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# Effects of the technical ingredient clomazone and its two formulated products on aquatic macrophytes☆

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ABSTRACT

One active ingredient can be a component of different types of formulations of pesticides, while the toxicity of its formulations may vary depending on various constituents used in the mixture. The present study focuses on evaluating the effects of the active ingredient clomazone and its formulations (Rampa® EC and GAT Cenit 36 CS, both containing 360 g a.i./l of clomazone) on non-target aquatic macrophytes. The two formulation types differ in their active ingredient release and presumed environmental impact. In order to cover different ecological traits, two species of aquatic macrophytes – the floating monocot *Lemna minor* and the rooted dicot *Myriophyllum aquaticum*, were used as test models. The results of this study revealed differences in the sensitivity of tested plants to clomazone. Based on the most sensitive parameters, *M. aquaticum* proved to be more sensitive than *L. minor* to the technical ingredient and both formulations. The species sensitivity distribution (SSD) approach that was tried out in an attempt to create a higher tier step of risk assessment of clomazone for primary producers indicates that tests on rooted macrophytes can add value in risk assessment of plant protection products. The capsule formulation of clomazone was less toxic than the emulsion for *L. minor*, but more toxic for *M. aquaticum*. The most toxic for *L. minor* was the emulsifiable concentrate formulation Rampa® EC, followed by technical clomazone ($EC_{50}$ 33.3 and 54.0 mg a.i./l, respectively), while the aqueous capsule suspension formulation GAT Cenit 36 CS did not cause adverse effects. On the other hand, the most toxic for *M. aquaticum* was the formulation GAT Cenit 36 CS, followed by technical clomazone and the formulation Rampa® EC, demonstrating a greater effect of the capsule formulation.



## 1. Introduction

Modern agricultural practice inevitably relies on pesticide application. In the EU countries, 473 active ingredients are currently approved for use in crop production (EC, 2020). It is estimated that global annual consumption of pesticides is ~4 million tons (Ortiz-Hernández et al., 2013) and herbicides make the most broadly used group (40%) (Grube et al., 2012). After pesticide application, a portion of the applied product reaches target organisms (pests), while >95% spreads in other parts of the environment – air, soil, water and non-target organisms (Gavrilescu, 2005; Arias-Estévez et al., 2008; Miller and Spoolman, 2011). Pesticides are applied after formulating into a product – a mixture of one or more active ingredients (a.i.) and a range of co-formulants used to enhance the effectiveness, stability and other desirable pesticide properties (Knowles, 2005; Cox and Surgan, 2006; Gašić and Orešković, 2006; Arias-Estévez et al., 2008; Solomon et al., 2013). Co-formulants may exert biological or chemically activity which can result in increase or decrease of pesticide toxicity after the formulation process (Cedergreen and Streibig, 2005; Cox and Surgan, 2006; Pereira et al., 2009; Queirós et al., 2018). Co-formulants are often considered or declared as inert ingredients because they are not formulation segments that are directly responsible for the mode of action. This could lead to underestimation of the toxicity of a formulation, since

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its co-formulants can exert even higher toxicity than the active ingredient itself (Zhu et al., 2014; Mesnage et al., 2015; de Brito Rodrigues et al., 2019). Formulation composition varies depending on brands produced in different countries and, since information about co-formulants is often classified as confidential, it is rarely disclosed on product labels (Cox and Surgan, 2006; Mesnage et al., 2015). Finally, depending on formulation composition (formulations with the same active ingredient but different sets of co-formulants), the toxicity of end-use products can be extremely variable.

To address the issue of toxicity of an active ingredient and different formulations, two commercial herbicides, Rampa® EC (Galenika Fitofarmacija, Belgrade, Serbia) and GAT Cenit 36 CS (GAT Microencapsulation AG, Ebenfuth, Austria), were selected in the present study. Both formulations contain clomazone as active ingredient (nominally the same amount) and are currently in use. Clomazone (2-[(2-chlorophenyl)methyl]-4, 4-dimethyl-3-isoxazolidinone) is a carotenoid biosynthesis inhibitor from the isoxazolidinone herbicide group, used for control of annual broadleaf weeds and grasses (Ferhatoglu and Barrett, 2006) with annual consumption in USA only of ~500 t (US EPA, 2007a). The formulation Rampa® EC is an emulsifiable concentrate (EC) – a very simple formulation system which contains a blend of two emulsifiers (10% or less), the a.i. and an organic solvent. The volatility of clomazone and its potential to damage non-target plants is an important limitation for its use (Schreiber et al., 2015, 2016). Efforts to reduce the negative environmental impact of pesticides have led to the development of new technologies based on gradual release of active ingredients (Knowles, 2008). Capsule formulations deliver significant performance advantages over standard emulsifiable concentrates (EC). The capsule wall encloses the active ingredient dissolved in a small amount of organic solvent, protecting it from volatilization and other unwanted processes while the release rate can be controlled by varying particle size, wall thickness and porosity of the wall. That way significant improvement in environmental and operator safety can be achieved. The capsules are suspended in water with surfactants, which ensures product stabilization and easy formation of suspension after dilution with water. Capsule suspensions (CS) are very complex systems that need many different surfactants and other chemicals for production (Lee and Nicholson, 2003; Casana Giner et al., 2012; Bristow and Wu, 2015; Mesnage and Antoniou, 2018). On the basis of capsulation formulations performance and advantages, the aqueous capsule suspension GAT Cenit 36 CS was chosen as the other formulation type for our study.

Clomazone is highly water soluble (1102 g/l), weakly sorptive (Kd = 1.5–7.4) and has a relatively low n-octanol-water partition coefficient (Kow = 2.5) which indicates its hydrophilic character. These physicochemical properties of clomazone altogether with its widespread use reflect on its potential impact on contamination of water bodies (Van Skoj and Tjeerdema, 2014). Clomazone residues in surface water bodies in agricultural areas have been detected in concentrations ranging 0.03–8.9 μg/l (Zanella et al., 2002; Dutra de Armas et al., 2007; Marchesan et al., 2007; Becker et al., 2009; Sauco et al., 2010; Struger et al., 2011; Adeniyi et al., 2017; Stout et al., 2018; Caldas et al., 2019; Montagner et al., 2019), while its concentration detected in a draw well in Brazil was 4.4 μg/l (Sousa et al., 2018). Effect assessment of clomazone and its formulated products (capsule suspension formulation type) in aquatic primary producers has been performed for authorization requirements (EFSA, 2007). This assessment showed that the diatom algae *Navicula pelliculosa* was the most sensitive species to clomazone with $EC_{50}$ = 0.136 mg/l, while the $EC_{50}$ for *Raphidocelis subcapitata* was 2.0 mg/l for the active ingredient and 29.8 mg a.i./l for the formulation. According to available data, the median effective concentration in macrophytes varies from 10.2 mg/l to 55.6 mg/l in *Lemna* sp., and to 129.6 mg/l in *Azolla caroliniana* (Michael et al., 2004; EFSA, 2007; US EPA, 2007b; Silva et al., 2012; Della Vechia et al., 2016). However, no studies on rooted macrophytes have been reported so far, either for the active ingredient or formulated products.

Assessment of herbicide impact on non-target aquatic macrophytes is an integral part of the pesticide registration process and it is one of the required risk assessment (RA) steps. For this purpose, the commonly used macrophyte species is *Lemna minor*. According to the guidance on tiered RA (EFSA, 2013), when *Lemna* sp. and algae do not manifest sensitivity in exposure to a herbicide ($EC_{50}$ > 1 mg/l) additional testing with a rooted macrophyte may be required, and *Myriophyllum* sp. is on the list of recommended species. One of the advantages of *Myriohyllum* sp. tests is the possibility of applying a more ecologically relevant system with water and sediment. For years back, efforts have been made towards improvement, optimisation and standardization of methods for testing rooted macrophytes (Knauer et al., 2006; Maltby et al., 2010; Teodorović et al., 2012; Tunić et al., 2015). According to Vonk and Kraak (2020), out of 18 standardized protocols for primary producers testing, 9 are with algae, 5 with floating macrophyte species (namely *Lemna* sp.) and only 4 with submerged/emergent species (among which 3 freshwater macrophytes as model species – *Myriohpyllum aquaticum*, *M. spicatum* and *Glyceria maxima*).

In this study we evaluated the effects of the technical ingredient and differently formulated clomazone products with the same content of active ingredient on two aquatic macrophyte species – the floating *Lemna minor* and the rooted *Myriophyllum aquaticum*. Our major goals were to: a) assess species sensitivity to clomazone, b) highlight differences in effects of the technical grade substance and its formulations and c) evaluate the addition of rooted macrophyte species in RA. The formulations selected for this study are up-to-date regarding their use and efficacy, and represent older (emulsifiable concentrate – Rampa® EC) and newer (capsule suspension – GAT Cenit 36 CS) formulation types. Major differences between the two formulation types concern their composition (solvent, co-formulants) and controlled release of the active ingredient in the capsule suspension formulation type.

## 2. Materials and methods

### 2.1. Chemicals

The declared purity of the technical ingredient (CAS 81777-89-1, Shenzhen Yancheng Chemicals Co., China) used in this study is >95% (the technical ingredient is a material resulting from a manufacturing process comprising the active ingredient, together with associated impurities). Two commercial products (formulations) – Rampa® EC (emulsifiable concentrate, Galenika fitofarmacija, Belgrade, Serbia, hereafter refered to as Rampa) and GAT Cenit 36 CS (aqueous capsule suspension, GAT Microencapsulation AG, Ebenfurth, Austria, hereafter referred to as GAT) used in this study had the content of clomazone nominally 360 g/l. The highest test concentrations were prepared by diluting the technical ingredient and formulations in respective standard test medium (Steinberg test medium ISO 20079, (OECD 221, 2006) for *L. minor*; Smart and Barko test medium OECD 239, 2014 for *M. aquaticum*). The prepared solutions were further diluted by standard test medium to make the final series of test concentrations. All concentrations are expressed as mg of clomazone active ingredient (a.i.) per liter of standard test medium.

Concentrations of the active ingredient in all solutions were analyzed using a HPLC-DAD Shimadzu Prominence coupled with

PDA detector (Shimadzu, Japan) on a ZORBAX Eclipse XDB-C18 column, 150 × 4.6 mm, 5 μm (Agilent) at 30 °C. Analyses were performed at the beginning of experiments and when the solutions were renewed using a method described by Niell et al. (2010), with modifications as described in Stevanovic et al. (2017).

### 2.2. Test organisms

A laboratory culture of *L. minor* was maintained in 500 ml plastic pots in Steinberg medium (ISO, 20079, 2005). *M. aquaticum* was cultured on standard artificial sediment (ISO 16191, 2013) and watered with semi-concentrated Steinberg medium (1:1 v/v, Steinberg medium: distilled water). Laboratory cultivation conditions were the same for the two macrophyte species: temperature (24 ± 2 °C) and constant light intensity (85–135 μE $m^{-2}s^{-1}$; checked regularly with a digital Lux meter Testo 545).

### 2.3. Test design and evaluation

Tests with L. *minor* were conducted following the growth inhibition test (OECD 221, 2006). A total of 10–11 fronds per pot (triplicates and controls in 4–6 replicates) were exposed for 7 days to a series of concentrations (3.3, 10, 30, 90, 270 and 810 mg/l) of the active ingredient and two commercial formulations of clomazone. Plants were exposed in semi-static conditions, with test solution renewal after 72 and 120 h. In order to calculate growth inhibition, total frond number (FN), fresh weight (FW) and total frond area (FA) were measured at the beginning and the end of experiment, and also on days when medium renewal was carried out. Fronds were counted, fresh weight was measured on an analytical scale (after gently removing solution from plants) and total frond area was determined by image analysis (Adobe Photoshop, ver. cs. 6).

Toxicity tests with *M. aquaticum* were run in static conditions in a sediment-water system, according to the ring test guideline (OECD, 2011). Apical fragments (6 ± 1 cm) from laboratory plants were planted in plastic pots containing standard artificial sediment, pots were placed in 2 l glass beakers and Smart & Barko medium (OECD 239, 2014) was added. After a three-day adaptation period, the plants were exposed to the same series of concentrations of active ingredient and two clomazone formulations for a period of 7 days. There were three to six control replicates while treatments were tested in triplicates. Shoot length above sediment (SLS), total shoot length (TSL), whole plant fresh weight (FW) and root fresh weight (RFW) were determined. Parameters were measured at the beginning and the end of the experiment, while SLS was also measured after 72 h. The three-day adaptation/rooting period of plants in experimental pots, from DAT -3 till DAT 0 (the beginning of the exposure phase) makes it impossible to measure FW and TSL of the same plants measured at the end of the test. In order to evaluate these parameters, additional five pots with plants were added at the beginning of the test and used only for initial measurements (DAT 0) and comparison to measurements at the end of the tests (DAT 7). Relative growth rate in control and treatments was calculated and inhibition of growth based on RGR of parameters in treatments compared to control was assessed.

### 2.4. Statistical analysis and risk assessment refinement step

Statistical analysis was conducted using Graph Pad Prism version 5 (Graph Pad Software, USA) and STATISTICA 7 (StatSoft, USA). The $EC_{50}$ (concentration causing a 50% inhibition of growth) values were calculated based on nonlinear logarithmic regression of concentration-response curves constructed on the basis of measured concentrations. Response in concentration-response curves being percent inhibition in average relative growth rate of a growth variable. The relative growth rates (RGR) of measured endpoints and growth inhibition (%) of treatments vs. control were calculated in accordance with the OECD 221 (2006) and OECD 239 (2014) guidelines. In order to compare the effects of treatments and controls, a one-way ANOVA was performed. When statistical significance in RGR was found, post hoc multiple comparison by Dunnett's method was used to evaluate further differences between the test concentration and controls.

A two-way ANOVA (STATISTICA 7, StatSoft, USA) was performed in order to compare the effects of treatments with the same concentration of a.i. clomazone between the technical ingredient and formulations. Transformation of growth inhibition was performed based on the following formula:

$$X = \sqrt{(Xi + b)}$$

where: X – square root transformed percentage of growth inhibition, Xi – % of inhibition normalized by control and b – correction factor.

When statistical significance was found, a post hoc multiple comparison by Duncan's method was used to evaluate further differences in inhibition between the treatments with technical ingredient and formulations.

Species sensitivity distribution (SSD) was modeled using the lowest toxicity values of clomazone for *L. minor* and *M. aquaticum* in this study and available literature data (Supplementary material, Table S1) using the software ETX 2.1 (RIVM, Netherlands). The hazardous concentration for the specified fraction (5%) of aquatic primary producers was calculated as the median (HC5) and lower level estimate (LL HC5). Regulatory acceptable concentrations (RAC) were determined using a corrected assessment factor (AF) 3 (recommendation by EFSA, 2013) or 5 (proposed by ETX) for HC5 and 0.44 for the LL HC5.

Predicted environmental concentrations (PEC) step 1 and 2 were modeled using Steps 1–2 in FOCUS, version 3.2, and step 3 using FOCUS TOXSWA 4.4.3 (FOCUS, 2001; Beltman et al., 2014; Beltman, 2015). Since PEC values for formulations could not be estimated, they were calculated only for the active ingredient clomazone. Modeling was based on the application recommendation for clomazone in Serbia: one treatment (preemergence) in soybean, application rate 270 g a.i./ha. The results of PEC step 3 modeling represent a more realistic scenario, since it covers topography, soil type, meteorological conditions, surface waterbody type, etc. Out of 10 predefined FOCUSsw scenarios, only two were representative (runoff scenario Bologna – R3 and runoff scenario Roujan – R4) for soybean clomazone application in the South European zone. The results of PEC modeling are summarized in the Supplementary material (S2 a-d).

## 3. Results

Chemical analysis indicated that the measured concentrations of clomazone in solutions with the technical ingredient (measured purity 95.3%) and formulations Rampa (measured concentration 363.2 g/l, nominal 360 g/l) and GAT (measured concentration 358.4 g/l, nominal 360 g/l) did not differ greatly from nominal concentrations (<20%) and were stable during tests. The $EC_{50}$ results shown in this paper are derived from measured concentrations of the active ingredient. Measured concentrations from the solutions are shown in Supplementary results (S3a, b) and tables of results (S4, 5, 6) have information on both, the nominal and measured, concentrations.

### 3.1. Species sensitivity to clomazone

Clomazone, a.i. and two formulated products, significantly reduced the growth of aquatic macrophytes (Supplementary material, Table S4, S5). A statistically significant reduction in *L. minor* growth was found in almost all treatments of the technical ingredient and the formulation Rampa (Supplementary material, Table S4). In plants exposed to the formulation GAT, no significant growth reduction was detected in treatments with lower exposure concentrations (<90 mg a.i./l). Significant reduction in growth rate of *M. aquaticum* (based on SLS, TSL and RFW) was recorded in all treatments with the active ingredient and formulations, except the lowest tested concentration of Rampa (Supplementary material, Table S5, S6). Hormetic stimulation was observed regarding *M. aquaticum* root formation. The technical ingredient and formulation Rampa stimulated root growth in treatments with the two lowest tested concentrations but with no statistical significance compared to control. Absence of roots was registered in plants exposed to the highest concentration of the technical ingredient and in all treatments with ≥30 mg a.i./l of the formulation Rampa. However, in plants exposed to the formulation GAT significant reduction in root growth was noted only in treatment with the highest tested concentration, while roots were present in all treatments.

In general, the rooted macrophyte *M. aquaticum* was considerably more sensitive than the floating species *L. minor* in exposure to the technical ingredient, as well as both formulations (Table 1). This applies to all parameters except *M. aquaticum* RGR FW in the test with Rampa. The $EC_{50}$ values for *L. minor* parameters ranged from 51.3 to 643.9 mg/l for RGR FN, 38.7–369.7 mg/l for RGR FW and 33.3–321.0 for RGR FA. The $EC_{50}$ values for *M. aquaticum* ranged from 1 to 16.6 mg/l for plant length parameters, 1.2 to >810 mg/l for FW. The $EC_{50}$ values based on RFW were calculated only in tests with *M. aquaticum* and were 80.5, 27.4 and 300.3 mg/l for clomazone, Rampa and GAT, respectively.

### 3.2. Effects of the technical ingredient and formulations

The results showed different toxicity of the two formulation types of clomazone to the test plants. The capsule formulation was less toxic than the emulsion to *L. minor*, but more toxic to *M. aquaticum* (except based on RFW $EC_{50}$).

All parameters of *L. minor* (FN, FW and FA) showed a similar pattern of response to different formulations. The plant was most sensitive to Rampa and the least sensitive to GAT. Medium sensitivity to the active ingredient was observed compared to the formulations. Based on $EC_{50}$ values the most sensitive parameter for *L. minor* was FA, with $EC_{50}$ 54 mg a.i./l for the technical ingredient, and 33.3 and 321.0 mg a.i./l for the formulations Rampa and GAT, respectively (Table 1). Rampa was statistically more and GAT less toxic to *L. minor* than the technical ingredient (Fig. 1).

Based on the $EC_{50}$ for *M. aquaticum*, the principal parameter suggested by the protocol (OECD, 2011), shoot length above

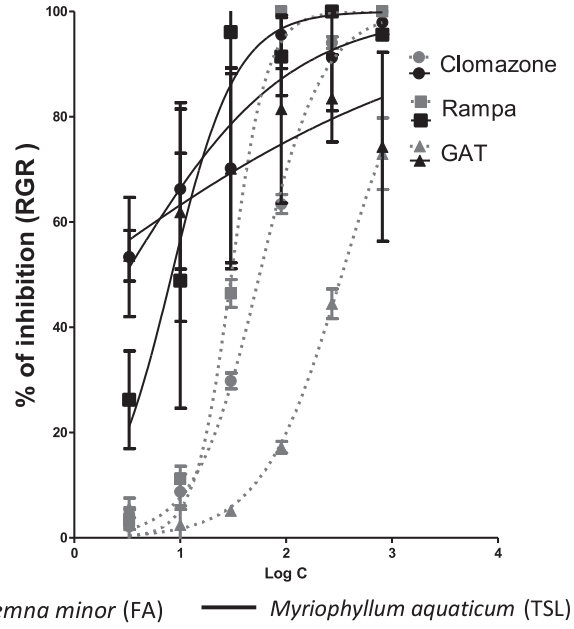


**Fig. 1.** Regression curves of *L. minor* growth inhibition (%) based on total frond area (RGR FA) and *M. aquaticum* based on total shoot length (RGR TSL) after 7-day exposure to the technical ingredient clomazone and formulations Rampa and GAT. Each point represents mean values with standard deviation.

**Table 1**
Median inhibitory concentrations ($EC_{50}$) and concentrations causing 10% growth inhibition ($EC_{10}$) (95% confidence intervals), no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) after a 7 day exposure of *Lemna minor* and *Myriophyllum aquaticum* to clomazone and its formulations Rampa and GAT.

| | mg a.i./l | *L. minor* | | | M. aquaticum | | | |
|---|---|---|---|---|---|---|---|---|
| | | RGR FN | RGR FW | RGR FA | RGR SLS | RGR TSL | RGR FW | RFW |
| Clomazone | $EC_{50}$ [a] | **99.2** ± 4.4[D] (90.6–108.5) | **59.9** ± 4.5[C] (52.8–68.0) | **54.0** ± 4.4[C] (50.3–58.0) | **16.6** ± 5.9[K] (8.3–33.4) | **3.3** ± 6.3[J] (1.4–7.7) | **1.2** ± 102.2[J] (0.0–1068.0) | **80.5** ± 5.0[L] (57.5–112.5) |
| | $EC_{10}$ [a] | 36.3 (30.4–43.4) | 13.9 (10.4–18.7) | 12.8 (10.8–15.1) | – | – | – | 27.2 (14.6–51.1) |
| | NOEC | 10[b] (8.2[a]) | <3.3[b] (3.0[a]) | 3.3[b] (3.0[a]) | – | – | – | 30[b] (29.0[a]) |
| | LOEC | 30[b] (31.5[a]) | 3.3[b] (3.0[a]) | 10[b] (8.2[a]) | 3.3[b] (3.6[a]) | 3.3[b] (3.6[a]) | 3.3[b] (3.6[a]) | 90[b] (83.9[a]) |
| Rampa | $EC_{50}$ [a] | **51.3** ± 4.3[E] (48.8–54.0) | **38.7** ± 4.5[D] (34.8–43.0) | **33.3** ± 4.4[C] (30.9–36.0) | **8.7** ± 4.7[J] (6.6–11.3) | **9.3** ± 4.8[J] (6.8–12.8) | > **810**[L] (191.6–47459.0) | **~27.4**[K] (very wide) |
| | $EC_{10}$ [a] | 24.1 (22.3–26.1) | 16.4 (13.0–20.6) | 14.2 (11.5–17.5) | 2 | 1.8 | – | 26.5 |
| | NOEC | 10[b] (10.9[a]) | <3.3[b] (3.6[a]) | 3.3[b] (3.6[a]) | 3.3[b] (3.7[a]) | 3.3[b] (3.7[a]) | 3.3[b] (3.7[a]) | 10[b] (11.2[a]) |
| | LOEC | 30[b] (32.8[a]) | 3.3[b] (3.6[a]) | 10[b] (10.9[a]) | 10[b] (11.2[a]) | 10[b] (11.2[a]) | 10[b] (11.2[a]) | 30[b] (33.6[a]) |
| GAT | $EC_{50}$ [a] | **643.9** ± 4.6[D] (536.5–772.7) | **369.7** ± 4.5[C] (328.0–416.7) | **321.0** ± 4.5[C] (285.6–360.7) | **8.5** ± 7.2[J] (2.9–24.8) | **1.0** ± 21.8[J] (0.03–33.9) | **7.1** ± 9.8[J] (1.2–42.0) | **300.3** ± 5.0[K] (195.3–461.7) |
| | $EC_{10}$ [a] | 66.2 (45.7–86.5) | 41.3 (31.6–53.8) | 47.3 (36.4–61.7) | – | – | – | 154.9 (90.4–258.2) |
| | NOEC | 30[b] (28.6[a]) | 30[b] (28.6[a]) | 30[b] (28.6[a]) | 3.3[b] (2.9[a]) | – | – | 270[b] (233.2[a]) |
| | LOEC | 90[b] (85.9[a]) | 90[b] (85.9[a]) | 90[b] (85.9[a]) | 10[b] (8.6[a]) | 3.3[b] (2.9[a]) | 3.3[b] (2.9[a]) | 810[b] (699.5[a]) |

RGR – Relative growth rate.
$EC_{50}$ obtained from single experiments and are shown with standard deviations and confidence intervals (95%).
Same letters in row mark no statistically significant differences between $IC_{50}$ values.
[a] Measured concentrations.
[b] Nominal concentrations.

sediment (RGR SLS), responded similarly to Rampa and GAT (Table 1). If we choose total shoot length (TSL) (which was either the most sensitive or did not significantly differ from the most sensitive parameter) for toxicity comparison, no major differences were found in toxicity between the technical ingredient ($EC_{50}$ = 3.3 mg a.i./l) and the formulation GAT ($EC_{50}$ = 1.0 mg a.i./l), while Rampa ($EC_{50}$ = 9.3 mg a.i./l) was statistically less toxic than both. Regression curves for the most sensitive parameters for both macrophyte species are shown in Fig. 1.

Another aspect that has been evaluated is species dependent sensitivity. The most prominent difference in species sensitivity was registered in exposure to the formulation GAT as its $EC_{50}$ for *M. aquaticum* was found to be 300 times lower than it was for *L. minor*. This result is based on the ratio between the most sensitive parameters in the two macrophyte species. If this parallel is set for FW as a common parameter, the difference is lower, but still remarkable (50 times lower $EC_{50}$ for *M. aquaticum*). Comparison of $EC_{50}$ was done for the most sensitive parameters (FA for *L. minor*; TSL for *M. aquaticum*) in Graph Pad Prizm and results indicated that *M. aquaticum* was significantly ($p < 0.01$) more sensitive than *L. minor* to the technical ingredient, as well as the two formulations (Fig. 1).

The two-way ANOVA was used to confirm differences in the toxicity of equal concentrations of the technical ingredient and formulations to the two species. The common endpoint fresh weight (FW) was used for comparison. The results indicated the highest toxicity of Rampa to L. *minor*, with no differences compared to the technical ingredient, in treatments with higher concentrations (Fig. 2a). The formulation GAT caused significantly lower growth inhibition than both the technical ingredient and the other formulation. Conversely, in experiments with *M. aquaticum* no differences in growth inhibition were observed between the technical ingredient and GAT, while the formulation Rampa was less toxic in treatments with lower concentrations (Fig. 2b).

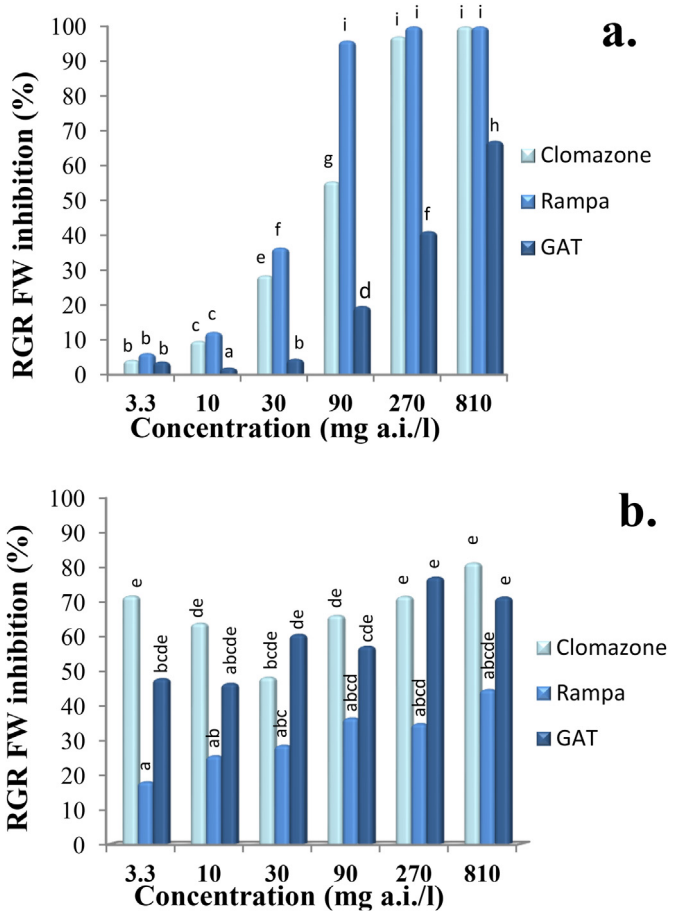


**Fig. 2.** Growth inhibition intensity (%), comparison based on RGR fresh weight for: a) *L. minor* and b) *M. aquaticum* after a 7-day exposure to the technical ingredient clomazone and formulations Rampa® EC and GAT Cenit 36 CS. [1] same letters mark no statistically significant differences. *Note: Please use Fig.3 in black and white for printing.

### 3.3. Rooted aquatic macrophytes in risk assessment for plant protection products

The results obtained in this study, along with literature data, were used for species sensitivity distribution (SSD) analysis (Fig. 3). Species sensitivity assessment was examined using the most sensitive parameter, and in tests with *L. minor* it was FA, and TSL for *M. aquaticum*. Risk was assessed comparing regulatory acceptable concentration (RAC) with the predicted environmental concentration (PEC). PEC values for step 1 and 2 generated in FOCUS were 0.067 and 0.027 mg/l, respectively. The median estimate of the HC5 was 0.16 mg/l, while the lower level estimate HC5 (LL HC5) was 0.005 mg/l. Risk assessment guidance (EFSA, 2013) recommends an assessment factor (AF) of 3, while the ETX software proposes an assessment factor of 5 for the median HC5 and 0.44 for the LL HC5. The calculated RAC value for the median HC5 was 0.032 mg/l (AF = 5), i.e. 0.052 mg/l (AF = 3), and even though the risk was not acceptable for step 1 PEC, it was for step 2 PEC (PEC < RAC). When risk is assessed on the basis of LL HC5 values, the calculated RAC (0.011) is below PEC step 2. In other words, the risk based on LL HC5 is unacceptable at this step. Based on the results of FOCUS TOXSWA step 3 scenarios – R3 and R4 were modeled as representative and were 0.00673 and 0.00572 mg/l, respectively. Finally, RA of clomazone for primary producers was demonstrated to be acceptable at PEC step 3 (both scenarios).

## 4. Discussion

### 4.1. Differences in sensitivity of two macrophyte species

The results obtained in this study showed higher sensitivity of *M. aquaticum* in comparison to *L. minor* to the technical ingredient clomazone and its formulations. However, comparison of sensitivity based on $EC_{50}$ values for RGR FW should be considered with caution because of the very wide confidence intervals which were a

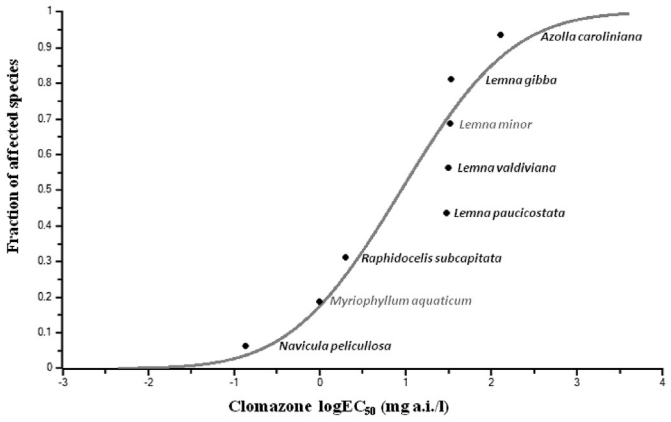


**Fig. 3.** Species sensitivity distribution (SSD) for aquatic primary producers: clomazone. *Note: Please use Fig.1 in black and white for printing

result of higher variability of *M. aquaticum* FW in treatments and lower statistical power of the test based on this parameter. High variability of weight growth parameters was reported in the international ring test on *Myriophyllum* sp. (Ratte and Ratte, 2014 – cit. Tunic et al., 2015). Namely the average coefficient of variation (CV%) in 10 laboratories for RGR DW (dry weight) in controls was 32.9%, and the average minimal detectable difference in estimates based on this endpoint was 42.1% (the higher the value, the lower the power of the test). In experiments with the technical ingredient, the $EC_{50}$ value obtained for *M. aquaticum* (1.2 mg/l) was 50x lower than for *L. minor* (54.0 mg/l). There is no other study with clomazone that can support our findings, but higher sensitivity of *Myriophyllum* sp., in comparison to *Lemna* sp., has been reported for various other herbicides (Turgut and Fomin, 2002; Cedergreen et al., 2004; Tunić et al., 2015). Turgut and Fomin (2002) examined the sensitivity of *M. aquaticum* to 17 pesticides (of which 14 herbicides) and concluded that for the most tested substances sensitivity was similar to *Lemna* sp. Nevertheless, *M. aquaticum* showed higher sensitivity toward auxin simulators (2,4-D; dicamba; dichlorprop), pyridate and some herbicides from the sulfonylurea group (metsulfuron-metyl, thifensulfuron-meryl and triflusulfuron-metyl). Higher sensitivity of *Myriophylum* sp. (*M. aquaticum* and *M. spicatum*) to auxin simulators, compared to *L. minor*, was confirmed in the Tunić et al. (2015) study. Interestingly, Cedergreen et al. (2004) demonstrated lower toxicity of *M. spicatum* compared to *L. minor* in exposure to metsulfuron-metyl. Two studies (Turgut and Fomin, 2002; Cedergreen et al., 2004) demonstrate different sensitivity among macrophytes of the same family and highlight that a universally most sensitive species does not exist. Based on results of the comparative study of six herbicides (*L minor*, *M. aquaticum*, *M. spicatum*), Tunić et al. (2015) concluded that none of the three examined species can be marked as the most sensitive. Differences in the sensitivity of macrophyte species can be attributed to pesticide properties but may also result from a variety of reasons concerning the biology and ecology of macrophytes (Maltby et al., 2010). Dobbins et al. (2010) highlighted that differences in species sensitivity, with regard to herbicides that target various aspects of photosynthesis, might be due to different photosynthesis pathways of species (i.e. whether it is a C3 or a C4 plant). The aquatic environment, as well as the terrestrial, is home to macrophytes with diverse photosynthesis pathways, such as C3, C4, CAM and C3–C4 pathways (Xu et al., 2012). Although most monocotyledonous plants are C4 plants, *Lemna* sp. are C3 plants (Filbin and Hough, 1985; Esquivel et al., 2000), with 3-phosphoglyceric acid as the initial $CO_2$ fixation product during photosynthesis. Literature data on *Myriophyllum* sp. photosynthesis features are diverse. Some authors claim that *Myriophyllum* sp. has a C3 (Salvucci and Bowes, 1983) or a C4 photosynthesis pathway (Wang et al., 2019), while others found that it exhibits characteristics of both C3 and C4 plants (Stanley and Naylor, 1972; Van et al., 1976). The C4 carbon cycle is an addition to the C3 photosynthesis pathway and it ensures higher rates of photosynthesis (Xu et al., 2012). Considering that clomazone inhibits isoprenoid biosynthesis (by inhibiting the reaction between glyceraldehyde 3-phosphate and pyruvate) we speculate that it could be the reason for the higher sensitivity of *M. aquaticum* in our experiments. In addition to this, *L. minor* has a stunted root, while *M. aquaticum* has a stronger root with high uptake potential. Since clomazone uptake occurs by root, the combination of higher root uptake and greater photosynthesis rates in *M. aquaticum* uphold the results obtained in this study. A number of researchers that compared the sensitivity of different aquatic macrophytes to herbicides (Turgut and Fomin, 2002; Cedergreen et al., 2004; Tunić et al., 2015) found that a universally most sensitive species does not exist. Our results are in accordance with these findings and emphasize the importance of taking into account different groups of primary producers when estimating herbicide effects.

### 4.2. Differences in toxicity between the technical ingredient and formulations

Tested plants reacted differently after exposure to the active ingredient and formulations of clomazone. The formulation Rampa caused higher inhibition of growth rate of *L. minor* compared to the clomazone technical ingredient. GAT was the least toxic to *L. minor* ($EC_{50}$ = 321.0 mg/l). On the other hand, the technical ingredient and both formulations caused high inhibition of *M. aquaticum* growth, with GAT being unexpectedly the most toxic based on all parameters except RFW. Different toxicity of formulations, compared to their active ingredients, has been described by many authors (Mesnage et al., 2015; Queirós et al., 2018; de Brito Rodrigues et al., 2019). Compared to the clomazone technical ingredient, Rampa exhibited higher or similar toxicity in tests with *L. minor* and *M. aquaticum*, respectively. According to the material data sheets for Rampa (MSDS, 2013), its constituents besides a.i. (36.4% w/w clomazone) mostly include an organic solvent (57.3% w/w petroleum, Solvesso 100) and less than 10% of undisclosed co-formulants. Toxicity of organic solvents to aquatic animals has been well-documented, but our knowledge regarding aquatic plants is lacking. Solvents and co-formulants may enhance the availability and uptake of the active ingredient, which may explain the registered toxicity to *L. minor*. Capsule suspensions are a new generation of formulations with their active ingredient encapsulated and gradually released. By default, capsule suspensions should be less toxic than the emulsifiable concentrate formulation, just as it was observed in the *L. minor* tests. An interesting finding of our study was that the GAT formulation demonstrated high toxicity to *M. aquaticum*, and the difference in toxicity to the two species was considerable (300x lower $EC_{50}$ for *M. aquaticum* compared to *L. minor*). The observed differences could only partially be explained by differences in species sensitivity, while the other reason is most likely related to formulation composition. A number of researchers associate variable toxicity of formulations (compared to their active ingredients or different formulation types) with the use of co-formulants (Howe et al., 2004; Zhu et al., 2014; Mesnage et al., 2015). Under existing laws and policies, the impact of co-formulants on environmental fate, metabolism or toxicity of end-use formulations is ignored and usually information on its content is protected as confidential data (Cox and Surgan, 2006; Mesnage et al., 2019). Similarly, the formulation GAT is a very complex system (capsule suspension formulation) that needs support of many co-formulants to be stable (Knowles, 2005). The active ingredient in formulation GAT makes one third of total product volume, while the rest (66.2% w/w) is a mixture of undisclosed co-formulants (MSDS, 2011). The most complete understanding of formulation toxicity would be gained if separate assessments of the active ingredient, co-formulants and final formulation would be performed. Still, such studies are rare since co-formulants are rarely identified. A study by de Brito Rodrigues et al. (2019) testing the active ingredient glyphosate, its formulation and the surfactant POEA (polyethoxylated tallow amine) demonstrated that the surfactant was the most toxic for developing zebrafish embryos. A similar study was performed using human cells, and higher toxicity of a glyphosate formulation and co-formulants was shown, compared to the active ingredient (Defarge et al., 2016). Studies are usually based on formulation assessment and it is impossible in such research to distinguish if co-formulants are directly responsible for toxic effects, or toxic effect is

due to interactions with the active ingredient (Cox and Surgan, 2006). For instance, some adjuvants (usually mixer of the surfactants) can be toxic themselves, but even when that is not the case they can increase pesticide toxicity by increasing the uptake rate (Mesnage et al., 2015). Also, some surfactants can reduce surface tension and allow water soluble active ingredients to penetrate the lipid-based plant cell walls and membranes (Mesnage et al., 2019). Taking into consideration that many surfactants are needed to produce a CS formulation, the observed toxicity of GAT in *M. aquaticum* could be the result of complex interactions between the active ingredient clomazone, the co-formulants and plant surface. Contrary to *M. aquaticum,* which is exposed via its whole surface, *L. minor* is exposed only dorsally, while the ventral side of fronds is on the surface, not exposed to chemicals. The conclusion of the peer reviewed pesticide RA of the active substance clomazone (EFSA, 2007) contains concerns regarding the appropriateness of *Lemna* sp. as test organism since it seemed not to be sensitive to the formulated product (which was also a capsule suspension formulation). The results obtained in our study are consistent to that report. On the other hand, Della Vechia et al. (2016) found that the same formulation (Gamit® CS) was more toxic to *L. minor* than the one tested in our study. In the mentioned study, plants were submitted to disinfection in sodium hypochlorite solution (2%) prior to clomazone exposure, hence it is possible that the higher sensitivity of plants happened due to stress or potential cell damage. Investigation of clomazone formulated as an emulsifiable concentrate has not been performed up to date and this is the first such report. Our results show that the toxicity of the active ingredient and formulations to the tested aquatic macrophytes can be ranked in the following order from the most to least toxic: formulation Rampa > technical ingredient clomazone > formulation GAT for *L. minor,* and formulation GAT = technical ingredient clomazone > formulation Rampa in *M. aquaticum* assays.

Meredith et al. (2016) highlighted that capsulated pesticide formulations have a potential to change the way the active ingredient interacts with the environment and biota, thus limiting active ingredient specific properties (such as $K_{ow}$ or $DT_{50}$) in estimating environmental persistence, mobility, bioconcentration potential, etc.

### 4.3. Added value of rooted macrophyte species in RA refinement step

Studies of clomazone toxicity to aquatic macrophytes have been reported since 2004 (Michel et al., 2004; EFSA, 2007; Silva et al., 2012). Based on literature data (Jonsson et al., 1995; Michel et al., 2004; EFSA, 2007) and this study results *L. minor* is the least sensitive *Lemna* species to technical clomazone, less sensitive than *L. paucicostata* and *L. gibba*. Nevertheless, these differences in sensitivity are not great and may result from differences in maintenance conditions or interlaboratory species sensitivity. Compared to *Azolla caroliniana,* however, *L. minor* is more sensitive to clomazone. To the best of our knowledge, literature presents only data from studies with floating species. The results of our study are the first report on clomazone effects in rooted aquatic macrophytes.

The revision of the Guidance document on aquatic ecotoxicology (EC, 2002) and the publication of the Guidance on tiered RA for plant protection products for aquatic organisms in edge-of-field surface waters (EFSA, 2013) were important steps in improving the constantly evolving RA process.

Generally, aquatic plants have been neglected for regulatory purposes, in comparison with faunal species. Current regulation on pesticides with herbicidal modes of action claims obligatory acute and chronic testing with fish and aquatic invertebrates and obligatory testing with *Lemna* species and algae (green and non-green). Additional testing with rooted macrophyte species (preferably *Myriophyllum*) is advised in case of insensitivity ($EC_{50}$ > 1 mg/l) of standard test species. Lewis and Thursby (2018) imposed an important question: How certain can results be from two phytotoxicity tests in representing the sensitivity of a diverse aquatic plant community?

One of the challenges in SSD modeling is a relatively large number of tested species for its successful application. The inclusion of *M. aquaticum* for clomazone effect assessment contributed to SSD modeling and gave an insight in potential adverse effects on aquatic primary producers. The results obtained in this study, along with literature data, meet the requirement for SSD analysis – 8 species of primary producers. RA based on lower level estimate HC5 (LL HC5) and PEC values on the basis of the worst case scenario (step 1 and 2) demonstrated a high risk for primary producers, while the more realistic fate and behavior pesticide model (step 3) indicated that no unacceptable adverse effects on aquatic primary produces will occur after clomazone application. Still, if we compare the LL HC5 value (0,005 mg/l) with clomazone concentrations detected in surface waters (Marchesan et al., 2007), there are indications of potential adverse effects on a fraction (5%) of the most sensitive species.

## 5. Conclusion

Our study brings the first report on the negative impact of clomazone on rooted aquatic macrophytes. The two tested macrophyte species responded differently to the technical ingredient and formulations of clomazone. Results of our study, along with literature data on species sensitivity, endorse a notion that floating aquatic macrophytes are not by default the most sensitive species to represent all aquatic primary producers and that the inclusion of rooted species in RA is highly recommended and justified. With the inclusion of *M. aquaticum*, one of the results of the SSD modeling was the LL HC5 value in the range of environmentally relevant clomazone concentrations. This indicates that the hazardous threshold in the environment could be exceeded and the number of threatened species could be higher than the defined acceptable fraction (5%). The toxicity of formulations to the tested aquatic macrophyte species differed significantly, indicating that co-formulants may have contributed to this change. Differences were noted in relation to species tested, as well. The clomazone capsule formulation was less toxic to *L. minor*, but more toxic to *M. aquaticum* (except regarding RFW) than the emulsifiable concentrate. Different responses of the two test species to one conventional and another newer formulation indicates that estimates regarding newer formulations should also be made with caution.

## Author contributions

Marija Stevanović: Investigation, Formal analysis, Validation, Writing – original draft preparation, Visualization, Dragica Brkić: Supervision, Writing – review & editing, Tanja Tomić: Investigation, Validation, Writing – review & editing, Varja Mihajlović: Investigation, Validation, Tijana Đorđević: Validation – chemical analysis, Slavica Gašić: Supervision, Conceptualization, Resources, Writing – review & editing

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2021.116753.

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