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Original Research

Comparison of Activity and Persistence of Microbial Insecticides Based on *Bacillus thuringiensis israelensis* and *Bacillus sphaericus* in Organicly Polluted Mosquito-Breeding Sites

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Abstract

Environmental factors can influence the effectiveness of microbial insecticides based on spores and crystal proteins of *Bacilus thuringiensis israelensis* (*Bti*) and *B. sphaericus* (*Bs*) in mosquito control programs. The impact of water quality and sunlight on the activity and persistence of commercially available microbial larvicidal formulations based on *Bti* and *Bs* was investigated within irrigation fields in the Wrocław area. Bioassays were conducted in field- and semi-field conditions using *Ochlerotatus caspius* [Pallas] larvae. The survey on the persistence of *Bti* and *Bs* strains after the application of microbial insecticides based on *Bti* and *Bs* was carried out by isolating both bacilli strains from the sediment found in sewage channels. The results showed different activity patterns in larval mortality, but no significant differences between dosages after both biocides application. It was observed that natural sunlight decreases the effectiveness of *Bti* and *Bs*. The modeling of the *Oc. caspius* larvae survival data after *Bti* application showed a substantial difference between the proportion of mosquito larvae mortality and survival rate in sunlit and shaded conditions. In sunlit conditions, the activity of *Bs* was also reduced, but the mortality effect demonstrated significant interaction between time and exposure habitats. The presence of bacilli in sewage channels showed their occurrence in sediment. However, the great number of *Bti* and *Bs* strains among the total spore-forming bacilli in environmental samples was insignificant, ranging from 3.5% to 6.1%.

Keywords: *Bacillus thuringiensis israelensis*, *Bacillus sphaericus*, mosquito larvae, wastewater, biocide activity, biocide persistance

Introduction

Due to globalization and climate change, mosquito vectors of human disease pose a constant threat to many countries. Recent interceptions of indigenous and exotic mosquitoes with the potential to vector serious mammalian diseases have highlighted the need for control products and/or eradication programs [1, 2]. The following environmental impacts of any pesticide that could impact mammalian and non-target safety should be considered: effect on the environment, persistence and occurrence in the natural environment, and possible host resistance [3, 4]. In many mosqui-

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to-control programs, commercially available products based on *Bacillus thuringiensis israelensis* (*Bti*) and *B. sphaericus* (*Bs*) have been determined to be extremely safe environmentally. Therefore, it was considered to have commercial potential as a control agent for mosquito and black fly vector-borne diseases (i.a. malaria, dengue, West Nile fever, onchocerciasis), as well as nuisance mosquito species in West Africa, the USA, and Europe [5, 6]. About 300 tons of *Bti* have been applied in West Africa alone with no indications of deleterious effects on human health or non-target organisms. No occurrences of resistance within host populations have been reported. Therefore, *Bti* has been used in areas considered as environmentally sensitive [7].

Bti is a gram-positive, rod-shaped, spore-forming soil bacterium that often exhibits insecticidal properties [3]. This bacillus produces protein toxins that are concentrated in a parasporal bodies, called the protein crystal (δ-endotoxins). The insecticidal effect is caused by the parasporal crystal, which usually contains four major proteins (58, 68, 125, and 135 kDa). *Bti* is highly pathogenic against mosquitoes (*Culicidae*) and black flies (*Simuliidae*), and has some virulence against certain other *Diptera*, especially *Chironomidae* (midges). The *Bti* spores and parasporal crystals must be ingested by the larval stage of the target organism to cause mortality [5]. The toxin binds to a receptor on the midgut cell wall, resulting in pore formation in the cell and leading to death of the larva. There appears to be a synergistic interaction between up to four proteins, resulting in a highly complex mode of action, toxicity to mosquito larvae, and no resistance development. Research on constant exposure of *Aedes vexans* [Meigen] in the field for over 25 years in Germany resulted in no difference in the level of resistance in exposed and unexposed populations [8]. Similar results were reported for black flies [9]. The risk of resistance to bacterial toxins is inversely proportional to the complexity of the mode of action, which is definitely less complex with the second spore-forming bacterium – *B. sphaericus*. The efficacy of *Bs* is based on parasporal protein inclusions, which are located in coated "spore crystal complex" [5]. In contrast to *Bti*, *Bs* has a binary toxin consisting of two different molecular weights: 51.4 kDa and 41.9 ka. Both are required for a high level of mosquitocidal activity. Another protein toxin of about 100 kDa is also produced. It is not homologous either to the binary toxin or to *Bti* toxins, which are considered environmentally safe.

Apart from different susceptibilities of various mosquito species to *Bti* and *Bs*, there are other biotic and abiotic factors that could potentially influence the efficacy of microbial control agents. These include temperature, water quality, sunlight intensity, developmental stage, mosquito larval density, the presence of sediments, and vegetation, as well as associated filter feeding non-target organisms [11-14].

Wrocław has conducted a mosquito-control program for several years, based mainly on the use of pyrethroids. In 1998, the city implemented a new biorational mosquito control strategy. It includes mapping mosquito-breeding sites, assessing the productivity of the breeding-sites, and monitoring the dynamics of larval and adult mosquito populations. The use of microbial insecticides and environmental management practices is an important element in an overall mosquito control strategy [15]. Since 2007, the routine mosquito-control operation has been carried out within irrigation fields, located along the Odra River, that are used as an alternative sewage purification system [16]. More than 100 km of channels distribute sewage into meadows for wastewater purification. The meadows support the development of high populations of floodwater (*Oc. caspius*, *Ae. vexans*) and domestic (*Culex pipiens/torrentium*) nuisance-mosquito species.

In order to economically control of floodwater mosquitoes (mostly *Oc. caspius* in organic polluted conditions) and address environmental concerns, the present study examines the influence of sunlight exposure on the activity and persistence of *Bti* and *Bs* in wastewater and sewage sediments after the biocide's application. It is crucial to understand the impact of environmental factors concerning the selection of an appropriate formulation and its dosage, as well as the optimal timing for application during routine field treatments to control mosquito larvae within organic polluted habitats.

Experimental Procedures

General Description of the Study Area

The irrigation fields were constructed in 1890 in the floodplains of the Odra River in Wrocław, Poland, to provide natural sewage purification for ~60,000 m^3 of sewage per day in a total area of 1,100 [17]. The system consists of sewage reservoirs, sewage canals, ancient river meanders, meadow infiltration basins, and underground drainage pipe systems. After infiltration in the meadows, an underground drainage system collects the purified water, which then flows into channels that release the effluent into the Odra River.

Application of Biocides into the Artificial Microcosm Test Series

The evaluation of the activity and residual effect of two microbial control agents was conducted in microcosm test series in June 2009. Forty plastic buckets (each with a diameter of 30 cm) were filled with 10 L of filtered wastewater and placed outdoors for 14 days in the irrigation fields (51°11'19. 65"N/16°58'27. 59"E).

Two of the commercially available microbial larvicidal formulations were tested: VectoBac® WDG (water dispersible granule formulation of *Bti* with a potency of 3,000 ITU/mg, lot: 140-885-PG) and VectoLex® (water dispersible granule with a potency of 650 Bs ITU/mg, lot: 142-446-PG). Two different application rates of each product were applied once as follows:

a) 200, 800 g/ha of VectoBac® WDG (=1.4 and 5.6 mg per bucket).
b) 400, 1,600 g/ha of VectoLex® WDG (=2.8 and 11.2 mg per bucket).

The dosage of each product was replicated in four repetitions where 32 containers were treated with different dosages of the products and eight containers served as controls. All 40 containers were evenly placed in sunlit and shaded conditions. Based on the capacity of the containers, pre-weighted amounts of VectoBac® WDG and VectoLex® WDG were suspended in 500 ml of water and evenly distributed on the surface using a one-litre hand-held sprayer. Sixteen containers were treated with the same product and their dosages were exposed to sunlight. An additional 16 containers were kept in the shade. Before treatment, 30 third-instar larvae of *Oc. caspius* were placed in each container. The activity of the microbial-control agents was assessed by determining larval mortality after 24 hours. Every other day, all cadavers and living larvae or pupae were removed from the containers and replaced with 30 larvae. The tests were conducted for four weeks. For the evaluation of efficacy and residual control, only the third and fourth instar larvae were considered because early instars could survive for several days after eclosion and prior to consuming a lethal dose of the larvicide.

Water-quality parameters, including pH, ammonium, nitrite, nitrate, phosphate, and chlorine, were assessed using Viscolor ECO (Macherey-Nagel GmbH&Co. KG., Germany) and ACQUAMERC (Merck Chemicals, Germany) sets. The conductivity was recorded using a conductivity-measurement apparatus (HANNA instruments, Germany).

Isolation of *Bti* and *Bs* Strains from Sewage Sediment

The isolation of *Bti* and *Bs* strains from the sludge samples was carried out in March, May, and October 2007 as a separate study. Sludge samples were periodically collected from the irrigation channels (51°10'13. 89"N/16°58'27.81"E and 51°08'59. 82"N/16°59'51.54"E) in order to analyze the influence of the commercially available products (VectoBac® WDG and VectoLex® WDG) used to control *Cx. pipiens/torrentium* larvae on the bacterial populations occurring in sediment within sewage channels. One ml of each sample was suspended in 10 ml of sterile, distilled water, and then 1ml of the supernatant fluid was heated at 80ºC for 10 min in a water bath [18]. The liquid samples, before and after pasteurization, were inoculated on Mossel agar with egg yolk (Merck, Germany) and nutrient agar (BioCorp, Poland) plates. Both types of agar plates were incubated at 30ºC for 72 hours. During bacteriological analysis, the total amount of bacteria and spore-forming bacteria (cfu/ml) were determined. The endospore-forming bacilli, mainly mannitol- and lecithinase-negative or mannitol-negative and lecithinase-positive strains, were identified according to Claus (1986) and Lacadet et al. (1999) using a biochemical test (BioMerieux, France) and a phase-contrast microscope to detect the parasporal bodies of *B. thuringiensis* [19, 20].

Data Analysis

To estimate the time periods after *Bti* and *Bs* applications in which 10%, 50%, 90%, and 95% mosquito larvae are able to survive in natural sunlight and shaded conditions, the analysis of covariance (ANCOVA) was used in a scheme of General Linear Model with binomial errors. During the analysis, the following explanatory variables were taken under consideration: the number of days after application of the microbial-control agents, dosage, and the active ingredient of the insecticide, as well as the location of the containers. The logistic function for VectoBac® WDG and quadratic function for VectoLex® WDG was used to model the proportion of living larvae to the 30 larvae that were placed in the containers after each 24 hours. All data were analyzed using R 2.2.1 version and R's "base" as well as "MASS" packages (R Development Core Team, 2005) [21].

Results

Selected chemical and physical parameters of the wastewater collected from the mosquito-breeding site indicated high levels of organic pollution. The following water-quality parameters were determined: pH, 7.5; conductivity, 1,256 μS; chlorine anions, 120 mg/liter; ammonium, 1.0 mgNH_4/liter; nitrite, 0.1 mgNO_2/liter; nitrate, 5.0 mgNO_3/liter; and phosphate, 5.0 mgPO_4/liter. The temperature of the wastewater measured at the beginning of the test was 21ºC.

The results of the activity of the microbial-control agents against mosquito larvae tested within artificial microcosm series are summarized in Table 1 and in Figs. 1 and 2. The application of VectoBac® WDG (*Bti* formulation) showed no significant difference between dosages 200 and 800 g/ha (p=0.547) in mosquito larvae reduction, but this mortality effect was significantly higher when compared to the control (p=0.00617). The modeling of the *Oc.*

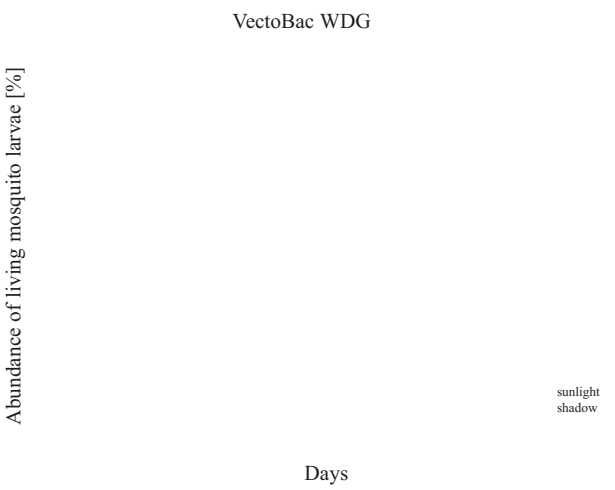

Fig. 1. Dynamics of the VectoBac® WDG activity in the sewage conditions due to sunlight and shadow exposure.

Table 1. Survival rates of *Oc. caspius* larvae after VectoBac® WDG and VectoLex® WDG applications in sunlight and shadow exposure conditions.

Survival rate of *Oc. caspius* larvae [%]	Number of days after biocide application			
	Bti		*Bs*	
	sunlight exposure	shadow exposure	sunlight exposure	shadow exposure
10	2.4	11.2	-	-
28	-	-	-	6.9
39	-	-	6.4	-
50	9.4	15.2	-	-
90	16.3	25.3	-	-
95	18.7	27.5	-	-

caspius larvae survival data clearly showed a substantial difference between the proportion of mosquito larvae mortality and survival rate in the sunlit and shaded containers. According to the Fig. 1, natural sunlight lowers the efficacy of *Bti*, which means that much more larvae are able to survive in containers exposed to sunlight (odds ratio=16, p=0.035). During this time, the effectiveness of VectoBac® WDG was reduced (p=0.019). However, no statistically significant interaction between time and exposure conditions (p=0.714) was observed. This means that the process of loss of the insecticidal proteins effectiveness has the same rate in both sunlight and shadow exposure conditions. The probability of mosquito larvae survival is increased 1.35 times per unit of time (=one day in this study).

In the containers exposed to sunlight, the number of mosquito larvae was greatly reduced by VectoBac® WDG over two days, and only ten percent of the specimens were unaffected (Table 1). However, in shaded conditions, the same survival rate was maintained for 11 days. After two weeks, the larval survival rate was assessed at 90%. After 27 days, the efficacy of *Bti* was the lowest in containers exposed to sunlight and the survival rates increased by 95%.

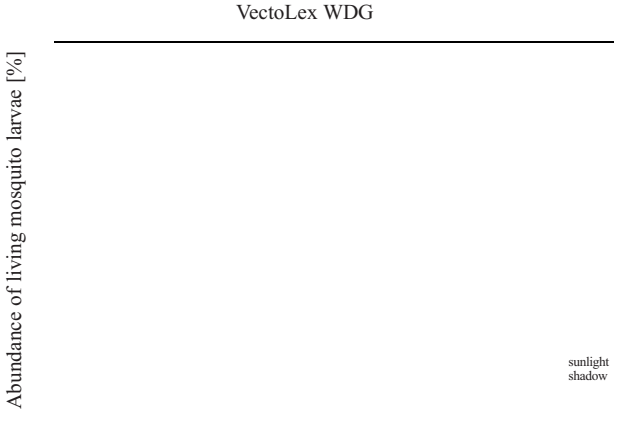

Fig. 2. Dynamics of the VectoLex® WDG activity in sewage conditions due to sunlight and shadow exposure.

After the application of VectoLex® WDG, there were no significant differences in larval mortality between dosages of 400 and 1,600 g/ha (p=0.082). However, the VectoLex® WDG efficacy is higher after two days post treatment. Over the following days, effectiveness dropped and the percentage of mosquito larvae increased (Fig. 2).

The modeling of the *Oc. caspius* larvae survival data showed that the activity of *Bs* is also reduced in sunlight, but the mortality effect depends on time, showing significant interaction between time and exposure conditions (p=0.000534). This means that during the first six days after VectoLex® WDG application, its efficacy remains constant in both sunlight and shade (Fig. 2). However, during this time the probability of larvae survival in containers exposed to sunlight is higher than in containers placed in shadows.

In the sunlight-exposed containers, the number of mosquito larvae was strongly reduced by VectoLex® WDG after 6.4 days and 6.9 days post-application in shaded conditions (Table 1). The probability of mosquito larvae survival ranged from 28% to 39% in shaded and sunlit exposure conditions, respectively.

Isolation of *Bti* and *Bs* Strains from the Sewage Sediments

The results of qualitative characteristics of spore-forming and non spore-forming bacteria occurrence within sediment samples collected from control and tested channels are summarized in Table 2.

During the study period, the relative proportion of the number of spore-forming to non-spore-forming bacteria in sludge samples was small. The percentage of endospore-production bacteria in each of the sludge samples was related, regardless of the origin of the samples (control or treated channels) and the sampling period. These percentages ranged from 0.12% to 0.82% for the control channels, and from 0.48% to 1.7% for the channels where biocides were used (Table 2). The investigation carried out after the application of microbial control agents in the drainage ditches within irrigation fields showed that the qualitative change within the populations of bacteria from *Bacillus* spp. was insignificant. The percentage of inclusion-forming *Bti* strains to the total number of bacteria in the sludge samples ranged from 0.026% to 0.027% in March and May 2007. In October 2007, the percentage of spore-forming *Bs* strains among the bacteria was assessed at the level 0.1%. More *Bs* strains were isolated from the tested samples (*Bs* index=0.15 in October 2007) than *Bti* strains (*Bti* index=0.034 and 0.055 in March and May 2007 respectively). In the control channels, no *Diptera*-specific *Bt* and *Bs* strains were detected.

Discussion

Our study indicated the presence of *Bti* or *Bs* spore-forming strains in sewage channels after application of commercially available microbial larvicidal formulations. However the great number of these strains among the total spore-forming bacilli were insignificant and ranged from

Table 2. Spore-forming bacilli occurrence within sludge samples collected from the irrigation channels before and after biocides applications.

Investigation period	Origin of sample	Total viable of bacteria [cfu/ml]	Total number of spore-forming bacteria [cfu/ml] (%)*	Percentage of *Bti* strains to the bacilli/ to total No. of bacteria [%]	Percentage of *Bs* strains to the bacilli/ to total No. of bacteria [%]	Index of isolates** (No. of total colonies bacilli examined)	
						Bti	*Bs*
March 2007	Control channel	1.6×10^6	3.2×10^3 (0.2)	0	ND	0 (87)	ND
	Tested channel	1.6×10^6	1.2×10^4 (0.75)	3.52 (0.026)	ND	0.034 (117)	ND
May 2007	Control channel	6.0×10^6	7.2×10^3 (0.12)	0	ND	0 (40)	ND
	Tested channel	3.1×10^6	1.5×10^4 (0.48)	5.55 (0.027)	ND	0.055 (54)	ND
October 2007	Control channel	3.5×10^6	1.9×10^4 (0.82)	ND	0	ND	0 (38)
	Tested channel	2.9×10^6	4.9×10^4 (1.7)	ND	6.1 (0.10)	ND	0.15 (41)

*percentage of bacilli among the total number of bacteria,
**index was calculated as the No. of colonies *Bti* or *Bs* strains divided by the total number of spore-forming bacterial colonies examined, ND – not determined.

3.5% to 6.1% during our investigation. Therefore, the negative impact on bacterial population abundance within the sediments occurring in tested sewage channels could not be confirmed. Most *Bacillus* species are widely distributed in nature. Spore-forming bacilli are usually found worldwide in soil, water, food, stored agricultural products, insects, and insect-breeding environments or the plant materials [19]. Both *Bt* and *Bs* were isolated with different frequency from various sites in the natural environment, in particular from soil, water, and plants, as well as from wastewater sludge [22]. Their occurrence is not necessarily related to the natural habitat due to the passive distribution and persistence of spores. Most *Bt* strains were also found in freshwater, brackish, and marine sediments in different parts of the world [23]. The distribution of *Bt* isolates throughout the world, like *Bs*, was variable, depending on the geographical origin of samples and the source of materials from which they were isolated [23, 24]. For example, the distribution of *Bt* strains in the soil samples of many countries in Europe and South America was considerable: 83.9% and 93.5%, respectively [25]. Quesada-Moraga et al. showed that the percentages of *Bt* strains among the *B. cereus/B. thuringiensis* group could range from 6.5% to 10.75% in soil samples collected from different parts of Spain [24]. Meanwhile, Dias et al. detected only 2.23% *Bs* and 1.6% *Bt* in soil samples of different origin from urban, agricultural, forested and horticultural areas in Argentina. More isolated strains were identified as *B. cereus* (66%) and other *Bacillus* spp. (30.2%) [22].

This study also shows that *Bti* and *Bs* WDG are effective and environmentally friendly tools for mosquito control under extreme conditions in larval habitats, including high organic pollution. The efficacy and activity of formulation of biopesticides based on *Bti* against the mosquito vectors were estimated by many investigations under field conditions. The manner of the biopesticide's application, as well as the types of habitats treated, are greatly important to persistence and maintain the activity of crystalliferous *B. thuringiensis* strains in the environment. The laboratory test showed that the effectiveness of aqueous and granule VectoBac products lasted for 1-4 days in polluted habitats such as cesspits, U-drains, and cement tanks, whereas the same biopreparations remained effective for 2-9 days in stream pools [26].

According to Boisver, the activity of *Bti* or *Bs* against target organisms can be influenced by environmental factors such as organic pollution, water temperature, and the presence of colloidal particles [27]. Water pollution, especially in the presence of free chlorine in the water, which was also detected in our study, can inhibit or destroy the endotoxin. A clear inverse correlation has been observed between the amount of chlorine in the water and larval mortality by Sinegre et al. [28]. Apparently, in the presence of organic and inorganic particles and/or floating materials, fewer toxin particles are ingested per unit of time than in the absence of extraneous materials. Moreover, the availability of *Bti* crystals is decreased by their adsorption onto suspended particles followed by a slow sedimentation. In cases of high larval density and/or water pollution, higher rates of application will be necessary for mosquito larvaiciding [29, 30]. However, the high efficacy of minimum dosages of various formulations of *Bti* and *Bs*, including VectoBac® WDG, VectoBac® 12 AS, and VectoLex® WDG in an irrigation ditch system against larvae of *Cx. p. pipiens,* was confirmed by Rydzanicz et al. [17]. The recommendation to use the minimum effective dosage may facilitate a more cost-effective use of microbial-control agents due to the lower cost of materials. In our microcosm tests, it was also

shown that the activity of microbial-control agents depends strongly on exposure conditions. This indicates that the long-term effect of microbial insecticides can be achieved only in covered (without sunlight), contained, breeding habitats with limited organic content and substrate. According to Rydzanicz et al., the lack of long-term activity of *Bti* dispersed on the water surface within natural mosquito-breeding sites in our study area may be explained by a combination of the specific abiotic or biotic features of the study area, such as exposure to sunlight, water column mixing from wind, or the combined effect of these factors and highly polluted water and extremely high densities of mosquito larvae [17]. Increasing sunlight lowers the efficacy of *Bti* and *Bs*, as was shown earlier in laboratory conditions [10].

Our study also demonstrated that in contrast to *Bs*, the persistence of *Bti* crystal proteins in natural conditions is short. It is well known that the toxicity of *Bti* lasts only a few days at most, and efficacy can be reduced within 24 hours [31]. The lower sensitivity of *Bs* may result from the fact that the protein of the bacterium is enclosed in the exosporium, whereas the δ-endotoxin of *Bti* is uncoated. It is possible that a coated spore-crystal complex is more tolerant to ultraviolet light than that the uncoated protein. This feature is also responsible for the slow mode of action of products based on *Bs* and its potential to persist under certain field conditions [6]. In laboratory study it was shown that the presence of mosquito larval cadavers in water contributes to the maintenance of toxic levels of *Bs* [5]. Larval cadavers seem to contain all the nutrients necessary both for vegetative multiplication of the bacteria and for toxin synthesis associated with the sporulation process. The germination of *Bti* in the gut of *Aedes/Ochlerotatus* larvae was also experimentally demonstrated. Laboratory studies in France with 4th-instar larvae of *Ae. aegypti* showed that suspensions prepared from 2 preparations of *Bti* retained their activity against the larvae for only 3-5 days, but this period could be extended by leaving dead larvae in the medium [32]. In our microcosm test series all cadavers and living larvae or pupae were removed every other day and replaced with untreated larvae to eliminate the residual activity of both microbial control agents.

The duration of action of *Bti* and *Bs* against larvae of *Anopheles stephensi* was also evaluated in Russia [33]. The activity of each product depended on the initial concentration of spores. The optimal concentration of spores was determined to be at least 10^5 spores/ml for *An. stephensi* larvae. The number of larvae/test containers also influenced the efficacy and duration of activity. The more larvae present in test vessels, the shorter the duration of kill. The presence of dead larvae in the test vessel also increased the duration of action by *Bti* as much as four times compared to test vessels where the dead larvae were removed.

Conclusions

The examinations conducted show that the activity of microbial insecticides in natural conditions is strongly reduced by sunlight. Therefore, this feature has to be taken under consideration and the number of biocide applications should be tailored to the mosquito larval development in each particular breeding site. Despite the high organic contamination occurring in the mosquito breeding-sites located within irrigation fields in Wrocław, the application of the minimum dosage of microbial control agents based on *Bti* and *Bs* results in efficient lethal effect on floodwater mosquito larvae. The routine application of the commercially available microbial insecticides against mosquito larvae showed insignificant qualitative change within bacterial populations occurring in the sediments within sewage channels. This limits any environmental risk associated with the biocide's persistence.

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