THE SUSCEPTIBILITY OF *SIMULIUM VITTATUM* LARVAE (DIPTERA: SIMULIIDAE) TO *BACILLUS THURINGIENSIS* VAR. *ISRAELENSIS* IN THE LABORATORY[1]

R. L. FROMMER, S. C. HEMBREE, J. H. NELSON, M. REMINGTON AND P. H. GIBBS
US Army Medical Bioengineering R&D Laboratory, Fort Detrick, Frederick, Maryland 21701

ABSTRACT. The susceptibility of *Simulium vittatum* to varying concentrations of *Bacillus thuringiensis* var. *israelensis* (Abbott Lab. #6406–125 with an ITU/mg of 400–600) after 15, 30, 60, 90, 120 min and 24 hr of exposure was studied. A sharp decrease in LC50 (0.81 to 0.32 ppm) and LC90 (1.71 to 0.86 ppm) concentration values occurred when exposure times were increased from 30 to 60 min. Change in LC50 and 90 concentration requirements was minimal when exposure times were expanded beyond 60 min.

INTRODUCTION

The unavailability of chemical larvicides for use in black fly control in streams and rivers in the United States and the relative ineffectiveness of adulticides (Carestia et al. 1974a, b) have focused attention on the possible use of insect pathogens as larvicides.

Lacey and Mulla (1977) evaluated in the laboratory the susceptibility of field collected black fly larvae, *Simulium vittatum*, to 13 strains of *Bacillus thuringiensis*. However, even exposures to 10 ppm for 24 hr failed to produce 90% mortality with these strains. The high concentrations required and the difficulty of maintaining exposures in flowing water for 24 hr would seem to remove these strains from serious considerations as biological control agents for black flies.

The recently discovered *israelensis* variety of *Bacillus thuringiensis* (Goldberg and Margalit 1977, de Barjac 1978) has demonstrated high potential as a larvicide for black flies in both laboratory (Undeen and Nagel 1978, Undeen and Colbo 1980). Preliminary field trials (unpublished) conducted by this laboratory were of limited success and demonstrated the need to study in detail the concentration-time-response relationship between *B. t.* var. *israelensis* (*Bti*) and target organisms dwelling in flowing water habitats. These studies are neces-

[1] The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense. Use of proprietary names does not constitute indorsement.

sary because the agent applied to a flowing stream for a given period of time spreads out so that, as the distance downstream from the treatment site increases, longer periods of time are required for the concentration to pass a given point. This phenomenon indicates a reduction in concentration levels, but an increase in terms of duration of exposure. Thus, to determine effectiveness, the concentration must be defined in terms of both concentration and duration of exposure. Studies described below have been conducted for that purpose. Subsequent studies will determine the effects of the stream habitat on attenuation of the concentration as it moves downstream. Only when all of these factors are considered can an appropriate concentration be determined that will control black fly larvae for a given length of breeding habitat.

METHOD AND MATERIALS

The *Bti* used was an experimental, powdered formulation (400–600 International Toxic Units (ITU)/mg) provided by Abbott Laboratories, Lot No. 6406-125. Twenty milligrams of powder were placed in a Waring blender with 1 liter of distilled water and mixed at maximum speed for 1 min. No wetting agent (detergent) was added. This 20 ppm stock solution was then used to prepare serial dilutions. Following standard plate count procedures, total spores/mL were correlated with weight/volume (ppm) expressions of concentrations by further dilution and pipetting 1 mL of material onto plates of tryptose-blood agar base. Plate counts were replicated 8 times to permit statistical evaluation.

Larvae of *S. vittatum* were lab reared from eggs collected from several streams at Holston Army Ammunition Plant near Kingsport, Tennessee. Terrestrial grasses overhanging in the water and submerged aquatic vegetation (*Potomogeton crispus* L. and *P. pectinatus* L.) to which egg masses were attached were placed in petri dishes and held on wet ice for transportation and storage. For hatching and rearing, egg masses were placed in 15-liter bell jars containing 14 liters of aerated tap water. Water temperatures were ca. 22°C throughout rearing and testing. Food used during these studies was a 5 gm/liter suspension of Tetramin (95%)/Prolinn (5%). Fifty ml of the food suspension were added to each rearing container initially and at each water change. Constant water aeration and some movement was accomplished by using airstones along the bottom of each rearing jar. Glass rods 45 cm long × 0.32 cm in diameter were placed in the rearing jars to provide attachment substrates from which the larvae could be easily removed for testing. Twelve liters of aerated tap water were exchanged daily. Approximately 20 days were required for hatching and development of larvae to the 5th to 7th instar, the age used for testing.

Bioassays were conducted using four of the bioassay apparatuses described by Hembree et al. (1980). Each apparatus held 10 containers (480-mL waxed paper cups) inside of which rotated 120-mL plastic bottles. The bottles provided both movement of water and substrate for larval attachment. Larvae also attached to walls of test containers. The only aeration in the test containers was that occurring at the surface of the test medium as a result of the rotating plastic bottles. Sampling indicated that during 24-hr test operations, water in the test containers contained an average of 7.69 ppm dissolved oxygen. The saturation point of dissolved oxygen in water at 22°C is only slightly greater at 8.8 ppm.

Effects of the addition of food on larval feeding before treatment were considered a potential problem, since Gaugler and Molloy (1980) reported this could reduce the activity of *Bti* significantly. However, since food is available in the field environment prior to, during, and after any field treatment, a decision was made to provide food for larvae during and after laboratory testing.

To conduct a test, 180 mL of aerated tap water were added to each of the test

containers, which were then placed on the bioassay apparatus. The apparatus was turned on and adjusted to 120 rpm and 0.5 mL of food was added to each container. Larvae were removed from rearing containers, and those of appropriate size (5th to 7th instar), as described by Mulla and Lacey (1976), were selected. Ten larvae were added to each of the 10 test containers on each test apparatus used and allowed to acclimatize for 3 hr. Pretest data revealed no significant difference in control larval mortality when acclimatized for less than 24 hr. In early tests, larvae were examined after the acclimatization period and were found to have distributed themselves randomly on the inner surface of the test containers and on the outer surface of the rotating plastic bottle, indicating relatively homogeneous conditions within the test containers. Similarly, in these preliminary tests when mortality was assessed, dead larvae were found at all positions within each test cup and on the bottles for each treatment concentration and exposure time. After acclimitization, the desired quantities of *Bti* were added to the test containers in 20 ml of distilled water. Nine different concentrations were used for each duration of exposure, except for the 15 min and 24-hr exposures in which 5 and 7 dilutions were used, respectively. One control container was used on each apparatus.

To examine the possibility of reduction in spores/ml between initial *Bti* concentrations immediately preceding treatment from those during testing, samples of various concentrations from test cups, while on the bioassay apparatus during operation, were collected at timed intervals over a 30-min period. These results clearly demonstrated that there was no measurable settling nor were there any differences between spores/ml between initial treatment and during treatment.

Exposures were terminated after the desired time by removing the test containers one at a time and pouring out the bacterial suspension. The larvae remained attached to the bottle or to the walls of the test container. Two-hundred milliliters of aerated tap water and 0.5 ml of food were then added to each test container, which was immediately returned to the apparatus. Only a few seconds were required to terminate an exposure and return the refilled container to the apparatus. Accurate durations of exposure were provided by timing the treatments and terminating the exposures in the sequence in which the treatments were initiated. Undoubtedly traces of *Bti* remained, but this would have been diluted by a factor of at least 400:1 when clean aerated tap water was added. This effectively reduced the concentration of the highest concentration to 0.005 ppm, which was below the threshold of response to this material at all durations of exposures tested.

Mortality was assessed 24 hr after terminating exposures. The machines were turned off. Most of the larvae remaining alive became agitated and began to move around. Those that did not move were touched with the tip of an applicator stock and were judged dead if no movement resulted. Data were recorded as number dead per 10 larvae exposed.

Upon termination of testing all 120-mL plastic bottles were removed from each bioassay apparatus and individually hand washed in hot Alconox detergent tap water solution, rinsed, and air dried.

The statistical procedures utilized in analyzing the data were analysis of variance of a randomized block in conjunction with Duncan's Multiple Range Test (Duncan 1955), polynomial regression analysis (Hogben et al. 1971) and probit analysis (Barr et al. 1976).

Ten replications of each concentration of *Bti* were conducted with 15-min exposures, 20 replications at 30-min exposures, 10 replications at 60-, 90-, and 120-min exposures and 8 replications at 24-hr exposuires.

RESULTS AND DISCUSSION

The ranking of mean mortality rates (Table 1) shows no statistical difference

Table 1. Mean percent mortality rates of *S. vittatum* to varying concentrations of *Bti* at six time exposures.[1]

Treatment Concentrations								
ITU/mg[3]	Wt/Vol (PPM)	Estimated Mean SPORES/ml±CI[2]	15 Min Exposure	30 Min Exposure	60 Min Exposure	90 Min Exposure	120 Min Exposure	24 Hour Exposure
800–1200	2.0	$6.153\pm0.241\times10^4$		96.0 ± 3.2 a				
720–1080	1.8	$5.187\pm0.188\times10^4$		86.0 ±10.7 b				
640–960	1.6	$4.288\pm0.152\times10^4$	62.0±24.5 a	90.0 ± 5.2 a				
560–840	1.4	$3.453\pm0.137\times10^4$		73.0 ±12.3 c				
480–720	1.2	$2.691\pm0.144\times10^4$	53.0±20.5 a	75.0 ± 7.5 c	94.0± 6.0 a	95.0± 5.9 a	98.0± 3.0 a	
400–600	1.0	$2.014\pm0.154\times10^4$	53.0±17.6 a	77.0 ± 8.5 c	89.0± 6.2 a,b	92.0± 9.4 a,b	87.0±13.1 a	100.0± 0.0 a
320–480	0.8	$1.429\pm0.170\times10^4$		54.5 ±11.2 d	90.0± 5.8 a,b	95.0± 6.9 a	92.0± 6.6 a	
280–420	0.7	$1.172\pm0.169\times10^4$	31.0±18.3 b					
240–360	0.6	$9.517\pm0.109\times10^3$		26.5 ±13.3 e	82.0±14.4 b	91.0± 7.1 a,b	86.0±17.2 a	
200–300	0.5	$7.591\pm0.168\times10^3$	29.0±20.7 b					100.0± 0.0 a
160–240	0.4	$5.960\pm0.170\times10^3$		10.0 ±10.1 f	67.0±16.4 c	78.0±12.5 b	53.0±21.3 b	100.0± 0.0 a
120–180	0.3	$4.681\pm0.193\times10^3$			51.0±10.9 d	46.0± 8.4 c	41.0±28.7 c	91.6± 7.4 a
100–150	0.25	$4.158\pm0.220\times10^3$						12.5±10.6 b
80–120	0.20	$3.733\pm0.241\times10^3$			23.0±12.7 f	34.0±23.8 d	20.0±21.8 d	
50–75	0.125	$2.642\pm0.261\times10^3$						7.5±17.7 b
40–60	0.1	$2.728\pm0.269\times10^3$			2.0± 3.0 f	7.0± 4.8 f	4.0± 3.7 e	
20–30	0.05	$1.000\pm0.398\times10^3$			5.1± 6.1 f	11.0±11.9 d	2.0± 4.5 e	13.3±13.1 b
00–00	0.00	Control	0.0± 0.0 c	1.50± 1.8 g	1.0± 2.3 f	5.0± 7.7 d	1.0± 2.3 e	0.0± 0.0 c

[1] Percent mortality rates (number dead larvae/10 exposed) determined 24 hours following exposure, confidence intervals of the mean determined with 5% error. Means followed by the same letter are not significantly different at 5% error using analysis of variance of a randomized block with Duncan's Multiple Range Test.

[2] Spores/ml determined by standard plate count method. Each entry is the estimated mean spores/ml determined by polynomial regression analysis ($\sqrt{y} = \beta_0 + \beta_1 x + \beta_2 x^2 + \beta_3 x^3$, where $x = 1 + \log_{10}$ dose) based on 8 replications of plated spore counts per weight/volume expression (PPM). Confidence intervals of estimated mean spores/ml determined at 5% error.

[3] International Toxic Units/milligram (ITU/mg).

occurring within each of the 6 time exposures at both the higher and lower treatment concentrations. The remaining intermediate mean mortality rates are significantly different.

A possible explanation for these rankings is that larval mortality occurs within a very narrow range of treatment concentrations. The clustering effects at both the upper and lower ends of the concentration spectrum appear to be a function of a susceptibility threshold. This is most noticeable at the 24-hr exposure time, where larval mortality increases nearly eightfold from 0.25 to 0.30 ppm treatment concentration.

As Table 1 reveals, the estimated mean spores/mL were not equally partitioned between weight/volume classes. This phenomenon was possibly a result of inconsistent and imperfect suspension of *Bti* spores when the powdered formulation was mixed with water in preparation for making dilutions and/or normal variations existing in quantifying plated spore counts. An important factor to consider is that spores/ml may not be directly and consistently correlated with toxicity (Dulmage 1971). However, attempting to correlate these factors will contribute to an understanding of the relationship between treatment concentration and corresponding larval susceptibility. In the field environment, it will also assist in determining spore distribution following treatment application.

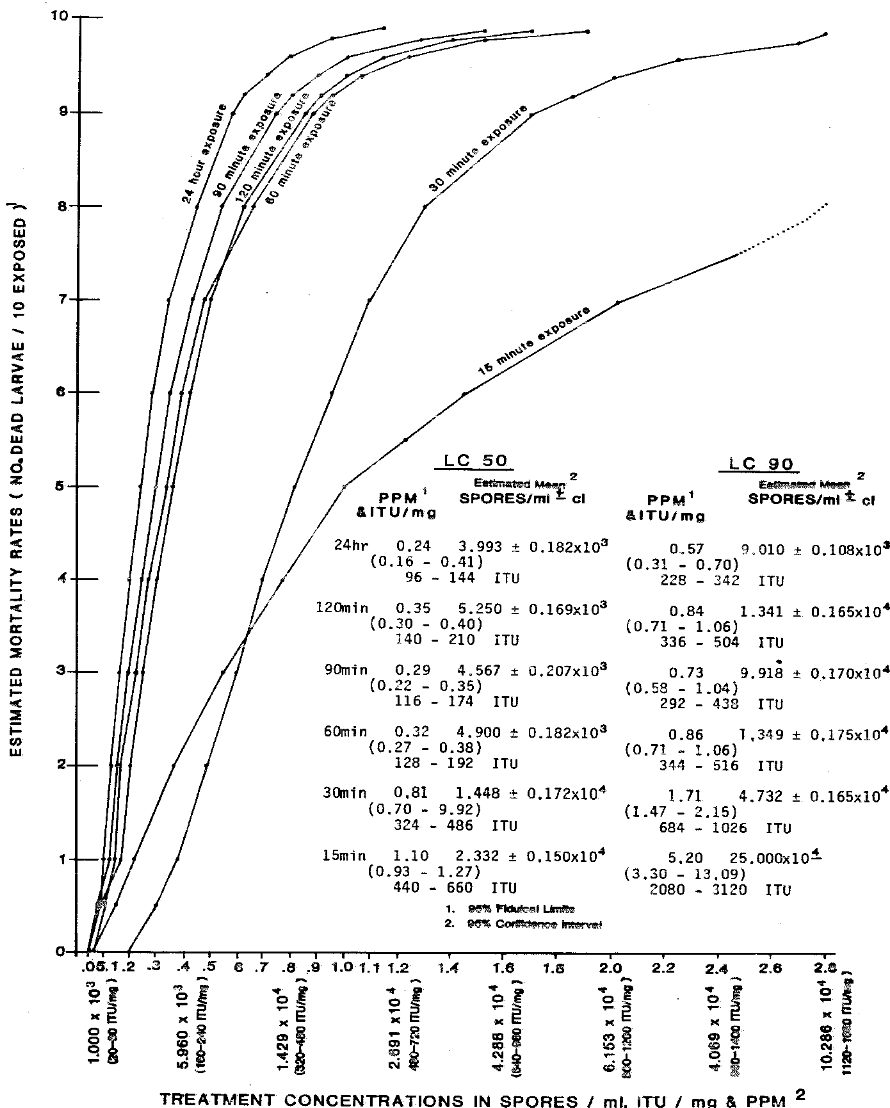
Figure 1 is a graphic presentation of Table 1 using probit analysis. The mortality of *S. vittatum* larvae observed after 15- and 30-min exposures was distinctly different from one another and from the exposures of longer duration. The results of exposures of longer duration are noticeably clustered together. The data indicate that larval exposures to *Bti* of durations longer than 60 min have little, if any, additional larvicidal effect. The LC50 and LC90 values, as illustrated in the insert on Figure 1, indicate increasing response of *S. vittatum* larvae to a given concentration of *Bti* as durations of exposure increase. As the exposure duration increases, the amount of *Bti* required to produce significant mortality decreases, with the sharpest decline occurring between 30- to 60-min exposures (Figure 2).

The fiducial limits should be considered as confidence zones or bands of the estimated mean dose of any particular concentration. However, doses were variable, as indicated by spores/ml. Therefore, when using laboratory data as a guide to field studies, the upper fiducial limits probably should be used to estimate concentration. Data from this study (Figure 2) indicate that minimum concentrations of 0.92 to 2.15 ppm of *Bti* should be used to achieve 50% to 90% mortality in larvae during a 30-min exposure. However, caution should be taken when using laboratory bioassay results for estimating field concentrations since actual concentration requirements for the field may be or are usually higher.

These findings differ somewhat from those reported by other workers. Laird et al. (1978) found 30-minute exposures of *Cnephia ornithophilia* (field collected) and *Simulium verecundum* (laboratory reared) to Research Unit Vector Pathology (RUNVP) *Bti* formulation resulted in LC50's of 1.5×10^3 spores/ml and 2.5×10^3 spores/ml and LC90's of 2.5×10^3 spores/ml and $5.6 = 10^3$ spores/ml, respectively. Thirty-minute exposures of *S. verecundum* using the Institute Pasteur Standard (IPS)-78 *Bti* (1000 UI/mg) powder required higher spores/ml for both LC50 and LC90, i.e., 5.0×10^4 spores/ml and 1.3×10^5 spores/ml, respectively.

However, the response of *S. damnosum* to the IPS-78 powder during a 30-minute exposure was similar to the findings reported here for *S. vittatum* with an LC50 of 1.5×10^4 spores/ml and an LC90 of 3.7×10^4 spores/ml (Undeen and Berl 1979).

Obviously, species differ in their response to the same material. Also, the same species respond differently to materials from different sources, even when concentration is quantified by spores/ml. The fact that concentrations presently can be standardized only by comparative bioassays reduces comparability of data



1.- Estimated mortality rate determined using probit analysis with log dose transformations

2.- See Table 1 Footnote 2 for explanation

Fig. 1. The susceptibility of *S. vittatum* to varying concentrations of *Bti* at 6 different time exposures.

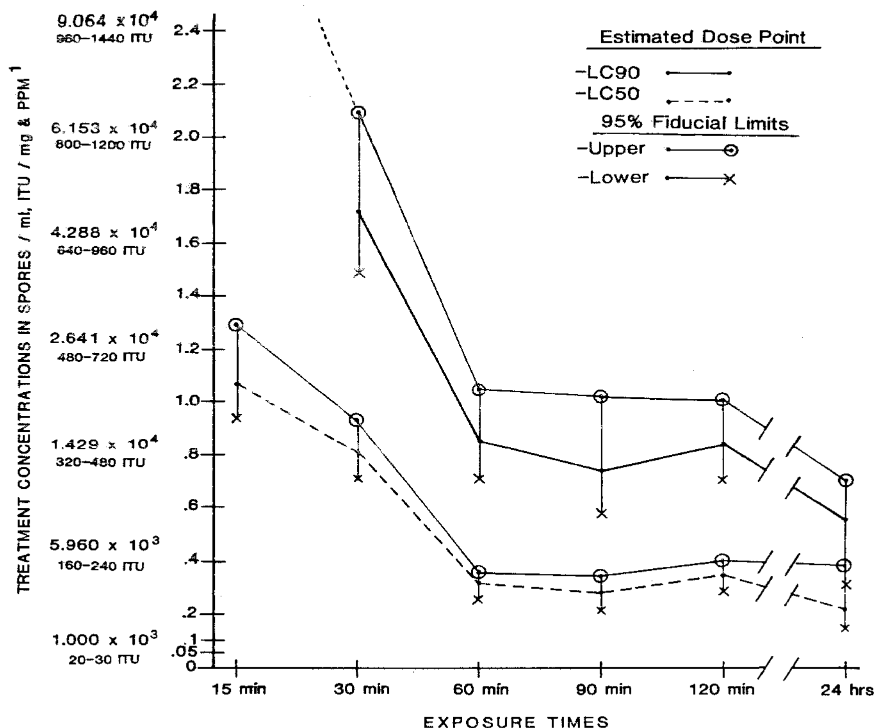


Fig. 2. The LC50's & LC90's of *Bti* to *S. vittatum* at 6 different time exposures.[2]

generated in different laboratories. This indicates that some materials are more reactive than others and presents the possibility of product improvement through strain selection and improving production and formulation methods.

References Cited

Barr, A. J., Goodnight, J. H., Sall, J. P. and Helwig, J. T. 1976. A user's guide to SAS 76. SAS Institute, Inc., Raleigh, NC.

Carestia, R. R., Frommer, R. L., Vavra, R. W. and Loy, V. A. 1974a. Field evaluation of black fly control-aerial applications. Mosquito News 34:330–332.

Carestia, R. R., Frommer, R. L., Vavra, R. W. and Loy, V. A. 1974b. Field evaluation of black fly control-ground applications. Proc. Calif. Mosq. Control Assoc. 42:93–95.

de Barjac, H. 1978. A new strain of *Bacillus thuringiensis* var. *israelensis* serotype 14. C.R. Acad. Sci., Paris. 286:797–800.

Dulmage, H. T. 1971. Production of delta endotoxin by eighteen isolates of *Bacillus thuringiensis*, serotype 3, in 3 fermentation media. J. Invertebr. Pathol. 18:353–58.

Duncan, D. B. 1955. Multiple range and multiple F tests. Biometrics 11:1–42.

Gaugler, R. and Molloy, D. 1980. Feeding inhibition in black fly larvae (Diptera: Simuliidae) and its effects on the pathogenicity of *Bacillus thuringiensis* var. *israelensis.* In press.

Goldberg, L. J. and Margalit, J. 1977. A bacterial spore demonstrating rapid larvicidal activity against *Anopheles sergentii, Uranotaenia unguiculata, Culex univittatus, Aedes aegypti* and *Culex pipiens.* Mosquito News 37:355–358.

Hembree, S. C., Frommer, R. L. and Remington, M. P. 1980. A bioassay apparatus for evaluating larvicides against black flies. Mosquito News. This number.

Hogben, D., Penvy, S. T. and Varner, R. N. 1971. Omnitab-II Users Reference Manual, NBS, Washington, DC.

Lacey, L. A. and Mulla, M. S. 1977. Evaluation of *Bacillus thuringiensis* as a biocide of black fly larvae (Diptera: Simuliidae). J. Invertebr. Pathol. 30:46–47.

Laird, M., Undeen, A. H., Colbo, M. H. and Nagle, W. L. 1978. Research unit on vector pathology. Memorial University of Newfoundland Annual Report. pp. 60.

Mulla, M. S. and Lacey, L. A. 1976. Feeding rates of *Simulium* larvae on particulates in natural streams (Diptera: Simuliidae). Environ. Entomol. 5:283–287.

Undeen, A. H. and Berl, D. 1979. Laboratory studies on the effectiveness of *Bacillus thuringiensis* var. *israelensis* de Barjac against *Simulium damnosum* (Diptera: Simuliidae) larvae. Mosquito News 39:742–745.

Undeen, A. H. and Colbo, M. H. 1980. The efficacy of *Bacillus thuringiensis* var. *israelensis* against black fly larvae (Diptera: Simuliidae) in their natural habitat. Mosquito News 40:181–184.

Undeen, A. H. and Nagel, W. L. 1978. The effect of *Bacilluis thuringiensis* ONR-60A strain (Goldberg) on *Simulium* larvae in the laboratory. Mosquito News 38:524–527.