Journal of the American Mosquito Control Association, 00(00):000–000, 2025

OPERATIONAL NOTE

FIELD APPLICATION RATE OF NATULAR® SC IN METRIC UNITS TARGETING *AEDES ALBOPICTUS*

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ABSTRACT. NATULAR®SC, mosquito larvicide, was laboratory and field-tested against *Aedes albopictus* to determine the application rate in metric units (μl/liter). The objective was to estimate in microliters (μl) the amount of formulated NATULAR SC to apply per liter of habitat water. Replicated dose response experiments were conducted on lab-reared third instar *Ae. albopictus* under controlled laboratory conditions followed by probit analysis. The median lethal dose, LD_{50}, was estimated to be 0.07 μl/liter (95% C.L. 0.054,0.094). Control mortality was less than 1%. The LD_{95} was estimated to be 0.54 μl/liter (95% C.L. 0.323,1.265). According to the World Health Organization Pesticide Evaluation Scheme (WHOPES) doubling the LD_{95} approximates the diagnostic dose and is an estimate of the field application rate. The next step consisted of outdoor field trials in 5-liter buckets. Twelve application rates ranging from 0.1 μl/liter to 2.0 μl/liter were tested. Results indicated 1 μl/liter was an optimum application rate. A major conclusion is no single best application rate will kill 100% of susceptible mosquito larvae. An optimal field rate balances efficacy with minimizing the concentration of pesticide. In every treatment a small probability of larval survival is always present. This is a compelling argument for rotating active ingredients according to their mode of action.

KEY WORDS Application rate, NATULAR®SC, spinosad

When a new product or formulation becomes available, one of the first things to do is to establish an insecticide resistance baseline for target mosquitoes. The baseline is the initial data point, the median lethal dose (LD_{50}) before target mosquitoes respond to selection pressure in the field. The baseline is the reference point for comparing mosquito response over time. Increases in the LD_{50} are evidence of developing insecticide resistance. This operational note establishes the baseline for NATULAR® SC.

Aedes albopictus (Skuse) is an important vector of arboviruses, including Zika, dengue, and chikungunya viruses, and has recently expanded its range in the eastern United States to New York and southern New England (Gloria-Soria et al. 2020). *Aedes albopictus* is a container habitat mosquito (Hawley et al. 1987). Cans, buckets, tree holes, flowerpots, and tires are all common habitats. The volume of these containers is measured in ml and liters, not acres. The NATULAR SC label states the field application rate in fluid ounces/acre. This note addresses that issue.

NATULAR SC was registered by the U. S. Environmental Protection Agency in January of 2021. The NATULAR label states the field application rate in the Imperial system, pounds per acre and fluid ounces per acre. Pounds per acre may be convenient for helicopter spraying of salt marsh mosquitoes, but microliters per liter (μl/liter) are more appropriate for treating container habitats. *Aedes albopictus* treatment sites are tree holes and tires with very low acreage. The objective was to estimate the LD_{50} and LD_{95} in μl/liter and to provide guidelines for field application rates in the Système International d'Unités (SI) system, the metric system.

The work reported here is an effort to promote insecticide resistance monitoring beyond the traditional Centers for Disease Control and Prevention (CDC) bottle bioassay to include "off-the-shelf" pesticides that every mosquito control program maintains on premises.

A sample of NATULAR SC (liquid suspension concentrate) was obtained from Clarke Mosquito Control Products (Roselle, IL) in late 2023. A formulated product was chosen over an analytical-grade active ingredients because that is what operational mosquito control programs use. During the winter of 2023, colonized *Ae. albopictus* third instars were used for range finding dose-response experiments to estimate the median lethal concentration, LD_{50}. One ml of NATULAR product was pipetted into 1 liter of deionized water to make the stock solution. The SI system will be used throughout this study. Serial dilutions were prepared. Range-finding bioassays determined mortalities greater than zero and less than 100%. Probit analysis enabled estimation of the median lethal dose LD_{50} and the LD_{95}. The LD_{95} is key to establishing the diagnostic dose. The diagnostic dose is defined by the World Health Organization Pesticide Evaluation Scheme (WHOPES) as twice the LD_{95} (WHO 1998). Chang and Su published a series of papers on their experiences with various formulations of NATULAR (Su and Chang 2012, Su et al. 2014). Their LD estimates were the starting point for bioassays. In addition, the Safety Data Sheet for NATULAR SC provided useful toxicological data

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that narrowed range finding. For example, the "no observable effect concentration" (NOEC) for water fleas, *Daphnia magna* (Straus) was 1.2 μg/liter or ≈ 1 ppm.

Based on published reports (Su and Chang 2012), range finding experiments began with concentrations at 0.01 μl/liter and the highest at 1.0 μl/liter. Focus was on colonized *Ae. albopictus*. This species is a container-inhabiting mosquito appropriate for liquid larvicide. The colony was established October 2019 from larvae collected in steel can ovitraps placed in a woodlot, Flemington, NJ 08822 [40.5075053 -74.8545709] that was a prime candidate for larviciding. The colony was maintained in an insectary with controlled light wavelength (5,000 degrees Kelvin), photoperiod (16 h light:8 h dark), and temperature (27 degrees C). Relative humidity was maintained by steel cans of deionized water lined with seed germination paper (Anchor Paper Co., Saint Paul, MN 55101). The paper also served as the oviposition substrate.

Dose-response experiments were replicated 4 times. Fresh stock solution was prepared for each replicate. Each bioassay used 5 or more concentrations that yielded mortality greater than 0% but less than 100%. When we report concentrations they are parts of NATULAR SC (the formulated product), not spinosad (the active ingredient). Sample size was approximately 100 larvae per concentration. Probit analysis allows the per cent mortality, the dependent variable (y-axis), to be expressed as a function of the concentration of insecticide, the independent variable (x-axis). This function allows us to estimate LC_{95} which, in turn, estimates the diagnostic dose for insecticide resistance testing according to WHOPES guidelines. The variance-covariance matrix was used to estimate the 95% confidence limits using the laptop program POLO SUITE (LeOra Software, Petaluma, CA) (Robertson et al. 2020). The project was subdivided into three phases: microcosm, mesocosm, and macrocosm. The microcosm studies were conducted in 250 ml final volume using disposable 532 ml SOLO® cups [Dart Container Corporation, Mason, MI 48854]. These studies were conducted indoors at 27 degrees C, using laboratory-reared *Ae. albopictus*. Results were scored every 24 h for 96 h. Mesocosm studies were conducted indoors at 27 degrees C in 5-liter buckets, using *Ae. albopictus* larvae in clean de-ionized water. Macrocosm studies were conducted outdoors under highly variable temperatures in an oak/maple wood lot where construction equipment was stored. Tractor tires, roadside guardrails, and steel girders peppered the site. Field-collected water containing leaves and other debris was used to fill the 5-liter buckets. The mosquito genera recorded outdoors included *Aedes*, *Culex*, *Anopheles,* and *Toxorhynchites*. Voucher mosquito specimens were prepared and archived in the Hunterdon County mosquito collection. Many other insect species were present: Chironomids, grasshoppers, leafhoppers, crickets, silphids, assorted beetles, etc. On one occasion, a drowned mouse was recorded in one outdoor bucket. A major difference between the mesocosm and macrocosm studies was that the number of dead larvae could be counted in the mesocosm studies because the water was "clean." This was impossible in the macrocosm studies that had "dirty" water, so either some alive or ALL DEAD were recorded.

PART 1

Microcosm dose-response bioassays were analyzed by probit. The results are shown in Table 1 and Fig. 1. The slope and intercept were subjected to a t-test. The value of the t-Ratio, $t_{.05[\infty]} = 1.96$, indicated that the slope was significant and that the treatment had an effect. The t-Ratio is not the same as a goodness-of-fit test, which tests how well the data fit the probit model. At $P = 0.05$, the Chi-square value was 7.73 (nonsignificant) for 3 degrees of freedom (df). Heterogeneity was 2.58 (χ^2 divided by df), which means the data fit the probit model (Robertson et al. 2020). There was mortality in the controls. At the lowest test concentration, 0.01 ul/liter, there was 1 dead larva in the treatment and 1 dead larva in the control. The Abbott's correction formula (Abbott 1925) was not applied. No other control mortality was recorded.

PART 2

The diagnostic dose calculation (2 X LD_{95}) yielded 1.08 μl/liter. Results of macrocosm trials suggest a field application rate of 0.9 μl/liter – 1.0 μl/liter (depending on temperature, instar, organic matter). Macrocosm studies were exploratory and not analyzed statistically because there was too much variation. The optimum field application rate was 1 μl/liter. The matrix was complex because there were too many variables impacting outcomes e.g., temperature, larval instars, mosquito species other than *Ae. albopictus*, other insect (and arthropod) species. Trials better described the macrocosm; there was too much variation to allow use of the term replicate. One way to reduce this variation was to use only third instars. Late fourth instars or pupae must be excluded because they are metabolically different than thirds because they do not eat. Ingestion of NATULAR is an important pathway in how the pesticide enters the body of the insect. Trials must be limited to a narrow temperature window.

PART 3

Mesocosm studies, which were done indoors during the winter 2024/2025, enabled us to corroborate results from the outdoor tests. Mesocosm studies (4, 5-liter buckets) showed that 0.9 μl/liter yielded 99.7% mortality (n = 849). Two live third instars were recorded at 24 h. There was no control mortality.

This project began with a question: What is the minimum amount of "off-the-shelf" NATULAR SC that will kill 100% of susceptible larvae in a known volume of water? The label states to apply 2.9 fluid ounces per acre. For container mosquitoes like *Ae. albopictus* volume is more important than surface area. The diagnostic dose is not the same thing as the 100% lethal

Table 1. Probit results.

NATULAR® SC July 31, 2024, Final			
Slope/Intercept t test > 1.96			
	Parameter	Standard Error	T-Ratio
Intercept	2.146	0.125	17.076
Slope	1.864	0.102	18.641

Chi Square Goodness of Fit Test

µl/liter	n	Respond	Expected	Residual	Probability	Standard Error
0.01	213	11	12.07	−1.07	0.056	−0.319
0.026	210	50	43.92	6.07	0.209	1.030
0.05	220	72	85.76	−13.76	0.389	−1.903
0.1	272	179	166.16	12.84	0.610	1.596
0.26	270	227	230.68	−3.68	0.854	−0.635

Variance Covariance Matrix

	Intercept	Slope
Intercept	0.015	0.011
Slope	0.011	0.010

Lethal Dose Matrix and Confidence Limits

LD	Lethal Dose (µl/liter)	Conf. Limit	90%	95%	99%
50	0.07	Lower	0.058	0.054	0.041
		Upper	0.087	0.094	0.127
95	0.54	Lower	0.362	0.323	0.239
		Upper	0.961	1.265	5.076
99	1.25	Lower	0.736	0.635	0.430
		Upper	2.729	3.972	26.832

Chi square = 7.739 (ns); $P = 0.05$; Degrees of freedom = 3; Heterogeneity = 2.579.

dose (LD_{100}) (Petersen et al. 2004). Because of statistical problems defining the LD_{100}, including the exceedingly large confidence limits, the diagnostic dose is defined as 2 times the LD_{95}, a value with tighter confidence limits.

What is the relationship of diagnostic dose to field application rate? That is the question addressed in the macrocosm studies. The hypothesis of the macrocosm studies is 2 X LC_{95} will kill 100% of susceptible third instar *Ae. albopictus*.

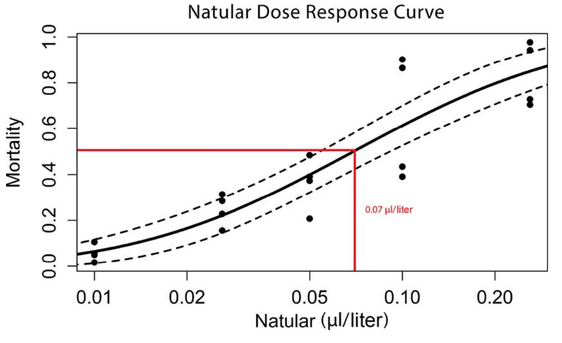

Fig. 1. Regression plot showing the estimation of the median lethal dose (LD_{50}).x-axis, log scale; y-axis, probit scale.

As a practical matter, the objective was to establish, by trial and error, the minimal amount of NATULARSC that will kill the maximal number of susceptible mosquito larvae in a tree hole, tire or similar habitat.

Probits or probability units are a means of transforming data from sigmoid dose response curves to give a straight line (Bliss 1934). Raw data of pesticide effects on insects usually yield an asymmetrical curve that is difficult to interpret, especially at values approaching 100% mortality, which tend to flatten to an asymptote. This problem can be resolved if percent mortality and pesticide concentration are transformed to units that plot on a straight line permitting the application of standard statistical techniques like linear regression.

Our original question asks about 100% kill. Probit analysis is based on the cumulative frequency distribution (Finney 1947). The normal frequency distribution extends from negative to positive infinity. There is no 0% or 100%. There is always a probability, however small, of an "outlier." The best one can do is optimize pesticide treatments, balancing effectiveness with minimizing pesticide use. The data presented here indicate that 1 µl/liter is an optimum concentration.

A major conclusion of this study is that there is no single best application rate that will kill 100% of susceptible mosquito larvae. An optimal field rate balances efficacy against minimizing the concentration of pesticide released into the environment. Some larvae will always survive treatment. This is not a pesticide failure. It is an intrinsic characteristic of the normal probability distribution and a compelling argument for rotating active ingredients according to their mode of action. This operational note establishes the baseline LD_{50} response of *Ae. albopictus* as 0.07 µl/liter (95% C.L. 0.054, 0.094).

The active ingredient in NATULAR SC is spinosad, which belongs to the Insecticide Resistance Action Committee (IRAC) Group 5 [nicotinic acetyl-choline receptor (nAChR) allosteric modulators] and could be applied in rotation with the larvicides *Bacillus thuringiensis israelensis* (*Bti*) (IRAC Group 11) and methoprene (IRAC Group 7).

Future work will focus on baseline data of mosquito species such as *Culex pipiens* (L).

This summer project was supported by the Northeast Regional Center for Excellence in Vector Borne Diseases at Cornell University in the form of an internship awarded to Abigail Golembiewski [summer internship SI-2024-098321]. Funding was provided by the Centers for Disease Control and Prevention (CDC) (NU50CK000633). Jack Petersen thanks Larry Hribar, Phil Lounibos, and Leonard E. Munstermann for helpful comments. The content of this research is solely the responsibility of the authors and does not necessarily represent the official views of the CDC.

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