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Research article

COMPARISON OF MOSQUITO LARVICIDAL FORMULATIONS OF DIFLUBENZURON ON *CULEX PIPIENS* MOSQUITOES IN BELGRADE, SERBIA

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Culex mosquitos are important infectious diseases vectors in temperate and tropical regions of the World, affecting nearly 350 million people in both developed and developing countries. Our approach was to "recycle" the well-established larvicide, and by studying the tablets, pellets and granules as floating or sinking formulations, we found a method to optimise the use of diflubenzuron against *Culex pipiens* mosquitoes in field conditions.

A standard WHO procedure was used to test the larvicide efficacy. The combined efficacy of all floating formulations was 10.7% higher than sinking preparations (*p*-value =0.002) and that maximised throughout the experiment on days 14 and 21, (*p*-values 0.012 and 0.008, respectively). All floating formulations kept their efficacies above 70% until day 21 of the experiment, while the mortality of sinking designs dropped significantly after day 14. The lowest efficacy was observed when sinking granules were used and the highest when floating tablets were applied in the canals. Only the floating tablets showed no significant changes in efficacy from day 1 to 21, but then that efficacy drops sharp until day 35.

Since the larvae spend most of their time on the surface of the slow-moving waters to breathe, and floating pellets and tablets are made of materials that can serve as food sources, we conclude that these formulations have a higher efficacy, at least in conditions of non- or slow-moving waters. This study shows the importance of a systematic approach to reformulation of old, already proven and environmentally safe larvicides which can control the mosquito populations and their spreading of various pathogens.

Key words: *Culex pipiens*, diflubenzuron, larvicide, mosquito

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INTRODUCTION

Culex mosquitos are important infectious diseases vectors in temperate and tropical regions of Americas, Europe and many parts of the Middle East and Northern Africa. The diseases these mosquitos transmit, according to WHO affect nearly 350 million people in both developed and developing countries worldwide [1]. *Culex* mosquitoes are the most important vector of West Nile virus (WNV) [2-4], St Louis encephalitis virus, as well as avian malaria, and filarial worms [5-7]. WNV has been reported in Serbia since 1970s through nationwide sero-epidemiological surveys and it was detected in *Culex pipiens* mosquitoes during a mosquito surveillance programme in 2010 [8,9].

The Belgrade area has very good environmental conditions for the development and survival of numerous mosquito species, among which the most common are *Culex* mosquitoes [10]. Belgrade is situated on two big rivers, and has more than 3000 km of drainage reclamation canals and other water surfaces, with a very poor city sewage system, and most of the households have septic tanks with overflow pipes that go directly into the canals in suburban areas. All of these make very good environmental conditions for the development mosquitoes in large numbers.

In Belgrade, for larvicidal treatment of mosquitoes, since 1970s, temefos in various formulations was used. In 2012 EU banned the use of organophosphate larvicides, which urged the need for the development of new, environmentally friendly substances. One of many, diflubenzuron has been used since 2012. Mainly, the larvicide was used in formulation with zeolite - to create the sinking granules, which were used predominantly, or corn-cob for floating granules.

Diflubenzuron is classified as an insect growth regulator (IGR) of the benzoyl urea family. It is nonselective chitin synthesis inhibitor, which interrupts the formation of the exoskeleton of target organisms and consequently larval growth. Given that resistance to existing larvicides is becoming more common throughout the World, and new active substances are not being introduced, it is necessary to improve the effect of existing approved larvicides by optimizing the new formulations that will allow them longer contact with larvae and a better effect.

This study was designed to evaluate the initial and residual activity of three floating and three sinking formulations with diflubenzuron in field conditions of slow-moving waters, against *Culex pipiens* mosquitoes.

MATERIAL AND METHODS

Formulation preparation: Floating and sinking formulations in the form of tablets, pellets and granules with diflubenzuron were prepared by using only organic compounds: zeolite for sinking and corncob for floating formulations. The active ingredient diflubenzuron was provided by TNJ Chemical Industry Co., Ltd. The concentration of the active substance in the product was 10% tablets, 5% pellets and 1% granules. The tablets were 4 cm in diameter, about 0.7 cm thick and weigh 10 g. The pellets were about 3 cm long and 0.5 cm in diameter, while the granules were up to 2 mm in size. The used quantity of both sinking and floating tablets, pellets and granules was calculated so that the concentration of 100 g/ha was used.

Study area: The experiments were done on two canals located near a pheasant farm on the left bank of the Danube river, near Belgrade. No larvicide treatment, in the previous 5 years had been performed on the selected canals.

The first canal has a total length of 850 m, while the second one is about 730 m long. The width of both canals in average is three meters. Both canals are closed on one end so that the flow of water in them is minimal. Canals are surrounded by arable land and, at the time of the study, wheat was planted. Both canals are overgrown on both sides with marsh vegetation where Narrow leaf cattail (*Typha angustifolia*, fam. *Typhaceae*) and sedges (*Carex* spp., Fam. *Cyperaceae*) predominated, and Duckweeds (*Lemna* spp., fam. *Lemnaceae*) on the water surface. On each canal, treatment was performed on areas at least 200 meters apart, with several different formulations. On the first canal, the following formulations were used: floating tablets, sinking tablets and floating pellets. On the second canal the formulations were used in the following order: sinking pellets, floating granules, and sinking granules. Each formulation was used on the area of 100m in the length of canal. For all formulations, the treated area was around 300 m^2, or 0.03 ha.

Application and dosage: The dose was determined based on WHOPES recommendations, and taking into account the type of habitat, as well as the number and type of larvae present, it was decided that the dose of active substance should be 100 g a.i./ha [11]. The application of the prepared formulations was done by manual dispersion. For the treatment of the planned area of 300 m^2 three sinking tablets (30 g), three floating tablets (30 g), 60 g of sinking pellets, 60 g of floating pellets, 300 g of sinking granules, and 300 g of floating granules were used.

Larval sampling: From each treated area, eight samples were taken about 10 m apart from each other. Sampling was done from the same marked places for each time point during the experiment. Larvae were collected with a standard 300 ml dipper. After taking the sample, larvae were poured into a plastic, four L container and filled with water up to two litters. A mosquito net was placed on each container to catch the adult mosquitoes that emerged. Only larvae of L_3 and L_4 developmental stages were left in the container and their mortality/emerging was recorded. Larval sampling was done on day one, seven, 14, 21, 28 and 35 post treatment.

Data analysis: The overall larval numbers were calculated, with the associated exact binomial confidence intervals (95 % CI). To compare the floating and sinking formulations the Student – T test was used. Further, analysis of variance (ANOVA) for the comparison among each formulation type. For the group differences between floating and sinking formulations in each time point separately, the univariate Student

T-Test, while differences in individual formulations were compared by using the ANOVA. To determine which variables in ANOVA bear the most differences, a post hock significance range test was performed using Tukey. HSD core function in R. All statistical analyses were performed using R (http://www.R-project.org/), using the tidyverse, lme4, ggpubr and rstatix packages [12].

RESULTS

The discovery and introduction of new larvicides to control the mosquito populations is very long, exhaustive and expensive process that very few dare to undertake. Regardless, the reports of mosquito larvicide resistance are becoming more and more frequent, so to be able to prevent transmission of infectious diseases for which these insects are vectors, the new methods for application of existing active compounds are necessary. We tested the efficacy of six diflubenzuron formulations (three floating and three sinking) on *Culex spp.* larvae in field conditions of slow moving or standing waters.

Floating larvicides have higher efficacy rate in standing waters

The combined efficacy of all floating formulations is significantly higher than sinking designs when all time points are combined (Fig 1) (p=0.002). Moreover, the mean efficacy of floating larvicides was 10.7% higher than sinking formulations (75.8% vs 65.1%, respectively) although the minimal values were similar (30.5% and 27.3%,

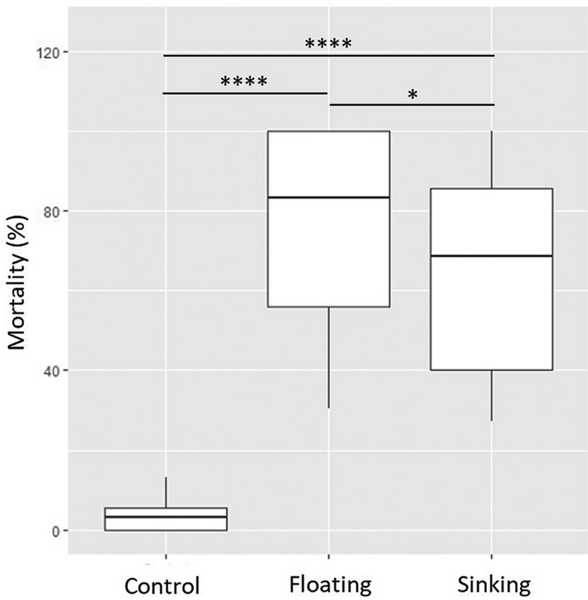

Figure 1. Difference in efficancy between floating and sinking formulations of diflubenzuron in field conditions

respectively) and the difference in standard deviation remained consistent (23.2 and 24.6, respectively).

The statistically significant differences in efficacy between floating and sinking formulations were observed throughout the whole experiment. The lowest differences were recorded at the beginning of the experiment, on day-1 where floating formulations were on average 2.3x more efficient than sinking formulations (p-value = 0.028 95% CI: 0.27 - 4.39). The highest differences were observed on days 14 and 21, when floating formulations were lethal for 13 times more larvae than sinking ones (12.9x and 13.02x respectively) (p-values 0.012 and 0.008, 95%CI 3.11 - 22.87 and 3.73 - 22.31, respectively) (Fig 2). The differences between floating and sinking formulations lowered at the conclusion of experiment on day 35 to 6.8x higher lethality of floating designs (p-value = 0.003, 95%CI 2.51 – 11.01) (Fig 2).

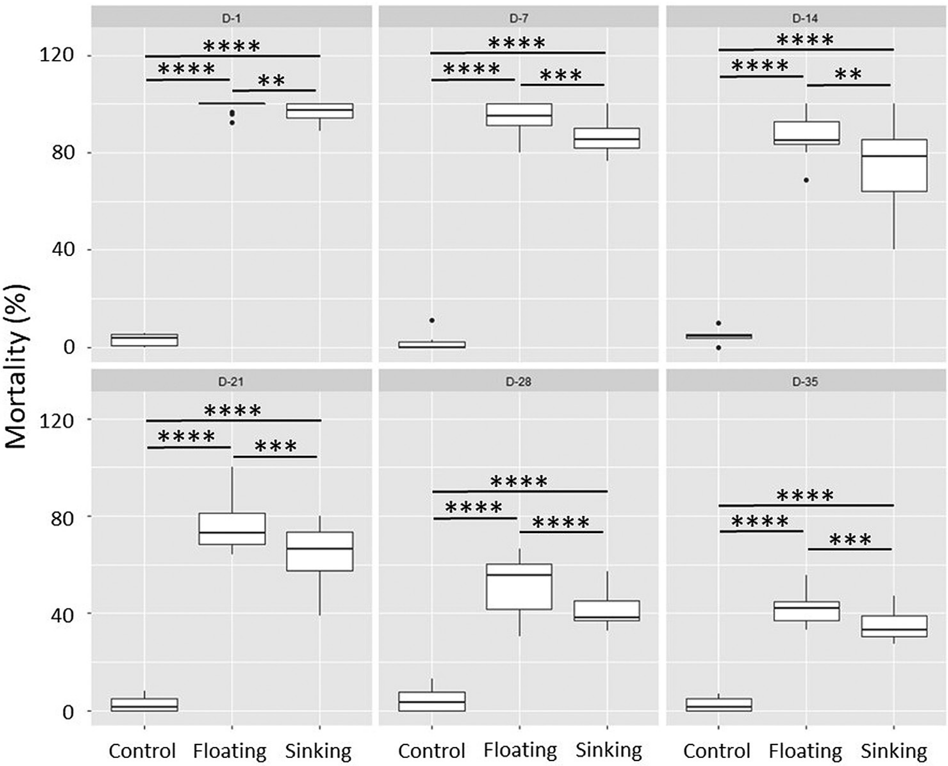

Figure 2. Difference in efficancy between floating and sinking formulations of diflubenzuron in field conditions throughout the course of the experiment

Both floating and sinking pellets lose their efficacy in the same time dependent manner

When the efficacy of different floating and sinking formulations was compared throughout the course of the experiment, the floating and sinking pellets showed the same time-dependent loss of efficacy (Fig 3). Therefore, there is no statistically significant difference in efficacy between floating and sinking pellets although the latter showed to be slightly less fatal for *Culex* mosquito larvae (*p*-value = 0.258). Contrary to the pellets, floating granules exhibited 12.4 times higher efficacy in all, but D-1, time points of the experiment (*p*-value = 0.047, 95%CI: 0.1 – 24.6). Floating tablets efficacy dropped the least during the first 21 days of the experiment (from 97% to 89%), contrary to sinking tablets which lost their efficacy by this time point as low as 72%, and were significantly less efficient throughout the experiment (12.9 times, *p*-value = 0.027, 95%CI: 1.5 – 24.3) (Fig 3). Areas under the curves (AUC) in Fig 3: the lowest efficacy was observed when sinking granules were used (AUC: 2048.1) and the highest when floating tablets were applied in the canals (AUC: 2750.62). Equally interesting, the highest difference in efficacy between floating and sinking formulations was observed in the tablet preparation, and the lowest in the efficacy of granules.

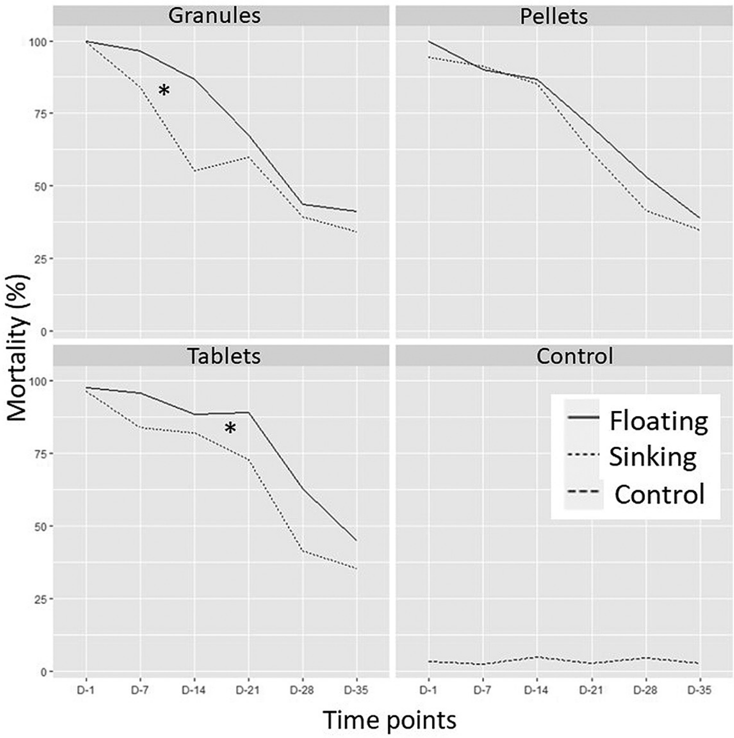

Figure 3. Efficancy of different diflubenzorone formulations throughout the five weeks experiment

Floating formulations keep high efficacy rates longer than sinking

All floating formulations (granules, pellets and tablets) keep their efficacy above 70% until day 21 of the experiment, while efficacy of the sinking design drops significantly after day 14 (Fig 4). All formulations show a statistically significant temporal loss in efficacy (Fig 4). In all, but floating tablets, the mean efficacy dropped below 60% until day 28, while at the conclusion of the experiment floating formulations had their mean efficacy above 45% (Fig 4). For neither of floating formulations, there was no statistically significant difference between day one, seven and 14, whereas the difference was statistically significant even between seven and 14 days for sinking granules (*p*-value =0.0003, 95%CI: -46.12 - -11.31). Of all, only the sinking tablets had significantly lower efficacy on seventh compared to the first day (*p*-value = 0.0338, 95%CI: -24.16 - -0.67), but then, there was no change in efficacy until 21st day of experiment. Only the floating tablets showed no significant changes in efficacy from day 1 to 21 (Fig 4), but then that efficacy drops sharply until day 35.

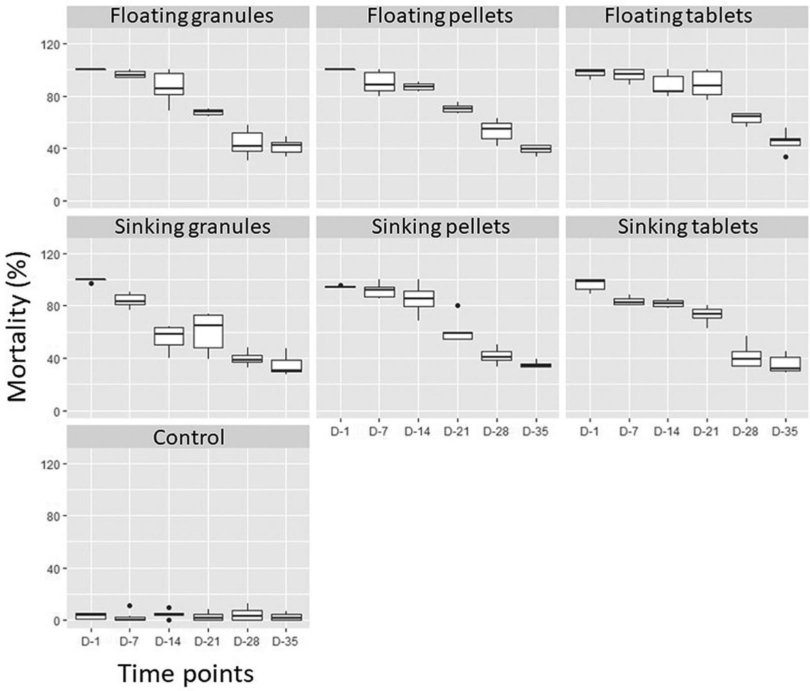

Figure 4. Efficancy of each diflubenzuron formulation in field conditions throughout the course of the experiment

DISCUSSION

Control of mosquito populations became the very thin line between the complete eradication of the species, environmental pollution and development of resistance in targeted species. At the same time, with global warming, many mosquito species are spreading their habitats, interestingly more and more to urban areas [13-16]. Official data from the WHO state that in the last fifty years only 40 new compounds against all pathogens and their vectors have been registered, while these diseases contribute to

25% human mortality around the globe (cardiovascular diseases contribute with 31% of the cases). At the same time, mosquitos and the diseases they transmit to animals represent a great economic, veterinary and public health threat. From the beginning of this year, eight new potential larvicides have been proposed worldwide [17-24]. However, all these compounds have to pass the long processes of safety evaluation, environmental sustainability, dosing, etc. Therefore, our approach was to "recycle" the old, well-established larvicides, and by studying the formulations, we found a method to optimise the use of diflubenzuron in field conditions of stagnating or slow-moving waters that never dry out. To the authors' knowledge, this is the first systematic approach to studying the different formulations of larvicides in field conditions.

Belgrade lays at the estuary of two big rivers, Sava and Danube. Both of these rivers in the city and its suburbs have big areas where the banks are not maintained and where rivers annually cause floods. In the same time, the network of over 3000 km of drainage and irrigation canals has been neglected for decades to such an extent that it became the recipient of sewage waters for local neighbourhoods. Meanwhile, with the global raise in annual temperatures the Belgrade area became an ideal ground for development of large mosquito populations, as well as pathogens they transmit [25]. Therefore, the isolated parts of the drainage and irrigation canals around Belgrade became the ideal field-testing ground for the efficacy of various larvicide formulations. Systematic approach and constant monitoring of the efficacy can assure the release of sufficient doses of larvicide, which is important for stopping the development of resistance to the active substance [26]. Diflubenzuron is a very effective insect growth regulator available for mosquito larvae control not only in Serbia, but worldwide [27-29].

In our study, the sinking formulations were made using zeolite clay. Zeolite, because of its non-binding to the active compounds, a slow steady degradation time and non-polluting characteristics, was earlier validated by our group as the material of choice for sinking larvicide formulations [10]. Our conclusion is that sinking formulations have the lowest efficacy, at least in the conditions of non- or slow-moving waters. These results can be explained by the fact that the larvae spend most of their time on the surface of the water due to breathing, and go down to the bottom only for food, if there is no food on the surface, and when they are scared [14-16,30-32]. Sinking formulations have a weaker residual effect compared to floating ones because the bottom of slow-moving waters is often muddy and full of detritus, so they become often covered with mud in a short time, which reduces the release of the active substance. Similar results to ours were obtained by Cetin at al., within the first 21 days of experiment [33]. On a day 28 in the closed septic tanks their sinking formulations maintain efficacy, whereas in our study that efficacy is lost in the open waters and continues to decline until the end of the experiment.

Unlike sinking, floating formulations remain on the surface of the water or in the upper layers where they make close contact with the larvae. Bearing in mind that pellets and tablets are made of ecological materials that do not pollute the environment, and can serve as food attracting larvae, they achieve much higher intake than granules. Msangi *et al.* (2011) [34] saw also higher efficacy of tablets compared to granules and powder when compared the different concentrations of diflubenzuron on larvae of *Anopheles* and *Culex* species. In their experiment, tablet concentrations kept a very low survival rate (below 0.5%) of the larvae throughout the 30 days of the experiment, whereas, granules initially had an efficacy slightly above 0.5%, which dropped only at the conclusion of the experiment. In our study we didn't test the powder formulations of diflubenzuron, although they showed very good residual effects over the 23 and 30 days of the experiments on *Aedes* spp and *Anopheles* spp, respectively in two separate studies of Chen *et al.* (2008) [35] and Msangi et al. (2011) [34]. Both of these studies also showed that after the time of 23 and 30 days, respectively, the efficacy of the powders drops significantly, so we opted for the formulations that will release diflubenzuron slower over a longer period of time. The higher efficacies of larger formulations, such as pellets and even larger tablets, can be explained by their production technology. In both cases, the active substance is added to the mixture before pelleting or tableting. This way, the active substance remains inside the pellets and tablets achieving a longer residual effect.

Some studies showed that sinking formulations are effective in controlling the mosquito larvae especially in fast running waters or semiarid water sources [36]. However, our experiments clearly show that floating formulations have much higher efficacy when applied on the open, slow-moving waters, used for irrigation, as pastures or even water reservoirs for urban and semi urban environments. Moreover, because of the food sources for larvae, floating pellets and tablets show much longer residual effects. This study shows the importance of a systematic approach to reformulation of old, already proven and environmentally safe larvicides which can control the mosquito populations and their spread of various pathogens.

The effects of diflubenzuron on non-target organisms depend on the species that was in contact with it. It does not pose a high risk of acute and chronic toxicity to mammals, fowl, fish, and wild birds, but it is highly toxic to both aquatic invertebrates and crustaceans [37]. Toxicity to other aquatic organisms should be taken seriously and the recommended doses and time between two applications should be strictly adhered to during aplication.

Authors' contributions

BP conducted field research, participated in the development of formulations and drafted the manuscript. ZK carried out the production of formulations and determined the method of application and dosage. RT participated in field research. ST participated in the design of the study and performed the statistical analysis. VDJ participated in statistical analysis. MDJ conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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POREĐENJE FORMULACIJA LARVICIDA NA BAZI DIFLUBENZURONA NA KOMARCIMA *CULEX PIPIENS* U BEOGRADU, SRBIJA

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Komarci iz roda *Culex* su značajni vektori infektivnih bolesti u umerenim i tropskim regijama sveta, zahvatajući skoro 350 miliona ljudi kako u razvijenim, tako i u siromašnim zemljama. Naš pristup je podrazumevao "recikliranje" već postojećeg larvicida, a pravljenjem plivajućih i tonućih formulacija tableta, peleta i granula pronašli smo metod da optimizujemo upotrebu diflubenzurona u terenskim uslovima za suzbijanje *Culex pipiens* komaraca.

Za testiranje efikasnosti larvicida upotrebljena je standardna procedura WHO. Kombinovana efikasnost svih plivajućih formulacija je iznosila 10.7% više od tonućih (*p*-value =0,002) i maksimalnu vrednost je postigla 14. i 21. dana (*p*-values 0,012 i 0,008). Sve plivajuće formulacije zadržale su efikasnost iznad 70% do 21. dana eksperimenta, dok je mortalitet primenom tonućih formulacija značajno opadao posle 14 dana. Najniža efikasnost je zabeležena kod tonućih granula, a najviša kod plivajućih tableta. Samo plivajuće tablete nisu pokazale značajnu razliku u efikasnostiod 1. do 21. dana, ali im efikasnost opada značajno nakon 35. dana.

Imajući u vidu da larve veći deo svog vremena provode na površini vode zbog disanja, a da su plivajuće tablete i pelete napravljene od prirodnih materijala koji mogu larvama poslužiti i kao hrana, zaključili smo da ove formulacije imaju veću efikasnost u uslovima sporotekućih voda. Ova studija pokazuje značaj sistematskog pristupa reformulisanju starih, već dokazanih larvicida koji mogu suzbiti populaciju komaraca i širenje bolesti.