

# Fly ash based *Bacillus thuringiensis* var. *israelensis* formulation for use against *Culex quinquefasciatus*, the vector of filariasis in natural ecosystems

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## ABSTRACT

**Background & objectives:** Fly ash is produced in huge quantities by the various thermal power stations in India. This thermal waste has been employed as a carrier material in the preparation of a biopesticidal water dispersible powder (WDP) formulation for use against mosquitoes. In the present investigation, this newly developed fly ash based WDP formulation was evaluated in natural breeding habitats of mosquito.

**Methods:** Fly ash based WDP formulation of *Bacillus thuringiensis* var. *israelensis* (VCRC B17) was evaluated for its efficacy and residual activity in aquatic habitats supporting breeding of *Culex quinquefasciatus*, the vector of lymphatic filariasis in Neyveli Township, Neyveli Lignite Corporation, India for a period of one month.

**Results:** At an application rate of 10 kg/ha, the WDP was effective for five days regardless of the habitat, and provided 80–100% reduction in larval abundance of *Cx. quinquefasciatus*.

**Interpretation & conclusion:** The study indicates that for continued control of immature density and prevention of adult emergence, a weekly application of this formulation is necessary. This study also showed that fly ash based formulations can be used for immediate control of mosquitoes in different types of habitats and has also brought out a new avenue for the utilization of coal ash.

**Key words** *Bacillus thuringiensis* var. *israelensis*; biopesticides; *Culex quinquefasciatus*; field evaluation; fly ash; mosquito control

## INTRODUCTION

Fly ash, a byproduct produced during the combustion of coal in thermal power plants is produced in huge quantities in India. Indian coal on an average has 40–45% ash content and hence every 100 tonnes of coal burnt generates 40 tonnes of ash. The 70 or more coal based thermal power plants in India generates about 160 million tonnes of ash every year<sup>1</sup>. Several studies have been carried out on different aspects of fly ash and its utilization like brick making, cement making, land reclamation, filling up of mines and removal of dyes. Only a little of it is being utilized in different ways. Its recent and potential use in agriculture as a manure<sup>2–7</sup> has opened a new ray of hope, while exploratory tests carried out at our institute has indicated yet another usage—as a carrier for biopesticidal formulations.

Recent initiatives by the pesticide regulatory departments of European and North American governments and also Indian government have significantly renewed interest in biopesticide technologies as alternatives for pest management following plans to deregister many chemi-

cal pesticides within the next 10 yr. The literature is abundant with studies on screening for microorganisms with attributes of biopesticidal activity. However, very few of the authors have considered formulating the microorganisms with commercial applications in mind<sup>8</sup>.

Vector Control Research Centre (VCRC), Puducherry, India has been involved in the development of biopesticides for use against various mosquito vectors of diseases such as bancroftian filariasis, Japanese encephalitis, malaria, dengue and chikungunya. Many entomopathogenic organisms have been isolated and explored, the chief among them being an isolate of *Bacillus thuringiensis* var. *israelensis* (VCRC B17). This strain was isolated during the 1980s<sup>9</sup> and the WHO reference centre (Pasteur Institute, Paris) has rated its activity as a mosquito larvicide to be on par with the then available standard strain, IPS80. Ever since then, several studies have been conducted to prove its efficacy both in laboratory and field conditions<sup>10–12</sup>. The bacterium is safe to mammals and non-target organisms found coexisting with mosquito larvae<sup>13–15</sup>.

In this study, a water dispersible powder (WDP)

formulation developed using this *Bti* isolate and fly ash as the carrier material was taken up for dose fixation studies followed by medium-scale field trial in all larval habitats of *Culex quinquefasciatus* (Diptera: Culicidae), the vector of lymphatic filariasis in Block-21 of the Neyveli Lignite Corporation, South India for a period of one month.

## MATERIAL & METHODS

### *Safety to non-target organisms*

This formulation was used for field trials after ensuring its safety to non-target organisms found in association with mosquito larvae, viz. crustaceans, insects, mayfly nymphs, water beetles, fishes, snails and tadpoles.

### *Small-scale field trial in natural breeding sites*

The WDP formulation developed at VCRC using *Bti* (VCRC B17) as an active ingredient and fly ash as carrier material in the ratio of 4:5 with 1% binding agent was used for field trials. Preliminary evaluation of this formulation was carried out in aquatic habitats of Puducherry and Cuddalore, namely cesspits during February to March, 2010 to arrive at the optimum dosage of the WDP formulation which can be used for the medium scale field trials at Neyveli.

Cesspits usually collect sullage water from the households and are rich in organic matter. The water surface area in these pits ranged between 0.5 and 2 m<sup>2</sup>. Out of 24 cesspits selected, six were kept as control and six each were treated at the dosages of 5, 10 and 15 kg/ha respectively. Required amount of the WDP was weighed, added to a knapsack compression sprayer along with the required quantity of water, shaken vigorously and sprayed to the surface of the cesspits. The pH and temperature of the water ranged from 6.7–8 and 27–30°C, respectively.

In all the cesspits, pre-treatment immature density was monitored at biweekly intervals for two weeks prior to treatment. From each cesspit, four dipper samples (each 350 ml) were examined on each day of sampling. Samples collected were transferred to trays, immatures counted by instars and returned to the habitats. Immature density measurement on the day of treatment was followed by post-treatment density monitoring, 24 h after the spray and thereafter every alternate day, i.e. on Days 3, 5 and 7 followed by weekly observations till the density reached the pre-treatment level. The mean number of larvae and pupae collected/dip was calculated for each day of sampling and for each treatment as well as for control. The I and II instars were considered together as early instars and III and IV instars as late instars.

Percentage reduction in larval and pupal densities of *Cx. quinquefasciatus* during post-treatment was calculated using formula of Mulla *et al*<sup>16</sup>. The differences between treatments were compared by analysis of variance (ANOVA) with dosage and number of days as independent factors. The ANOVA was carried out after transforming the percentage reduction to arcsine values. The SPSS statistical package<sup>17</sup> version 21.0 for Windows was employed for the analyses.

### *Selection of optimum field application dosage*

From the dosages tested against *Cx. quinquefasciatus* in the small-scale field trials, the minimum dosage at which the maximum effect was achieved was selected as the optimum field application dosage for the medium scale field trials. The frequency of larvicidal treatment is determined based on the reappearance of IV instar larvae or pupae.

### *Medium-scale field trial at Neyveli*

The trial was conducted in Block 21 of the Neyveli Township, Neyveli Lignite Corporation, Neyveli, Tamil Nadu, India during August 2010. Preliminary surveys were carried out during July 2010 to select suitable breeding sites. The WDP formulation was evaluated against immatures of *Cx. quinquefasciatus* in three types of breeding habitats, namely cesspits, unlined drains and tar storage tanks. The WDP suspension was prepared and applied at 10 kg/ha, the dose found optimal during the small-scale field trial.

*Cesspits:* A total of 14 cesspits were present in this block, of which four were kept as control and the remaining were treated. From each cesspit, four dipper samples were examined on each day of sampling. The depth of the water was 10–15 cm and water surface area in these pits ranged from 0.79–3.63 m<sup>2</sup>. The average temperature and pH of the habitat water recorded during the trial period were 29.4°C and 8.4 respectively.

*Drains:* Two drains of length 175 and 45 m were present in this area. These drains contained slow moving sullage water of depth 5–10 cm with high content of organic matter. The drain of 45 m length was left untreated as control while the other was divided into stretches of 12 m length. Each stretch was considered to be a separate entity and used for treatment. Fifteen dipper samples were examined for every 12 m segment and the immature density was recorded as mentioned earlier. The average temperature and pH of the water were 30°C and 8.6 respectively.

*Tar storage tanks:* Tar used for laying of roads is kept stored under water in huge cement tanks of capacity

2 x 5 x 0.5 m to prevent drying. These tanks support breeding of *Cx. quinquefasciatus*. Out of nine total tanks, three were kept as control and the rest were treated with the WDP at the same dose mentioned earlier. Four dipper samples were taken from four corners of the tank. The average temperature and pH of the water were 30°C and 8 respectively.

In all the habitats, pre-treatment density was monitored at biweekly intervals for two weeks prior to treatment. Samples collected were transferred to trays, immatures were counted instar wise, and returned to the habitats. Immature density measurement on the day of treatment was followed by post-treatment density monitoring 24 h after the spray and thereafter every alternate day, *i.e.* on Days 3, 5, 7, *etc.* The appearance of IV instar larvae was followed by the next round of spray 48 h later. In this way, the cesspits and tar tanks received a total of IV and V rounds of continuous spraying. However, the drains started overflowing due to unprecedented rains which occurred on Day 7 of initiation of the trial, and therefore only II rounds of spraying could be done in this habitat.

The data were analyzed separately for habitats such as drains, cesspits and tar storage tanks. The difference in immature densities (early instars and late instars and pupae) among treated and untreated habitats (cesspit, drain and tar storage tanks) were compared by ANOVA test. In the ANOVA, test days and experimental groups (treated/untreated) were considered as independent factors. The 95% confidence interval (CI) for the larval densities was used to compare the difference in larval densities over different days of observation.

## RESULTS

### Evaluation of WDP formulation

**Small-scale field trials in natural breeding sites:** The pre-treatment densities of the early instar larvae in the cesspits treated with the three dosages, 5, 10 and 15 kg/ha were 52.4, 47 and 44.1/dip, while in untreated it was 49.3/dip. On Day 1 post-treatment, the reduction of larval densities (early instars) was 91, 92.4 and 93%, respectively at the three dosages (Fig. 1). On Day 3 post-treatment, the reduction was >70% at 15 kg/ha, whereas at 5 and 10 kg/ha, the reduction was >59%. On Day 7, the reduction of early instars was <60% at all the three dosages. The pre-treatment densities of the late instar larvae were 73.6, 64.3 and 64.4/dip, while in untreated it was 63.7/dip. On Day 1 post-treatment, the reduction of larval densities (late instars) was 96.1, 94.3 and 96.9%, respectively at the three dosages (Fig. 1). On Day 3 post-treatment, the reduction was >97% at 15 kg/ha, whereas

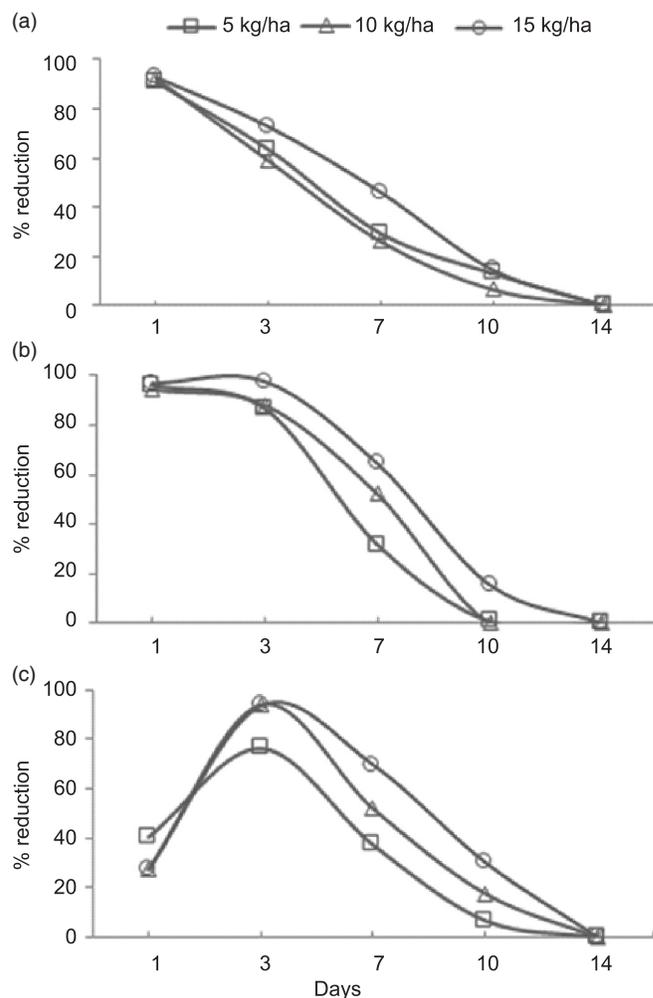


Fig. 1: Efficacy of fly ash based water dispersible powder (WDP) formulation on (a) Early instar, (b) Late instar, and (c) Pupae of *Culex quinquefasciatus* in cesspits.

at 5 and 10 kg/ha, the reduction was >86%. On Day 7, the reduction of late instars was <65% in all the three dosages, after which the larval density started increasing and reached the pre-treatment level on Day 10 post-treatment. The pre-treatment density of pupae was 16.5, 14.3 and 17.4/dip, respectively at the 5, 10 and 15 kg/ha dosages in the treated pits, while in the control pit it was 16.6/dip. On Day 1 post-treatment, only a marginal decrease was noticed. However, by Day 3 post-treatment, 76.3, 94 and 93.6% reduction was obtained in the pits treated at the three dosages respectively. The density started increasing by Day 7 and reached the pre-treatment level by Day 10.

The three dosages caused remarkable reduction in the immature stages. They did not vary significantly in reducing the early instars [ $F = 1.32$ ,  $df = (2, 85)$ ,  $p = 0.27$ ], late instars [ $F = 0.84$ ,  $df = (2, 85)$ ,  $p = 0.43$ ] and pupae [ $F = 0.28$ ,  $df = (2, 85)$ ,  $p = 0.75$ ]. The interaction effect of

dosages by days was also non significant ( $p > 0.05$ ). The difference in larval and pupal density was significant on different days of observation [early:  $F = 45.35$ ,  $df = (4, 85)$ ,  $p < 0.01$ ; late: ( $F = 47.04$ ,  $df = (4, 85)$ ,  $p < 0.01$ ; pupae:  $F = 17.53$ ,  $df = (4, 85)$ ,  $p < 0.01$ ]. Regardless of the dosages used, effective larvicidal and pupicidal activities of the WDP formulation were observed by 24 and 48 h respectively. However, at 10 kg/ha, 94% pupal reduction was observed up to Day 3. Hence, 10 kg/ha was selected as the optimal dosage for the medium scale field trials.

*Medium-scale field trial at Neyveli*

The mean population density (average of days -17, -10, -4 and 0) of larvae and pupae recorded in the three types of habitats prior to WDP treatment are given in Table 1.

**Cesspits:** At 24 h post-treatment, the density of early instar stages was brought down by 76.3% (Fig. 2). However, by Day 3, a gradual resurgence was noticed reaching pre-treatment level by Day 7. While the density of late instars was reduced by 95%, the level of reduction further increased to 99.3% on Day 3 and 86.6% on Day 5, after which the population started building up to 73.7% on Day 7 (Fig. 2). The same trend of reduction and resurgence was observed with all the IV rounds of spray. In case of pupal stages, by Day 1 only 63.1% reduction was observed which reached 100% on Day 3. By Days 5 and 7, only a slight increase in the pupal population was noticed. Reduction of >90% was maintained throughout the IV rounds of spray.

Results of the ANOVA test showed that abundance of immature stages differed significantly ( $p < 0.001$ ) between *Bti* treated and untreated habitats over a period of

Table 1. Mean population density±SE of larvae and pupae recorded in the three types of habitats prior to WDP treatment

Habitats/Immature stages	Mean density ± SE (Average of Days -17, -10, -4 and 0)	
	10 kg/ha	Untreated
<b>Cesspits</b>		
Early instars	22.9 ± 4.7	29.9 ± 8.6
Late instars	30.3 ± 6.8	20.2 ± 5.6
Pupae	5.6 ± 1.3	4.1 ± 1.4
<b>Tar storage tanks</b>		
Early instars	21.2 ± 2.2	17.4 ± 1.4
Late instars	20.2 ± 2	17.2 ± 1.3
Pupae	1 ± 0.4	1.5 ± 0.5
<b>Drains</b>		
Early instars	10 ± 1.7	9.2 ± 2
Late instars	9.6 ± 1.8	8.3 ± 1.9
Pupae	2.3 ± 0.6	0.6 ± 0.3

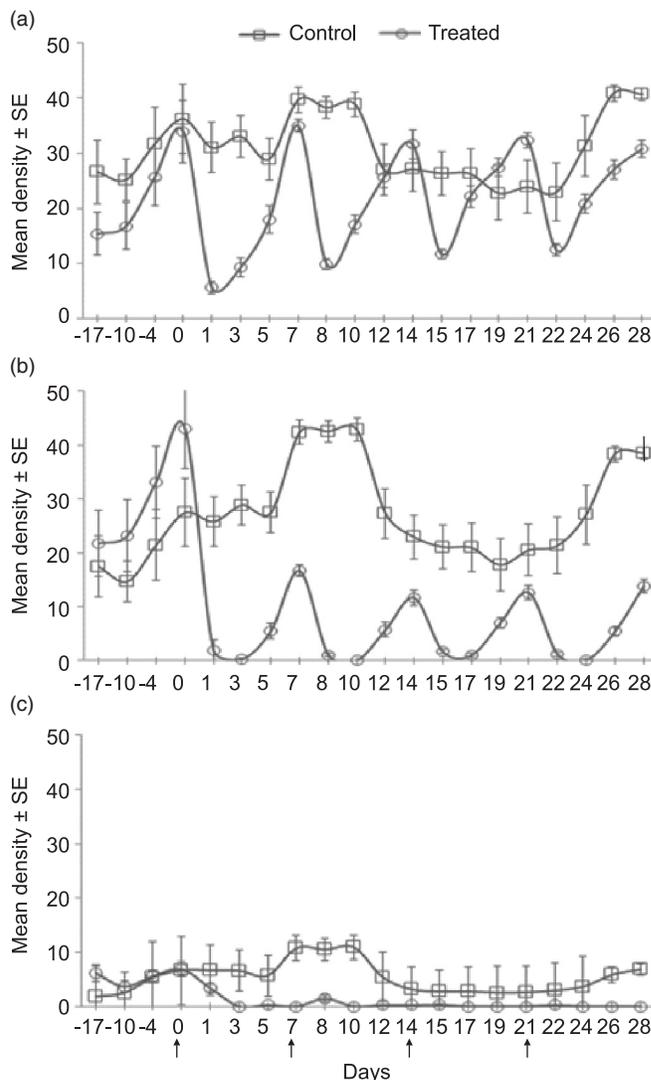


Fig. 2: Mean density of (a) Early instar, (b) Late instar, and (c) Pupae of *Culex quinquefasciatus* in cesspits treated with fly ash based WDP formulation.

time. Interaction effect of the treatment and days: early instars:  $F = 2.56$ ,  $df = (19, 956)$ ,  $p < 0.001$ , late instars:  $F = 4.801$ ,  $df = (19, 956)$ ,  $p < 0.001$ , pupae:  $F = 6.55$ ,  $df = (19, 956)$ ,  $p < 0.001$  found to be significant. Comparison of larval densities over different days of observation showed that the density on pre-treatment days was not significant ( $p > 0.05$ ) in treated and untreated habitats. But the density in treated was significantly ( $p < 0.05$ ) lower and not overlapped to untreated habitats on post-treatment days up to three days. This trend was seen during further spraying also.

**Drains:** After 24 h of treatment, the density of early instar stages was brought down by 80.9% (Fig. 3). However, by Day 3, a gradual resurgence was noticed reaching pre-treatment level by 7th day. While the density of late instars was brought down by 97% on Days 1 and 3,

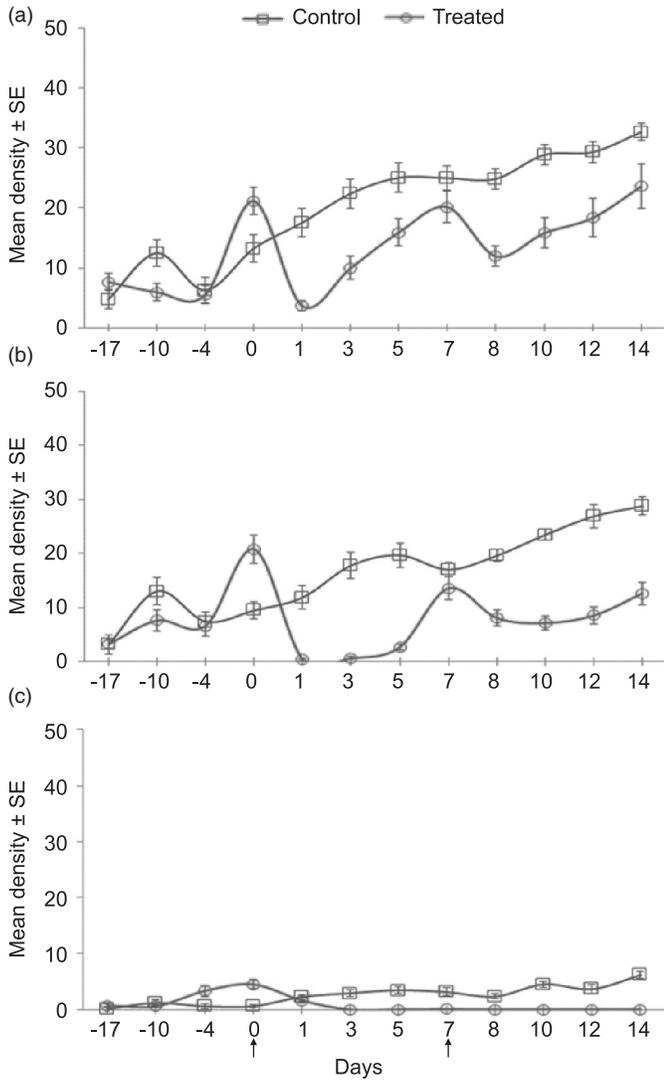


Fig. 3: Mean density of (a) Early instar, (b) Late instar, and (c) Pupae of *Culex quinquefasciatus* in drains treated with fly ash based WDP formulation.

the population started resurging by 5th day (88.3%) and by 7th day, the population reached half the pre-treatment level (31.3%). The percent reduction noticed during the II round of spray was lesser than that noticed during the I round. The results of the III round of spray could not be followed due to unprecedented rains during this time. With the pupal stages, by Day 1 only 79.8% reduction was noticed. However, this reached 100 and 99.4% on Days 3 and 7 respectively. After Day 7 no pupa could be recorded throughout the study period in the drains.

The ANOVA test showed that abundance of immature stages differed significantly ( $p < 0.0$ ) between *Bti* treated and untreated habitats over a period of time. Interaction effect of the treatment and days—early instars:  $F = 6.12$ ,  $df = (11, 336)$ ,  $p < 0.001$ , late instars:  $F = 15.88$ ,  $df = (11, 336)$ ,  $p < 0.001$ , pupae:  $F = 22.07$ ,  $df = (11, 336)$ ,

$p < 0.001$  found to be significant. Comparison of larval densities over different days of observation showed that the density on pre-treatment days was not significant ( $p > 0.05$ ) in treated and untreated habitats. But the density in treated habitats were significantly ( $p < 0.05$ ) lower and not overlapped to untreated habitats on post-treatment days up to five days.

**Tar storage tanks:** The density of early instars was brought down by 99.8% on Day 1 and 87.5% on Day 3. However, by Day 5 itself, a gradual resurgence was noticed reaching pre-treatment level by Day 8 (57.3%). Almost complete mortality (99%) of late instar stages was noticed by 24 h of treatment (Fig. 4). The reduction in late instars were maintained at 100% up to Day 5 after which resurgence was noticed at 94.3% on Day 8, resulting in IV instar stages on Day 14 (71.6%). The II, III and IV rounds of spray were done at seven days interval while the V round of spray was done on Day 9. With all the treatments, there was a 100% decline of late instar stages

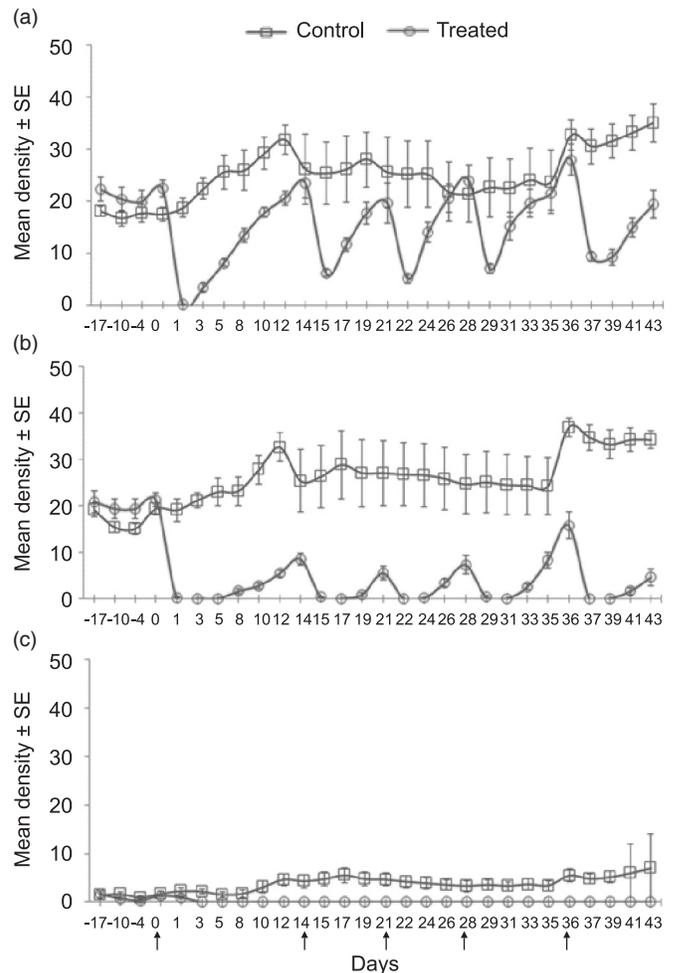


Fig. 4: Mean density of (a) Early instar, (b) Late instar, and (c) Pupae of *Culex quinquefasciatus* in tar storage tanks treated with fly ash based WDP formulation.

up to three days, followed by a slow build-up of the population. Among the pupal stages, even though a slight decline in the population was noticed by Day 1, 100% control of pupal development was observed throughout the 43 days observation of this habitat.

The ANOVA test showed that abundance of immature stages differed significantly ( $p < 0.001$ ) between *Bti* treated and untreated habitats over a period of time. Interaction effect of the treatment and days: Early instars:  $F = 3.72$ ,  $df = (27, 670)$ ,  $p < 0.001$ ; Late instars:  $F = 7.58$ ,  $df = (27, 670)$ ,  $p < 0.001$ ; and Pupae:  $F = 5.44$ ,  $df = (27, 670)$ ,  $p < 0.001$  found to be significant. Comparison of larval densities over different days of observation showed that the density on pre-treatment days was not significant ( $p > 0.05$ ) in treated and untreated habitats. But the density in treated were significantly ( $p < 0.05$ ) lower and not overlapped to untreated habitats on post-treatment days up to five days. This level of significance was maintained during further application of WDP formulation.

## DISCUSSION

Fly ash has found immense use in agriculture in recent years due to the presence of macro- and micro-nutrients<sup>4, 18-20</sup>. However, this is the first study wherein this material has been effectively used in the preparation of a biopesticidal formulation, water dispersible powder, to replace the commonly used carrier material, namely charcoal, plaster of paris, etc. When this WDP formulation was tested in cesspits, drains and tar storage tanks, the reduction in larval population ranged from 90–100% in all the habitats within 24 h. This reduction was continued up to 72 h after which a gradual increase in the larval population was observed. By Day 5, larvae reappeared but the larval density was lower than that during pre-treatment period. Application of the subsequent dose was necessary by seventh day to suppress the larval population. With the pupal stages, the reduction noticed within 24 h was not highly significant. However, by 72 h, 100% reduction was noted which was continued throughout the study period in all the habitats.

The WDP formulation was found effective at 1 g/m<sup>2</sup> (10 kg/ha) in all the habitats tested without showing any difference in the expression of toxicity. This is in conformity with the observation of many others who found *Bti* to be effective in both polluted and non-polluted habitats<sup>21-23</sup>. In one study, Kumar *et al*<sup>24</sup> successfully used *Bti* H-14, strain 164, at 1 g/m<sup>2</sup> in controlling the breeding of *An. stephensi* Liston at construction sites, abandoned overhead tanks, and curing tanks in Goa, India. Another

study in Chennai, India showed that fortnightly application of *Bti* (strain 164, serotype H-14) at 1 g/m<sup>2</sup> surface area in waterways resulted in significant reduction in both immature and adult densities of *Cx. quinquefasciatus*<sup>25</sup>. In all the habitats, this formulation was effective in completely suppressing the immature population initially, *i.e.* by 24–72 h respectively. However, it did not exhibit residual activity and this is in agreement with many other studies conducted with different types of water dispersible formulations prepared using the same bacterial isolate and other carrier materials<sup>12, 21, 26-28</sup>. Similar observation has been made with powder formulations prepared using other bacterial species<sup>29-30</sup>, which have been attributed to several environmental factors<sup>31-32</sup> including high temperature<sup>33</sup>. Such polluted habitats are subjected to constant disturbance by frequent and intermittent inflow of sullage water, which might have accelerated the settling of the active ingredient into the bottom mud<sup>34</sup>, thus, making the toxin unavailable for ingestion by the mosquito immature stages which usually spend more time at the top layers of water.

Earlier field evaluations carried out with the wettable powder (WP) formulation of this bacterial isolate (VCRC B17) using another carrier material during different periods of the year (dry and wet season) showed that the activity was unaffected by climate<sup>21, 30</sup>. Further, this formulation proved to be highly effective in polluted and non-polluted habitats supporting breeding of culicine and anopheline mosquito species<sup>26-27</sup>.

The effect obtained on the pupal stages is however not direct because being the transition stage between the larvae and adult they do not feed. The reduction observed on these stages is due to the impact on the larval population which has subsequently resulted in diminishing the formation of the pupal stages. With many of the commercial WPs of *Bti* tested in the field against *Anopheles* and *Culex* vectors, only high level of initial control could be achieved and the residual activity was only for a week<sup>28, 35</sup>. Hence, the study indicates that for continued control of the immature population and prevention of adult emergence, a weekly application of this formulation is necessary.

The present study clearly demonstrates the effectiveness of fly ash based WDP formulation of *Bti* in controlling mosquitoes in urban areas and townships. These results were found to be comparable to that obtained with WDP formulations of *Bti* prepared using other carrier materials. Hence, the fly ash based WDP formulation being safe to associated non-target fauna like fishes, notonectids, water bugs and beetles (unpublished data) can be used safely in combination with other biocontrol agents for mosquito control.

## CONCLUSION

A dose of 10 kg/ha of fly ash based water dispersible powder formulation (WDP) is recommended for control of *Cx. quinquefasciatus* mosquitoes in highly polluted habitats like cesspit, drains, *etc.* With biopesticides gaining wide importance in recent times in the wake of maintaining a safe environment, such use of fly ash in the making of biopesticidal formulations will help not only in its utilization but also in protecting the environment where it is applied, as it has proved to be safe to non-target organisms and mammals. This study has thus brought out a new avenue for the utilization of coal ash.

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