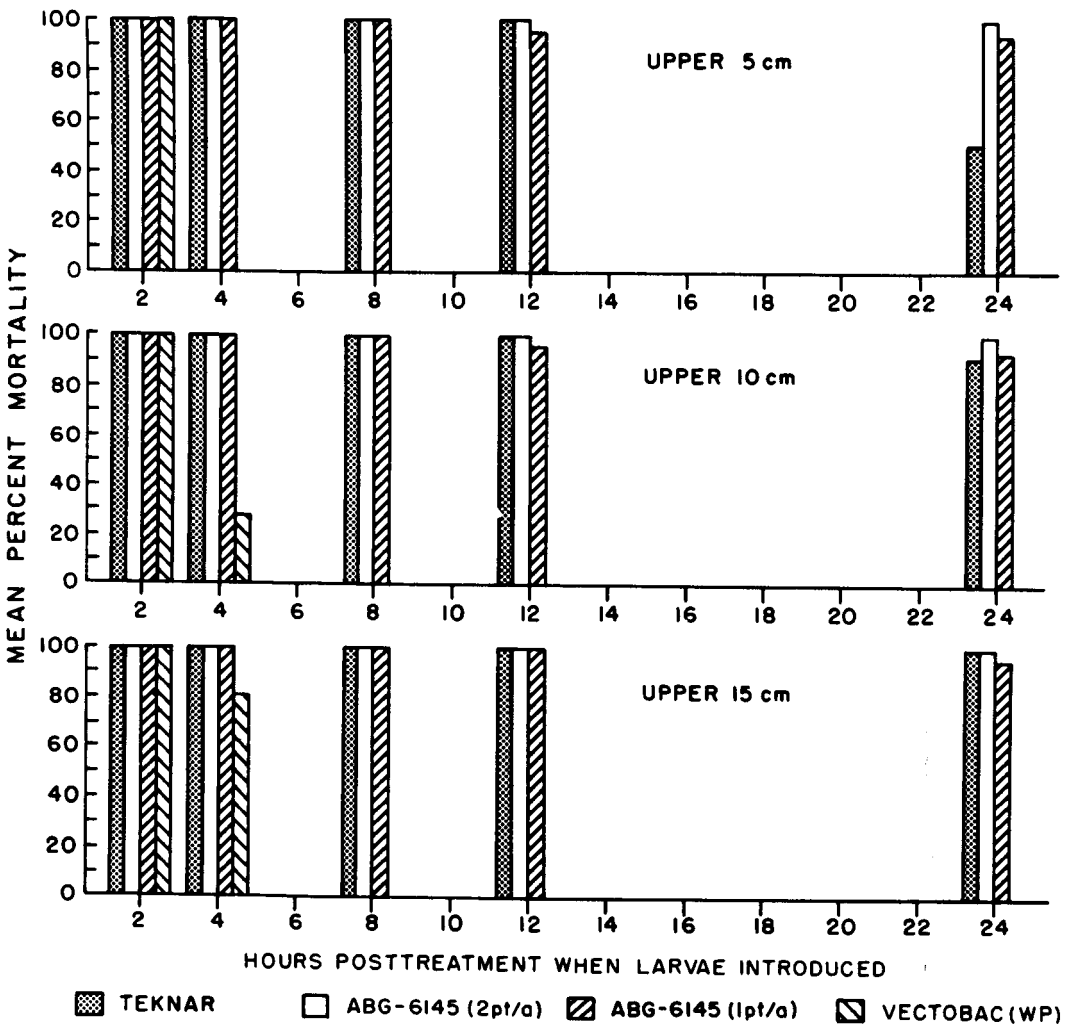

Fig. 3. Comparison of settling rates of Vectobac and two ABG-formulations in the water column using 4th-instar *Aedes aegypti* larvae. Settling rates are depicted as mean percent mortality of larvae confined to fixed depths (5, 10, 15 cm) following introduction at 1–24 hr posttreatment. Treatments are aqueous suspensions of *Bacillus thuringiensis* (H-14) (ABG-6145) at 1.0 and 2.0 pt/acre and a wettable powder (WP) at 1.0 lb/acre compared with Teknar (1.0 pt/acre) used as a standard. Results for the three granular formulations of ABG-compounds (ABG-6138, ABG-6140, ABG-6141) are not shown; each of these products caused 100% mortality at all depths throughout the 24-hr test period.

the test chamber is then measured rather than the settling rate from the surface.

Semi-floating formulations present a more difficult situation. Our suggested procedure is to apply the material to the water surface before inserting the inner cylinder. Wait for the desired posttreatment interval at which time any material still floating at the surface can be temporarily removed (e.g., with forceps or pipette). Then insert the inner cylinder and adjust it to the appropriate depth following which the floating material should be replaced on the surface and the mosquito larvae added. Care should be taken to minimize disturbance of the water column during this procedure.

Observations on the vertical movement of *Ae. aegypti* and *Cx. pipiens* larvae revealed distinctly different activity profiles (Table 1). *Culex pipiens* larvae spent 89% of their time in the upper 3 cm of water compared to only 43% of the time for *Ae. aegypti*. *Aedes aegypti* tended to actively ascend and descend the column and spent more than 25% of the time near the bottom of the test chamber, compared to less than 4% of the time at the bottom by *Cx. pipiens*. Never were more than 70% of the *Ae. aegypti* larvae at the surface simultaneously; conversely, there was never less than 70% of the *Cx. pipiens* larvae at the surface at the same time.

These data support the general observation

Table 1. Activity profile for *Aedes aegypti* and *Culex pipiens* larvae (4th-instar) based on the time spent at various depths of a water column during 1-hr observation periods.

Depth (cm)	Percent time spent at various depths			
	Aedes aegypti		*Culex pipiens*	
	Mean	Range	Mean	Range
0–3	43.2	(40.0–49.2)	89.3	(88.3–90.0)
3–6	11.9	(10.3–13.8)	3.3	(2.8–3.8)
6–9	9.4	(7.8–10.5)	2.3	(1.5–3.2)
9–12	9.7	(9.5–10.0)	1.5	(0.7–2.0)
12–15	25.7	(19.7–30.0)	3.8	(2.8–4.5)

that larvae of different mosquito genera exhibit different feeding patterns relative to water depth. Whereas *Anopheles* spp. feed almost exclusively at the water surface, *Culex* spp. tend to feed just below the surface in the upper few centimeters of water. *Aedes* spp. are more typically bottom feeders, spending a significant portion of their time actively moving between the water surface and the substrate where they graze. In the case of *Ae. aegypti* reported here, 31% of the time was spent in transit between the surface and bottom of the chamber, i.e. between depths of 3 and 12 cm (Table 1).

CONCLUSIONS

This technique can be used not only to measure the settling rates of different *B.t.* (H-14) products and formulations but also to provide an index of suspension properties and, in the case of granular materials, dispersion rates from the bottom of the water column. Furthermore, this laboratory method can help determine the effectiveness of various formulations against different target species either directly by testing those species or indirectly by knowing their feeding behavior relative to water depth.

The described apparatus provides a simple, inexpensive means for indirectly quantifying the amount of *B.t.* (H-14) at different water depths following *B.t.* (H-14) application. Tests are easily replicated and, based on the studies we have conducted, provide consistently repeatable results. The variance in mortality is extremely low such that 10 mosquito larvae per test are more than adequate, provided they are the same instar and age. By standardizing the water-column size (e.g., 30 cm depth), mosquito species and instar (e.g., 4th-instar *Ae. aegypti*), and the depths at which larval mortality is determined (e.g., 5-cm increments from the surface), this procedure could be used routinely at any location to provide good comparative data on product performance.

The selection of a particular formulation of *B.t.* (H-14) for controlling mosquitoes in the field should take into account the target species involved and its activity profile relative to depth of feeding. To be effective against *Anopheles* larvae, the material must remain suspended for an extended period at the water surface, allowing adequate exposure time for larvae to ingest a lethal dose. Aqueous suspensions and floating or semi-floating formulations of *B.t.* (H-14) should be best suited for controlling both *Anopheles* and *Culex* larvae. Materials which settle more rapidly are likely to be better for controlling *Aedes* larvae and other bottom-feeding species. As for a general-use product in which the target species is not defined or which involves multiple species with different feeding behaviors, a material which remains suspended throughout the water column is likely to provide the most satisfactory results.

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