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ICE GRANULES CONTAINING ENDOTOXINS OF MICROBIAL AGENTS FOR THE CONTROL OF MOSQUITO LARVAE—A NEW APPLICATION TECHNIQUE

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ABSTRACT. This study investigated a new method for delivering microbial mosquito control agents into aquatic sites as ice granules for mosquito control. Solutions containing powder formulations of *Bacillus thuringiensis israelensis* (*Bti*) or *Bacillus sphaericus* were transformed into ice pellets (named IcyPearls) using a special ice-making machine. This new technique was demonstrated to have the following advantages over *Bti* sand granules: 1) the *Bti* ice pellets melted on the water surface and released the microbial crystals there; 2) the control agent remained inside the ice pellets during the application and were not lost by friction in the spraying equipment; and 3) the ice formulation resulted in increased swath widths, significantly reducing the cost of application. In large field tests, IcyPearls have been applied at dosages of 5 and 10 kg/ha containing 400 g as well as 100, 200, and 400 g of VectoBac® WDG (3,000 ITU/mg), respectively, against larvae of *Aedes vexans*. Mortality rates of 91–98% were achieved.

KEY WORDS Mosquito control, ice granules, microbial control agents

INTRODUCTION

In recent years, several reviews addressing the formulation of entomopathogenic bacteria have been published (Beegle et al. 1990, Burges and Jones 1998, Couch 2000). The most common formulations available to users are aqueous suspensions, wettable powders, water-dispersible granules (WDGs), corncob granules, pellets, briquettes, and fizzy tablets. Sand granules are generally prepared on site by the applicator using a cement mixer.

For almost 2 decades, *Bacillus thuringiensis israelensis* (*Bti*) and *Bacillus sphaericus* have been used successfully as biological control agents against mosquitoes in Germany (Becker 1997). In the Upper Rhine valley, 100 cities and municipalities along a 310-km stretch of the River Rhine have joined forces in the German Mosquito Control Association (KABS) to combat these insects—mainly *Aedes vexans* (Meigen)—over a 600-km^2 breeding zone on the Rhine floodplain. The overall goal of the KABS is to control mosquitoes while conserving biodiversity and maintaining stability of ecosystems. As a result of this stability and biodiversity, natural mortality factors of mosquitoes that might escape treatment are enhanced (Becker 1997). Microbial control tools are integrated with environmental management (e.g., improving ditch systems to control water levels and provide permanent habitats for aquatic predators, such as fish) to achieve this objective.

The control of *Aedes* mosquitoes by GMCA/KABS is based mainly on the use of *Bti* products. The area treated regularly is 10–20% of the potential breeding area of 600 km^2, depending on the extent of flooding, and involves a team of approximately 400 people. To treat the first and second larval instars, less than 200 g of 3,000 ITU/mg of *Bti* water-dispersible granules (VectoBac® WDG, Valent BioSciences Corporation, Libertyville, IL) or 0.5–1.0 liters of liquid concentrate (1,200 ITU/mg) are dissolved in 9–10 liters of filtered pond water for each hectare treated. This is applied using backpack sprayers. For deeper sites or when later instars are present, the dosages are doubled. During the worst floods, a third to half of the area were treated with *Bti* granules dispersed by helicopter. In the years prior to 2001, almost 200,000 ha of mosquito breeding sites had been treated successfully with about 100 tons of *Bti* soluble powder and fluid concentrates. The powder formulations have been used to produce about 1,200 tons of *Bti* sand granules to be applied to swamps by helicopter or ground application. The application of microbial control agents has resulted in a reduction of the mosquito populations of more than 90% each year, based on the results of adult trap counts. No evidence of harmful impacts on the environment associated with treatments has been observed.

In this program, sand has been used as the carrier for technical powder formulations. A mixture of 50 kg fire-dried quartz sand (grain size 0.9–2 mm), 0.8–1.4 liters of vegetable oil (as a binding agent), and 0.8–1.6 kg of *Bti* powder (potency ~8,000 ITU/mg) usually has been sufficient to treat 2–3 ha either by a helicopter equipped with a Simplex spreader or by hand. This technique was developed in 1983, and since then, more than 1,000 tons of *Bti* sand granules have been dispersed, mostly by helicopter, over the breeding grounds, with very effective results.

However, this technique does have disadvantages. In particular, due to friction within the aerial application equipment, some of the active agent is lost during the application process. Also, due to their weight, the *Bti*-laden sand granules tend to sink to the bottom of the breeding sites. Thus, some of the active agent does not remain in the feeding zone of the mosquito larvae, causing a need for increased dosages.

In 1997, a new method of delivering the active material, called the *Bti* ice granule, was developed to overcome these disadvantages. After a short development period, this technique was successfully tested and patented. The method involved creating a suspension of *Bti* powder and water and transforming it into ice pellets (named IcyPearls) using a special ice-making machine.

Bti ice granules needed to effect the transformation of the suspension from liquid to solid form can be produced in one of two ways. The first method uses a conventional icemaker. The disadvantage of this method is that the ice is wet as it leaves the machine. If the ice pellets are then stored below freezing point, the surface water causes them to freeze together so they are no longer able to be dispersed. Therefore, this method requires an additional processing stage to dry the granules. This additional stage involves passing the granules through a liquid nitrogen bath after they have left the icemaker. This freezes the remaining water film, ensuring that the granules remain dispersible. The 2-stage process is relatively expensive and slows production. The shape of granules made by this method is cubic or cylindrical, with a diameter of 4 mm and a length of 6 mm.

To lower costs, speed production, and produce granules of optimum size, improved ice making techniques were sought. This article describes a new method of ice-pellet production and presents results of experimental applications of these insecticide-containing pellets.

In this study, the efficacy of this new technique has been assessed against floodwater mosquitoes.

MATERIALS AND METHODS

Special equipment was constructed to transform water into ice using only liquid nitrogen. Transportation and aerial application of the frozen granules also required the development of special equipment so that the granules could be dispersed efficiently and homogeneously over mosquito breeding sites. To reduce the rate of melting of the ice pellets, an insulated cover for the application bucket was constructed out of 60-mm-thick polystyrene foam. The application bucket was attached to a helicopter by means of a sling load harness.

Dispersal of the granules was achieved by means of a rotating disk. When the apparatus was switched on, the granules were fed onto a rotating disk with deflectors that caused the granules to be thrown outward by centrifugal force. Because the friction produced by ice was negligible, the circular acceleration resulted in a much wider swath than could be achieved with conventional granules.

Production of the ice pellets: Droplets of a *Bti* water suspension were rapidly frozen in a circulating nitrogen bath. The method used a special technique to regulate the circulation of liquid nitrogen along a channel, about 420 mm wide, in the upper section of the equipment. The speed of flow was determined by the slope of a channel so that, at a nitrogen height of 20 mm, it was about 1 m/sec. The aqueous *Bti* solution was fed into this liquid nitrogen stream, drop by drop, using a drip feed device. The device consisted of a closed container. On the underside of this container, there were 200 syringes, 10 mm apart, arranged in 5 rows. The *Bti* solution was pumped into the closed container and dripped out via the syringes. The size of the droplets depended on the diameter of the syringe needle and the rate of droplet production depended on the quantity of fluid in the container, which could be controlled as needed. The grain size of the IcyPearls averaged 4 mm (volume 33 mm^3). Upon application, about 30 grains of this size were deposited per m^2 when 10 kg/ha were applied.

By regulating the rate of droplet production into the flow of liquid nitrogen, it was possible to prevent the frozen droplets from sticking together. The granules were selected and carried away from the liquid nitrogen by a metal conveyor belt and stored in a refrigerated container/room until used.

Different concentrations of *Bti* were prepared as follows: Three mixtures were prepared using 10 liters of tap water mixed with 100, 200, and 400 g of VectoBac WDG, respectively, each for 1 ha. One additional mixture was prepared using 5 liters of tap water mixed with 400 g of VectoBac WDG to treat 1 ha.

IcyPearls were produced from these suspensions using the liquid nitrogen process described above. The following IcyPearls rates were then tested.

First treatment site: Ten kilograms IcyPearls/ha containing 100 g VectoBac WDG (a total of 30 kg of this mixture was prepared to treat 3 ha).

Second treatment site: Ten kilograms IcyPearls/ha containing 200 g VectoBac WDG (a total of 30 kg of this mixture was prepared to treat 3 ha).

Third treatment site: Ten kilograms IcyPearls/ha containing 400 g VectoBac WDG (a total of 50 kg of this mixture was prepared to treat 5 ha).

Fourth treatment site: Five kilograms IcyPearls/ha containing 400 g VectoBac WDG (a total of 25 kg of this mixture was prepared to treat 5 ha).

Vegetation

A dense canopy consisting of poplar trees (*Populus canadensis* Moench), bushes (*Viburnum opulus* L.), reed (*Phragmites australis* Trin.), and nettles (*Urtica dioica* L.) was present in the test sites.

Sampling

At each treatment site, four plots of about 100 m^2 were chosen for sampling. At each plot, 10 dips were taken with a WHO standard dipper (350 cm^3). The number of larvae of each instar were counted and recorded before treatment as well as 24 and 48 h after treatments. The larval density in an untreat-

Table 1. Effect of *Bti* IcyPearls granules applied at a dosage of 10 kg/ha containing 100 g Vectobac WDG (30 ITU/mg) = 0.3×10^9 ITU/ha.[1]

Plot	Number of larvae/10 dips Pretreatment	24 h	48 h
1	49	0	0
2	65	0	0
3	39	5	5
4	91	19	16
Average	61	6	5.25
SE	±22.69	±8.98	±7.54
Reduction rate		90.2%	91.4%

[1] *t*-Value + degrees of freedom (df): 4.66; df = 6 (all *t*-values are referring to the 48-h evaluation); niveau of significance: P = 1%.

Table 2. Effect of *Bti* IcyPearls granules applied at a dosage of 10 kg/ha containing 200 g Vectobac WDG (60 ITU/mg) = 0.6×10^9 ITU/ha.[1]

Plot	Number of larvae/10 dips Pretreatment	24 h	48 h
1	53	0	0
2	61	0	0
3	74	2	2
4	47	7	5
Average	58.75	2.25	1.75
SE	±11.67	±3.30	±2.36
Reduction rate		96.2%	97.0%

[1] *t*-Value: 9.57; df = 6; niveau of significance: P = 0.1%.

ed plot was used as a control. Data were statistically analyzed according to Student's *t*-test (Köhler et al. 1984).

Mosquito species

Species composition in the test sites was as follows: for treatment sites 1 and 2, *Ae. vexans* L_2/L_3 (85%) and *Culiseta annulata* (Schrank) L_1–L_4 (15%); for treatment sites 3 and 4, *Ae. vexans* L_2/L_3 (90.9%), *Ochlerotatus sticticus* (Meig.) L_2/L_3 (4.7%), and *Aedes rossicus* D.G.M./*cinereus* (Meig.) L_2/L_3 (4.4%).

Application

The granules were applied by means of the above-described insulated bucket equipped with a rotating device (seeder) and operated by the pilot in a Bell 47 helicopter. The rotation speed was 600 rpm. The seeder used in this study produced a swath of 30 m, which was measured in dynamic tests. The correct amount of granules for each treatment site was measured into the seeder to obtain the desired dosages. The speed of the helicopter was 60 km/h. The height of application was 10 m above vegetation, in total, 50 m above the breeding sites.

RESULTS

Results are summarized in Tables 1–4. Application of 100, 200, and 400 g of VectoBac WDG in 10 kg of IcyPearls per hectare resulted in mortality rates of 91.4, 97, and 99.9%, respectively, at 48 h after the application. The differences between the application of 100 and 200 g of WDG in 10 kg of IcyPearls differed significantly (P = 5%), but there was no significant difference between the application of 200 and 400 g of WDG/ha. Application of 400 g of WDG in 5 kg of IcyPearls per hectare resulted in 97.6% mortality. This was not significantly different from the mortality achieved with the same rate of active ingredient in 10 kg of IcyPearls per hectare.

DISCUSSION

The ice-granule application method is very effective against mosquito larvae even at the low rates of 100–200 g of VectoBac WDG per hectare, which is equivalent to an application doses of 0.3 to 0.6×10^9 ITU/ha. These doses are approximately one third of the ITU dose normally used in corncob-based treatments for floodwater species. Effective control at these lower doses is most likely due to: a) the *Bti* ice pellets melting on the water surface and releasing the toxins in the feeding zone of the mosquito larvae; b) the toxins remaining inside the ice pellets and not being lost by friction in the spraying equipment, as is the case with *Bti* sand granules used; c) excellent penetration of the vegetation being achieved due to the pearl-like shape and specific weight of the granules; and d) excellent dispersion of WDG in water and the low rates of settling of the active ingredients.

For the routine treatments, KABS decided to use 7.5 kg of IcyPearls per hectare containing 250 g of VectoBac WDG (0.75×10^9 ITU/ha; 48 IcyPearls m^2). All treatments were successful, and almost 100% mortality was achieved at all sites. It is likely that a dosage of 5 kg of IcyPearls containing 250

Table 3. Effect of *Bti* IcyPearls granules applied at a dosage of 10 kg/ha containing 400 g Vectobac WDG (120 ITU/mg) = 1.2×10^9 ITU/ha.[1]

Plot	Number of larvae/10 dips Pretreatment	24 h	48 h
1	65	0	0
2	220	0	1
3	267	0	0
4	757	9	0
Average	327.25	2.25	0.25
SE	±299.21	±4.5	±0.5
Reduction rate		99.3%	99.9%

[1] *t*-Value: 2.18; df = 6; niveau of significance: not significant.

Table 4. Effect of *Bti* IcyPearls granules applied at a dosage of 5 kg/ha containing 400 g Vectobac WDG (240 ITU/mg) = 1.2×10^9 ITU/ha.[1]

Plot	Number of larvae/10 dips		
	Pretreatment	24 h	48 h
1	567	0	18
2	38	0	4
3	78	2	0
4	223	7	0
Average	226.5	2.25	5.5
SE	±240.51	±3.30	±8.54
Reduction rate		99%	97.6%

[1] *t*-Value: 1.84; df = 6; niveau of significance: not significant.

g of VectoBac WDG will be enough to treat breeding sites with unpolluted water (rice fields, floodplains); in polluted or very densely vegetated areas, higher dosages might be necessary.

The usual swath size for fluid concentrates and commercial corncob granules from equipment used in Germany is less than 15 m. The IcyPearls delivery equipment tested and used in this study produced swaths of more than 30 m with IcyPearls. Thus, costs for aerial application may be significantly reduced. Under cold-storage conditions, ice granules can be stored without any degradation of the active ingredients for a long time. Caking of the ice granules will occur after prolonged periods of storage; the granules can easily be restored to free-flowing condition by stomping on the ice in the storage containers.

Based on the experience in the KABS program, the production costs for ice granules are much lower than the costs for sand and commercially available corncob granules. Comparative costs may differ in other locations, depending on energy costs and the cost of commercial *Bti* formulations. In 2001, the water level of the Rhine River was extremely high, causing wide-spread floods and breeding of floodwater mosquitoes. This situation was easily handled by treating more than 12,000 ha with ice granules. More than 20,000 ha have been treated since 1997, saving several hundred thousands of dollars compared with the expense of the more costly *Bti* sand granules. Usually the granules are packed in plastic boxes containing 22.5 kg of ice granules for the treatment of 3 ha. The plastic boxes are transported in refrigerated trucks to the various landing sites of the helicopters. For each application flight, 5 plastic boxes are filled in the application bucket in order to treat 15 ha per flight.

A prerequisite for the successful use of ice granules—either carrying *Bti* or *B. sphaericus* toxins—is the availability of special ice machines, a sufficient supply of fluid nitrogen, and efficient application systems. This technique has the potential to reduce application costs of *Bti* and *B. sphaericus* treatments significantly.

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