BioAssay

BioAssay, 19, 2024: ba19002
doi: 10.37486/1809-8460.ba19002
e-ISSN: 1809-8460
Open Access Full Text Article

Research Article

New formulation of *Bacillus thuringiensis israelensis* for the control of *Culex quinquefasciatus* Say, 1823 (Diptera, Culicidae) in an effluent treatment pond

Alex da S. Bocaleti[1], Valtir Y. T. de Almeida[1], Eduarda A. de Lima[1], Renan R. Schorr[2], Francisco de A. Marques, Mario A. N. da Silva[2], Gislayne F. L. T. Vilas-Boas[1], João A. C. Zequi[1]

[1]Universidade Estadual de Londrina, Londrina, PR, Brazil. [2]Universidade Federal do Paraná, Curitiba, PR, Brazil.
Corresponding author: zequi@uel.br

Edited by: Elio C. Guzzo

Received: October 01, 2024. Accepted: November 25, 2024. Published: December 23, 2024.

Abstract. *Culex quinquefasciatus* Say, 1823 (Diptera, Culicidae) has relevance in health due to their role in transmitting pathogens that cause diseases such as Bancroftian filariasis, West Nile virus, Rift Valley fever, Saint Louis encephalitis, Zika virus and Mayaro virus. Subsequent monitoring and control of these vectors are crucial to minimize the circulation of these pathogens. *Bacillus thuringiensis* subspecies *israelensis* de Barjac, 1978 (Bti) is a bacterium that produces toxins with specific entomopathogenic action, distinguishing it from synthetic insecticides due to its high specificity and lower susceptibility to resistance. This study aims to evaluate the effectiveness of new Bti-based formulations for controlling *C. quinquefasciatus* mosquitoes under field conditions. Product BR 101 demonstrated satisfactory efficacy for up to five days, achieving a peak population reduction of 94.97% on the third day post-application, followed by a gradual population recovery. The product proved effective for *C. quinquefasciatus* control without affecting abiotic factors, offering an efficient option for reducing vector density with minimal impact on non-target fauna due to its selectivity.

Keywords: Biological control; Bioinsecticide; Culicidae; Cry; Entomopathogens.

Introduction

The Culicidae are distributed across practically all habitats, being more predominant in tropical and subtropical regions. These environments, characterized by higher precipitation rates and high temperatures, combined with disordered urban growth and the pollution of rivers and ditches, become a suitable environment for the development, proliferation, and adaptive success of these insects (Forattini 2002; Rueda 2008; Araújo et al. 2018; Lima-Camara et al. 2016). *Culex quinquefasciatus* Say, 1823 (Diptera, Culicidae) larvae colonize effluent treatment ponds more effectively due to the abundance of organic matter, which facilitates the maintenance of a high larval population density (Zequi et al. 2014).

Females of this genus tend to feed on human blood, which makes them significant vectors for several arboviruses. Thus, *C. quinquefasciatus* is an anthropophilic species considered an important vector of several pathogens, including the nematode *Wuchereria bancrofti* (Cobbold, 1877) (agent of Bancroftian filariasis), the West Nile virus, and a potential vector of Rift Valley fever, Saint Louis encephalitis, and Mayaro virus (Lopes et al. 2019). In Brazil, in 2023, lymphatic filariasis linked to *C. quinquefasciatus* suggested imminent eradication, with only a few isolated cases remaining in the Northeast region of the country (Brasil 2023). In 2024, Brazil eliminated filariasis as a public health problem (WHO 2024). However, this species is also a potential transmitter of other arboviruses, such as Zika virus (Guo et al. 2016; Guedes et al. 2017) and Oropouche virus (Cardoso et al. 2015). Several cases of Oropouche have been registered in Brazil (up to Epidemiological Week 31 of 2024, 7,497 cases), an unprecedented fact in the world, with two deaths occurring in the state of Bahia and there is notification and investigation of suspected cases of Oropouche in pregnant women involving congenital anomalies or fetal deaths (Brasil 2024).

According to the Surveillance Guide for *C. quinquefasciatus* published by the Ministry of Health in 2011, control measures for this mosquito include actions such as public education, periodic cleaning of reservoirs, vegetation control along riverbanks, and the use of chemical agents such as insecticides based on pyrethroids, organophosphates, and carbamates. However, the continuous use of these synthetic insecticides has led to resistance, rendering them ineffective and ultimately resulting in their reduced or suspended use (Brasil 2011; Lopes et al. 2019).

Biological control using *Bacillus thuringiensis* subsp. *israelensis* de Barjac, 1978 (Bti) was adopted by the Ministry of Health to replace organophosphate insecticides and growth regulators when the larval population shows resistance to these products according to (Brasil 2019). Due to its high specificity, capacity to reduce environmental contamination, and absence of risks to human health, the use of these entomopathogens presents advantages over other insecticides, which promote resistance selection and cause damage to the environment (Angelo et al. 2010; Bravo 2018).

The toxicity of Bti to larvae manifests through the production of proteins from this crystalline delta-endotoxin, resulting in the formation of a protoxin, which is produced during the bacterial sporulation phase. For the toxic effect to occur, it is necessary for this protoxin to be solubilized in alkaline pH in the larva's intestine, leading to the formation of Cry proteins (Silva et al. 2011). Cry protein crystals cause paralysis of the digestive system in *Culex* species, leading to death due to starvation, widespread muscular paralysis, and septicemia. The Cyt protein acts synergistically with the Cry protein, promoting the disruption of the lipid bilayer of the cell membranes of this insect, thereby amplifying its toxicity (Angelo et al. 2010).

The present study aimed to evaluate the effectiveness of a new formulation with 2,700 International Toxin Units (ITU) per milligram (BR 101) for the biological control of *C. quinquefasciatus* in an effluent treatment pond environment in Cambé, Paraná, Brazil. Additionally, it also sought to establish correlations between abiotic factors and the persistence of Bti under field conditions.

Material and Methods

For the formulation of the bioinsecticide, colonies were inoculated into a liquid NYSM medium (Myers & Yousten 1980) and incubated overnight at 30°C with agitation. Subsequently, 300 mL of the culture were transferred to the bioreactor (Allbiom – AIIMic LAB SCR07 – twin) supplied with 6 L of NYSM medium and maintained at 30°C ± 2, 300 RPM, pH 7.0, with a constant air supply of 1 L/min per liter of medium until complete sporulation (~36 hours). The fermented product was centrifuged at 13,700 × g RCF at 10°C, for 15 minutes (Sorvall - RC-5C), discarding the supernatant. The solid content was frozen at -20ºC and subjected to lyophilization (Liotop - L101) for 24 hours.

Given the egg deposition characteristics of *C. quinquefasciatus* on the water surface of ponds, the bioinsecticide was formulated as dispersible granules. Lyophilized spores and crystals of Bti BR101 were combined with a specific excipient and distilled water. The resulting mixture was then sieved through a 24-mesh sieve and placed in an oven at 35°C for 30 minutes. After this period, the mass was sieved again using a 60-mesh sieve, to obtain the dispersible granules in water.

The efficacy of the formulation was tested between 10/29/2019 and 11/27/2019 in an effluent treatment lagoon located in a crude soybean oil company in Cambé, Paraná, Brazil (23°17'06" S and 51°15'15" W) colonized by *C. quinquefasciatus*, which has an area of 665 m² and receives waste from the oil production (Fig. 1A). Fifty grams of the formulation were applied using a device specifically produced for the application of the product developed in this study. Therefore, a 50 mL Falcon tube was punctured, attached to a handle and filled with the powder formulation, which was evenly sprayed along the perimeter of the pond, within one meter from the edge, where *C. quinquefasciatus* larvae normally colonize and feed (Fig. 1B).

Six sampling points were designated around the perimeter of the pond. Abiotic parameters were measured on each sampling day, with a thermo hygrometer placed adjacent to the pond for atmospheric temperature and relative humidity readings. A multiparameter instrument (Hanna - HI9828) was employed to assess pH, conductivity, total dissolved solids, salinity, and water temperature at points 1, 3, and 5 (Fig. 1C). A population fluctuation assessment was conducted at the onset of the experiment at zero hours, and subsequently at 1, 2, 3, 5, 7, and 15 days, when a new application of the product was carried out, starting a second 15-day test. Larvae were collected using a 1.5 mm mesh aquatic insect net, employing a one meter drag from the pond interior to the edge along the surface (Fig. 1D). Collected larvae were preserved in labeled vials containing 90% alcohol and stored at room temperature in the Medical Entomology Laboratory following Zequi & Lopes (2007).

The permanent SISBIO/IBAMA license (23093) and the company's authorization to collect mosquitoes from the effluent treatment lagoon were used to collect mosquitoes. Specimens were deposited at the Zoology Museum of the State University of Londrina.

Results and Discussion

The initial population within the pond, depicted in Fig. 2 as Sampling 1, was determined by examining six sample points, amounting to 3,182 larvae. Subsequently, with the application of the formulated product along the entire perimeter of the pond, a notable decline in population was observed. Within the initial 24-hour period following the application, there was an observed reduction of 33.7%. By the second day, this reduction escalated to 79.07%, culminating in a decrease of 94.97% of the larvae population by the third day. From the fifth day onwards, a population recovery reaching 47.74% was observed, indicating a process of larval restoration that lasted until the seventh day, resulting in a total number of larvae greater than that initially observed. On the fifteenth day, the first sampling ended and the second began, accompanied by a new application of the formulation (0-day of the second test). On this day, a population of 1,693 specimens were observed.

Figure 1. Field experiment for *C. quinquefasciatus* larvae in a wastewater treatment pond of a soybean oil company in Cambé-PR, Brazil. (A) Wastewater treatment pond area as the test zone. (B) Deployment and application of the formulated product. (C) Assessment of water abiotic parameters with the aid of multiparameter devices. (D) Larvae collection using a net.

After the second application, depicted in Fig. 2 as Sampling 2, we observed a reduction in population, with a decrease of 73.36% within the initial day. Between the second and the fifth days, there was a slight increase in population; however, it remained below the initial population observed on day zero, showing reductions of 40.28%, 29.71%, and 21.38%, respectively. From the seventh day onward, there was an increase in the accumulation of debris in the pond, which facilitated the resurgence of larvae.

Considering the mortality relationship, the product was applied during the first repetition following an average precipitation of 32.8 mm, remaining rain-free until the seventh day, which may have contributed to more effective control. During the second application, precipitation occurred from the time of application through day two, potentially aiding in the mechanical transport of the formulation and larvae via runoff, consequently reducing the mortality rate up to day five, similar to the first replication (Fig. 2).

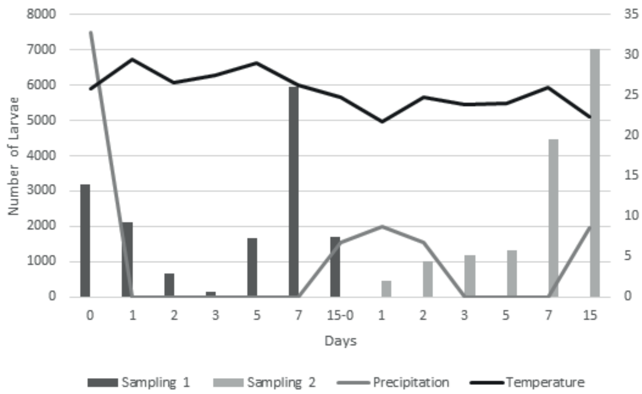

Figure 2. The total number of *C. quinquefasciatus* larvae in the effluent treatment pond, colonized and sampled during the period from 29/10/2019 to 27/11/2019, under the application of the formulated BR101 formulation, was correlated with temperature (°C) and precipitation (mm) variables. The study included two 15-day replicates.

Throughout the field experiment, the mean temperature recorded was approximately 26.94°C, coupled with a relative humidity of 44.87%. These measurements exhibited ranges from 14.6 to 37.9°C for temperature and 15% to 95% for relative humidity, respectively. Notably, great variation in abiotic parameters was observed in pH, conductivity, and total dissolved solids (TDS) (Tab. 1). This variation in abiotic factors is attributed to the variable contribution of waste from the company into the pond during the period, resulting in variable changes.

The efficiency of the used formulation showed satisfactory results for 5 days in both tests. The first factor that may have contributed to this result was the occurrence of rain on the 0-day in the first test and between the 0 and 2-day in the second test. Furthermore, the great quantity of organic material may have diminished the effectiveness of the product, as presented by other authors (Gaugler & Molloy 1980; Boisvert & Boisvert 2000; Lacey 2007), due to the adsorption of Bti-crystals to the organic matter and an increase in suspended food options. However, filtration feeding can enhance the intake of suspended Bti products (Fry-O'Brien & Mulla 1996; Dawson et al. 2019), as assessed by Zequi et al. (2014) with the Bti-based bioinsecticide AquaBac® XT, which remains suspended for a longer period and achieved the maximum control rate at 2 L/ha, a key characteristic of formulated BR101.

Table 1. Mean values of abiotic parameters and minimum and maximum values between the two application replicates of the BR101 formulation in an effluent treatment pond in the field with *C. quinquefasciatus* larvae from 29/10/2019 to 27/11/2019.

Abiotic parameters	Sampling 1	Sampling 2
pH	7.19 b* (5.07-8.92)	9.39 a (7.94-11.58)
Water temperature (ºC)	30.87 a (21.59-34.44)	31.02 a (26.76-11.58)
	2021 b (1087-2999)	2879.52 a (2111-3958)
TDS (mg/L) Conductivity (µS cm^{-1})	911.44 b (492-1486)	1292.71 a (975-1728)
Atmospheric temperature (ºC)	28.11 a (18.3-37)	25.77 b (14.6-37.9)
Relative humidity (%)	44.75 a (15-91)	45 a (15-95)

*Means followed by the same letter in the row do not differ from each other by the t-test at the 5% level

Other issues that can affect the effectiveness of Bti in waste ponds include water flow, which gradually carries Bti-products through mechanical runoff, and the occurrence of deposited substances that can degrade Bti toxin, such as chlorine, an important component in both supply water and cleaning (Sinegre et al. 1981; Rydzanicz et al. 2010).

Effluent treatment lagoons are complex environments and simply increasing concentrations of Bti-based products, as highlighted by Mulla et al. (2003), may not be sufficient to control *C. quinquefasciatus* in these environments. Thus, other factors should also be considered, such as temperature, solar radiation, precipitation, and vegetation, as pointed out by Lacey (2007).

This study presents the results of two tests carried out in an effluent treatment lagoon with a new Bti-based product in the form of dispersible granules for the control of *C. quinquefasciatus* larvae. Therefore, we developed a specific apparatus for applying the product, which allowed a wide distribution throughout the entire perimeter of the lagoon which can be used by other dispersible granule products. The findings suggest that our new formulation demonstrates efficacy in controlling *C. quinquefasciatus* larvae in open environments with water flow and organic matter input. However, its application in effluent and waste treatment ponds always requires a highly specific and cautious approach, in which case it should be applied weekly for efficient control.

Acknowledgments

We thank Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for their financial support.

Funding Information

The project was developed with financial support from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) under public call 440385/2016-4 CNPq/CAPES/MS-Decit, and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - financing 001.

Authors' Contributions

ASB: Investigation, Validation, Visualization, Writing – original draft, Writing – review & editing; VYTA: Investigation, Writing – original draft; EAL: Investigation, Writing – original draft, Writing – review & editing; RRS: Investigation, Writing – original draft; FAM: Investigation, Writing – original draft; MANS: Investigation, Writing – original draft; GTV-B: Conceptualization, Methodology, Writing – review & editing; JACZ: Conceptualization, Funding acquisition, Methodology, Project administration, Validation, Visualization, Writing – review & editing.

Conflict of Interest Statement

All authors have declared that there is no conflict of interest.

References

Angelo, E. A.; Vilas-Bôas, G. T.; Castro-Gómez, R. J. H. (2010) *Bacillus thuringiensis*: general characteristics and fermentation. *Semina: Ciências Agrárias,* 31: 945-958. doi: 10.5433/1679-0359.2010v31n4p945

Araújo, Y. C.; Roque, R. A.; Tadei, W. P.; Zequi, J. A. C. (2018) Controle de *Aedes* (*Stegomyia*) *aegypti* (Linnaeus, 1792) (Diptera: Culicidae) aclimatados em diferentes temperaturas e níveis de gás carbônico utilizando *Bacillus thuringiensis israelenses*, *Saccharopolyspora spinosa* e Piriproxyfen. In: Carvalho, Y. C. S. *Patologia das doenças,* pp. 59-71. Belo Horizonte: Atena.

Boisvert, M.; Boisvert, J. (2000) Effects of *Bacillus thuringiensis* var *israelensis* on target and nontarget organisms: a review of laboratory and field experiments. *Biocontrol Science and Technology,* 10: 517-561. doi: 10.1080/095831500750016361

Brasil (2009) Ministério da Saúde. Secretaria de Vigilância em Saúde. Departamento de Vigilância Epidemiológica. Diretrizes Nacionais para a Prevenção e Controle das Epidemias de Dengue. https://www.gov.br/saude/pt-br/centrais-de-conteudo/publicacoes/svsa/dengue/diretrizes_nacionais_prevencao_controle_dengue.pdf/view. Access on: 24.ix.2024.

Brasil (2011) Ministério da Saúde. Secretaria de Vigilância em Saúde. Guia de Vigilância para *Culex quinquefasciatus*. http://www.gov.br/saude/pt-br/centrais-de-conteudo/publicacoes/svsa/culex-quinquefasciatus/guia_vigilancia_culex_quinquefasciatus.pdf/view. Access on: 8.v.2024.

Brasil (2019) Ministério da Saúde. Secretaria de Vigilância em Saúde. Departamento de Imunização e Doenças Transmissíveis. Coordenação-Geral de Vigilância de Arboviroses. Recomendações para Manejo da Resistência de *Aedes aegypti* a Inseticidas. https://www.gov.br/saude/pt-br/centrais-de-conteudo/publicacoes/estudos-e-notas-informativas/2019/nota-informativa-no-1032019-cgarbdeidtsvsms.pdf. Access on: 24.ix.2024.

Brasil (2023) Ministério da Saúde. Secretaria de Vigilância em Saúde. Doenças Socialmente Determinadas: saiba mais sobre a Filariose Linfática. http://www.gov.br/saude/pt-br/assuntos/noticias/2023/junho/doencas-socialmente-determinadas-saiba-mais-sobre-a-filariose-linfatica. Access on: 7.v.2024.

Brasil (2024) Ministério da Saúde. Secretaria de Vigilância em Saúde. Coordenação-Geral de Vigilância de Arboviroses. Nota Técnica conjunta nº 135/2024-SVSA/SAPS/SAES/MS. Brasília. https://www.gov.br/saude/pt-br/centrais-de-conteudo/publicacoes/notas-tecnicas/2024/nota-tecnica-conjunta-no-135-2024-svsa-saps-saes-ms. Access on: 30.ix.2024.

Bravo, A. (2018) Biodiversity of Cry toxins produced by *Bacillus* *thuringiensis* and evolution of resistance to these toxins in different insect pests. *Toxicon,* 149: 98. doi: 10.1016/j.toxicon.2018.02.010

Cardoso, B. F.; Serra, O. P.; Heinen, L. B. S.; Zuchi, N.; Souza, V. C.; Naveca, F.G.; Santos, M. A. M.; Slhessarenko, R. D. (2015) Detection of Oropouche virus segment S in patients and in *Culex quinquefasciatus* in the state of Mato Grosso, Brazil. *Memórias do Instituto Oswaldo Cruz,* 110: 745-754. doi: 10.1590/0074-02760150123

Dawson, D.; Salice, C. J.; Subbiah, S. (2019) The efficacy of the *Bacillus thuringiensis israelensis* larvicide against *Culex tarsalis* in municipal wastewater and water from natural wetlands. *Journal of the American Mosquito Control Association,* 35: 97-106. doi: 10.2987/18-6771.1

Forrattini, O. P. (2002) *Culicidologia Médica*. Vol 2. São Paulo: Edusp.

Fry-O'Brien, L. L.; Mulla, M. S. (1996) Effect of tadpole shrimp, *Triops longicaudatus* (Notostraca: Triopsidae), on the efficacy of the microbial control agent *Bacillus thuringiensis* var *israelensis* in experimental microcosms. *Journal of the American Mosquito Control Association,* 12: 33-38.

Gaugler, R.; Molloy, D. (1980) Feeding inhibition in black fly larvae (Diptera: Simuliidae) and its effects on the pathogenicity of *Bacillus thuringiensis* var. *israelensis. Environmental Entomology,* 9: 704-708. doi: 10.1093/ee/9.5.704

Guedes, D. R.; Paiva, M. H.; Donato, M. M.; Barbosa, P. P.; Krokovsky, L.; Rocha, S. W. S.; Lasaraiva, K.; Crespo, M. M.; Rezende, T. M.; Wallau, G. L.; et al. (2017) Zika virus replication in the mosquito *Culex quinquefasciatus* in Brazil. *Emerging Microbes & Infections,* 6: e69. doi: 10.1038/emi.2017.59

Guo, X.-X.; Li, C.-X.; Deng, Y.-Q.; Xing, D.; Liu, Q.-M.; Wu, Q.; Sun, A.-J.; Dong, Y.-D.; Cao, W.-C.; Qin, C.-F.; Zhao, T.-Y. (2016) *Culex pipiens quinquefasciatus*: a potential vector to transmit Zika virus. *Emerging Microbes & Infections,* 5: e102. doi: 10.1038/emi.2016.102

Lacey, L. A. (2007) *Bacillus thuringiensis* sorovariety *israelensis* and *Bacillus sphaericus* for mosquito control. *Journal of the American Mosquito Control Association,* 23: 133-163. doi: 10.2987/8756-971x(2007)23[133:btsiab]2.0.co;2

Lima-Camara, T. N. (2016) Emerging arboviruses and public health challenges in Brazil. *Revista de Saúde Pública,* 50: 36. doi: 10.1590/s1518-8787.2016050006791

Lopes, R. P.; Lima, J. B. P.; Martins, A. J. (2019) Insecticide resistance in *Culex quinquefasciatus* Say, 1823 in Brazil: a review. *Parasites & Vectors,* 12: 591. doi: 10.1186/s13071-019-3850-8

Mulla, M. S.; Thavara, U.; Tawatsin, A.; Chomposri, J.; Su, T. (2003) Emergence of resistance and resistance management in field populations of tropical *Culex quinquefasciatus* to the microbial control agent *Bacillus sphaericus*. *Journal of the American Mosquito Control Association,* 19: 39-46.

Myers, P. S.; Yousten, A. A. (1980) Localization of a mosquito-larval toxin of *Bacillus sphaericus* 1593. *Applied and Environmental Microbiology,* 39: 1205-1211. doi: 10.1128/aem.39.6.1205-1211.1980

Rueda, L. M. (2008) Global diversity of mosquitoes (Insecta: Diptera: Culicidae) in freshwater. *Hydrobiologia,* 595: 477-487. doi: 10.1007/s10750-007-9037-x

Rydzanicz, K.; Sobczynski, M.; Guz-Regner, K. (2010) Comparison of activity and persistence of microbial insecticides based on *Bacillus thuringiensis israelensis* and *Bacillus sphaericus* in organicly polluted mosquito-breeding sites. *Polish Journal of Environmental Studies,* 19: 1317-1323.

Silva, M.; Furigo Junior, A.; Furlan, S. A.; Souza, O. (2011) Production of bio-insecticide *Bacillus thuringiensis* var *israelensis* in semicontinuous processes combined with batch processes for sporulation. *Brazilian Archives of Biology and Technology,* 54: 45-52. doi: 10.1590/S1516-89132011000100006

Sinegre, G.; Gaven, B.; Vigo, G. (1981) Contribution to standardization of laboratory tests on experimental and commercial formulations of the serotype H-14 of *Bacillus thuringiensis*. II – influence of temperature, free chlorine, pH, and water depth of biological activity of primary powder. *Entomologie Medicale et Parasitologie,* 19: 149-155.

WHO - World Health Organization (2024) Brazil eliminates lymphatic filariasis as a public health. WHO newsroom. http://www.who.int/news/item/01-10-2024-brazil-eliminates-lyphatic-filariasis-as-a-public-health-problem. Access on: 01.x.2024.

Zequi, J. A. C.; Lopes, J. (2007) Biological control of *Culex* (*Culex*) *saltanensis* Dyar, (Diptera, Culicidae) through *Bacillus thuringiensis israelensis* in laboratory and field conditions. *Revista Brasileira de Zoologia,* 24: 164-168. doi: 10.1590/s0101-81752007000100020

Zequi, J. A. C.; Santos, F. P.; Lopes, J. (2014) Control of *Culex quinquefasciatus* and *Cx. saltanensis* (Diptera: Culicidae) with *Bacillus thuringiensis israelensis* in wastewater treatment lagoons. *Revista Colombiana de Entomología,* 40: 98-103.