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EVALUATION OF *BACILLUS THURINGIENSIS* SUBSP. *ISRAELENSIS* AND *BACILLUS SPHAERICUS* COMBINATION AGAINST *CULEX PIPIENS* IN HIGHLY VEGETATED DITCHES

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ABSTRACT. Among the few mosquito larvicides available in the market, *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) and *B. sphaericus* (*Bs*) represent the most environmentally safe alternatives. The combination of the 2 products is known to overcome their specific limitations by producing a synergistic effect. The aim of the study was to assess the effect and persistence of a single treatment with a granular *Bti* + *Bs* formulation on highly vegetated ditches in northeastern Italy that represents the primary rural larval sites for *Culex pipiens*, the primary vector of the West Nile virus in Europe. The analysis takes into account the nonlinear temporal effects on the population dynamics of larvae and pupae. The results showed a dramatic reduction in mosquito larval abundance 24 h posttreatment (93%) and was effective against larvae up to 22 days (100%). The residual effect after 28 days was 99.5%, and a limited residual effect was observed after 39 days (31.2%). A reduction in pupal density was observed after 4 days (70%) and was >98% from days 14 to 28 posttreatment, persisting for up to 39 days (84% after 39 days). The results demonstrate the effective use of the *Bti* + *Bs* formulation against *Cx. pipiens* in vegetated ditches in rural areas. Our modeling framework provides a flexible statistical approach to predict the residual effect of the product over time, in order to plan a seasonal intervention scheme.

KEY WORDS *Bacillus*, control, *Culex pipiens*, larval reduction, larvicide, mosquito

INTRODUCTION

Larval control is a key component of integrated mosquito control management, particularly in temperate regions (Marrama and Schaffner 2012). However, few larvicide products are currently available. The commercial introduction of new products is hindered by the time and financial investment required for basic research and product development, registration, and commercialization. In Europe, insect growth regulators (e.g., S-methoprene, pyriproxyfen, diflubenzuron; hereafter IGRs) are the only chemical commercial products available for larval mosquito control (EU 2012; regulation 528/12). Insect growth regulators are widely applied due to their persistence (with a maximum persistence for 3-4 weeks with diflubenzuron and a minimum for 1-2 weeks with s-methoprene.), which reduces implementation costs where multivoltine or sequentially appearing target species are present. However, there are increased reports of resistance to IGRs (e.g., diflubenzuron) in Mediterranean populations of *Culex pipiens* (Linnaeus): France (Fotakis et al. 2020), Turkey (Guz et al. 2020), and Italy (Porretta et al. 2019), with particular reference to Italy where resistance has been phenotypically observed (Grigoraki et al. 2017). Physical products, such as monomolecular films that induce the drowning of eggs and suffocation of larvae and pupae (Mbare et al. 2014), are increasingly used. However, they may affect nontarget species and are only effective on larval sites with limited vegetation that breaks their monomolecular surface (Garrett and White 1977, Nayar and Ali 2003).

Bacterial larvicides (e.g., *Bacillus thuringiensis* subsp. *israelensis* de Barjac, *B. sphaericus* Neide, and their combinations), which produce toxins lethal to the larvae of Diptera (e.g., mosquitoes, fungus gnats, and black flies), are very specific, resulting in lower environmental impact, and thus are preferred in natural habitats (Boisvert and Boisvert 2000, Lacey 2007). The United States Environmental Protection Agency and the European Union have published guidelines for the assessment of risks associated with the use of microbial pesticides (EPA 2000, EU 2012).

Bacillus thuringiensis israelensis (hereafter *Bti*) is largely used against mosquitoes in rural areas, particularly in nonpolluted water (e.g., gutters, ditches). It shows higher persistence than in water with high levels of organic matter which binds with toxins, leading to more rapid denaturation (Mulla 1990, Russell et al. 2009), thus increasing implementation costs due to the need for frequent applications. So far, no resistance to *Bti* formulations has been

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Fig. 1. Left: A typical rural ditch hosting mosquito larvae and pupae in Padua Province (Italy). Right: Collector sampling for mosquito immature stages using dipper.

documented in mosquito populations even after long and widespread field applications (Becker 1997, Regis and Nielsen-LeRoux 2000). In contrast, *B. sphaericus* (hereafter *Bs*) is utilized predominantly in organically rich habitats and has been shown to have longer residual activity and higher tolerance to organic pollution (Mulla et al. 1999) than *Bti* (Lacey 2007). However, *Bs* is effective against a narrower range of mosquito species than *Bti*, and a potential for resistance development has been observed in the *Cx. pipiens* complex (Rao et al. 1995, Yuan et al. 2000).

Combinations of *Bti* and *Bs* (hereafter *Bti* + *Bs*) present the desirable attributes of both bacterial species in a single formulation: they are effective in clear and highly organic waters and show a greater persistence than *Bti* alone (28 days compared with 7–14 days of *Bti*). Moreover, they were shown to act synergistically, enhancing the toxicity against some mosquito species, such as *Culex quinquefasciatus* Say and *Aedes aegypti* (L). Free-flowing granular formulations with a narrow particle size are available for direct application by hand or granule spreaders (WHO 2016, EPA 2018).

The *Bti* + *Bs* combination effectiveness and persistence have been tested in small- and large-scale field trials on a variety of mosquito species (WHO 2016) (e.g., *Ae. aegypti*, *Ae. albopictus* (Skuse), *Cx. pipiens*, *Cx. quinquefasciatus*) depending on the type of treatment (e.g., manual, or aerial application), and habitats such as catch basins (Anderson et al. 2011, Guidi et al. 2013), water tanks (Cetin et al. 2015), and natural or artificial rainwater (Su 2008). However, very few data are available on vegetated larval sites. In polluted water ditches in India, *Cx. quinquefasciatus* larval and pupal densities were significantly reduced for 8–15 days at dosages of both 500 and 1,000 mg/m^2 (WHO 2016); in Brazilian fishponds, *Anopheles darlingi* (Root) larval abundance was reduced for up to 7 days after an application of *Bti* + *Bs* (Fontoura et al. 2020). Notably, the shorter time of the residual effect reported in Brazilian fishponds may potentially be due to the overlapping of the rainy season in the Amazon, when heavy rainfall raises the water level of larval habitats, diluting the product.

Traditionally, the experimental design to establish the efficacy of insecticide includes a comparison of a treated versus an untreated area. Most of the field and laboratory *Bti* + *Bs* experiments evaluate the reduction of larvae/pupae pre- and posttreatment, using Mulla's equation (Mulla et al. 1971). However, such an approach does not consider the temporal dynamics of the larvae/pupae population along with other abiotic factors that could have had an impact during the course of the experiment.

We carried out a case–control, longitudinal field experiment to assess the persistence of the lethal effect of the *Bti* + *Bs* combination in highly vegetated ditches along roads in a rural area in northeast Italy (Fig. 1). These ditches collect water from rainfall, civilian dwellings, and agricultural/industrial activities, and represent the main larval sites for *Cx. pipiens*, not only a high-nuisance species in the country, but also the primary vector of the West Nile virus (WNV), a pathogen responsible for hundreds of autochthonous human cases in Europe each year. The results are expressed as a function of larval/pupal abundance after treatment, including their nonlinear temporal dynamics, which allow a better assessment of the treatment's effectiveness and persistence under field conditions than conventional analytic approaches.

MATERIALS AND METHODS

Study area and experimental procedures

Twelve ditches of various depths between 0.4 and 1 m, hosting mosquito larvae, were selected in the rural area of the municipality of Brugine (45°17′47.731″N, 11°59′42.406″E; province of Padua, northeast Italy) (Fig. 1, left panel). The selected ditches had not been treated with larvicide for at least 1 year.

The experiment was carried out from June 6 to July 15, 2019. Five randomly selected ditches (treated sites) were treated by distributing 15 g/10 m^2 of a granular formulation of *Bti* (4.7% p/p) and *Bs*

(2.9% p/p) (Vectomax FG®; INDIA Industrie Chimiche srl, Padua, Italy) manually on the water surface. The remaining 7 ditches were left untreated.

Larval and pupal sampling was carried out using a 500-ml standard telescopic dipper in both treated and untreated sites at the beginning of the experiment and at 7 additional time points (i.e., 24 h and 4, 7, 14, 21, 28, and 39 days posttreatment) (Fig. 1, right panel). In each ditch, 2 dippings were carried out close to each bank and 1 dipping in the central part, waiting 30 sec between each dipping. The collected water was transferred into a white bucket, and mosquito larvae and pupae were counted. Morphological identification of Culicidae genera was carried out only for 3rd and 4th instars. A subsample of larvae was reared to adult stage and identified to species (Severini et al. 2009).

Statistical methods

The abundance of larvae and pupae in treated and untreated sites was assessed by a generalized additive mixed model (GAMM) in a Bayesian framework. The response variable (the total number of specimens collected in a ditch at a given time point) is assumed to follow a negative binomial distribution with mean μ, dispersion parameter θ, and logarithmic link function. Four smoothing functions modeling the temporal dynamic during the experiment (O'Sullivan spline with 3 internal knots) were considered in the GAMM framework: one for larvae at the treated site, one for pupae at the treated site, one for larvae at the untreated site, and one for pupae at the untreated site. Moreover, we included 4 dichotomous variables identifying which life stage (larva or pupa) and site (treated or untreated) the observed number of specimens belonged to. This model structure makes it possible to quantify independently the nonlinear temporal effect of the number of specimens at the 2 sites. The number of days of field experiment (40 days) was standardized (after subtracting its mean value and dividing by its standard deviation) to improve the numerical stability of the model (Schielzeth 2010). Finally, we modeled the individual variability of ditches (which represent the observational unit of our experiment and were resampled over 40 days) across sites as a random effect term in the GAMM, whose equations are the following:

$$Y_{i,j} \sim NB(\mu_{i,j}, \theta)$$

$$E(Y_{i,j}) = \mu_{i,j}; Var(Y_{i,j}) = \mu_{i,j} + \frac{\mu_{i,j}^2}{\theta}$$

$$\begin{aligned}\log(\mu_{i,j}) = \beta_0 + \beta_1 X^{P,T} + \beta_2 X^{L,U} + \beta_3 X^{P,U} \\ + f_{L,T}(Day_{i,j}) + f_{P,T}(Day_{i,j}) \\ + f_{L,U}(Day_{i,j}) + f_{P,U}(Day_{i,j}) + \varepsilon_j\end{aligned}$$

$$\varepsilon_j \sim Norm(0, \sigma^2)$$

where $Y_{i,j}$ is the total number of specimens (either larvae or pupae) collected during sampling i ($i = 1, \ldots, 8$) in the jth ditch ($j = 1, \ldots, 11$); β_0, β_1, β_2, and β_3 are model parameters, with β_0 representing the average value of larvae in the treated site; X are dichotomous variables identifying if $Y_{i,j}$ are either pupae (P) or larvae (L) in the treated (T) or untreated (U) site; $f()$ is the smoothing function on the number of days on 1st sampling (Day); and ε_j is the ditch random effect with mean 0 and variance σ^2. Diffuse normal priors were used for regression parameters, while Cauchy priors were used for standard deviation parameters (Gelman 2006). Finally, model assumptions were checked by graphical inspection of model residuals. To estimate the model parameters, 3 Markov chains were built running 50,000 iterations with a burn-in of 40,000 and a thinning rate of 10. The reduction in larvae and pupae density in the treated site was calculated by the following equation:

$$\text{Reduction } (\%) = \frac{e^{\mu_{i,j}} - e^{\mu_{1,j}}}{e^{\mu_{1,j}}}$$

where $\mu_{i,j}$ is the predicted mean value of either larvae or pupae only in the treated site, as obtained from the GAMM model. All analyses were carried out using the R and R2jags (Su and Yajima 2020) statistical software packages.

RESULTS

A total of 16,843 mosquito larvae and 5,774 pupae were collected during 7 sampling periods after the treatment of ditches with *Bti* + *Bs* (i.e., 1,421 larvae and 1,319 pupae in the treated ditches and 16,843 larvae and 4,455 pupae in the untreated ditches). In the treated ditches, the number of larvae and pupae collected ranged from 0 to 360 and from 1 to 65, respectively, while for untreated sites these ranges were slightly higher (20–380 and 25–130, respectively). The temporal dynamics of larvae and pupae collected in treated and untreated ditches at each time interval is shown in Fig. S1. The morphological identification of a subsample of adults emerged from collected larvae suggests the exclusive presence of *Cx. pipiens* in the ditches included in the study. Precipitation and Temperature data were obtained by the Regional Environmental Agency (http://www.arpa.veneto.it/bollettini/meteo60gg/Staz_111.htm). The average daily temperatures ranged between 19.8°C and 33.6°C, and the rainfall was negligible (precipitation = 1.31 mm during the whole study period) . The overall percentage of samplings with zero specimens after treatment is estimated to be 74.3% and 49.7% for larvae and pupae in the treated site and 7.9% and 11.9% in the untreated site, respectively. Figure S2 shows the percentage of samplings with zero specimens in the 7 collections after treatment.

The GAMM results show a comparable larval and pupal abundance in treated and untreated sites before

Table 1. Expected mean abundance (log-link scale) of mosquito larvae and pupae in ditches treated with a combination of *Bacillus thuringiensis* subsp. *israelensis* and *B. sphaericus* and untreated ditches, as estimated by a generalized additive mixed model based on a Bayesian framework, estimated mean values, and 95% credible confidence intervals of model parameters.

Parameter	Mean	SE	2.5%	97.5%
Treated—larvae (intercept)	0.730	0.265	0.186	1.251
Treated—pupae	0.039	0.130	−0.216	0.283
Untreated—larvae	3.069	0.338	2.364	3.703
Untreated—pupae	1.805	0.350	1.051	2.471
Negative binomial size parameter ($1/\theta$)	1.079	0.058	0.968	1.197
Random effect of ditches	0.586	0.154	0.359	0.943

treatment, and a significantly lower abundance in the treated site compared with the untreated site after treatment (Table 1). The estimated differences between smoothers in treated and untreated sites (Fig. S3) highlighted that the significantly lower abundance persistence is up to 28 and 32 days after the product application for larvae and pupae, respectively. Figure 2 shows the 4 estimated nonlinear smoothers representing the temporal dynamics of sampled larvae and pupae in the treated and untreated sites. In the treated sites, a significantly sharp decrease in larval abundance is observed immediately (24 h) after the larvicide application, whereas the decrease of pupae is delayed by 4 days. An increase in larval and pupal abundance is observed after 21 days in treated sites, and larvae reach levels comparable to those observed in untreated sites slightly before pupae (28 versus 32 days posttreatment).

The mean net reduction of larval abundance was evident in all treated ditches 24 h posttreatment (92.95%). No live larvae were sampled up to 22 days after treatment, and the reduction (99.5%) was significant for up to 28 days. A residual effect was observed for up to 39 days posttreatment (31.2% reduction). As expected, based on the need for the toxins to be ingested in order to exhibit a lethal effect on pupal density was observed after 4 days (70.1%). Pupal reduction was >98% from 14 to 28 days posttreatment and persisted up to the end of the experiment (84% after 39 days) (Fig. S4 and Table 2). The estimated variance of the random effect variable (i.e., ditches) in GAMM is 0.586 (95% CI 0.359–0.943), indicating the presence of some heterogeneity among ditches.

DISCUSSION

We report the results of a field experiment conducted to test the persistence of a single application of *Bti* + *Bs* granular formulation on *Cx. pipiens* mosquitoes in highly vegetated rural ditches in Italy. The effectiveness of *Bti* + *Bs* has been rarely evaluated in this kind of habitat, despite their suitability as optimal breeding sites for this mosquito species, primary vector of the WNV. The result of the statistical analysis provided strong evidence of a reduction across time in the relative abundance of larvae and pupae in treated ditches.

A comparison of the results obtained with the existing literature is hampered by differences in the type of treated larval habitats, target species,

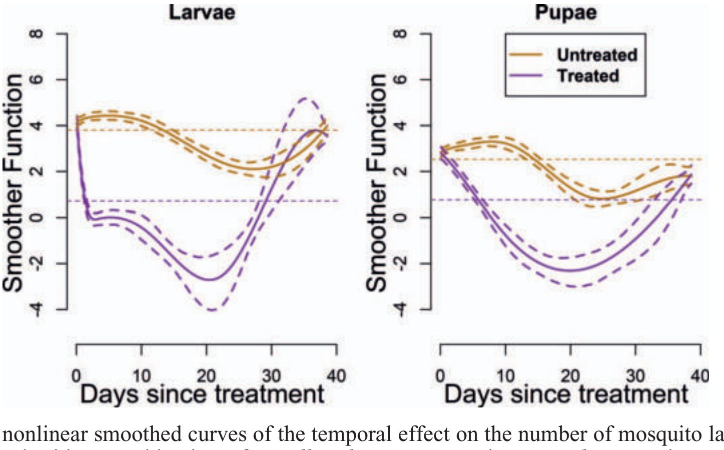

Fig. 2. Estimated nonlinear smoothed curves of the temporal effect on the number of mosquito larvae (left) and pupae (right) in ditches treated with a combination of *Bacillus thuringiensis* subsp. *israelensis* and *B. sphaericus* (*Bti* + *Bs*) (purple) and untreated ditches (orange). Solid lines = posterior mean in log-link scale estimated by generalized additive mixed model (GAMM) model. Dashed lines = 95% credible confidence interval. Horizontal dashed lines = estimated posterior mean for the smoother.

Table 2. Percent reduction in mosquito larvae and pupae abundance in ditches with abundant vegetation that were treated with a combination of *Bacillus thuringiensis* subsp. *israelensis* and *B. sphaericus*. Percentages are based on the results of a generalized additive mixed model. N = number of collected larvae and pupae.

Days posttreatment	Reduction, % (N)	
	Larvae	Pupae
24 h	92.95 (174)	21.3 (722)
4	98.46 (103)	70.14 (351)
7	98.44 (22)	89.1 (23)
14	99.5 (21)	98.71 (21)
21	100 (0)	99.3 (1)
28	99.5 (27)	98.97 (7)
39	31.77 (1,074)	83.96 (197)

experimental designs, and statistical approach. In fact, most of the available studies have assessed the impact of *Bti* + *Bs* on mosquito abundance only during the day of field sampling using the Mulla's equation (Mulla et al. 1971)

$$100 - \left(\frac{Control\_pre}{Treated\_pre} \times \frac{Treated\_post}{Control\_post} \times 100\right)$$

which does not allow for a proper evaluation of effectiveness over time. In addition, we applied Mulla's formula (Table S1) to our data, obtaining similar results to those of our statistical model.

Our results are similar to those obtained in wetlands in the USA, where a single aerial application of *Bti* + *Bs* (8.9 kg/ha) was effective against *Cx. tarsalis* Coq. larvae and pupae for up to 28 days posttreatment (Dritz et al. 2011). We estimated a higher efficacy for *Bti* + *Bs* compared with a previous evaluation in Brazilian fishponds (Fontoura et al. 2020), where a single application (10 kg/ha) substantially reduced (>95%) anopheline larval densities for 7 days. However, Fontoura et al. (2020) reported residual effects for up to 21 days after reapplication of *Bti* + *Bs* (20 kg/ha). Our results, obtained from rural ditches, estimated *Bti* + *Bs* to be less effective with respect to other studies conducted in different settings, such as a septic tank in Turkey (Cetin et al. 2015) and urban catch basins in the USA (Anderson et al. 2011) and Switzerland (Guidi et al. 2013). The results obtained have a great operational value, by predicting the residual effect of *Bti* + *Bs* over time. This is particularly relevant in some areas of Europe (Italy, France, Spain, and Switzerland [EU regulation 528/12]) where only 8 *Bti* + *Bs* treatments are allowed during each season. According to model results, a treatment every 3 wk would prevent the emergence of *Cx. pipiens* adults for 5–6 consecutive months. However, treatments at 28-day intervals would reduce the emergence of 99.5% of adults in the treated sites. Notably, the model did not include abiotic variables (such as temperature and/or rainfall) or biotic ones (such as ecological differences among the ditches tested), which were included as random effects accounting for unexplained variability by model predictors. Future studies could exploit the model to evaluate the interaction between larvicide and a wide range of different climatic conditions. Finally, our results support the effectiveness of *Bti* + *Bs* granular formulations for the treatment of vegetated ditches that represent the main rural breeding sites of *Cx. pipiens* in some regions of northeastern Italy where WNV is endemic.

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