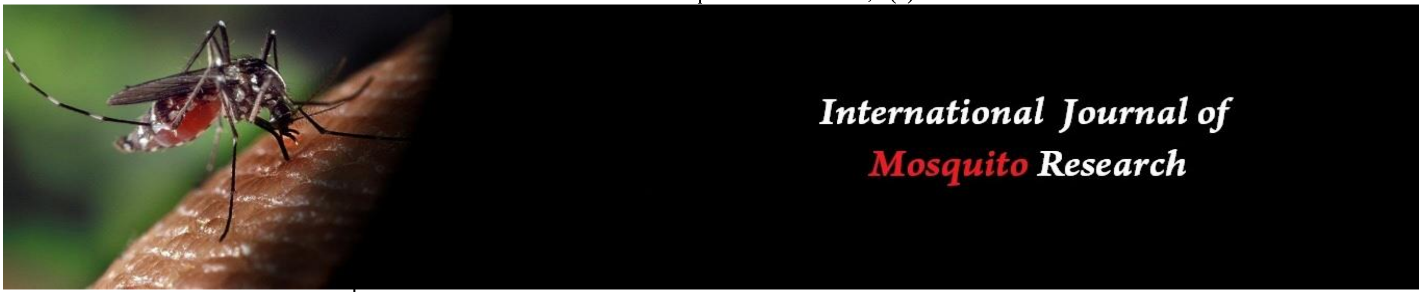

ISSN: **2348-5906**
CODEN: **IJMRK2**
IJMR 2021; 8(1): 58-64

www.dipterajournal.com
Received: 28-11-2020
Accepted: 30-12-2020

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Silica granules to embed *Bacillus thuringiensis israelensis:* Biological control of mosquito larvae

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Abstract
A new application technique for the control of mosquito larvae under tropical conditions was conducted in Cebu City, Philippines. The study used silica granules to evaluate a totally new technique to embed, store, and release the endotoxins of microbial agents. A bacterium, *Bacillus thuringiensis israelensis* was embedded in silica granules. It further assessed the efficacy of the embedded bacterium in silica granules (product) under laboratory and field conditions. Six concentrations were prepared. Each of the concentration was tested with 25 3rd instar larvae of *Aedes aegypti*. Results show that in laboratory test, LC_{50}, LC_{90} and LC_{99} of 0.593, 2.226 and 6.543 granules are needed to eliminate 50%, 90% and 99%, respectively of the mosquitoes exposed to the product. Moreover, the number of granules continues to increase until the 16th day after treatment except for the LC_{90} and LC_{99} that it subsides during the 16th day. Field test shows LC_{50}, LC_{90} and LC_{99} of 0.290, 2.640 and 15.975 granules which are needed to terminate 50%, 90% and 99% of mosquito larvae. The product is proven to kill the larvae of mosquito with mortality rate that varies in relation to the number of granules applied and to the number of days the granules present in the water. Further, it is proven to be environmental friendly, less expensive, easy to produce and apply.

Keywords: *Aedes aegypti*, *Bacillus thuringiensis israelensis***,** mosquito, silica granules, biological control, dengue control

1. Introduction
Mosquitoes (family Culicidae) are at the center of worldwide medical entomological research primarily because of their importance as vectors of dangerous diseases, such as malaria, dengue, yellow fever, encephalitis, lymphatic filariasis and chikungunya. They play a major role in life quality and health of humans. Mosquitoes are tiny insects but the damage they incur on mankind is enormous [1].

Of all disease-transmitting insects, the mosquito is the greatest menace. There are over 3,200 species of mosquitoes in the world. Of this figure, approximately 1,000 species are considered as vectors or carriers of pathogens and about 60 are considered dangerous because they are responsible for transmission of pathogens that can kill humans. Some species belonging to the top 60 are; *Aedes aegypti* (main carrier of dengue and yellow fever viruses), *Anopheles gambiae* and *An. funestus* (vectors of malaria and filariasis), *Culex pipiens* complex (responsible for filariasis and arboviruses), and *Aedes albopictus* (vector of epidemic chikungunya) [2].

Malaria is endemic in 91 countries, with about 40% of the world's population at risk. By undermining the health and working capacity of hundreds of millions, it is closely linked to poverty and stunts social and economic development. Up to 500 million cases occur every year, 90% of them in Africa, and there are up to 2.7 million deaths annually [3].

Dengue is the world's most important mosquito-borne virus disease, with 2500 million people worldwide at risk of infection and 20 million cases a year in more than 100 countries. In 1995, the worst dengue epidemic in Latin America and the Caribbean for 15 years struck at least 14 countries, causing more than 200 000 cases of dengue fever and almost 6000 cases of the more serious dengue haemorrhagic fever [3].

Many major cities of the world, especially in the Americas, are at risk of potentially devastating epidemics of yellow fever because they are infested with *Aedes aegypti* mosquitos which can transmit the disease.

Lymphatic filariasis (elephantiasis) infects about 120 million people in tropical areas of Africa, India, South-East Asia, the Pacific Islands and South and Central America [3].

Mosquitoes are extremely successful organisms due to their ability to adapt to a wide range of habitats. They are found throughout the world, except in deserts and permanently frozen areas. Mosquito larvae colonize a wide range of water bodies, temporary and permanent, highly polluted as well as clean, large or small, stagnant or flowing, even the smallest accumulations such as water-filled buckets, flower vases, old tires, hoof prints or leaf axils [2].

Like all Diptera, mosquitoes exhibit complete metamorphosis. All mosquitoes need aquatic habitats for their development. After hatching they pass through four larval instars, and a pupal stage where the transformation to the adult takes place. Most species are un-autogenous, it means after copulation the females have to take a blood- meal to complete egg development [2].

Adult mosquitoes vary greatly in their bionomics, e.g. concerning the host seeking, biting and migration behavior and strategy for reproduction. It is the medical importance and the troublesome behavior of mosquitoes that has aroused the interest of scientists.

Humans have to compete with insects for many of his fundamental needs, and for this reason battle lines have been drawn out since prehistoric days. There is evidence that humans have been engaged in war with pests for more than 4000 years [4]. Astonishingly, although the conflict is old, its dynamics and the behavior of its main participants are still not well understood. Even the objectives, at least of man, are poorly defined. Only in recent decades have people begun to ask fundamental questions, and to search for sophisticated methods which would respect both nature, and human needs.

In the early days man used inorganic materials to combat insect pests. The Sumerians apparently used sulphur compounds to control insects well before 2500 BC. The Chinese in 1200 BC used plant-derived fumigants as well as mercury and arsenic compounds as insecticides [5]. Most of the methods and materials usually based on superstition and folklore were useless. Frustration with insurmountable insect problems often resulted in the use of illogical methods to overcome the difficulties of plagues and/or damages caused.

When millions of humans are killed or disabled annually from insect borne diseases, and the World toll of insects, vector borne diseases, weeds, and rodents is estimated at S 100 billion annually, it becomes apparent that the control of various harmful organisms is essential for the future development of human health, agriculture and industry. In the process of accommodating these vital human requirements, pesticides have consequently become an indispensable part of the global process. In the 1940s the chemical era was opened with the discovery of synthetic organic insecticides, and a totally new concept of insect control began.

When mosquito populations are exposed to selection pressure from insecticides, they may become resistant. Meaning to say, they developed ability in a strain of insects to tolerate doses of toxicants which would prove lethal to the majority of individuals in a normal population of the same species [6].

Biological Control is the broadest sense, is defined as the reduction of the target population by the use of predators, parasites, pathogens, competitors or toxins from microorganisms [7].

Biological control aims to reduce the target population to an acceptable level and at the same time to avoid side-effects to the ecosystem. As far as mosquito control is concerned, biological control measures should integrate the protection of humans from mosquitoes with the conservation of biodiversity, while avoiding toxicological and eco-toxicological effects. As a result, the regulatory power of the ecosystem is maintained by protecting the existing community of mosquito predators.

Mosquitocidal bacteria have been known since the early 1960s when the first strains of Bacillus sphaericus with larvicidal activity were discovered [8]. However, these strains were not sufficiently toxic to merit commercial development. The discovery of the gram-positive, endospore-forming soil bacterium *Bacillus thuringiensis* ssp. *israelensis* (*Bacillus thuringiensis israelensis*) in the Negev desert of Israel in 1976 and of the potent strains of *B. sphaericus* in recent years have inaugurated a new chapter in the control of mosquitoes and blackflies [9-11].

The study aims to evaluate a totally new technique to embed, store, and release the endotoxins of microbial agent.

Specific aims:

To assess:

1. the efficacy of new product in laboratory and field conditions by applying different concentrations against the larvae of *Aedes aegypti,* and;
2. the new product in terms of effectivity and cost benefit, compared to the established products available in the market for the control of mosquito larvae.

2. Materials & Methods

2.1 Laboratory Test

2.1.1. Range Finding Test (Assessment of the potency and the minimum effective dosage in the laboratory)

The silicate that was embedded with endotoxin of *Bacillus thuringiensis israelensis* was evaluated against the larvae of mosquito under laboratory conditions. Bioassays were conducted according to World Health Organization (WHO) guidelines (World Health Organization 1981) at 6 different concentrations with controls in 3 replicates.

Twenty five early 4th instar larvae of *Aedes aegypti* (Linn.) in 2 ml of water were added to plastic disposable cups filled with 148 ml of distilled water. Larvae were not fed during the tests. Mortality readings of larvae were taken after 24 h of treatment until a 50% mortality rate was achieved. After obtaining the desired mortality rate (50%), another batch of mosquito larvae were released in the same preparation until a zero mortality rate of the larvae was achieved. The LC_{50}, LC_{90}, and LC_{99} values were calculated using logit and probit analysis.

2.1.2. Definitive Test

Six different concentrations based on the most promising result from the range finding test were tested. The same procedure as in the range finding test was applied.

2.2. Field Test

Assessment of the optimum effective dosage in the field

Based on the results achieved in the laboratory, the optimum effective dosage for field applications was determined. Twenty one plastic containers (each has 100L water capacity) that were naturally infested with *Aedes aegypti* larvae were used in the study. Four to six increasing concentrations were applied. All trials were carried out in triplicate with control, and the concentration was chosen to produce at least 3 larval mortalities between 20 and 95% for computerized LC_{50}, LC_{90}, and LC_{99} values.

Before the application of the new product, the following parameters were observed: no. of 1st and 2nd larval instars, 3rd and 4th larval instars as well as pupae; the location of a container (shaded or un-shaded); a container covered or uncovered; a container painted or unpainted. Additionally, a sign “Do not Touch” was posted in each container to avoid usage of water.

Mortality rate of the larvae will be evaluated in every 24 hours after the treatment and then on every 2 days until a zero percent mortality rate will be achieved. If re-infestation of more than 10 3rd instar larvae, re-application of the product was applied. The data obtained from laboratory and field tests were calculated and analyzed by using Probit and Logit models.

3. Research Environment

The study was conducted in Cebu which is located at the center of the archipelago (9º25’ N and 11º30’ N and between 123º25’ E) about 400 miles south of Manila. The topography of Cebu is characterized by narrow coastlines, limestone plateaus, and coastal plains but with predominant rolling hills and rugged mountain ranges traversing the northern and southern lengths of the island. The climate is relatively moderate, having no distinct wet and dry season with a temperature range of between 23-33ºC (73-91º F). The coolest temperatures occur in January and the warmest in May. Cebu has a population of about 3.5 million with the city proper accounting for 718,821.

4. Results & Discussions

Table 1: Mortality Rate Distribution among Mosquito Larvae in the Laboratory After the first 24 hour Exposure to the New Product Application.

Days After Granule Application	Probability Model	Slope	Intercept	LC_{50}	LC_{90}	LC_{99}
Day 1	Logit (P)	4.195	0.796	0.646	2.158	8.047
	Probit (P)	2.232	5.506	0.593	2.226	6.543
Day 4	Logit (P)	3.781	0.584	0.701	2.670	11.499
	Probit (P)	2.043	5.384	0.649	2.748	8.920
Day 7	Logit (P)	3.065	0.493	0.690	3.597	21.797
	Probit (P)	1.713	5.320	0.650	3.641	14.832
Day 10	Logit (P)	3.457	0.202	0.874	3.776	18.653
	Probit (P)	1.972	5.145	0.843	3.823	13.115
Day 13	Logit (P)	3.071	-0.043	1.033	5.363	32.367
	Probit (P)	1.858	4.972	1.035	5.070	18.508
Day 16	Logit (P)	3.839	-0.460	1.318	4.924	20.744
	Probit (P)	2.212	4.744	1.305	4.956	14.706

As displayed above in table 1, the LC_{50} of 0.646 during the first day of application of the new product implies that 0.646 granules are needed in order to terminate 50% of the mosquitoes using the logit model, while an LC_{90} of 2.158 signifies that we need only 2.158 granules of the new product to extinguish 90% of the mosquitoes. Furthermore, an LC_{99} of 8.047 means that we need 8.047 granules in terminating 99% of the mosquitoes. These values are increasing as the number of days is increasing after the treatment of the new larvicide. Similarly, as pointed out in the table above, an LC_{50} of 1.318, LC_{90} of 4.924 and an LC_{99} of 20.744 indicate that 1.318 granules, 4.924 granules and 20.744 granules are needed respectively to remove 50%, 90% and 99% of the mosquito larvae after the first 24-hour exposure to the solution during the 6th day after larvicide treatment. Also the table shows the LC_{50}, LC_{90} and LC_{99} of 0.593, 2.226 and 6.543 granules are needed to eliminate 50%, 90% and 99% of the mosquitoes exposed using the probit model.

Likewise these values keep increasing as the days after the application of the new product are increasing. As denoted from the result presented, the number of granules continues to increase until the 16th day after treatment except for the LC_{90} and LC_{99} that it subsides during the 16th day.

Table 2: Mortality Rate Distribution among Mosquito Larvae in the Laboratory After the first 48-hour Exposure to the New Product Application.

Days After Granule Application	Probability Model	Slope	Intercept	LC_{50}	LC_{90}	LC_{99}
Day 2	Logit (P)	4.087	1.440	0.444	1.532	5.915
	Probit (P)	2.087	5.870	0.383	1.575	4.988
Day 5	Logit (P)	4.730	0.969	0.624	1.819	5.845
	Probit (P)	2.501	5.604	0.574	1.867	4.885
Day 8	Logit (P)	2.883	1.056	0.430	2.487	16.878
	Probit (P)	1.519	5.658	0.369	2.572	12.530
Day 11	Logit (P)	3.568	0.751	0.616	2.543	11.949
	Probit (P)	1.969	5.460	0.584	2.614	8.871
Day 14	Logit (P)	3.409	0.381	0.773	3.410	17.225
	Probit (P)	1.969	5.235	0.759	3.400	11.540
Day 17	Logit (P)	2.623	0.245	0.807	5.550	45.543
	Probit (P)	1.568	5.151	0.801	5.260	24.383

As exhibited in the table above, the LC_{50} of 0.444 during the second day of exposure to the new product denotes that 0.444 granule are needed of the new product in order to eliminate 50% of the mosquitoes using the logit model while an LC_{90} of

1.532 means that 1.532 granules of the new larvicide are required to extinguish 90% of the mosquitoes exposed. Moreover an LC_{99} of 5.915 signifies that 5.915 granules of the new treatment are needed to remove 99% of the mosquito larvae exposed.

It is quite apparent from the results that these treatment requirement increases as the number of days after the new product application increases. Specifically, during the 17th day after the treatment application, 0.807, 5.550 and 45.543 granules are required to extinguish 50%, 90%, and 99% of the mosquito larvae after the 48-hour exposure to the solution with the new product application. Likewise, it is also manifested in table 2 above the LC_{50}, LC_{90}, and LC_{99} of 0.383, 1.575 and 4.988 granules respectively as requirement to terminate the 50%, 90%, and 99% of the mosquitoes exposed after the first 48-hour of the new product application using the probit model. Similarly, these values are increasing all throughout the experiment until the 17th day after the larvicide treatment which demonstrates its diminishing power to extinguish the mosquitoes exposed.

Table 3: Mortality Rate Distribution among Mosquito Larvae in the Laboratory After the first 72-hour Exposure to the New Product Application.

Days After Granule Application	Probability Model	Slope	Intercept	LC_{50}	LC_{90}	LC_{99}
Day 3	Logit (P)	All mosquitoes or 100% of them were terminated after 72-hour of treatment exposure.				
	Probit (P)					
Day 6	Logit (P)	All mosquitoes or 100% of them were extinguished after 72-hour of treatment exposure.				
	Probit (P)					
Day 9	Logit (P)	2.406	1.880	0.165	1.355	13.451
	Probit (P)	1.190	6.111	0.1164	1.391	10.509
Day 12	Logit (P)	4.240	1.055	0.564	1.860	6.840
	Probit (P)	2.254	5.639	0.521	1.928	5.604
Day 15	Logit (P)	3.810	0.969	0.557	2.101	8.947
	Probit (P)	2.085	5.584	0.525	2.161	6.854
Day 18	Logit (P)	2.393	0.825	0.452	3.746	37.637
	Probit (P)	1.317	5.520	0.403	3.788	23.5415

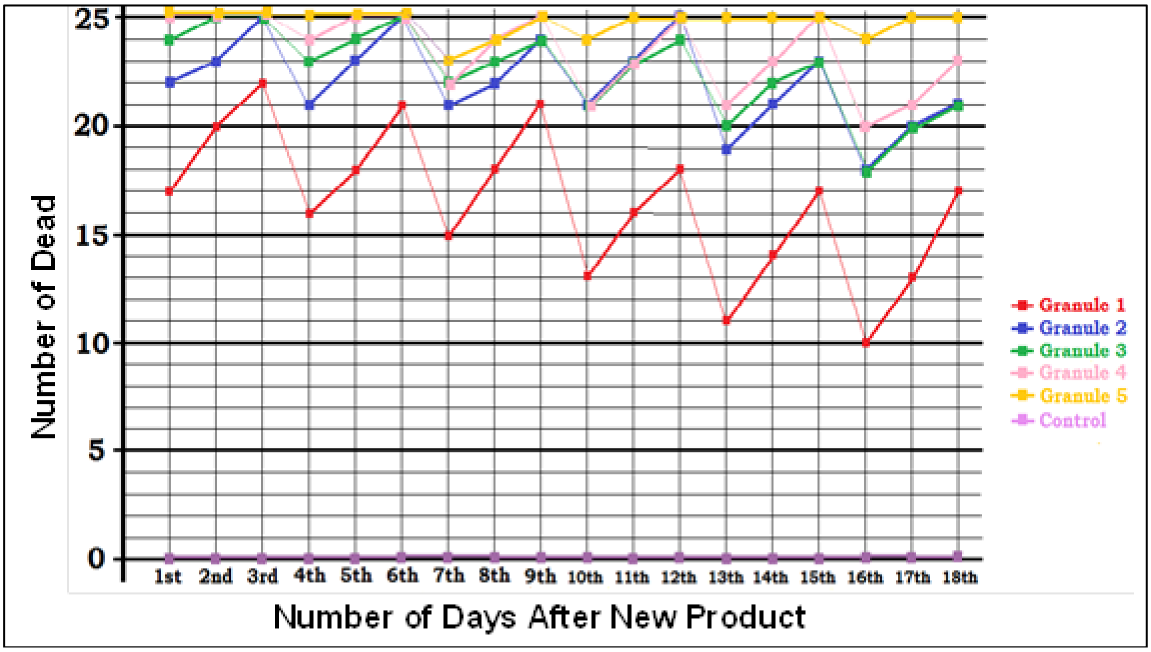

Fig 1: Mortality Distribution Among the Six Batches of 25 Mosquito Larvae Exposed to the New Product Application in the Laboratory of 18 Days Across Five Different Granule Concentrations

As displayed above in table 3 and figure 1, all mosquitoes or 100% of them were terminated after the 72-hour of treatment exposure. This simply means that none of them survived after the 72-hour exposure to the new product application. Even when the new set of 25 mosquito larvae were exposed to another 72-hour starting from Day 3 of treatment application of the new larvicide, none of them survived, which demonstrates the 100% capacity of the treatment in all of the 5 different concentrations.

However, its capability to exterminate mosquito larvae started to subside in the third set of new 25 mosquito larvae exposed to the granule product application. The result shows the LC_{50}, LC_{90}, and LC_{99} of 0.165, 1.355 and 13.451 granules respectively are the needed requirement to extinguish 50%, 90%, and 99% of the exposed mosquito Larvae to the new product using the logit model which are also very close to the LC_{50}, LC_{90}, and LC_{99} of 0.116, 1.391 and 10.509 granules respectively as the needed requirement to terminate 50%, 90% and 99% of the mosquito larvae using the probit model. However, these values are also increasing but in a slower fashion as compared to the first 24-hour and 48-hour exposure to the new product. This simply manifest that the longer is the exposure the more susceptible are the mosquitoes to be extinguished.

Table 4: Mortality Rate Distribution among Mosquito Larvae in Field after the First 24-hour Exposure to the New Product Application.

Days After Granule Application	Probability Model	Slope	Intercept	LC_{50}	LC_{90}	LC_{99}
Day 1	Logit (P)	2.553	1.156	0.352	2.557	22.231
	Probit (P)	1.336	5.718	0.290	2.640	15.975
Day 4	Logit (P)	2.534	0.941	0.425	3.130	27.662
	Probit (P)	1.355	5.595	0.364	3.214	18.971
Day 7	Logit (P)	2.268	0.581	0.555	5.162	58.897
	Probit (P)	1.274	5.375	0.508	5.146	33.994
Day 10	Logit (P)	2.343	0.465	0.633	5.489	57.930
	Probit (P)	1.311	5.309	0.581	5.514	34.530
Day 13	Logit (P)	2.483	0.036	0.967	7.419	68.537
	Probit (P)	1.428	5.043	0.934	7.376	39.784
Day 16	Logit (P)	3.492	-0.535	1.423	6.068	29.443
	Probit (P)	2.002	4.700	1.412	6.162	20.489

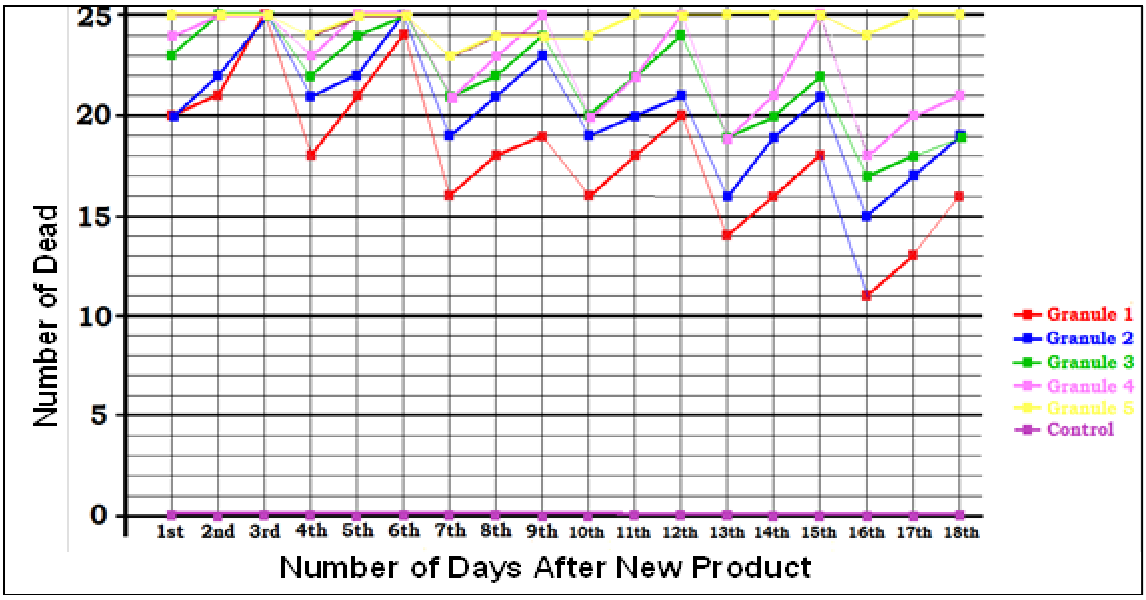

Fig 2: Mortality Distribution Among the Six Batches of 25 Mosquito Larvae Exposed to the New Product Application in the Field of 18 Days Across Five Different Granule Concentrations

As shown in table 4 and figure 2, the LC_{50} of 0.352 during the first day of application of the new product implies that we need 0.352 granules in order to terminate 50% of the mosquitoes using the light model while an LC_{90} of 2.557 indicates that we need 2.557 granules of the product to terminate 90% of the mosquitoes. Likewise an LC_{99} of 22.231 shows that we need 22.231 granules in eliminating 99% of the mosquitoes exposed. These values are increasing in the later days after the treatment of the new product. As gleaned from the table, an LC_{50} of 0.967, LC_{90} of 7.419 and LC_{99} of 68.537 indicated that 0.967 granule, 7.419 granules and 68.537 granules are needed respectively to terminate 50%, 90% and 99% of the mosquito larvae after the first 24-hour exposure to the solution during the 13th day after treatment.

The table above also shows the LC_{50}, LC_{90} and LC_{99} of 0.290, 2.640 and 15.975 granules which are needed to terminate 50%, 90% and 99% of mosquito larvae using the probit model. These values are increasing as the days after treatment are increasing. It is quite apparent from the result that the number of granules continues to increase until the 16th day after treatment except for the LC_{90} and LC_{99} that it decreases during the 16th day.

Table 5: Mortality Rate Distribution among Mosquito Larvae in the Field after the First 48-hour Exposure to the New Product Application.

Days After Granule Application	Probability Model	Slope	Intercept	LC_{50}	LC_{90}	LC_{99}
Day 2	Logit (P)	2.038	1.690	0.148	1.774	26.626
	Probit (P)	1.021	6.015	0.101	1.823	19.251
Day 5	Logit (P)	2.719	1.472	0.288	1.848	14.082
	Probit (P)	1.383	5.898	0.224	1.893	10.785
Day 8	Logit (P)	2.330	0.912	0.406	3.559	38.040
	Probit (P)	1.273	5.570	0.356	3.621	23.979
Day 11	Logit (P)	2.385	0.858	0.437	3.641	36.855
	Probit (P)	1.291	5.547	0.377	3.707	23.899
Day 14	Logit (P)	1.761	0.585	0.466	8.237	189.392
	Probit (P)	1.032	5.366	0.442	7.704	79.209
Day 17	Logit (P)	2.175	0.083	0.916	9.383	118.848
	Probit (P)	1.302	5.058	0.903	8.703	55.208

As depicted in the table 5, the LC_{50} of 0.148 during the second day of exposure to the new product means that we need 0.148 granule of the new product in order to terminate 50% of the mosquito larvae using the logit model while an LC_{90} of 1.774 indicates that we need 1.774 granules of the treatment to terminate 90% of the exposed mosquito larvae. Moreover, an LC_{99} of 26.626 shows that we need 26.626 granules in eliminating 99% of the mosquito larvae exposed. These values are increasing as the days after treatment are increasing. It is quite apparent in the 17th day after the application of the new product, that we need 0.916, 9.383 and 118.848 granules of the treatment to eliminate 50%, 90% and 99% of the mosquito larvae after the 48-hour exposure to the solution with the new product application. Likewise, the table also displays the LC_{50}, LC_{90} and LC_{99} of 0.101, 1.823 and 19.251 granules which are needed to terminate the 50%, 90% and 99% of the mosquitoes using the probit model after the first 48-hour of exposure to the treatment. However, these values continue to increase until the 17th day after the treatment except for LC_{99} that it increases until the 14th day but decreases during the 17th day.

Table 6: Mortality Rate Distribution among Mosquito Larvae in the Field after the First 72-hour Exposure to the New Product Application.

Days After Granule Application	Probability Model	Slope	Intercept	LC_{50}	LC_{90}	LC_{99}
Day 3	Logit (P)	Mosquito larvae were 100% terminated at this day				
	Probit (P)	Mosquitoes were 100% terminated at this day				
Day 6	Logit (P)	1.969	2.585	0.049	0.635	10.486
	Probit (P)	0.956	6.463	0.030	0.646	7.999
Day 9	Logit (P)	2.968	1.308	0.363	1.994	12.809
	Probit (P)	1.537	5.794	0.305	2.078	9.939
Day 12	Logit (P)	3.255	1.165	0.439	2.075	11.317
	Probit (P)	1.674	5.729	0.367	2.137	8.990
Day 15	Logit (P)	2.254	0.917	0.392	3.697	42.828
	Probit (P)	1.273	5.567	0.359	3.642	24.107
Day 18	Logit (P)	1.707	0.563	0.468	9.072	230.521
	Probit (P)	1.001	5.354	0.443	8.448	93.379

As gleaned in table 6, all or 100% the mosquito larvae were terminated during the third day of exposure to the solution with the new product application. However, when another set of mosquito larvae were exposed to the third (3rd day, after 72 hour exposure to the treatment, an LC_{50}, LC_{90} and LC_{99} of 0.049, 0.635 and 10.486 granules respectively of the new product were needed to terminate 50%, 90% and 99% of the mosquito larvae using the logit model. These values are almost closer to the LC_{50}, LC_{90}, and LC_{99} of 0.030, 0.646 and 7.999 respectively when using the probit model in estimating the needed concentrations for the treatment in eliminating 50%, 90% and 99% of the mosquito larvae after 72 hour exposure to the new product. It is also quite noticeable in the data shown above that the LC_{50}, LC_{90} and LC_{99} are increasing proportionally to the increasing days after the new product application. This simply means that we need 0.443, 8.448 and 93.379 granules of the new product in terminating 50%, 90% and 99% of the new set of mosquito larvae during the 18th day which is 72-hour exposure to the new product application.

It could be shown in the study that the granules have a very effective residual effect. The efficacy varies according to the dosage of the granules and the number of days exposure [12]. The larvicidal efficacy is caused by the parasporal crystal of the Bti that contains four major proteins (27,65,128 and 135 kDa) [13]. Bti lacks the ability to reprocess in insect populations. Normally, as reported for some of the more lethal insecticides for mosquito control, Bti persists for days rather than months. It indicated that a primary powder formulation of Bti (serotype H-14) had almost no residual effect on mosquito larvae beyond application, although the delta-endotoxin remained chemically stable in neutral and acid waters [14].

Agitation, UV, sedimentation, quality of water, pollutants, conditions of environment such as pH and temperature are some factors affecting the presence of Bti [15]. Agitation is the most important parameter in maintaining the presence of Bti cells and the bioavailability of the Bti toxin [16]. In their finding, sediment proved to decrease the effectivity of the 3rd instar larve of *Ae. aegypti* by increasing the presence of the Bti. It did not significantly extend the duration of the efficacy for controlling mosquito larvae if too much dosages of Bti were applied, but treated populations were never attained to the same degree due to increased predation and lack of breeding places [17].

4. Conclusion

The environmentally product is effective in killing the larvae of *Aedes aegyti* mosquitoes in both laboratory and field tests.

5. Acknowledgement

The author thanks Cebu Normal University for funding the research.

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