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Diffusion of *Bacillus thuringiensis* bacteria and their effect on aquatic invertebrates in the Nemunas River after using VectoBac 12AS preparation

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Blackfly control with the microbiological preparation VectoBac 12AS was started in Lithuania in 1999. This preparation is based on bacterium *Bacillus thuringiensis* var. *israelensis*. The VectoBac 12AS is applied at a single point from a bank prominence from the year 2000. Investigations were carried out in April–June of 2006 and 2007. The bacteria were found in the Nemunas River 164 km downstream the point of application of the larvicide 3 days after the application. This showed that VectoBac 12AS reached this segment of the river in 3 days. The density of *B. thuringiensis* bacteria decreased downstream from the point of application of the preparation. The highest density of bacteria was found in blackfly larvae, lower densities were found in the ground and on water plants. The effect of VectoBac 12AS on nontarget invertebrates was estimated in study sites from the point of application up to Druskininkai. Using the method of application from one point, this distance of the river was affected by the highest doses of preparation, and its effect on nontarget organisms could be seen in this part of the river. The usage of the preparation had no effect on nontarget invertebrates in the Nemunas River. Significant differences in Chironomid density were detected only in one study site, 6 km downstream the point of application of the larvicide. At a distance of 14 km from the point of application and downstream the river, no differences in the density of Chironomid larvae were detected.

Key words: bacterium, *Bacillus thuringiensis* var. *israelensis*, blackflies, aquatic invertebrates

INTRODUCTION

Outbreaks of bloodsucking blackflies began in the 70s of the 20th century in the south-eastern part of Lithuania. Blackfly control with the microbiological preparation VectoBac 12AS was started in Lithuania in 1999. This preparation is used in the Nemunas River till now. The VectoBac 12AS is based on bacterium *Bacillus thuringiensis* var. *israelensis*. *Bacillus thuringiensis* produces parasporal crystals during sporulation (Federici et al., 1990). These crystals are composed of proteins which are activated in insects by alkaline intestinal pH and digestive enzymes. The parasporal crystals have no contact toxity and must be ingested to exert their toxic action, which includes lysis and disintegration of midgut epithelial cells (Chilcott et al., 1990). The serovariety *Bacillus thuringiensis* var. *israelensis* is particularly active on Diptera of the suborder Nematocera, so preparations made of *Bacillus thuringiensis* var. *israelensis* are specific, effective and not dangerous for the environment or man (Ali, 1981; Ходырев, 1990; Волжинский et al. 1990).

From 4200 to 8500 kg of VectoBac 12AS have been used in the Nemunas River each year. The discharge of the Nemunas River during the usage of the preparation was from 152 to 363 m^3/s. The first year 1999, the preparation was poured out from the ship (Bartninkaitė et al., 2006). VectoBac 12AS is applied at a single point from a bank prominence from the year 2000. The abundance of bloodsucking blackflies decreased more than 10 times in the segment of the river up to 100 km during this period (Bartninkaitė et al., 2006). Data on the diffusion peculiarities of the preparation along the Nemunas River were not published. Investigations on the effect of VectoBac 12AS application on aquatic invertebrates downstream Druskininkai were carried out in 2001 and 2004 (Bernotienė, 2001; Bernotienė, Višinskienė, 2006). No effect on nontarget organisms was detected in the Nemunas River downstream Druskininkai (Bernotienė, 2001; Bernotienė, Višinskienė, 2006). The segment of the river between the point of application and Druskininkai has not yet been investigated. Using the method of application of the preparation in one point, this segment of the river was exposed to the highest doses of the preparation. The effect on nontarget organisms could be seen in this part of the river.

The aim of the work was to estimate the diffusion of VectoBac 12AS along the river and to determine the effect of the preparation on water invertebrates near the point of its application.

MATERIALS AND METHODS

Investigations were carried out in April–June of 2006–2007. In these years application of the preparation was made in one point

(Varviškė, E23°47'14" N53°54'01"). The treatment was repeated two weeks after the first application in the same point to affect the blackfly larvae that had immigrated downstream the river from Belarus in 2006.

Investigations were carried out in Varviškė (15 m upstream the point of application of the preparation), Bugieda (6 m downstream the point of application, E23˚47'55" N53˚56'06"), Gerdašiai (14 km downstream the point of application, E23˚53'46" N53˚56'54"), Druskininkai (26 km downstream the point of application, E23˚59'41" N54˚01'36"), Liškiava (34 km downstream, E24˚03'19" N54˚04'43"), Merkinė (56 km downstream, E24˚10'46" N54˚09'26"), Alytus (96 km downstream, E24˚03'14" N54˚24'02"), Birštonas village (164 km downstream the point of application, E24˚00'51" N54˚35'24").

Samples of aquatic plants, ground, water, and blackfly larvae for microbiological investigations were taken in four study sites (Druskininkai, Merkinė, Alytus and Birštonas) in 2006. They were taken before the application of the preparation, 3 days and 14 days after the first application and 3, 15 and 32 days after the second application of the preparation in 2006. Aquatic plants, ground, water and blackfly larvae were sampled in two study sites (Merkinė and Alytus) 3 days and 7 days after the application of the preparation in 2007. *B. thuringiensis* bacteria were isolated by usual microbiological methods (Bluzmanas, 1970). Samples were kept 45 min. at 50 °C to kill all nonsporic bacteria. After that, in sterile conditions, 1 g of material was put into 100 ml of sterile water, and after 10 min of shaking 1 ml of suspension was sowed on meat peptone agar (MPA). Every sample was sowed into 5 plates. Plates were incubated at the 28 °C for 7 days. The developed *Bacillus* colonies were counted, and bacterial smears from each colony were prepared. The colonies with inclusion bodies were assigned to *B. thuringiensis*. 483 plates were sowed and 700 bacterial smears were investigated.

The abundance of blackfly larvae was estimated on water plants (*Glyceria maxima* (Hartman) or *Butomus umbellatus* L.). We collected 3 samples at a time. Blackfly larvae were collected in Varviškė, Gerdašiai, Druskininkai, Liškiava, Merkinė and Alytus before the application of the preparation, as well as 3 days and 7 days after the application of the biological larvicide in 2006–2007.

Abundance of Chironomid larvae living on water plants like blackfly larvae was investigated in 2006 between Druskininkai and Alytus. The effect of the preparation on aquatic invertebrates was estimated in study sites chosen between the point of application and Druskininkai (Varviškė, Bugieda, Gerdašiai, Druskininkai) in 2007. Samples of zoobenthic invertebrates were collected by the kick-sampling method in two 0.1 m^2 areas at each study site. Aquatic invertebrates were collected before and 7 days after the application of the biological preparation.

For statistical treatment, average, standard error and standard deviation were calculated. To estimate the differences between the abundance of bacteria or the abundance of water invertebrates, the t test for dependent samples was used.

RESULTS AND DISCUSSION

Microbiological investigations

Before the application of the preparation, samples of ground, water, water plants and blackfly larvae were taken in the Nemunas River near Druskininkai and Alytus (2006), Varviškė and Bugieda (2007) to detect whether *B. thuringiensis* bacteria could be found before the application. *B. thuringiensis* bacteria were found only in blackfly larvae in Druskininkai (2006) and in Bugieda (2007). *B. thuringiensis* were extracted from dipterous insects, so it can be found in the bodies of blackflies (Ходырев, 1990). 0.4 ± 0.24 was the highest density of *B. thuringiensis* in blackflies before the application of the preparation (2006, Druskininkai).

Three days after the application of VectoBac 12AS, *B. thuringiensis* bacteria were found in all study sites of the river (Fig. 1). The highest density was found in blackfly larvae, lower densities being found in soil and on water plants. The density of *B. thuringiensis* bacteria decreased downstream from the point of application of the preparation (Fig. 1).

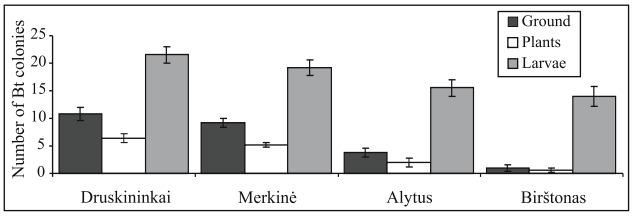

Fig. 1. Abundance of *B. thuringiensis* in different study sites 3 days after the application of preparation, 2006. Mean ± SD

The fact that *B. thuringiensis* bacteria were found in the Nemunas River in the last study site (Birštonas), i. e. 164 km downstream the point of application of the preparation, showed that in 3 days bacteria covered a segment of the river more than 150 km longe. The density of *B. thuringiensis* bacteria in the ground near Birštonas was on average 10.8 times less than in Druskininkai.

The density of *B. thuringiensis* bacteria in blacklfy larvae was high in all segment of the river (Fig. 2). In Birštonas, it was 1.6 times lower than in Druskininkai. It even increased 14 days after the application (Fig. 2) and did not decrease after 22 days.

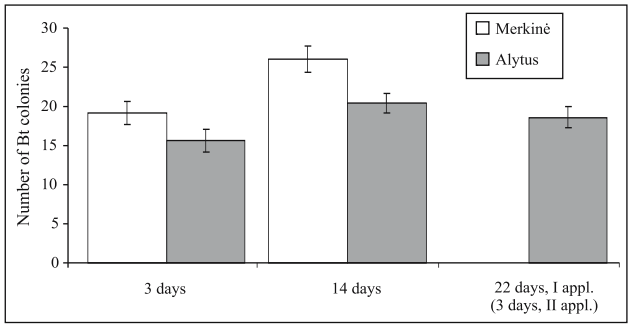

Fig. 2. Abundance of *B. thuringiensis* bacteria in blackfly larvae after the usage of the preparation, 2006 (Merkine 22 days – no data.) Mean ± SD

The abundance of *B. thuringiensis* bacteria decreased in soil 1.9–4.3 times within 4 days (from the 3rd to the 7th day) after the application of the preparation in 2007 (Fig. 3). These differences were statistically significant ($t = 3.64$, $p < 0.01$ in Merkinė and $t = 5.27$, $p < 0.001$ in Alytus). The abundance decreased 14 days after the application 1.1–3.3 times (2007); 34 days after the first application (or 15 days after the second application) *B. thu-*

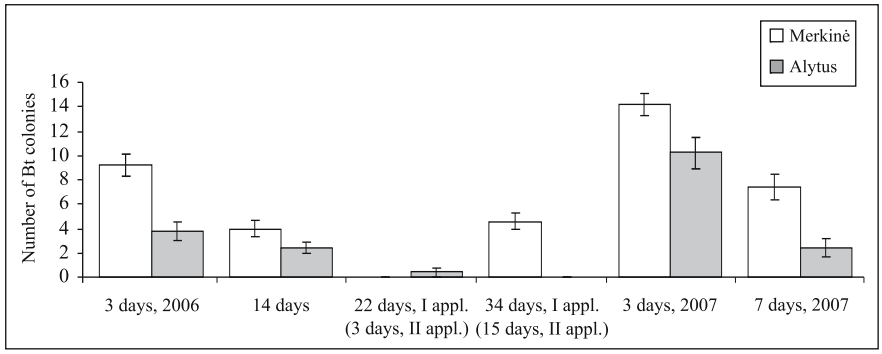

Fig. 3. Abundance of *B. thuringiensis* bacteria in the ground after the usage of the preparation, 2006–2007. Mean ± SD

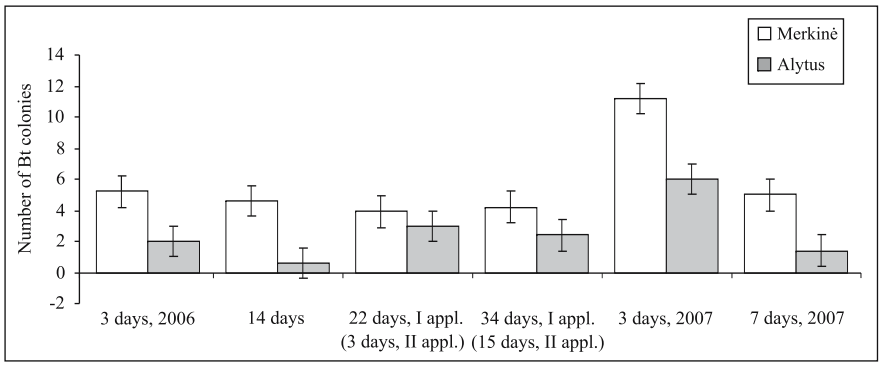

Fig. 4. Abundance of *B. thuringiensis* bacteria on water plants after the usage of the preparation, 2006–2007. Mean ± SD

ringiensis bacteria were not found in the Nemunas River downstream Merkinė. The density of *B. thuringiensis* bacteria was higher in 2007 than in 2006 in the ground 1.5 to 2.7 times, and these differences were statistically significant ($t = 2.81$, $p < 0.05$ in Merkinė and $t = 4.32$, $p < 0.01$ in Alytus).

Abundance of *B. thuringiensis* bacteria on water plants was lower than in soil, and it did not decrease so rapidly (Fig. 4); 32 days after the application of the preparation the density of *B. thuringiensis* on water plants was 0.6 ± 0.4 in Merkinė and 0.4 ± 0.3 in Alytus. This density is very similar to that in blackfly larvae before the application. The density of *B. thuringiensis* bacteria on water plants was from 2.2 to 3 times higher in 2007 than in 2006, and the difference was statistically significant in both study sites ($t = 3.59$, $p < 0.01$ and $t = 3.17$, $p < 0.05$). This could be explained by the fact that a different amount of VectoBac 12AS preparation was used in different years (4200 and 5700 kg) and the discharge of the Nemunas River also varied (171 and 153 m^3/s, respectively). So, the density of the preparation was 1.6 times higher in 2007 than in 2006.

Investigations on blackfly (*Diptera: Simuliidae*) larvae

The abundance of blackfly larvae differed in different study sites before the application of the preparation. It varied between 129 ± 22 to 730 ± 290 larvae / dm^2 of water plant surface. We calculated the mortality of blackflies in study sites 3 days and 7 days after the application. (Fig. 5). The mortality of blackflies was close to 100% 34 km downstream the point of application. The mortality of blackfly larvae increased 7 days after the application of the preparation. So, the effect of VectoBac 12AS on blackfly larvae could be seen at a distance of more than 90 km downstream from the point of its use in 7 days.

Investigation on other aquatic invertebrates

Investigations of different researchers (Liber et al., 1998) have shown that *B. thuringiensis* var. *israelensis*, the active ingredient of the preparation VectoBac 12AS, can affect some larvae of chironomids (Diptera: Chironomidae). Investigations of chironomid larvae, which live on water plants like blackfly larvae, were carried out in 2006. Larvae of *Polypedilum breviantenatum* Tschern., *Rheotanytarsus* sp., *Tanypus* sp., *Tanytarsus* gr. *gregarius* Kieff. were counted on water plants before and 7 days after the application of the preparation. Material was collected in Druskininkai, Merkinė and Alytus. The abundance of chironomids was 83.3 ± 13.6 larvae on 1 dm^2 of water plant surface before the application. It increased to 105.2 ± 63.08 larvae on 1 dm^2 of water plant surface after the application. These

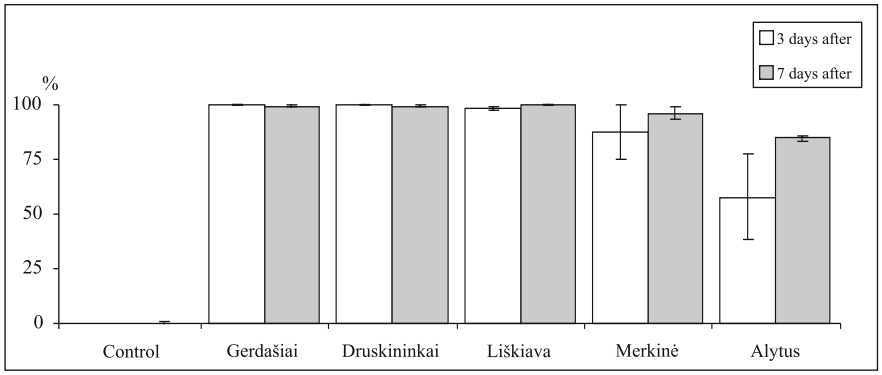

Fig. 5. Blackfly mortality in different study sites after the application of VectoBac 12 AS, 2006–2007. Mean ± SD

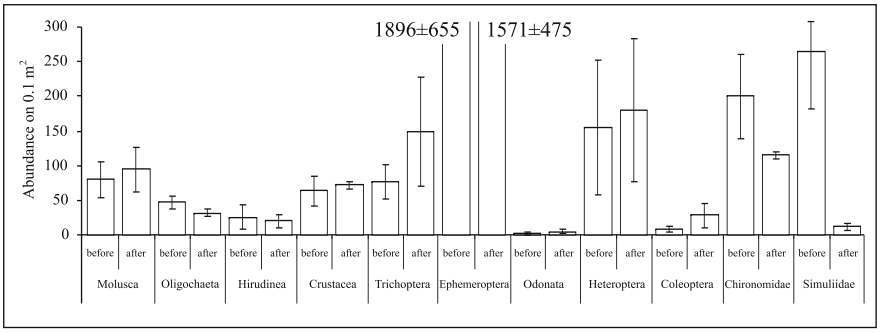

Fig. 6. Abundance of aquatic invertebrates in the Nemunas River before and after the application of preparation VectoBac 12AS, 2007. Mean ± SE

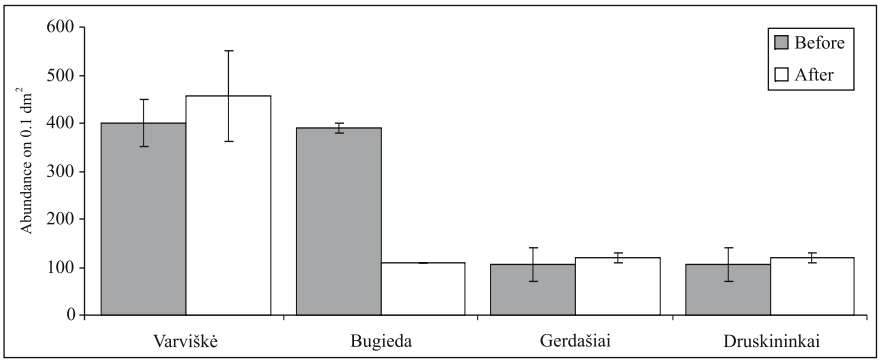

Fig. 7. Density of Chironomidae in different study sites in the Nemunas River before and 7 days after the application of VectoBac 12AS, 2007. Mean ± SD

differences were not statistically significant. So, the abundance of chironomids did not depend on the application of VectoBac 12AS downstream Druskininkai.

Investigations of all water invertebrates were carried out in 1999 (Bernotienė, 2001), 2004 (Bernotienė, Višinskienė, 2006) in the Nemunas River before and after application of VectoBac 12AS. Investigations were made downstream Druskininkai. No effect on water invertebrates was detected. We investigated the density of water invertebrates between the point of application of the preparation and Druskininkai in 2007. Using the method of application from one point, this segment of the river was affected by the highest doses of the preparation, and the effect on nontarget organisms could be seen in this part of the river.

During the studies in the Nemunas River, 64 taxa of macroinvertebrates were identified: Mollusca (6 taxa), Hirudinea (4), Crustacea (4), Oligochaeta (1), Arachnida (1), and Insecta: Ephemeroptera (11), Diptera (11), Trichoptera (10), Coleoptera (7), Heteroptera (4), Odonata (3), Plecoptera (1), Megaloptera (1). No statistically significant differences between the density of Mollusca, Oligochaeta, Crustacean, Hirudinea, Heteroptera, Ephemeroptera, Odonata, Trichoptera, Coleoptera in each study site and in all segment between the point of application and Druskininkai were detected before and 7 days after the application (Fig. 6). The density of Trichoptera and Coleoptera even increased after the application, but these differences were not statistically significant and were related to natural life cycles of insects, because the number of these insects increased also in the control point.

Only differences in the density of blackfly larvae before and after application were statistically significant ($t = 3.179$, $p < 0.05$). A statistically significant differences in the density of chironomids in one study site (Bugieda) was detected before and 7 days after the application ($t = 28$, $p < 0.05$). In other study sites, the abundance of chironomids did not differ ($p > 0.05$) (Fig. 7).

Laboratory studies have shown that different species of chironomids respond differently to *B. thuringiensis* bacteria (Garcia et al., 1980). Orthocladiinae and Tanytarsini are the most sensitive groups (Liber et al., 1998). So, the preparation can influence chironomidae larvae in a short segment (up to 6 km downstream) from the point of application of the preparation in the Nemunas River. At a distance of 14 km from the point of application, no differences in the density of chironomid larvae were detected (Fig. 7).

Future investigations on chironomid larvae will supplement our knowledge on their relationship with *Bacillus thuringiensis* bacteria.

CONCLUSIONS

The preparation VectoBac 12AS used in the Nemunas River in one locality (Varviškė) covers the segment of the river longer than 150 km in 3 days after its usage. VectoBac 12AS affected blackfly larvae up to 100 km downstream from the point of application of the preparation.

The highest abundance of *B. thuringiensis* bacteria was detected in blackfly larvae, and lower numbers were detected in the ground and on water plants after the application of the preparation. The abundance of *B. thuringiensis* bacteria decreased gradually downstream from the point of VectoBac 12AS application.

The use of the preparation had no effect on nontarget invertebrates in the Nemunas River. Statistically significant differences in the density of chironomids in one study site 6 km downstream the point of application of the preparation was detected 7 days after its use. At a distance of 14 km from the point of application and downstream the river, no differences in the density of Chironomid larvae were detected.

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BACILLUS THURINGIENSIS BAKTERIJŲ SKLIDIMAS IR POVEIKIS VANDENS BESTUBURIAMS NEMUNE PO PREPARATO VectoBac 12AS PANAUDOJIMO

S a n t r a u k a

Kraujasiurbių upinių mašalų gausumui reguliuoti Nemune nuo 1999 m. iki šiol naudojamas biologinis preparatas VectoBac 12AS. Preparato aktyvusis pagrindas yra sporas formuojančios *Bacillus thuringiensis* var. *israelensis* bakterijos bei jų formuojami parasporaliniai kristalai. Nuo 2000 m. preparatas išpilamas vienoje Nemuno vietoje, o jo efektyvumas stebimas pasroviui nuo preparato panaudojimo vietos.

Tyrimai, atlikti 2006–2007 m., parodė, kad per 3 dienas po preparato panaudojimo *Bacillus thuringiensis* bakterijų randama tyrimų taške, esančiame 164 km pasroviui nuo preparato panaudojimo vietos. Preparatas efektyviai veikia upinių mašalų lervas apie 100 km atkarpoje pasroviui nuo jo panaudojimo vietos.

Didžiausias *Bacillus thuringiensis* bakterijų gausumas nustatytas upinių mašalų lervose, mažesnis gausumas – grunte ir ant vandens augalų. Bakterijų gausumas palaipsniui mažėja pasroviui nuo preparato panaudojimo vietos.

Preparato poveikis kitiems vandens bestuburiams buvo tirtas atkarpoje nuo preparato panaudojimo vietos iki Druskininkų. Būtent šis upės ruožas paveikiamas didžiausių preparato koncentracijų supilant jį vienoje vietoje. Nebuvo nustatyta jokio preparato poveikio vandens bestuburiams nei viename tyrimų taške. Tik Nemune ties Bugieda (6 km pasroviui nuo preparato panaudojimo vietos) nustatytas statistiškai patikimas uodų trūklių lervų sumažėjimas. Kitame tyrimų taške (14 km pasroviui nuo preparato panaudojimo vietos) uodų trūklių lervų gausumas prieš panaudojant preparatą ir jį panaudojus nepakito.

Raktažodžiai: bakterijos *Bacillus thuringiensis* var. *israelensis*, upiniai mašalai, vandens bestuburiai