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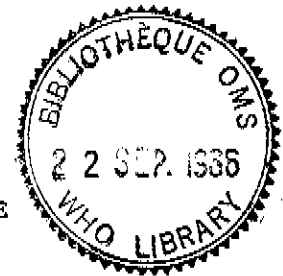
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INFORMAL CONSULTATION ON THE DEVELOPMENT  
OF BACILLUS SPHAERICUS AS A MICROBIAL LARVICIDE



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This report contains the collective views of an international group of experts convened by the UNDP/WORLD BANK/WHO SPECIAL PROGRAMME FOR RESEARCH AND TRAINING IN TROPICAL DISEASES (TDR). It does not necessarily reflect the views of TDR/WHO. In the interests of rapid communication it has been submitted to only minimal editorial revision. Moreover, any geographical designations used in the report do not imply the expression of any opinion whatsoever on the part of TDR or WHO concerning the legal status of any country, territory, city or area or of its authorities concerning the delimitation of its frontiers or boundaries.

Ce rapport exprime les vues collectives d'un groupe international d'experts réuni par le PROGRAMME SPECIAL PNUD/BANQUE MONDIALE/OMS DE RECHERCHE ET DE FORMATION CONCERNANT LES MALADIES TROPICALES (TDR). Il ne représente pas nécessairement les vues du TDR/OMS et, en vue d'une diffusion accélérée, il n'a pas été l'objet d'une mise en forme particulièrement soignée. En outre, les noms géographiques utilisés dans le présent rapport n'impliquent, de la part du TDR ou de l'OMS, aucune prise de position quant au statut juridique de tel ou tel pays, territoire, ville ou zone, ou de ses autorités, ni quant au tracé de ses frontières.

## 1. INTRODUCTION

Over the past decade, resistance to chemical insecticides continued to spread among important mosquito and blackfly vectors. In the same period, fewer new chemical insecticides were developed to replace those that had become ineffective. As a result, it became imperative that alternative vector control agents and methods be developed.

Of the various potential microbial agents for vector control, bacteria continue to hold the most potential for further development as larvicides.<sup>1</sup> During the past five years, Bacillus thuringiensis H-14 (B.t. H-14) has been successfully developed as an operational larvicide and has proved of considerable importance in vector control programmes based on larvicides, particularly the Onchocerciasis Control Programme (OCP) in West Africa. B.t. H-14 is relatively ineffective in polluted water and its residual activity in most habitats is limited to a few days after treatment. There is evidence, however, that one or more strains of B. sphaericus may be effective in such habitats, particularly against species of Culex, and that this bacterium may have a longer duration of efficacy due to persistence or recycling.

An informal consultation was held in Geneva, Switzerland, from 7 to 11 October 1985, to critically assess the diverse data available on B. sphaericus. The consultation was opened by Dr A.O. Lucas, Director of the UNDP/WORLD BANK/WHO Special Programme for Research and Training in Tropical Diseases (TDR) and Dr N.G. Gratz, Director of the WHO Division of Vector Biology and Control, who stressed the urgent need for the development of more effective agents for controlling vectors of the major tropical diseases.

## 2. CHARACTERISTICS OF BACILLUS SPHAERICUS

### 2.1 Strains and Isolates

B. sphaericus is an aerobic, spore-forming bacterium found commonly throughout the world in soil and aquatic environments. Both insecticidal and non-insecticidal strains are known. Early studies focused on strain 1321 (SSII-I), isolated from mosquito larvae in India, but most recent work has concentrated on strains 1593, 2297 and 2362, isolated, respectively, from Indonesia, Sri Lanka and Nigeria. Altogether 186 strains have been isolated, of which 45 show some toxicity to mosquito larvae and provide 100% mortality in 48 hours at a concentration of  $10^7$  cells/ml. These isolates are maintained in the WHO Collaborating Centre at the Institut Pasteur, Paris,\* and are available, together with a reference catalogue, to interested scientists.

The various strains of B. sphaericus have been divided by serology into 44 serotypes. The pathogenic strains fall into five subgroups based on their H-antigen and seven subgroups based on sensitivity to various phages. At present, it is not possible to distinguish between these strains using conventional biochemical tests for identifying bacteria. On the basis of toxicity to mosquito larvae, as determined by bioassay, the various pathogenic strains of B. sphaericus can be divided into three major groups with, respectively, low, moderate and high toxicity, but the grouping depends on (a) test species (Culex species are the most sensitive to B. sphaericus, followed by Anopheles species, followed by the aedine species, of which some, such as

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Aedes aegypti, are very insensitive, while others such as A. melanimon, have been shown to be quite sensitive; Psorophora and Mansonia have also been shown to be highly sensitive); (b) bioassay conditions; (c) the nature and conditions of the bacterial culture medium.<sup>2</sup> In contrast to B.t. H-14, existing isolates of B. sphaericus are virtually nontoxic to blackflies. Specific levels of activity exhibited by the different toxic strains of B. sphaericus against mosquitos are noted and discussed in section 3, below.

## 2.2 Mode of Action

At present, the molecular mode of action of B. sphaericus is not known. However, in studies of the histopathology caused by B. sphaericus in mosquito larvae, the midgut epithelium appears to be the primary site of action, at least initially. After ingestion of sporulated cells of insecticidal strains, the epithelium distends and the gut is paralysed. This is followed by epithelial cell lysis and larval death.

Studies of the various insecticidal strains of B. sphaericus, particularly 1593 and 2297, and more recently 2362, have demonstrated that high toxicity is routinely associated with the presence of parasporal, proteinaceous inclusions, which vary in size depending on the isolate. Toxic activity, however, has also been shown to be associated with the spore, cell wall and cytoplasm of vegetative cells immediately prior to sporulation.

Studies on the isolation and identification of the toxin responsible for larval death have provided sound evidence that the primary toxin is a protein with a molecular weight of 43-55K. Immunological studies using antibodies raised against this protein have shown that a similar protein exists in all of the most toxic strains, e.g. 1593, 2297 and 2362. A single DNA fragment complementary to a gene or gene fragments encoding this protein (cloned from 1593) hybridizes strongly with DNA from all toxic strains, but only weakly or not at all with nontoxic strains, providing further evidence that all toxic strains of B. sphaericus contain a similar larvicidal toxin. This toxin is resistant to many proteases and to temperatures and pH levels within the ranges found under most field conditions. However, it can be destroyed in the laboratory above 80°C and at pH values above 9.5.

There is some evidence that the toxin of B. sphaericus is produced as a protoxin digested to a toxin form and perhaps activated by midgut conditions. The activated, cytotoxic and insecticidal B. sphaericus toxin is not toxic to cultured mammalian cells nor to blood cells, in contrast to the solubilized toxin of B.t. H-14. The B. sphaericus toxin has also a much more limited spectrum of activity against mosquito species than the B.t. H-14 toxin. As mentioned above, A. aegypti larvae and all blackfly larvae are insensitive to the toxin. The reasons for this are not clear at present, but there is some evidence from studies with A. aegypti that larvae of this species possess midgut proteases that inactivate the toxin soon after ingestion. Cell culture studies should be useful in determining the basis for the spectrum of activity, since cultured cells of Aedes spp. are significantly less sensitive to the toxin than are those of Culex quinquefasciatus.

## 2.3 Bioassay

A standardized bioassay, similar to that developed for B.t. H-14, has been developed for determining the potency of B. sphaericus preparations: the toxicity of preparations of unknown potency is compared with that of a standard preparation with an arbitrarily defined potency. The bioassay employs fourth-instar Culex pipiens pipiens as the test insect (and is described in detail in Annex II). Fourth-instar larvae of C. quinquefasciatus can be used as an alternative.

To date, two standard powders have been developed for use in bioassays: (1) RB 80, which is based on isolate 1593, serotype H-5a5b, and has a defined potency of 1000 toxic units (TU) per mg; and (2) SPH 84, which is based on isolate 2297, serotype H-25, and has an established potency of 1500 TU/mg in relation to RB 80. Both of these standards were formulated from dried, sporulated cultures of the designated B. sphaericus strains, and tests of their stability, particularly with regard to heat, have shown that they are stable.

#### 2.4 Potential for Improved Strains

Insecticidal B. sphaericus strains have been found in dead mosquitos, other aquatic insects and soil samples from mosquito habitats. Recently, new strains have been isolated from insects, including adult blackfly (2362), lepidopterous larvae and grasshoppers. Some of these strains are highly toxic to mosquito larvae but not to the insects from which they were isolated. This suggests that other potentially useful strains may be found in systematic isolations from soil samples, insects other than mosquitos, and water samples. Methods for the isolation of these bacteria utilizing media which specifically promote the growth of B. sphaericus have been recommended (Annex III).

The isolation of substrains from currently existing strains also holds promise for increasing strain toxicity. This technique, coupled with repeated passage of the bacterium through mosquito larvae, has led to the isolation of the highly toxic strain 1593M. A method for the initial screening of many isolates has been suggested (Annex III). This involves culture of selected bacterial colonies in small volumes of medium in tubes and assay of each tube against a single cup of susceptible larvae using a volume of water which provides a discriminating dosage of bacterial culture (e.g. 1:100 dilution).

There is some evidence that the high potency of the selected strains is due to the presence of multiple copies (3-5) of the biocide gene. In view of this evidence, the possibility exists that strains with even higher copy numbers and greater toxicity may be found.

To develop B. sphaericus strains with a mixture of desirable traits, such as broader spectrum of action, longer persistence under field conditions and greater recycling potential, genetic engineering techniques may be useful.

### 3. LABORATORY AND FIELD EVALUATIONS

Most of the laboratory and field studies on efficacy carried out to date have emphasized evaluations of primary powders and experimental formulations of isolates 1593, 2297 and 2362. The primary powders were prepared by several laboratories, including the United States Department of Agriculture Research Laboratory (USDA), Brownsville, TX, USA; Abbott Laboratories, North Chicago, IL, USA; the Central Drug Research Institute, Lucknow, India; and Solvay & Company, Brussels, Belgium. The results, discussed below, were presented as summaries of laboratory and field trials reported at the meeting and will be published subsequently by those who carried out the evaluations. No literature references are therefore available at the present time.

#### 3.1 Results of Laboratory and Field Trials

In general, both the laboratory and field evaluations showed that strain 2362 was the most effective against all species and instars tested, followed by 1593, which in turn was more effective than 2297 (Tables 1, 2 and 3). Variations from this general pattern, obtained in some field trials, appeared to be related to type of formulation and percentage of active ingredient. Although this assessment is by no means definitive because of the preliminary nature of the trials, there was general agreement that formulations of 2362 were the most effective, particularly in the field.

Table 1. Toxicity (mg/l) of *Bacillus sphaericus* isolates 1593, 2297 and 2362 to fourth instars of selected mosquito species in the laboratory (at 48 hr)<sup>a</sup>

Species	1593			2297			2362		
	LC50	LC90	LC90	LC50	LC90	LC90	LC50	LC90	LC90
<u>Culex quinquefasciatus</u>	0.03	0.043 (RB 80) <sup>b</sup>	0.047 <sup>c</sup>	0.035 <sup>c</sup>	0.047 <sup>c</sup>	0.005 <sup>d</sup>	0.005 <sup>d</sup>	0.009 <sup>d</sup>	0.009 <sup>d</sup>
<u>Culex gelidus</u>	0.006	0.022 (RB 80) <sup>b</sup>	nt	nt	nt	0.021	0.021	0.09 (BS 48) <sup>e</sup>	0.09 (BS 48) <sup>e</sup>
<u>Culex modestus</u>	nt	nt	0.008 <sup>c</sup>	0.002 <sup>c</sup>	0.008 <sup>c</sup>	0.003 <sup>c</sup>	0.003 <sup>c</sup>	0.007 <sup>c</sup>	0.007 <sup>c</sup>
<u>Anopheles albimanus</u>	0.08	0.21 (RB 80) <sup>b</sup>	0.21 <sup>c</sup>	0.085 <sup>c</sup>	0.21 <sup>c</sup>	0.062 <sup>d</sup>	0.062 <sup>d</sup>	0.18 <sup>d</sup>	0.18 <sup>d</sup>
<u>Anopheles gambiae</u>	nt	0.7 <sup>c</sup>	nt	nt	nt	nt	nt	0.7 <sup>c</sup>	0.7 <sup>c</sup>
<u>Anopheles quadrimaculatus</u>	0.08	1.91 (RB 80) <sup>b</sup>	1.77 <sup>c</sup>	0.09 <sup>c</sup>	1.77 <sup>c</sup>	0.06 <sup>d</sup>	0.06 <sup>d</sup>	1.77 <sup>d</sup>	1.77 <sup>d</sup>
<u>Psorophora columbiae</u>	0.012 <sup>c</sup>	0.089 <sup>c</sup>	nt	nt	nt	0.01 <sup>d</sup>	0.01 <sup>d</sup>	0.02 <sup>d</sup>	0.02 <sup>d</sup>
<u>Mansonia uniformis</u>	0.057	0.242 (RB 80) <sup>b</sup>	5.0	5.0	nt	0.123	0.123	0.417 (BS 48) <sup>e</sup>	0.417 (BS 48) <sup>e</sup>
<u>Aedes nigromaculis</u>	0.1 <sup>c</sup>	nt	25.0	25.0	nt	0.03 <sup>d</sup>	0.03 <sup>d</sup>	0.05 <sup>d</sup>	0.05 <sup>d</sup>

<sup>a</sup> This table gives only an approximate indication of the relative toxicity of the three isolates to selected species of mosquitoes.

<sup>b</sup> RB 80 is a primary powder prepared by the Institut Pasteur, Paris, France.

<sup>c</sup> Primary powder prepared by Dr H. Dulmage, United States Department of Agriculture, Brownsville, TX, USA.

<sup>d</sup> Primary powder prepared by Abbott Laboratories, North Chicago, IL, USA.

<sup>e</sup> Primary powder prepared by Solvay & Co., Brussels, Belgium.

nt not tested

### 3.1.1 Laboratory evaluations

In laboratory studies, 1593, 2297 and 2362 all proved toxic to species of Culex, Anopheles, Mansonia, Psorophora (Table 1) and Culiseta. However, none of the isolates was significantly toxic to Aedes aegypti, although A. melanimon and A. triseriatus were susceptible, which indicates that B. sphaericus may prove useful as a larvicide against some aedine mosquitos.

The experimental 2362 powder (Abbott Laboratories, ABG-6184) had the highest efficacy against Culex quinquefasciatus, with an LC<sub>90</sub> of about 0.01 mg/litre for fourth instars. With formulated materials, the flowable concentrate of 2362 ((Solvay & Co., BSP-1) was the most effective experimental formulation, with an LC<sub>90</sub> of 0.038 mg/litre against fourth instars of the same species. This is a very high level of efficacy for a product in a preliminary stage of development, as can be seen by comparing the efficacy of experimental B.t. H-14 preparations against C. quinquefasciatus and Anopheles albimanus during a similar stage of development (Table 2).

Table 2. Comparative toxicity (LC<sub>90</sub> mg/l) of experimental formulations of Bacillus thuringiensis H-14 and B. sphaericus 2362 against Culex quinquefasciatus and Anopheles albimanus

Species	<u>B. thuringiensis</u> H-14		<u>B. sphaericus</u> 2362	
	Bactimos(WP) <sup>a</sup>	ABG-6108D <sup>a</sup>	ABG-6184 <sup>b</sup>	Solvay BSP-1 <sup>c</sup>
<u>Culex quinquefasciatus</u>	0.28	0.41	0.01	0.038
<u>Anopheles albimanus</u>	0.23	0.46	0.18	1.10

<sup>a</sup> Wetttable powder (50% primary powder)

<sup>b</sup> Primary powder

<sup>c</sup> Flowable concentrate (12% primary powder)

### 3.1.2 Field evaluations

Field evaluations were conducted in several habitats, in organically enriched or clear water, and in shaded habitats or in full sun against natural populations of Culex spp., Mansonia spp., Anopheles spp. and Psorophora columbiae. Effective control (95-100%) was achieved by two primary powders of the 2362 isolate (USDA and Abbott Laboratories), the 2297 isolate and a flowable concentrate (Solvay & Co. BSP-1) of 2362 at 0.25 kg/ha against the Culex spp. The flowable concentrate provided 82% reduction of A. quadrimaculatus in mature rice fields at 1.0 kg/ha applied aerially with a Beecomist ULV generator. Virtually 100% control of Psorophora columbiae was achieved with a Beecomist-applied flowable concentrate at 0.5 kg/ha in recently flooded rice fields. (Results of these and other trials are given in Table 3.)

Results of field tests of the primary powders and flowable concentrate\* indicate that, in general, the primary powders were slightly more active than

\* It should be noted, however, that the flowable concentrate of 2362 (BSP-1) is only 12% active ingredient (primary powder).

Table 3. Efficacy of experimental formulations of Bacillus sphaericus isolates 1593, 2297 and 2362 against selected mosquito species in the field<sup>a</sup>

Species	kg/ha required for 90% reduction		
	1593	2297	2362
<u>Culex quinquefasciatus</u>	0.25 <sup>b</sup>	0.25 <sup>b</sup>	0.25 <sup>c</sup>
<u>Culex tarsalis</u>	0.22 <sup>b</sup>	nt	0.22 <sup>b</sup>
<u>Culex modestus</u>	nt	0.10 <sup>b</sup>	0.10 <sup>c</sup>
<u>Psorophora columbiae</u>	0.50 <sup>b</sup>	0.25 <sup>b</sup>	0.11 <sup>d</sup>
<u>Aedes melanimon</u>	0.75 <sup>b</sup>	nt	0.56 <sup>c</sup>

- <sup>a</sup> This table gives only an approximate indication of the relative toxicity of the three isolates to selected species of mosquitos.
- <sup>b</sup> Primary powder prepared by Dr H. Dulmage, United States Department of Agriculture, Brownsville, TX, USA.
- <sup>c</sup> Flowable concentrate (12% primary powder) prepared by Solvay & Co., Brussels, Belgium.
- <sup>d</sup> Primary powder prepared by Abbott Laboratories, North Chicago, IL, USA.
- nt Not tested

the flowable concentrate and that Culex spp. were more easily controlled than Anopheles. When preparations of the 1593 and 2362 isolates were applied to sewage effluent at concentrations of 7.5 and 8.2 mg/l, respectively, they resulted in 94 and 96% reduction of A. gambiae.

Some of the most impressive results achieved in the field to date with experimental formulations of B. sphaericus have been obtained against Culex quinquefasciatus in highly polluted waters in the Ivory Coast and in the United Republic of Tanzania. In the latter country, for example, the BSP-1 formulation of 2362 provided effective control of C. quinquefasciatus in cess-pits and latrines for as long as six to ten weeks, when applied at a rate of 10 g/m<sup>2</sup>. This is a high rate of application, but should the price of B. sphaericus be similar to that of B.t. H-14, B. sphaericus would appear to be cost-effective against strains of C. quinquefasciatus resistant to chemical insecticides (Table 4).

In addition to efficacy against C. quinquefasciatus, the BSP-1 and ABG-6184 preparations of 2362 have been shown to be relatively effective against Mansonia uniformis, an important vector of Brugian filariasis in South-East Asia. At a rate of 1 kg/ha, both these formulations provided an 80% reduction of larval populations for as long as 14 days after application.

### 3.2 Persistence versus Recycling

Persistence of B. sphaericus is defined here as the presence in the environment of the spore/crystal complex containing larvicidal toxin.

Table 4. Comparison, between Bacillus sphaericus and chlorpyrifos, of estimated cost of one year's control of Culex quinquefasciatus in the United Republic of Tanzania

	<u>B. sphaericus</u>	Chlorpyrifos
<u>Conditions</u>		
Formulation	BSP-1 (12% primary powder)	EC (480 g/l)
Dosage	10 g/m <sup>2</sup> = 0.010/m <sup>2</sup>	1 g/m <sup>2</sup> = 0.0010/m <sup>2</sup>
Frequency of treatment	9 rounds per year	24 rounds per year
Surface treated	500 m <sup>2</sup>	500 m <sup>2</sup>
<u>Cost US \$</u>		
Staff & equipment	378	1008
Control agent*	360	180
Total	738	1188

\* Price per litre of chlorpyrifos US \$15 and B. sphaericus US \$8 (estimated price based on that of equivalent formulation of B. thuringiensis H-14).

Recycling refers to the replication and sporulation of the bacterium in mosquito cadavers or in their aqueous environment, with subsequent larvicidal activity in the same habitat. Residual larvicidal activity may be due to the persistence of sufficient and accessible toxin and/or the recycling of the bacterium. Even though the amount of accessible B. sphaericus toxin may decrease steadily in certain habitats, prolonged control may continue due to the extremely high susceptibility of hatching larvae.

In India, larvicidal activity persisted against C. quinquefasciatus for six to ten weeks in clear shallow water in shade and in direct sunlight. In another study against the same species in Arizona, USA, control persisted for one week or less in clear shallow water in sunlight and up to four weeks in clear water in shade. In organically enriched habitats, control persisted for variable lengths of time depending on dilution by rainfall and the presence of ovipositing females. Larvicidal activity persisted for less than a week in septic ditches heavily inundated with rainwater and for over four weeks in sewage tanks in the presence of continued oviposition. Prolonged residual activity in polluted habitats in the presence of continued oviposition may have been due to proliferation of the bacterium in larval cadavers. In clear water in woodland pools with less dense larval populations, residual activity was probably due to sufficient accessible larvicidal toxins. Simulated studies in earthen jars using primary powders and formulations of B. sphaericus (strain 2362) against C. quinquefasciatus in tap water, with concentrations ranging from 10 to 200 mg per 40 litres of water, provided up to approximately four weeks of residual control.

When tested against natural populations of Mansonia (at 1 litre or 1 kg/ha), the flowable concentrate and primary powder provided good residual control. At day 7 and day 14 after treatment, the overall reduction of Mansonia larvae was 90.1 and 80.4%, respectively. Using application rates of 0.1-0.5 kg/ha of a flowable concentrate of 1593M against A. culicifacies and A. subpictus, one week of control was obtained.

Although it is clear that B. sphaericus recycles in nature and grows well in larvae cadavers, there is no confirmation at present that it recycles at levels high enough to provide effective long-term vector control. In fact, most evidence indicates that in situations where control is achieved for several weeks, residual activity is due to persistence of spores in the larval feeding zone rather than to recycling. As with many chemical insecticides, the residual activity or persistence of B. sphaericus is proportional to the rate of initial application.

Information on the factors influencing persistence is sparse, but preliminary studies indicate that extremes of pH and high levels of organic pollution, of ions and of solar radiation can influence residual activity.

### 3.3 Potential Use of B. sphaericus in Vector Control

B. sphaericus shares constraints with B.t. H-14 in being effective only against larvae and in having to be ingested by larvae to be lethal. Its apparently longer impact on larval populations would however reduce the number of applications needed for continuous, satisfactory vector larval control in endemic areas where oviposition occurs daily and the egg-to-adult life-cycle is relatively short under the prevailing high temperatures. Even in highly polluted habitats, such as cesspits and sewage dumps, B. sphaericus is effective against C. quinquefasciatus, which is a disease vector and nuisance pest in urban areas. Larviciding is an important component of integrated strategies against mosquito breeding in urban areas, and B. sphaericus could well prove to be a safe, economical and appropriate antilarval agent in this situation. Its use by unskilled personnel or local populations may be practicable. The results of laboratory, simulated field and actual field evaluations of B. sphaericus in various parts of the world highlight the potential of the agent against C. quinquefasciatus, which is responsible for over 80 million cases of Bancroftian filariasis globally.

An immediate priority, therefore, is to evaluate B. sphaericus further to determine its role as a safe, economical, antilarval component for use in integrated control programmes against C. quinquefasciatus in urban areas. Based on present knowledge, strain 1593 or 2362 should be selected for further development, with large-scale production of formulations, such as wetttable powders, liquid concentrates and granules, for comparative field evaluations against C. quinquefasciatus, followed by larger-scale trials in endemic and nonendemic urban areas.

A protocol for the field evaluation of B. sphaericus formulations is provided in Annex IV.

## 4. PRODUCTION

### 4.1 Media and Fermentation Conditions

B. sphaericus lends itself well to propagation by conventional fermentation techniques, and preparations of good potency and viability can be prepared. Conventional, relatively inexpensive, post-fermentation recovery techniques are quite satisfactory. Although studies are not complete, it appears that B. sphaericus strain 2362 is especially suited to fermentation

production. The most cost-effective way to produce B. sphaericus is by submerged fermentation in deep tanks.

The fermentation of B. sphaericus, though conventional, does pose certain mechanical or physical problems, which must be resolved by fermentation studies. For example, the water must not be too hard; its temperature must be carefully controlled to maintain the desired temperature in the fermentation medium; water used in this medium must not be polluted with wastes or chemicals toxic to the bacillus; the water recovered from the completed fermentation must not be allowed to pollute the surrounding environment; and the power supply must be reliable. Within these limitations, however, B. sphaericus fermentation is straightforward. The fermentation must be tailored to suit the final formulation planned for the product. Although liquid preparations are often highly effective, shipment of liquids, such as flowable concentrates, is generally more expensive per unit of field activity than shipment of dry formulations. The particle size of fermentation media must be sufficiently small to pass standard nozzles in delivery apparatus.

B. sphaericus can be grown in media containing a variety of proteinaceous substances. Unfortunately, it does not use carbohydrate as a carbon and energy source, and proteinaceous substrates may be more expensive and in some areas more difficult to obtain. A specific strain may not respond in the same way to different protein sources: it has been found that strain 2362 continues to produce toxin at high protein concentrations, whereas toxin production by strain 1593 is suppressed as the protein level is increased (Table 5).

Table 5. Effect of increasing protein concentration on sporulation and toxin formation by Bacillus sphaericus 1593 and 2362

Supplement	Strain 1593		Strain 2362	
	Spores/ml	LC <sub>50</sub> (ng/ml)*	Spores/ml	LC <sub>50</sub> (ng/ml)*
none	3.5 × 10 <sup>8</sup>	1.1	1.3 × 10 <sup>9</sup>	0.01
2% tryptone	4.6 × 10 <sup>5</sup>	41.4	9.5 × 10 <sup>8</sup>	0.03
4% tryptone	9.9 × 10 <sup>5</sup>	33.5	3.6 × 10 <sup>8</sup>	0.04
6% tryptone	1.1 × 10 <sup>5</sup>	136.0	6.4 × 10 <sup>8</sup>	0.04

\* Washed and resuspended bacterial cells bioassayed against L2 Culex quinquefasciatus. Assay read after 48 hours.

B. sphaericus has an absolute requirement for biotin and thiamin, but these may be supplied by complex medium ingredients, such as yeast extract or corn steep liquor. Cations, such as Mn<sup>2+</sup> and Ca<sup>2+</sup>, favour sporulation and associated toxin formation. Adequate cations may be present in local water supplies or can be added if sporulation seems poor.

It is possible to produce highly toxic cells with relatively short fermentation times. When the sporangia have developed, as indicated by the presence of swollen cells, they can be harvested, if heat-resistant spores are not needed. It is not yet known whether a high level of spores within the product is necessary for long-term larval control. Very toxic products containing low levels of spores could be efficiently prepared either by short-term batch or by continuous fermentation. Although adequate aeration and agitation are necessary for the growth of these bacteria, the optimum

level of oxygen required is not clear. A shift from air (0.8 litre air/litre medium/min) to pure oxygen at the same rate did not enhance toxicity nor did a decrease to 0.4 litre air/litre medium/min adversely affect toxicity in strain 2362.

Strain 2362 is able to produce toxin at higher temperatures than strain 1593 (Table 6). This may be important for fermentation in tropical countries, where the supply of fermentor cooling water may be limited.

A useful, detailed description of fermentation requirements and equipment can be found in "Guidelines for the Production of Bacillus thuringiensis H-14".<sup>3</sup> These apply equally to the production of B. sphaericus.

Table 6. Effect of growth temperature on sporulation and toxin formation by Bacillus sphaericus 1593 and 2362

Temperature (°C)	Strain 1593		Strain 2362	
	Spores/ml	LC <sub>50</sub> (ng/ml)*	Spores/ml	LC <sub>50</sub> (ng/ml)*
25	3.2 x 10 <sup>8</sup>	0.1	9.7 x 10 <sup>8</sup>	0.07
29	2.2 x 10 <sup>8</sup>	0.6	1.0 x 10 <sup>9</sup>	0.05
35	3.5 x 10 <sup>6</sup>	9.6	6.9 x 10 <sup>8</sup>	0.04

\* Washed and resuspended bacterial cells bioassayed against L2 Culex quinquefasciatus. Assay read after 48 hours.

#### 4.2 Formulations

The effectiveness of B. sphaericus strains depends partly on the target species, environmental conditions and level of toxin present in spore preparations, and partly on formulation and application method. Industry has produced a wide variety of formulations of B.t. H-14 to control a number of mosquito and blackfly species in a multitude of habitats. Until last year, industrial interest in the commercial development of B. sphaericus was low. Recently, primary powders and a flowable concentrate of B. sphaericus have been produced by industry and by the United States Department of Agriculture. The flowable concentrate formulation has facilitated aerial application of undiluted material with a ULV generator for the control of Anopheles and Psorophora larvae in rice fields. It has also provided good control of culicines in polluted habitats due to a slower settling rate than is observed with primary powders. Small-scale formulation of briquettes, pellets and granules have provided additional material for evaluation. These formulations have resulted in effective and sustained control of several susceptible mosquito species in various environmental settings.

#### 4.3 Local Production

##### 4.3.1 Current status

Several countries where tropical diseases are endemic have developed some technical capability for the production of B.t. H-14. Although the media employed for B. sphaericus and B.t. H-14 are different, the fermentation parameters and facilities are to a large extent interchangeable. In view of the urgent need for control of insecticide-resistant strains of C. quinquefasciatus in many tropical countries, particularly in the filariasis control

programmes, it becomes essential that local production of B. sphaericus be initiated whenever possible.

Considerable work has been carried out in India and in Thailand on the production of B. sphaericus 1593, and laboratories in Ghana, Nigeria and the Philippines have experience in the production of B.t. H-14 relevant to the production of B. sphaericus. Fermentation media based upon meat extract and yeast extract have been used in the Indian programmes, and less expensive nutrients originating from by-products of other bacterial fermentations have been used in Thailand. The use of blood-based media has been tested in Nigeria. Concern exists regarding the reproducibility of fermentations using some raw materials. To prevent contamination of fermentations, the same standards of equipment sterilization and site sanitation that are applied in industrialized countries must be applied to facilities in developing countries.

#### 4.3.2 Quality control

Quality control is critical to effective and uniform control programmes. The standard RB 80 available from the WHO Collaborating Centre at the Institut Pasteur, Paris, should be used to assess the final potencies of fermentation batches. Careful evaluation of the cost of production vs. the potency of samples will be required. In addition, the persistence and recycling potential of B. sphaericus may also require evaluation when the bacterium is produced under different conditions. Thus, quality control after production is needed, followed by observations of efficacy in the field by experienced personnel.

### 5. SAFETY

#### 5.1 Effects on Non-Target and Beneficial Fauna

An important consideration in the development of pest control agents is the assessment of the safety spectrum of candidate materials to non-target and beneficial fauna coexisting with and exerting regulatory pressure on disease vectors. This requirement is applicable to both chemical and biological control agents. The environmental safety of biological control agents is measured not by the immediate response of groups or individuals but by the effect on populations of various taxa (both predators and detritus feeders) cohabiting with target species.

A number of studies on the effects of B. sphaericus strains on non-target predacious organisms have been conducted. Emphasis has been placed on insects and crustaceans commonly found in mosquito breeding sites. Additional data have also been gathered on fish found in mosquito breeding sites and on honey-bees which might visit treated sites and drink water treated with this bacterial agent.

So far, no adverse effects of B. sphaericus have been noted on dominant non-target and beneficial fauna coexisting with mosquito larvae. Larvicidal rates of B. sphaericus (1593 and 2362 primary powders) had no adverse effects, over seven to eight weeks, on populations of the crustaceans Eulimnadia taxana, ostracods in the genera Cyprinotus and Cypridopsis, the cladoceran Moina rectirostris, the copepod Cylops vernalis and several other species of crustacea. The macroinvertebrate fauna, including members of Anisoptera, Zygoptera and Ephemeroptera, were not adversely affected, nor were species in the families Corixidae, Notonectidae, Dytiscidae, Hydrophilidae and Chironomidae. In some limited studies, larvae of Culicoides midges (Ceratopogonidae) were affected at dosages much higher than those needed for mosquito control.

Tests with aqueous suspensions of B. sphaericus (SSII-1 and 1593), using high rates of application, produced no acute mortality in honey-bees (Apis

mellifera), nor did they affect their life span. Similarly, rates as high as a hundredfold the larvicidal rate of 1593M showed no adverse effects on fish coexisting with mosquitos.

In aquatic habitats supporting mosquitos, a large number of predacious macroinvertebrates exist together with the target insects. Although field treatments with B. sphaericus (1593 and 2362) caused no noticeable effects, a laboratory trial was carried out, in which B. sphaericus-infected mosquito larvae were offered as the only source of food to several predacious organisms. Culex larvae (L4) exposed to 1000 mg/l (100 times the larvicidal rate) were offered for several days to predators, such as dragon-flies, damsel fly naiads and the notonectid Notonecta unifasciata: the full daily food requirement of these predators was provided by larvae whose guts were filled with a lethal dose of B. sphaericus (2362, BSP-1). Predation on treated larvae did not induce any acute adverse effects nor alter the developmental rates of the predacious organisms.

Similarly, predation by larvae of several species of Toxorhynchites (family Culicidae) on larvae of C. quinquefasciatus exposed to B. sphaericus (2013, 1593) at higher than larvicidal rates produced no adverse effects. Larvae of one species (Toxorhynchites rutilus rutilus), fed on larvae of C. quinquefasciatus exposed to 50-100 times the LC<sub>100</sub> concentration of B. sphaericus (1593), suffered significant mortality at this extremely high concentration. When T.r. rutilus was exposed to 20 mg/l of strain 2297 in the presence of prey larvae, no adverse effects were noted.

From the data currently available, no effects of B. sphaericus strains (SSII-1, 1593, 1593M, 2362, 2297) have been noted at rates of application used for effective larviciding. This bacterial agent is highly selective. Nevertheless, additional studies are needed to confirm the safety of B. sphaericus to non-target organisms.

## 5.2 Mammalian Safety

Although safety testing of B. sphaericus has not been exhaustive, a significant amount of mammalian safety data exists. The data are reassuring and indicate that several entomopathogenic B. sphaericus strains are not hazardous to mammals. Further studies are indicated, as noted in the recommendations below (section 7), but the consultation was reassured that not only the efficacy of B. sphaericus but also its lack of significant hazards warranted further development of this agent.

De Barjac used strains 1593 (serotype H-5a5b)<sup>4</sup> and 2297 (serotype H-25) for: subcutaneous and intraperitoneal injections in mice; intracerebral injections in mice; intragastric administration in mice and rats; inhalation and percutaneous application in mice; anaphylaxis tests in guinea-pigs; serial intraperitoneal injections in mice; repeat feeding in mice; and a search for virulence or persistence in heart blood of mice. Doses of  $1 \times 10^7$  to  $1 \times 10^8$  viable bacteria per animal were used. Media were either nutrient broth or MBS medium. Neither mortality nor signs of clinical illness were observed. No changes in behaviour nor depression of body weight gain were observed; no macroscopic lesions were found at necropsy. B. sphaericus was not re-isolated from the animals at the end of the experiments; there was no evidence of its persistence in blood nor of increasing virulence upon repeated animal passages.

Increases in spleen or liver weight were detected in some animals given B. sphaericus intravenously or intraperitoneally. The organs were not examined histologically. An alkaline extract of the spore/crystal complex of strain 2297 was injected into mice without effect.

Shadduck and colleagues could not demonstrate any mortality or clinical illness in animals given B. sphaericus strains 1593 (serotype H-5a5b), SSII (serotype H-2) and 1404 (serotype H-2) grown on synthetic media.<sup>5</sup> Subcutaneous administration of  $7 \times 10^9$  B. sphaericus strain 1404 resulted in a subcutaneous abscess in one of the five mice injected, but all other mice were unaffected by the injection of this or smaller doses of strain 1404 or 1593. Intraperitoneal injection of rats with  $3 \times 10^8$  viable B. sphaericus strain 1404 or similar doses of strain 1593 or equal doses of the autoclaved organisms produced no detectable lesions.

Intracerebral injection of mice, rabbits and rats produced lesions in animals given either viable or autoclaved B. sphaericus, and the organism was recovered several times from rats given high intracerebral doses of each of the three strains. Intraocular injection of B. sphaericus at several different doses also resulted in the recovery of organisms. Lesions occurred in animals given either autoclaved or viable organisms. None of the three strains was a significant eye irritant in the standard rabbit eye irritation test. In an experiment to measure intracerebral replication of strain 1593, only 600 bacteria per 100 mg of wet brain tissue were recovered three days after injection of  $5 \times 10^5$  viable organisms, and by day 5 the number had fallen to fewer than 10 bacteria per 100 mg. No more than 30 bacteria per 100 mg of brain were ever recovered after day 3, and by day 14 the brains were sterile.

Several other laboratories have carried out limited animal safety tests and no untoward effects have been noted.

Taken together, these studies show that high doses of B. sphaericus strains 2297, SSII, 1404 and 1593 are without effect when given by conventional routes to mammals. Injected intracerebrally, they can produce mild lesions in the brains of rats and, injected intraocularly, more severe lesions in the eye of rabbits, but the presence of lesions in the brain and eye following injection of autoclaved preparations indicates that the lesions may result from the injection of high concentrations of foreign proteins. B. sphaericus is capable of surviving in mammalian tissue but is cleared rapidly. Since lesions occurred only at the highest doses in the most vulnerable mammalian target sites and were in large measure the result of injection of foreign material, the evidence indicates that the isolates of B. sphaericus studied to date are avirulent for mammals; it seems highly unlikely that they pose any hazard to man.

Although there is no evidence that B. sphaericus is a human or animal pathogen, organisms belonging to the B. sphaericus group have been recovered from human lesions. In all likelihood, the frequency of these recoveries will increase as a result of the increased use of B. sphaericus, and the intense scrutiny given to patients will result in an increased chance of finding infection by latent microorganisms. B. sphaericus has not been shown to be an allergen or severe irritant, but many materials which, like B. sphaericus, are fine powders of organic material, can be irritant to mucous membranes or can occasionally induce immediate or delayed hypersensitivities. It seems probable that these reactions will be reported in some individuals frequently exposed to high concentrations of B. sphaericus powders.

There is strong evidence that B. sphaericus is safe. Nevertheless, further studies are necessary, as noted below.

## 6. CONCLUSIONS

6.1 The most toxic B. sphaericus isolates available are more effective than B.t. H-14 against species of Culex, Mansonia and some species of anopheline mosquitoes and on this basis alone warrant further development and evaluation.

6.2 Of the existing isolates of B. sphaericus, isolates 1593, 2297 and 2362 show considerable potential for development as cost-effective larvicides. Based on fermentation studies and field evaluations, 2362 is at present the best candidate for further development.

6.3 Existing effective B. sphaericus isolates are toxic to most mosquito species but are nontoxic to some species (e.g. A. aegypti) and to blackfly larvae. Use of existing isolates will therefore be restricted to mosquito control programmes.

6.4 Experimental primary powders and formulations of 1593, and particularly of 2362, prepared by industry and the USDA have proved two to five times more effective as larvicides against Culex and some Mansonia species than existing B.t. H-14 formulations. There is also limited laboratory evidence studies that B. sphaericus formulations are also more effective than those of B.t. H-14 against some anophelines.

6.5 In contrast to B.t. H-14, experimental formulations of B. sphaericus 2362 (BSP-1) at rates of  $10 \text{ g/m}^2$  ( $2 \times 10^{10}$  spores/g) controlled insecticide-resistant strains of Culex quinquefasciatus breeding in cesspits and pit latrines for at least six weeks and, at some sites, as long as ten weeks after a single application. Thus, B. sphaericus has the potential for development as a larvicide with significant residual activity, for use in polluted waters against insecticide-resistant vectors of filariasis.

6.6 Existing B. sphaericus isolates/strains are amenable to fermentation/production with existing technology and facilities used for the production of B.t. H-14.

6.7 Currently available formulations of B. sphaericus are effective as larvicides, but their efficacy could possibly be increased by: (a) isolation of more toxic strains and strains with a broader host spectrum; (b) development of better formulations tailored to a wider variety of habitats and for longer periods; and (c) the use of genetic engineering/recombinant DNA technology.

6.8 Currently available evidence is insufficient to determine definitely whether the recycling of B. sphaericus in treated habitats contributes significantly to the documented residual activity of experimental formulations. At present, it appears that residual activity is due primarily to persistence. Further studies are required to determine whether B. sphaericus recycles at levels high enough to provide effective long-term vector control.

6.9 Studies on the fermentation of strain 2362 indicate that maximum toxin production is achieved just prior to spore formation. Thus, it may be possible to reduce costs by terminating fermentation prior to spore maturation, provided it is ultimately demonstrated that the contribution of the spores to toxicity and residual activity is minor.

6.10 Existing formulations of B. sphaericus are stable and maintain their efficacy for at least six months.

6.11 All current evidence indicates that B. sphaericus is a larvicide highly specific for mosquitos. There are no data indicating that B. sphaericus is toxic or pathogenic to mammals, other vertebrates or non-target invertebrate organisms.

## 7. RECOMMENDATIONS

7.1 Based on preliminary indications that B. sphaericus, particularly isolate 2362, is an effective larvicide for Culex quinquefasciatus and that

appropriate formulations have significant residual activity, it is recommended that more extensive field trials of the most effective formulations be carried out against selected mosquito species. Trials should focus on major vectors of filariasis, such as species of Culex and Mansonia, and vectors of malaria, including Anopheles albimanus, A. gambiae and A. culicifacies. Preference should be given to further evaluation of formulations based on isolate 2362.

7.2 The search for more effective strains of B. sphaericus should be continued, with emphasis on the isolation of more toxic strains and strains with a broader host spectrum. Attempts should be made to obtain isolates that are highly toxic to species of Anopheles.

7.3 The use of classical genetic techniques and recombinant DNA technology to engineer more toxic strains of B. sphaericus should be considered.

7.4 Biochemical and physical factors that affect the fermentation of B. sphaericus and influence sporulation and toxin production should be further investigated.

7.5 The development of more effective formulations, including flowable concentrates, wettable powders, granules, pellets and sustained-release formulations for use in a variety of habitats, should be pursued. The participation of industry in this endeavour should be encouraged. The development of more stable formulations is of high priority.

7.6 The local production and/or formulation of B. sphaericus in countries where tropical diseases are endemic should be encouraged. Emphasis should be placed on the development of cheap and efficient media based on locally available ingredients, and on safe production and use. The assistance of other TDR Components and other United Nations agencies, such as the United Nations Industrial Development Organization, should be sought when appropriate to encourage the effective local development of larvicides.

7.7 The precise nature of the residual activity observed in situations where B. sphaericus is applied should be elucidated. The question of whether B. sphaericus can recycle at levels high enough to produce effective long-term vector control should be resolved.

7.8 Field trials should be conducted to determine the importance of viable spore counts on the persistence of larvicidal activity and recycling.

7.9 Because the toxin produced by B. sphaericus is different from that of B.t. H-14 and is not active against blackfly larvae, studies of its mode of action should be continued.

7.10 Continued emphasis should be placed on ensuring that existing and new formulations of B. sphaericus are safe to mammals and other non-target organisms. Further safety tests should be carried out on new formulations, as indicated, to assure that modifications of media or other growth conditions have not produced alterations that affect safety. Strain 2297 (serotype H-25) should be tested more extensively, since this strain is entomologically and serologically distinct from strain 1593 (serotype H-5a5b). On the other hand, strain 2362 (serotype H-5a5b) is very similar to strain 1593, and safety data collected on 1593 can be extrapolated to 2362. Inclusion of proper control animals (or patients) and inocula in all experiments is crucial to allow investigators to distinguish between background illnesses or lesions, effects of B. sphaericus as a viable microbiological entity, and its ability to act as a foreign body or nonspecific irritant. It is recommended that irradiation (UV, gamma) rather than heat be used to produce an inactive control inoculum, since irradiation is less likely to denature proteins and inactivate toxins.

7.11 Reference strains and antibiotic sensitivity data should be supplied to appropriate government or reference laboratories in regions where B. sphaericus is intended for use. Human and animal isolates should be compared to reference strains and submitted to the WHO Collaborating Centre at the Institut Pasteur in Paris, to be serotyped. The availability of this service should be widely publicized.

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\*\* Available from: World Health Organization, HQ/VBC, 1211 Geneva 27, Switzerland. The full document number should be cited as in the bibliographical reference.

ANNEX I

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## ANNEX II

BIOASSAY METHOD FOR THE TITRATION OF BACILLUS SPHAERICUS  
PREPARATIONS WITH RB 80 STANDARD

The bioassay is based on a comparison of mosquito larval mortality produced by a B. sphaericus 1593 standard with mortality caused by the test sample. Using Culex pipiens pipiens larvae, the activity of the standard has been assigned the value of 1000 toxic units (TU) per mg.

The standard powder (50 mg) is weighed and placed in a 20-ml penicillin-type flask, to which is added 10 ml deionized water and 15 glass beads (86 mm diameter). The contents are thoroughly homogenized by shaking on a crushing vibrator machine (e.g. Dangoumau type) for 10 min at 700 strokes/min. From this homogenate, a stock solution is made in a test-tube (22 mm diameter) by adding 0.1 ml of the homogenate to 9.9 ml deionized water, followed by maximum agitation on a Vortex agitator for a few seconds. The concentration in the stock solution is 50 mg standard/litre.

To carry out assays, 148 ml deionized water is placed in each of a series of plastic cups. To each cup, 25 early L4 larvae of C.p. pipiens in 2 ml water are added by means of a Pasteur pipette. With precision pipettes or micropipettes, 120, 90, 60, 30, 24 and 15  $\mu$ l aliquots of stock standard solution are added to the cups to obtain final concentrations of 0.04, 0.03, 0.02, 0.01, 0.008 and 0.005 mg/l, respectively, of the standard. Two or four cups are used for each concentration and for the control, which contains only 150 ml deionized water and 25 larvae in each cup.

A small amount of food (e.g. ground mice biscuit) is added to each cup in order to avoid excessive mortality.

Early L4 larvae are used, as they are more representative of the total susceptibility of the target population and easier to handle. In bioassays with Anopheles spp. (e.g. stephensi), which are less susceptible than Culex spp., L3 larvae should be used. A small amount of food should be added daily, especially if the assay is extended to 72 hr.

For bioassay of preparations of unknown activity, an initial homogenate is made, and comparable dilutions are prepared as for the RB 80 standard. The range of dilutions should exceed that of the standard in order to obtain a reliable regression line. Time can be saved by first making a range-finding bioassay, with widely spaced concentrations of test preparation. The data obtained can be used to fix the subsequent concentrations in an exact bioassay and as a partial replicate of this bioassay.

Each bioassay series should preferably involve at least 400 larvae for the standard and 100 larvae for the control; for preparations of unknown activity, 500 to 1000 larvae should be used. All tests should be conducted at  $25 \pm 2^\circ\text{C}$ .

In bioassays with C.p. pipiens, mortality is determined at 24 and 48 hr and is based mainly on the counting of live larvae. Because B. sphaericus acts more slowly than E.t. H-14, higher mortality is most often observed from 24 to 48 hr and even after 72 hr. Therefore, 48-hr LC<sub>50</sub> values are usually quoted. If pupation occurs, the pupae should be removed and their numbers deducted from the number of larvae initially placed in the cup.

With the slower reacting Anopheles, it may be necessary to wait 72 hr and determine both the 48-hr and the 72-hr LC<sub>50</sub>. When control mortality exceeds 5%, the mortalities of treated groups should be corrected according to Abbott's formula. Tests with control mortality higher than 10% should be discarded. Mortality-concentration regression lines should be drawn on gaussian logarithmic paper. Based on the LC<sub>50</sub> values of standard and unknown preparations, the titre of the latter is determined by the following formula:

$$\text{titre of unknown (TU per mg)} = \frac{1000 \text{ TU} \times \text{LC}_{50} \text{ of standard}}{\text{LC}_{50} \text{ of unknown preparation}} \quad (\text{on } \underline{\text{C.p. pipiens}} \text{ or other species})$$

For increased accuracy, bioassays should be repeated on at least three different days and the standard deviation calculated.

If SPH 84 standard is used instead of RB 80 standard, the same range of concentrations may be used. The titre of SPH 84 has been determined to be 1500 TU C.p. pipiens per mg, with reference to RB 80.

ANNEX III

TECHNIQUE FOR THE SELECTIVE ISOLATION OF INSECTICIDAL  
BACILLUS SPHAERICUS FROM SOIL SAMPLES

1. Samples are collected using sterile or disposable tools (e.g. plastic spoons) and placed into sterile vials or new plastic bags. Samples (1 g) are weighed out using sterile tools on new weighing papers.
2. Samples are dispersed by shaking in 1 ml of sterile distilled water or saline solution and pasteurized (80°C for 12 min).
3. From each pasteurized sample, several flasks of NYSM medium<sup>1</sup> are inoculated, using 0.1 ml of the pasteurized sample per flask.
4. Flasks are incubated at 28°C and 150 rpm for 48 hr. At the end of this time, the growth in the flasks is inspected for the presence of typical B. sphaericus spores (spherical, terminal or subterminal -- the latter two causing the sporangium to swell).
5. Samples (1 ml) from each flask are pasteurized, as above.
6. These samples are plated on selective agar medium,<sup>2,3</sup> Plates are incubated at 28-30°C for 24-48 hr. Colonies with characteristic B. sphaericus morphology (circular, tan, cratered centre) are selected and numbered individually. Bacteria from each colony are observed microscopically using an oil immersion (X100) objective. Colonies exhibiting typical B. sphaericus spore morphology are noted, as is the presence or absence of parasporal inclusions.
7. Colonies which appear to be B. sphaericus are inoculated into fresh NYSM flasks, which are incubated for 8 hr as before.
8. Cultures are bioassayed against Culex pipiens pipiens or C. quinquefasciatus larvae (using the method shown in Annex II). Any culture demonstrating insecticidal activity is streaked onto a jar for isolation, then recultured and assayed, and a viable cell count carried out.

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ANNEX IV

PROTOCOLS FOR LABORATORY AND FIELD EVALUATIONS

B. sphaericus, a spore-forming organism, has been shown to manifest a high level of activity against mosquitos in the genera Culex, Aedes, Anopheles, Mansonia and Psorophora. Most of these are widely distributed and some are vectors of pathogenic organisms. The following protocol is provided to facilitate field trials for the evaluation of experimental and commercial B. sphaericus formulations.

A. Preliminary Field Trials

When promising strains, preparations and formulations become available, they should first be tested in simulated field conditions. Breeding sites that can be treated include jars, barrels, tanks, soak pits and small pools or ponds, etc. The following procedure should be employed for gathering data in simulated field conditions.

1. Select several identical breeding sites; determine pretreatment larval population density by dipping or by another appropriate technique, taking at least four samples per site.
2. Assign sites to various treatments, including controls, which are left untreated. Perform each treatment at least twice. Several dosages of each formulation should be tested.
3. Apply liquids, granular, pellet or other formulations evenly, using any suitable available equipment. The rate of application should be expressed per unit area or per unit volume. Application rates of active material, formulations, or both, should be carefully recorded.
4. Take post-treatment samples two days later and at weekly intervals after treatment for asynchronous species. For synchronous species, such as Aedes and Psorophora, only a single two-day sample need be taken.
5. Larval counts should be divided into 1st/2nd and 3rd/4th instars. Pupal counts should also be recorded.
6. Counts of non-target organisms present should be included in these evaluations.
7. Data from each replicate should be averaged for each treatment and interval, and the percentage reduction in 3rd/4th instars calculated.
8. If there are no natural populations in the experimental units, laboratory or field-collected larvae can be used in sentinel cages.
9. Data on biotic and abiotic parameters, such as vegetation, presence of predators, extent of sunlight, water temperature and quality, pollution level, water flow and dilution, should be gathered.
10. It is desirable that the data for treatments and controls be analysed statistically and the significance of means tested. It is important that

average trends of untreated target and non-target fauna be established and plotted over the whole period during which B. sphaericus remains effective.

B. Small-Scale Field Trials

After evaluation of B. sphaericus strains and formulations under simulated field conditions, promising powders or formulations should be tested in small plots in actual field situations. The same basic requisites, sampling regimen and evaluation parameters apply. In small-scale field trials, the plot's surface size can vary from one to several square metres. For the larger size of plots, formulations may have to be applied by pressurized spray cans or broadcasters of solid formulations. It is important that several dosages of each formulation are tested, so that the lowest effective rate for a given species and situation is determined.

To assess persistence of action, it is desirable to compare one, five, or ten times the effective dosages to determine how high dosages may influence the period of control. For some species and in some situations, higher rates of application are justified to obtain long-lasting control.

C. Large-Scale Field Trials

In these trials, large expanses of breeding sites, such as rice paddies, underground storm drains, freshwater and salt marshes, cisterns, cesspits and other major sources of mosquitos, should be treated with B. sphaericus formulations. Ground and aerial equipment may be employed in these trials. Control plots or areas should be sampled along with treated plots or areas. In these trials it is not necessary to use numerous application rates: the rate providing 100% reduction of larvae in small-scale trials should be used. Alternatively, for long-lasting control, the laboratory LC<sub>100</sub> rate may be increased by five, ten or more times if necessary.

The procedures and evaluation steps used under simulated field conditions will suffice for large-scale field trials. However, additional information on the physical condition of formulations, ease of application and suitability of application equipment should be noted and recorded. It is very important to observe and describe the details of the experimental plots, particularly when collecting data on various biotic and abiotic parameters.

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