

Article

Cyromazine on Ecologically Friendly Biodac Carrier as a Larvicidal Agent: Evaluation of Its Efficacy in Mosquito Control of *Culex pipiens*

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Abstract

Culex pipiens is a widespread mosquito species with high ecological plasticity that thrives in urban, peri-urban and rural aquatic habitats. It is a major vector of West Nile virus (WNV), contributing to virus transmission among bird reservoirs and serving as a bridge vector for transmission to humans and mammals, which can result in neuroinvasive disease and fatalities. Controlling its populations reduces biting nuisance and associated economic and health burdens, making vector management essential for effective public health protection. Available methods to control this species are limited and require significant improvement because conventional strategies are often short-term, non-specific and ecologically problematic. The present study evaluated the efficacy of cyromazine granules on the Biodac carrier in laboratory, semi-field (in barrels) and field experiments (in canals). Content of cyromazine was 0.5 or 2%. Two formulations were tested: granules coated with stearate and uncoated granules. The highest efficacy was demonstrated by application of 2% cyromazine, both coated and uncoated, compared to the cyromazine with 0.5% active substance. Cyromazine showed high efficacy in the control of *Cx. pipiens* ranging from 85.8% to 100% in the laboratory, 68.1% to 100% in the semi-field and 48.1% to 98.8% in the field conditions (depending on the formulation applied), enabling long-lasting suppression of juvenile stages. In the laboratory, 53 days post-treatment, the residues were still present in the water. In the field experiment (in canals) the population reduction was recorded up to the 56th day post-treatment.

Keywords: environment; house mosquito; insect growth regulators; mosquito control; public health



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1. Introduction

Mosquitoes (Diptera, Culicidae) are widely distributed insects, present in all biogeographical regions. Their juvenile stages (larvae and pupae) develop in various aquatic habitats of different sizes, ranging from very clean ones to those loaded with decomposing organic matter. Mosquitoes are considered to be the most important group of hematophagous arthropods. In most of their species, females take blood meals from humans, mammals, and birds, as a prerequisite for successful egg maturation and offspring production [1]. Consequently, they have a significant vector role in transmitting pathogens

that cause diseases with frequent fatal outcomes, such as malaria, West Nile fever, dengue fever, yellow fever, chikungunya, and others [2].

The genus *Culex* is considered among the most ecologically plastic mosquitoes, owing to its ability to develop in a wide range of aquatic habitats—from organically polluted waters to complex biocenoses [1]. Such ecological breadth, combined with the reduced availability of biocides and their intensive application, creates favorable conditions for the selection of resistant populations.

In urban areas, mosquito species that breed in close proximity to human dwellings represent a particular problem. In Serbia, *Culex pipiens* Linnaeus, 1758 (Diptera: Culicidae) (common house mosquito) has high significance, as it has been responsible for the occurrence and spread of a viral disease caused by the West Nile virus for the last 13 years. The first major outbreak occurred in 2012, while endemic establishment and recurrent outbreaks were detected in the following years, resulting in the highest number in Europe in 2013 and the second highest in 2018 (302 and 415 human cases, respectively) [3–5]. The number of listed cases is high in relation to the population size in Serbia. Since 2019 low to moderate virus circulation has been recorded, with exceptions in 2022 (226 human cases and 12 deaths) when Serbia had the third highest number of WNV cases in Europe [6]. However, it is still not predictable when significant increases in case numbers may occur again.

In Serbia, *Aedes (Stegomyia) albopictus* (Skuse, 1894) (Diptera: Culicidae), the Asian tiger mosquito, has become a significant invasive species and a competent vector for various pathogens [7,8], alongside the native *Cx. pipiens*. While both species frequently coexist in urban biotopes such as artificial containers, road drains, and manholes, *Cx. pipiens* exhibits a broader ecological plasticity, also inhabiting larger sites like drainage canals [9,10]. This partial overlap in their larval habitats suggests that targeted control during the aquatic developmental phase could simultaneously reduce populations of both species.

The mosquito control strategy in Serbia and its neighboring countries remains primarily reliant on chemical interventions, specifically the application of synthetic insecticides targeting adult populations (adulticides) [11]. However, this approach often fails to achieve long term efficacy, largely due to rapid larval development and the overlapping generations' characteristic of species such as *Cx. pipiens* and *Ae. albopictus* [12]. Furthermore, heavy reliance on adulticides does not align with the One Health concept, as it can lead to adverse effects on human and animal health, as well as broader environmental degradation [13,14]. Consequently, there is an urgent need to develop integrated strategies that minimize unnecessary chemical application and mitigate their ecotoxicological impact [15].

Controlling these mosquito species at their juvenile stages through the proper insecticide selection is of essential importance for public health as it prevents the emergence of adult mosquitoes that act as nuisances and potential carriers of pathogens. Frequent use of biocides with the same mode of action allows mosquitoes to develop resistance to a large number of insecticidal active substances, especially when it comes to conventional biocides from the groups of chlorinated hydrocarbons and organophosphates [16].

Global surveillance has confirmed that resistance is widespread across the genera *Anopheles*, *Aedes*, and *Culex* for all four major chemical classes. These cases are categorized by their mode of action (MoA) as defined by the IRAC classification [17]. Within the group of acetylcholinesterase (AChE) inhibitors, resistance to organophosphates has been documented in approximately 50 *Anopheles*, 14 *Aedes*, and 18 *Culex* species, while carbamate resistance has been reported in at least 45 *Anopheles*, nine *Aedes*, and 10 *Culex* species [18]. The trend is even more pronounced regarding voltage-gated sodium channel modulators: pyrethroid resistance occurred in over 60 *Anopheles*, 15 *Aedes*, and 18 *Culex* species, while organochlorine resistance is present in approximately 55 *Anopheles*, 12 *Aedes*, and 15 *Culex* species [18,19].

Given that adulticides are the most available method for mosquito control in Serbia, it is imperative to introduce new products that would reduce the populations of the mentioned species in juvenile stages.

The escalating development of insecticide resistance remains the primary challenge in global mosquito control efforts [20]. Given the limitations of traditional chemical classes, there is a critical need for alternative compounds with novel modes of action. Cyromazine (N-cyclopropyl-1,3,5-triazine-2,4,6-triamine; CAS RN: 66215-27-8) represents a promising candidate in this context [21]. As an Insect Growth Regulator (IGR), cyromazine interferes with the molting process and chitin deposition (by disrupting ecdysone signaling, leading to death during the larval-to-pupal transition), offering a unique mechanism of action that differs significantly from conventional neurotoxic insecticides [22,23]. Specifically, it has been shown to lack cross-resistance with pyrethroids, carbamates, and organophosphates, making it a potentially vital tool for managing resistant populations [24,25].

Originally used for dipteran control in agriculture, cyromazine's high selectivity has made it increasingly relevant in integrated vector management. It is highly effective against *Cx. pipiens* and *Ae. albopictus*, where it inhibits adult emergence and reduces the reproductive potential of surviving adults [26,27]. Due to its stability and unique target site, cyromazine is now a key component in resistance-breaking strategies for modern mosquito control programs [28,29]. This unique biochemical pathway and established efficacy in diverse environments justify its evaluation as an effective larvicide in the present study.

In addition to the larvicidal application, the potential activity of cyromazine against adult mosquitoes has also been explored. Studies on *Cx. pipiens* and *Ae. epacticus* Dyar & Knab, 1908 (Diptera: Culicidae) showed that oral intake of cyromazine via sugar-based feeders reduced female fecundity and prolonged blood meal digestion time, without affecting oviposition behavior, sex ratio, or lifespan [30]. The authors reported that such exposure was sufficient to inhibit the larval development from laid eggs, which opens the possibility of integrated application of cyromazine aimed at adult stages as well.

Preventing adult emergence and consequently breaking the pathogen transmission chain is of crucial importance in countries endemic for a certain disease, as is the case with WNV in Serbia. The specificity of mosquito biology itself, as well as coexistence of their juvenile stages with other living organisms in aquatic habitats, requires special attention and caution when selecting the means to suppress their population.

Therefore, the aim of our study was to present an ecologically and economically acceptable method for larval control of *Cx. pipiens*, applicable in future mosquito control programs in Serbia and neighboring countries. For that purpose, the biological efficacy and formulation persistence of an IGR with the active substance cyromazine on the Biodac carrier (biodegradable granules) was tested.

2. Materials and Methods

The cyromazine efficacy evaluation was carried out during a three-year study in laboratory, semi-field and field experiments. The evaluation procedure was performed in accordance with WHO guidelines [31].

2.1. Test Insects

In the laboratory and semi-field conditions individuals from a mosquito colony of *Cx. p. biotype molestus* were used. The colony was established and had been continuously maintained in the Laboratory of Medical and Veterinary Entomology (LME) of the Faculty of Agriculture in Novi Sad. Mosquitoes used in this research belonged to a colony isolated from potential contact with biological or chemical agents that could interfere with the process of obtaining results in the present study. For the field experiment, the efficacy was

assessed in drainage canals on a wild population of *Cx. pipiens* (the species present in canals were identified in LME).

2.2. Larvicide: Cyromazine

The treatment of larvae in all three phases was performed by applying cyromazine granules on a Biodac carrier (producer and supplier was DUOHEM d.o.o., Nova Pazova, Serbia). The granulated Biodac carrier was obtained from recycled biodegradable cellulose. The content of the active substance in the technical product that was used to produce this formulation of granules was 97%.

Cyromazine interferes with the hormone system in insects, which consequently impacts on cuticle sclerification and nucleic acid metabolism.

2.3. Laboratory Experiment

Three formulations of cyromazine granules, with the following % of active substance, were used:

1. 2% cyromazine granules on Biodac;
2. 0.5% cyromazine granules on Biodac;
3. 0.5% cyromazine granules on Biodac, coated with stearate (to enable slow release of active substance and to increase its persistence).

A dose of 10 kg/ha was applied. The initial dose (10 kg/ha) was given by the granule producer. Following the results obtained in the laboratory experiment, the dose was modified in accordance with the field conditions.

An experiment was performed in plastic trays of 21.1 × 21.1 cm (water surface 445.21 cm²), with a volume of 2 L, filled with 1.7 L of conditioned tap water. Twenty-five larvae of L₂–L₃ instars per tray were used. The granules were applied manually, spreading them onto the water surface. In the control trays larvae were not treated. Treatment and control were performed in four replicates. During the experiment, larvae were fed with fish food (Tetramix, Preljina, Serbia), daily. Mortality of larvae, pupae and adults (unsuccessfully molted) and the number of eclosed adults were recorded daily. The experiment was performed at room temperature. Water temperature in the trays was 23 ± 3 °C during this experiment.

The evaluation tests aimed to assess the effective dose for controlling mosquito larvae, but also the persistence/duration of granule efficacy. The duration of action was assessed by reintroducing new larvae into the already treated water after the previously introduced group of larvae had completed development (adults eclosed) or died in any stage. The larval reintroduction was repeated for up to a period of 53 days, during which larvae were reintroduced on 11th, 26th and 40th post-treatment days. When the water became completely turbid, the experiment was terminated, as water turbidity in some mosquito species can cause mortality.

To assess the IGR efficacy, it was necessary to monitor the impact on the metamorphosis and transformation into a pupa, as well as the degree of functionality and mortality of pupae. By careful monitoring of all developmental stages and examining individuals under a stereomicroscope, all changes that further affect the dysfunction of the next stage of development or malformations were recorded. All procedures for assessing the development of larvae, pupae and adults were equally monitored in the control part of the experiment.

After the experiment ended (53rd post-treatment day), analysis of the water from the rearing trays was performed. The content of cyromazine in the water was determined by reverse-phase high-performance liquid chromatography (Agilent Technologies, Santa Clara, CA, USA), using UV detection at 230 nm and external standardization. A

composite sample was formed for each granule formulation by taking 250 mL of water per replicate (total 1 L/formulation).

Afterward, the experiment was repeated using only the cyromazine formulation with the highest efficacy (containing 2% active substance).

2.4. Semi-Field Experiment

The experiment was carried out in the botanical garden at the Faculty of Agriculture in Novi Sad, Serbia during 2014 and 2015. The test was performed in 30 L volume barrels, 82 cm wide at the bottom, 94 cm in the middle and 57 cm at the top and 16.5 cm at the opening. The barrels simulated artificial water containers that are frequently inhabited by populations of *Cx. pipiens* but also *Ae. albopictus* in urban and rural areas. The applied dose was calculated according to the water surface, which was similar to the surface of the water in the plastic containers used in laboratory conditions. Considering that the given concentration of cyromazine in a water volume of 30 L resulted in a low mortality, the dose in each barrel was increased 15 times to increase the concentration. According to the results obtained in the laboratory, in the semi-field test, only granules with 2% cyromazine were used. Both stearate-coated and uncoated cyromazine on Biodac were evaluated. Both formulations were performed in three replicates (three barrels for each formulation). A control barrel in which the larvae were only fed and inspected was in three replicates as well.

Barrels were filled with conditioned water to the top. Larvae (50 larvae/barrel) of L₂–L₃ instars were added to the water. The application of granules on the water surface was done manually. Larvae were fed daily with fish food (Tetramix, Preljina, Serbia). To prevent oviposition by wild females from the field and to prevent release of the mosquitoes from the experiment, the barrel's opening was covered with an improvised lid made of 5 L plastic bottles with the bottom removed to fit over the barrel opening. Standard metal WHO cages were placed on the top of the bottle opening to prevent the release of adults into nature (Figure 1). The barrels were inspected in 24 h intervals. Eclosed adults were determined by counting the number of pupal exuviae. After no live individuals remained in the barrels, new larvae were reintroduced (50 larvae/barrel).



Figure 1. The barrel with the cover and WHO cage in which the semi-field experiment was performed.

The study lasted 17 days, from 23 August to 9 September. The average water temperature in the barrels was 18.8 ± 1.7 °C. The experiment was repeated in the following year and lasted from 29 July to 13 August (15 days). The average water temperature was 24.1 ± 1.8 °C.

2.5. Field Experiment

The drainage canals were selected in a semi-urban area in Novi Bečej (45°36'3.96" N 20°8'29.54" E), Serbia. Canals of the same type were selected for control and treatment. The canals were positioned in front of houses on both sides of the street and serve to collect rainwater, as well as household wastewater from kitchen sinks. The canal designated for the treatment was 40 m long and 1.5 m wide. The canal was divided into four parts that were observed separately, and a length of 3×10 m was used for treatment (3×15 m² surface). Identified plant species in the canals were: *Setaria viridis* (L.) P. Beauv., *Plantago lanceolata* L., *Carex hirta* L., *Sonchus oleraceus* L., *Cynodon dactylon* (L.) Pers., *Poa annua* L., *Agropyron repens* (L.) P. Beauv., *Cichorium intybus* L., *Lemna minor* L., *Rumex crispus* L., *Potentilla reptans* L., *Polygonum aviculare* L., *Trifolium repens* L. and *Setaria glauca* (L.) P. Beauv.

The field trials were performed as three identical experiments in the same type of canal, following the same procedure to treat the mosquitoes and to evaluate cyromazine granules: the first experiment in 2014, the second in 2015 and the third in 2015 but after the second ended. The temperature and the precipitation data for 2014 and 2015 are given in Supplementary Table S1 [32].

According to the laboratory and semi-field tests, the formulation of 2% cyromazine on the Biodac carrier was selected for the field experiment during 2014. In this phase 15 kg of granules/ha was applied. The granules were applied manually on each replicate in the selected canal (10×1.5 m), ensuring even distribution across the water surface. A higher dose (15 kg) compared to the one applied in the laboratory (10 kg) was applied due to presence of organic matter in the canal which could decrease the efficacy of granules. Sampling was done on each meter of the canal (one sample/m² of length). The same procedure was repeated in all parts of the canal. Juvenile stages were sampled with a standard WHO dipper of 350 mL in volume. Number of egg batches, larvae (classified into two categories: L₁–L₂, and L₃–L₄) and pupae were recorded. After counting the individuals in the dipper, samples were returned to the canal. In the control canal, no larvicides were applied, and only the natural dynamics of the juvenile mosquito population were monitored. The control canal was 20 m long and 1.5 m wide. This canal was divided into two parts, and sampling was carried out following the same procedure as in the treated canal. The juvenile population was first inspected before treatment, and then the canal was treated with cyromazine on the same day. Monitoring was performed on the 4th, 7th and 21st post-treatment days. The experiment was performed in August. Water temperatures were measured on the days of canal inspections and always in the period from noon to 2 p.m. The measured water temperatures were 27.3 °C (before treatment), 28.0 °C (4 post-treatment days), 29.5 °C (7 post-treatment days) and 24.0 °C (21 post-treatment days), on average 27.2 ± 2.32 °C.

The same experiment was repeated in the following year (2015) in the same canals. The population in the canal was recorded before treatment and on the 3rd post-treatment day and subsequently at weekly intervals up to 38 days post-treatment. In the canal treated with cyromazine and in the control canal inspection was performed until the 38th post-treatment day. The study lasted from 2 June to 9 July. The mean water temperature during that period in the cyromazine-treated canal was 26.4 ± 4.8 °C, while in the control canal it was 26.5 ± 4.4 °C. The temperatures were measured between 10 and 11 a.m. during the inspections of juveniles in the canal.

The third repetition of the field experiment was performed in the same year (2015) and lasted for 91 days. The procedure was the same as described for the previous two field experiments, except for the duration that was longer.

The average water temperature in the canals during the entire duration of the experiment (91 days in total) was 28.50 ± 3.69 °C. The total amount of precipitation during the

91 days of monitoring was 188.7 mm of rain. Precipitation was registered 46 days out of a total of 91. The maximum average air temperature during this period was 30.81 ± 7.19 °C, and the minimum was 22.75 ± 5.87 °C.

2.6. Statistical Analysis

All statistical analyses were performed in Statistica 13.2 (Tibco Software, Palo Alto, CA, USA) software package.

In the laboratory experiments the efficacy evaluation of the applied cyromazine was presented by the cumulative mortality percentage of the exposed population for each post-treatment day compared to the pre-treatment population. When the mortality of the control population was <5%, the efficacy of the applied cyromazine was calculated by mortality: $\text{mortality (\%)} = (\text{number of dead} / \text{total treated}) \times 100$. The same formula was used to calculate the natural mortality of the control group. However, if the mortality of the control population was between 5 and 20%, the following formula was applied [33]: $\text{corrected mortality (\%)} = ((\% \text{ mortality in treatment} - \% \text{ mortality in control}) / 100 - \% \text{ mortality in control}) \times 100$.

The obtained mortalities were transformed into $\arcsin\sqrt{\text{mortality}}$. The transformed mortalities of the treated mosquito population were compared with the mortalities of the control population by analysis of variance (ANOVA) and Duncan's test at a 95% confidence interval ($p < 0.05$). Median lethal time (median lethal time LT50, LT90 and LT99) was calculated for all applied cyromazine formulations in the laboratory using probit regression analysis [34].

In the semi-field experiment the cyromazine efficacy in barrels was presented by the total and average percentage of adult inhibition eclosion (eclosion inhibition, IE): $\text{IE (\%)} = 100 - (T * 100C)$ where T = percentage of successfully eclosed adults from the treated barrel and C = percentage of successfully eclosed adults from the control barrel.

In the field experiment the cyromazine efficacy was evaluated by the % reduction of the exposed population for each post-treatment day compared to the pre-treatment population. Population reduction percentages were determined by Mulla's formula [35]: $\text{reduction (\%)} = 100 - ((C1/T1) * (T2/C2)) * 100$ where C1 = pre-treatment control population, C2 = post-treatment control population, T1 = pre-treatment treated population, and T2 = post-treatment treated population.

The $\arcsin\sqrt{\text{reduction}}$ transformation was performed. The significance of mortality was compared with the mortality in the control canal using a general linear model (GLM) and repeated measurements ANOVA, and significant differences in mortality by day were determined by Duncan's post hoc test with a 95% confidence interval. Population dynamics was also analyzed using a GLM, repeated measurements method, and Duncan's post hoc test ($p < 0.05$).

3. Results

3.1. Laboratory Experiment

Culex p. biotype molestus showed high sensitivity to all three tested granule formulations of cyromazine (Figure 2A–D, Table 1). Cyromazine granules showed high efficacy causing high mortality of treated individuals during the period of 53 days. When 0.5% uncoated cyromazine granules were applied, the maximum mortality was on average 95.7%, for all four larval introductions. When 0.5% coated cyromazine granules were applied, the mortality was 92.1%, while applying 2% cyromazine granules resulted by mortality of 100%. Both types of granules with 0.5% cyromazine showed high efficacy on the first introduced larval population (10th post-treatment day), causing mortality of 97.8% (uncoated granules) and 99.6% (coated granules). An efficacy decrease of granules with

0.5% was recorded in the 2nd introduced larval population (period 12–20 post-treatment days). After ten days the efficacy of cyromazine granules (0.5%) decreased from 97.8 to 85.8% for uncoated granules and from 99.6 to 91.30% for coated ones. However, high efficacy was recorded after 41 post-treatment days, when the uncoated granules achieved mortality of 95.7%, while the coated granules caused a mortality rate of 92.1%. Granules with 2% cyromazine maintained a consistently high efficacy (mortality 100%) during all 53 post-treatment days. Maximum mortality of exposed mosquitoes was achieved on average in ten days with uncoated granules with 0.5%, followed by 11 days with the application of coated granules with 0.5%, and the granules with the highest cyromazine content (2%) acted the fastest, with 100% mortality in the entire experiment achieved on average in 9 days. Although a high mortality rate was achieved, the exposure period of the fourth introduced group of individuals lasted the longest compared to the previous three. While maximum mortality was achieved in 8–10 days in the first three larval exposure periods, in the last (the fourth) larval introduction the maximum mortality rate was achieved in 13 days for all three formulations. When using 0.5% cyromazine (coated and uncoated granules), part of the exposed larval population managed to molt to the next developmental stage (pupal stage, at which point mortality occurred), while in the case of using 2% cyromazine, no larvae pupated during 53 days of the experiment. During the first exposure of larvae (1–10 post-treatment days), the efficacy of coated and uncoated granules did not differ significantly ($p = 0.501$). However, the mortality caused by the 2% cyromazine granules was significantly higher compared to the one caused by 0.5% cyromazine, uncoated ($p = 0.023$) and coated ($p = 0.008$). Mortality in all treated trays significantly differed from the mortality of the control group ($p = 0.008$).

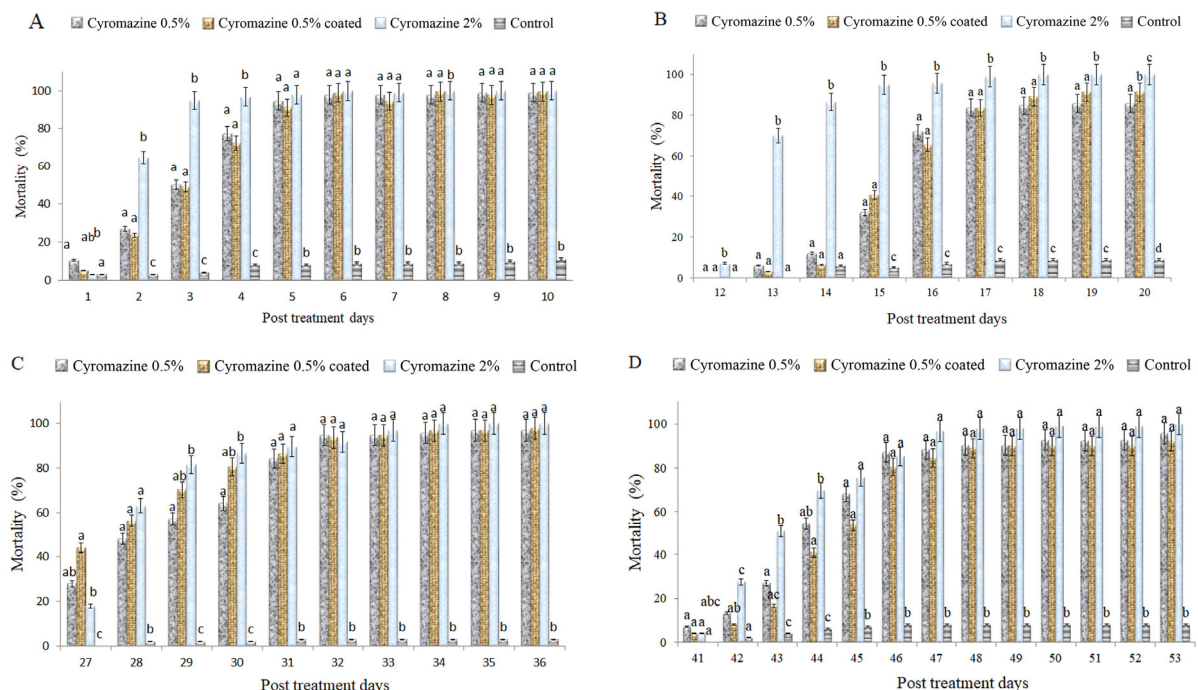


Figure 2. (A–D) Mean cumulative mortalities of *Cx. p. biotype molestus* juvenile stages after treatment with granules: 0.5% cyromazine coated and uncoated and 2% cyromazine during 2013 (ANOVA and Duncan's post hoc test, $p < 0.05$. Different letters indicate significant differences between the compared categories): (A) = the first; (B) = the second; (C) = the third and (D) = the fourth introduced group of larvae.

Table 1. Mean cumulative mortality (%) of *Cx. pipiens* larvae 1–53 days after cyromazine treatment.

Cyromazine granules (%)	Post-treatment days												
	1	2	3	4	5	6	7	8	9	10			
	Days of larval exposure to cyromazine												
	1	2	3	4	5	6	7	8	9	10			
Cyromazine 0.5	10.3	26.8	50.0	77.2	94.6	97.8	97.8	97.8	97.8	97.8			
Cyromazine 0.5 coated	5.2	23.7	49.0	72.8	91.3	99.2	96.7	99.7	97.8	99.6			
Cyromazine 2	3.1	65.0	94.8	96.7	97.8	99.9	98.9	100	–	–			
Control	3.0	3.0	4.0	8.0	8.0	9.0	9.0	9.0	10.0	11.0			
	Post-treatment days												
	12	13	14	15	16	17	18	19	20				
	Days of larval exposure to cyromazine												
	1	2	3	4	5	6	7	8	9				
Cyromazine 0.5	0	6.0	11.5	31.6	72.0	83.5	84.6	85.8	–				
Cyromazine 0.5 coated	0	3.0	6.3	40.0	65.6	83.5	89.0	91.3	–				
Cyromazine 2	7.0	70.0	86.5	94.7	95.7	98.9	100	–	–				
Control	0	0	6.0	5.0	7.0	9.0	9.0	9.0	9.0				
	Post-treatment days												
	27	28	29	30	31	32	33	34	35	36			
	Days of larval exposure to cyromazine												
	1	2	3	4	5	6	7	8	9	10			
Cyromazine 0.5	28.0	48.0	57.1	64.3	84.5	94.9	94.9	95.9	96.9	96.9			
Cyromazine 0.5 coated	44.0	56.1	70.4	80.6	86.6	93.8	94.9	96.9	96.9	98.0			
Cyromazine 2	18.0	63.3	81.6	86.7	90.0	91.8	96.9	100	–	–			
Control	0	2.0	2.0	2.0	3.0	3.0	3.0	3.0	3.0	3.0			
	Post-treatment days												
	41	42	43	44	45	46	47	48	49	50	51	52	53
	Days of larval exposure to cyromazine												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Cyromazine 0.5	7.0	13.3	27.1	54.6	67.7	87.0	88.0	90.2	90.2	92.4	92.3	92.4	95.7
Cyromazine 0.5 coated	4.0	8.2	16.7	41.2	53.8	80.4	84.8	89.1	90.2	90.2	90.1	90.2	92.1
Cyromazine 2	4.0	27.6	51.0	70.1	75.3	84.8	96.7	97.8	97.8	98.9	98.9	98.9	100
Control	0	2.0	2.0	2.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0

In the second part of the experiment (12–20 post-treatment days), no significant difference was detected between the efficacy of coated and uncoated 0.5% cyromazine granules ($p = 0.051$), but they did differ significantly ($p < 0.001$) compared to granules with 2% cyromazine and to the control group. Mortality of the third introduced group of larvae (27–36 post-treatment days) did not differ significantly. The situation changed in the last part of the experiment (41–53 post-treatment days) when the efficacy of 0.5% cyromazine in the two formulations did not differ significantly from each other ($p = 0.296$); 2% cyromazine granules did not differ significantly from uncoated 0.5% cyromazine granules either ($p = 0.090$) but differed significantly from the coated 0.5% cyromazine granules ($p = 0.015$). There was a highly significant difference in the mortality between the control and treated groups ($p < 0.001$).

By comparing the mortality caused in four different groups of mosquitoes, over 53 days, it was determined that the efficacy of the products changed as a function of time

($p = 0.002$). The efficacy of uncoated 0.5% cyromazine granules was significantly reduced in the second introduction of larvae compared to the first, and the efficacy was also significantly reduced in the last introduction of larvae (41–53 post-treatment days) compared to the mortality of the initially exposed group (1–10 post-treatment days) ($p = 0.026$). The same situation was observed when analyzing the efficacy of the coated 0.5% cyromazine granules. The efficacy was significantly reduced in the second introduction of larvae ($p = 0.012$) and the initial efficacy of the granules differed significantly from the efficacy in the last period of larval exposure ($p = 0.010$). The 2% cyromazine formulation showed greater efficacy compared to the previous two types of granules. The high efficacy of the granules (100% larval mortality) lasted for three introductions (1–36 post-treatment days).

Regardless of the mortality of the entire exposed population (100%) that was achieved during the fourth introduction of larvae, the efficacy differed significantly compared to the first and second introductions of larvae ($p = 0.002$), as well as the third one ($p = 0.008$), because in the fourth introduction the granules required much more time to achieve the mortality of the entire exposed population which may indicate a reduction in the granule efficacy.

Water analyses by chromatography showed that during 53 days the highest cyromazine content was retained in trays with 2% cyromazine granules followed by the trays with 0.5% uncoated granules. Almost two times less cyromazine was detected in trays where coated 0.5% cyromazine granules were applied compared to trays with uncoated 0.5% cyromazine granules (Table 2).

Table 2. Cyromazine residues in water at 53 days post-treatment.

Granules	Dose g/Tray	Dose g/L	Initial (Nominal) Concentration a.s. (mg/L)	Residues at 53 Days µg/L (mg/L)
Cyromazine 0.5	0.045	0.026	0.13 mg/L	18.18 (0.018)
Cyromazine 0.5 coated	0.045	0.026	0.13 mg/L	10.32 (0.010)
Cyromazine 2	0.045	0.026	0.52 mg/L	22.34 (0.022)

According to the obtained results which indicated the achieved efficacy of two formulations with different active substance contents, the formulation of granules based on 2% cyromazine was selected. The analysis was then repeated in the following year (Table 3).

Table 3. Mean cumulative mortality (%) of *Cx. pipiens* larvae 1–61 days after treatment with cyromazine (2%).

Cyromazine granules (%)	Post treatment days												
	1	2	3	4	5	6	7	8	9	10	11	12	13
	Days of larval exposure to cyromazine												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Cyromazine 2%	5.1	9.2	41.8	60.4	74.7	88.2	91.0	87.3	94.2	94.1	97.7	98.8	100
Control	1.0	2.0	2.0	9.0	10.0	10.0	11.0	14.0	14.0	15.0	15.0	15.0	15.0

Cyromazine granules (%)	Post treatment days																
	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
	Days of larval exposure to cyromazine																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Cyromazine	1.0	5.0	16.7	39.7	49.5	66.3	70.0	71.1	74.7	79.3	79.1	82.6	90.7	94.1	96.5	98.8	100
Control	0	0	4.0	6.0	7.0	10.0	10.0	10.0	13.0	13.0	14.0	14.0	14.0	15.0	15.0	15.0	15.0

Table 3. Cont.

Cyromazine granules (%)	Post treatment days										
	35	36	37	38	39	40	41	42	43	44	45
	Days of larval exposure to cyromazine										
	1	2	3	4	5	6	7	8	9	10	11
Cyromazine	0	13.4	23.6	69.7	69.7	69.7	69.7	80.7	80.7	80.7	100
Control	0	3.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0	11.0

Cyromazine granules (%)	Post treatment days														
	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
	Days of larval exposure to cyromazine														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Cyromazine	0	27.0	27.0	67.0	78.7	85.1	89.4	93.6	96.8	97.8	98.9	100	100	100	100
Control	0	0	0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0

Table 4 presents the median lethal time, i.e., the time required to cause 50%, 90% and 99% mortality (lethal time: LT₅₀, LT₉₀ and LT₉₉; at confidence intervals of 0.05) of exposed mosquito larvae for the three cyromazine formulations. Formulation with 2% cyromazine acted twice as fast compared to both evaluated formulations with 0.5% cyromazine.

Table 4. Mean LT of *Cx. pipiens* larvae treated with cyromazine granules, 1–53 post-treatment days, expressed in days, with confidence intervals (0.05).

Mean LT 1–10 post-treatment days			
	LT ₅₀	LT ₉₀	LT ₉₉
Cyromazine 0.5	2.9 (2.1–13.6)	5.2 (4.4–6.7)	–
Cyromazine 0.5 coated	3.2 (1.9–4.1)	5.5 (4.5–7.8)	7.4 (6.0–11.5)
Cyromazine 2	1.8 (1.3–2.2)	3.0 (2.5–4.4)	4.6 (3.5–8.8)
Mean LT 12–20 post-treatment days			
	LT ₅₀	LT ₉₀	LT ₉₉
Cyromazine 0.5	4.8 (3.9–5.0)	–	–
Cyromazine 0.5 coated	4.5 (4.1–4.8)	7.1 (6.5–8.1)	–
Cyromazine 2	1.8 (1.5–2.1)	3.3 (2.9–4.1)	5.5 (4.4–8.0)
Mean LT 27–36 post-treatment days			
	LT ₅₀	LT ₉₀	LT ₉₉
Cyromazine 0.5	2.1 (1.6–2.5)	6.3 (5.2–8.3)	–
Cyromazine 0.5 coated	1.5 (1.2–1.7)	5.4 (4.8–6.3)	–
Cyromazine 2	1.9 (0.3–2.7)	4.8 (3.9–6.6)	7.3 (5.8–11)
Mean LT 41–53 post-treatment days			
	LT ₅₀	LT ₉₀	LT ₉₉
Cyromazine 0.5	3.7 (3.2–4.1)	8.9 (7.8–10.4)	–
Cyromazine 0.5 coated	4.4 (3.8–4.9)	9.9 (8.6–11.9)	–
Cyromazine 2	2.9 (2.7–3.1)	6.2 (5.8–6.7)	11.4 (10.2–12.9)

Typical manifestation for IGR substances, caused by cyromazine in this experiment, are given in Figure 3. In the photo on the left and in the middle, the pupae died due to unsuccessful molting processes in which the larval exuvia stayed attached to the distal part of the pupal abdomen. In the photo on the right, the adult was unable to eclose. Its legs were kept in the pupal exuvia, causing the adult to die.



Figure 3. The pupae that failed to molt completely from larval to pupal stage ((left) and (middle)) and adult that failed to complete eclosion (right).

3.2. Semi-Field Experiment

The effects of uncoated granules with 2% cyromazine on the Biodac carrier and stearate-coated granules with 2% cyromazine on the Biodac carrier on larval/pupae development were recorded daily. The results are shown in Tables 5 and 6. No change was observed in the individuals exposed until the fifth post-treatment day. Therefore, the results from the 5th to the 14th post-treatment day are presented. In barrels treated with uncoated granules a high reduction of adult eclosion was recorded (83.0%) while treatment with coated granules resulted in a lower reduction in eclosion with an average of 68.1%. Uncoated granules of 2% cyromazine showed higher efficacy compared to coated granules. In the second repetition, both formulations had higher efficacy. No individuals in the treated water successfully molted to the adult stage. In the control barrels, the IE was on average 20.0%, while in all treated barrels a complete effect with 100% mortality was achieved. Immediately after the application, a negative characteristic of the formulation was noted. Upon contact with water the granules clustered in the central part of the barrel opening. This phenomenon is considered undesirable as it prevents uniform application over the targeted water surface. On the 11th day post-treatment, all uncoated granules containing 2% cyromazine were still clustered and floating on the water surface, whereas all coated granules were distributed across the entire water surface.

Table 5. Number of alive larvae, pupae, eclosed adults and % of inhibited eclosions of adults from the barrels where 2% cyromazine granules on Biodac carrier (uncoated and coated) in semi-field conditions (barrels) were applied (the first experiment). The numbers are given as mean values of three replicates.

	Post-treatment days														
	5			7			8			9			10		
	AL	EA	ΣA	AL	EA	ΣA	AL	EA	ΣA	AL	EA	ΣA	AL	EA	ΣA
Cyromazine uncoated	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cyromazine coated	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Control	0	1	0	10	1	1	9	6	7	6	12	19	5	11	30
	Post-treatment days														IE (%)
	11			12			13			14					
	AL	EA	ΣA	AL	EA	ΣA	AL	EA	ΣA	AL	EA	ΣA			
Cyromazine uncoated	1	0	0	3	0	0	8	8	8	0	0	8		83.0	
Cyromazine coated	0	0	0	15	0	0	0	15	15	0	15	15		68.1	
Control	4	10	40	2	5	45	1	1	46	0	1	47		6.0	

AL = alive pupae; EA = eclosed adults; ΣA = sum of adults; IE = inhibited eclosion of adults.

Table 6. Number of alive larvae, pupae, eclosed adults and % of inhibited eclosions of adults in the barrels where 2% cyromazine granules on Biodac carrier (uncoated and coated) in semi-field conditions (barrels) were applied (the repeated experiment). The numbers are given as mean values of three replicates.

	Post-treatment days														
	5			7			8			9			10		
	AL	EA	ΣA	AL	EA	ΣA	AL	EA	ΣA	AL	EA	ΣA	AL	EA	ΣA
Cyromazine uncoated	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cyromazine coated	0	0	0	0	0	0	0	0		0	0	0	0	0	0
Control	0	0	0	0	0	0	5	0	7	18	0	0	0	0	0
	Post-treatment days														
	11			12			13			14					
	AL	EA	ΣA	AL	EA	ΣA	AL	EA	ΣA	AL	EA	ΣA	IE (%)		
Cyromazine uncoated	0	0	0	0	0	0	0	0	0	0	0	0	100		
Cyromazine coated	0	0	0	0	0	0	0	0	0	0	0	0	100		
Control	14	18	18	15	10	32	8	0	32	0	8	40	20.0		

AL = alive pupae; EA = eclosed adults; ΣA = sum of adults; IE = inhibited eclosion of adults.

3.3. Field Experiment

All granules sank to the canal bed (into the mud) immediately after application, suggesting siltation over time and resulting in a cyromazine efficacy reduction.

The biological efficacy of 2% cyromazine granules was verified by the degree of suppression of the natural juvenile population during the specified post-treatment days in relation to the pre-treatment population and reduction in the control canal. The evaluation efficacy of cyromazine included only older larvae (L₃ and L₄) in the post-treatment period because the granules could not reach the early instars (L₁–L₂) when granules were applied. This evaluation system eliminated all young larvae that were hatched daily due to the permanently available water surface for oviposition by the wild population of *Cx. pipiens* females which continuously lay eggs in available water containers during the summer season.

The population reduction rate in the experiment carried out in 2014 (the first field experiment) 21 days after treatment ranged from 79.4% to 98.9% in the three canal sections. The average mortality was 87.1%. Although the population in the canal designated for treatment was higher in the pre-treatment period than in the control, a significant difference was not found ($p = 0.763$). Statistical analysis revealed a highly significant difference between the treated and control groups on the 21st post-treatment day ($p < 0.001$). Cyromazine reduced the population only on the 21st day after treatment, while in the periods of four and seven post-treatment days a significant difference between the control and the treatment was not recorded ($p = 0.597$ and $p = 0.117$). In the control canal, the population was slightly reduced four days after treatment. Reduction was 10.2%. This reduction was not statistically significant compared to the pre-treatment population ($p = 0.443$). However, seven days after treatment the control group of individuals increased significantly ($p < 0.001$) compared to the pre-treatment population. Twenty-one days after the treatment, the population was significantly reduced ($p < 0.001$) compared to the pre-treatment population.

When the experiment was repeated in 2015 (the first repetition), the cyromazine granules showed high efficacy from the third post-treatment day (71.3%) when a significantly higher reduction was recorded compared to the control (21.0%). Seven days after treatment with cyromazine when the reduction was 87.7%, the granules provided maximum efficacy with mortality in individual repetitions up to 99.6%. After 27 post-treatment days the

efficacy of this cyromazine formulation was reduced to 57.9% and one week later to an average of 48.2% (38 days after treatment). The effect of cyromazine was not statistically significant compared to the untreated population at 27 days after treatment (Table 7). It is important to note that the high % of population reduction in the control canal could be caused by different factors (for example, by air temperature) in the environment where the experiment was conducted.

Table 7. Reduction of the *Cx. pipiens* population in the post-treatment period compared to the pre-treatment population in canal treated with 2% cyromazine and in the control canal in Novi Bečej (the second field experiment) in 2015, calculated by Mulla's formula.

IGR	Population reduction (%) on post-treatment days						
	3	7	14	21	24	27	38
Cyromazine	71.3	87.7	48.1	71.9	79.0	57.9 *	48.1 *
Control	21.0	1.4	42.5	21.4	40.2	38.0	39.7

* The efficacy of cyromazine demonstrated a false decrease (days 27 and 38) because the population in the control naturally declined which can be explained by many environmental factors.

The experiment was repeated in the same year (2015), at the same site. The biological efficacy of cyromazine was determined in the treated canal, expressed through population reduction, where the average population size on post-treatment days was compared with the average population before treatment (Table 8). On pre-treatment days the populations in the canal selected for treatment with cyromazine were significantly higher than in the control canal ($p < 0.01$ in the first canal and $p = 0.006$ in the second canal). Cyromazine significantly reduced the population in the treated canal during all three months of monitoring, and the maximum efficacy was recorded 35 days after treatment (reduction of 98.4%). After 63 post-treatment days, the population reduction was below 50%. Further observation is not considered because the population in the control canal was significantly reduced (63.8%). After the activity of cyromazine no significant difference ($p = 0.352$) was recorded between the populations in the treated and control canals which indicates a significant reduction of the treated population compared to the population before treatment. The efficacy of 2% cyromazine is presented in Table 8.

Table 8. Mortality of *Cx. pipiens* in canals treated with 2% cyromazine granules and in the control canal in Novi Bečej in 2015 (the third field experiment).

	Mortality (%) on post-treatment days													
	2	4	7	14	21	35	42	49	56	63	70	77	84	91
Cyromazine *	89.23	54.94	95.93	92.58	74.98	98.85	87.64	96.98	90.88	46.09	74.14	92.97	64.87	–
Control	+	+	+	+	+	+	15.94	0	17.39	7.25	63.77	42.03	97.1	89.86

* In the canal treated with cyromazine after 63 post-treatment days mortality was no longer taken into account due to very high population decline in the control; + population growth in the canal compared to pre-treatment.

4. Discussion

The results of this study suggest that the IGR cyromazine, formulated on the Biodac carrier, may have potential under laboratory conditions. The mortality profile indicates that the mode of action is closer to chitin synthesis inhibitors than to juvenile hormone analogs [24], which is consistent with the occurrence of larval deformities [36,37]. At a concentration of 0.5%, some of the larvae reached the pupal stage, while the concentration of 2% completely prevented that transition [38].

Under semi-field and field conditions, efficacy showed variability in relation to temperature and hydrological parameters. The performance of Biodac granules was closely associated with the physical characteristics of the habitat. The applied granules initially floated,

enabling contact with the surface water layer where larval feeding predominantly occurs; however, after approximately 10 days, they sank, and their sedimentation was observed.

The uncoated 2% cyromazine formulation achieved 82.98% inhibition of adult eclosion, thereby fulfilling the efficacy criteria established by the World Health Organization [31], while coated formulations did not consistently reach the 80% threshold. In some cases, population decline was influenced by the seasonal temperature drop, which confirms the importance of environmental factors [39]. Nevertheless, siltation and submergence of granules decreased the release of the active substance, resulting in a decrease in overall efficacy. In some cases, population decline was influenced by seasonal temperature drops, which confirms the importance of environmental factors [39].

The safety profile of cyromazine remains a subject of debate. Although potential risks associated with its metabolite melamine have been reported [40,41], the majority of available studies indicated low toxicity to mammals and birds [42,43]. Data on effects on non-target aquatic organisms are heterogeneous, ranging from non-toxic or mildly toxic effects on *Daphnia magna* Straus, 1820 (Diplostraca: Daphniidae) [44,45] reported by the European Union [46], highlighting sensitivity in certain aquatic invertebrates.

Although mosquito resistance to cyromazine has not yet been confirmed, it has been documented in the house fly *Musca domestica* Linnaeus, 1758 (Diptera: Culicidae) [47,48] and in *Lucilia cuprina* (Wiedemann, 1830) (Diptera: Culicidae) [49], underscoring the need for rational and carefully managed application strategies. Compared to other IGR compounds, cyromazine is considered somewhat less effective, but with a more favorable toxicological profile and good efficacy against pyrethroid- and carbamate-resistant populations [24]. Sustained but controlled release from the Biodac carrier may further reduce concentration peaks and associated selection pressure, while sustaining an effective dose over a longer period.

Finally, the 2% cyromazine formulation showed the highest and most consistent larvicidal potential, particularly under laboratory conditions, whereas field results emphasized the importance of formulation type, temperature, and habitat characteristics. To enhance the persistence of cyromazine in the aquatic environment, various controlled-release formulations have been developed, including lignin-based matrices coated with ethylcellulose [50] and low-density polyethylene systems with a polyurethane coating [51]. Prolonged and uniform release of the active substance may ensure a more stable larvicidal effect while reducing the need for frequent applications.

However, the results of the present study indicate that the coating of 2% cyromazine granules on the Biodac carrier with stearate did not lead to an improvement in biological efficacy compared to uncoated granules. Likewise, no statistically significant difference was observed between coated and uncoated formulations containing 0.5% active substance. Although, after 53 days in water, a lower concentration of cyromazine was recorded in the coated granules (10.32 µg/L) compared to the uncoated ones (18.18 µg/L), confirming the gradual release as well as the degradation/leaching of the active substance, this difference did not result in a proportional increase in efficacy. These findings suggest that delayed release alone is not a sufficient condition for enhanced biological performance, but that the formulation efficacy is strongly influenced by ecological factors and interactions with the substrate.

Nevertheless, although the physical properties of the Biodac carrier impose certain limitations under field conditions, it plays a key role in optimizing the biological efficacy, persistence, and environmental safety of cyromazine. This highlights the need for further formulation development aimed at improved controlled release and better dispersion in the aquatic environments.

5. Conclusions

The long-term use of conventional chemical compounds imposes the need for the evaluation of other agents with selective action and more acceptable ecotoxicological effects to introduce sustainable mosquito control strategies in these areas by potential inclusion in future programs. Based on the research conducted, the following conclusions were made. The tested formulations of insect growth and development regulator granules based on 2% cyromazine on the Biodac carrier showed high efficacy in controlling the species *Cx. pipiens* in laboratory conditions. The content/amount of cyromazine was directly proportional to the efficacy of the tested granules. Granules with 2% cyromazine provided greater efficacy compared to granules with 0.5%. The formulation of cyromazine granules (2%) applied at a dose of 15 kg/h provided a high level of suppression of larval stages in artificial water containers, but the application of this IGR had limited possibilities in natural mosquito breeding habitats due to the poor flotation of the formulation. Although the formulation of granules with 2% cyromazine provided suppression of the treated population in canals, the use of these granules is more recommended in breeding sites or in biotopes with solid bottoms, such as those made of concrete. The reason for this conclusion is the silting of the granules due to the inability to float, which is further reflected in the reduced efficacy of the applied IGR.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/environments13050262/s1>, Table S1: Mean monthly air temperature and total monthly precipitation in Novi Bečej [32].

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