

INTERPRETATION OF DENSITY DEPENDENT DATA IN COMPARISONS OF *BACILLUS THURINGIENSIS* VAR. *ISRAELENSIS* FORMULATIONS¹

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ABSTRACT

The effect of larval density on estimates of effectiveness of larvicides with *Bacillus thuringiensis* var. *israelensis* (BTI) as the active ingredient (a.i.) was evaluated. Bioassays with *Aedes aegypti* (L.) fourth instars showed that the toxic effect of a standard powder (IPS-78) and a wettable powder formulation (Bactimos®) was independent of volume because the effect of varying the water volume in test vessels was usually negligible and had no obvious relationship to larval mortality. Data were best interpreted as a function of dose (i.e., the absolute amount of a. i.) and the number of larvae in each test vessel rather than as a function of concentration (dose rate). This indicated that accurate comparisons of acute toxicity estimates derived from different tests with differing numbers of larvae (i.e., differing densities) can be made if results are reduced to and expressed as the EC₅₀/larva.

Key Words: BTI, Biological Control, Mosquito Larvicide, Potency Test, Efficacy Testing.

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INTRODUCTION

The discovery of *Bacillus thuringiensis* (Berliner) var. *israelensis* de Barjac (Goldberg and Margalit, 1977; de Barjac, 1978)⁶, which is highly effective for control of mosquito larvae, was followed by rapid development of commercially produced larvicide formulations. To verify potency of these formulations, the World Health Organization (WHO) developed a general protocol (de Barjac and Larget, 1979) for a bioassay with *Aedes aegypti* (L.) as the test species.

The purposes for measuring potency are for quality control and verification of trade and label claims. Potency conveys little that is relevant to performance or overall effectiveness of different BTI formulations because bioassay procedures for measuring potency are not designed for making direct comparisons of formulations or for estimating field performance. Therefore, extensive laboratory and field testing to judge general effectiveness normally precedes regulatory labeling and marketing of new formulations.

At the present time, standardized versions of the WHO potency protocol [(Rishikesh and Queleennec (1983); McLaughlin et al. (1984)] have been adopted.

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 view of the Department of the Army or the Department of Defense. Use of proprietary names does not constitute endorsement.

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⁶ Henceforth referred to as BTI.

However, no standard protocols for evaluating the effectiveness of BTI or other larvicide formulations appear to have gained wide acceptance among entomologists, who test aquatic, filter-feeding macroinvertebrates, although some proposals exist [U.S. Environmental Protection Agency (USEPA), 1977; Molloy, 1982]. The need for more standardization of practices is emphasized by (1) the degree of detail necessary for specifying bioassay test procedures that yield precise, reproducible results (Buikema et al., 1982); (2) regulatory guidelines (USEPA, 1982) for meeting data requirements for biological insecticides.

Sinegre et al. (1981) showed that the performance of BTI formulations in laboratory bioassays was influenced by larval density. In the following study, the density effect was studied further to determine how test results from different BTI formulations might be compared and interpreted when test conditions were different.

MATERIALS AND METHODS

Bioassays of the standard BTI powder, IPS-78, were conducted in eight different volumes of water (0.10, 0.15, 0.25, 0.40, 0.50, 0.60, 0.80 and 1.00 l) with 25 mosquito larvae in each treatment vessel. Bioassays were also conducted with 50 larvae in 0.10, 0.25, 0.50, 0.75 and 1.00 l of water. The test was replicated eight times. Bioassays of a commercial wettable powder formulations of BTI (Bactimos) were conducted in five volumes of water (0.10, 0.25, 0.50, 0.75 and 1.00 l). The density of larvae in each test volume was 1/10 ml.

Stock suspensions (1 mg/ml) of BTI were agitated in a rotating knife blender for two minutes and then mixed with a magnetic stirrer while aliquots for each of the treatments (six/bioassay) were withdrawn with a micropipette.

Boro-silicate glassware was used for all test vessels. Test vessels were selected so that diameter and depth increased proportionately. Within the 0.1 - 1.0 l range of test volumes, vessel diameter increased from 2.4 - 4.0 in (6.1 - 10.2 cm) and depth increased from 2.4 - 4.9 in (6.1 - 12.5 cm). All volumes and densities were tested simultaneously. Distilled water was used in all tests to minimize possible loss of toxin activity caused by carbonate reversal (Schnell and Nickerson, 1983).

All bioassays were performed with *A. aegypti* larvae that were hatched within a two-hour interval from eggs submerged in deoxygenated water under a partial vacuum and reared at 25° C at a density of 500/ml on an infusion of liver powder, brewer's yeast and ground swine chow. After 96 hours, early fourth instars were selected for use in bioassays.

Immobilization (i.e., the failure of larvae to swim when disturbed) was used as an indicator of mortality and results were recorded from bioassay treatments after 24 hours. Other procedures followed those of the WHO susceptibility test (Brown and Pal 1971).

Data were analyzed to estimate EC_{50} values⁷. Computations were made using the maximum likelihood estimate based on the probit model using SAS® PROC PROBIT⁸.

⁷ Effective Concentration, 50% endpoint; terminology follows that of the American Society for Testing and Materials, 1987.

⁸ SAS Institute (1985). SAS is the registered trademark of SAS Institute Inc., Cary, NC, USA.

RESULTS AND DISCUSSION

Results of IPS-78 tests (Table 1) indicated that the response of assay larvae was primarily a function of available toxin and independent of the volume of water in treatment vessels. This was indicated by the similarity of EC_{50} estimates for some tests in which larval density was constant. For example, the EC_{50} for 25 larvae in 0.25 l (0.1659 mg/l) was nearly identical with the estimate for 50 larvae in 0.50 l (0.1666 mg/l). For the Bactimos study, in which larval density was the same for all bioassays, all EC_{50} estimates were nearly identical (Table 2). The consistency was also apparent from the similarity of probit line slopes ($3.83 \leq m \leq 4.09$) derived from the Bactimos data. There was greater variation in the probit line slopes ($2.53 \leq m \leq 4.19$) derived from data from IPS-78 bioassays, in which larval density varied. A collateral observation of interest was that the increase in water depth and surface area in the larger test volumes apparently had no impact on the strict density dependent relationship exhibited by the Bactimos test results.

Table 1 . Toxicity of IPS-78* to *Aedes aegypti* (L.) larvae at various densities.

Volume (l)	No. of Larvae	EC_{50} (FL)
0.10	25	0.2545 (0.2394, 0.2695)
0.10	50	0.3511 (0.3377, 0.3650)
0.15	25	0.2194 (0.2065, 0.2329)
0.25	25	0.1659 (0.1547, 0.1791)
0.25	50	0.2106 (0.2012, 0.2217)
0.40	25	0.1441 (0.1336, 0.1583)
0.50	25	0.1258 (0.1043, 0.1473)
0.50	50	0.1666 (0.1603, 0.1732)
0.60	25	0.1075 (0.0927, 0.1224)
0.80	25	0.0872 (0.0820, 0.0927)
1.00	25	0.0673 (0.0629, 0.0719)
1.00	50	0.0933 (0.0844, 0.1224)

* International standard reference powder of BTI (1,000 IU/mg)

EC_{50} : Effective concentration (mg/l), 50% endpoint; FL: 95% fiducial limits; IU: International units of toxicity.

Table 2. Toxicity of Bactimos* to *Aedes aegypti* (L.) larvae at a uniform (1 larva/10 ml) density.

Volume (l)	EC_{50} (FL)
0.10	0.0516 (0.0416, 0.0610)
0.25	0.0529 (0.0502, 0.0551)
0.50	0.0484 (0.0414, 0.0551)
0.75	0.0502 (0.0469, 0.0534)
1.00	0.0521 (0.0471, 0.0570)

* Lot No. 2351 (3,500 IU/mg)

EC_{50} : Effective concentration (mg/l), 50% endpoint; FL: 95% fiducial limits; IU: International units of toxicity.

That results were density dependent, indicated that larvae ingested virtually all the BTI toxin available to them regardless of the concentration/water volume in individual bioassays. This explanation was supported by previously reported data about the filter feeding dynamics of mosquito larvae. In a study of *A. aegypti*, Misch *et al.* (1987) showed that fourth instars were capable of concentrating BTI by a factor of 10,000.

Within the range of small volumes used in the bioassays for the studies described here, the concentration would have varied at most by a factor of 10 when an identical amount of BTI was introduced into each test volume. Such a concentration difference was apparently inconsequential when encountered by the highly efficient feeding of *A. aegypti* larvae, leaving differences in larval density as the only important factor influencing test results.

Similar findings have been reported for studies of other filter-feeding species exposed to insecticides in suspended particulate form. Schmidt and Weidhaas (1959) found a density dependent relationship for DDT toxicity in mosquito larvae. Although the DDT was initially present as a colloidal suspension, its affinity for adsorption onto suspended particulates (Fredeen *et al.*, 1953) apparently made ingestion the primary route of entry.

Styrlund (1974) demonstrated a density effect for another insect pathogen (*Bacillus sphaericus*) in suspension and Wraight *et al.* (1982) found that some differences between results from laboratory and field trials of BTI against *Aedes stimulans* (Walker) were attributed to density variations.

However, the impact of density dependence on the performance of *B. thuringiensis* has sometimes been overlooked. Hall *et al.* (1977) tested the effectiveness of 127 strains of *B. thuringiensis*, but compared results derived from bioassays conducted at two different larval densities without adjusting for its effect. Mulligan *et al.* (1980) assessed persistence using larvae as sentinels in bioassays of BTI-treated habitat water, but failed to approximate the initial field site (catch basin) larval density in bioassay vessels. Had the toxin persisted in the water column, it was unlikely that it would have been detected because larval density in bioassays was much higher than that of the initial field density.

Farghal *et al.* (1983) found that larval density was the determining factor influencing failures of a BTI formulation applied at the maximum recommended rate as part of some operational control efforts. This indicated that label claims for some BTI formulations may not always be accurate for adequate control of some species when maximum field densities occur.

Singere *et al.* (1981) first determined the additive effect of larval density on observed BTI toxicity using laboratory bioassays conducted under a variety of conditions. They concluded that tests must be conducted under strictly defined conditions in which both the volume of water and the number of larvae are constant. Results reported here corroborated their data but indicated that water volume and/or the number of test larvae could vary provided that larval density was constant. Nevertheless, the narrow restrictions recommended by Sinigre *et al.* (1981) appear to be appropriate and necessary for standardized potency protocols to ensure accuracy because the objective is to characterize toxin activity in quantitative terms. When the relative performance and overall effectiveness of different BTI formulations is of interest however, varying some procedures in different laboratory studies should not necessarily invalidate a comparison of results if toxicity estimates are adjusted for the density effect (i.e., express the effective dose rate as the EC₅₀/larva).

Equilibration of the density effect might also be useful in some field performance studies. For habitats in which larval density is relatively easy to estimate (e.g., tree holes, catch basins, vernal pools), simulation of field density in laboratory bioassays might eliminate some of the guesswork involved in deriving application rates for initial field tests — a costly, labor intensive process. The elimination of bias caused by density differences might also facilitate the identification of some extrinsic factors influencing field performance and make possible the development of a method to compare laboratory and field performance of a biological control agent such as the efficiency index proposed by Burges (1973) for some non-aquatic insect pests.

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