REDUCTION OF MORTALITY RATES OF *BACILLUS THURINGIENSIS* VAR. *ISRAELENSIS* AQUEOUS SUSPENSIONS DUE TO FREEZING AND THAWING

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ABSTRACT. When studying the behavior (carry, dispersion, persistence) of *Bacillus thuringiensis* var. *israelensis* (*B.t.i.*) formulations used in the treatment of rivers or streams for black fly control, a large number of samples containing small quantities of *B.t.i.* are required for proper analysis. Freezing is a useful procedure to prevent enzymatic alteration or bacterial growth in samples before bioassays are to be performed. Using *Aedes atropalpus* neonate larvae, we studied the effect of freezing and thawing of *B.t.i.* aqueous suspensions by looking at mortality response parameters such as the slope and the LC_{50} of the probit regression. Initial concentration values of 1, 5, 10 and 20 mg/liter at the moment of freezing of the *B.t.i.* suspensions did not significantly affect toxicity. The number of freeze-thaw cycles greatly increased the LC_{50} values without much change to the slope of the log-probit regressions. We derived an equation that allowed us to compensate for the loss of toxicity of a given *B.t.i.* sample, knowing the number of freeze-thaw cycles.

INTRODUCTION

Due to the lack of direct methods for measuring the presence of the *B.t.i.* toxic crystals (physical, chemical or immunological), the toxicity (potency) of field samples is interpreted mainly from spore counts (Undeen and Colbo 1980, Frommer et al. 1981a, 1981b; Merritt et al. 1989, Matanmi et al. 1990) or bioassays (Lacey and Lacey 1981). When using a bioassay, direct toxicity of a given sample is measured in mg/liter of formulation or in units per ml. Studies of the behavior (persistence, dispersion) of *B.t.i.* formulations in rivers, streams or ponds involves collecting a large number of samples which cannot be processed immediately. Thus field samples may have to be kept for variable periods of time before assaying. In such cases, samples are usually frozen or kept at 4°C in order to prevent or minimize enzymatic deterioration of the crystals or bacterial growth.

Unfortunately, the stability of *B.t.i.* toxic crystals has only been investigated for temperatures ranging from 10 to 35 °C (Mulligan et al. 1980, Ignoffo et al. 1981, Sinègre 1981, Guillet et al. 1982), at 50 °C (Ignoffo et al. 1982), and at 80 °C (Dempah[2]). These authors have found that the toxic activity of dry powders or formulations was remarkably stable at those temperatures.

Because of the absence of literature on the effect of freezing and thawing on *B.t.i.* suspensions, we examined the freeze-thaw procedure to see if it would affect the toxic activity of the crystals, and to determine how it may modify parameters such as the LC_{50} and the slope of probit mortality curves.

MATERIALS AND METHODS

To observe the decrease in mortality and the effect of different initial concentrations of *B.t.i.*, we prepared a series of suspensions which were frozen and thawed up to 4 times and subsequently diluted and bioassayed. From the results we were able to work out a simple equation to compensate for the loss of activity of frozen-thawed *B.t.i.* aqueous suspensions.

Bioassays: Bioassays were performed under laboratory conditions (20–22 °C) using *Aedes atropalpus* (Coq.) neonate larvae because freshly hatched organisms offer greater physiological synchronicity and greater sensitivity (Ibarra and Federici 1987). The method used was based upon the one described by these authors, in which a single neonate larva was placed in an individual well of a microtiter plate (96 holes) and then exposed to various chemical concentrations. To minimize experimental variability, all samples were assayed in 3 replicates of 32 larvae, using eggs of the same degree of maturity. Mortality counts were made after 24 hours. This technique permitted us to process up to 20 microtiter plates per day.

Freeze-thaw procedure: Experiments were conducted to assess if *B.t.i.* suspensions prepared at different initial concentrations would give the same mortality response when diluted

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[2] Dempah, J. 1979. Essais de *Bacillus thuringiensis israelensis* sur les moustiques. Rapport. D. E. A. Entomologie Medicale, Fac. des Sciences, Paris XI, et Lab. ORSTOM, Bondy, France.

Table 1. Schedule of bioassays that were performed on 32 larvae on successive days, August 6, 7 and 8, or in triplicates of 32 larvae on September 17, 24 and 25. The concentration (mg/liter) of *Bacillus thuringiensis* var. *israelensis* suspensions at the time of freezing is presented in brackets.

	Number of freeze-thaw cycles				
Test no.	0	1	2	3	4
1	Aug. 6–8 (1)	Aug. 6–8 (1)	—	—	—
2	Aug. 6–8 (5)	Aug. 6–8 (5)	—	—	—
3	Aug. 6–8 (10)	Aug. 6–8 (10)	—	—	—
4	Aug. 6–8 (20)	Aug. 6–8 (20)	—	—	—
5	Sep. 17 (20)	Sep. 17 (20)	Sep. 17 (20)	Sep. 17 (20)	Sep. 17 (20)
6	—	—	Sep. 24 (20)	Sep. 24 (20)	Sep. 24 (20)
7	—	—	Sep. 25 (20)	Sep. 25 (20)	Sep. 25 (20)

and bioassayed, and to evaluate if a single freeze-thaw cycle of those *B.t.i.* suspensions would affect the mortality response. Concentrations of 1, 5, 10 and 20 mg/liter of *B.t.i.* were prepared in distilled water using Teknar HPD® (Zoëcon lot no. 0080227). These concentrations cover the range suggested by the company for treating black fly larvae in streams. Each suspension was divided into 2 equal volumes and each of the 4 concentrations was kept either at 4°C or frozen once at −25°C then thawed at room temperature. We ended up with 2 identical series of 4 *B.t.i.* concentrations that were either unfrozen or frozen-thawed once. Before the bioassay, each preparation was diluted in distilled water for testing mortality response at a final concentration of 25, 50, 100 and 200 μg/liter. These concentrations were selected following preliminary neonate larvae bioassays to cover a range of mortality from 10 to 90%. The bioassays were performed on 3 successive days (August 6–8) as presented in Table 1.

Since the results from this first series showed that all bioassays with once frozen or unfrozen samples had similar mortality response curves whatever their initial concentration, another series was prepared at 20 mg/liter. This concentration was selected so that enough toxic activity would be left after multiple freeze-thaw cycles. Equal volumes of this suspension were frozen-thawed 0, 1, 2, 3 and 4 times. Each of these samples was then diluted at testing concentrations varying from 50 to 750 μg/liter before bioassays. To ensure the validity of probit analysis, we tried to obtain 3 concentration values on either side of the LC_{50}.

One additional series of bioassays was performed in triplicates for unfrozen and frozen once samples (Table 1, test 5). For two, three and four times frozen samples, bioassays were done in triplicates for 3 different days (September 17, 24 and 25) as shown in Table 1.

Statistical analysis: Each set of bioassays was analyzed by probit analysis (Finney 1971). The percentage of mortality (in probit units) is expressed by a linear relation, determined by a maximum likelihood procedure, as a function of the logarithm of the concentration (in μg/liter). Natural mortalities were taken into account using Abbott's formula (Abbott 1925).

To demonstrate the independence of mortality responses for a given number of freeze-thaw cycles performed on samples at different concentrations at the time of freezing, we used the statistical approach developed by Hong et al. (1988). This approach consisted in the determination of a single probit line called "grand probit" (GP) from multiple toxicity test data. To achieve this, the individual probit lines (for a given number of freeze-thaw cycles) were pooled, and using a parallel line technique (Finney 1971, Hubert 1984), a common slope and LC_{50} were calculated for the grouped data. Then χ^2 tests were used to confirm the hypothesis on the homogeneity of the individual probit slopes (parallelism) and LC_{50}. If the calculated χ^2 values (for each parameter) were lower than the critical χ^2 values, it indicated at the 95% confidence level, that the probit slopes were parallel and the LC_{50}s were homogenous. These statistics were used also to demonstrate parallelism of single probit lines obtained when grouping all

tests for 0, 1 and 2 freeze-thaw cycles, and with 3 and 4 cycles.

RESULTS

Freezing at 4 different concentrations: In Table 2, rows A and B, we give the calculated χ^2 values for parallelism and LC_{50} homogeneity tests. Row A represents the tests performed on the probit lines (maximum likelihood) obtained from unfrozen *B.t.i.* suspensions prepared at 1, 5, 10 and 20 mg/liter. Row B represents χ^2 values for parallelism and LC_{50} homogeneity tests for the same concentrations, but frozen once. Since the critical χ^2 value at 4 d.f. is 9.49 for a 95% confidence level (Zar 1984) and that the χ^2 values of rows A and B are below this value, we can conclude that whatever the concentration at the time of freezing was, the probit lines are parallel and the LC_{50}s are homogenous. Furthermore, the low χ^2 values for unfrozen suspensions (row A) indicate that the neonate bioassay technique is reliable. There seems to be a difference in the behavior of the *B.t.i.* suspensions after one freezing cycle. This can be seen by the greater χ^2 values after freezing the suspension once compared with unfrozen suspensions (Table 2, row B compared with row A) and by a greater dispersion of the mortality values around the grand probit line (Fig. 1B compared with Fig. 1A).

Freeze-thaw treatment: In Table 2 rows C, D and E, we present the χ^2 values for parallelism and the LC_{50} homogeneity test after multiple freeze-thaw cycles of *B.t.i.* suspensions prepared at 20 mg/liter. Since the critical χ^2 value at 2 d.f. is 5.99 for a 95% confidence level (Zar 1984) and that all calculated χ^2s are below that value, it indicates that the separate tests for a given number of cycles have similar slopes and LC_{50} values; ensuring the reliability of the grand probit grouping.

Table 2. Chi-square validation tests for parallelism and homogeneity of the LC_{50}s after the maximum likelihood for individual tests. Values in rows A and B represent the analysis of the probit lines obtained with preparations unfrozen or frozen once at an initial concentration of 1, 5, 10 and 20 mg/liter. Rows C, D, and E are the results from a preparation of 20 mg/liter frozen-thawed 2, 3 or 4 times. Rows F, G and H are the analyses of the groupings of the various tests.

	Number of cycles	Number of tests	Parallelism (χ^2)	Homogeneity (χ^2)
A	0	5	0.74	2.62
B	1	5	8.80	4.21
C	2	3	1.06	2.08
D	3	3	1.57	1.43
E	4	3	5.86	2.74
F	0-1-2-3-4	19	36.85	—
G	0-1-2	13	11.37	—
H	3-4	6	7.58	—

In Fig. 1F the grand probit (GP) lines after 0, 1 and 2 cycles on the upper part have similar slope values, and the GP lines after 3 and 4 cycles share a similar slope value. After all the assays (0, 1, 2, 3 and 4 cycles) were combined (Table 2, row F), the analysis of parallelism failed because the calculated χ^2 (36.85) is higher than the critical value of 28.87 (18 d.f.) for the 95% confidence level (Zar 1984). After grouping the tests for 0, 1 and 2 freeze-thaw cycles (Table 2, row G), we can see that the lines are parallel (critical $\chi^2 = 21.03$, calculated $\chi^2 = 11.37$). Furthermore, grouping the tests for 3 and 4 cycles (Table 2, row H) also shows that this group of lines are parallel (critical $\chi^2 = 11.07$, calculated $\chi^2 = 7.58$). We calculated an average slope value of 2.024 for 0, 1 and 2 cycles which is statistically different from 2.453 after 3 and 4 freeze-thaw cycles.

The absence of overlapping of the LC_{50} values presented in Table 3, indicates that each freeze-thaw treatment is directly and significantly modifying the median lethal concentration value. Furthermore, the 95% CIs are all within 7% of the LC_{50} values. Figure 2 shows the effect on the LC_{50} (n) when the number of freeze-thaw cycles (n) is increasing. We have modeled this increase of LC_{50} by the exponential equation,

$$LC_{50}\ (n) = \alpha\ 10^{\beta n} \qquad (1)$$

where n = 0, 1, 2, 3 or 4. After calculation of the regression line ($R^2 = 0.99$) on the dependant variable log (LC_{50} (n)), we obtained the estimated parameters: $\alpha = 79.22$ and $\beta = 0.15$. The parameter α corresponds to the predicted LC_{50} (0) (unfrozen), and β is the rate of increase of the LC_{50} after freezing.

Correction of mortality after n freeze-thaw cycles: For each number of cycles (n), we have a probit line given by,

$$Y_n = a_n + b_n X \qquad (2)$$

where Y_n is the mortality expressed in probit units, a_n is the intercept, b_n the slope of the probit line and X is the logarithm of the concentration. It is known (Hubert 1984) that the LC_{50} is given by,

$$\text{Log}\ (LC_{50}\ (n)) = (5 - a_n)/b_n \qquad (3)$$

then (2) can be written as,

$$Y_n = 5 + b_n\ (X - \log\ (LC_{50}\ (n))). \qquad (4)$$

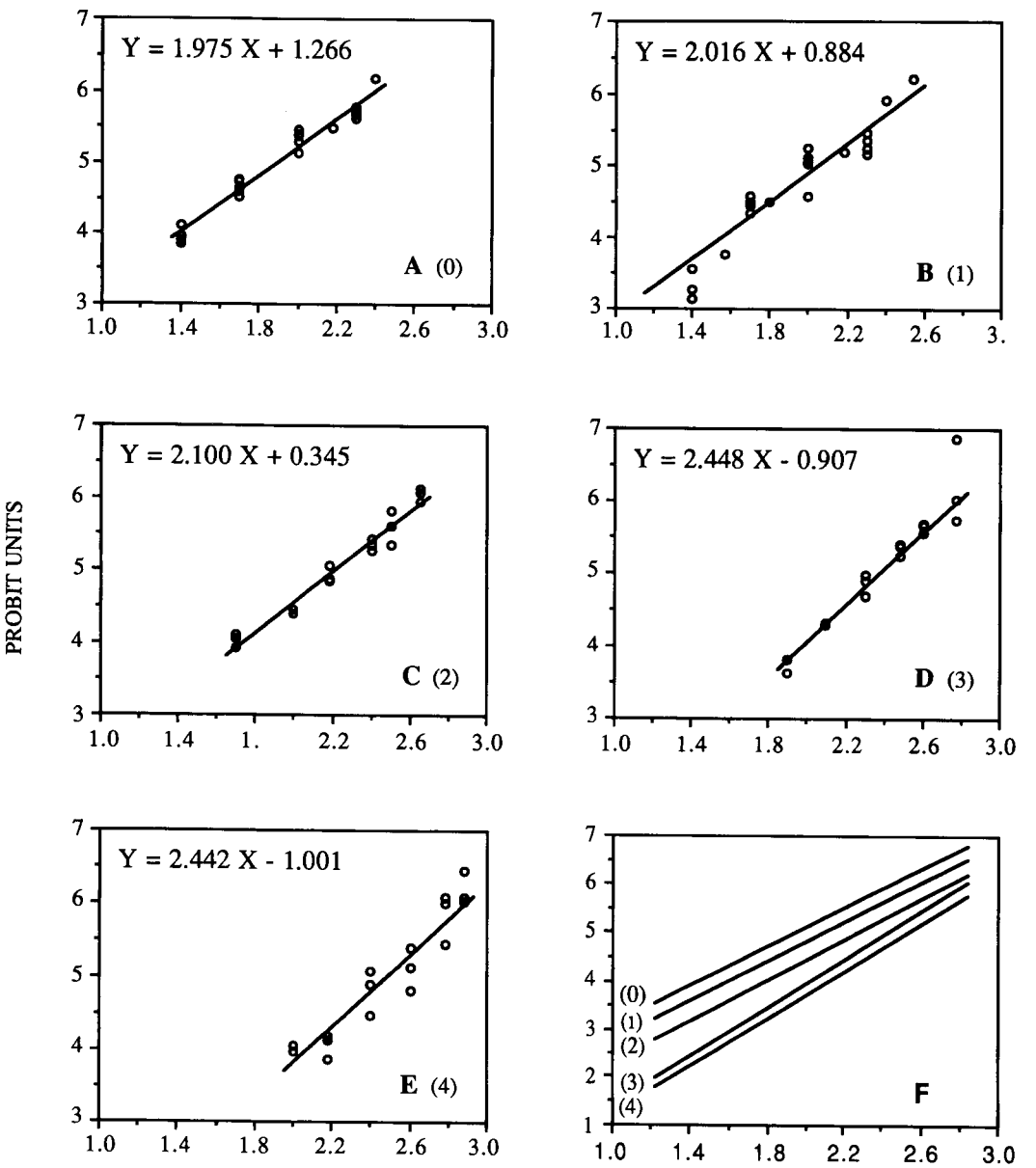

Fig. 1. Grand probit lines of mortality responses to *Bacillus thuringiensis* var. *israelensis* suspensions submitted to (0), (1), (2), (3) and (4) freezing cycles. Assays for 0 and 1 cycle (Figs. A and B) consisted of 5 different tests, performed on preparations frozen at 1, 5, 10 and 20 mg/liter. Preparations of 20 mg/liter were frozen 2, 3 and 4 times and tested 3 times (Figs. C, D and E). Figure F displays all the grand probit lines to better visualize the parallelism and the LC_{50} shift after multiple freeze-thaw cycles.

Table 3. Probit equations, LC_{50}s and 95% confidence intervals obtained from the grand probit analysis incorporating multiple data tests (5 separate tests for 0 and 1 freeze-thaw cycle, 3 separate tests for 2, 3 and 4 cycles).

Cycle	Probit equation	LC_{50}	95% confidence interval
0	$Y = 1.975\ X + 1.266$	77.77	72.64–83.27
1	$Y = 2.016\ X + 0.884$	109.99	102.49–118.04
2	$Y = 2.100\ X + 0.345$	164.50	153.26–176.56
3	$Y = 2.488\ X - 0.907$	236.79	222.90–251.55
4	$Y = 2.422\ X - 1.001$	300.08	281.71–319.65

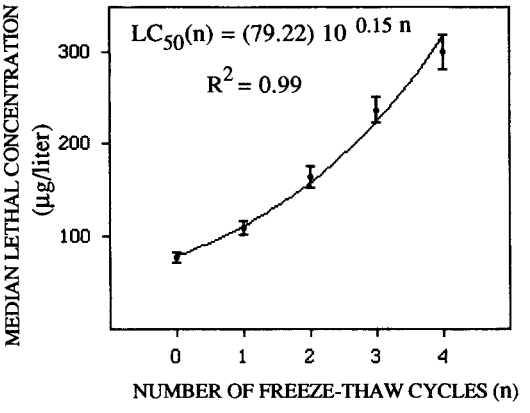

Fig. 2. Relation between the median lethal concentration and the number of freeze-thaw cycles.

Using equation (1) and the parallelism of the probit lines for n = 0, 1 and 2, with their calculated common slope m = 2.024; after rearranging (4) it becomes,

$$Y_n = 5 + m\ (X - \log\ (\alpha\ 10^{\beta n}))$$

or

$$Y_n = 5 + m \cdot (X - (\log\ (LC_{50}\ (0)) + \beta n)). \quad (5)$$

From this relation, the loss of mortality (in probit units) for a given concentration, after n freeze-thaw cycles compared to an unfrozen one, is expressed by,

$$Y_0 - Y_n = m\ \beta\ n \quad (6)$$

where Y_0 is the mortality for the unfrozen suspension. In our case, using Teknar HPD and *Ae. atropalpus* neonate larvae as test organisms, we obtained $Y_0 \approx Y_n + 0.3$ n (0.3 being the product of the probit slope (m) by the rate of increase of the LC_{50} after freezing (β)).

DISCUSSION

Since sporeless *B.t.i.* formulations are now available, there will be a greater tendency for using bioassays or direct toxicity tests to measure *B.t.i.* activity in streams or ponds. Regardless of the technique, numerous field samples will have to be frozen and subsequently tested for toxicity. It is then important to assess the effect of freezing on *B.t.i.* aqueous suspensions.

The tests performed on the hypothesis that the regression lines are parallel for the preparations at different concentrations values (Table 2, rows A and B), indicate that the concentrations (in the range considered here) at the time of freezing do not have to be taken into account. This means that the field samples frozen at different initial concentrations will follow the same pattern. On the other hand, the results of freezing-thawing indicate that the number of cycles has a direct influence on the magnitude of the *B.t.i.* toxicity alteration (Table 3 and Figs. 1 and 2). Indeed, for a single freeze-thaw cycle the LC_{50} value of 109.99 µg/liter represents a 24% reduction in mortality. At the extremes, after 4 freeze-thaw cycles, the LC_{50} value is increased from 78 to 300 µg/liter, indicating a 6.25 fold decrease in mortality. In concrete terms, a concentration giving 50% mortality would give less than 8% mortality after 4 freeze-thaw cycles.

Figure 1 shows a greater dispersion of individual experimental values around the GP line after 4 freeze-thaw cycles. Because the manipulations were done in the same manner for all the samples, this increased variability suggests that the properties of our *B.t.i.* preparations could have been modified by the freezing treatment, making it more difficult to be resuspended after thawing. Freezing may promote flocculation (or aggregation) of *B.t.i.* crystals. If such is the case that only one or a few crystals are sufficient to kill a larva, flocculation of crystals could create a large particle that would only kill a single larva instead of many, thus reducing the mortality even though the concentration is not modified. In addition, if the aggregates are large enough, they could exceed the range of particle sizes that a specific instar is able to ingest. In such a case, the reduction of activity following freeze-thaw

cycles, could be less severe on larger larvae such as a 4th instar.

Freezing damage to the *B.t.i.* toxic protein could also account for the reduction of mortality. During freezing, substantial enhancement of solute concentration occurs in the liquid within the layer surrounding the ice nucleus (Steponkus 1984). Depending on the nature of the solute, it could induce a local change of pH and modify the active site of the toxin crucial for binding to the plasma membrane of cells (Sarjeet et al. 1989). In a largely accepted mechanism of action proposed by Knowles et al. (1989), initial binding of the δ-endotoxin is a necessary step, thus a modification of affinity or binding capability will likely reduce the potency of a given preparation.

Regardless of the process involved, our results clearly indicate an important loss of toxic activity after an aqueous suspension of *B.t.i.* has been frozen and thawed. These observations should be an incentive to include an appropriate procedure to bioassay previously frozen material.

Adding the necessary additional samples sufficient to get the estimated parameter of reduction (β) in equation (6), and using the procedure described in "Correction of mortality after n freeze-thaw cycle," we demonstrated that it is possible to calculate the toxic activity that would originally be present, from results obtained with previously (and repeatedly) frozen-thawed material. Equation (6) shows that the loss of mortality (in probit units) for a given suspension, after n freeze-thaw cycles compared to an unfrozen one, is almost 0.3 n. A different correction factor would be expected if the bioassays are performed using different instars or species, or if a different *B.t.i.* formulation is being studied, because the rate of increase of the LC_{50} after freezing is an intrinsic factor of a formulation. The procedure used in this experiment could be useful for deciding the proper technique for managing the storage and handling of a large number of *B.t.i.* samples.

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