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par

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Etude de la dispersion et de la perte du larvicide biologique particulaire, *Bacillus thuringiensis* serovar. *israelensis* , sur les substrats benthiques et dans la zone hyporhéique des cours d'eau.

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Le document ci-joint présente les résultats de mon travail de recherche effectué dans le cadre d'une maîtrise scientifique (classe B) en Sciences de l'Environnement (programme 3403). Le but de cette recherche était d'étudier les causes de la perte de l'insecticide biologique *Bacillus thuringiensis* serovar. *israelensis* (*B. t. i.*) en rivières. Pour se faire, il était nécessaire de mesurer la dispersion du larvicide (*B. t. i.*) dans l'eau de rivière, puis de déterminer si la toxine de *B. t. i.* se retrouvait dans l'eau de la nappe hyporhéique ou associée aux substrats benthiques tels la mousse, l'herbe, le périphyton et les sédiments.

Problématique

Le *B. t. i.* est un larvicide biologique de plus en plus utilisé lors des programmes de contrôle des insectes piqueurs. La ou les toxines responsables de son activité sont produites par la bactérie au moment de la sporulation. Suite à sa production en fermenteur la ou les toxines sont préparées en diverses formulations en y ajoutant des produits et des additifs propres à favoriser la dispersion, la miscibilité avec l'eau, la conservation ainsi que l'ingestion par les larves. Ce dernier paramètre est primordial car il est reconnu que le mécanisme d'intoxication par le *B. t. i.* est déclenché dans un milieu hautement alcalin (pH≈10), ce qui est le cas de l'intestin moyen des larves de diptères. Ce mode d'action particulier explique la très haute spécificité du *B. t. i.* et du même coup, le distingue des insecticides de contact.

Lors de la préparation du larvicide, on s'efforce d'obtenir un maximum de toxines associées aux éléments de formulation sous forme particulaire de taille optimale allant de 35 à 50 µm. Il est maintenant reconnu que ce

gabarit rencontre les critères de sélectivité alimentaire des organismes cibles.

Le *B. t. i.* offre une spécificité marquée pour les larves de certains diptères (mouches noires et moustiques), car contrairement aux produits chimiques, il ne produit que très peu d'effets sur la faune non-cible. D'autres événements contribuent à augmenter l'utilisation du *B. t. i.*. En effet, il s'agit souvent de l'unique alternative pour contrer les foyers de résistance aux produits chimiques, et de plus il est parfois le seul produit autorisé par législation, sur certains territoires.

Cependant, sous certaines conditions d'application, ce larvicide biologique offre dans les cours d'eau, des performances médiocres en raison d'une portée réduite. La portée d'un produit (larvicide) se définit comme étant la distance en aval du point d'épandage à laquelle un taux de mortalité souhaité (normalement 95%) est maintenu. Une portée réduite aura pour conséquence la multiplication des sites d'épandage le long du cours d'eau à traiter, ce qui augmentera le coût des opérations de contrôle. Par opposition, les produits chimiques (de nature soluble) se caractérisent par des portées pouvant aller à des dizaines de km alors que le *B. t. i.*, dans les pires conditions ne portera qu'à des dizaines de mètres.

Il existe une corrélation entre la faible portée du *B. t. i.* et certaines caractéristiques des rivières, tels le faible débit, la densité de la végétation aquatique, le rapport élevé entre la surface des substrats et le volume d'eau. Cependant, à notre connaissance, aucune étude n'a eu pour objet de déceler la présence du *B. t. i.* associé à différents substrats benthiques ou à tout autres éléments de rivière. Nous considérons les divers

compartiments que sont les substrats tels la mousse (*Platylonella lescurii*), les herbes (*Gramineae sp*), le périphyton, les sédiments et la zone hyporhéique, comme pouvant jouer un rôle de réservoirs (puits) dans lesquels une partie de la fraction toxique du *B. t. i.* soit susceptible d'être déplacée et retenue, au détriment du volume d'eau courante accessible aux larves de mouches noires, organisme cible du *B. t. i.* en eau courante. Ce travail prétend contribuer à une meilleure compréhension du comportement du *B. t. i.* en rivières, à identifier les causes de la perte puis mesurer l'importance relative de ces dernières.

Résumé des résultats de l'étude

Les résultats de l'étude et les mises au point techniques nécessaires à sa réalisation ont donné lieu à la rédaction de trois articles.

Un premier, article intitulé "Loss of *B. t. i.* larvicidal activity and its distribution in benthic substrates and the hyporheic zone of streams" rédigé en conformité avec le "Journal canadien des sciences halieutiques et aquatiques" (Canadian Journal of Fisheries and Aquatic Sciences) présente les conclusions se rapportant de manière spécifique à la problématique. Cette étude confirme que les doses de *B. t. i.* (Tableau 1), calculées à partir de l'aire sous les courbes des Figures 1b, 1d et 1f, diminuent de manière exponentielle (Figure 2) en aval du point d'épandage. Les divers substrats benthiques contribuent à retenir une partie des cristaux toxiques (Tableau 4). Par ordre croissant d'importance, les contributions des substrats sont de 5% pour les sédiments et le périphyton combinés, de 10% pour la mousse seule, alors qu'aucune perte significative n'a pu être correlée à la présence d'herbes. Les larves, ainsi que l'ensemble des

organismes filtreurs présents dans le cours d'eau au moment de l'application du *B. t. i.* peuvent contribuer pour 5% de la perte, et cela en prenant des taux d'ingestion et des densités de population extrêmes. La principale cause du retrait rapide de *B. t. i.* est imputable à sa pénétration dans la couche hyporhéique. Les concentrations en larvicide des échantillons d'eau provenant de sondes à des profondeurs de 15, 20, 30 cm (Figure 3) sous le lit de la rivière, (Figure 3), montrent que la zone hyporhéique contribue à elle seule, pour plus de la moitié du *B. t. i.* perdu., à débit hyporhéique égal à celui du cours d'eau (Tableau 2).

L'ensemble des résultats de cette partie de recherche indique que la mesure directe du *B. t. i.* par essais sur des larves néonatales de moustiques a permis de mesurer la perte de larvicide sur un cours d'eau et de déceler la présence de toxicité associée aux substrats benthiques et à la zone hyporhéique jusqu'à une profondeur de 65 cm (Tableau 3). Cela permet de saisir l'importance des interactions de la zone hyporhéique en regard du matériel particulaire et d'ouvrir des voies en vue de l'amélioration des formulations de *B. t. i.*.

Ayant de nombreux échantillons à analyser, nous devions avoir recours à la congélation afin d'en minimiser la détérioration. Nous avons mesurer l'effet de la congélation et celui de la présence de matière en suspension sur des solutions aqueuses de *B. t. i.*, semblables aux échantillons de terrain. Les résultats de cette deuxième partie sont présentés dans l'article intitulé "Effects of Freezing and Field-Collected Substrates on the Efficacy of *Bacillus thuringiensis* serovar. *israelensis* as Determined Through Bioassays in the Laboratory" dont la rédaction se conforme aux exigences de la revue "Biocontrol Science and Technology".

Afin de démontrer l'effet de la congélation et celui de la concentration en *B. t. i.* au moment de la congélation, des solutions de 1, 10, 100 et 1000 mg/l ont été préparées congelées et décongelées 0, 1, 2 et 3 reprises, puis testées à une concentration finale de 50 µg/L. Les résultats de le Tableau 1 indiquent que la concentration au moment de la congélation n'a pas d'effet sur la mortalité alors que chaque cycle gel-dégel additionnel impose une baisse de mortalité des échantillons. En valeurs absolues, les mortalités passent de 100% à 64% suite à la première congélation, puis à 46% à la deuxième congélation, alors que la troisième réduit la mortalité à 38% de la valeur initiale. Ces valeurs indiquent que la perte de mortalité est de forme exponentielle négative.

Les essais, sur larves de moustiques, de solutions contenant une concentration fixe de *B. t. i.*, mais des concentrations en seston pouvant excéder 5 fois la valeur normale dans le cours d'eau étudié, indiquent qu'il existe une corrélation entre la perte de mortalité et l'augmentation de la quantité de matière en suspension (Figure 2). De manière à mesurer la présence de toxines de *B. t. i.* associées aux végétaux, le protocole expérimental implique de broyer 500 mg de plantes dans 20 ml d'eau. Nous avons fait une série de solutions de concentration allant de 750 à 5000 µg/L de *B. t. i.* toutes préparées avec 12,5 mg/L de mousse broyée. La pente et la concentration médiane létale (CL_{50}) de cette droite standard, obtenues par analyse probit, servent de point de comparaison avec les paramètres d'une droite standard préparée avec la même substance, mais sans la mousse. La CL_{50} passe de 52 à 2282 µg/L en présence de la mousse alors que les pentes sont semblables (Figure 3). Ceci indique que la présence d'une grande quantité de matière solide dans

les échantillons de *B. t. i.* ne modifie pas le mécanisme d'action de la toxine, puisque la pente de la droite n'est pas changée, alors que la CL_{50} augmente par un facteur de 44 fois.

Devant l'importance de l'effet provoqué par la congélation d'une solution aqueuse de *B. t. i.*, pouvant initialement produire 60% de mortalité, nous avons voulu faire une étude plus approfondie de l'effet du gel/dégel sur des solutions de *B. t. i.*. Cette fois, le protocole expérimental incluait l'analyse de séries de solutions de *B. t. i.* non-congelées ou congelées 1, 2, 3 et 4 fois. Les tests effectués à partir de ces séries de solutions ont permis de révéler l'effet du gel sur les paramètres des droites probits (pentes des droites et CL_{50}). Les résultats de cette troisième partie se retrouvent dans l'article intitulé "Reduction of mortality rates and CL_{50} of *B. t. i.* aqueous suspensions due to freezing and thawing" rédigé suivant les exigences de la revue "Journal of the American Mosquito Control Association". Les résultats de le Tableau 2, ligne B, confirment que la concentration d'une solution au moment de la congélation est sans effet. Cette ligne regroupe des droites probit complètes faites à partir de solutions congelées à 1, 5, 10, et 20 mg/L. Un test de χ^2 est utilisé pour mesurer le parallélisme et l'homogénéité des CL_{50} des droites probits obtenues à partir des différentes préparations, mais pour un même nombre de cycles gel-dégel. Les χ^2 de la ligne F indique que les 19 droites obtenues ne sont pas toutes parallèles entre elles, alors que les χ^2 des lignes G et H montrent que les 13 droites obtenues après 0, 1 et 2 cycles de gel-dégel sont parallèles entre elles tout comme les 6 droites obtenues après 3 et 4 congélations. Ce regroupement des courbes 0-1-2 et 3-4 est visible sur la Figure 1F. On voit aussi l'augmentation de la CL_{50} à mesure que le nombre de cycle

gel/dégel est augmenté. Les valeurs de la CL_{50} du groupe de test pour un nombre donné de cycle gel-dégel, sont présentée au Tableau 3. La figure 2 montre que la CL_{50} augmente de façon exponentielle en fonction du nombre de cycles. Cette partie démontre qu'à chaque congélation, un échantillon contenant du *B. t. i.* perd de sa toxicité.

L'ensemble des travaux de terrain et des travaux de laboratoire dont cette recherche a fait l'objet contribuent à une meilleur connaissance du *B. t. i.*. Nous avons démontré que la présence de matière en suspension et la congélation de solutions de *B. t. i.*, influençait la réponse des essais sur larves de moustiques. Dans le futur, on aura intérêt à tenir compte de ces deux paramètres lors de la préparation de protocoles expérimentaux afin de valider les résultats des essais biologiques. La détection de toxines de *B. t. i.* associées à certains substrats benthiques et dans la nappe hyporhéique viennent élucider les causes de la perte rapide du *B. t. i.* en eau courante. L'importance relative des pertes est susceptible d'orienter les travaux visant à améliorer les formulation de ce produit. L'importance de la rétention de *B. t. i.* dans la zone hyporhéique aura sans doute un impact sur les travaux concernant le transport et la rétention de matériel particulaire en rivière.

Loss of *Bacillus thuringiensis* var. *israelensis* Larvicidal Activity and its Distribution in Benthic Substrates and the Hyporheic Zone of Streams

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Loss of *Bacillus thuringiensis* var. *israelensis* Larvicidal Activity and its Distribution in Benthic Substrates and the Hyporheic Zone of Streams

Abstract

Numerous field trials have indicated that the effectiveness of *Bacillus thuringiensis* var. *israelensis* (*B. t. i.*)is limited by a poor carry in streams. So far the mechanisms responsible for the removal of toxicity from a stream have not been identified. Two streams were treated with *B. t. i.* and experiments were conducted in order to monitor the transfer of *B. t. i.* toxicity from the channel water to different compartments of the stream habitat. These included benthic substrates such as sediments, periphyton covering bedrocks, vegetation (moss and grass) and water samples taken from hyporheic probes (15, 35 and 65 cm deep). To measure the *B. t. i.* toxic activity, all samples were analyzed by bioassays using mosquito neonate larvae. *B. t. i.* was detected in water samples taken from the probes, and the results indicate that shallow underflow could account for a significant fraction of *B. t. i.* loss. Another fraction of the loss can be accounted for by other compartments of the stream habitat, as confirmed by successfull monitoring of toxicity associated to benthic substrates.

Introduction

Shortly after being isolated by Goldberg and Margalit (1977), *Bacillus thuringiensis* var. *israelensis* (*B. t. i.*) was developed as a microbial insecticide and commercialy formulated into primary

powders, wettable powders, aqueous concentrates, emulsions and granular formulations. It is currently applied in rivers, streams and ponds as a larvicidal control agent for blackflies and mosquitoes. A proteinaceous crystal (δ-endotoxin) produced during sporulation of *B. t. i.* is responsible for its activity. A widely accepted model for its mode of action, proposed by Knowles et al. (1989), involves the binding of the toxin(s) to receptors of the epithelial midgut cells of the larvae. The ingestion of the product by the target organisms has been recognized as the first and necessary step of the intoxication process. In order to meet the requirements of larval selective feeding due to cephalic fan ray sizes and spacing (Kurtak 1978), this biocide is made into particulate formulations (Molloy et. al. 1984). Studies conducted on the periphytic fauna (nontarget organisms) demonstrated the high specificity of the *B. t. i.* toxin (Back et al. 1985, Merrit et al. 1989). This product is also biodegraded under natural conditions so it is not accumulated along the trophic chain. These facts make the product a suitable alternative to traditional insecticides when environmental concerns prevent the use of chemicals or to overcome cases of resistance to the latter.

Many field and laboratory trials have showed that *B. t. i.* formulations offer a much shorter carry distance in rivers than that observed when chemical larvicides are being used. The carry of a larvicide is the distance downstream of the application point at which a given efficacy (usually 95% mortality) is maintained. If this distance is reduced, the application points have to be closer (thus more numerous) along a given stretch of river. This, in turn, has a

major drawback on the economics of a control program. So far we know that the carry is reduced under certain field conditions, but to our knowledge nobody has ever looked at the distribution of the *B. t. i.* toxic activity simultaneously in the main-channel water of a stream and in the natural surrounding stream compartments (vegetation, periphyton, sediments, hyporheic zone). Physical, chemical or immunological methods to detect the presence of the proteic crystals in field samples have serious background problems and do not give a true indication of the toxic activity. Furthermore, field mortality counts reflect an observed and final toxicity useful in a very specific situation, the one prevailing at the time of the experiment. Because of the preceeding, the study of the behavior of *B. t. i.* in flowing water has been interpreted mainly from spore, viable or total cell counts (Undeen and Colbo 1980, Frommer et al. 1981a & 1981b, Merritt et al. 1989) or from larval mortality (Lacey & Lacey 1981, Lacey and Undeen 1984). Although it is generally assumed that spores disperse in water in a similar manner as the toxic fraction of *B. t. i.*, Ohana et al. (1987) have demonstrated that spore counts and toxicity were discordant in the presence of mud. Recently Merritt et al. (1989) concluded that "direct spore counts in river water samples may not accurately reflect *B. t. i.* carry or toxicity".

The literature supplies useful information on the behavior of *B. t. i.* in rivers. Results of field trials conducted in Newfounland streams, having flow rates ranging from 200 to 3400 $L \cdot min^{-1}$, showed a positive correlation between downstream carry and stream flow

rates, the carry being longer with higher discharge values (Colbo and Undeen 1980). This hypothesis of positive correlation of carry and discharge as been widely accepted (Gaugler and Finney 1982, Undeen and Lacey 1982, Riley and Fusco 1990). But Lacey and Undeen (1984) in a comparative study of 3 *B. t. i.* formulations (Teknar WDC (Sandoz), Vectobac WP (Abbott) and Bactimos WP (Biochem)) stated that, according to their results, the observed carry was not influenced by different discharges, although they admitted that the difference in discharge values may have been insufficient to produce a significant difference in the carry of the larvicide.

Undeen and Colbo (1980) also suggested that the large substrate-surface-area/volume ratio inherent in small streams could be the main factor of removal of *B. t. i.* by stream flora and fauna. It introduced the notion that flow rates alone could only be used as crude estimators of the carry and that accurate means of evaluating the stream substrate surface area were needed to predict the carry more accuratly. The density of vegetation in a stream is directly related to the total interacting surface and some shared the opinion that vegetation (and other natural substrates) afforded a significant contribution to the removal of toxic activity from the channel water (Fromer et al.1981b, Lacey and Undeen 1984, Undeen et al. 1984).

Following laboratory evaluations of a commercial primary powder formulation, Sinègre et al. (1981) suggested that sedimentation of the active particles could be responsible for reducing the efficacy of *B. t. i.* formulations, but Molloy et al. (1984) indicated that the settling rate of Teknar® in still water was less than 5 $mm \cdot h^{-1}$. To

prevent sedimentation, an oil based formulation of Teknar® was developed, it was believed that keeping as much active moiety near the surface of the water would provide a better carry. The study of Lacey and Heitzman (1985) on the efficacy of seven flowable concentrate formulations failed to indicate an improved performance of this type of formulation compared to aqueous concentrates.

Althought many hypotheses have been put forward to explain the loss of *B. t. i.* toxicity in flowing water, nobody has ever looked for the presence of *B. t. i.* in sediments, periphyton or aquatic vegetation and none estimated their contribution in reducing the carry of this larvicide.

Whatever the mechanism of removal of the *B. t. i.* toxic crystal, it is possible to estimate contributing field parameters. The behavior of the concentration of larvicide along the stream can be modelled by a diffusion-transport equation (Taylor 1953, Khalig 1978). Chalifour et al. (1990) and Chalifour and Delfour (1991) successfully integrated this relation into an optimization model of insecticide treatments with *B. t. i.* in rivers.

Numerous studies have indicated that there is an important exchange of water between the main channel of a stream and a zone immediately below it. This part of the stream is called the hyporheic zone and it contains an interstitial viable habitat (Stanford and Ward 1988). It is characterized as being spatially limited to no more than a few meters, in most cases centimeters, below and away from the river channel. This zone has been studied in the perspective of solute

exchange and transport (Bencala 1983, Triska et al. 1989 and 1990) and with regard to its denitrification potential (Duff and Triska 1990). Our study attempted to assess the presence of *B. t. i.* toxic activity in this compartment of river habitat.

Field trials were conducted in two streams, and we used bioassays to study the behavior of a *B. t. i.* based formulation injected into the streams and to measure the relative role that the different stream compartments play in the removal of the *B. t. i.* toxic crystals.

Study site Fraser

Studies were conducted in June 1990, October 1990 and June 1991 at the outlet of Petit Lac Fraser, in the Réserve Saint-Maurice, in the southern Laurentian region of the province of Québec (46° 56' N), (73° 04' W). The lake empties through a small cascade (1.9 m high) and the stream flows rapidly in small pools and riffles with an average slope of 4% over the studied section that runs from 20 to 215 m from the outlet. The stream bed is quite diversified, consisting of sandy areas to small pebbles and large rocks. An injection station (injection point) was located 20 m from the outlet and the open-channel sampling stations were located downstream from the injection point at 30, 107 and 215 m in 1990 and a hyporheic probe was driven to a depth of 15 cm at 75 m. In June 1991 an open-channel sampling station was located at 75 m together with a hyporheic probe driven to a depth of 20 cm. In October 1990 three probes reaching down to 15, 35 and 65 cm were driven side by side at this same location. Moss (*Platylonella lescurii*) covered more

than 50% of the substrate surface in the first 70 m of the stream but less than 10% of the remaining section. Aquatic vegetation (gramineae family) was present at a few locations along the first 100 m section. Periphyton covered the cobbles and pebbles to a variable extent according to current velocity and the shading afforded by the forest cover. Small pools, where sediments settles, were found in various locations along the last 100 m stretch.

Study site Boitel

In 1991 the studies performed were extended to a second stream. Ruisseau Boitel is the outlet of Lac Boitel (46° 57' N), (73° 02' W), also located in the Réserve Saint-Maurice. This stream flows turbulently for 1.5 km in a steep-sided valley shaded by mixed forest. A uniform gradient precludes the presence of pools. The stream bed is composed of rock material ranging from gravel and sand to large boulders (somewhat larger than the average substrate of Ruisseau du Petit Lac Fraser) and the moss coverage is minimal compared to the former site. The injection point was located about 10 m from a small riffle wich indicated the beginning of the lake outlet. An open-channel sampling station together with a 30 cm deep hyporheic probe was located at 876 m.

Materials and methods

Field trials

Four field experiments were conducted in order to assess a) the dispersion of *B. t. i.* toxicity (measured by bioassays) in open

channel, b) its presence in the hyporheic zone, and c) its association with natural substrates such as periphyton, vegetation and sediments.

1) Starting in June 1990, we measured the carry of *B. t. i.* in the Fraser stream and looked for toxic activity transfer from the channel water onto natural substrates such as: periphyton, sediments, moss and grass, and into the water of the hyporheic zone. Sampling was performed before, during and after a 20 min injection of larvicide into the open-channel water.

2) We ran a second experiment in October 1990. Following a 10 min injection of larvicide into the open-channel water, we sampled the moss in order to investigate the dynamics of *B. t. i.* toxic crystal sorption/desorption process (by the moss) over a 5 h period. Simultaneously, we sampled water from hyporheic probes at various depths (15, 35 and 65 cm) to obtain information on the penetration and displacement of *B. t. i.* beneath the stream bed.

3) In June 1991 we injected *B. t. i.* into the Fraser stream for 20 min and an open-channel sampling station was located beside the hyporheic probe at 75 m.

4) In June 1991 we injected *B. t. i.* into the Boitel stream for 20 min to assess the presence of *B. t. i.* in the hyporheic zone as far as 876 m from the injection point.

Stream discharge measurements

The flow rates were calculated on each day of field experiments. Water velocity was measured using a Pygmy current meter (Kahl

Scientific Instrument Corp., California, U.S.A.). The total cross section of the stream was separated into 30 cm wide sub-sections, the water velocity was measured at mid-depth of each sub-section. The average velocity was used to calculate the partial flow of a given sub-section, they were then summed to give the total stream discharge. The discharges at the time of experiments were 150 $L \cdot sec^{-1}$ in the Fraser stream in June 1990, 175 $L \cdot sec^{-1}$ in October 1990, 225 $L \cdot sec^{-1}$ in June 1991, and 375 $L \cdot sec^{-1}$ in the Boitel stream in June 1991. These figures were used to decide on the appropriate mass of *B. t. i.* and rhodamine needed so that they would be detected at all stations using bioassays and a fluorometer. In addition, the discharge figures were necessary to assess the total mass of larvicide passing at a given station in order to calculate the loss in mass unit. The mass of *B. t. i.* passing throught a given section of the stream is the product of the dose times the discharge of the stream, where the dose is the product of the *B. t. i.* concentration by the total time of passage (mg/L x min or $mg \cdot min \cdot L^{-1}$).

Hyporheic probes

The hyporheic probes were made of a 4 cm (ID) steel pipe mounted with a solid cone shaped tip welded to the bottom, and perforated with 35 small holes in its lower 8 cm. At least 24 hours before the experiments, they were driven to the desired depth (from the center of the perforations), and flushed (using a bilge pump) until samples of clear water could be retrieved with a 50 ml syringe mounted with the appropriate length of 3 mm (ID) Tygon tubing.The length of the

sampling apparatus was adjusted to reach down to the level of the center of the perforations.

Injection procedure

Injections of rhodamine WT as well as *B. t. i.* were performed using a 20 L Mariotte bottle. This technique allowed us to make the addition of rhodamine WT or *B. t. i.* at a steady rate throughout the dripping period. We adjusted the bottle outlet so the desired mass of product would drip at a rate of 1 liter per minute ($L \cdot min^{-1}$). The injection was done in the main channel of the stream far enough upstream of the first sampling station to ensure complete vertical mixing of the product before it reached it. All sampling schedules were synchronized with the beginning of the injection period.

Rhodamine WT tracings

In order to maximize the information that we can get out of a set of *B. t. i.* experiments, a preliminary tracing with the fluorescent dye rhodamine WT (Crompton &Knowles, Pennsylvania, U.S.A., the stock solution is 200 $g \cdot L^{-1}$) was performed to estimate the time of passage and the diffusion pattern of the larvicide in the stream and in the hyporheic zone. We used 325 ml (June 1990) and 75 ml (June 1991) of rhodamine WT in the Fraser stream, and 125 ml in the Boitel stream, June 1991. These volumes were adequate to visually locate the position (time wise) of the rhodamine slug at all stations and to produce a fluorescent signal in samples taken from the hyporheic probes and from the open-channel water. After the tracing, the samples were immediatly analysed for rhodamine

content using a Turner fluorometer (model 110). The results were used to decide on the final sampling schedule to be followed during the *B. t. i.* treatment. The rhodamine and the *B. t. i.* treatments were performed on successive days making discharge measurements each morning to ensure that no significant changes had occured during the night.

Larvicide (*B. t. i.*) tracings

The tracings were performed by injecting a mass of *B. t. i.* (Teknar HPD®, Zoëcon lot # 0080227, 3000 *Aedes aegypti* units per mg, used throughout this study) sufficient to produce 100% mortality of the black fly larvae over the entire studied segment. It consisted of 2.84 and 2.50 L in the Fraser stream in June 1990 and 1991 respectively, and 4.70 L in the Boitel stream (June 1991). Artificial substrates made of undulated plastic tiles were installed at different locations 2-3 days before treatment to permit larval attachment and after treatment, to assess the mortality in the stream.

Sampling procedure

Rhodamine samples were collected by hand in the open channel by plunging a 30 ml glass bottle into the water at the desired time. They were drawned from the hyporheic probes using a 50 ml syringe by pulling the plunger gradually over a 6 to 10 seconds time period, and placed in glass bottles. Water samples containing *B. t. i.* were obtained in a similar manner but they consisted of 125 ml volumes collected in plastic disposable containers.

Substrates were collected in different fashions according to their nature. The top layer of sediments was collected by suction through a 7 mm (ID) Tygon tubing. Periphyton samples were obtained by shaking for a minute a given volume of pebbles (500 ml) with 200 ml of water in a 1.5 L plastic container. This method was prefered over hand-brushing of individual stones because it allowed a random sampling of substrates that included stones we would not necessarily be able to hold and brush. Vegetation (moss and grass) was sampled by hand and the material placed in 18 oz sterile sampling bags (Whirl-Pak). All samples were collected 60 to 90 minutes after the passage of the *B. t. i.* slug. Before injection, stream water and samples of all types were collected to serve as controls. In addition a sample of the *B. t. i.* solution from the Mariotte bottle was taken back to the laboratory and diluted before freezing. Dilutions of known concentrations were used to make a standard bioassay curve of *B. t. i.* with neonate larvae.

Preservation and treatment of samples

B. t. i. samples were kept in coolers before being placed at -25°C (samples of June 1990), or kept in a refrigerator at 4°C (samples of October 1990 and June 1991). The frozen samples were thawed out at 4°C the day before bioassaying. The necessary volume of liquid samples (15 to 20 ml) were transferred to test tubes and the samples were returned to the refrigerator or to the freezer after the assays were completed. All the tubed samples were allowed to stand at room temperature for 2 hours before bioassays, to prevent a cold

shock to the test organisms. Dilutions were made to generate mortalities between 10 and 90%.

Samples containing sediments and periphyton were exposed to the larvae undiluted or diluted as needed. By comparing the mortalities to a standard curve we obtained the *B.t.i.* concentration (in $\mu g \cdot L^{-1}$ of formulation) of the samples, By filtration (0.22 μm) and drying at 100 °C for 24 hours we measured the mass of solids by volume units of samples, this allowed us to transform these mortality figures in mass units of *B.t.i.* per mass unit of substrates. Samples of 500 mg of the strained vegetal substrates (moss or grass) were crushed in 20 ml of stream water using a 30 ml tissue grinder (Potter-Elvehjem). The resulting preparation was exposed to the larvae after appropriate dilution.

Bioassays

In order to make sure that we would really be following the toxin on different substrates and in the hyporheic zone, we used bioassays. In the present study, bioassays were performed under laboratory conditions using either *Aedes triseriatus* (Say) or *Aedes atropalpus* (Coquillett) neonate larvae. This choice was prompted by the fact that freshly hatched organisms offer a greater physiological synchronicity and a greater sensitivity. The method used was based on the one described by Ibarra and Federici (1987) in which a single neonate larva is placed in an individual well of a microtiter plate (96 holes) and then exposed to dilutions of the samples to be tested. In order to minimize experimental variations, all samples were assayed

in triplicates of 32 larvae, performed on 3 successive days using eggs of the same degree of maturity. The mortality counts were made after 24 hours of contact. The mortality obtained with field samples was then compared with a standard curve (made with *B.t.i.* in stream water) in order to determine the concentration of *B.t.i.* toxic activity (expressed in µg of formulation per liter) present in the unknown samples.

To illustrate the increased sensitivity of neonates, it is worth mentioning that for a series of *B.t.i.* suspensions prepared in distilled water, the median lethal concentration (LC_{50}) is 10 to 12 times smaller than with early 4^{TH} instar larvae. This technique not only allowed us to determine small amounts of *B.t.i.* in various samples, but also permitted us to process up to 8 samples per day including a standard curve.

Statistical analysis

Each set of bioassays was statistically analyzed using probit analysis (Finney 1971). The percent 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 $\mu g \cdot L^{-1}$). Natural mortalities are taken into account using Abbott's formula. From the regression line, we were able to convert the mortality rate obtained for a given sample into concentration of *B.t.i.* formulation.

Results

Figure 1 (1a to 1f) shows the results obtained at the 3 open-channel stations after the rhodamine WT and the Teknar® HPD injections.on the Fraser stream in June 1990. Figure 1a represents the results of the rhodamine WT tracing and Figure 1b, the results of the *B. t. i.* tracing at the first station, located 30 m downstream from the injection point. The rhodamine and the *B. t. i.* curves (1a and 1b) are located around the same position on the time scale, this is also true for the pairs of curves (Fig 1c-1d and 1e-1f) representing the rhodamine WT and *B. t. i.* tracing at the second (107 m) and third (195 m) stations. At 107 m (1c and 1d), we can see a reduction in concentration values of both curves and a spreading out or trailing on the time scale. This spreading and trailing is even more prononced at the last station (195 m, Fig 1f) where the *B. t. i.* concentrations reach a maximum of 5 $mg \cdot L^{-1}$ compared to 16 and 10 $mg \cdot L^{-1}$ at the first and second stations, respectively. The maximum concentrations of rhodamine WT are 375, 325 and 275 $\mu g \cdot L^{-1}$ at the 30, 107 and 195 m stations, respectively.

From these figures, we can calculate the dose (in $mg \cdot min \cdot L^{-1}$) for each open-channel water station by estimating (numerically) the area under the concentration curve. The product of the dose by the measured discharge (in $L \cdot min^{-1}$) at the time of the treatment gives the quantity of product in mass unit (mg or kg) that passed at each station (Table 1). Knowing that we injected a total of 2.84 kg of *B. t. i.*, the percentage of loss of larvicide at the different stations can

be calculated and are presented in the far right column. We can see that 30 meters downstream from the injection point (station 1) 15% of the larvicide failed to be detected in the open-channel water. At the 107 m and the 195 m stations the loss was 46% and 67%, respectively. According to a one-dimensional diffusion-convection model, the decrease of the dose over the 3 stations respects a negative exponential curve (Fig 2a). After a natural logarithmic transformation of the dose (Fig 2b) and using a linear regression procedure we obtained a removal length (x_r) of 174 meters. This coefficient (x_r) corresponds to the rate of decrease of the dose in the exponential model and it is a function of the removal coefficient and the mean velocity of the one-dimensionnal advection-diffusion equation (Khalig, 1978). The individual contributions of the different compartments considered in the present paper (moss, grass, hyporheic zone, periphyton and sediments) are contributing to this removal coefficient.

The importance of the hyporheic zone in the removal of *B. t. i.* was never studied before. Figure 3a and 3b shows the results of rhodamine WT and *B. t. i.* sampling in a 15 cm deep hyporheic probe located 75 m from the injection point (Fraser, June 1990). Both were found underneath the stream bed. Compared to the curves of the open-channel stations (Fig 1a to 1d) we observed that the maximum concentrations are lower, the curves are delayed and trailed longer. Compared to the *B. t. i.* concentrations at the first 2 open-channel stations (16 and 10 $mg \cdot L^{-1}$), the maximum in this hyporheic probe was 1.7 $mg \cdot L^{-1}$. Since *B. t. i.* was found moving

under the stream bed for the first time, this experiment was repeated in June 1991 on the same stream. A probe reaching a depth of 20 cm and an open-channel sampling station were located side by side at 75 m downstream from the injection point. It allowed us to superpose both, open-channel and hyporheic curves in Fig 3c and 3d. Again we found the same type of curve in the open-channel water and the *B. t. i.* concentrations in the hyporheic compartment reached about 20% of the maximum value in the open-channel. In addition, we can see that *B. t. i.* curve is delayed and trailed behind after the passage of the slug in the open-channel (Fig 3d). In June 1991, we ran a similar experiment in the larger Boitel stream with an open-channel and a hyporheic sampling station (30 cm deep) located 876 m from the injection point (Fig 3e and 3f). The maximum concentration of *B. t. i.* in the hyporheic probe is reduced and its arrival is delayed as compared to the open-channel station. Although the samples were drawned from a greater depth, the maximum *B. t. i.* concentration in the hyporheic samples reached 75% of the maximum value of the open channel compared to 20% of the maximum open channel *B. t. i.* concentration when samples were drawned from 20 cm deep, on the Fraser stream (Fig 3d).

To calculate the corresponding doses of *B. t. i.* that passed at a sampling depth in the hyporheic zone in order to assess the mass of *B. t. i.* moving under the stream, we would need to know the corresponding discharge of this hyporheic compartment. But to get that estimation is very tedious and offers a limited confidance in the results. It depends on the volume of the hyporheic zone which can

easily exceed the volume of the open channel. In their study, Stanford and Ward (1988) estimated the volume of the hyporheic habitat on a studied section of the Flathead River (USA), to be 3 x 10^8 m^3 compared to 1.22 x 10^5 m^3 for the stream channel. Triska et al. (1989) estimated that the interstitial water volume of a studied section of Little Lost Man Creek (USA) was at least equal to, or probably greater than the surface water volume. In addition, the heterogeneity in porosity and permeability of the stream bed material is a source of variation in water retention and conductance, that make the measurement of the hyporheic discharge an extremely difficult task and thus irrelevant to the scope of this study.

From the results of the 1990 trial, we calculated the dose going through the 15 cm probe and then calculated hypothetical masses of *B. t. i.* that would have passed throught the hyporheic probe according to different discharge values (Table 2). During this tracing experiment the discharge in the open channel was 150 $L \cdot s^{-1}$, if we assume a 300 $L \cdot s^{-1}$ discharge in the hyporheic zone this would produce a 0.94 kg loss of *B. t. i.* which is greater than the total loss measured between the first and second stations (0.86 kg). But at the stream discharge value, the hyporheic compartment would account for 55% of the total loss found between the first two stations (Table 2).

In October 1990, we performed an additional experiment with hyporheic probes driven to 15, 35 and 65 cm depths in the Fraser stream to analyse the *B. t. i.* penetration in the stream bed. Results in Table 3 show that following the beginning of the injection, it took

less than 5 min for the larvicide to reach down to 15 cm under the stream bed, less than 10 min to reach 35 cm and was detectable in the sample collected at 60 min from the 65 cm probe. The maximum hyporheic *B. t. i.* concentration was 2.98 $mg \cdot L^{-1}$ at 15 cm compared to 25 $mg \cdot L^{-1}$ the open channel. Samples from the deeper probes reached 0.40 and 0.36 $mg \cdot L^{-1}$ suggesting that the water exchanges between the main channel and the hyporheic zone were significant in the first 15 cm but decreased sharply between 20 and 35 cm at this particular location (considering the 20 cm probe in June 1991).

The mortalities observed for *B. t. i.* toxicity associated with benthic substrates are presented in Table 4. Among the vegetation collected, *B. t. i.* toxic crystals were found in much greater quantity on the moss than on the grass, the mortality rates being as high as 100% and as low as 7%, respectively. In fact, the mortalities obtained with the grass collected after the stream had been treated with *B. t. i.* are similar to the controls (Table 4), indicating that this type of vegetation is not retaining much of the toxic crystals.

Because of the importance of the moss coverage in the first section of the Fraser stream (more than 50%) and thus, the large interacting surface it represented, we monitored (October 1990) the dynamic association/dissociation of the *B. t. i.* toxicity with this substrate. The results of this test are presented in Fig. 4. We can see that following an injection of *B. t. i.* at a rate of 25 $mg \cdot L^{-1}$ for 10 minutes in the open channel, the moss sampled in the stream at 50 m became more and more toxic reaching a maximum 45 minutes after the beginning of the injection. The *B. t. i.* concentration values presented for the

moss samples are the results of bioassays after crushing 500 mg of strained moss in 20 ml of water. At the maximum concentration value (45 min) bioassays results indicated that the 20 ml suspension contained 0.12 mg of *B. t. i.* ; considering that 0.12 mg of *B. t. i.* was associated with 500 mg of moss, then as much as 240 mg of *B. t. i.* was present for each kg of strained moss.

After that peak, the toxic activity decreases slowly to half of that maximum value four hours later. This indicates a relatively strong but reversible association of the larvicide with this natural substrate. In order to account for the entire loss of the 0.84 kg of *B. t. i.* that occured between the first and second open-channel stations, a density of 25 $kg \cdot m^{-2}$ of moss would have been necessary over that section of the stream. We estimated the density of the moss coverage over this section to be no more than 2 to 3 $kg \cdot m^{-2}$. At such density this substrate alone would account for 10% of the loss.

The asssays performed on periphyton samples gave mortalities as high as 82% at 30 m and 30% at 107 m (Fraser, June 1990), indicating that this substrate is also interacting with *B. t. i.* (Table 4). If we make the hypothesis that the maximum rate of *B. t. i.* association with this substrate is the same than the one calculated for the moss (240 $mg \cdot kg^{-1}$), and considering an average periphyton density of 0.2 $kg \cdot m^{-2}$ (dry mass) over the stream bed surface area between the first and second station (77 m by an average width of 1.82 m), we come-up with a total of 6.7 g of *B. t. i.* formulation that would have been removed by this substrate. Even with a periphyton

density as high as 1 $kg \cdot m^{-2}$, this benthic substrate alone would account for only 4% of the *B. t. i.* removal.

The mortalities from the moss and periphyton samples taken from both streams in 1991 are significant but somewhat lower than the year before. This is consistant with the fact that the doses injected in 1991 were lower than the one in 1990. There was little toxicity associated with the sediments (Table 4). Due to the profile of the stream, the pools in which we sampled sediments were located at 142, 187 and 202 m downstream of the injection point. These distances do not seem to be the sole factor of the low mortality rates because the moss sampled at 205 m on the same occasion gave 83% mortality.

Discussion

For the first time, direct measurement of *B.t.i.* concentrations (in $mg \cdot L^{-1}$ of a formulation) using bioassays was performed to monitor the carry and the loss of this larvicide in a stream. The use of the neonate bioasssay procedure guaranteed that we were really looking at the dispersion of the toxic fraction of the larvicide and not a component (spore or viable count) of the formulation.

Because we intended to elucidate where the *B.t.i.* toxic activity was migrating to, the outlet of Petit Lac Fraser, a stream with a short removal length (x_r) of 174 m, fulfilled our needs. This length value represents the distance at which the dose will be 37% of the initial dose. It is an instant picture of a treated stream and can vary according to stream profile and parameters such has discharge rate, density of natural substrates and the extent of the hyporheic zone. This removal length is also indicative of the field carry potential of a given formulation. In the prevailing conditions on the Fraser in June 1990, the loss of *B.t.i.* toxic crystal was distributed over a relatively restricted substrate-area, allowing it to be detected. After analysing the open-channel water sampled at the three stations, we calculated that, along the first 174 m stretch downstream of the injection point, 1.79 kg of *B.t.i.* formulation (63% of the injected mass) had been transfered from the open-channel water to other compartments of the stream habitat.

Significant fractions of the missing *B.t.i.* were recovered in some compartments of the stream habitat. This is the first time that

hyporheic probes were used to study *B.t.i.* and we found an important portion of the dose under the stream bed, thus incapable of killing black fly larvae. We estimated that, sediments and periphyton would each account for about 1% of the loss, moss (*Platylonella lescurii*) could take up to 10% of the lost larvicide and the hyporheic zone accounted for 55%, or more, of the total loss. The remaining portion of the loss (≈30%) could be attributed to underestimation of some contributions or that some were overlooked. For example, although the measured surface of the section was 140 m^2, the true bed surface in contact with the *B. t. i.* toxic crystals was actually much higher. Even if we could measure this parameter it still would account for a small percentage of the total removal.

It is difficult to evaluate the true contribution of the sediments in the *B. t. i.* removal because this substrate could be interacting with the larvicide not only at its interface with the channel water but at different levels as the water flows through the channel before it reaches the hyporheic zone. At the time of the experiment we were suspecting only a surface interaction, that is why we did not sample deeper into the sediments. Even if it has a small *B. t. i.* retention capacity, compared to moss and periphyton, the density of sediments in the stream (considering not only the surface but the entire volume of it) could make this substrate play a role at least equivalent to the periphyton in the removal of *B. t. i.* activity.

In agreement with Frommer et al. (1981a, 1981b) who found that the presence of vegetation did not change mortality in a *B. t. i.*

treated stream, the smooth ribbon-like grass leaves did not retain much of the *B.t.i.* toxicity while the large surface moss did, suggesting that the surface area of a given substrate is influencing its retaining capability. We do not know yet if the *B.t.i.* is retained to substrates by a mechanical or chemical mechanism but a test using different *B.t.i.* formulations would help to elucidate this matter. Although a few authors have hypothesized that benthic substrates could play a role in the removal of *B. t. i.* to explain its poor carry, this is the first time that actual *B. t. i.* toxicity has been found to be associated with some substrates, thus making it unavailable for ingestion by filtering black fly larvae. Our results also confirmed the data obtained by Back et al. (1985) who found that streams treated at high dosage with *B. t. i.* induced a large mortality of *Blephariciдae*, a periphyton grazing Diptera.

The hyporheic discharge estimation alone could make up the difference, the 55% contribution of this zone is estimated by taking an equal discharge value as the one in the open channel (150 $L \cdot s^{-1}$), but at 200 $L \cdot s^{-1}$ (Table 2) this compartment would account for 74% of the loss. The other compartments could hardly make-up for the difference, even if their importance were greater than estimated.

Some of the larvicide is removed by the filtering action of the black fly larvae present in the stream. A study performed by Morin et al. (1988b) on the same stream indicated that the ingestion rate of the black fly larvae was around 10^{-3} $mm^3 \cdot min^{-1}$. Taking into account that the passage of the *B. t. i.* slug took no more than 30 min, a total of 2.8×10^7 larvae feeding continuously during that period would

have been necessary to remove the entire 0.84 kg of *B. t. i.* that was lost over this section. We estimated that the larval density at the time of the experiment was less than 1 larva per cm^2, compared to a density of 20 larvae·cm^{-2} that would be needed to account for the entire removal of the larvicide. According to these figures, the entire mass of material ingested by the larvae during the experiment would account for 5% of the total loss. According to Ross and Wallace (1983) and Morin et al. (1988a), ingestion of total seston by filter-feeders would only account for less than a few percent of the *B. t. i.* injected.

The river bed material (sand and debris) is likely to act as a filter for the water flowing through it. The trials performed with hyporheic probes at different depths showed that an heterogenous permeability prevails in the riverbed material. It appears that the permeability varied with the location of the probes and is a reflection of the morphological differences between the two streams. At a depth of 20 cm in the Fraser stream (June 1991), the maximum concentration reached 20% of the open-channel concentration (Fig. 3d) compared to 75% at a depth of 30 cm in the Boitel stream in June 1991 (Fig. 3f), and the trial of fall 1991 revealed that the speed of diffusion varied with depth, *B.t.i.* had reached the 15 cm probe after 5 minutes, the 35 cm probe after 10 minutes while it took an hour to get to a depth of 65 cm (Table 3). If indeed the river bed material acts as a filter, we expect the larger particules to be retained in the first layer and that only finer size particules are allowed to make it as far as 65 cm. The *B.t.i.* trapped in this filter-like material would

be prevented from coming back to the channel water thus accounting for the exponential loss of larvicide that we observed. It could also explain why only low concentrations of *B.t.i.* made it to the 35 cm probe. We believe that the rushes of water in small turbulent streams could flush away filter forming material (vegetation, debris, sand, etc.), thus keeping a certain portion of the hyporheic zone "open" to the main channel. Larger and slower flowing rivers are likely to allow the formation of a less permeable frontier between the open channel and the hyporheic zone, thus reducing the water exchange and keeping more of the larvicide available to the target organisms (blackfly larvae) thus contributing to a longer carry.

In conclusion

The neonate larval technique was successfully used to monitor dispersion curves of *B.t.i.* in streams and to measure directly the loss of toxicity. It was also a very useful method to estimate the *B.t.i.* toxic activity in different compartments of the stream. Spore counts, have been considered as a standard technique to monitor *B.t.i.* toxicity but now that the preparations tend to be mainly sporeless, this sensitive, rapid and field adaptable method should be used as a standard procedure in field work involving *B.t.i.*

The contribution of the hyporheic zone to the *B.t.i.* dispersion and loss in rivers had not been previously considered. The results obtained indicate that this compartment plays a major role in *B. t. i.* removal compared to natural substrates.

B. t. i. formulations have not really improved over the years, as far as downstream carry is concerned. Compared with chemical larvicides which, being solubles, have a much better rate of exchange between the hyporheic zone and the open channel water, *B. t. i.*, a particulate larvicide, is most likely being trapped in this hyporheic zone.

Whether or not *B. t. i.* formulations can be improved to reduce their interactions with benthic substrates and to have a better exchange between the two zones remains to be seen but our results indicate that these factors should now be taken into consideration.

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Undeen, A. H., and L. A. Lacey. 1982. Field procedures for the evaluation of *Bacillus thuringiensis* var. *israelensis* (serotype 14) against black flies (*Simuliidae*) and nontarget organisms in streams. Misc. Publ. Entomol. Soc. Am. 12: 25-30.

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Table 1. Calculated doses of rhodamine WT and *B. t. i.* that passed at the open-channel stations. The corresponding masses of *B. t. i.*, obtained from the product of the dose times the stream discharge (150 $L \cdot s^{-1}$), are presented in fourth column. The percent loss of larvicide at the three distances, downstream from the injection point are showed in the fifth column.

Station distance (m)	Rhodamine dose ($mg \cdot min \cdot L^{-1}$)	*B. t. i.* dose ($mg \cdot min \cdot L^{-1}$)	*B. t. i.* mass (kg)	*B. t. i* loss (%)
0	7.22	315.6	2.84	0
30	7.00	267.0	2.40	15
107	6.67	171.5	1.54	46
195	6.26	103.2	0.93	67

Table 2. Calculated masses of *B. t. i.* that would have passed by the 15 cm deep hyporheic probe located at 75 m (Fraser, June 1990), according to different underflow discharge values. An actual dose of 53.05 mg·min·L^{-1} was calculated for this probe. The loss of *B. t. i.* between the 30 and 107 m (1^{st} and 2^{nd} stations) being 0.86 kg , the losses in percent of this value corresponding to different discharge figures are presented.

Discharge ($L \cdot s^{-1}$)	Mass (kg)	Loss (%)
200	0.64	74
150(1)	0.47	55
75	0.24	28
35	0.11	13

(1) discharge at the time of experiment

Table3. Concentration of larvicide in samples taken from hyporheic probes driven at three different depths in order to assess the penetration and displacement of toxicity underneath the stream bed. It followed a 10 min injection of *B.t.i.* in the open-channel water. The probes were located at 75 m in the Fraser stream, October 1990.

Depth (cm)	15	35	65
Times (min)	Concentrations in $mg \cdot L^{-1}$		
0	0.00	0.00	0.00
5	2.49	0.00	*
10	*	0.18	*
15	2.98	0.30	0.00
20	*	0.30	*
25	0.98	*	*
30	0.44	*	0.00
40	*	0.40	*
45	0.40	*	0.00
60	0.48	0.36	0.36
80	*	0.31	0.30
90	0.44	*	*
100	*	0.17	0.22
120	0.02	*	0.02
300	0.02	*	0.02

* no sample was collected at that time

Table 4. Percentage of mortality from bioassays performed on benthic substrates in order to detect the presence of *B.t.i.* toxic cystals 60 to 90 minutes after the passage of a *B.t.i.* slug.

Elements	Location	Date	Control substrates	Treated substrates
Moss	Fraser 30m	June 1990	1	98
	Fraser 107m	June 1990	1	100
	Fraser 205m	June 1990	1	83
	Fraser 50m	June 1991	0	33
	Boitel 20m	June 1991	1	64
Grass	Fraser 75m	June 1990	3	1
	Fraser 205m	June 1990	3	7
Periphyton	Fraser 30m	June 1990	0	82
	Fraser 107m	June 1990	0	30
	Fraser 30m	June 1991	0	4
	Boitel 67m	June 1991	1	68
Sediments[1]	Fraser 142m	June 1990	2	10
	Fraser 187m	June 1990	2	3
	Fraser 202m	June 1990	2	7

[1] Because of the stream profile, we could not collect sediments at stations above 142 m.

Fig. 1. Results of the rhodamine WT (left) and the *B. t. i.* (right) tracing at 3 stations located at 30, 107 and 195 meters from the injection point. Each experimental point is the result of sample analysis using a fluorometer for rhodamine and neonate bioassays for *B. t. i.* . They are presented in $\mu g \cdot L^{-1}$ and in $mg \cdot L^{-1}$, respectively.

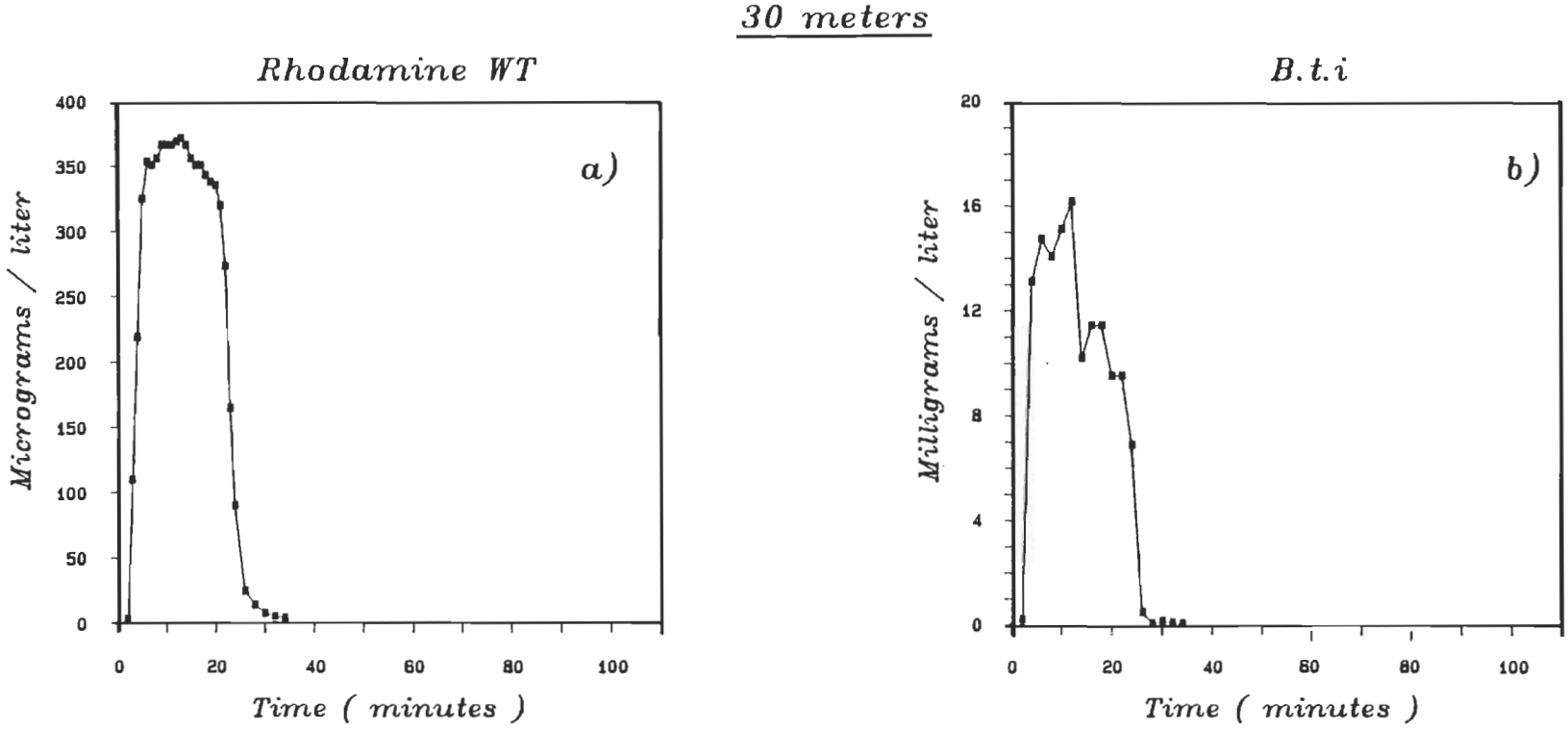

30 meters
Rhodamine WT
a)
Micrograms / liter
400
350
300
250
200
150
100
50
0
0
20
40
60
80
100
Time (minutes)
B.t.i
b)
Milligrams / liter
20
16
12
8
4
0
0
20
40
60
80
100
Time (minutes)

Figure 1

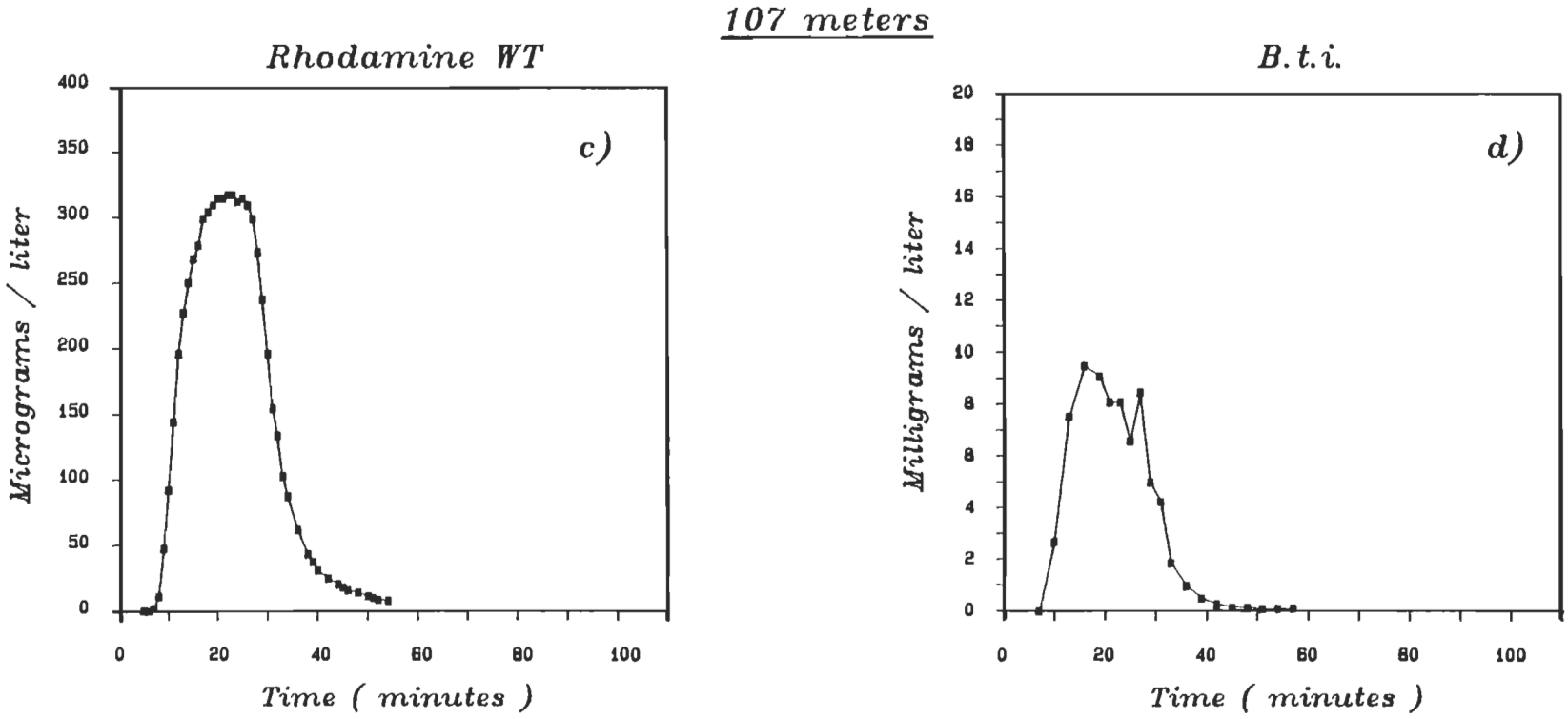

107 meters
Rhodamine WT
c)
Micrograms / liter
Time (minutes)
B.t.i.
d)
Milligrams / liter
Time (minutes)

Figure 1

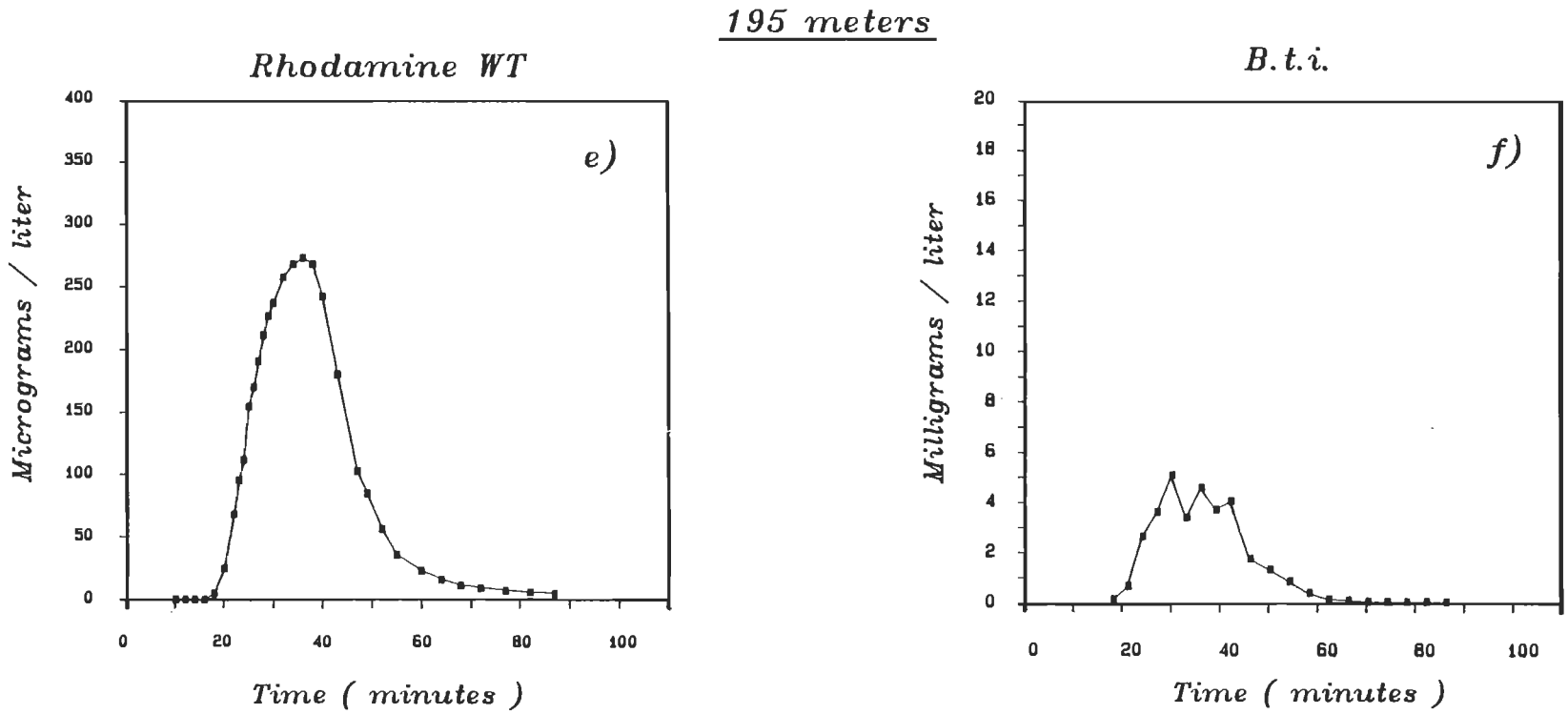
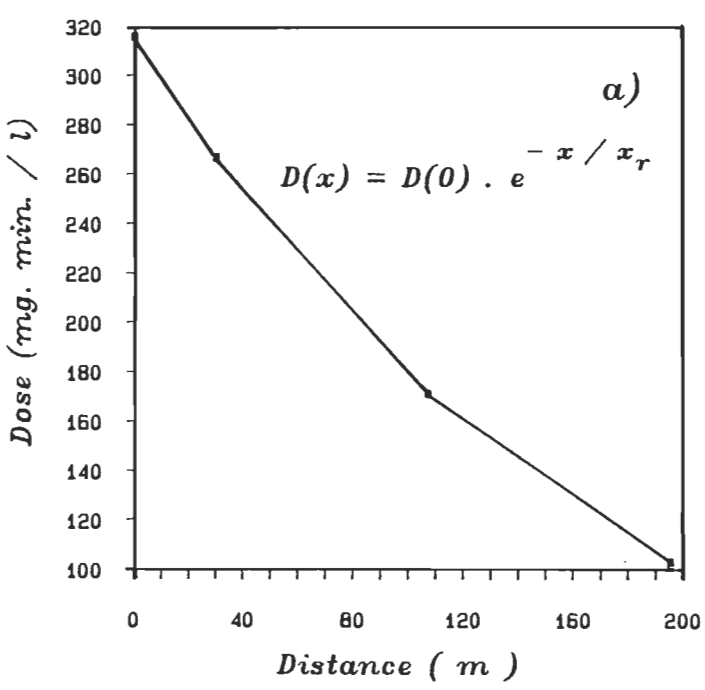

195 meters
Rhodamine WT
e)
Micrograms / liter
400
350
300
250
200
150
100
50
0
0
20
40
60
80
100
Time (minutes)
B.t.i.
f)
Milligrams / liter
20
18
16
14
12
10
8
6
4
2
0
0
20
40
60
80
100
Time (minutes)

Figure 1

Fig. 2. The left side (a) presents the doses mesured at the injection point and at the three open-channel stations, plotted against their respective sampling position (in meters). The natural log of these doses against the distances and the removal length (x_r) calculated from the regression line are presented in (b).


a)
$D(x) = D(0) \cdot e^{-x/x_r}$
Dose (mg. min. / l)
320
300
280
260
240
220
200
180
160
140
120
100
0
40
80
120
160
200
Distance (m)

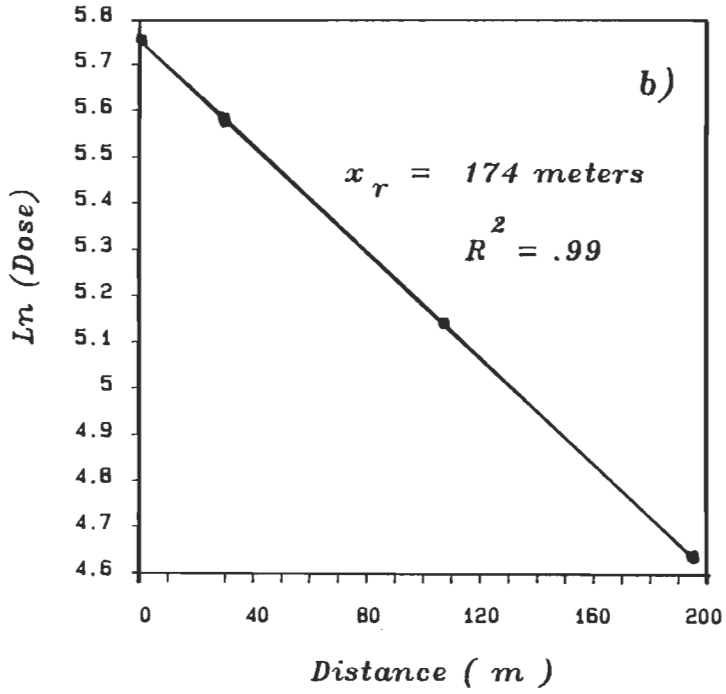

b)
x_r = 174 meters
R^2 = .99
Ln (Dose)
5.8
5.7
5.6
5.5
5.4
5.3
5.2
5.1
5
4.9
4.8
4.7
4.6
0
40
80
120
160
200
Distance (m)

Figure 2

Fig.3. Rhodamine WT (a) and *B. t. i.* (b) concentrations in samples drawn from a 15 cm deep hyporheic probe located at 75 m in the Fraser stream, June 1990. At the same location in June 1991, rhodamine WT (c) and *B. t. i.* (d) concentration, were drawn from a 20 cm deep probe and in the open-channel water above. In (e) we can see the passage of the rhodamine WT simultaneously in the open-channel and in the hyporheic zone at a station located 876 meters from the injection point in the Boitel stream, June 1991. In (f) we can see the results of the *B. t. i.* tracing at this location.

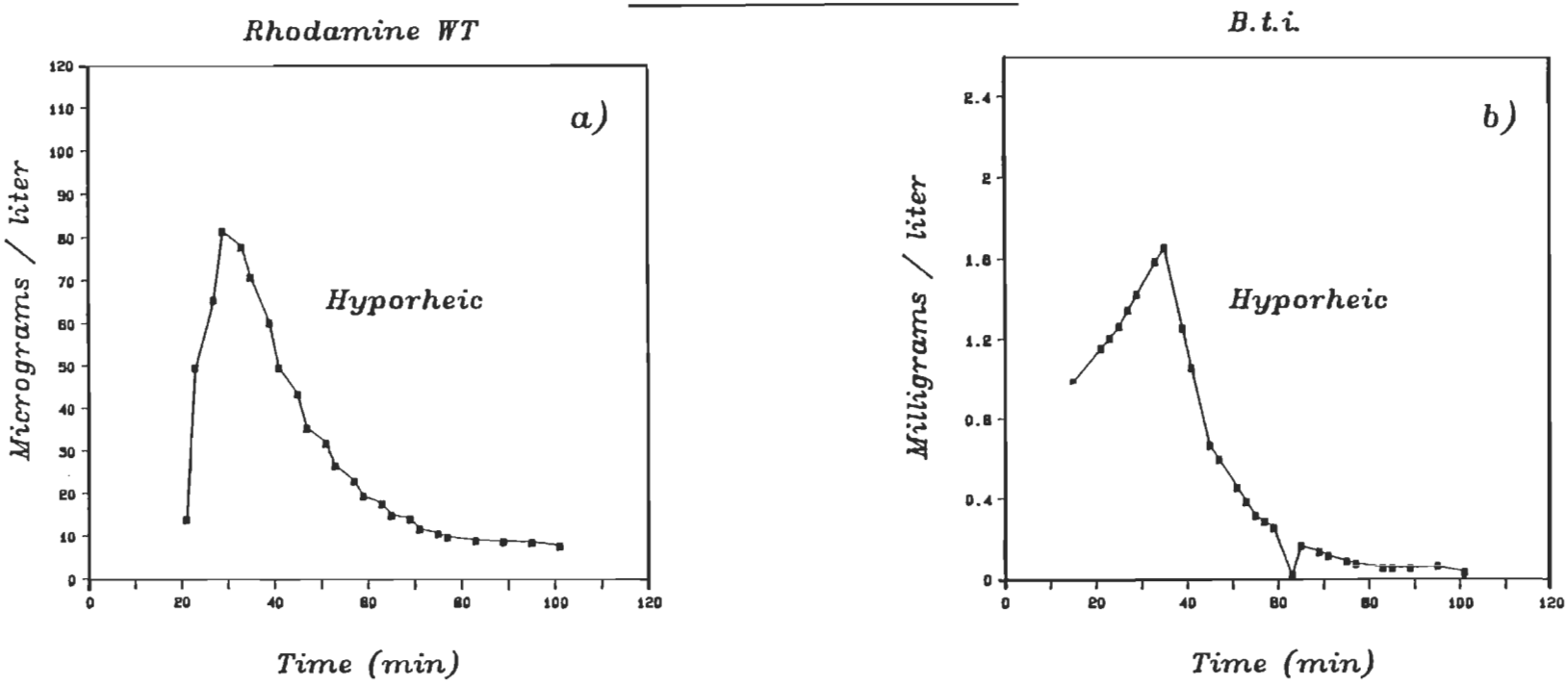

Fraser 75 m (1990)
Rhodamine WT
a)
Micrograms / liter
Hyporheic
Time (min)
B.t.i.
b)
Milligrams / liter
Hyporheic
Time (min)

Figure 3

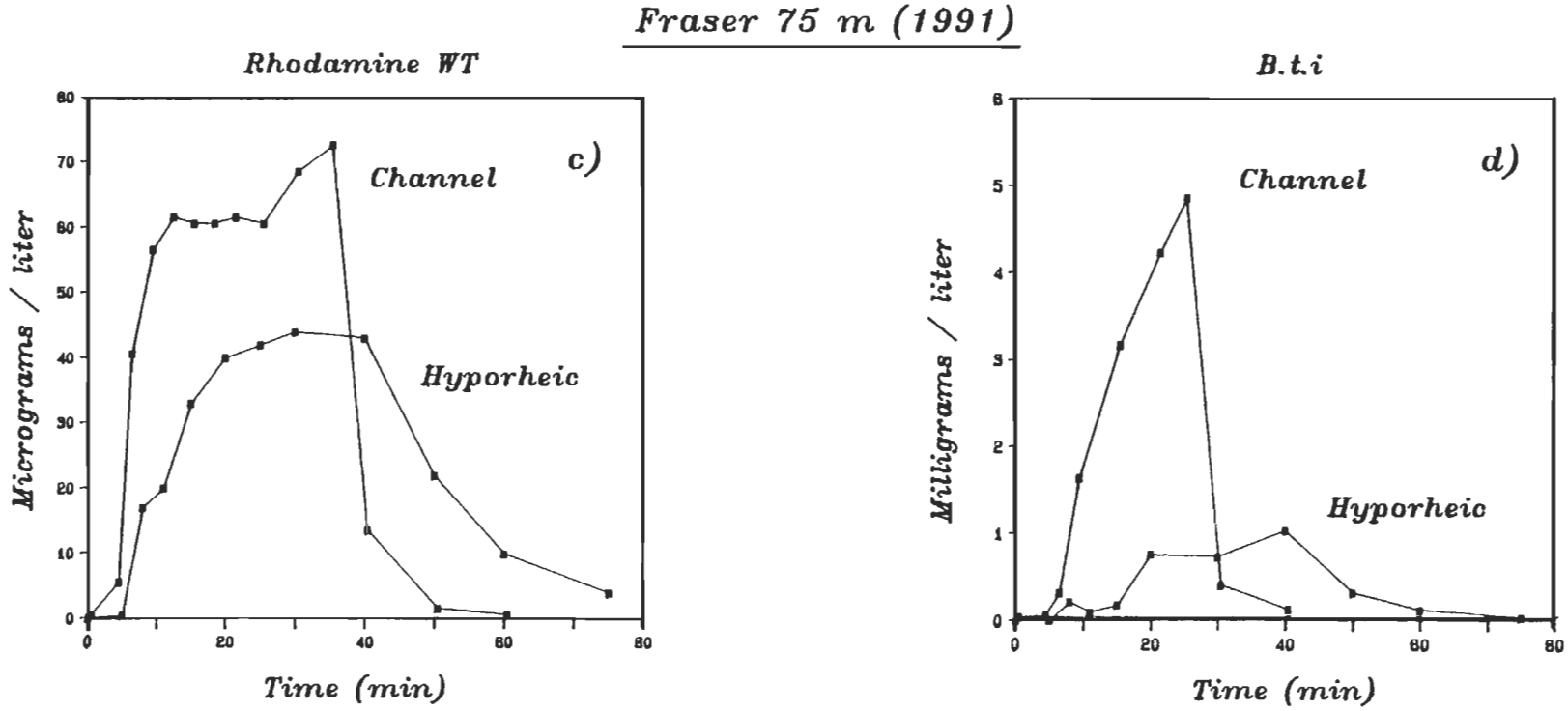

Fraser 75 m (1991)
Rhodamine WT
c)
Channel
Hyporheic
Micrograms / liter
Time (min)
B.t.i
d)
Channel
Hyporheic
Milligrams / liter
Time (min)

Figure 3

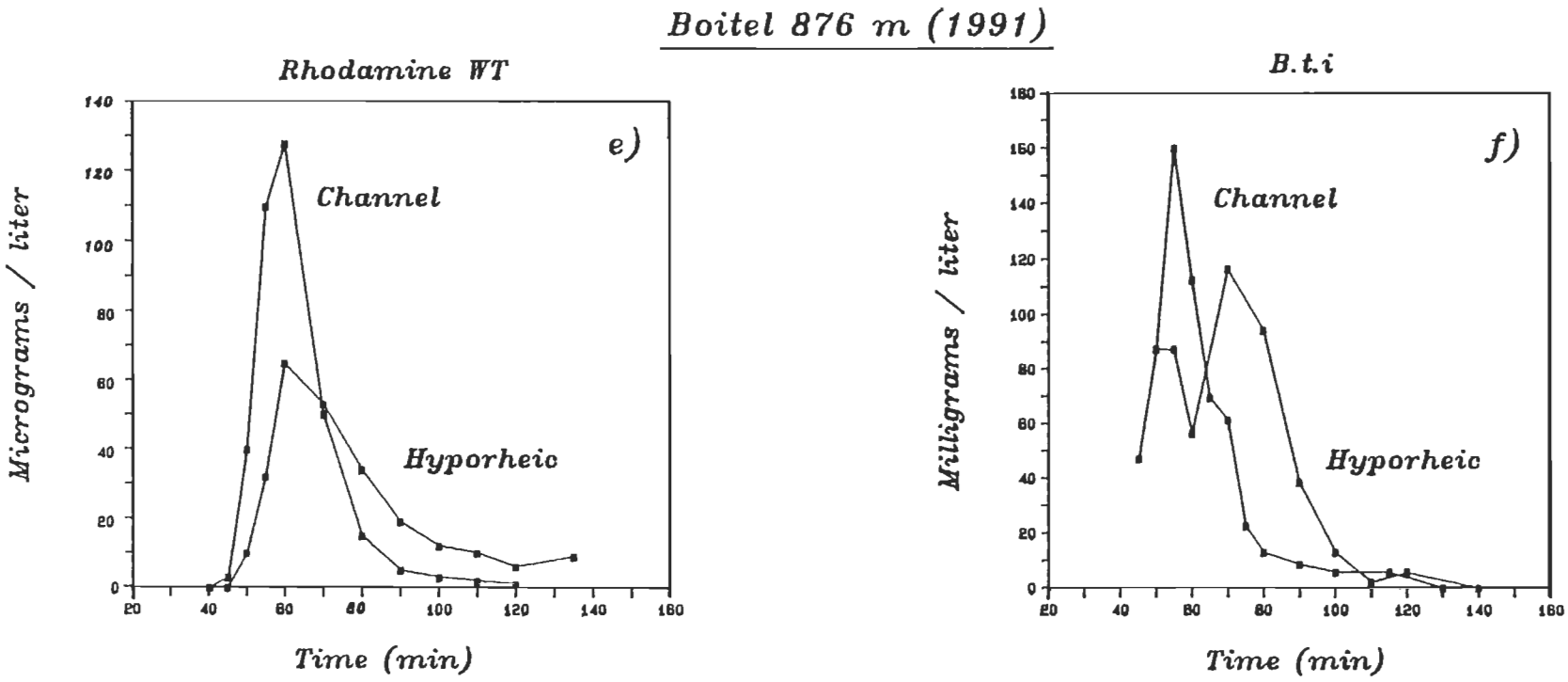

Boitel 876 m (1991)
Rhodamine WT
e)
Channel
Hyporheic
Micrograms / liter
Time (min)
B.t.i
f)
Channel
Hyporheic
Milligrams / liter
Time (min)

Figure 3

Fig. 4. Dynamic of the *B. t. i.* attachment/detachment process to moss following a 10 minute injection of *B. t. i.* in the open-channel water. The toxicity of the moss is presented in $mg \cdot L^{-1}$ following bioassays on preparations of crushed moss in water. The maximum rate of toxicity associated with the moss (arrow) has been converted in mg of *B. t. i.* formulation per kg of strained moss.

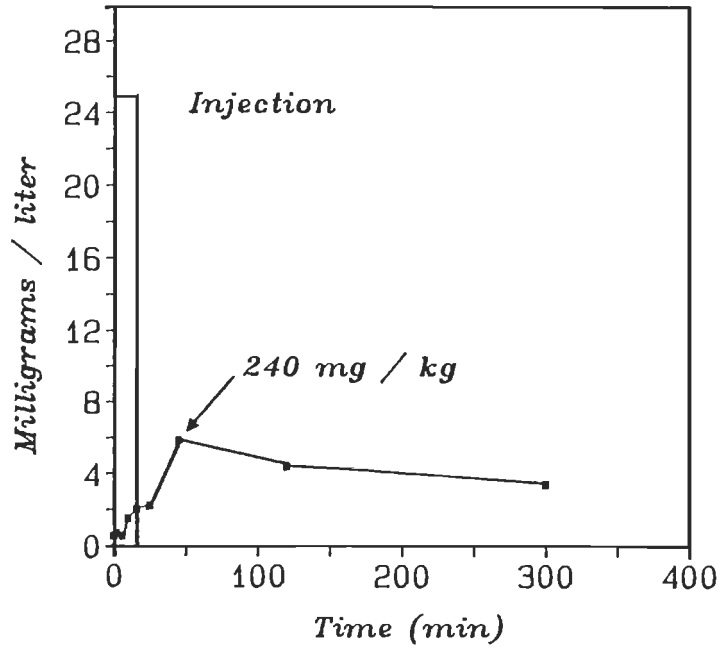

28
24
20
16
12
8
4
0
Injection
240 mg / kg
Milligrams / liter
0
100
200
300
400
Time (min)

Figure 4

Effects of Freezing and Field-Collected Substrates on the Efficacy of *Bacillus thuringiensis* serovar. *israelensis* as Determined Through Bioassays in the Laboratory

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Keywords: *Bacillus thuringiensis* , freezing, toxicity loss, bioassay.

Running title: Alteration of *B.t.i.* toxicity in field samples.

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ABSTRACT

Numerous field trials have been undertaken to better understand the behaviour (persistence, dispersion etc.) of *Bacillus thuringiensis* serovar. *israelensis* (*B.t.i.*) formulations when treating rivers or streams for blackfly control. After an extensive sampling of water and natural substrates (periphyton, sediments, moss), freezing is a useful procedure to prevent enzymatic deterioration or bacterial growth in samples before bioassays are to be performed. Using *Aedes triseriatus* neonate larvae, we quantified the effect on potency of freezing and thawing of *B.t.i.* suspensions at operational field concentrations. In addition, as samples varied in their content of natural substrates we tested the hypothesis that the presence of such suspended solids affected the mortality response of larvae. Our results showed that these parameters are of significant importance and should be accounted for when comparing bioassays performed on previously frozen or turbid samples.

INTRODUCTION

Since the discovery of *Bacillus thuringiensis* serovar. *israelensis* (*B.t.i.*) (Goldberg & Margalit, 1977), its activity against mosquito and blackfly larvae has been well documented (Undeen & Nagel, 1978; Gaugler & Finney, 1982; Molloy *et al.*, 1984). Due to the lack of direct methods for measuring the presence of the toxic crystals (physical, chemical or immunological methods) the toxicity (potency) of field samples was interpreted mainly from spore counts (Undeen & Colbo, 1980; Frommer *et al.*, 1981a & 1981b; Merritt *et al.*, 1989; Matanmi *et al.*, 1990) or bioassays (Lacey & Lacey, 1981). When using bioassay procedures, 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 to prevent or minimize, enzymatic deterioration of the crystals or bacterial growth. But 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.*, 1981a; Sinègre, 1981; Guillet *et al.*, 1982), at 50 °C (Ignoffo, 1982). These authors have found that the toxic activity was remarkably stable under these experimental conditions.

The presence of natural substrates (e. g. particulate material) has been shown to decrease toxicity by altering the feeding behavior of larvae and/or interaction with *B.t.i.* crystals, (Mulla *et al.*, 1982; Van Essen & Hembree, 1982; Dupont & Boisvert, 1986; Merritt, 1987; Morin *et al.*, 1988). The purpose of most of these studies was to find the effect of suspended matter, sediments or vegetation on the residual efficacy of *B.t.i.* primary powders or formulations in the field. In our study, as we intended to assess the

movement of *B.t.i.* from open channel water into different compartments of the stream habitat, such as sediments, aquatic vegetation and epilithic periphyton (microfloral growth covering rocks), it was decided to determine whether these materials, collected in the field could affect larval mortality under laboratory conditions. The effect of water turbidity and the presence of suspended matter has also received considerable attention in the past (Ignoffo *et al.*, 1981b; Standaert, 1981; Ramoska *et al.*, 1982; Silapanuntakul *et al.*, 1983; Guillet *et al.*, 1985; Karch *et al.*, 1991). These authors unanimously agreed that a reduction of potency was correlated with the presence of suspended matter.

We are seeking to study where the loss of *B.t.i.* larvicidal activity occurs when treating a river for blackfly control. In such a study, stream water samples, periphyton and vegetation (moss and herb) have to be frozen until they are tested for larvicidal activity. Because of the lack of literature on the effect of freezing and thawing on *B.t.i.* samples containing various amounts of suspended matter, periphyton or moss, we undertook a study to determine if these conditions would affect the toxic activity of the samples, and if so, to quantify the loss of activity in order to be able to determine the original amounts of activity present in samples before freezing or in the absence of suspended matter.

MATERIALS AND METHODS

Bioassay

In the present study bioassays were performed under laboratory conditions using *Aedes triseriatus* (Say) neonate larvae. This choice was prompted by the fact that freshly hatched larvae offer a greater physiological synchronicity and a greater sensitivity to *B.t.i.*. The method used was based on the one described by Ibarra and Federici (1987) in which a single neonate larva is placed in an individual well of a microtitre plate (96 holes) and then exposed to dilutions of the samples to be tested. In order to minimize experimental variations, all samples were assayed in triplicates using 32 larvae for each treatment, performed on 3 successive days using eggs at the same stage of maturity. Mortality counts were made after 24 h and statistically analyzed using probit analysis (Finney, 1971; Hubert, 1984). The mortality obtained was then compared with a standard curve (prepared with dilutions of *B.t.i.* in stream water) in order to estimate *B.t.i.* toxic activity (expressed in µg of formulation per litre) present in the unknown samples.

To illustrate the increased sensitivity of neonate larvae, it is worth mentioning that for a series of *B.t.i.* suspensions prepared with Teknar HPD® (Zoëcon lot # 0080227, used throughout this study) in distilled water, a median lethal concentration (LC_{50}) of 50 $\mu g \cdot L^{-1}$ was obtained compared with a LC_{50} of 600 $\mu g \cdot L^{-1}$ for early 4^{th} instar larvae. This technique not only allowed us to determine small amounts of *B.t.i.* in various samples, but also permitted us to process up to 8 samples per day including a standard curve.

Freeze-thaw procedure

In order to quantify the effect of freezing and thawing on *B.t.i.* toxicity, a series of *B.t.i.* suspensions was prepared at 1, 10, 100 and 1000 $mg \cdot L^{-1}$ in distilled water. Each one was separated in four equal volumes and the following procedure was applied to each of the four concentrations. One sample was kept at 4°C, a second was frozen once (-25°C) then thawed and kept at 4°C, a third was frozen-unfrozen twice and kept at 4°C and a fourth sample was frozen-unfrozen three times and kept at 4°C. We then ended up with four identical series of *B.t.i.* suspensions that were frozen and thawed 0, 1, 2 and 3 times. These preparations were then diluted for final testing at a concentration of 50 $\mu g \cdot L^{-1}$. This concentration was selected following preliminary neonate larvae bioassays, indicating around 50-60% mortality after 24 h of contact. Furthermore, at this level, changes in potency are most-clearly demonstrated when using probit analysis (Finney, 1971; Hubert, 1984).

Suspension of natural substrates

Since we were dealing with various amounts of suspended matter in field samples, two different series of solutions were prepared to assess the modification to *B.t.i.* toxicity brought about by solids suspended in water. A first series of suspensions was prepared at 50 $\mu g \cdot L^{-1}$ of *B.t.i.* and with different concentrations (0.2, 0.6, 1.2, 2.4, 4.1 and 5.5 $mg \cdot L^{-1}$) of suspended material. Suspended material was obtained by the following procedure. It was determined by filtration through a 0.22 µm type GS filter followed by drying 24 h at 100°C, that untreated stream water contained 2 $mg \cdot L^{-1}$ of dried matter. The water was then centrifuged at 4 000 g for 30

min at 4°C and the pellet resuspended in distilled water to make a stock solution at 12 mg·L^{-1}. From this suspension, various dilutions were made, to provide six solutions containing the same initial amount of *B.t.i.* (50 µg of Teknar HPD per litre) but with variable amounts of suspended matter. These preparations were tested without freezing.

Preliminary observations had indicated that after field application not only was there a small amount of *B.t.i.* associated with moss present in a stream, but that moss greatly affected the mortality response when bioassayed. Because of these observations, a second series of *B.t.i.* standard concentrations ranging between 750 and 5 000 µg·L^{-1}, was prepared, each containing 12.5 g·L^{-1} of the moss *Platylonella lescurii* (Sull). Moss was field collected, strained through a sieve to remove excess water, weighed, finely crushed and then added to the various suspensions of *B.t.i.*. Again, these preparations were tested unfrozen. In order to measure the anticipated masking effect of the crushed moss on the mortality response, the probit line, representing the mortality (in probit units) as a function of the log of the dose (in µg·L^{-1} of Teknar HPD), was compared with the response by larvae to a series of *B.t.i.* standards prepared with stream water only.

RESULTS

Bioassay results after freeze-thaw cycles

The results presented in Table 1 indicate that the four series of frozen-thawed solutions behaved according to the same pattern, regardless of the three orders of magnitude (1 to 1 000 mg·L^{-1}) of the tested concentrations. A decrease of toxicity can be observed after each of the freeze-thaw cycles for all concentrations at which the *B.t.i.* suspensions

were frozen (first 4 columns). The pooled results of the four series computed in the fifth column, showed a reduction in mortality of 22% over the first freeze-thaw cycle and a reduction of 11 and 5% for the second and third freeze-thaw cycles, respectively. The data was normalized by dividing all four mean mortality figures by the mean (61%) obtained without freezing. This normalization is shown in Fig 1, where the decrease after various freeze-thaw treatments is 36, 18 and 8% respectively.

Table 1:

Figure 1:

We fitted a negative exponential curve to these values to represent the loss of mortality after each freeze-thaw and we obtained the relation: $y = 93.9 * 10^{-0.14\,n}$ ($R^2 = 0.97$, $P < 0.05$), where y is the mortality rate and n the number of freeze-thaw cycles.

Bioassay results with suspended matter and a fixed concentration of *B.t.i.*

Results from bioassays with a constant concentration of *B.t.i.* and a concentration range of suspended material showed that the presence of the material was correlated with the loss of potency (Fig 2). We observed that the samples containing higher levels of suspended material displayed the lowest mortality. Although the six values are not perfectly aligned, the tendency of such solids to decrease toxicity is statistically significant. A

simple linear correlation test at a confidence degree of more than 95% ($P < 0.05$), confirmed that there is a linear relationship between the two variables.

Figure 2:

The regression line obtained is described by the relation: $y = 66.7 - 3.0\ x$ ($R^2 = 0.69$, $P < 0.05$), where y is the mortality (%) and x the amount of dried suspended matter in $mg \cdot L^{-1}$. When the portion of the curve covering our experimental values is considered as linear, then the addition of the first 5 $mg \cdot L^{-1}$ of suspended matter to a *B.t.i.* suspension with an initial potency achieving 67% mortality, produces a reduction to 52% mortality. This represents a 22% drop of potency. The results indicate that the presence of 22 $mg \cdot L^{-1}$ of dried suspended matter (or more) in *B.t.i.* suspensions of 50 $\mu g \cdot L^{-1}$ or less, would be sufficient to lower the sensitivity of the assay below the detection limit.

Bioassay results with vegetation and variable concentrations of *B.t.i.*

To assess the way in which standard curves of mortality rates versus the log of concentration of *B.t.i.* could be affected by the presence of a constant amount (12.5 $g \cdot L^{-1}$) of stream moss, the results from *B.t.i.* standards prepared with this vegetation is compared to *B.t.i.* standards prepared without moss (Fig 3). The most striking effect produced by the addition of the 12.5 $g \cdot L^{-1}$ of crushed moss is the shift of the LC_{50} from 52

(95% confidence interval (CI): 46-58) to 2 282 $\mu g \cdot L^{-1}$ (95% CI: 2 085-2 497), a 44 fold increase.

Figure 3:

Furthermore, the equations of these curves are respectively $y = 1.9 x + 1.14$ without moss and $y = 2.2 x - 3.11$ with moss; where y is the probit and x the log of the *B.t.i.* concentrations. Because the accepted values of slopes for *B.t.i.* assays range from 1.5 to 6 (Ibarra & Federici 1987), the value of the slope obtained when moss is present is an indication that the bioassays are still valid under such conditions. Controls with and without moss gave 1% and 0% mortality rates and both curves were fitted by maximum likelihood probit using Abbott's formula to take into account the low levels of natural mortality. The parallelism of these two probit lines was tested using the method described by Hubert (1984). A χ^2 value of 1.823 was calculated, indicating that the lines did not differ significantly in their parallelism and that the presence of the moss did not affect the mode of action of the *B.t.i.* toxic crystal.

DISCUSSION

Our results indicated that freezing and thawing of *B.t.i.* suspensions decreases the toxic activity of this larvicide. We suspect that freezing could promote flocculation (or aggregation) of *B.t.i.* crystals. If such is the case, we can conceive that, if only one or a few crystals are sufficient to kill a

larva after ingestion, then following an agregation of 10 or 50 crystals, this lump if ingested by a neonate would only kill a single larva instead of many. This would cause an apparent decrease in potency of a sample. In addition, if the aggregates are large enough that they exceed the range of particle sizes that a specific instar is able to ingest, the reduction of activity after freezing, could be less apparent on older larvae, such as a 4^{th} instar. Regardless of the process involved, the first freeze-thaw cycle caused a 36% loss of toxicity. The results of the effect of freezing and thawing on *B.t.i.* suspensions indicate that if frozen material is to be used for eventual bioassay testing, appropriate procedures (choice of instar, number of cycles) should be included in the protocol so that the mortality obtained can be transformed into a corrected toxicity before freezing of the samples.

Our observations that toxicity is correlated with the presence of suspended material in *B.t.i.* preparations are consistent with previous studies. According to Guillet *et al.*, (1985), a sharp decrease in efficacy of *B.t.i.* formulations is observed when water turbidity increases. Standaert (1981), Mulla *et al.*, (1982), Ramoska *et al.*, (1982) and Silapanuntakul *et al.*, (1983), all noticed a reduction in mortality when *B. thuringiensis* H-14 was prepared with pond water compared to distilled water. Karch *et al.*, (1991) concluded that a Vectobac® 12-AS aqueous suspension of *B.t.i.* had a poor efficacy against *Culex quinquefasciatus* (Say) larvae in polluted gutter water. Ignoffo *et al.*,(1981b) found that about half of the *B.t.i.* activity was bound by 2% pond water solids and concluded that this reduction could be due to *B.t.i.* crystals binding to organic materials in sediments, rather than inactivation of *B.t.i.* by the solids *per se* . Compared to the above results we have shown that the presence of small amounts (less

than 5.5 mg·L^{-1}) of suspended material can have a significant effect on the dose-mortality response of a *B.t.i.* preparation. Furthermore, the presence of crushed moss at 1.25% (12.5 g·L^{-1}) reduced larval mortality to a level 44 times less than obtained without moss. Whether this reduction is caused by binding to natural substrates or degradation of the toxic crystals is not known, but these observations are of great importance especially if the persistence of *B.t.i.* toxic activity (i.e. activity of crystals only) is monitored in bodies of water containing variable quantities of suspended material. Indeed, most authors have not taken this into account in their persistence studies. It could be that the persistence of *B.t.i.* toxic crystals in water is probably much longer than has previously been reported.

This study has shown that the loss of *B.t.i.* potency related to the presence of suspended material is of importance and should be accounted for when analysing field samples. It could cause significant variation when comparing field efficacy of *B.t.i.* preparations between different countries; for example, African river waters are usually much more turbid than North American stream waters, resulting in a reduced efficacy.

In conclusion, we have found that samples containing *B.t.i.* toxic crystals will give a variable dose-mortality response based on the quantity of material present in the samples and whether the samples are frozen or not. All the relationships that we have found indicate a lowering of *B.t.i.* potency. This could have a significant effect on conclusions derived from persistence, absorption or efficacy studies of *B.t.i.* formulations.

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Table 1: Percent mortality of *Aedes triseriatus* (± SE) observed after freeze-thaw cycles of *B.t.i.* suspensions prepared at different concentrations. All samples were assayed at the same final concentration of 50 $\mu g \cdot L^{-1}$. All controls were less than 3%.

Number of freeze-thaw cycles	Concentrations in $mg \cdot L^{-1}$				Mean	Normalized
	1	10	100	1000	Value	Value
0	55 (±4)	65 (±8)	50 (±8)	73 (±8)	61 (±12)	100 (±20)
1	42 (±5)	36 (±8)	33 (±2)	45 (±7)	39 (±7)	64 (±11)
2	28 (±8)	22 (±5)	34 (±6)	29 (±7)	28 (±7)	46 (±12)
3	19 (±11)	26 (±5)	27 (±5)	20 (±7)	23 (±7)	38 (±12)

Figure 1: Decrease in larval mortality (normalized value ± SE) induced by a *B.t.i.* preparation at 50 $\mu g \cdot L^{-1}$ after freeze-thaw cycles.

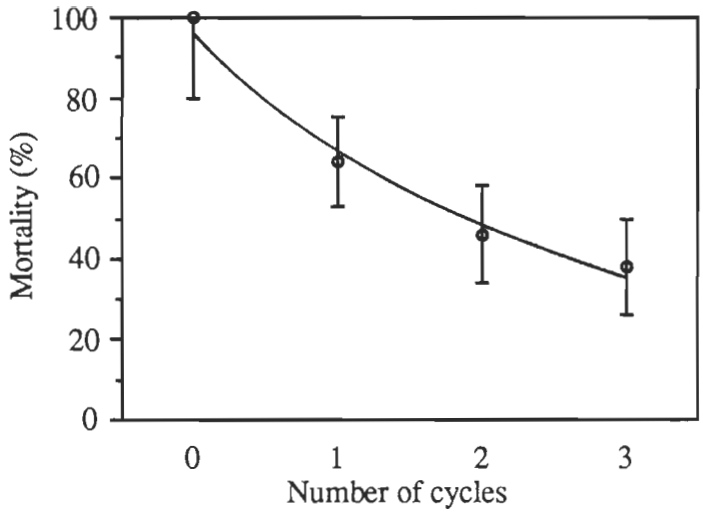

Figure 1

Figure 2: Percentage mortality (± SE) of neonate *Aedes triseriatus* larvae fed with aqueous *B.t.i.* suspension (50 $\mu g \cdot L^{-1}$) containing various concentrations ($mg \cdot L^{-1}$) of suspended solids.

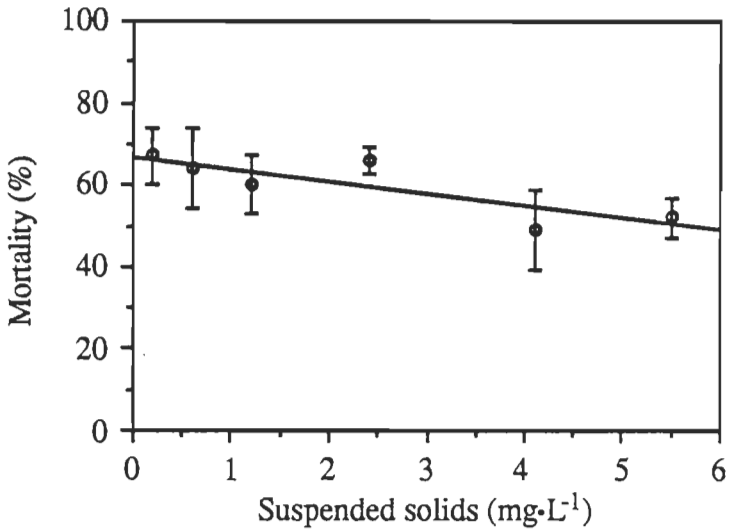

Figure 2

Figure 3: Probit response to *B.t.i.* preparation tested in the presence of 12.5 $g \cdot L^{-1}$ of moss (•) or in the absence of moss (o).

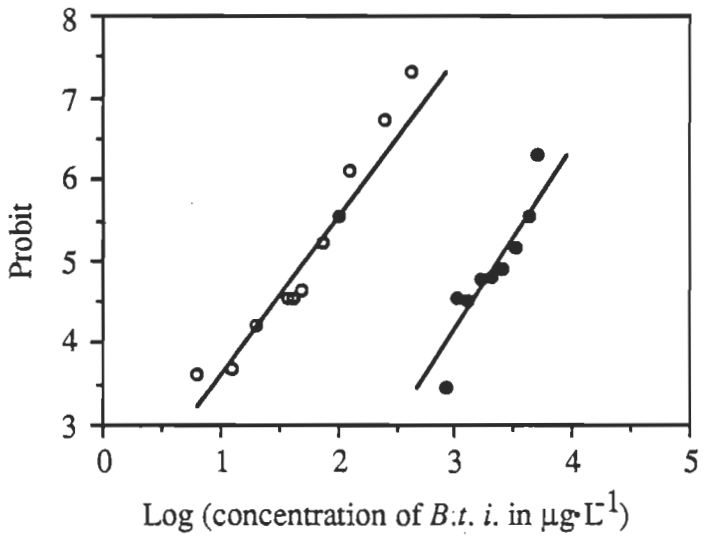

Figure 3

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Reduction of Mortality Rates of *Bacillus thuringiensis* var *israelensis* Aqueous Suspensions Due to Freezing and Thawing

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Running title: Effect of Freezing on *B.t.i.* Aqueous Suspensions

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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 of 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 *Bacillus thuriengiensis israelesis* (*B.t.i.*) toxic crystals (physical, chemical or immunological methods) 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, a 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 to 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 conducted a study to establish if the

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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.

freeze-thaw procedure would affect the toxic activity of the crystals, and to determine how it might modify parameters such as the LC_{50} and the slope in 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-thawed up to four 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* (Coquillett) neonate larvae because freshly hatched organisms offer a greater physiological synchronicity and a 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. In order 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. The 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 and bioassayed, and to evaluate if a single freeze-thaw cycle of those *B.t.i.* suspensions would affect the mortality response. *B.t.i.* concentrations of 1, 5, 10 and 20 mg/liter were prepared in distilled water using Teknar HPD® (Zoëcon lot # 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 either kept at 4°C or frozen once at - 25°C and then thawed out 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 bioasssay, each preparation was diluted in distilled water for testing mortality response at final concentrations 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.

The results from this first series showed that all bioassays with samples unfrozen or frozen once had similar mortality response curves whatever their initial concentration, although there was some decrease in mortality after freezing, thus another experiment was prepared using a 20 mg/liter concentration, 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 triplicate for unfrozen samples and samples frozen once (Table 1, test 5). For samples frozen two, three and four times, bioassays were done in triplicate on three different days (September 17-24-25) as presented in Table 1.

Statistical analysis : Each set of bioassays was statistically analyzed using 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 the Abbott's formula (Abbott 1925).

To demonstrate the independance of mortality responses for a given number of freeze-thaw cycles performed on samples at different concentration 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 a "grand probit" (GP) from multiple toxicity test data. To achieve this, the individual probit lines (for a given number of freeze-thaw cycle) 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 of 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} 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 four different concentrations : In Table 2 line A and B we present the calculated χ^2 values for parallelism and LC_{50} homogeneity test. Line 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/l. Line 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 of line 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. Furthemore the low χ^2 values for unfrozen suspensions (line A) are an indication that the neonate bioassay technique is reliable. There seem 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 to unfrozen suspensions (Table 2, line B compared to line A) and by a greater dispersion of the mortality values around the grand probit line (Fig. 1B compared to Fig. 1A).

Freeze-thaw treatment : In Table 2 line C, D and E we present the χ^2 values for parallelism and LC_{50} homogeneity test after multiple freeze-thaw cycles of *B.t.i.* suspensions prepared at 20 mg/l. 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^2 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.

In Fig. 1F we can see that the grand probit (GP) lines after 0, 1 and 2 cycles on the upper part have similar slope values, and the GP lines after cycles 3 and 4 share a similar slope value. After the totality of the assays (0, 1, 2, 3 and 4 cycles) were put together (Table 2 line 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 a 95% confidence level (Zar 1984). After grouping the tests for 0, 1 and 2 freeze-thaw cycles (Table 2, line 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, line 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 than 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, we can see that the 95% CI 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 modeled this increase of LC_{50} exponentially by the 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,

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

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

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

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

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

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

The fact that there are sporeless *B.t.i.* formulations now available will increase the necessity of using bioassays or direct toxicity tests to mesure *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 concentration values (Table 2 line A and B) indicated 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 be affected according to 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 Fig. 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. To illustrate, a concentration giving 50% mortality would give less than 8% 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 homogeneously after thawing. It is suspected that freezing promotes flocculation (or aggregation) of *B.t.i.* crystals. If such is the case, we can conceive that if only one or a few crystals are sufficient to kill a larva, flocculation of crystals would create a large particle that would only kill a single larva instead of many, thus reducing the mortality although 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 4^{th} instar.

Freezing damage to the *B.t.i.* toxic proteins could also account for the reduction of mortality. During freezing, substantial enhancements of solute concentration occur 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 domain 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, the results presented here 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 relation (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) tells us 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 is 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 to decide the proper way to manage storing and handling of a large number of *B.t.i.* samples.

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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.

Test no.	Number of freeze-thaw cycles				
	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)

Table 2. Chi-square validation tests for parallelism and homogeneity of the LC_{50}s after the maximum likelihood for individual tests. Values in line 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. Line C, D and E are the results from a preparation of 20 mg/liter frozen-thawed 2, 3 or 4 times. Line F, G and H are the analyses of the grouping 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	----

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. 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 (Fig 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 (Fig C, D and E). Figure F displays all the grand probit lines together in order to better visualize the parallelism and the LC_{50} shift after multiple freeze-thaw cycles.

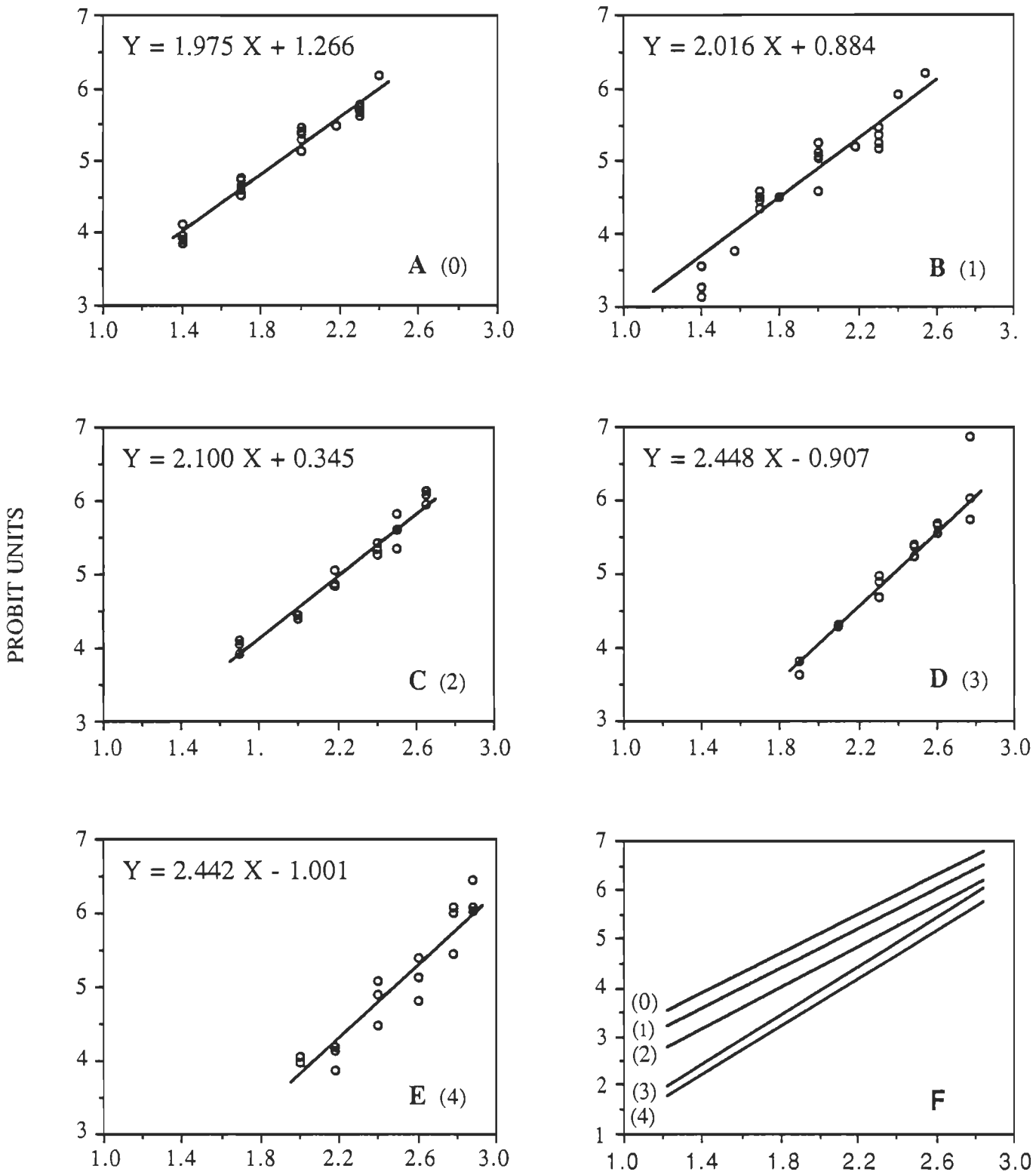
Y = 1.975 X + 1.266
A (0)
Y = 2.016 X + 0.884
B (1)
Y = 2.100 X + 0.345
C (2)
Y = 2.448 X - 0.907
D (3)
Y = 2.442 X - 1.001
E (4)
(0)
(1)
(2)
(3)
(4)
F
PROBIT UNITS
LOG OF B. T. I. CONCENTRATION (µg/liter)

Figure 1

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

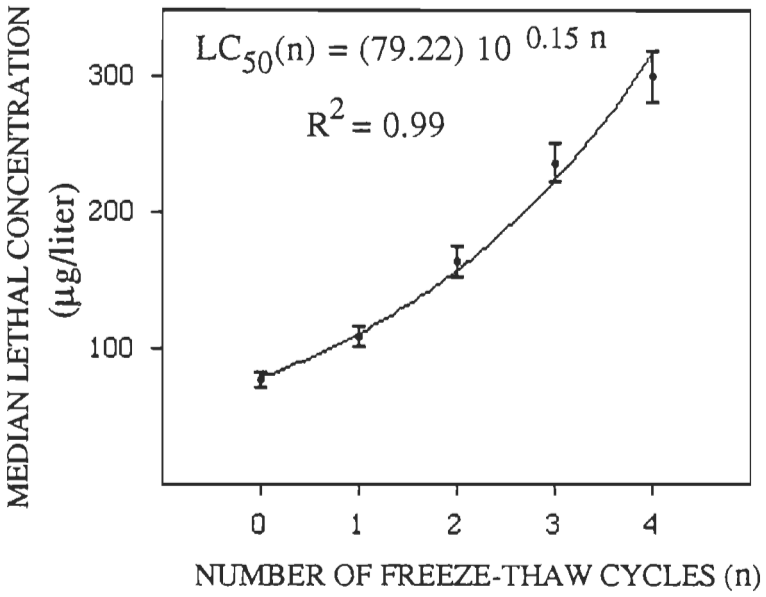
$LC_{50}(n) = (79.22)\ 10^{\ 0.15\ n}$
$R^2 = 0.99$
MEDIAN LETHAL CONCENTRATION (µg/liter)
300
200
100
0
1
2
3
4
NUMBER OF FREEZE-THAW CYCLES (n)

Figure 2