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STERILIZATION OF *BACILLUS THURINGIENSIS ISRAELENSIS* PRODUCTS BY GAMMA RADIATION

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ABSTRACT. This study examines the effect of routine gamma radiation based on cobalt 60 on the viability, mosquito larvicidal activity, and density of bacillus spores in the soil. Although 1 g of unirradiated powder of *Bacillus thuringiensis israelensis* (*Bti*) contains on average 6.2×10^9 spores, no spores survived radiation doses of 20.6 kGy and higher. Radiation at a dose of 20–25 kGy caused a 20–30% reduction in the effectiveness of *Bti* powder against mosquito larvae. In areas treated with unirradiated *Bti* material on average twice a year, soil contained 700,000 to 44 million spores per gram. In areas treated with irradiated *Bti* products, either no *Bti* spores or fewer than 100,000 were found per gram of soil. A radiation dose of 25 kGy fulfills the requirements of killing all spores in a *Bti* product and maintaining the effectiveness of the product in routine treatments. No viable spores remain in water used for household purposes or irrigation of garden areas when irradiated *Bti* fizzy tablets are used in water containers. Irradiation of *Bti* products fulfills the requirements of drinking water regulations and thus allows these products to be used widely.

KEY WORDS *Bacillus thuringiensis israelensis*, mosquito control, radiation, sterilization, drinking water

INTRODUCTION

Hundreds of tons of products based on *Bacillus thuringiensis* are used yearly against lepidopteran pest species in agriculture and forests, as well as against mosquitoes and black flies, without significant adverse effects on nontarget species (Becker and Margalit 1993). In Germany, *Bacillus thuringiensis israelensis* (*Bti*, serotype H-14) has been used widely since 1981. During this period, the national mosquito control program carried out by the German Mosquito Control Association (KABS) treated almost 200,000 ha of mosquito breeding sites with about 100 tons of *Bti* wettable powder and fluid concentrates. Powder formulations also have been used to produce approximately 1,200 tons of *Bti* granules to apply to wetlands by helicopter or ground equipment (Becker 1997). Since 1992, several million effervescent tablets (registered as Culinex tablets) have been used annually against larvae of *Culex pipiens* L., *Culiseta annulata* (Schrank), and *Anopheles plumbeus* Stephens, which usually breed in large numbers in artificial containers such as rainwater butts, water catchments, and cesspools in settlements. In river floodplains, many mosquito breeding sites occur in areas set aside for drinking water catchments. People often collect rainwater in containers to irrigate plants intended for human consumption. Effervescent *Bti* tablets have proven to be a suitable tool for control of mosquito larvae in these containers (Becker et al. 1991; Kroeger et al. 1995). Although *Bti* is considered safe (WHO 1999), people prefer bacilli- or spore-free products to avoid the risk of contamination. Because German authorities allow only *Bti* products that do not contain living bacilli or their spores, an asporogenous mutant of *Bti* was used to produce the tablets for control of *Culex*. During the fermentation process, the yield of this asporogenous mutant was not as good as expected and the stability of the mutant was also questionable. As a result, KABS decided to use a commercial, high-potency, *Bti* product based on the wild-type *B. thuringiensis* H-14. To comply with German law, the KABS decided to use gamma radiation to sterilize the microbial control agents. This study examines the effect of routine gamma radiation on viability of bacillus spores, mosquito larvicidal activity, and environmental consequences.

MATERIALS AND METHODS

Radiation process

Irradiation was accomplished with cobalt 60 (Co 60) in a type JS-8500 device (NORDON International Inc., Kanata, Ontario, Canada). The equipment, which has a capacity of 2 million Ci, was operated by Willy Ruesch AG (Kernen, Germany). For the continuous irradiation process, each irradiation carton was registered and labeled with the following information: date of registration, batch number, desired radiation dose, and registration number. The units were automatically transferred from a storage conveyor into the irradiation chamber, where they were circulated on 2 levels, traveling twice around the Co 60 radiation source. The master clock for the duration of the radiation was set according to the required radiation dose as well as on the actual Co 60 strength. In this study, radiation doses of 5, 10, 15, 20, 25, 30, and 35 kGy were chosen. Red Perspex dosimeters (type 4034, Harwell Dosimeters, Didcot, UK) with external dimensions of $10 \times 30 \times 3$ mm, sealed in aluminum foil, were used to check the absorbed routine radiation dose. The accuracy of the dosimeters was ±5%.

Bactimos® wettable powder (batch BIQI 0003, Novo Nordisk, Bagsvaerd, Denmark) with an activity of 3,500 ITU/mg was used for the irradiation.

For each radiation dose, 4 polyethylene bottles were each filled with 100 g of *Bti* wettable powder. Four bottles remained unirradiated as controls.

Assessment of radiation effect on spore viability

To determine the number of spores after irradiation with different doses, four 0.1-g samples of powder were taken under sterile conditions from each of the irradiated flasks of Bactimos wettable powder and transferred into 1 ml of sterile distilled water in Eppendorf microtubes. After homogenization with a Vortex (Heidolf Instruments GmbH, Schwabach, Germany), 100 µl of the solution was removed from each container with a Drigalski spatula and smeared onto nutrient agar. The agar dishes were prepared with agar and nutrient broth (comprised 1 g of meat extract, 2 g of yeast extract, 5 g of peptone, and 5 g of sodium chloride; Fluka AG, Sigma Aldrich Chemie GmbH, Munich, Germany) and distilled water. Altogether, 17 g of agar and 13 g of nutrient broth were measured into each liter of distilled water. The liquid agar (pH 7) was placed in an autoclave for 15 min at 121°C and then poured into sterile standard petri dishes.

Each Eppendorf microtube provided smears for 4 agar dishes (4 × 100 µl). For each radiation dose, 64 plates were inoculated and incubated for 48 h at 30°C and each was inspected after 24 and 48 h to check bacterial growth. As a control, 8 agar dishes that had been treated only with distilled water were added to each irradiated batch.

The bacterial colonies each were examined occasionally with a light microscope during the 48-h period to see whether spores or crystals were forming. The number of *Bti* colonies was counted. The experiment was begun with the powder that had been subjected to 35 kGy of radiation. Materials exposed to smaller doses were diluted to keep the number of spores within countable limits.

Assessment of radiation effect on larvicidal activity of *Bti*

Bioassays were carried out with powders exposed to different levels of radiation to investigate the reduction in effectiveness because of damage to the protein crystals. The bioassays followed World Health Organization guidelines (WHO 1981). Accordingly, 50 mg of each sample of Bactimos powder was weighed and poured into a 20-ml penicillin flask, then 10 ml of deionized water and 15 glass beads (6-mm diameter) were added. This suspension was homogenized for 10 min at 700 strokes/min in a shaker. Then 0.4 ml of the initial suspension was added to 39.6 ml of deionized water in a test tube. This was agitated for a few seconds by a Vortex-type agitator at maximum speed. Depending on the concentration required, 15–1,500 µl of homogenized and diluted Bactimos wettable powder suspension was added to 200-ml plastic cups, previously filled with 148 ml of distilled water. Twenty-five 4th-stage larvae of *Aedes aegypti* (L.) were added to each cup with a Pasteur pipette. Range-finding bioassays were made to save time, with only 2 widely spaced concentrations of the test materials. The results were used to estimate the concentrations needed in the final assay to obtain a reliable regression line.

Four cups were used for each concentration and the control. The latter were filled with 150 ml of deionized water. All tests were conducted at 25°C (±1°C).

Mortality data were read and recorded after 24 and 48 h by counting both dead and living larvae. Pupae were removed when they emerged and were not included in the mortality count. When control mortalities exceeded 5%, the percentages observed in the treated containers were corrected according to Abbott's formula (Abbott 1925). Test series with control mortalities greater than 10% were discarded. The results were subject to log-probit analysis (Finney 1971, Raymond 1985) and median lethal concentrations were determined.

Assessment of *Bti* spore density in areas treated with irradiated and unirradiated *Bti* products

In the spring of 1996, 25 soil samples were extracted in each of 8 areas in 2 geographically separate regions (region A: on the German east side of the Rhine River; region B: on the French west side of the Rhine River). In region A since 1983, an average of 2–4 treatments with irradiated *Bti* products were made annually after periods of floods; whereas in region B, similar treatments were made but with unirradiated *Bti* products. Regions A and B were separated from each other by more than 50 km. In each region, 5 soil samples were taken in 4 of the areas treated, at each of 5 locations. The same number of soil samples was taken in nearby untreated areas. The untreated samples were chosen so that they could not have been contaminated accidentally by *Bti*.

The soil samples were taken with a metal tube (square section, 10 × 10 cm) that was hammered 5 cm into the soil. The sample was sliced off and retrieved. The basic sampling pattern at each location was a 10 × 10-m square. A soil sample was taken in each corner of the square and 1 was taken in the center. Each sample was placed in an individual plastic container and thoroughly stirred. Two grams of earth were taken from each sample and combined in a plastic bag. These 10-g composite soil samples were again mixed thoroughly and stored at room temperature, taking care to clearly mark each area of origin of the 80 samples.

Soil sample treatment: From each sample, 1 g of soil was weighed and placed in a screw-top glass test tube together with 10 ml of sterilized distilled

water. The samples were well shaken and then placed in a hot water bath at 80°C for 10 min. Kalfon et al. (1986) showed that a heat shock of 80°C for 10 min was sufficient to kill bacilli and spores that were not heat resistant, but would not eliminate heat-resistant microorganisms. After cooling, the test tubes were placed in a refrigerator at 5°C, to prevent any new growth of heat-resistant spores.

Counting of the Bti *spores:* To isolate the heat-resistant bacilli in the 80 samples, a series of 10-fold diluted samples was prepared. In each case, 0.1 ml of aqueous suspension from the test tubes was mixed with 0.9 ml of sterile distilled water. The process was repeated with each dilution until a dilution of 10^{-6} was achieved. Then 0.1 ml of the undiluted sample as well as similar amounts of the 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-6} diluted samples were smeared onto each of 3 nutrient agar dishes and incubated at 30°C for 48 h. Examination and counting of the bacterial colonies followed. The dilution at 10^{-3} seemed to be best for the isolation of *Bti.* Colonies with growths typical of *B. thuringiensis* were examined with an optical phase-contrast microscope for crystal and spore development.

Bacillus thuringiensis colonies were relatively easy to recognize by their characteristic growth pattern. These colonies were usually white and displayed a typical sheen. Accurate identification required use of a phase-contrast microscope. For this, a small quantity of the colony being examined was removed from the agar with a wooden toothpick and placed on a slide in a drop of water. Evidence of spores and crystals then was observed with the phase-contrast microscope. The larvicidal activity of the identified *B. thuringiensis* strains was determined through bioassays with mosquito larvae. Ten milliliters of sterile distilled water and 10 3rd instars of *Aedes vexans* (Meigen) were placed in a 20-ml plastic beaker. A syringe of bacteria from the bacterial colonies was placed in an Eppendorf container already containing 1 ml of sterile distilled water. After homogenization with a Vortex, the suspension was divided into 3 samples in plastic beakers. The tests on each colony were divided into 3 stages. Three beakers with distilled water and mosquito larvae acted as a negative control. As the positive control, a 3-fold experiment with a bacterial suspension was undertaken with agar-fed bacteria of the IPS-82 standard (Institut Pasteur, Paris, France). The mortality was determined after 20 min and 24 and 48 h. If mortality exceeded 20% in the negative control, the experiment was rejected. After identification of the *Bti* colonies, the number of *Bti* spores per gram of soil was determined for each of the test areas, taking into account the various dilutions. Most of the spores and crystal-developing bacteria (*B. thuringiensis* isolate) were sent to the Pasteur Institute, Paris, for determination of their serotype.

Table 1. Effect of gamma irradiation on spore viability of a wettable powder (WP) formulation of *Bacillus thuringiensis israelensis.*

Doses (kGy)	Viable spores/mg WP	SD
0	6,191,379,000	7,717,996,000
5.7	1,178,750	400,612
10.9	104,936	24,016
15.6	5,584	4,418
20.6	0	0
24.9	0	0
30.1	0	0
35.4	0	0

RESULTS

Assessment of radiation effect on spore viability

The radiation doses measured with the dosimeter were 5.7, 10.9, 15.6, 20.6, 24.9, 30.1, and 35.4 kGy. As expected, a negative correlation was found between radiation dose and viability of the bacillus spores. Table 1 and Fig. 1 show this correlation. On average, 1 g of unirradiated *Bti* powder contained 6.2×10^9 spores. However, spore viability was greatly reduced when the powder was subjected to radiation at 15.6 kGy. No spores survived radiation doses of 20.6 kGy and higher.

Assessment of radiation effect on larvicidal activity of *Bti*

The results of the bioassays show that irradiation with Co 60 can cause a reduction in the effectiveness of the *Bti* powder (Table 2). Although 90% of toxicity remained after a dose of 10 kGy, increasing the dose to 20–25 kGy lowered the effectiveness by 20–30%, and after a dose of 35 kGy, this was further reduced to 50%. The mean activity values were plotted against radiation dose and fitted by means of linear regression analysis to yield a correlation coefficient $r = 0.921$ (99% confidence; Fig. 2).

Bti spore density in areas treated with irradiated and unirradiated *Bti* products

Altogether, about 3,000 individual colonies were examined with a phase-contrast microscope for evidence of spores and crystals. From these, 238 were identified as *B. thuringiensis* isolates, of which 142 were checked for their serotype classification. One hundred and three isolates were mosquitocidal and were identified as *Bti.* The spore counts per area based on the results of the classification of the bacilli by individual soil samples are documented in Table 3.

In the French region B, which was treated with unirradiated *Bti* material twice a year on average,

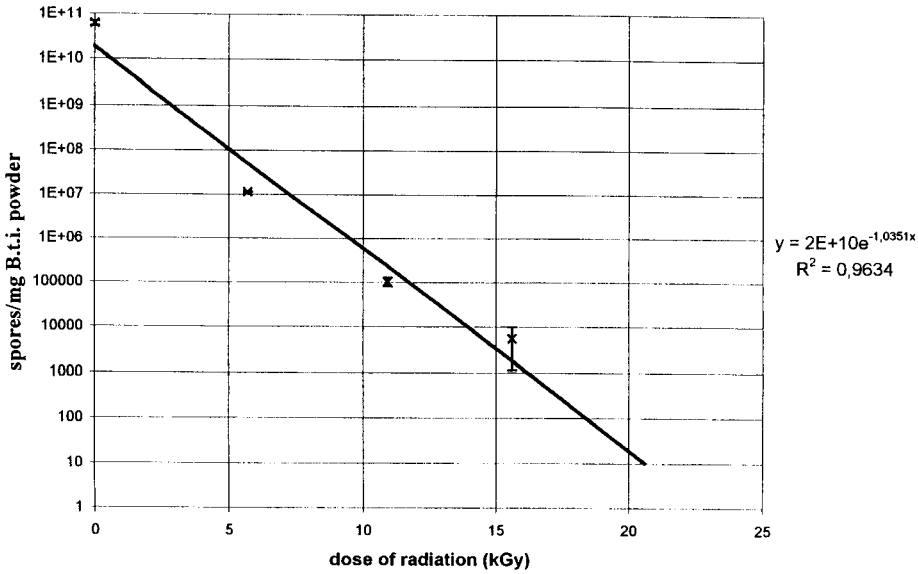

Fig. 1. Number of viable *Bacillus thuringiensis* var. *israelensis* spores after irradiation with various doses of gamma radiation.

between 700,000 and 44 million *Bti* spores were found per gram of soil. In the untreated areas, as well as in region A, which was treated with the irradiated *Bti* products, either no *Bti* spores (all untreated and 3 of the treated areas in A) or fewer than 100,000 *Bti* spores were found per gram of soil (1 treated area in Germany and 2 untreated areas in France).

DISCUSSION

Lacey and Smittle (1985) demonstrated a negative correlation between radiation dose and spore viability as well as between radiation dose and larvicidal activity. One goal of the research reported here was to determine the maximum dose for routine sterilization of *Bti* products that would maintain the effectiveness of the product. The results suggest that this dose is about 20 kGy.

In Germany, the usual radiation dose for *Bti* products is 25 kGy. Independent authorities check the sterility of the product. All investigations carried out to date have found no living spores in the product. Radiation of *Bti* products used in routine applications has not reduced their apparent effectiveness. A lowering of effectiveness by up to 20% would be allowable in any case. Routine treatment usually involves high doses of the *Bti* product to overcome the potential for development of resistance, high larval densities, or pollution of the breeding waters (Becker et al. 1992). A relatively

Table 2. Effect of gamma irradiation on the mosquito larvicidal activity against *Aedes aegypti* of a wettable powder formulation of *Bacillus thuringiensis israelensis*.

Doses (kGy)	LC_{50}[1] (range)	Toxicity (%)
0	0.0370 (0.0338–0.0405)	100
5.7	0.0419 (0.0359–0.0489)	88
10.9	0.0389 (0.0355–0.0426)	95
15.6	0.0487 (0.0447–0.0530)	76
20.6	0.0461 (0.0415–0.0513)	80
24.9	0.0524 (0.0449–0.0612)	71
30.1	0.0604 (0.0551–0.0662)	61
35.4	0.0736 (0.0668–0.0812)	50

[1] LC_{50}, median lethal concentration expressed in mg/liter; 95% fiducial limits in parentheses.

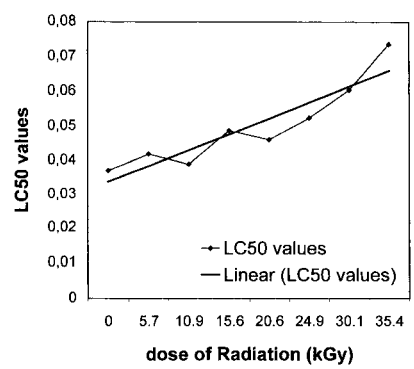

Fig. 2. Influence of various doses of gamma radiation on the median lethal concentrations.

Table 3. Number of *Bacillus thuringiensis* var. *israelensis* (*Bti*) spores per gram of soil in areas treated with radiated (region A) and unirradiated (region B) *Bti* formulations (Bactimos wettable powder).

Region	Treated[1]	*Bti* irradiated[2]	Mean no. spores/g	SD
Germany, A				
Ketsch	+	+	0	0
Lingenfeld	+	+	0	0
Philippsburg	+	+	0	0
Gernsheim	+	+	26,600	27,969
Ketsch	−		0	0
Lingenfeld	−		0	0
Philippsburg	−		0	0
France, B				
Mothern	+	−	708,250	20,800
Seltz 1	+	−	3,250,000	2,569,626
Seltz 2	+	−	44,333,333	19,629,910
Niederlaueterbach	+	−	1,997,000	2,886,751
				0
Fort Louis	−		0	0
Salmbach 1	−		0	72,419
Salmbach 2	−		40,000	55,698
Salmbach 3	−		46,600	

[1] +, treated; −, untreated.
[2] +, treated with irradiated *Bti*; −, treated with unirradiated *Bti*.

small reduction in effectiveness would be acceptable and is necessary for acceptance of routine *Bti* treatments by the regulatory authorities in Germany.

Regular treatment with unirradiated *Bti* products has been shown to lead to a significant increase of *Bti* spores in soils. This should not be regarded as a significant risk, because many studies, including this one, show that *Bti* is a naturally occurring bacteria in soil (DeLucca et al. 1981; Ohba and Aizawa 1986; Seelena et al. 1995). In 1 study, *B. thuringiensis* was found in 785 of 1,115 soil samples (Martin and Travers 1989). This indicates that *B. thuringiensis* is a habitual soil-dwelling organism. *Bacillus thuringiensis* often is found in association with *Bacillus cereus,* although *B. cereus* is found more frequently (Meadows 1993). The only known difference between *B. thuringiensis* and *B. cereus* lies in the production of protein crystals that are encoded by plasmids. In any case, the increase in the density of spores that occurs when unirradiated *Bti* product is applied results in a change in the soil microfauna. Approximately 10^{-4} spores per gram of soil would be applied with every application. Because bacteria are killed by exposure to ultraviolet radiation and by contact with other microorganisms and invertebrates that live in the soil, a natural regulation of relative populations will occur. Engler et al. (1980) found that 1% of the spores remained in mud 2 months after an application of *Bti* in mosquito-breeding surface waters. Petras and Casida (1985) found that the numbers of *Bti* spores decreased by a factor of 10 in the 1st 2 weeks after spraying and thereafter remained more or less constant over a period of about 8 months. An increase in numbers of spores after germination does not seem to be typical. Spores usually increase only in soils that are rich in nutrients (Krieg 1986). However, mosquitocidal bacteria also can germinate within the cadavers of mosquitoes (Aly et al. 1985, Becker et al. 1995, WHO 1999).

Dust containing viable bacteria spores can be produced and inhaled during the application of *Bti* powder products. However, this associated risk is negligible. Over the last 20 years, many hundreds of tons of unirradiated *Bti* products have been used worldwide every year, without any instances of illness associated with their application. Irradiation of the product may increase its safety.

Gene technology has potential for improvement of *Bti* products. Sterilization of gene-manipulated products may be essential for acceptance by the general public of such products applied in the environment.

An important advantage of irradiated *Bti* products is that application to potable water containers will not add viable spores to water used for household purposes or irrigation of garden areas. Although, according to present knowledge, viable *B. thuringiensis* spores are safe for humans, sterilization allows an extra level of safety and increased acceptance of microbial insecticides by the public. Irradiation of *B. thuringiensis* products fulfills the requirements for drinking water and thus allows *Bti* products to be used widely in water catchment areas.

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REFERENCES CITED

Abbott WS. 1925. A method of computing the effectiveness of an insecticide. *J Econ Entomol* 18:265–267.

Aly C, Mulla MS, Federici BA. 1985. Sporulation and toxin production by *Bacillus thuringiensis* var. *israelensis* in cadavers of mosquito larvae (Diptera: Culicidae). *J Invertebr Pathol* 46:251–258.

Becker N. 1997. Microbial control of mosquitoes: management of the upper Rhine mosquito population as a model programme. *Parasitol Today* 13:485–487.

Becker N, Djakaria S, Kaiser A, Zulhasril O, Ludwig H. 1991. Efficacy of a new tablet formulation of an asporogenous strain of *Bacillus thuringiensis israelensis* against larvae of *Aedes aegypti*. *Bull Soc Vector Ecol* 16:176–182.

Becker N, Margalit J. 1993. Control of dipteran pests by *Bacillus thuringiensis*. In: Entwistle P, Bailey MJ, Cory J, Higgs S, eds. Bacillus thuringiensis*: its uses and future as a biological insecticide* Sussex, United Kingdom: John Wiley & Sons. p 147–170.

Becker N, Zgomba M, Ludwig M, Petric D, Rettich F. 1992. Factors influencing the activity of *Bacillus thuringiensis* var. *israelensis* treatments. *J Am Mosq Control Assoc* 8:285–289.

Becker N, Zgomba M, Petric D, Beck M, Ludwig M. 1995. Role of larval cadavers in recycling processes of *Bacillus sphaericus*. *J Am Mosq Control Assoc* 11:329–334.

DeLucca AJ, Simonson JG, Larson AD. 1981. *Bacillus thuringiensis* distribution in soils of the United States. *Can J Microbiol* 27:865–870.

Engler S, Morawwcsik J, Schnetter W, Krieg A. 1980. Pilot-Versuch zur Bekämpfung von Stechmückenlarven im Freiland mit einem UV-behandelten Präparat von *Bacillus thuringiensis* var. *israelensis*. *Anz Schaedlingskde Pfanzenschutz Umweltschutz* 53:181–184.

Finney DJ. 1971. *Probit analysis* 3rd ed. Cambridge, United Kingdom: Cambridge Univ. Press.

Kalfon A, Lugten M, Margalit J. 1986. Development of selective media for *Bacillus sphaericus* and *Bacillus thuringiensis* var. *israelensis*. *Appl Microbiol and Biotech* 24:240–243.

Krieg A. 1986. *Bacillus thuringiensis,* ein mikrobielles Insektizid: Grundlagen und Anwendung. In: Broennimann A, Colhoun J, Heitefuss R, Kern H, eds. *Beihefte zur Phytopathologischen Zeitschrift* Berlin, Germany: Paul Parey Scientific Publishers. p 191.

Kroeger A, Dehlinger U, Burkhardt G, Anaya H, Becker N. 1995. Community based dengue control in Columbia: people's knowledge and practice and the potential contribution of the biological larvicide *B.t.i.* (*Bacillus thuringiensis israelensis*). *Trop Med Parasitol* 46:241–246.

Lacey LA, Smittle BJ. 1985. The effect of gamma radiation on spore viability and mosquito larvicidal activity of *Bacillus sphaericus* and *Bacillus thuringiensis* var. *israelensis*. *Bull Soc Vector Ecol* 10:98–101.

Martin PAW, Travers RS. 1989. Worldwide abundance and distribution of *Bacillus thuringiensis* isolates. *Appl Environ Microbiol* 55:2437–2442.

Meadows MP. 1993. *Bacillus thuringiensis* in the environment: ecology and risk assessment. In: Entwistle P, Bailey MJ, Cory J, Higgs S, eds. Bacillus thuringiensis*: its uses and future as a biological insecticide* Sussex, United Kingdom: John Wiley & Sons. p 193–213.

Ohba M, Aizawa K. 1986. Distribution of *Bacillus thuringiensis* in soil of Japan. *J Invertebr Pathol* 47:277–282.

Petras SF, Casida LE. 1985. Survival of *Bacillus thuringiensis* spores in soil. *Appl Environ Microbiol* 50:1496–1501.

Raymond M. 1985. Presentation d'un programme d'analyse log-probit pour micro-ordinateur. *Cah ORSTOM Ser Entomol Med Parasitol* 22:117–121.

Seleena PH, Lee L, Lecadet MM. 1995. A new serovar. of *Bacillus thuringiensis* possessing 28a28c flagellar antigenic structure: *Bacillus thuringiensis* serovar. *jegathesan,* selectively toxic against mosquito larvae. *J Am Mosq Control Assoc* 11:471–473.

WHO [World Health Organization]. 1981. Report of informal consultation on standardization of *Bacillus thuringiensis* H-14. TDR/BCV/B.T.H-14/811, WHO/VBC/81-828. Geneva, Switzerland: World Health Organization.

WHO [World Health Organization]. 1999. Microbial pest control agent *Bacillus thuringiensis* Environmental Health Criteria 217. Geneva, Switzerland: World Health Organization.