

Research article

STATISTICAL EVALUATION OF THE LARVICIDAL EFFECT OF DIFLUBENZURON ON *CULEX PIPPIENS* LARVAL STAGES

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Diflubenzuron is increasingly used in areas where mosquito larvae developed resistance to other insecticides. In our community diflubenzuron is not used to control mosquito larvae. Two formulations of 1% diflubenzuron (on corn-cob EF-1, and zeolite EF-2) were tested on *Culex pipiens* L (larvae) on one canal in the Belgrade suburb area. The effect was followed for seven weeks after application of the formulations. Formulation EF1 achieved a reduction in mosquito L₁L₂ larvae between 23.9% and 89.4%. The change was statistically significant the 21st and 28th day ($p < 0.001$), 35th and 42nd day ($p < 0.01$) and 49th day ($p < 0.05$). The maximal reduction obtained by formulation EF2 was 69.1%. The accomplished reduction was significant on the 28th and 42nd day ($p < 0.001$), 35th day ($p < 0.01$) and 21st ($p < 0.05$). Both formulations have maintained a good residual effect on the lower developmental larval stages. Maximum reduction achieved by EF1 on L₃L₄ larvae was 97.4%. Reduction of larvae was high between the 7th and 42nd day (66.4 - 97%). Statistically significant values were recorded on the 21st, 28th and 35th day. Formulation EF2 achieved a reduction of 99.5%. A statistically significant reduction in the value of mosquito larvae was obtained on the 14th, 21st, 28th, 35th and 42nd day.

Between the two used formulations there was no significant difference in the number reduction of lower larval stages, but for the higher larval stages EF1 proved to be more efficient.

Key words: diflubenzuron, formulation, cob, zeolite, larvae, mosquito, *Culex pipiens* L.

INTRODUCTION

Mosquitoes are vital insect species regardless of changing environmental and climatic factors, host species and habitat status. Although part of their life cycle is related to the aquatic environment, mosquitoes are biological vectors of numerous known and still

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undiscovered infectious agents [1-5]. The most successful form of protection from the transmission of agents are lack of contact with mosquitoes and reduction of their numbers [6,7]. This is not easy when one takes into consideration their diversity, breeding potential, size and mobility. Reduction of the number of mosquitoes can be achieved through changes of conditions for their maintenance on the site, use of protective clothing, nets, and repellents [8,9]. During the suppression of mosquito larvae with larvicides of chemical and microbial origin, as well as growth regulators, resistance can develop. Resistance can be accelerated due to an unplanned and uncontrolled use of these substances. Even before the appearance of organic insecticides, resistance to inorganic insecticides was registered in 12 species of arthropods [10]. In 1946 resistance was detected in two species of insects, in 1980 in 150 species, and in 1990 resistance was detected in 198 species. According to the World Health Organization (1992) resistance was disclosed in 56 species of mosquitoes of the genus *Anopheles*, 19 species of mosquitoes of the genus *Aedes* and 20 mosquito species of the genus *Culex*. Resistance was detected in larvae and adult individuals [11]. By 2008 resistance was detected in 553 arthropod species, of which 202 species are of public health importance [12].

The Belgrade environment offers conditions for the development and survival of a number of mosquito species among which a few are primary vectors of malaria, dirofilaria and some arboviral infections [13-16]. In the Belgrade district the presence of potential malaria carriers *Anopheles* mosquitoes has been previously described [15]. On Belgrade city territory in year 2012 in *Culex pipiens* mosquitoes the presence of West Nile virus was recorded [17]. In the earlier period adult mosquito forms were controlled with malathione based compositions, permethrine, lambdacyhalothrine and deltamethrine [18]. The long term use of organophosphate larvicides in the Belgrade area, as well as lack of information pertaining the resistance to larvicides, and their removal from the list of allowed biocides had influenced the need to study the effects of alternative substances for the control and elimination of mosquito larvae [19-21]. In the recent years there is an increased interest in studies on the larvicidal effects of diflubenzuron based formulations. These formulations are recommended by the World Health Organization [7,22] and the European Commission for Biocides.

In this paper we have studied the larvicidal effects of 1% diflubenzuron applied on two different carriers in a granular formulation with a granule diameter of 0.7 and 2.0mm. The aim of our trial was to study the efficacy of the tested formulations on different mosquito larval stages in order to assess their potential application in mosquito control programs in the Belgrade area.

MATERIAL AND METHODS

Two granulated 1,0% diflubenzuron 1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl)-urea formulations (96% a.i. Radon China Product Chemistry) were tested on *Culex pipiens* *L.* mosquito larvae in a dose of 100g a.i. per hectare of treated water surface. In the

experimental formulation 1 (EF 1) the difluobenzene was corncob in the form of 0.7mm granules. The carrier used in experimental formulation 2 (EF 2) was zeolite in the form of 2mm granules. The percentage share of diflubenzuron in the formulations was determined by gas chromatography and for both formulations measured 1.0%.

The trial was carried out in the Belgrade area on a hydromelioration channel on the Danube left bankshore (44 ° 53 '29.7 "N 20 ° 27 '20, 61" S; altitude 70m, Borca canal). The channel was 820m long, 2.5m wide and 0.4-0.6m deep. The water was slow-moving and rich in organic waste material. For the here presented study three 100m sections of the channel were selected with a 100m buffer zone between each of the sections. The first 100m section, with an area of 250m², was taken as the control. Between the control area and the area treated with EF1 (250 m²) there was an untreated buffer zone whose area was 250 m², also. Concurrently, between the EF1 treated zone and the EF2 treated zone there was an untreated 250m² zone. The areas treated with formulations EF1 and EF2 were subjected to 250g preparation based on 1% difluobenzuron (2.5g a.i. diflubenzuron per 250 m² water surface). Prior to treatment (August 2012) 8 water samples from every previously mentioned section were taken at 10 m intervals. Each sample volume was 250ml in order to assess the number of larvae, their stage of development and the species of larvae present. Mosquito species were determined on the basis of morphological characteristics of the developmental stages of larvae according to the identification key described by Utrio [23]. Water temperature was measured using a Trotec[®] BT-20 thermometer and air temperature by Kestrel 4000[®] weather station during the treatment and at each control.

Before the treatment water samples were taken in order to monitor the following parameters: biological oxygen demand (BOD₅) - method with ion selective electrodes using a portable oximeter DO6 + - EUTECH and cooling thermostat with ET 618-4; chemical oxygen demand (COD) - Dihromat/H₂SO₄ method, total nitrogen - persulphate digestion method, free ammonia - salicylate method, nitrates - method with chromotropic acid, sulfates - method with barium sulphate turbidity, phosphates - a method with ammonium molybdate and water hardness- method using a photometer with metalphthalein Multidirect - LOVIBOND and Thermoreactors RD 125 - LOVIBOND; turbidity was determined by using the formazin standard Fotometar checkdirect turbidity - LOVIBOND, pH value was determined using a pH meter - EUTECH; dissolved oxygen was measured using a portable oxymeter DO6 + - EUTECH.

The experiment started on the 10th August 2012 with the application of EF 1 and EF 2 on both waterbanks along the observed water sections in a width of 50cm. Both experimental surfaces were treated with 250g 1.0% diflubenzuron. Testing the

efficiency of the above formulations on mosquito larvae was performed at: 24h, 48h, 72h, 7, 14, 21, 28, 35, 42 and 49 days from treatment. Throughout the experiment eight water samples were taken at each location with a dipper in a volume of 250ml per sample. The larvae were counted and grouped according to different stages of development.

Estimation of the efficiency of the described diflubenzuron formulations was done according to the developmental stage of the larvae, earlier L₁ and L₂ stages were observed separately from the late L₃L₄ stages, according to the formula:

$$E = 100 - \frac{C_1}{T_1} \cdot \frac{T_2}{C_2} \cdot 100$$

E= reduction effect, expressed in percent

C₁ = number of larvae in the control channel before treatment

T₁ = number of larvae in the control channel after treatment

T₂ = the number of larvae in the treated channel before treatment

C₂ = number of larvae on the control channel after treatment

Statistical analysis of the obtained results included descriptive statistical parameters. These parameters enabled us to describe and interpret the obtained experimental results. In the process of testing and describing the statistically significant differences analysis of variance was applied. Kolmogorov-Smirnov Test for normality was used to determine whether sample data were normally distributed. As the obtained data was not normally distributed and the data depth series was small Kruskal-Wallis non parametric variance analysis was performed. Individual comparisons were performed with the Mann-Whitney U test and Dunn's multiple comparisons test. Level of significance was set at 5, 1, and 0.1%. Correlation analysis defined the strength of the dependence of the analyzed parameters expressed as Pierson's coefficient of linear correlation (r_{xy}). Statistical analysis of the obtained results was carried out with the aid of the statistical package PASW Statistics 18 and MS Excel.

RESULTS

Evaluation of the efficacy of two formulations based on 1.0% diflubenzuron on corncob and zeolite was carried in the Belgrade suburb district on a channel infested with *Cx. pipiens* L. mosquito larvae (Picture 1). The presence of overflowing septic tanks into the canal where the experiment was organized influenced the change of water quality and the increased presence of organic and inorganic substances that contributed to the development of *Cx. pipiens* L. mosquito larvae. The tested water samples measured increased concentration values of ammonia, total nitrogen, organic phosphorus and hardness compared to the average values measured for ammonia, total nitrogen and organic phosphates concentrations, as well as water hardness (Table 1).



Picture 1. Geographical coordinates of the location on which the study was carried out

Table 1. Some indicators of water quality in the Belgrade suburb district (Borča water canal)

Type of analysis	Values obtained	Marginal values of pollutants
Nitrates	0 mg/l	3 mg/l
Ammonia	12.4 mg/l	0,3 mg/l
Total nitrogen	60 mg/l	3 mg/l
Sulfates	99.7 mg/l SO ₄	100 mg/l
Phosphates	10.1 mg/l	0,1 mg/l
Chemical O ₂ consumption	71 mg /l O ₂	15 mg /l O ₂
Biological O ₂ consumption	4.38 mg/l O ₂	do 5 mg/l O ₂
Dissolved oxygen	1.2 mg/l	7 mg/l
pH	7.2	6,5-8,5
Water temperature	22.8°C	15-30 °C *

* optimal temperature for development of larvae

Throughout the experiment regular measurements of water and air temperature were taken. During the trial there was no statistical difference ($p > 0.05$) in water temperature between the diflubenzuron treated (EF1 and EF2) sections and the control. Water temperature in the canal was in the range between 15.1 and 24.6 °C (Figure 2). Air temperature varied between 8.5°C in the morning hours and maximal 39.5 °C in the daytime (Figure 1). No statistically significant differences ($p > 0.05$) were recorded between the treated areas (EF 1 and EF 2) and the control section of the canal.

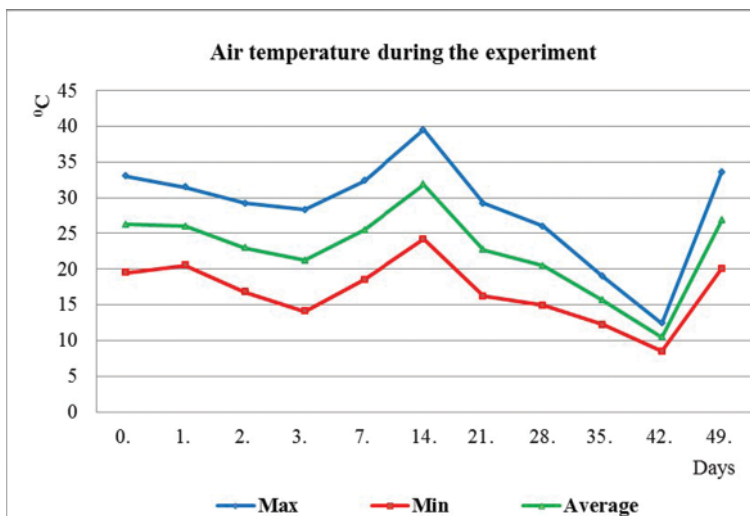


Figure 1. Air temperature during the experiment

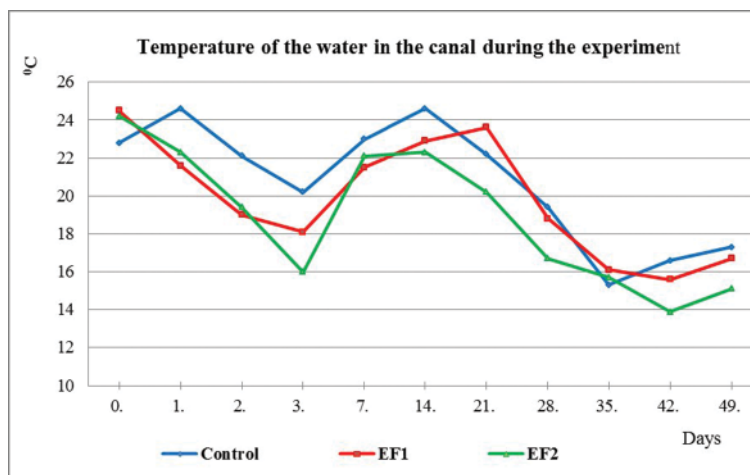


Figure 2. Temperature of the water in the canal during the experiment

The degree of correlation between the presence of mosquito larval stages and water temperature was established according to Pierson-s coefficient of linear correlation. A significant correlation was described between larval stages L_3L_4 in the control section ($p < 0.05$) and L_1L_2 in the EF1 and EF2 sections ($p < 0.05$). (Table 2.).

A reduction on the number of lower developmental *Cx. pipiens* larval stages (L_1 and L_2) in the treated water sections (EF1 and EF2) was registered the first day after treatment. Formulation EF1 induced a reduction of 48.7% and formulation EF2 induced a decrease by 47.7%.

Table 2. Correlations between developmental stages of *Cx. pipiens* mosquitoes and water temperature

CANAL	L ₁ L ₂	L ₃ L ₄	
CONTROL	r= 0.4540 p= 0.161	r = 0.6689 p = 0.024	p< 0,05
EF1	r= 0.6621 p= 0.026	r = 0.4320 p = 0.185	p<0,05
EF2	r= 0.702 p= 0.016	r = 0.4752 p = 0.140	p<0,05

The correlations are significant at p< 0.05 L- larvae

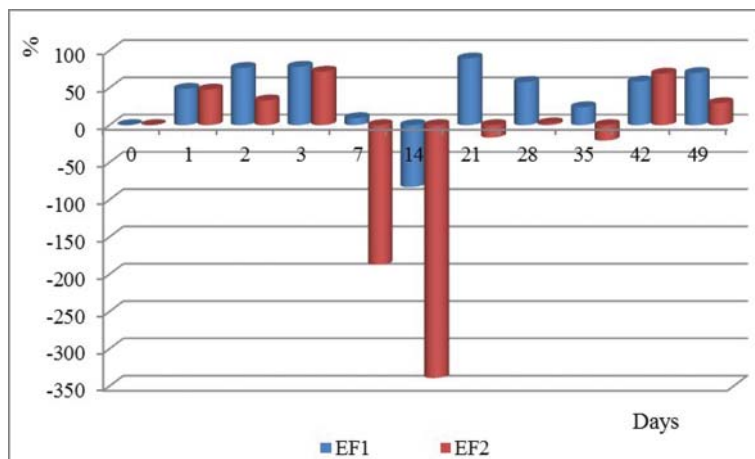


Figure 3. Reduction of the number of L1, L2 larvae due to EF1 and EF2 treatment, expressed as percents

The reduction in the number of larvae in the EF1 treated section increased between the first and third day from 48.7% to 78.2%. In the same three-day period EF2 treatment resulted in a decrease in reduction on the second day. During the first three days the reduction rate was 47.7% the first day, 33.2% the second day, and 71.6% the third day after EF2 treatment. Formulation EF1 induced a reduction in the number of larvae on the 7th and 14th day after treatment, thereon it increased to a maximum on the 21st day when it measured 89.4%. (Figure 3.)

Formulation EF2 resulted in a negative reduction in the number of larvae on days 7, 14, 21, and 35. On days 28, 42, and 49 the same formulation resulted in a positive reduction which ranged between 2.36% and 69.01%.

EF1 formulation showed better results during the experimental 7 weeks in the number reduction of *Cx. pipiens* L. lower larval stages compared to EF2. Both formulations showed a residual activity on L₁, L₂ mosquito larvae during the trial period.

Analysis of the significance relative to day zero in the water treated with formulation EF1 measured significant values on day 3 (p<0.05), 21, 28, 35, 42 and 49 (p<0.01).

Statistical analysis of the larvicidal effect of formulation EF2 relative to day zero was significant ($p < 0.05$) on day 21, and ($p < 0.01$) on days 21, 35, and 42.

Analysis of the statistical significance in L_1 , L_2 larvae number reduction for both formulations, showed that formulation EF1 significantly ($p < 0.01$) reduced the number of larvae on the first day when compared to the control untreated section. Comparisons of the reduction numbers throughout the trial period showed no statistical significance ($p > 0.05$). (Figure 4).

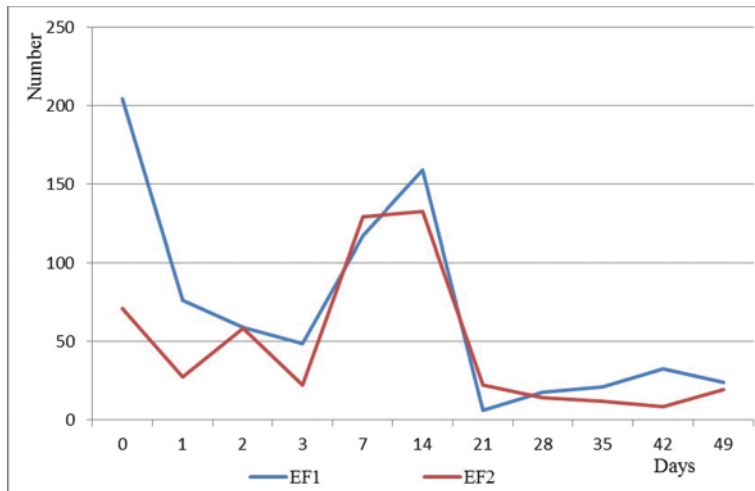


Figure 4. Comparison of the effects of EF1 and EF2 formulations on the reduction of the number of L1, L2 larvae

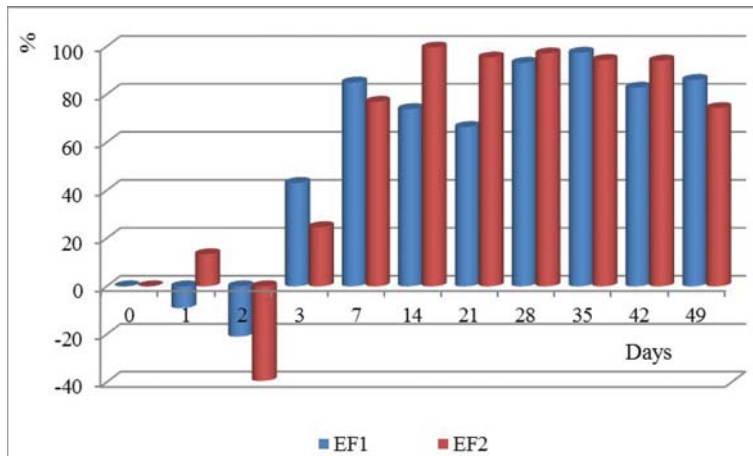


Figure 5. Reduction of the number of L3, L4 larvae due to EF1 and EF2 treatment, expressed as percents

Higher developmental stages of *Cx. pipiens* larvae (L_3 , L_4) treated with EF1 and EF2 the first and second day after treatment did not show a positive reduction in the number of larvae. Such reduction was recorded on the third day after treatment of the

EF1 treated canal sector, when it reached 43.1% compared to day zero. In the canal sector treated with EF2 the reduction in the number of larvae on the first day after treatment was 13.4%. In the following days the EF1 treated mosquito larvae showed a reduction in their number which ranged from 66.6% to 97.4%. In the same period the EF2 treated larvae showed a reduction which ranged from 74.3% to 99.5%. From the aspect of residual effects on L_3 , L_4 developmental stages both formulations showed a residual effect throughout the experimental period. (Figure 5.)

Statistical analysis of the number of present L_3 , L_4 mosquito larvae compared to day zero in the control untreated water showed significantly lower values on days 21 ($p < 0.01$), 35 and 42 ($p < 0.05$) after treatment.

Analysis of the statistical significance relative to day zero in waters treated with EF1 showed significantly higher values obtained on days 14 and 49 ($p < 0.05$), and 21, 28, 35 and 42 ($p < 0.01$).

Statistical analysis of the larvicidal effect of the formulation EF2 compared to day zero showed statistically significant effects on days 14, 21, 28, 35, and 42 ($p < 0.01$).

Statistical analysis of the differences in larvae (L_3 , L_4) number reduction between the two formulations confirmed that EF2 formulation compared to the control significantly reduced the number of larvae the first, 7th, 14th and 42nd day ($p < 0.05$) and 21st, 28th, 35th and 49th day ($p < 0.01$). EF1 formulation significantly reduced the number of L_3L_4 larvae the 2nd and 3rd day ($p < 0.05$) and on the 14th, and 31st ($p < 0.01$) compared to the control group. Only on the 3rd day of the experiment a significant difference ($p < 0.05$) between formulation EF1 and EF2 was recorded. Comparison of the reduction of the number of larvae (L_3L_4) showed no significant differences during the remaining time periods ($p > 0.05$). (Figure 6).

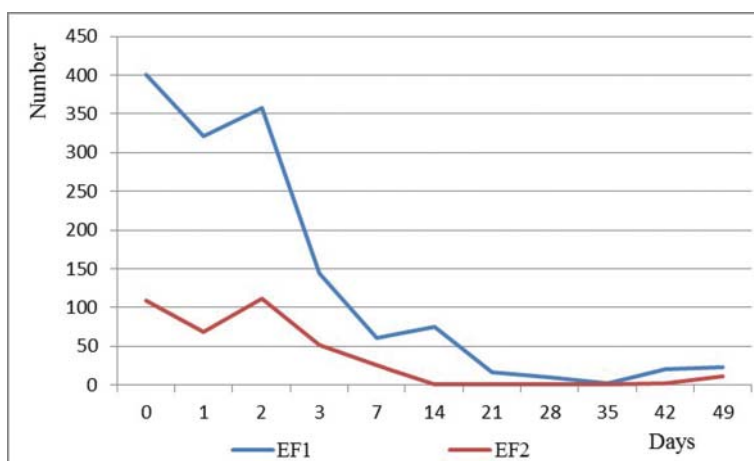


Figure 6. Comparative view of the effects of EF1 and EF2 formulations on the number of L_3, L_4 larvae

DISCUSSION

The large number of aquatic habitats, as well as the fauna and flora-composition and environmental factors in the Belgrade area are optimal for the development of many different types of mosquito of the genera *Culex*, *Anopheles*, *Aedes* and *Culiseta*. Protection of people and animals during the season of high mosquito activity is impossible without the use of mosquito larvicides and adulticides.

Continuous application of synthetic insecticides to combat mosquito larvae (temefos, pirimiphos-methyl) without checking the status of resistance, as well as legislation that precludes their use were the basis for their replacement with alternative insecticides. In areas where these insecticides are used as alternatives, insect growth regulators are implemented with success. In our environment, insect growth regulators are not used for the control mosquito larvae.

The use of insect growth regulators is not recent [1,7]. The emergence of resistance in arthropods important to the health of humans and animals has accelerated the research of mechanisms of resistance to synthetic insecticides, as well as ways to avoid it by means of synthesis of new alternative substances [24-28]. Insect growth regulators in relation to synthetic insecticides, have lower toxicity to the environment and to non-target organisms [7,29].

Diflubenzuron is an insect growth regulator that inhibits the synthesis of chitin in mosquito larvae during molting by inhibiting chitin synthase enzymes, thus acting on the development of different biological stages. The larvae after ingestion of diflubenzuron can not complete the molting cycle which consequently leads to uncompleted biological development and death. The use of diflubenzuron for the suppression of mosquito larvae was recommended by the World Health Organization in 1982 [1].

Research of the product's different formulations, usually 25% WP, 2% granules and tablets containing diflubenzuron was done before the WHO recommendations. Studies were done under laboratory and field conditions for different environments and with different types of mosquito larvae [29-31]. In these studies diflubenzuron has given good results for both initial and residual effects.

According to numerous studies WHO (1997) recommended the use of diflubenzuron in the form of 0.5% granules and 25% soluble powder at a dose of 25 -100 g/ha a.i., with toxic effects on mosquito larvae 1 to 4 weeks depending on water pollution [32]. The recommendation is for clean water 25-50 g/ha a.i. and for polluted water 50-100 g/ha. Numerous studies followed different habitats of the larvae of mosquitoes that preceded the WHO recommendations for the use of diflubenzuron at different concentrations and for different types of mosquito larvae. For drinking water, water canisters, and potable water wells, the recommended dose is 0.25 mg diflubenzuron/L [21].

The World Health Organization (2006) in two of its reports recommends the use of diflubenzuron in the first report at a dosage of 25-100 g/ ha in the form of WP [7], and in the second report 2% G (granules) in a dose of the active substance of 25-100 g/ha resulting in a residual effect of a few weeks [22].

Taking into account the recommendations of the World Health Organization and the formulation of the conditions of their use, our research and the obtained results can be compared to the research conducted in similar environments as far as water quality is concerned [31,33]. There are no studies that relate to the type of carrier diflubenzuron as in our study of corncob and zeolite.

Mosquitoes are holometabolic insects. Their life cycle from the egg to the imago lasts depending on water temperature between 10 and 14 days. During the season *Cx. pipiens* L mosquitoes have more generations, the number depending on weather conditions. The number of larvae depends on the length of the life cycle and environmental conditions. The number of individual larval stages depends on the changes from one larval stage to the other, and later on, on the development of the imago from the pupa stage [8]. This might explain the rise and fall of the number of individuals in each developmental stage occurring in the water environment.

In this study which involved all larval stages the mortality of the larvae was low during the first days after treatment, but the percentage increased as time passed by. This means that both formulations have a poor initial effect, but a good residual effect which is in agreement with previous results published by a number of authors [34,35].

In our study under field conditions the 1.0% diflubenzuron formulations displayed a good toxic effect on the larval stages of *Cx pipiens* L. during the 7 week trial. This coincides with the results obtained by a number of authors working in different geographical regions [36-38]. The effect of 2% diflubenzuron granules was determined in specific habitats [34]. Effects on the eradication of *Cx. pipiens* L and *Ae. albopictus* S. larvae which last for up to 6 weeks were obtained with the application of 2% diflubenzuron in granules and tablets in water pools [39,40].

Studies on the effects of diflubenzuron in different aquatic environments have shown that diflubenzuron has a wide range of effects and its use is justified for water environments in which resistency is registered due to long term use of some larvicides.

The tested 1% diflubenzuron formulations (EF1 and EF2) due to the results obtained on the reduction of *Cx. pipiens* L larvae in the initial and residual periods during the 7 weeks field trial period have shown that these formulations are a reliable mean of mosquito larvae control.

Statistical analysis has established a significant difference ($p < 0.05$ and $p < 0.01$), when compared to the untreated control, in the reduction of present larvae, especially L_3L_4 , on the surfaces where the tested formulations EF1 and EF2 were applied.

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STATISTIČKA EVALUACIJA LARVICIDNOG EFEKTA DIFLUBENZURONA NA RAZLIČITE RAZVOJNE STADIJUME LARVI KOMARACA VRSTE *CULEX PIPPIENS*

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U sredinama gde je kod larvi komaraca došlo do pojave rezistencije na insekticide, sve učestalije se koristi diflubenzuron. Za suzbijanje larvi komaraca u našoj sredini diflubenzuron nije korišćen. Ispitane su dve formulacije 1% diflubenzurona (kočanka i zeolit) na komarcima vrste *Culex pipiens* L. (larve) na jednom kanalu u okolini Beograda. Efekat je praćen sedam nedelja posle primene formulacija. Formulacija EF1, ostvarila je redukciju larvi L₁L₂ komaraca između 23,9 i 89,4%, statistički značajno 21. i 28. dana p<0.001, 35. i 42. dana p<0.01 i 49. dana p<0.05. Maksimalna redukcija larvi na formulaciji EF2 bila je 69,1%, statistički značajno 28. dana i 42. dana p<0.001, 35. dana p<0.01 i 21. dana p<0.05. Obe formulacije ostvarile su dobar rezidualni efekat na niže razvojne stadijume. Maksimalna redukcija larvi L₃L₄ stadijuma na formulaciji EF1 iznosila je 97,4%. Redukcija larvi bila je visoka između 7. dana i 42. dana (66,4-97%), statistički značajne vrednosti su ostvarene u 21. dana, 28. dana i 35. dana, a kod formulacija EF2 maksimalna ostvarena redukcija brojnosti larvi je iznosila 99,5%. Statistički značajne vrednosti redukcije brojnosti larvi komaraca dobijene su u 14. dana, 21. dana, 28. dana, 35. dana i 42. dana. Između formulacija ne postoji značajna statistička razlika u vrednostima redukcije nižih stadijuma larvi, ali je kod viših EF1 bila efikasnija.