

## Impact of Serial Dilutions of a Biolarvicide Formulation on Gut *Bacillus* spp Progression and Mortality of *Culex quinquefasciatus* Mosquito Larvae

\*Oduola AO<sup>1</sup>, Adesoye AO<sup>1</sup>, Ande AT<sup>1</sup>, Omojasola FP<sup>2</sup>, Adelaja OJ<sup>1</sup>, Ahmed El-Imam A<sup>2</sup>

### ABSTRACT

Conventional pesticides are the focal and mainstay tools being used in the control of mosquitoes. However, some of these pesticides are known to cause acute and long term health problems in man. Moreso that they constitute to the existing environmental pollution, there is therefore an urgent need to explore the effectiveness of biolarvicides which is usually considered as a safe alternative to these conventional pesticides. A thorough understanding of the fate and efficacy of biolarvicide formulations in mosquito breeding sites open to water fluctuations is required for effective implementation of larval source management programmes. This study investigated the colony progression and impact of *Bacillus* sp on the larval gut of *Culex quinquefasciatus*. Twenty 3<sup>rd</sup> instar larvae of *C. quinquefasciatus* mosquitoes were introduced into each of the three replicates of plastic beakers containing one litre of serial dilutions ( $1 \times 10^6$ ,  $1 \times 10^7$ ,  $1 \times 10^8$  and  $1 \times 10^9$ ) of VectoMax<sup>®</sup> CG concentrations. Mean larval mortality of mosquitoes in each concentration was calculated hourly over a 24-hour period. The concentration that induced 100% mortality under the shortest exposure period was chosen as the dosage to be evaluated for its colony forming units in the larval gut exposed. Surface sterilization of mosquito larvae and work space was carried out following standard techniques after which the mosquito guts were dissected and plated on nutrient agar. Discrete colonies on each of the replicated plates were counted using colony counter. The time taken to achieve 100% mortality of *Culex quinquefasciatus* after exposure to  $10^6$ ,  $10^7$  and  $10^8$  serial dilutions of the larvicides increased from 6-10 hours with an increasing dilution ratio. The least concentration of the microbial larvicide ( $10^9$ ) induced 96 % mortality in the *Culex* population at 16 hours of exposure. The significant mean count of *Bacillus* sp found in the gut of *Culex quinquefasciatus* and the lethal impact at reduced concentrations have shown that VectoMax<sup>®</sup> CG could be a useful Larval Source Management tool in habitat settings open to water fluctuations.

**Keywords:** *Culex quinquefasciatus* larval control, *Bacillus* spp, Ilorin North Central, Nigeria

### INTRODUCTION

Larval Source Management (LSM) is a vector control strategy with historical success of being used to target outdoor and indoor biting mosquito species at their immature stages<sup>1,2</sup>. It therefore becomes a viable and important component of Integrated Vector Management (IVM) of mosquito borne diseases<sup>3,4</sup>. The use of microbial larvicide formulations as a larval source management tool has a wide acceptance in many countries of the world because of its non-chemical nature<sup>5</sup>. This is less likely to result in the massive scale of insecticide resistance development associated with the use of several synthetic insecticides<sup>6</sup>. The eco-friendliness of microbial larvicides and their ability to prevent emergence of adult mosquitoes responsible for transmission of debilitating vector borne diseases offer them an advantage over other mosquito control strategies. VectoMax<sup>®</sup> CG is a mosquito

biolarvicide formulation that combines toxins from *Bacillus thuringiensis* subsp. *israelensis* and *Bacillus sphaericus*, to control *Culex quinquefasciatus* found in diverse breeding sites such as sewage, effluents, lagoons, abandoned ponds, septic ditches, animal, waste lagoons, impounded, wastewater associated with, fruit and vegetable and roadside<sup>7</sup>. *Culex quinquefasciatus* is a mosquito, dipteran and major vector of lymphatic filariasis. *Bacillus thuringiensis* subsp. *israelensis* is broad spectrum by activity and it has a rapid control effect and low potential for resistance development<sup>5</sup>. *Bacillus sphaericus* has an extended residual activity and an encouraging performance in polluted water which is the preferential breeding habitats of *C. quinquefasciatus*<sup>5,8</sup>. Combined bio-larvicides have been reported to be effective in the reduction of adult malaria vector population densities through larval source reduction<sup>9-11</sup> hence, these biolarvicides have potentials to be used in the control of disease transmitting mosquitoes. In Nigeria, mosquito breeding sites with varying characteristics in size, depth and water volume inflow abound, all of which are reported to

Department of Zoology<sup>1</sup>, Department of Microbiology<sup>2</sup>, P.M.B. 1515, University of Ilorin Ilorin, Nigeria.

\*Corresponding author: dayooduola@yahoo.co.uk

influence the performance and residual activity of biolarvicides<sup>12</sup>. In view of these, a laboratory investigation was conducted to evaluate the possible fate of lower concentrations of VectoMax® CG biolarvicide on larval mortality and *Bacillus* spp colony progression in the gut of *Culex quinquefasciatus* larvae. This study provides a laboratory evidence of the effectiveness of lower concentrations of VectoMax® CG formulation in the control of Culicine mosquitoes.

## MATERIALS AND METHODS

### Details and Source of Bio-larvicide

VectoMax® CG is a granular formulation developed by Valent BioSciences Corp., USA. Its constituents are, *Bacillus thuringiensis* subsp.*israelensis* serotype H-14, strain AM65-52, fermented solid spores toxins (4.7%) and *Bacillus sphaericus* serotype H5a 5b strain 2362, fermented solids spores and insecticidal toxins (2.7%). The VectoMax® CG evaluated in this study was obtained from the Molecular Entomology and Vector Control Research Laboratory of the Nigerian Institute of Medical Research Lagos.

### Mosquito Larvae rearing and preparation of Nutrient Agar

Larvae of *Culex quinquefasciatus* were sourced from an open field mosquito breeding sites in Tanke Ilorin, Nigeria following standard mosquito collection techniques<sup>13</sup>. The 3<sup>rd</sup> larval instar stages were identified and separated for the experiment in the Entomology Laboratory. The microbiological procedures in this study were carried out at the Microbiology Laboratory, University of Ilorin, Nigeria. The nutrient agar used for the colony count was prepared according to the manufacturer's instructions.

### VectoMax® CG Formulation and Dilution

One grain of VectoMax® CG weighing 0.001g was dissolved in 1litre of sterile distilled water and serially diluted to give the following concentrations;  $1.0 \times 10^{-6}$  g/L,  $1.0 \times 10^{-7}$  g/L,  $1.0 \times 10^{-8}$  g/L and  $1.0 \times 10^{-9}$  g/L. The content of each concentration was subsequently transferred to correspondingly labeled containers containing one litre of borehole water. Three replicates of each concentration were prepared for the assays along with a control experiment without VectoMax® CG formulation.

### Assay for Mortality and Gut Bacteria Count in Exposed Larvae

Twenty 3<sup>rd</sup> instar larvae of *C. quinquefasciatus* mosquitoes were introduced into each of the three replicates of plastic beakers containing one litre created for each concentrations of VectoMax® CG biolarvicide. Mean larval mortality of mosquitoes in each concentration was calculated hourly over a 24-hour period. The concentration that induced 100% mortality under the shortest exposure period was chosen as the dosage to be evaluated for its colony forming units in the larval gut. One live and one dead mosquito larvae were randomly removed from each of the exposure replicates. The number of colonizing *Bacillus* spp in the gut of the larvae was determined at 0, 2, 4 and 6 hours after exposure to bio-larvicides and compared with the control set up without larvicide application.

### Determination of the LC<sub>50</sub> and LC<sub>90</sub>

The Probit analysis was applied to determine the lethal concentrations that will induce fifty (LC<sub>50</sub>) and ninety percent (LC<sub>90</sub>) mortality of the test population<sup>14</sup>.

### Surface Sterilization of Mosquito Larvae and Work Space

Phosphate-buffered saline (PBS) adjusted to pH 7.4 was used as surface-sterilizer to prevent larval contamination during dissection. Each sampled mosquito larvae (dead or alive) was aseptically transferred to a sterile disposable petri dish cover, rinsed serially for 1 minute in 1% NaOCl and swirled in sterile PBS (81mM NaHPO<sub>4</sub>, 19mM NaHPO<sub>4</sub> 15mM NaCl, pH7.4). This was again rinsed in 70% ethanol and PBS for 10 seconds and 1 minute respectively. The last PBS wash was then retained for evaluation of efficiency of the surface sterilization process. All worktops were repeatedly sterilized by wiping with 70% ethanol to prevent contamination of plates and ensure aseptic conditions. A bunsen burner flame was used to prevent aerial contamination of plates and tubes.

### Larval Dissection and Culture Techniques

This was carried out under a dissecting microscope, in a double glass slide containing cold, sterile PBS. The entire gut of the larva was aseptically separated and transferred into a test tube containing 10 ml of sterile PBS. This was

replicated twice. The content of the tube was stirred thoroughly with a glass rod near a Bunsen flame, using spread plate method. Aliquots of 0.01ml from the last PBS wash of the larva were also plated in nutrient agar as controls for the surface sterilization process.

### Total Bacterial count

Discrete colonies on each of the replicated plates were counted using colony counter. The average of the counts of the duplicate plates was determined and was multiplied by the dilution factor of 0.01 to give the number of colony forming units per ml of the larva gut solution (Cfu/ml) and by a dilution of  $10^{-3}$  for the total number of organisms per larval gut.

### Isolation and Identification of Bacteria

One ml of the PBS- larval gut inoculum was seeded onto a sterile Nutrient Agar (NA) plate and incubated at 37°C for 24 hours. After incubation, representative colonies were picked aseptically and smeared on clean, grease-free glass slide and heat-fixed by passing a flame. Gram staining was done as described by Fawole and Oso<sup>15</sup>. The isolated bacteria were further characterized by spore staining, oxygen relationship and motility testing. Biochemical characteristics were determined based on different biochemical tests such as test for coagulase, catalase, oxidase, urease, indole, methyl red, citrate utilization ONPG and triple sugar fermentation as described by Fawole and Oso<sup>15</sup>. Identification was done using the Manual of Holt *et al.*<sup>16</sup>.

## RESULTS

### Mortality rates of mosquito larvae exposed to varying concentrations of VectoMax® CG biolarvicide.

The mortality results showed that third instar larvae of *Culex quinquefasciatus* were susceptible to all the serial dilutions ( $10^{-6}$ ,  $10^{-7}$ ,  $10^{-8}$  and  $10^{-9}$ ) of the VectoMax® CG achieving  $\leq 100\%$  mortality within 6-16 hrs of application (Figure 1). The  $10^{-6}$  and  $10^{-7}$  concentration accounted for the rapid mortality (1 hour post exposure) observed in this study. Likewise, these concentration also accounted for the highest mean mortality 20( $\pm 0.00$ ), just after 6 and 8 hours of exposure to VectoMax® CG application respectively (Table 1). It was observed that the time taken to achieve total mortality under each concentration increased with decreasing concentration. For instance, while  $10^{-6}$  concentration of the biolarvicide achieved total mortality at the 6<sup>th</sup> hour of exposure, the same was achieved at the 8<sup>th</sup> and 10<sup>th</sup> hour with  $10^{-7}$  and  $10^{-8}$  concentrations respectively (Table 1). At the lowest concentration, of  $10^{-9}$  of VectoMax® CG, a peak mean mortality 19.2( $\pm 1.2$ ) was attained at 16 hours and this was the maximum mortality. In addition, no mortality was recorded for the control experiment (Table 1). A log - probit analysis to determine the concentration of VectoMax® CG required to induce both fifty percent and ninety percent mortality ( $LC_{50}$  and  $LC_{90}$ ) of larvae (Figure 2 and 3) were  $\geq 2.89 \times 10^{-8}$  and  $\geq 3.39 \times 10^{-7}$  respectively.

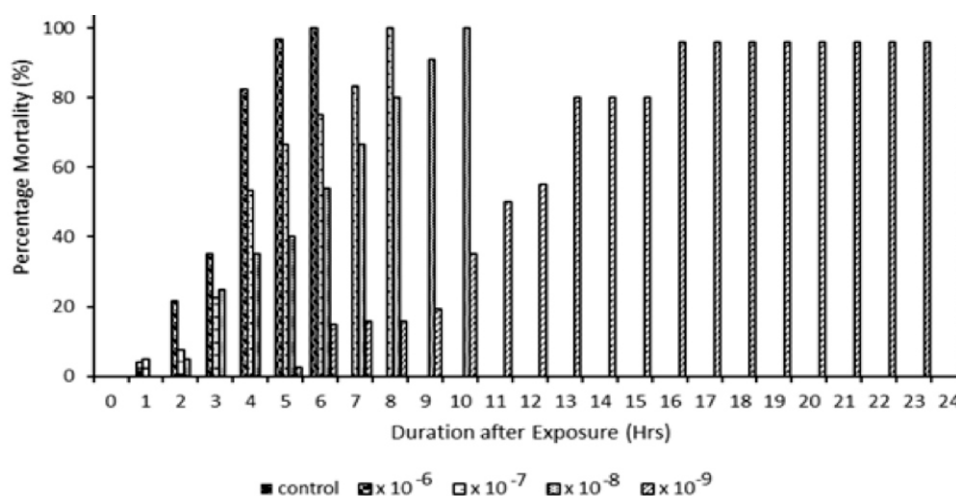


Figure 1: Percentage Mortality of Mosquito Larvae ( $\pm$ SD) Over 24 hour Exposure to VectoMax® CG

**Table 1: Mean mortality of *C. quinquefasciatus* larvae over a 24-hour exposure to varying concentration of VectoMax® CG**

| Duration<br>of Exposure<br>(Hrs) | Mean Larvae Mortality at varying concentrations |            |            |            |           |
|----------------------------------|-------------------------------------------------|------------|------------|------------|-----------|
|                                  | Control                                         | $10^{-6}$  | $10^{-7}$  | $10^{-8}$  | $10^{-9}$ |
| 0                                | 0.00±0.00                                       | 0.00±0.00  | 0.00±0.00  | 0.00±0.00  | 0.00±0.00 |
| 1                                | 0.00±0.00                                       | 0.80±0.02  | 1.00±0.00  | 0.00±0.00  | 0.00±0.00 |
| 2                                | 0.00±0.00                                       | 4.33±0.57  | 1.50±0.20  | 1.00±0.00  | 0.00±0.00 |
| 3                                | 0.00±0.00                                       | 7.00±0.00  | 4.50±0.20  | 5.00±0.00  | 0.00±0.00 |
| 4                                | 0.00±0.00                                       | 16.50±0.20 | 10.67±0.33 | 7.00±0.00  | 0.00±0.00 |
| 5                                | 0.00±0.00                                       | 19.33±0.57 | 13.33±0.57 | 8.00±0.00  | 0.50±0.20 |
| 6                                | 0.00±0.00                                       | 20.00±0.00 | 15.00±0.00 | 10.00±0.20 | 3.00±0.00 |
| 7                                | 0.00±0.00                                       | 16.67±0.33 | 13.33±0.57 | 3.16±0.17  |           |
| 8                                | 0.00±0.00                                       | 20.00±0.00 | 16.00±0.00 | 3.16±0.17  |           |
| 9                                | 0.00±0.00                                       |            | 18.20±0.10 | 3.83±0.21  |           |
| 10                               | 0.00±0.00                                       |            | 20.00±0.00 | 7.00±0.00  |           |
| 11                               | 0.00±0.00                                       |            |            | 10.00±0.00 |           |
| 12                               | 0.00±0.00                                       |            |            | 11.00±0.00 |           |
| 13                               | 0.00±0.00                                       |            |            | 16.00±0.00 |           |
| 14                               | 0.00±0.00                                       |            |            | 16.00±0.00 |           |
| 15                               | 0.00±0.00                                       |            |            | 16.00±0.00 |           |
| 16                               | 0.00±0.00                                       |            |            | 19.2±1.20  |           |
| 17                               | 0.00±0.00                                       |            |            | 19.2±1.20  |           |
| 18                               | 0.00±0.00                                       |            |            | 19.2±1.20  |           |
| 19                               | 0.00±0.00                                       |            |            | 19.2±1.20  |           |
| 20                               | 0.00±0.00                                       |            |            | 19.2±1.20  |           |
| 21                               | 0.00±0.00                                       |            |            | 19.2±1.20  |           |
| 22                               | 0.00±0.00                                       |            |            | 19.2±1.20  |           |
| 23                               | 0.00±0.00                                       |            |            | 19.2±1.20  |           |
| 24                               | 0.00±0.00                                       |            |            | 19.2±1.20  |           |

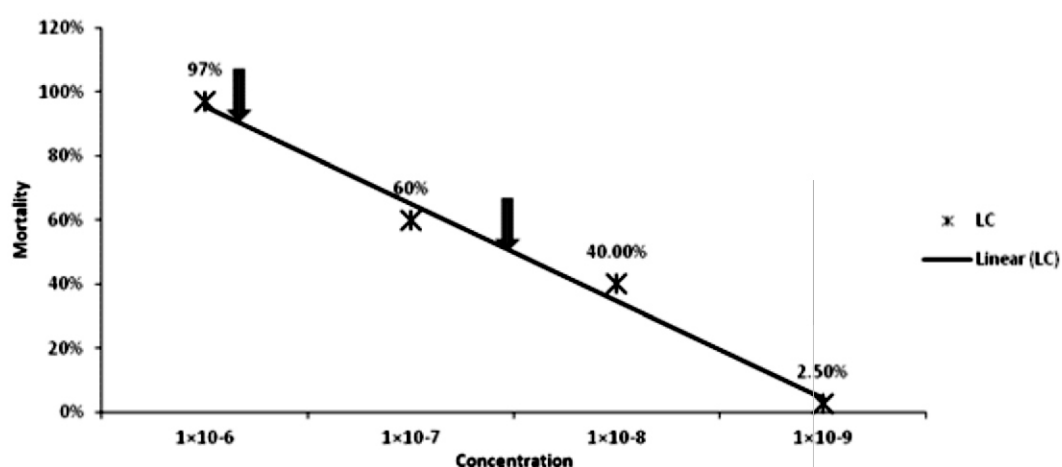


Figure 2: Lethal Concentration of Vectomax at the Fifth Hour of Larval Exposure in the Laboratory.

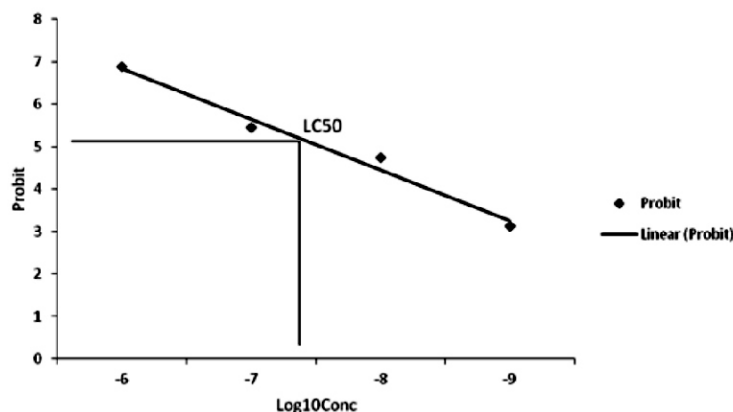


Figure 3: Log -Probit Mortality of *Culex quinquefasciatus* larvae exposed to varying concentration of VectoMax<sup>®</sup>

Intercept: 14.025

Slope: -7.54

LC<sub>50</sub>:  $2.89 \times 10^{-8}$

Intercept: 14.025

Slope: -6.47

LC<sub>90</sub>:  $3.39 \times 10^{-7}$

#### Load of *Bacillus* spp in gut of treated and untreated larvae of *C. quinquefasciatus*

The result of this study showed that the gut of unexposed population of *Culex quinquefasciatus* (control) had a mean count of *Bacillus* spp load ranging between  $1.26 \times 10^3$  ( $\pm 0.10 \times 10^3$ ) -  $1.86 \times 10^3 \pm (2.10 \times 10^3)$  cfu/ml (Table 2). However, these values did not significantly

differ ( $P > 0.05$ ) from each other at 0, 2, 4 and 6 hours exposure duration (Table 2). These loads were however significantly lower ( $P < 0.05$ ) than those in the exposed larval population. The mean count of *Bacillus* spp ( $1.10 \times 10^3$  -  $4.90 \times 10^4$  cfu/ml) in the gut of the live larvae increased with the passage of time (0 - 6 hours) (Table 2). This was further justified with a significant reduction in the count ( $4.66 \times 10^3$  -  $1.2 \times 10^3$  cfu/ml) of *Bacillus* spp in the non - feeding dead mosquito larvae (Table 2). Higher and significant count ( $P < 0.05$ ) of *Bacillus* spp was observed in the gut of dead at 0-2hrs compared to the control experiment. Similarly significantly higher count of *Bacillus* spp ( $4.90 \times 10^4$  cfu/ml) were observed in the gut of living mosquitoes after 4-6 hrs of exposure.

Table 2: Mean Count of *Bacillus* spp from the Gut of Third Instar Mosquito Larvae Exposed to  $10^{-6}$  g/L concentration of VectoMax<sup>®</sup> CG<sup>®</sup>

| Duration (hrs) | Mean Count of <i>Bacillus</i> (cfu/ml)     |                                            |                                            |
|----------------|--------------------------------------------|--------------------------------------------|--------------------------------------------|
|                | Control                                    | Live Larvae                                | Dead Larvae                                |
| 0              | $1.86 \times 10^3 \pm 2.10 \times 10^{3a}$ | $1.10 \times 10^3 \pm 1.00 \times 10^{3a}$ | $4.66 \times 10^3 \pm 2.10 \times 10^{3b}$ |
| 2              | $1.44 \times 10^3 \pm 1.10 \times 10^{3a}$ | $3.10 \times 10^3 \pm 0.10 \times 10^{4b}$ | $2.09 \times 10^3 \pm 0.20 \times 10^{3b}$ |
| 4              | $1.26 \times 10^3 \pm 0.10 \times 10^{3a}$ | $4.60 \times 10^4 \pm 1.00 \times 10^{4b}$ | $1.70 \times 10^3 \pm 1.00 \times 10^{3a}$ |
| 6              | $1.86 \times 10^3 \pm 2.10 \times 10^{3a}$ | $4.90 \times 10^4 \pm 0.00 \times 10^{4b}$ | $1.2 \times 10^3 \pm 1.00 \times 10^{3a}$  |

Mean carrying the same superscript alphabet along the row are not significantly different at  $P = 0.05$  values. Values are mean *Bacillus* count on the replicated plate.

## DISCUSSION

In this study, the total mortality observed in *Culex quinquefasciatus* larvae at all the serial dilutions attest to the efficacy of VectoMax® CG biolarvicide at very low concentrations. Similar studies have reported a 100% reduction of larvae in first 24 hours of application of *Bti*<sup>11</sup>. The rapid response of VectoMax® CG initiating death after 1 hour of application confirms the fast acting potentials of toxins produced from *Bacillus thuringiensis israelensis* and *Bacillus sphaericus* which are the active constituents. The significant mean count of *Bacillus* spp found in the gut of the treated larvae groups compared with the control group evidently demonstrated increased ingestion over time. This became evident when the number of *Bacillus* spp in the larval gut reduced with the passage of time after death. Higher mean count of *Bacillus* spp in the gut of dead larvae sampled between the 0-2 hours of exposure may have resulted from the voracious filter feeding habits and subsequent exposure to the toxins produced by the bacteria. The higher bacteria gut content found in the treated live larvae at 4 and 6 hours of exposure is either indicative of some form of tolerance reported in wild *Culex* population to the *Bacillus* sp<sup>17</sup> or this may have been the cumulative effect of ingested *Bacillus* spp from VectoMax® CG and those reported to colonise the gut of mosquito larvae from the natural habitat<sup>18</sup>. The *Bacillus* load observed in the gut of unexposed mosquito population is an indication of the existence of the natural fauna of *Bacillus* spp in the wild. Similarly significantly higher counts of *Bacillus* spp ( $4.90 \times 10^4$  cfu/ml) were observed in the gut of living mosquitoes after 4-6 hrs of exposure. This did not translate into mortality showing that the mortality was not always induced by higher densities of *Bacillus* spp. This is suggestive of the progressive feeding activity of the larvae on the VectoMax® CG and wild *Bacillus* spp. This could be a case of larval tolerance to the corresponding toxin concentration or that the required toxin threshold to induce mortality was yet to be reached. Characterising the different strains of *Bacillus* spp present using molecular techniques would have helped to tag the progression of the strains that make up the population. Alternatively further studies should consider using enzyme linked immunosorbent assays to profile and characterize the types and fate of toxins produced<sup>19</sup>. This will further improve the understanding on the functionality of the various species of *Bacillus* that are likely to be present in the mosquito gut.

Tetreau *et al.*<sup>20</sup> have reported the production of new crystal toxins different from that in the commercial mixture. This is important as these are the determinants of the toxicity of *B. thuringiensis*. Contrary to the report of traces of resistance development noticed in *Culex quinquefasciatus* exposed to the *B. sphaericus* larvicide<sup>21</sup>, the LC<sub>50</sub> and LC<sub>90</sub> determined in the laboratory also showed that VectoMax® CG was effective at lower concentrations inducing mortality within 6-8 hours of exposure. There are very scanty information on the effect and fate of low concentrations of this biolarvicide on mosquito larvae. Available reports have reported its low persistence and rapid loss of its insecticidal activity under field conditions yet; several authors have also suggested the possibility of it being recycled under specific conditions<sup>22,23</sup>. Hence establishing its effectiveness under low concentrations is an added value to the existing knowledge that it can be recycled. This piece of information is of valuable importance and attests to the efficacy of the biolarvicides in breeding sites that are prone to cyclical flooding.

## CONCLUSION

VectoMax® CG biolarvicide can therefore be included as one of the larvicides for larval source management because of its potential of being effective in the control of mosquito larvae at low concentrations.

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## Conflicts of Interest

The authors declare no conflict of interest.

## REFERENCES

1. Killeen GF, Fillinger U, Knols BGJ. Advantages of larval control for Africa malaria vectors: low mobility and behavioural responsiveness of immature mosquito stages allow high effective coverage. *Malaria J.*, 2002;1:8.
2. Fillinger U, Lindsay SW. Larval source management for malaria control in Africa: *myths and reality Malaria J.*, 2011;10:353
3. Beier J, Keating J, Githure J, Macdonald M, Impoinvil D, Novak R. Integrated vector

- management for malaria control. *Malaria J.* 2008; 7 (Suppl 1): S4-10.1186/1475-2875-7-S1-S4
4. Castro MC, Tsuruta A, Kanamori S, Kannady K, Mkude S. Community-based environmental management for malaria control: evidence from a small-scale intervention in Dar es Salaam, Tanzania. *Malaria J.*, 2009; 8
5. Lacey LA. *Bacillus thuringiensis* Serovariety *israelensis* and *Bacillus sphaericus* for Mosquito Control. *J Am Mosq Cont Assoc* 2007; 23(sp2):133-163.
6. Hemingway J, Ranson H. Insecticide Resistance in Insect Vectors of Human Disease. *Ann Rev Ent*, 2000; 45:369-389.
7. Després L, Lagneau C, Frutos R. Using the Bio-Insecticide *Bacillus thuringiensis israelensis* in Mosquito Control, Pesticides in the Modern World - Pests Control and Pesticides Exposure and Toxicity Assessment,. Margarita Stoytcheva (Ed.), *In Tech*, 2011; ISBN: 978-953-307-457-3.
8. Lacey LA. Persistence and formulation of *Bacillus sphaericus*. In e Barjac, H. and D. Sutherland, editors. eds. *Bacterial Control of Mosquitoes and Black Flies: Biochemistry, Genetics, and Applications of Bacillus thuringiensis israelensis and Bacillus sphaericus*. University Press New Brunswick Rutgers, 1990;284-294
9. Skovmand O, Bauduin S. Efficacy of a granular formulation of *Bacillus sphaericus* against *Culex quinquefasciatus* and *Anopheles gambiae* in West African countries. *J Vec Borne Dis*, 1997; 22:43-51.
10. Shililu J, Tewolde G, Brantly E, Githure J, Mbogo C, Beier J, et al.. Efficacy of *Bacillus thuringiensis israelensis*, *Bacillus sphaericus* and temephos for managing *Anopheles* larvae in Eritrea. *J Am Mosq Control Assoc*, 2003;19:251-8.
11. Dambach P, Louis VR, Kaiser A, Ouedraogo S, Sié A, Sauerborn R, Becker N. Efficacy of *Bacillus thuringiensis* var. *israelensis* against malaria mosquitoes in northwestern Burkina Faso. *Parasit Vectors*, 2014; 7:37.
12. Kiarie-Makara MW, Dong-Kyu L. Comparative Larvicidal and Residual Activity of *Bacillus thuringiensis* var. *israelensis* and Temephos (1.0 % Sg) In Granules Against *Culex pipiens pallens*(Diptera: Culicidae) In Semi-Field Tests. *IOSR J Pharm Biol. Sci.*, 2016; 11:24-32.
13. Service MW. Mosquito Ecology: Field sampling methods. 2nd Edition Elsevier 1993; *Appl. Sci, London New York*.
14. Finney DJ. *Probit Analysis*. University Press, Cambridge London:1979;68-72.
15. Fawole MO, Oso BA. Laboratory Manual of Microbiology. *Spectrum Books Ltd. Ibadan*, 2007; 127pp.
16. Holt JG, Kreg NR, Sneath, PHA, Stanley JT, William ST. *Bergey's Manual of Determinative Bacteriology*. William and Wilkins, Baltimore, USA; 1994; 8<sup>th</sup> Ed., 1130pp.
17. Boyer S, Paris M, Jago S, Lemperiere G, Ravanel P. Influence of insecticide *Bacillus thuringiensis* subsp. *israelensis* treatments on resistance and enzyme activities in *Aedes rusticus* larvae (Diptera: Culicidae) *Biol Control*, 2012; 62:75-81.
18. Desiely H, Silver G, Pidiyar V, Alberto L. First Isolation of microorganisms from the gut of *Aedes aegypti* (Diptera: Culicidae) *Mem Inst Cruz*, 2007; 102(8):201-233.
19. Palm CJ, Donegan K, Harris D, Seidler RJ. 1994. Quantification in soil of *Bacillus thuringiensis* var *kurstaki* delta-endotoxin from transgenic plants. *Mol. Ecol.*1994; 3:145-151.
20. Tetreau, G, Alessi, M, Veyrenc, S, Périgon, S, David, J.-P., Reynaud, S et al. (2012). Fate of *Bacillus thuringiensis* subsp. *israelensis* in the Field: Evidence for Spore Recycling and Differential Persistence of Toxins in Leaf Litter. *Appl and Env Microb*, 78(23), 8362-67.
21. Guillaume T, Mattia A, Sylvie V, Sophie P, Jean-Philippe D, Stéphane R et al., Fate of *Bacillus thuringiensis* subsp. *israelensis* in the Field: Evidence for Spore Recycling and Differential Persistence of Toxins in Leaf Litter. *Appl and Env. Microb*, 2012;78: 8362-67.
22. Tilquin, M, Paris, M, Reynaud, S, Despres, L, Ravanel, P, Geremia, R. A et al., Long Lasting Persistence of *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) in Mosquito Natural Habitats. *PLoS ONE* 2008; 3(10).
23. de Melo-Santos MAV, de Araujo AP, Rios EMM, Regis L. Long lasting persistence of *Bacillus thuringiensis* serovar *israelensis* larvicidal activity in *Aedes aegypti* (Diptera: Culicidae) breeding places is associated to bacteria recycling. *Biol. Control*, 2009; 49:186-191.