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Efficacy of the Microbial Larvicide VectoMax®G against *Anopheles gambiae* s.l. and *Culex* spp. Larvae under Laboratory and Open Field Trial Experiments in the City of Yaoundé, Cameroon

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Abstract

Background: With the rapid expansion of insecticide resistance limiting the effectiveness of insecticide-based vector control interventions, integrated control strategies associating larviciding could be appropriate to improve current control efforts. The present experimental study assesses laboratory and field efficacy of the larvicide VectoMax®G on *Anopheline* and *Culicine* larval stages in Yaoundé. **Methods:** The effect of the larvicide VectoMax®G, a combination of *Bacillus thuringiensis* var. *israelensis* (*Bti*) and *Bacillus sphaericus* (*Bs*), on larval development was assessed during both laboratory and open field trial experiments. Laboratory experiments permitted the evaluation of five different concentrations with four replicates/experiments. Laboratory experiments were conducted with *Anopheles coluzzii* "Ngousso" and *Culex quinquefasciatus* laboratory strains. Open field trials were conducted using sixteen plastic containers with a diameter of 0.31 m buried in an array of four rows with 4 containers each. Distance between rows and between containers in a row was 1 meter. This experiment permitted to test the

effect of the microbial larvicide VectoMax®G under operational application conditions on field mosquito populations. **Results:** The time to induce 100% mortality after exposure to serial concentrations of the larvicide varied according to the dose from 4 - 12 hours for *An. coluzzii* and 6 - 9 hours for *Cx. quinquefasciatus* in laboratory experiments. Measurements of the residual activity indicated that all VectoMax®G concentrations were still active after 35 days and killed 86% - 100% of larvae. Lethal dose of VectoMax®G killing 50% of larvae was estimated at 5.24×10^{-8} mg/m^2 for *An. coluzzii* and 1.25×10^{-8} mg/m^2 for *Cx. quinquefasciatus*. The lethal concentration inducing 95% mortality was estimated at 3.13×10^{-7} mg/m^2 for *An. coluzzii* and 2.5×10^{-8} mg/m^2 for *Cx. quinquefasciatus*. Open field trials tests indicated that sub-lethal concentrations of VectoMax®G successfully killed 100% *An. gambiae* s.l. larvae within 24 hours, while with *Culex* spp. larvae, 100% mortality was recorded after 48 hours post-treatment. Natural recolonization of water containers by larvae was recorded between 3 and 6 days respectively after the treatment with sublethal doses. Late instar larvae were recorded 5 and 6 days after treatment. When the jars were treated with reference dosage or supra doses of VectoMax®G, recolonization of water containers was observed six days after treatments. No pupae of both species were found 6 and 7 days post-treatment. **Conclusions:** The study indicated high efficacy of the microbial larvicide VectoMax®G against *Anopheline* and *Culex* larvae. Microbial larvicides such as VectoMax®G could be appropriate for controlling mosquito population particularly in areas experiencing high insecticide resistance or outdoor biting mosquitoes.

Keywords

VectoMax®G, *Bacillus thuringiensis* var. *israelensis*, *Bacillus sphaericus*, *Anopheles gambiae* s.l., *Culex* Mosquitoes, Yaoundé, Camreroon

1. Background

Vector control in Africa heavily relies on insecticide-based interventions such as Long-lasting insecticide nets (LLINs) and indoor residual spraying (IRS) [1]. However, the effectiveness of these measures is affected by a certain number of limits including the rapid expansion of insecticide and behavioural resistance in vector populations [2] [3] [4]. To minimize the dependence on chemical insecticides, there is an urgent need to explore alternative measures for mosquito control. One such alternative control approach is to include larviciding as an additional intervention in urban settings [5] [6]. In Africa, the use of larval source management (LSM) as an additional tool for integrated vector management (IVM) has become increasingly requested in different epidemiological contexts [7]. Larviciding has been at the forefront of control strategies that successfully eliminated malaria in many places [8].

Larviciding is a vector control intervention that consists of regular application in standing water collections of chemical or biological insecticides in order to

kill mosquito larvae. VectoMax®G is a mosquito biolarvicide formulation that combines toxins from *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) and *Bacillus sphaericus* (*Bs*), to control *Anopheline* and *Culex* found in diverse breeding sites. The advantages of biolarvicide as compared to chemical compounds are their effectiveness at relatively low doses, safety to humans and non-target organisms and the fact that they reduce insecticide selection pressure [9] [10]. In addition, *Bti* has a broad spectrum that could be targeted and has a rapid control effect and low potential for resistance development in the field [11] [12]. Moreover, to become resistant to *Bti*, an individual must develop resistance mechanisms to each of the four toxins, namely Cry4Aa, Cry4Ba, Cry11Aa, and Cyt1Acontained in each *Bti* spore [13]. Nowadays, there have been no reports of resistance to *Bti* in mosquito populations despite it having been applied for decades in several countries [13] [14]. However, some biotic and abiotic factors such as mosquito species, the rate of ingestion, the density and age of the larvae, the temperature and the organic matter content have been reported to affect the efficacy of biolarvicide formulations in the field [12] [15]. Unlike *Bti*, which requires clean water to be effective, *Bs* can provide good control of larvae in polluted water which is the preferential breeding habitats of *Culex* [12] [16] [17]. *Bacillus sphaericus* has an extended residual activity. However, there have been several reports suggesting development of resistance to *Bs* in many places [18] [19] [20], indicating that resistance management strategies are needed in operational programmes that use *Bs*. A mixture of *Bti* and *Bs* may represent a potentially effective approach and may prevent emergence of resistance to biolarvicides [21]. In this study, the effect of the larvicide VectoMax®G combining *Bacillus thuringiensis israelensis* strain AM65-52 and *Bacillus sphaericus* strain 2362 formulation (VectoMax®G) on *Culex* and *Anopheline* larvae was tested to determine the effectiveness of different doses and residual effect of the larvicide in both laboratory and open field trial experiments.

2. Materials and Methods

2.1. Study Area

Laboratory tests were performed at the Malaria Research Laboratory of OCEAC (Organization for the Coordination of the fight against endemic diseases in Central Africa). Standardized open field trials were carried out in Ekounou, a central district of the city of Yaoundé. The characteristics of Yaoundé have been presented in previous study [22].

2.2. Mosquito Populations

Laboratory assays were carried out with 3rd instar larvae of *An. coluzzii* "Ngousso" and *Cx. quinquefasciatus* laboratory colonies. These mosquito strains were originally colonized from *Anopheles* s.l. and *Culex* spp. larvae collected respectively in 2006 and 2017 in the city of Yaoundé and maintained at the OCEAC insectary.

All mosquito larvae used in the laboratory experiments were reared at room temperature of 25˚C - 27˚C, 80% relative humidity. Larvae were reared in 20 × 30 cm white rectangular plastic containers filled with spring water. Larvae were fed by adding a pinch of crushed TetraMin® Baby fish food spread evenly on the water surface once daily.

The open field trials were conducted with larvae from wild *An. gambiae* s.l. and *Culex* spp. females that naturally oviposited or were added in the experimental containers.

2.3. Details and Source of Bio-Larvicide

VectoMax®G is a granular formulation developed by Valent BioSciences Corporation, Illinois, USA. Its constituents are *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) serotype H-14, strain AM 65 - 52 (45 g/kg), fermented solids spores toxins 4.7% primary powder (p/p) and *Bacillus sphaericus* (*Bs*) strain 2362, ABTS-1743 (27 g/kg), fermented solids spores and insecticidal toxins (2.9% p/p) granular, titrating 50 *Bs* international toxic units per mg (UTI/mg).

2.4. Laboratory Assays

The granular formulations of VectoMax®G were tested in the laboratory to determine the minimum effective dosages, from the recommended dose by the manufacturer. A total of 50 to 200 third instar larvae were exposed in different plastic containers containing different concentration of the larvicide. Test concentrations were obtained after sequential dilution of recommended dose by the manufacturer (500 mg/m^2). The formulation of VectoMax®G was weighed and sprinkled on the water surface of the containers.

The bioassays were run with five different concentrations of VectoMax®G (ranging from 500 to 25 mg/m^2). Each experiment contained a control (distilled water only). The experiments were run in four replicates at the same time and the entire experiments were carried out on three different occasions. All trials were conducted at ambient temperatures ranging from 25˚C to 27˚C and larvae were not fed during the experiments.

To determine laboratory efficacy of biolarvicide, mean larval mortality of mosquitoes in each concentration was calculated over a 12 hour period. Third instars (laboratory strain) of *An. coluzzii* and *Cx. quinquefa*sciatus were used.

Concerning the assessment of the residual activity of VectoMax®G at different doses, only *An. coluzzii* "Ngousso" larvae were introduced into the test containers at day 0 post-treatment. Different mosquito larval batches were tested at day 1, day 3, day 7, day 14, day 28, and day 35. Larval mortality was recorded 24 hours after experiment with each dose and dead larvae were removed. Moribund larvae were considered as dead and included in the analyses. When mortality exceeded 10% in the controls, the experiment was discarded and repeated.

2.5. Open Field Trials

Open field trials with VectoMax®G were performed between July and September

2018, corresponding to the dry season and the beginning of the rainy season. Artificial ponds were created following the experimental design described in Fillinger *et al.* [23]. Sixteen plastic containers with a diameter of 0.31 m each and about 30 cm long were buried into an array of four rows with 4 containers each. Distance between rows and between containers in a row was 1 meter. Soil from known *Anopheles* breeding sites was added to each container, providing a standardized environment with suitable breeding conditions for mosquitoes. The containers were filled with water from a nearby well. Each container received about 18 litres of water, representing an average water depth of 0.28 m. The habitats were then left open during two weeks for mosquito oviposition/colonization. In order to prevent the emergence of the malaria vector, all containers were carefully screened for pupae once daily and any pupae present were removed.

Experiments were implemented fourteen days after the jars were set up to allow wild females to oviposit in the containers. Of the 16 containers buried, four served as controls, and each row of four containers was treated with a given concentration of the VectoMax®G. The doses evaluated were calculated using as reference the minimal reference dosage of 500 mg/m^2 recommended by the manufacturer irrespective of the actual water depth and this was done to simulate operational procedures. Two different ranges of concentrations were assessed: the first assays included both sub-lethal and doses equivalent or above the reference minimal dosage with the following doses tested 928.38; 398 and 199 mg/m^2. In the second set of experiments, the following concentrations (all above the minimal reference dosage) were evaluated 1021.22; 1525.2 and 2042.44 mg/m^2. Prior to experiments, 80 *Anopheles* (60 first and second instar and 20 third and fourth) and 20 *Culex* spp. (10 first and second instar and 10 third and fourth) field-collected larvae were placed in each container. *Anopheles* s.l. and *Culex* spp. larvae were collected from surrounding natural habitats for which recent studies indicated that 91.1% of anopheline larvae were *An. coluzzii* and 8.9% *An. gambiae* s.s. [24] and 79.4% *Cx. quinquefasciatus* [25].

The respective concentrations of biolarvicide (VectoMax®G) were applied evenly on the water surface of each container, by hand. All containers were examined daily and larval count was performed in all 16 containers, pupae were removed. Immature mosquitoes were classified in three categories: early instars (first and second), late instars (third and fourth) and pupae. All larvae were classified to genus and development stage and then returned to their respective containers.

2.6. Data Analyses

From the bioassay results, lethal concentration (LC50 and LC95) values were determined using log-probit regression analysis in WINDEL software version 32. LC50 represents the concentration of the larvicide killing 50% of larvae and LC95% is the concentration of the larvicide killing 95% of larvae.

The percentage reduction in larval mosquito densities was calculated using the formula of Mulla [26] which takes into account natural changes (for instance through predation) occuring at the same level and rate in both treated and untreated sites:

$$\text{Percentage reduction} = 100 - (C1/T1 \times T2/C2) \times 100$$

where C1 and C2 are the average number of larvae in the control containers pre- and post-treatment; T1 and T2 are the average number of larvae in treated containers before and after treatment. The average number of all larval instars, late instars, and pupae in the control and treatment containers were compared daily by non-parametric Kruskal-Wallis One-way ANOVA on ranks (a = 0.05) using R version 4.0.2 software.

3. Results

3.1. Laboratory Experiments

Mortality Rates of Mosquito Larvae Exposed to Different Concentrations of VectoMax®G

Larval mortality was determined after 12 hours exposition to VectoMax®G as a ratio of death and exposed larvae. The mortality results showed that third instar larvae of *An. coluzzii* and *Cx. quinquefasciatus* were susceptibles to all serial dilutions of the VectoMax®G with 100% mortality recorded within 4 - 12 hours and 6 - 9 hours after exposition respectively (Table 1 and Table 2). It was observed that the time taken to achieve 100% mortality varied according to the larvicide concentrations. With concentration of 500 mg/m^2 (representing the minimal

Table 1. Mean mortality rate of *Anopheles coluzzi* larvae (Lab strain) after 12-hour exposure to varying concentration of VectoMax®G.

Duration of exposure (Hours)	Mean Larvae Mortality of *An. coluzzi* at varying concentrations ± SE					
	Control	25 mg/m^2	50 mg/m^2	125 mg/m^2	250 mg/m^2	500 mg/m^2
0	0.00 ± 0.00	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	38.25 ± 13.33	39.75 ± 18.65	79.00 ± 18.36	70.00 ± 20.36	85.50 ± 6.20
2	0.00 ± 0.00	70.75 ± 15.14	66.00 ± 16.04	85.25 ± 14.42	79.50 ± 18.19	98.00 ± 2.00
3	0.00 ± 0.00	90.00 ± 6.12	83.00 ± 7.82	97.50 ± 2.50	91.25 ± 8.75	99.75 ± 0.25
4	0.00 ± 0.00	94.00 ± 3.46	90.50 ± 6.18	99.00 ± 1.00	97.25 ± 2.75	100.00 ± 0.00
5	0.00 ± 0.00	95.50 ± 2.63	92.50 ± 5.42	100.00 ± 0.00	100.00 ± 0.00	
6	0.00 ± 0.00	97.00 ± 1.73	94.75 ± 3.20			
7	0.00 ± 0.00	97.50 ± 1.50	95.00 ± 3.00			
8	0.00 ± 0.00	98.25 ± 1.18	95.00 ± 2.87			
9	0.00 ± 0.00	98.25 ± 1.18	96.25 ± 2.25			
10	0.00 ± 0.00	98.50 ± 0.96	97.00 ± 2.12			
11	0.00 ± 0.00	99.25 ± 0.48	98.50 ± 0.87			
12	0.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00			

Table 2. Mean mortality rate of *Culex quinquefasciatus* (Lab strain) after 12-hour exposure to varying concentration of VectoMax®G.

Duration of exposure (Hours)	Mean Larvae Mortality of *Culex quinquefasciatus* at varying concentrations ± SE					
	Control	25 mg/m²	50 mg/m²	125 mg/m²	250 mg/m²	500 mg/m²
0	0.00 ± 0.00	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	0.00 ± 0.00	0.00 ± 0.00	8.50 ± 2.10	14.50 ± 2.90	17.75 ± 2.59
2	0.00 ± 0.00	56.50 ± 8.93	53.00 ± 7.19	80.25 ± 6.93	88.00 ± 4.40	82.50 ± 12.52
3	0.00 ± 0.00	70.75 ± 3.99	76.75 ± 4.84	89.25 ± 4.09	91.75 ± 3.14	84.25 ± 12.77
4	0.00 ± 0.00	88.75 ± 3.35	90.00 ± 2.34	94.25 ± 2.17	95.50 ± 2.63	86.00 ± 12.67
5	0.00 ± 0.00	93.50 ± 1.94	94.00 ± 2.83	97.00 ± 1.08	96.25 ± 2.78	86.00 ± 12.67
6	0.00 ± 0.00	95.25 ± 1.80	97.25 ± 0.95	99.25 ± 0.48	97.25 ± 2.43	100.00 ± 0.00
7	0.00 ± 0.00	97.25 ± 1.11	99.00 ± 0.41	100.00 ± 0.00	100.00 ± 0.00	
8	0.00 ± 0.00	98.75 ± 0.25	100.00 ± 0.00			
9	0.00 ± 0.00	100.00 ± 0.00				
10	0.00 ± 0.00					
11	0.00 ± 0.00					
12	0.00 ± 0.00					

reference dosage recommended by the manufacturer) a 100% mortality was recorded after 4 and 6 hours exposure in both species (Table 1 and Table 2). When sublethal concentrations such as 25 mg/m², total mortality of larvae exposed was obtained after 12 hours for *An. coluzzii* (Table 1) and after 9 hours for *Cx. quinquefasciatus* (Table 2). The VectoMax®G concentration of 6.59×10^{-8} mg/m² and 2.25×10^{-7} mg/m² represented the LC50% and LC95% doses for *An. coluzzii* while 1.29×10^{-8} mg/m² and 2.91×10^{-8} mg/m² represented the LC50% and LC95% doses for *Cx. quinquefasciatus*. Concerning laboratory residual activity, the results indicated that VectoMax®G formulation at 500 mg/m² minimal dose recommended by the manufacturer and WHO performed effectively against *An. coluzzii* up to 28 day with 86.33% ± 9.58% larval mortality. Mortality rates for each species at different doses are shown in Table 3. No dead was observed in control containers.

3.2. Open Field Trials

Anopheline and *Culicine* mosquito larvae were detected 6 to 7 days after the artificial habitat was set-up. Both early instars (L1 and L2 larvae) and late instar (L3 and L4 larvae) were recorded. The percentage reduction of *Anopheles* s.l. larvae following VectoMax®G application is shown in Table 4. Two rounds of treatments took place with each lasting more than 12 days. Different concentrations were tested during each round. The mean number of larvae including early, late instars and pupae in control and treated sites are shown in Figure 1. A 100% mortality rate was recorded within the first 24 hours after larviciding application. Over time in both treated and untreated containers, natural declines

Table 3. Residual activity of VectoMax®G against susceptible strain of *Anopheles coluzzii* "Ngousso" exposed at different time period.

Days	Mean mortality (%) of *An. coluzzi* ± SE					
	Control	25 mg/m²	50 mg/m²	125 mg/m²	250 mg/m²	500 mg/m²
1	0.00 ± 0.00	99.37 ± 0.47	100 ± 0.00	100 ± 0.00	100 ± 0.00	100 ± 0.00
3	0.00 ± 0.00	78.37 ± 12.35	99.75 ± 0.25	99.62 ± 0.24	99.87 ± 0.12	100 ± 0.00
7	0.00 ± 0.00	53.75 ± 26.43	99.75 ± 0.25	99.50 ± 0.20	99.37 ± 0.62	100 ± 0.00
14	0.00 ± 0.00	69.00 ± 17.37	98.50 ± 1.50	100 ± 0.00	100 ± 0.00	100 ± 0.00
28	0.00 ± 0.00	80.50 ± 11.29	99.25 ± 0.25	98.50 ± 0.64	97.00 ± 1.35	96.0 ± 1.29
35	0.00 ± 0.00	86.33 ± 9.58	99.67 ± 0.33	100 ± 0.00	99.67 ± 0.33	100 ± 0.00
40	-	-	-	-	-	-

-: No test because the water had dried in the containers.

Table 4. Average number of *Anopheles gambiae* s.l. larvae and reduction rate for different VectoMax®G concentrations after application in open field trials.

	Average number/container												Percentage reduction					
	Early instars				Late instars				Pupae				Early instars			Late instars		
Day	C	T1	T2	T3	C	T1	T2	T3	C	T1	T2	T3	T1	T2	T3	T1	T2	T3
0*	**60.0**	**60.0**	**60.0**	**60.0**	**20.0**	**20.0**	**20.0**	**20.0**	**0.0**	**0.0**	**0.0**	**0.0**						
1	56.0	0.0	0.0	0.0	15.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100	100	100	100	100	100
2	49.0	0.0	0.0	0.0	22.5	0.0	0.0	0.0	2.3	0.0	0.0	0.0	100	100	100	100	100	100
3	34.8	0.0	0.0	0.0	40.3	0.0	0.0	0.0	2.8	0.0	0.0	0.0	100	100	100	100	100	100
4	26.0	0.0	0.0	0.5	32.8	0.0	0.0	0.0	3.8	0.0	0.0	0.0	100	100	100	100	100	100
5	6.0	1.0	0.75	0.8	25.8	0.0	0.0	0.0	2.5	0.0	0.0	0.0	83	88	87	100	100	100
6	5.5	5.5	0.0	0.3	12.8	0.0	0.0	0.0	7.3	0.0	0.0	0.0	0	100	95	100	100	100
7	55.3	24	32.8	29	9.5	11.0	19.5	28.5	1.5	0.5	1.3	3.3	57	41	48	0	0	0
8	33.5	14.5	24.8	25.3	20.8	13.0	36.0	34.8	4.0	1.5	1.8	5.5	57	26	24	38	0	0
9	19.8	7.0	16.3	13.0	28.8	9.5	8.5	15.8	3.8	1.3	1.5	4.5	65	18	34	67	70	45
10	24.8	13.3	18.3	22.5	24.8	12.5	9.3	11.8	1.3	0.0	0.8	1.8	46	26	9	50	63	52
11	23.8	11.3	13.3	22.0	15.0	12.5	7.0	7.3	9.3	0.3	1.3	1.0	53	44	8	17	53	51
12	9.0	0.8	14.3	8.0	10.8	10.5	3.8	4.3	5.5	1.8	1.3	1.0	91	0	11	3	65	60
13	**60.0**	**60.0**	**60.0**	**60.0**	**20.0**	**20.0**	**20.0**	**20.0**	**0.0**	**0.0**	**0.0**	**0.0**						
14	45.5	0.0	0.0	0.0	19.8	0.0	0.0	0.0	3.0	0.0	0.0	0.0	100	100	100	100	100	100
15	30.5	0.0	0.0	0.0	20.5	0.0	0.0	0.0	4.3	0.0	0.0	0.0	100	100	100	100	100	100
16	22.3	1.0	0.0	0.0	18.8	0.0	0.0	0.0	6.8	0.0	0.0	0.0	96	100	100	100	100	100
17	15.3	6.8	0.0	0.0	18.8	0.0	0.0	0.0	3.5	0.0	0.0	0.0	56	100	100	100	100	100
18	9.0	16.5	0.0	0.0	15.5	0.0	0.0	0.0	4.8	0.0	0.0	0.0	0	100	100	100	100	100
19	5.8	5.8	5.8	1.0	6.8	0.5	0.0	0.0	8.3	0.0	0.0	0.0	0	0	83	93	100	100
20	11.8	9.0	5.8	2.0	4.5	0.5	1.3	0.0	1.3	0.0	0.0	0.0	24	51	83	89	71	100
21	31.0	22.0	26.3	19.5	35.3	13.0	21.8	21.3	4.3	2.3	7.3	4.0	29	15	37	63	38	40
22	22.5	13.75	12.3	5.5	26.0	7.8	22.5	21.8	14.0	1.8	2.3	1.0	39	45	76	70	13	16
23	16.3	9.5	5.8	9.8	23.3	6.5	18.8	16.8	6.5	2.8	4.0	2.8	42	64	40	72	19	28
24	11.5	13.8	6.0	9.3	17.8	4.8	11.3	8.3	5.8	0.8	4.3	4.8	0	48	19	73	37	53
25	4.8	16.8	3.5	8.0	17.3	5.3	7.3	7.3	3.8	0.8	4.5	4.3	0	27	0	69	58	58

Asterisks (*) indicate days with larvicide application. C = Control; T1, T2 and T3 **day 0*** ≠ T1, T2 and T3 **day 13*** (see **Figure 1**).

and increases of larval densities were observed. In the first round of larvicide treatment, a reduction rate of 100% for late instar larvae was observed for up to six days after treatment. VectoMax®G was very effective against the late instars reducing the population by 100% within 24 hours post treatment for all concentrations. Generally, the larvicide impact on the late instars remained high up to day 6 post treatment with VectoMax®G (Figure 1). VectoMax®G was effective against early instars of *Anopheles* s.l. with a reduction of 100% of the larval population within 24 hours. This effect lasted up to day 3 after application before recolonization of the sites occurs.

Though the VectoMax®G treatments resulted in 100% mortality of the early and late instars of *Anopheles* s.l. within 24 hours, an initial recolonization of treated sites by L1 larvae was generally observed three to four days after treatment. All concentrations tested, were equally effective up to 4 days post-treatment for early instar larvae and up to 6 days for late instars. During the first 6 days of round one and the first 8 days of round two, no pupae were found in the treated containers, meaning that the late instars were killed by the action of larvicide.

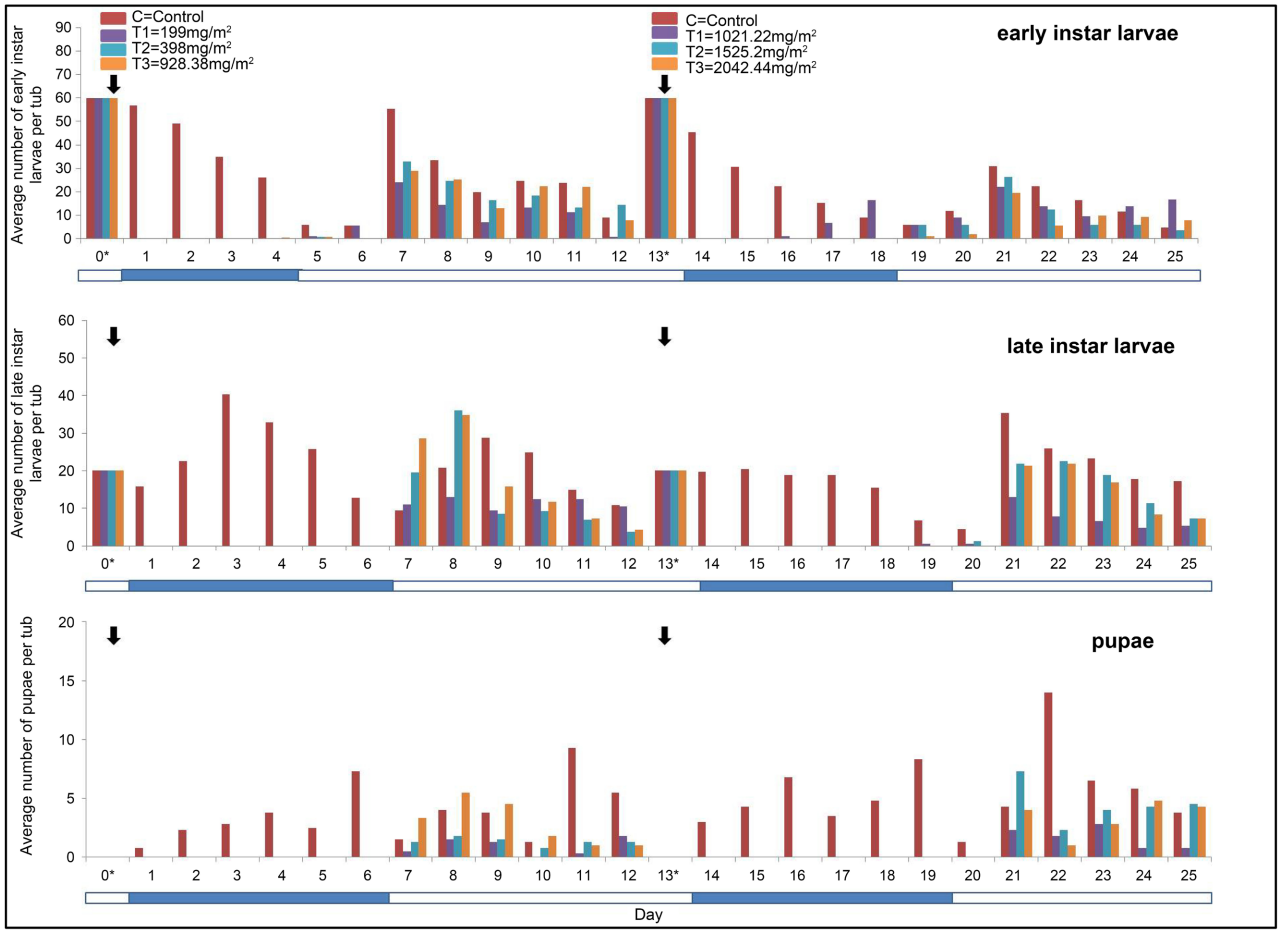

Figure 1. Population dynamics of early, late instars and pupae of *Anopheles gambiae* s.l. in open field trial experiments with VectoMax®G. Arrows indicate the date when we introduced a new batch of larvae in the containers. *Asterisks indicates VectoMax days application. White horizontal bars indicate no significant difference between treatment and control containers, blue bars do ($\alpha = 0.05$).

The effects of VectoMax®G on larval densities of *Culex* spp. and reduction rate compared to control containers is shown in Figure 2 and Table 5. Sublethal concentration of VectoMax®G was not found to be effective against late instars of *Culex* larvae 24 hours post treatment. A 100% reduction was obtained after 48 hours. When supra doses were used 100% reduction and no recolonization of sites was observed up to seven days post-treatment. VectoMax®G was effective against early instars of *Culex* spp. with a reduction of 81% - 100% of the larval population within 24 hours depending on concentration/doses. This effect lasted up to day 5 after application. More interesting, pupation levels were very low in the treated ponds (Figure 2), which is considered the most important parameter for efficacy assessment of larval control measures [27].

Table 5. Average number of *Culex* spp. larvae and reduction rate for different VectoMax®G concentrations after application in open field trials.

	Average number/container												Percentage reduction					
	Early instars				Late instars				Pupae				Early instars			Late instars		
Day	C	T1	T2	T3	C	T1	T2	T3	C	T1	T2	T3	T1	T2	T3	T1	T2	T3
0*	**10.0**	**10.0**	**10.0**	**10.0**	**10.0**	**10.0**	**10.0**	**10.0**	**0.0**	**0.0**	**0.0**	**0.0**						
1	9.0	0.0	0.0	0.0	7	4.0	0.8	0.3	1.0	1.3	1.5	0.3	100	100	100	43	89	96
2	5.75	0.0	0.0	0.0	6.5	0.0	0.0	0.0	1.75	0.0	0.0	0.0	100	100	100	100	100	100
3	2.75	0.0	0.0	0.0	6.25	0.0	0.0	0.0	0.75	.0.0	0.0	0.0	100	100	100	100	100	100
4	1.25	0.0	0.0	0.0	5.75	0.0	0.0	0.0	0.5	0.0	0.0	0.0	100	100	100	100	100	100
5	0.75	0.0	0.0	0.0	2.75	0.0	0.0	0.0	0.5	0.0	0.0	0.0	100	100	100	100	100	100
6	0.25	0.0	0.0	0.0	2.0	0.0	0.0	0.0	0.5	0.0	0.0	0.0	100	100	100	100	100	100
7	9.75	6.0	1.5	3.0	5.0	3.75	2.5	1.75	0.0	0.0	0.0	2.25	38	85	69	25	50	65
8	5.25	1.25	1.0	2.5	6.0	1.75	2.25	5.0	1.75	0.0	0.0	0.0	76	81	52	71	63	17
9	0.25	0.0	0.0	0.0	8.0	0.25	2.5	0.0	1.0	0.0	13.5	3.75	100	100	100	97	69	100
10	1.0	0.0	0.0	0.0	7.25	0.25	0.0	2.25	0.5	0.0	2.75	0.5	100	100	100	97	100	69
11	0.25	0.0	0.0	0.0	3.25	0.25	0.0	2.75	3.0	0.25	0.0	0.0	100	100	100	92	100	15
12	0.0	0.0	0.0	0.0	2.5	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0	0	0	100	100	100
13*	**10.0**	**10.0**	**10.0**	**10.0**	**10.0**	**10.0**	**10.0**	**10.0**	**0.0**	**0.0**	**0.0**	**0.0**						
14	4.75	0.0	0.0	0.0	12.5	0.0	0.0	0.0	1.0	0.0	0.0	0.0	100	100	100	100	100	100
15	4.5	0.0	0.0	0.0	8.25	0.0	0.0	0.0	1.75	0.0	0.0	0.0	100	100	100	100	100	100
16	2.75	0.0	0.0	0.0	7.0	0.0	0.0	0.0	1.5	0.0	0.0	0.0	100	100	100	100	100	100
17	1.75	4.5	0.0	0.0	5.0	0.0	0.0	0.0	0.5	0.0	0.0	0.0	22	100	100	100	100	100
18	2.75	10.75	0.0	0.0	7.0	0.0	0.0	0.0	1.5	0.0	0.0	0.0	87	100	100	100	100	100
19	39.25	2.75	0.0	0.0	0.5	0.0	0.0	0.0	0.75	0.0	0.0	0.0	97	100	100	100	100	100
20	50.0	2.5	0.25	0.0	4.75	0.0	0.0	0.0	1.0	0.0	0.0	0.0	98	100	100	100	100	100
21	54.25	4.5	3.5	2.25	39.25	7.0	5.5	3.0	0.0	0.0	0.0	0.0	89	92	95	85	88	93
22	20.0	0.75	0.25	0.5	57.5	1.5	1.75	1.5	1.75	0.0	0.25	0.25	94	98	96	97	96	97
23	5.75	2.5	0.0	3.5	55.25	1.5	0.5	1.0	3.5	0.0	0.25	0.0	21	100	0	96	99	97
24	1.5	2.25	0.0	0.0	45.25	1.25	0.25	1.5	3.5	0.0	0.0	0.0	0	0	0	96	99	95
25	0.0	0.5	0.0	0.0	38.75	2.0	0.25	0.5	3.0	0.25	0.0	0.25	0	0	0	0	0	0

Asterisks (*) indicate days with larvicide application. C = Control; T1, T2 and T3 **day 0*** ≠ T1, T2 and T3 **day 13*** (see Figure 2).

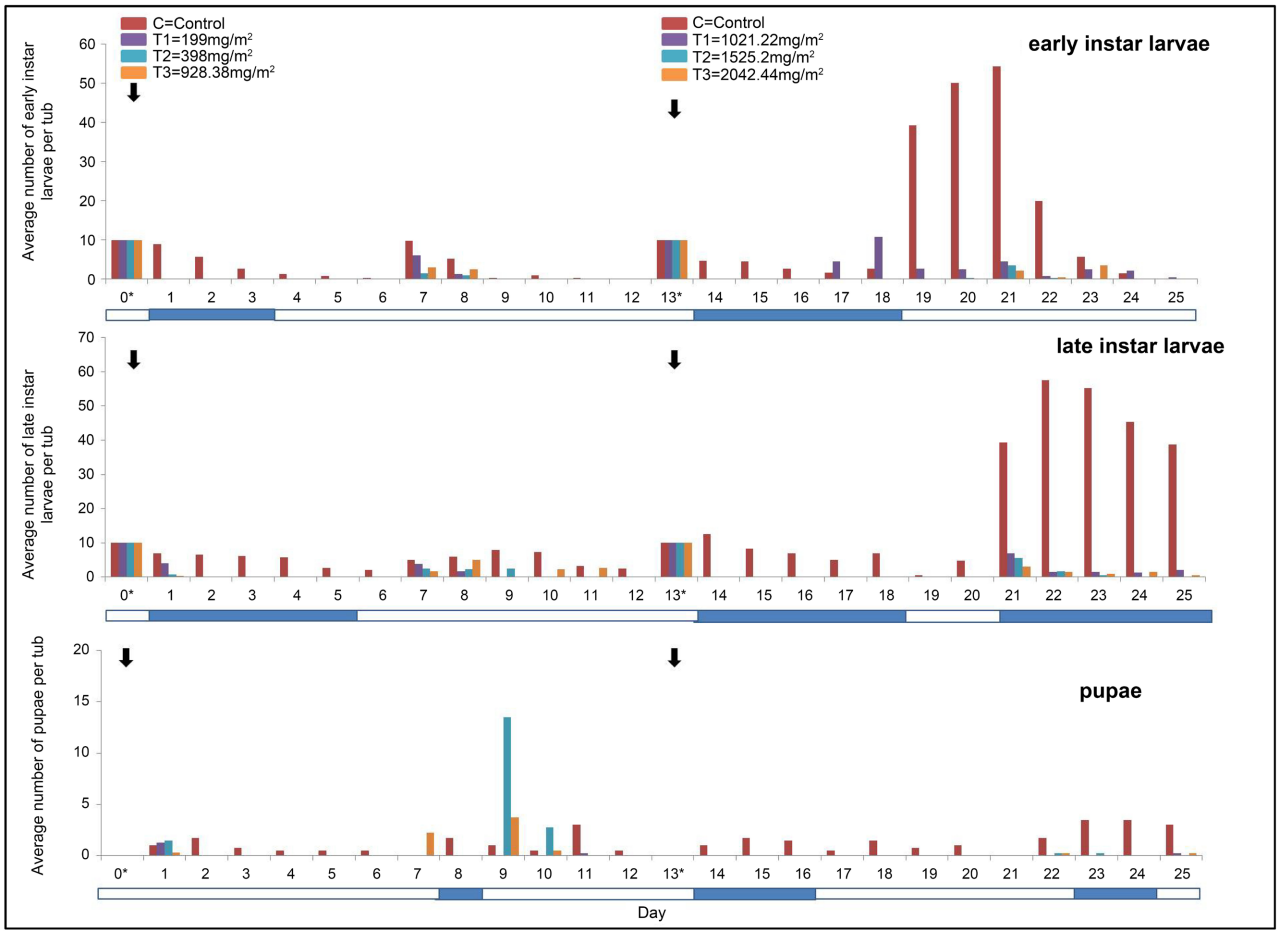

Figure 2. Population dynamics of early, late instars and pupae of *Culex* spp. in open field trial experiments with VectoMax®G. Arrows indicate the date when we introduced a new batch of larvae in the containers. *indicates VectoMax days application. White horizontal bars indicate no significant difference between treatment and control containers, blue bars do ($\alpha = 0.05$).

4. Discussion

In this study, several doses of VectoMax®G were used regarding the minimum reference dose recommended by the manufacturer, *i.e.*, 500 mg/m^2. Doses lower than the latter were used to estimate the effectiveness of the product in case of insufficient quantity or wrong weighing. Other doses greater than or equal to the standard dose were also used. The manufacturer recommends retreatment after 3 to 4 weeks under conventional weather conditions for normal and above normal doses and states that an appearance of stage 1, 2, or 3 larvae do not indicate a need for retreatment. Therefore, this study also examined whether the manufacturer's recommendations could be applied in our environmental context. The total mortality observed with *An. coluzzii* and *Cx. quinquefasciatus* larvae at all the serial concentrations attest to the efficacy of VectoMax®G larvicide at very low concentrations in the laboratory. Similar studies have reported a 100% mortality of larvae during the first 16 hours [28] and 24 hours [29] of application of VectoMax®G. The rapid response of VectoMax®G initiating death after 1 hour of application confirms the fast-acting potentials of toxins produced from *Bti*

and *Bs* which are the active constituents. However. VectoMax®G residual activity can last more than 35 days. Similar studies on *Aedes aegypti* have reported the temephos larvicide maintaining its effectiveness for the first 42 days in a laboratory in Peru [30], and in Malaysia, the same number of days were reported with *Bti* [31].

Concerning the open field trial, we recorded a very low natural colonization of the containers by mosquitoes; this could be due to the presence of several potential and natural breeding sites/water collections not far away from the study site. It is also important to point the fact that the size, depth, and colour of the water in the bucket would have probably influenced mosquito oviposition behaviour because the buckets used were white, 30 cm deep, and contained clear water during the first weeks. All the concentrations determined in the laboratory were subsequently tested in open field trials and revealed that these concentrations were equally effective up to 4 days post-treatment for both early and late instars and up to 7 days when considering the late instars only. The containers were exposed to a whole array of environmental factors such as rainfall, pollution, sunlight, similarly to other mosquito breeding places. The results of the control containers indicated that there is a steady supply of young instars from eggs, which were not affected by the larvicide. The observed fluctuations in larval populations have also been reported in other studies [27]. VectoMax®G, was found to reduce both early and late instar stages. Surprisingly, late instars stages were found to be more affected by treatment and retreatment of sites.

The different applications of VectoMax®G resulted in an effective reduction of the density of Anopheline and Culicine larvae and pupae (81% to 100%) with the normal and higher doses 24 hours after the treatment. Our results are similar to those of many studies such as the one conducted by Owolola *et al.* [32] in Lagos (Nigeria), where the small-scale field trial caused an effective inhibition of the emergence of *An. gambiae s.s.* and *Cx. quinquefasciatus* greater than 80%, as well as the study in Penang (Malaysia) by Ahmad *et al.* [33] reporting similar results on *Cx. quinquefasciatus* and *Ae. aegypti*. For sublethal doses, the efficacy of VectoMax®G was good on Anopheles larvae and pupae (96% - 100% reduction rate) but less important with *Culex* spp. (27% - 83% larval reduction). VectoMax®G was also found to be more effective on Anopheles larvae during the first day's post-treatment but less effective after one week. On the opposite, *Culex* spp. larvae were less affected during the first day post-treatment but displayed a high reduction rate several days after treatments. This could be explained by the difference in the feeding and resting behaviour between Anopheline and *Culex* mosquitoes [34] [35] [36]. Anopheles larvae feed mainly on the surface and generally only dive to escape from danger. They do not remain at depth as long as Culex spp. where the latter mainly feed. Because the larvicide crystals sediment after few days, they are during the first days more available to Anopheles which quickly consume the lethal quantity and then when they sediment they become available to *Culex* spp.

The moderate residual effect of VectoMax®G was recorded during field experiments. The current literature reports different findings on the residual effect of microbial larvicides, Kinde-Gazard and Baglo [37] reported 9 days before larvae reappeared after larvicide treatment. Kroeger *et al.* [38] found in a study carried out in Ecuador and Peru that the effect of treatment could last 7 to 10 days. In Eritrea, Shililu *et al.* [39] described an effect of up to three weeks of microbial larvicides. In Burkina Faso, Kenya, and Ghana, Dambach *et al.* [40], Fillinger *et al.* [23] and Nartey *et al.* [41] described an effect ranging between three and six days. Our findings are in line with these observations [42] and could be ascribed to a similar experimental setup. Small-scale trials in Goa, India with VectoMax®G showed 15 - 52 days, 10 - 42 days and 15 - 22 days of residual activity on larvae and pupae densities of *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus* respectively [32]. In a simulated field trial conducted with VectoMax®G in Nigeria conducted in cement tanks with clean water, it appeared that VectoMax®G could cause effective inhibition (>80%) of *An. gambiae* s.l. emergence for a period of 21 to 36 days. Application of VectoMax®G in polluted water tanks caused effective inhibition (>80%) of *Cx. quinquefasciatus* for 7 to 44 days [32]. In a simulated trial in Malaysia, VectoMax®G application in pots containing polluted water resulted in effective inhibition (>80%) of *Cx. quinquefasciatus* emergence for 7 - 14 days [33]. Based on these findings, the manufacturer recommends reapplication after 3 to 4 weeks under conventional weather conditions, which they believe is their average period of effectiveness. Several reasons can be given to explain the low residual effect of VectoMax®G observed in our study. It is possible that the counting procedure would have had a considerable impact on the larvicide residual effect. The fact of stirring the water to bring up the larvae and pupae hidden in the depths would have induced early sedimentation of the larvicide and its unavailability for Anopheline larvae which feed more on the surface. The sedimentation of the product is more accentuated if the depth is great. This is in line with the observation of Becker and Margalit [14] stating that the efficacy of the different formulations is influenced by the availability of VectoMax®G crystals in the first 10 cm of the surface of a water column. In the present study, the high-water depth (30 cm) is a possible explanation for the low persistence of the residual effect of the product. It has also been reported that the presence of a high concentration of chlorine and iron seems to reduce the toxic activity of VectoMax®G crystals [43]. Although being carefully washed, the containers may have retained chemical residues that could have reduced the persistence of the larvicide. It was also noted throughout the study that many competitors such as frog tadpoles, were regularly present in most containers. Although their numbers were not measured, they may be responsible to some extent for the reduced persistence of the product, as they consume the product and quickly make it unavailable to larvae. Organic pollution also acts on the effectiveness of the product by adsorption of the product crystals on organic particles, facilitating precipitation, which decreases their availability [44] [45] [46].

Despite these possible limiting factors, the present study highlighted the high efficacy of the biolarvicide VectoMax®G against Anopheline and Culicine larvae.

5. Conclusion

Our results strongly suggest that the microbial larvicide VectoMax®G has a high larvicidal effect on both *Anopheles and Culex* spp., the known vectors for *Plasmodium* and Lymphatic filariasis respectively. Given the high rate of malaria in Cameroon, successful and affordable vector control strategies, such as the use of microbial larvicides could be key for the successful elimination of malaria in urban settings.

Authors Contributions

KE and CAN conceptualized and designed the study; KE, DS, NE, BR, DDL, TA and S-CN performed the field experiments; KE performed laboratory experiments and statistical analysis. FG, NF, A-AP and WCS critically reviewed and amended the manuscript. KE and CAN interpreted, analysed data and wrote the manuscript with input from all authors. All the authors read and approved the final version.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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