Qualitative and quantitative changes in the mosquito fauna in the city of Szeged in 1999 and production, concentration and toxicity control of a *Bacillus thuringiensis* subsp. *israelensis* (Bti) product which is appropriate for mosquito control

Summary of PhD Thesis

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I. INTRODUCTION

Phylogeny indicates that mosquitoes appeared on the Earth 93 million years ago. At present maybe 45 species can be found in Hungary. Mosqitoes have long been unpleasant for humans because the female individuals of some species need to suck human blood for their reproduction. There are other mosquito species which prefer the blood of animals, birds, amphibians or reptilians. Mosquitoes may spread dangerous diseases through their blood-sucking.

The Culicidae fauna of Hungary were investigated over a long period by Mihályi and his co-workers. The mosquito assemblages in the Tisza Basin have been monitored by others too *(Mihályi et al., 1953; Mihályi, 1954; Zoltai, 1957; Tóth, 1977; Branka, 1984; Kertész, 1987)*, but no study has yet been reported on urban mosquitoes living in a disturbed habitat. One of the most important tasks in combating the problem caused by mosquitoes has always been to identify the entomological situation. Weekly chemical anti-mosquito treatment can lead to changes in the composition of the mosquito assemblages. The density of natural mosquito assemblages is influenced by the flood wave of the River Tisza and by the current rainfall. The mosquito assemblages in the Tisza Basin are similar to those of the urban mosquitoes in the early and wet springtime. In the course of an arid season, the multivoltine species accompany the several-generation mosquitoes. The individual numbers of the Culex genus can be markedly high in a season without rainfall. The species of the Culex genus lay their eggs on the surface of water in the form of rafts *(Mihályi, 1963)*.

People began to think about protection against mosquitoes only last century. For this purpose, floral poisons were utilized. These were effective, but expensive. Pesticides with neurotoxic effects (e.g. DDT) and later phosphoric-acid-esters (e.g. Diazinon and Gesarol M) have been used more recently. Anti-mosquito treatment must be applied prudently because these chemicals are additonally dangerous for aquatic and land animals *(Mihályi 1963)*.

There is considerable interest in the use of alternative strategies for insect control, such as biological treatment. Biological products obtained for *Bacillus thuringiensis ssp. israelensis (de Berjak, 1978),* and *Bacillus sphaericus (Meyer and Neide, 1904)* provide a possibility for effective protection without endangering the environment.

The serovar of *Bacillus thuringiensis* is increasingly used as an ecologically friendly anti-mosquito agent or for biological plant protection. According to *Klier and Rapoport* (*1987); Thanabalu at al. (1992)* and *Pramatha (2000),* in the course of plant protecion the genome of the δ-endotoxin is often built into the genetic stock of the plants.

Bacillus thuringiensis ssp. *israelensis* (Bti) bacteria can be successfully applied for the elimination of mosquito larvae. This is due to the fact that their spores contain a crystalline protein (δ-endotoxin) which, after entering the digestive system, starts to activate and kill the mosquito larvae (*Gill et al., 1992)*.

Insects closely related to mosquitoes can be endangered by an overdose of the Bti product. The death of these non-target organisms can be avoided by precise dosage of the Bti product.

Szmirnov et al. (1986) reported that the δ-endotoxin is formed next to the Bti spore during sporulation. The manual of *Bergey (1986)*, and the publication by *Szmirnov and et al. (1986)* state that the fermentation of the bacteria is promoted by the substrate of glycolysis, and inhibited by increase of the metal concentration of the medium. Sporulation can be stimulated either by the depletion of the nutrient of the medium, or by increase of the Ca^{2+}, Mg^{2+}, K^{+}, Mn^{2+}, Fe^{3+} and Zn^{2+} concentrations. The sporulation of immobilized bacteria is quicker, than that in a medium with free cells.

Formulation of the Bti suspension and its use for practical purposes are possible only with relatively concentrated systems. Many problems relating to the concentration and thickening of microorganism suspensions can be solved by using water-soluble polymers/polyelectrolytes. This is due to the high efficiency (addition of polymers in ppm amounts can drastically change the degree of cell aggregation), relatively low cost and simplicity of the flocculation process. Flocculation by polymers can be used for the concentration of cells either as an independent (main) method or as an auxiliary means of enhancing conventional phase separation processes such as sedimentation, filtration, centrifugation or flotation.

The Bti spores must be applied to the water in which the mosquito larva breed. The sprayed larvicide Bti product adheres to the vegetation. For its subsequent dissolution, the Bti spore suspension needs to be immobilized as heavier granules. The Bti spores suspended in the breeding water exert a toxic effect on mosquito larva.

The toxic effect of the Bti product does not depend on the breeding of the Bti bacteria because the δ-endotoxin is coded by a plasmid which is not part of the Bti chromosome. The larvicidal toxicity of the Bti product must be checked from time to time. It is important to determine the sensitivity of biological organisms to the Bti product.

The age of the mosquito larva is an important factor in the toxicity. *Federici (1995)* found that younger larva are more sensitive than older ones to Bti spores,.

The toxin of Bti spores acts selectively on the biting mosquito larva. It is currently believed that Bti bacteria are harmless to other aquatic organisms.

Aims:

- To survey the sites and densities of mosquitoes in Szeged.

- To produce an optimum Bti spore product.

- To concentrate the Bti spores from a relatively dilute Bti suspension.

- To produce a product containing Bti immobilized on a heavy granule.

- To monitor by biological tests the sensitivities of biological organism to the Bti product.

II. Methods

Ecological examinations were performed for the: morphological and ecological characterization *(Mihályi and Gulyás, 1963)* of mosquitoes collected by the human-trap method *(Erdős et al. 2001)*. Biological testing was based on MSZ: EN ISO 6341 (1998). This method was applied for quicker determination.by the examination of younger larva.

The toxicity tests on the mosquito larvae were performed by determining the inhibition of mobility. The initial efficient Bti concentration (24-hour EC50) was determined preventing, i.e. the concentration sufficient to kill 50% of the larvae. The breeding water served as medium for the larvae. For each Bti concentration, 30-75 individuals of 3^{rd} instant larvae of *Aëdes vexans* were used in 100 ml of local breeding water, with and without the addition of flocculant; the total volume of the test tube was 200 ml. Increasing amounts of the Bti product were added to the water containing the larvae. The tests were performed during 16/8 photoperiods at room temperature. A space without toxic vapour was ensured. After a 24-hour exposure, the number of killed individuals was counted and expressed as a percentage of the initial number of larvae. The retardation of the mobility of the larvae corresponding to a given concentration of the flocculant was likewise determined.

For individual observation, the mosquito larvae were placed in 3 ml holes in a tissue breeding plate. The results of individual observations and the test tube findings were similar.

The microbiological and biotechnological methods included the fermentation of Bti cells *(Smith, 1982)*, counting of the living cell number (diluted plaiting) *(Alföldi and Hegedűs, 1952)*, determination of the sporulation (by Schaeffer-Fulton aggressive painting) *(Arnold et al., 1992)*, determination of the dynamics of Bti culturing (diluted plaiting) *(Alföldi and Hegedűs, 1952)* and measurement of the pH *(Arnold et al., 1992)*.

The biochemical examinations involved determinations of free amino-acid content *(Hušek,* 1991), and determinations of total sugar content (anthrone-method) *(Ábrahámné et al., 2004)*.

The colloid chemical examinations related to the charge determination of water-soluble polymers (flocculation method) *(Szántó, 1987)*, performed under dynamic conditions in a flowing cell, with a PDA-2000 dispersion analyser (Rank Brothers, UK) that permits determination of the size of forming aggregates *(Gregory, 1988)*, determinations of optical density *(Arnold et al., 1992),* and determinations of the bound water content of the granules by gravimetry.

III. New results

I began my research work by determination of the mosquito assemblages in Szeged in the year 1999. I investigated and compared the springtime mosquito fauna (disturbed by urbanization) with that occurring later. The protection against mosquitoes can be planned effectively only after a knowledge of the constitution of the mosquito assemblages.

Typically, the widely used chemical insecticides endanger human health, kill non-pest insects and poison the environment. Health problems (for example, cancer) have been linked to pesticide accumulation in the groundwater. There is therefore increasing interest in the use of alternative strategies for insect control, such as biological treatment. I subsequently studied the fermentation of *Bacillus thuringiensis* ssp. *israelensis* (Bti) bacteria. This biological method is environmentally friendly, in contrast with chemical methods. The possibilities of breeding and spore-forming of Bti bacteria were investigated on a synthetic and a monocomponent culture medium. I optimized the main component of the synthetic medium. The good breeding of bacteria combined with good sporulation is difficult; severe conditions can result in good sporulation and not so good fermentation. I succeeded in producing a medium with good properties (T_{20}).

The Bti suspension is currently in the course of industrial production in large quantities. Formulation of this suspension and its use for practical purposes are possible only with relatively concentrated systems. Diluted suspensions of Bti bacteria (that are fermented microbiologically) can be effectively flocculated and concentrated by using polyelectrolytes, which are necessary for formulation.

Bti suspensions were studied in a form bound on a support. I compared the smooth carrier with porous-surfaced carriers.

Biological testing was employed to check on the toxicity of the Bti suspensions and Bti products. The sensitivities of biting mosquito larvae (*Aëdes vexans*) of different ages were compared in biological tests. The toxicity of the Bti spores was also investigated for the *Daphnia magna* Straus (Cladocera, Crustacea) species. This species is not related systematically to *Aëdes vexans* (Diptera, Culicidae).

1 Results on mosquito assemblages

1.1 Few mosquito species are present in the River Tisa flood area. Far from the River Tisza, the local conditions may result in a more diverse mosquito species composition. I found numerous woods-favouring *Aëdes sticticus* species and salt-favouring *Aëdes dorsalis* species besides *Aëdes vexans* species. After the stabilization of the optimum temperature, the Culex genus individuals emerged in force in the middle of June. The number of their individuals became dominant during the dry summer. *Culex pipiens molestus* dominated at nearly all sampling sites. This result can be explained by the impact of urbanization In the changed environment, the dominant species appeared at all collecting sites.

1.2 Fewer individuals of *Anopheles hyrcanus* were collected in Szeged. In earlier examinations, this species was not observed in the Tisza Basin, but it has recently been found in the mosquito assemblage of the Tisza Lake.

2 Results on *Bacillus thuringiensis* subsp. *israelensis* fermentation

2.1 I developed and studied a synthetic culture medium. A medium with a saccharose content resulted in $2.6*10^7$ n/ml living cells after fermentation 96 for hours, and in 100% sporulation after fermentation 144 for hours. The toxicity of the Bti spores was similar to that of VECTOBAC 12 AS.

2.2 I developed and studied a culture medium (T_{20}), which from all aspects was optimum. The number of Bti bacteria increased with appropriate efficacy (3-$5x10^7$ cells/ml) in this medium, and the vegetative cells formed toxic spores (99.9-100%) without an exogenous component in a short time. An advantage of this medium is that it can be produced through use of an industrial by-product at a relatively low price.

2.3 My examinations revealed that the log phase of the breeding dynamics of the Bti fermentation in the T_{20} medium demanded 20 hours. The corresponding period for other microorganisms is 5-10 hours.

2.4 I found that, up to the end of the vegetative phase, the concentrations of the amino-acids proline (Pro), methionine (Met) and tyrosine (Tyr) in the HL culture (Beef-extract nutrient culture) increased relative to the initial levels. By the end of fermentation (sporulation), all of the free amino-acids had been consumed from the HL culture.

2.5 The sterile T_{20} medium was alkaline (pH=9.06). At the beginning of fermentation, the pH decreased, but after a 16-hour fermentation the pH was slightly increased (pH=9.11). It is presumed that the pH decrease is caused by the organic acid produced by fermentation. The later pH increase in the T_{20} medium is most likely caused by the decomposition of amino-acids by desamination or alkaline fermentation of the Bti bacteria.

2.6 My examinations indicated that the most efficient reagent for the flocculation of Bti spore suspensions was the cationic polyelectrolyte SNFH528.

2.7 My experiments demonstrated that numerous anionic polymers can flocculate the diluted (1:50) Bti spore suspension. Among the anionic samples tested, the SNFH 149 polymer possessed the best flocculation activity.

However, it was further proved that the anionic polymer was not effective in flocculating the natural (non-diluted) Bti suspension. It may be supposed that anionic segments of the polymer are attached to positive functional groups of the cell surface, resulting in the formation of extended adsorbed layers of weakly deformed macromolecules that are able to bond several cells via polymeric bridges.

2.8 A more concentrated (0.1%) polyelectrolyte solution was found to possess a higher flocculation efficiency as compared with a more diluted one (0.01%).

2.9 Addition of the optimum polymer dosage in two equal portions enhanced the flocculation activity of the flocculant as compared with one-step addition.

2.10 Cationic polymers accelerated, while anionic polymers decelerated the sedimentation of natural (non-diluted) Bti suspensions.

2.11 The Bti spores separated from the carrier within 2 hours, and the product was just as toxic for 3rd instant larvae of *Aëdes vexans* as the product with free cells.

3 Results of biological toxicity testing

3.1 In the course of my examinations, the toxicity of the Bti spores was compared with that of the Bti vegetative cells. The Bti spores were 2600 times more toxic than the vegetative cells for 3rd instant larvae of *Aëdes vexans*. The vegetative Bti cells contain only the genetic code of δ-endotoxin, and the vegetative Bti cells are therefore not toxic.

3.2 The toxicity of the Bti suspension fermented in T_{20} culture was demonstrated to be the same as that of the commercial product VECTOBAC 12 AS.

3.3 The degree of toxicity of flocculated Bti suspensions for biting mosquito larvae was found to be in the same range as that for a non-flocculated suspension, and comparable with the effect of commercial VECTOBAC 12 AS. Flocculation is an effective method for the industrial production of a domestically fermented Bti preparation. The flocculation of the Bti suspension is good for concentration of the Bti product on an industrial scale, and does not affect the toxicity of the Bti product.

3.4 Toxicity of the Bti suspension was studied in a form bound on a support. The Bti spores quickly separated from the smooth carrier, and this product was just as toxic for 3rd instant larvae of *Aëdes vexans* as the Bti product with free cells. However, the storage of the Bti product is not solved.

3.5 The sensitivities of biting mosquito larvae (*Aëdes vexans*) of different ages were compared in biological tests. The 3rd instant larvae were 3 times more sensitive than 4th instant larvae to the Bti spores, and 7.6 times more resistant than 2nd instant larvae.

3.6 The toxicity of the Bti spores was also investigated for the *Daphnia magna* Straus (Cladocera, Crustacea) species. This species is not related systematically to *Aëdes vexans* (Diptera, Culicidae). The *Daphnia magna* species was 1500 times more resistant than the biting mosquito larvae to the Bti spores.

IV. Practical utility of the new results

1; I developed and studied a new Bti spore (T_{20}) product culture medium with appropriate efficacy (3-5x10^7 cells/ml). One advantage of this medium is that it can be produced through the use of an industrial by-product at a relatively low price. The vegetative cells quickly formed toxic spores (99.9-100%) without an exogenous component.

2; Cationic water-soluble polymers can also be applied for the concentration of biological native Bti suspensions. The flocculated Bti suspension preserves its larvicidal toxicity.

3; My results have demonstrated that the sensitivities of mosquito larvae of different ages differ. In my opinion it is necessary to determine the ages of mosquito larvae in breeding water. A full knowledge of the ages of the larvae permits application of the optimum Bti dosage against the mosquito larvae. An overdose of the Bti product can endanger other organisms besides biting mosquitoes.

The results of this dissertaion may well be of importance as regards the construction of the Vásárhelyi Terv. When the catastrophe-reservoir comes into being, new mosquito breeding will spring up.

V. Publications

Publications:

(1.) Szepesszentgyörgyi, Á., Bárány, S., Mécs, I. (2003): Flocculation of *Bacillus thuringiensis* ssp. *israelensis* suspension by polyelectrolytes. - microCAD 2003 International Scientific Conference. Proceedings, Ed. University of Miskolc, pp. 101-109. [IF: 0]

(2.) Szepesszentgyörgyi, Á., Bárány, S. (2003): Flocculation of *Bacillus thuringiensis* suspension and its role in formulation as anti-mosquito agent. - *CERECO '2003*. International Scientific Conference. Proceedings, Ed. University of Miskolc, pp. 203-214. [IF: 0]

(3.) Szepesszentgyörgyi, Á., Bárány, S., Mécs, I. (2005, in press): Flocculation of *Bacillus thuringiensis* var. *israelensis* suspension and its efficacy against mosquito larvae. - Acta Biologica Hungarica, 56, 1, [IF: 0,416]

(4.) Bárány, S., **Szepesszentgyörgyi, Á.** (2004, in press): Flocculation of cellular suspensions by polyelectrolytes. - Advances in Colloid a.Interfaces Science. [IF: 3,311]

(5.) Szepesszentgyörgyi, Á., Bárány, S., Skvala, J., Mécs, I. (2004, in press): Ba*cillus thuringiensis* szuszpenzió flokkuláltatása polielektrolitokkal és tenzidekkel. - Magyar kémiai folyóirat. [IF: 0]

Publication under judgement:

(6.) Szepesszentgyörgyi, Á., Otgonchimeg, R.: Seasonal changes in the mosquito fauna (Diptera, Culicidae) in the city of Szeged in 1999. - Tiscia an Ecological Journal. [IF: 0]

Posters:

(1.) Szepesszentgyörgyi, Á., Mécs, I.(1999): *Bacillus thuringiensis* (Bti) fermentáció – szúnyoggyérítés (1998-1999. results). – Budapest, Bay Zoltán Day. Nov 12, 1999.

(2.) Szepesszentgyörgyi, Á., Mécs, I.(2000): Szúnyogvizsgálat, *Bacillus thuringiensis* (Bti) fermentáció (1998-2000. results). – Szeged, Bay Zoltán Day. May 05, 2000.

(3.) Szepesszentgyörgyi, Á., Mécs, I.(2000): *Bacillus thuringiensis* (Bti) fermentáció – szúnyoggyérítés (1999-2000. results). – Budapest, Bay Zoltán Day. Nov 03, 2000.

(4.) Szepesszentgyörgyi, Á., Mécs, I.(2001): *Bacillus thuringiensis* (Bti) fermentáció – szúnyoggyérítés (2000-2001. results). – Budapest, Bay Zoltán Day. Nov 02, 2001.

(5.) Szepesszentgyörgyi, Á., Mécs, I.(2002): *Bacillus thuringiensis* (Bti) fermentáció – szúnyoggyérítés (2001-2002. results). – Budapest, Bay Zoltán Day. Okt 11, 2002.

Lectures:

(1.) Szepesszentgyörgyi, Á., Bárány, S. (2003): Flocculation of *Bacillus thuringiensis* ssp. *israelensis* suspension by polyelectrolytes. - microCAD 2003 International Scientific Conference. University of Miskolc, Miskolc, March 7-8, 2003.

(2.) Szepesszentgyörgyi, Á. (2003): A gyálai Holt-Tisza Feketeparti szakaszának csípőszúnyog faunája - Csongrád Megyei Természetvédelmi Egyesület, Szeged, Március 20, 2003.

(3.) Szepesszentgyörgyi, Á., Bárány, S., Mécs, I. (2003): Flocculation of *Bacillus.thuringiensis* suspension and its role in formulation as anti-mosquito agent. - *CERECO '2003*. International Scientific Conference. Miskolc, April 28-30, 2003.

(4.) Bárány, S., **Szepesszentgyörgyi, Á**. (2003): Flocculation of cellular suspensions using polyelectrolytes. - XVI. Europan Chemistry at Interfaces Conference, Vladimir, Russia, 14-18 July, 2003.

(5.) Bárány, S., **Szepesszentgyörgyi, Á. (**2003): Flocculation of cellular suspensions using polyelectrolytes. - 77th American Chemical Society Colloid and Surface Science Meeting, Atlanta, USA, June 15-18, 2003.