LYSINIBACILLUS SPHAERICUS BIOLARVICIDE, AN EFFICACIOUS TOOL IN THE CONTROL OF *ANOPHELES GAMBIAE* IN KUMASI, GHANA

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ABSTRACT

Malaria continues to be the leading cause of morbidity and mortality globally. In Ghana control of the vector involves multiple strategies. One of the strategies being adopted is the integrated vector management (IVM) control approach. However, there is evidence of growing resistance by malaria vectors in the country to several classes of insecticides. Therefore interest in alternatives to augment already existing interventions is high. We assessed the effectiveness of Water Dispersible Granular (WDG) formulation of *Lysinibacillus sphaericus (*also known as *Bacillus sphaericus)*, a biolarvicide, as a tool against *Anopheles gambiae* Giles complex larvae in the laboratory and controlled field study in Kumasi metropolis, Ghana. The Lethal Concentration (LC) of the biolarvicide to kill 50% (LC_{50}) and 95% (LC_{95}) of first generation (F_1) *Anopheles gambiae* larvae were determined in the laboratory by a range of bioassays. The optimum effective dosage of *Bs* VectoLex® formulations against naturally occurring *Anopheles gambiae* Giles complex larvae was determined in controlled field trials during the rainy and dry seasons. We show here that *Anopheles gambiae* Giles complex larvae were susceptible to the biolarvicide with LC_{50} and LC_{95} of 0.0027 mg/l and 0.0086 mg/l, respectively after 24 hours exposure in the laboratory. The 50 fold concentration of the LC_{95} were found to achieve 100% larval mortality within 24 hours post-application for up to 10 (dry season) to 12 days (rainy season) under field conditions. There was a highly significant difference ($p<0.0001$) in larvae population after addition of 0.5 mg/l as compared to the control for both seasons. The biolarvicide proved to be an efficacious tool in the reduction of *Anopheles gambiae* Giles complex larval population. Further studies need to be carried out to evaluate the impact this biolarvicide in terms of reduction in actual malaria incidence and also assess cost effectiveness of the biolarvicide in relation to other malaria vector control interventions.

Keywords: *Lysinibacillus sphaericus*, *Anopheles*, malaria, vector, biolarvicide, Kumasi.

Introduction

Malaria is holoendemic in Ghana, accounting for 38% of all outpatient illnesses and 36% of all admissions in hospitals and clinics in the country [1].

Its incidence in the country follows a particular ecological zoning, the highest occur in the forest areas followed by the coastal zone and then the northern savannah [2],

peaking shortly after the major rainy season (May to July) but markedly reduced during the dry season (November to February).

In order to control malaria, its vector abundance should be controlled [3]. Malaria vectors breed in clear, sunlit, temporary water bodies such as swampy areas for agriculture [3].

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Uncultivated and wastelands are increasingly being converted into vegetable farms in major cities of Ghana and thereby creates pockets of peri-urban malaria breeding-friendly environment within cities [4]. Farming activities in Kumasi are made possible by irrigation, utilising water from small streams and dug out wells all year round. Urban agriculture in Kumasi thus generates breeding sites for malaria transmitting *Anopheles gambiae sensu stricto* [4]. Breeding sites of Kumasi metropolis mapped by Nartey *et al*. [5], indicate that they are interspersed in several urban areas. As it is to be expected, malaria transmission is more intense for communities with large numbers of irrigated farms than the non-irrigated ones [6]. World Health Organization (WHO) in 2012 released a statement on the use of biolarvicides for malaria control in sub-Saharan Africa. It recognizes that biolarviciding should be considered for IVM but only in areas where breeding sites are '*few, fixed and findable*'[7]. There is thus a growing appreciation for the importance of biolarviciding in urban and periurban areas, where the breeding sites are likely few, easily identifiable and amenable to control [8].

In Ghana, malaria vector control measures are presently confined to Indoor Residual Spraying (IRS) and use of Long-Lasting Insecticidal Nets (LLINs) both advocated for by the World Health

Organization [9]. Vectors of malaria are becoming increasingly resistant to various classes of insecticides except just a few in Ghana [10], larval control might therefore be a tool to be added to the current interventions to lower the impact of the disease[11], however very little is known about the effectiveness of the use within West Africa and in particular the Ghana.

Larviciding using *Lysinibacillus sphaericus*, formerly called *Bacillus sphaericus* [12] has been reported to have minimal effect on non-target organisms and is safe to the user, exhibit very low risk of insect resistance and is environmentally friendly [13]. The strain *Bs* 2362 has particularly been shown to be effective against a variety of mosquito species worldwide [14-22].

As part of IVM control programme against malaria in Ghana preparations are far advanced to produce and implement large-scale application of biolarvicides although the country is relying largely on inconsistent evidence from elsewhere in Africa [21,22]. We are unaware of any studies of *Bs* as a larvicide of *Anopheles gambiae* mosquitoes studied in Ghana. Additionally, there have been conflicting reports on the ability of *Bs* to persist for some days to weeks in treated habitats [21-24]. In a bid to provide additional data and to clarify the exact role that *Bs* plays, we sought to investigate the residual activity of *Bs* on malaria vector. We therefore assessed the effectiveness of Water Dispersible Granular (WDG) formulation of *Bs* Vectolex® as a tool against *Anopheles gambiae* larvae in the laboratory and wild larvae in controlled field trials in the Kumasi metropolis, Ghana as well as its residual effect.

Materials and Methods

Study Area

The study was carried out at the premises of Kumasi Centre for Collaborative Research in Tropical Medicine (KCCR) of the Kwame Nkrumah University of Science and Technology (KNUST) Kumasi, Ghana (06°, 41°N 001°, 36° W). Kumasi is located in the tropical forest zone with the major rainy season from May to July and the dry season from November to February [25] (Figure 1)

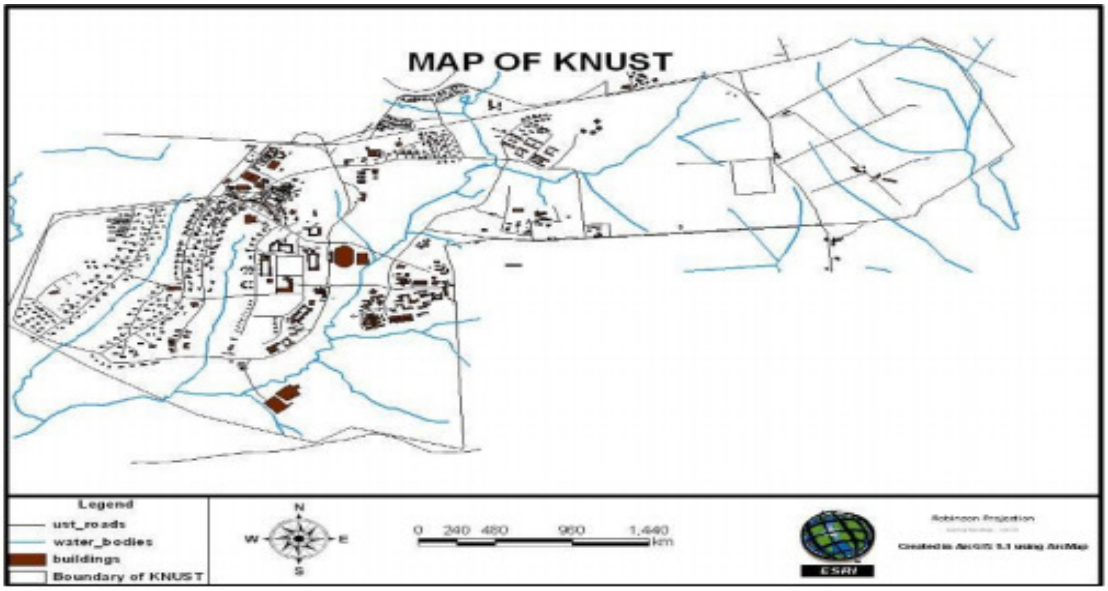

Figure 1: Map of Kwame Nkrumah University of Science and Technology showing larval sampling site (S) KNUST

Climatological Data

The temperature as well as rainfall patterns during the controlled field trials carried out in the rainy season (May to July 2011) and dry season (November to February) are shown in Figure 2 and 3.The rainy season was characterised by frequent rains, on 15 different occasions post application of formulation. The peak rainfall, 58.6mm occurred on the 19th day post-treatment with the biolarvicide (Figure 2). Temperatures during the rainy season ranged from a minimum of 19.2 ºC to a maximum of 29.5 ºC (Figure 2).

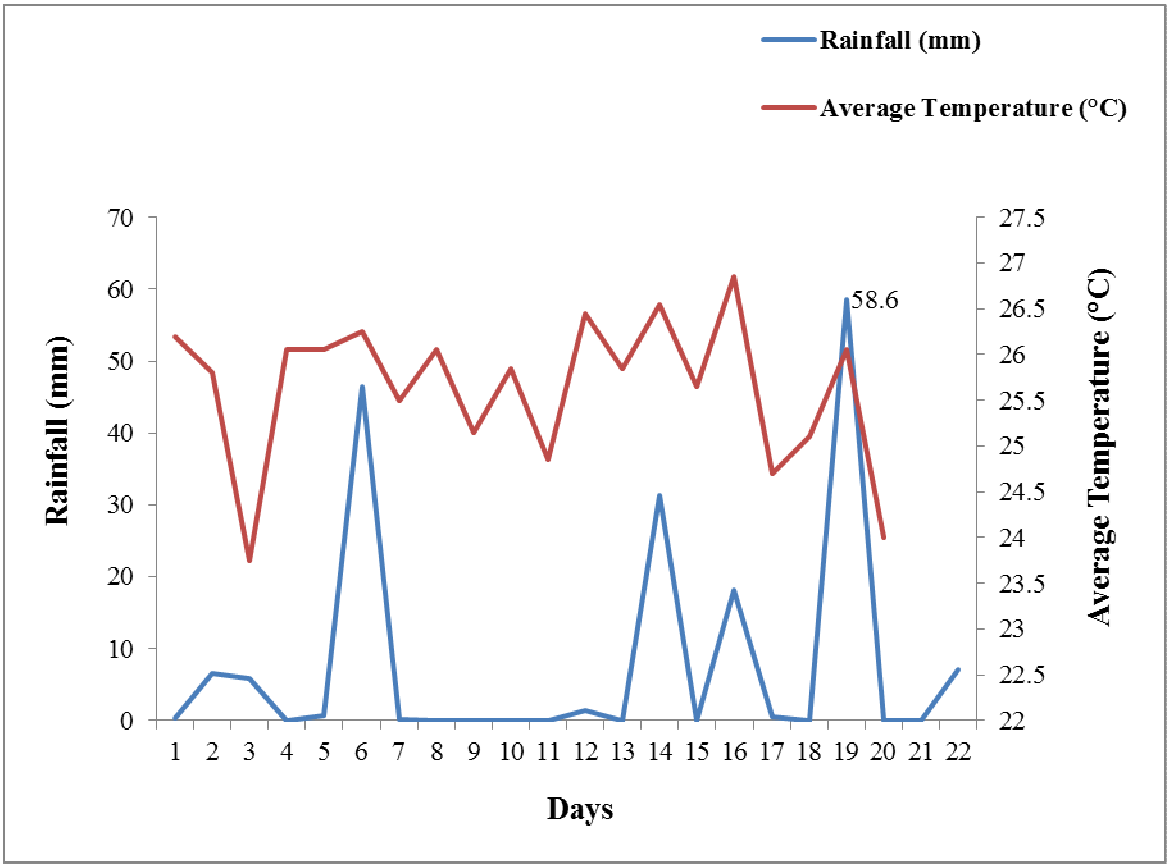

Figure 2: Daily rainfall and temperature pattern after application of the *Bs* in the rainy season

During the dry season period it rained five times after application of formulation and the peak rainfall was 11.6mm on the second day post-treatment (Figure 3). Temperature for the dry season ranged from a minimum of 21.6 ºC to a maximum of 33.5 ºC (Figure 3).

Laboratory Procedures

Mosquitoes

Anopheles larvae were sampled from their natural breeding sites in and around an irrigated leguminous farmland on KNUST campus and reared in the laboratory to adult stage. The larvae were fed by adding a pinch of ground Tetramin[@] (Tetra, Germany) fish food spread evenly on their habitat water surface twice daily to grow into adult. Adult male *Anopheles* mosquitoes were fed on 10% sugar solution soaked in cotton wool while the females anopheles mosquitoes were fed with blood meal from guinea pigs for the first generation (F1) larvae. The F1 larvae were reared in tap water. The adult *Anopheles* mosquitoes were used for morphological identification using the keys of Gillies and DeMeillon [26] while the F1 third and early fourth instar larvae were used for a series of bioassays.

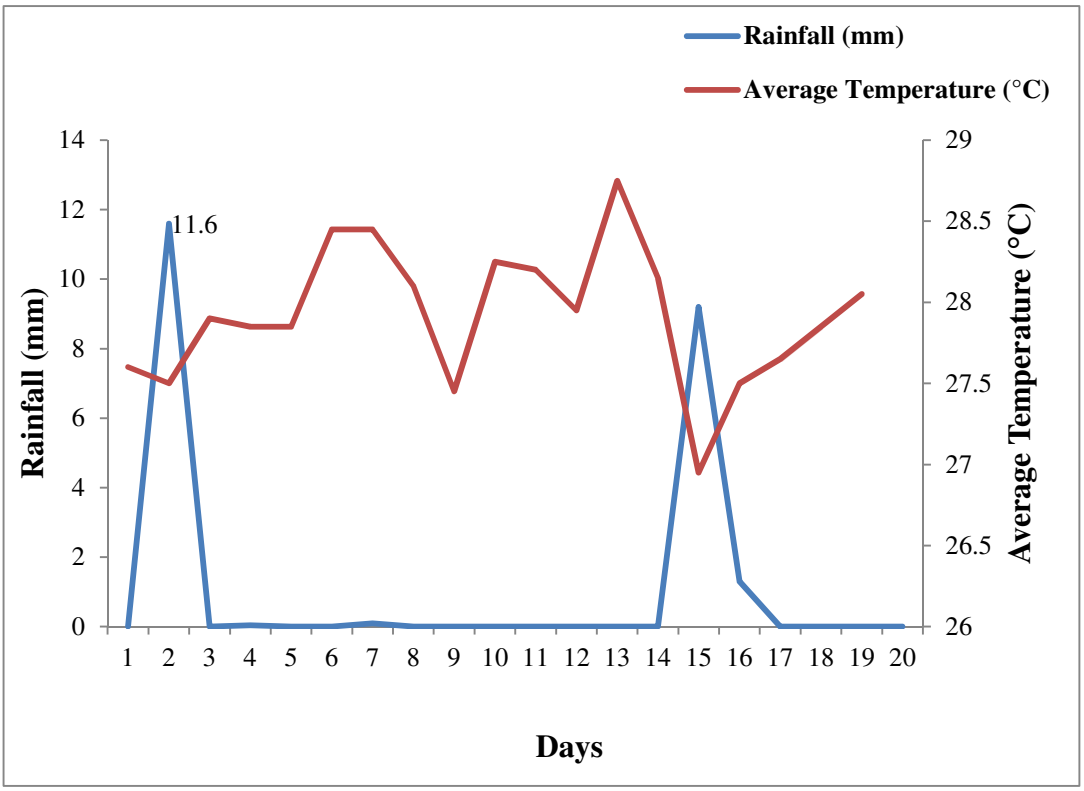

Figure 3: Daily rainfall and temperature pattern after application of *Bs* in the dry season

Biological Larvicide and Bioassay

The *Bs* serotype h5a5b, strain 2362, WDG formulation (VectoLex®, Valent Biosciences Corporation, Illinois, USA) was used. The Bioassay was carried out per standard testing procedures by the International Programme on Chemical Safety (IPCS) of WHO [27] Preliminary test were conducted by exposing, mosquito larvae to *Bs* concentrations ranging from0.0015mg/l to 0.05mg/l to determine the range of activity of the Bs formulation. The mortalities of the larvae recorded at concentrations; 0.01mg/l, 0.005mg/l, 0.004mg/l, 0.0035mg/l, 0.003mg/l, 0.0025mg/l and 0.002 mg/l were used to determine LC50 and LC95 respectively in accordance with WHO recommendation.

Fresh stock of 1% *Bs* Vectolex® was prepared, from which aliquots of the seven concentrations (0.01mg/l, 0.005mg/l, 0.004mg/l, 0.0035mg/l, 0.003mg/l, 0.0025mg/l and 0.002 mg/l) were made with distilled water in plastic disposable cups. We added to each cup twenty-five (25) third and early fourth first generation *Anopheles gambiae* larval instar containing the different test concentrations. Each experiment contained an untreated control. Four replicate cups were used for each concentration and control. The experiment was carried out on three different occasions under similar environmental conditions running for 24 hours. During this period larval mortality records were taken at 25±1°C. The experiment was discarded and repeated when mortality for the control exceeded 10%. This was to ensure that mortality in the larval population was due solely to the activity of the biolarvicide and not an external factor. In estimating the mortality the total dead larvae were counted. Larvae were declared as dead when they could not be induced to move by touching it with a Pasteur pipette.

Controlled Field Trials

In order to compare the performance of the larvicide during both seasons in terms of effective dose and residual activity, we conducted controlled field trials during the rainy and repeated in the dry seasons within the KNUST campus following the study design by

Fillinger *et al.*[21] and Majambere *et al*. [22]. Briefly, two adjacent beds of 20 x 2 m each were raised on a well sunlit area and 9 bowls of 0.45 m diameter and 0.26 m depth were fitted into each bed making 18 bowls in total. Six bowls were used as control, six for the 50 fold treatment concentration and the rest for 100 fold treatment concentration. We selected treatment and control bowls randomly using a web-based randomisation tool [28]. The distance from one bowl to the other was 1.5 m with each bowl filled with soil to 1/5th of its capacity and 10 litres of *Anopheles* habitat water all from an irrigated leguminous farmland on KNUST campus (Figure 1). This provided the required biotic and abiotic conditions for mosquito oviposition. The set up was left standing for 12days to allow for oviposition of female *Anopheles* mosquito and for development of these eggs to 3rd and 4th instar larvae.

Treatment concentrations used for the field trials were calculated on the basis of a standard water depth of 0.1m and a fixed surface area [29]. LC values represented minimum dosages only since laboratory assays were conducted under standardized conditions with less abiotic and biotic influences. Therefore under field conditions, formulation concentrations have to be increased up to several times the LC_{95} to obtain sufficient larval control [21]. The tested *Bs* WDG concentration was 50 more than the LC_{95} (0.5 mg/l). We added 100 fold of the LC_{95} (1.0mg/l) to ascertain the effectiveness and residual effect as compared to the 50 fold LC_{95}.

The *Bs* Vectolex formulation was then applied using a handheld sprayer to the whole water surface of each test bowl. Sampling larvae and pupae were done using a 350 ml standard dipper by taking 10 dips from different positions at the periphery and the centre of each bowl. After counting, larvae were returned to respective bowl while pupae were bred to adult stage for subsequent morphological identification. All pupae were collected with a Pasteur pipette early in the morning and late in the evening into petri dishes filled with water and placed in the rearing cages to emerge into adult. First and 2nd larval stages were grouped as early instars and the 3rd and 4th larval stages as late instars.

Daily recordings were done until the 12th day after which the monitoring was carried out every other day till the end of the test on the 22nd day. Counting was stopped after the 22nd day because the number of late instar larvae in the treated habitat reached numbers similar to or greater than that of the control. This procedure was carried out for both the rainy and dry seasons at ambient temperatures 23 to 29°C.

Data analysis

The LC_{50} and LC_{95} mortality data were analysed using the Simple Logistic Regression Model in SPSS version 16.0 (SPSS Inc., Chicago, USA).The average number of larvae and pupae collected per dip and the percentage reduction on each day of observation for each replicate in treatment and control were calculated using Microsoft Excel 2007.The normality of average number of larvae and pupae per dip in control and treatment bowls in field trials were carried out using D'Agostino & Pearson omnibus normality test. Based on the normality test results, all comparison were done using the non parametric Mann-Whitney test except for the comparison between the control and the treatment for the rainy season in which a parametric unpaired t test was used. All normality and comparison test were carried out using the GraphPad Prism 5. The percentage reductions for the mean numbers were calculated using the formula of Mulla *et al*. [30]: % Reduction = 100 - (C1/T1× T2/C2) ×100, C1 and C2 described the mean number of larvae in the control bowls for the pre- and the post-treatment, respectively. T1 and T2 denote the mean number of larvae in the treated tubs for the pre- and the post-treatment, respectively.

Ethical approval

The Committee for Human Research, Publications and Ethics (CHRPE) at the Kwame Nkrumah University of Science and Technology, Kumasi, Ghana gave ethical approval for the study. We sought for additional permission to use the premises of KCCR for the field trial from authorities at KCCR before proceeding.

Results

Identification of the *Anopheles* mosquitoes

Out of a total of 2284 *Anopheles* mosquito sampled during both the laboratory and controlled field study, 99.9% were identified morphologically as *Anopheles gambiae* Giles *complex* and 0.1% as *Anopheles funestus*.

Laboratory Bioassays

A 24-hour exposure of late instar *Anopheles gambiae* larvae to *Bs* resulted in LC_{50} (50% mortality) at 0.0027 mg/l and LC_{95} (95% mortality) at 0.0086 mg/l (Figure 2).

Figure 4: Set up for controlled field trials

Field Trials

Oviposition by Anophelines (89%) and Culicines (11%) mosquitoes were observed within 4 to 6 days after field trials had been set up. We pooled results for both genera together because Culicines made only 11% of the total population and the effect of *Bs* on both genera was the same.

Rainy season

Figures 5 and 6 show results of 0.5 mg/l and 1.0 mg/l concentrations of *Bs* VectoLex® formulation tested during the rainy and dry season respectively in an open field and the impact of each concentration on larval population as compared with controls.

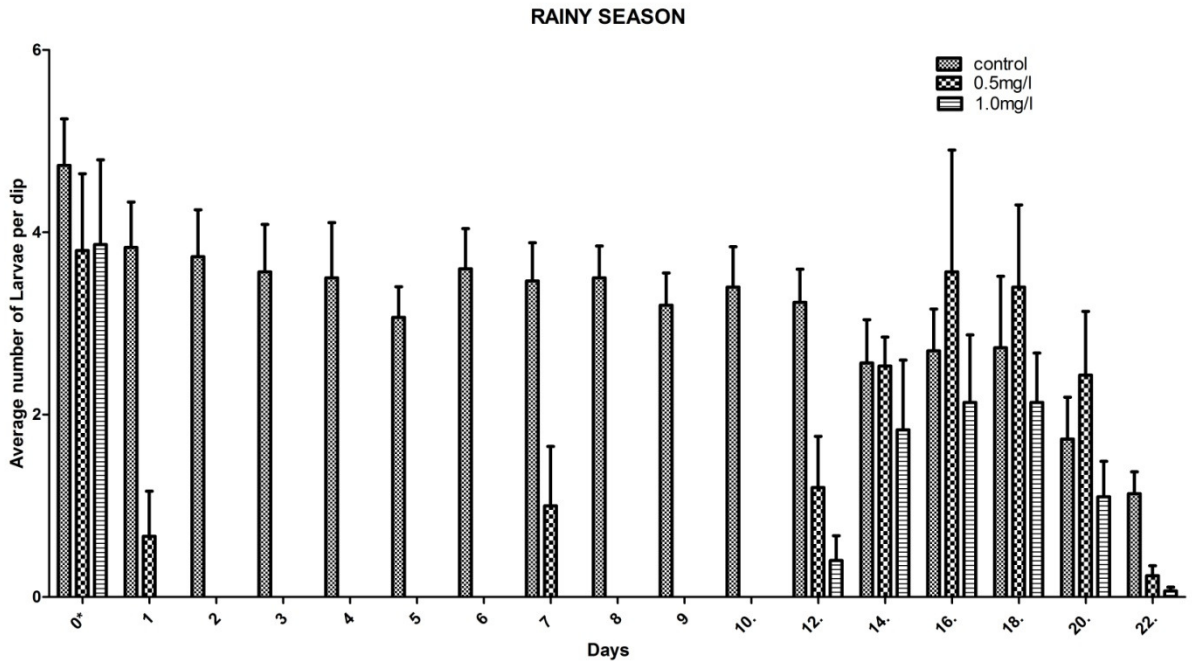

Figure 5: Average total larval numbers exposed to different concentrations of *Bs* in a controlled field trial during the rainy season

The average number of early and late instars larvae, per dip in the 18 bowls ranged from 3.9 to 4.7 before the *Bs* formulation was added. The 0.5 mg/l and 1.0 mg/l concentrations used recorded 100 % larval mortality from the first day of addition of formulation to the twelfth day. Therefore the residual effect lasted for 12 days for both concentrations. The only exception was incidental appearance of early instar larvae on the first (79.5%) and seventh day (66.9) which were due to freshly hatched larvae yet to feed or that had fed less than fifteen minutes hence alterations in the midgut were yet to start since *Bs* works as quick as 15 minutes after ingestion of its spore-crystal complex [31] all these early instar larvae died after 24 hours.

Overall, there was statistically significant difference between the control and each of the test concentrations, 0.5 mg/l and 1.0 mg/l ($p<0.0001$). There were however no statistically significant differences between the 0.5 mg/l and 1.0 mg/l treatment concentrations ($p=0.39$).

The average number of pupa per dip was 0 in all the bowls till day 14 at a *Bs* concentration of 0.5 mg/l and from then onwards 0-1 pupae till day 22. Similarly, the 1.0 mg/l treatment concentration resulted in no pupae per dip being recorded till day 12 followed by 0-3 till day 22. In the control bowls pupae numbers increased from zero to a range of 0-10 pupae by day 7 and then decreased gradually till day 22. There was no statistically significant difference ($p=0.95$) between the 0.5 mg/l and 1.0 mg/l treatment concentrations.

Dry season

The average numbers of *Anopheles* larvae per dip before treatment ranged 2.8 and 6.9 in all bowls. The impact of 0.5mg/l concentration of *Bs* Vectolex on the population of larvae and the percentage reduction in comparison with untreated bowls was also reported. There was a 100% reduction in all treatment bowls till day 9 with one exception, when one larva per dip was counted on day 7. The effect of the formulation on day 10 of a 0.5 mg/l and 1.0 mg/l concentration led to a reduction of 70.2% and 87% respectively for both early and late instar larvae.

Since numbers of larvae in controls continuously declined after day 10 no further reductions could be calculated for the treated bowls by day 22. Overall, there was statistically high significant difference between the control and each of the test concentrations ($p=0.0001$). There were however no significant differences between the 0.5 mg/l and 1.0 mg/l treatment concentrations ($p=0.765$).

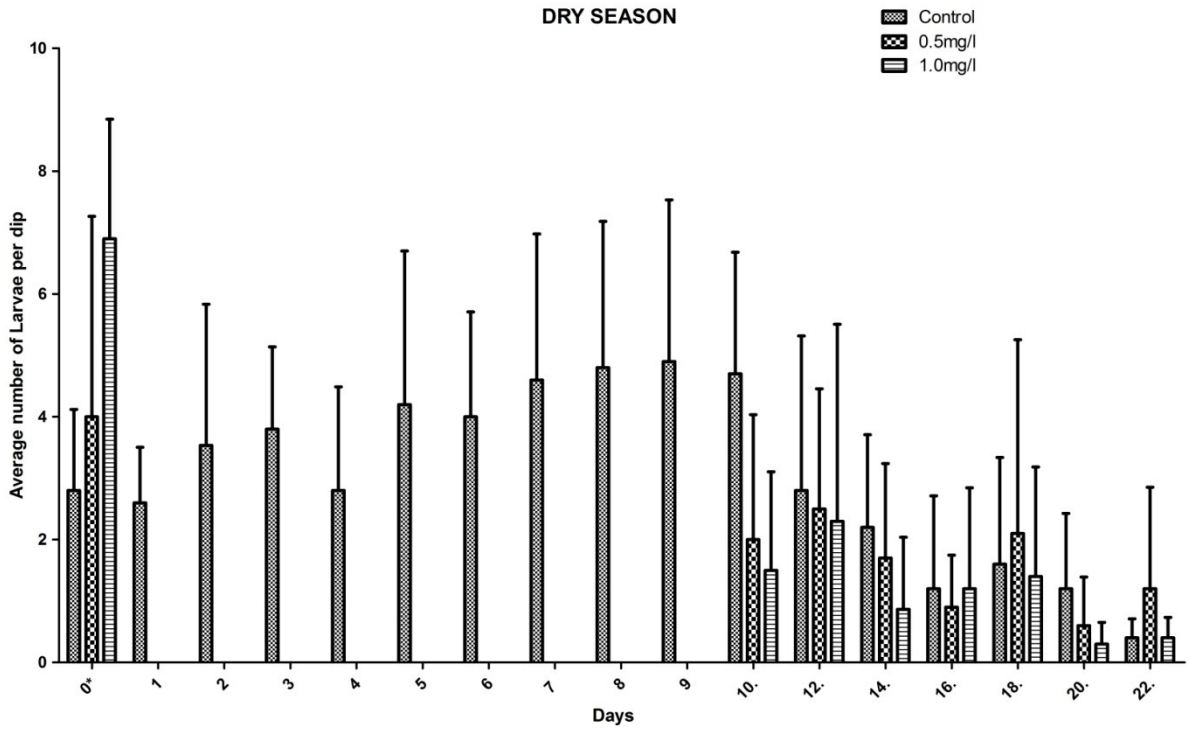

Figure 6: Average total larval numbers exposed to different concentrations of *Bs* in a controlled field trial during the dry season

At *Bs* concentration of 0.5 mg/l and 1.0 mg/l, there was no pupa recorded on day zero in all 18 bowls. Pupae emerged on day 12 for both treatment concentrations and disappeared afterwards. At *Bs* concentration of 0.5 mg/l, pupae re-emerged on day 16 with 0- 4 pupae per dip while at *Bs* concentration of 1.0 mg/l, pupae re-emerged on day 20. Thereafter an average of zero to two pupae were recorded till day 22. The number of pupae was increasing in control bowls from zero to a range of 0-12 by day 7. The number decreased gradually till the 22nd day. There were no significant differences between the 0.5 mg/l and 1.0 mg/l treatment concentrations (p=0.28) in terms of pupae mortality.

Discussion

The results of the study showed that larvae of the *Anopheles gambiae* of malaria in the Kumasi metropolis are highly susceptible to *Bs* under laboratory and field conditions with a relatively high residual effect of 10 days in the dry season and 12 days in the rainy season respectively.

Our LC values were similar to those of earlier studies [22,32] with slight differences occurring in comparison with the results of others [21,33]. These occurrences may be due to methodical differences where first generation *Anopheles* larvae were reared in tap water for our study instead of natural habitat water as in the case of Fillinger *et al*. [21] and laboratory reared first generation larvae instead of field collected larvae as reported by Majorie *et al.*[33].

In contrast to previous studies though [24,34], our study revealed a relatively prolonged residual effect of 10 days (dry season) and 12 days (rainy season). The duration of our residual effect however agrees with studies in USA, Cuba, Thailand and Kenya [18-21]. The application of larvicides with delayed residual effect such as *Bs* are expedient for malaria control programmes as they require less manpower and larvicide concentration. This by implication may likely be cost effective relative to other interventions hence appropriate to be included in an IVM programme. However, further studies would be required to explore the exact contribution in terms of cost. Fillinger and Lindsay [35]and Karch *et al*. [36] reported a higher residual activity by applying the *Bs* formulation repeatedly. To achieve maximum effect, reapplication of the *Bs* WDG formulation on mosquito larvae is therefore recommended to be carried out every 10-12 days during all seasons. One has to indicate that the effective use

of larvicides depends on application techniques, other biological agents within the ecological niche, duration of use and other environmental factors such as sunlight. We are of the view that in applying this larvicide particularly in the rainy season, one would have to consider all such factors in addition to cost and safety the agent is to non-targeted organisms .The higher control persistence observed in the rainy season may not be due entirely to the larvicide alone, but due to other factors such as the mechanical action of the rain on the larvae and pupae.

During the period of the study, it rained fifteen times in the rainy season and five times in the dry season. The rains decreased the temperatures of the water in the treatment and control bowls while the dry season had increasing temperatures and since high temperature reduce biolarvicidal activity of *Bs* formulation [37] the residual effect during the dry season were 2 days shorter than that of the rainy season. *Bs* exposed to prolonged sunlight reduces its activity. In as much as sunlight activity in the dry season may be cumulatively high *Bs* still showed residual effect because of its recycling activity in dead larvae[38]. Therefore, in the application of *Bs* as a larvicidal agent in the control of malaria vector all such physiochemical and biotic factors should be considered.

A heavy down pour of rain on day 19 post-treatment in the rainy season could be the reason for the decline in the larval and pupal population from 20th day post treatment. The direct impact of rain on the larvae and pupae as well as the washing away of the larvae by excess water in the bowls may have lowered their survival [39]. Pupae, when hit directly by rain, will eventually drown when their hydrostatic balance is compromised [40]. This then will reduce contact time with the agent larvicide and thereby reduce its efficacy.

We are of the view that it is possible to have had extended residual effect in the rainy season because larvae being washed away and pupae drowning cause reduction in contact time with the agent larvicide and thereby reduce its efficacy. We suggest that as a result of this inherent activity of the larvicide and the tendency to have torrential tropical rain, the application of this larvicide should take into consideration such factors as they do have the potential to limit its efficacy. Perhaps since incidence of Malaria is very high during the rainy season one would have to apply the larvicide strategically just after a major downpour. The difficulty though is that one is unable to predict the occurrence of such heavy downpour, which has a washing effect on the larvae and pupae while reducing the efficacy of the concentration of the larvicide.]

Early instar *Anopheles* larvae were counted on the 1st, 7th and 12thday post application of the *Bs* formulation during the rainy season. It is assumed, that oviposition continued but freshly hatched larvae died 24 hours later due to biolarvicidal activity of the *Bs* formulation. This changed from day 12 onwards, when increasing larval survival in the field trial were recorded. The decline in larval numbers in the control bowls coincided with the appearance of algae bloom and tadpoles, both conditions possibly rendering potential breeding sites no longer attractive for oviposition of *Anopheles gambiae* females [31]. Therefore the application of this particular larvicide should consider the presence of such competitive environmental fauna and flora that have the tendency to render the intervention ineffective.

In applying this finding to the general population we had to consider not only irrigated vegetable farms but also construction sites as we do have anecdotal evidence that they do contribute significantly to malaria vector populations and hence malaria transmission in the city [4]. A proposed larval control program in urban areas should therefore concentrate on areas with urban agriculture and include the sunlit water bodies targeting anophelines [3] and others like those with nutrient rich stagnant water and shady areas to target culicines [41].Whereas large-scale application of *Bs* is recommended we are unable to provide the complete picture in terms of the actual reduction in the number of malaria episodes occurring within the Ghanaian population. We therefore recommend further investigation that would translate these findings into larger human population to determine the actual reduction in malaria incidence within community settings in addition to determining the cost effectiveness of the intervention for policy action.

Conclusion

At recommended doses, *Bs* is effective and might prove efficacious in the control of malaria in combination with other recognised larvicides such as *B.thuringiensis var israelensis* and other

known interventions. Low concentrations of *Bs* are required to control the population of larvae of malaria vector in the field and thus can offer additional alternatives when combined with other interventions. We propose that based on its 10 to 12 days residual effect reported in this study, reapplication in a control intervention should be done every 10 to 12 days during a programme that involves the use of *Bs*. Additional studies are required to show the cost effectiveness of this intervention in community studies to see how this translates to actual reduction in malaria incidence.

Competing Interest

The authors declare that they have no competing interests

Authors' Contribution

SBA acquisition of data, analysis and interpretation of data and drafting the article revising it critically for important intellectual content

EOD drafting the article revising it critically for important intellectual content and final approval of the version for publication

TK analysis and interpretation of data and final approval of the version for publication

AA drafting the article revising it critically for important intellectual content

RN acquisition of data

JD revising it for intellectual content

NB conception and design of work drafting and final approval of the version for publication

SO drafting the article revising it critically for important intellectual content

KOD drafting the article revising it critically for important intellectual content and final approval of the version for publication

PS revising it critically for important intellectual content

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