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Toxicity of clomazone and its formulations to zebrafish embryos (*Danio rerio*)

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ABSTRACT

Herbicides are the most widely used group of pesticides but after reaching water bodies they are able to cause adverse effects on non-target organisms. Different formulations using the same active ingredient are frequently available, which raises the issue of potential influence of different formulation types on herbicide toxicity. The present study evaluated the toxicity and teratogenic effects of the active ingredient clomazone and its two formulations (Rampa® EC and GAT Cenit 36 CS, both containing 360 g a.i./l of clomazone) on zebrafish embryos. The crucial difference between the two formulation types is the way of active substance release. This investigation is the first report on zebrafish embryotoxicity of both clomazone and its formulations. The technical active ingredient and formulations caused mortality and diverse teratogenic effects, showing different levels of toxicity. The LC_{50} values for the technical ingredient, Rampa® EC and GAT Cenit 36 CS were 61.4, 9.6 and 92.5 mg a.i./l, respectively. Spontaneous movements in 22 hpf embryos decreased under exposure to both the technical ingredient and formulations. A significant number of underdeveloped embryos was detected after exposure to clomazone and Rampa® EC, while no underdevelopment was noted in embryos exposed to GAT Cenit 36 CS. Exposure to the technical ingredient and formulations led also to a series of morphological changes and interfered with the growth of zebrafish embryos. The EC_{50} based on detection of edemas, spine and tail tip deformations and gas bladder absence (120 hpf) was 12.1, 10.1 and 24.1 mg/l for technical clomazone, Rampa® EC and GAT Cenit 36 CS, while teratogenicity index (TI) based on LC_{50}/EC_{50} ratio was 5.1, 1 and 3.8, respectively. The data in this study showed that the emulsifiable concentrate formulation (Rampa® EC) caused statistically significantly higher toxicity, and the aqueous capsule suspension (GAT Cenit 36 CS) lower toxicity than technical clomazone. It indicates that different formulations with the same active ingredient may have different environmental impacts, which is why risk assessment based only on active ingredient toxicity might not be sufficient in terms of preventing formulation effects on the environment.

1. Introduction

Herbicides have a very important role in plant protection but their use also creates a risk to human health and the environment. Besides their many benefits, herbicides may affect non-target organisms, contaminate soil and water and have toxic effects in aquatic ecosystems. Clomazone (2-[(2-chlorophenyl)methyl]-4, 4-dimethyl-3-isoxazolidinone) is an isoxazolane herbicide which is used to control annual broadleaf weeds and grasses. The general mode of action of clomazone is inhibition of carotenoid biosynthesis (HRAC, 2017). Precisely, clomazone inhibits the formation of isoprenoids including carotenoids, plastoquinone, tocopherol and gibberellin hormones in higher plants (Ferhatoglu and Barrett, 2006). Owing to long and widespread use of clomazone for plant protection and its physicochemical properties, which have demonstrated a potential to contaminate water, its possible impact on aquatic organisms is of great concern (Crestani et al., 2007). Clomazone residues have been detected in many water samples collected from rivers in rice-growing regions, but also in sediments and wastewater, ranging from 0.03 to 1000 µg/l (Zanella et al., 2002; Marchesan et al., 2007; Becker et al., 2009; Sauco et al., 2010; Struger et al., 2011; Hug et al., 2014). Also, clomazone has been detected in fish liver and muscle samples (Lazartigues et al., 2011; Lazartigues et al., 2013a; Lazartigues et al., 2013b; Caldas et al., 2013) raising concerns over its potential adverse effects. As many other active ingredients of pesticides, clomazone is applied formulated into a suitable product. The effects of other formulation components on the fate, behavior and/or

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toxicity of pesticide active ingredients are not always fully considered even though they might be able to greatly enhance or lessen the toxicity of a formulation. Enhancement of the toxicity of substances after the formulation process is not unusual and has been reported for other pesticides (Beggel et al., 2010). In order to reduce the environmental impact of pesticides, new technologies based on gradual release of active ingredients have been developed (Knowles, 2008).

So far, toxicological properties of clomazone have been evaluated on adult fish as the main model in aquatic toxicity assessments. According to available data, the median lethal concentration (96 h LC_{50}) of clomazone for fish species varies from 7.3 mg/l in freshwater silver catfish (*Rhamdia quelen*), over 15.5 and 34 mg/l in rainbow trout (*Oncorhynchus mykiss*) and bluegill sunfish (*Lepomis macrochirus*), to 40.6 mg/l in estuarine sheepshead minnow (*Cyprinodon variegatus*) (Dos Santos Miron et al., 2005; EFSA, 2007; EPA, 2007; Nufarm, 2015). Most of the relevant studies focused on revealing biochemical and metabolic alterations (Dos Santos Miron et al., 2008; Cattaneo et al., 2012; Pereira et al., 2013). Inhibition of acetylcholinesterase activity (AChE) in brain and muscle tissue, gill lamellar edema and hepatocyte vacuolization were observed after exposure to clomazone in studies with *R. quelen* (dos Santos Miron et al., 2005; Crestani et al., 2007; Brum et al., 2014). Biochemical and metabolic alterations and oxidative stress were confirmed also for other species (*Cyprinus carpio, Prochilodus lineatus*, *Leporinus obtusidens*) exposed to clomazone (Moraes et al., 2007; dos Santos Miron et al., 2008; Cattaneo et al., 2012; Pereira et al., 2013; Murussi et al., 2015). However, no studies on embryotoxicity and teratogenicity of clomazone were reported. Also, no data are available on fish embryotoxicity of any formulation containing clomazone as its active ingredient.

In terms of the 3R concept (reduce-refine-replace), the zebrafish embryo toxicity test with *Danio rerio* (zFET) has been suggested as an alternative tool to examine the adverse effects of aquatic toxicants (Nagel, 2002; Braunbeck et al., 2005; Scholz et al., 2008). According to Belanger et al. (2013) FET test is a good alternative model to standard fish acute test, as it provides almost the same prediction of environmental hazard, while improving animal welfare. External fertilization, rapid development, ease of treatment, transparency during embryonic and larval development, all allow monitoring of morphological abnormalities and are some of the attributes making zebrafish embryo testing an adequately sensitive fish bioassay (Cook et al., 2005; Fraysse et al., 2006; De Esch et al., 2012; Pérez et al., 2013). By now, numerous studies of pesticide toxicity have been performed using zebrafish embryos (Görge and Nagel, 1990; Lin et al., 2013; Watson et al., 2014). Many of them have confirmed that exposure of zebrafish to pesticides leads to various nonlethal malformations: yolk sac and pericardial edema, spine and body curvature, heart rate and neural disorders, and craniofacial and swim bladder deformations (Cook et al., 2005; Wiegand et al., 2001; Lin et al., 2013).

The objective of this study was to investigate the effects of the herbicide clomazone and its two different formulation types: emulsifiable concentrate (old type of formulation) – Rampa® EC, and aqueous capsule suspension (CS) (newer type of formulation) – GAT Cenit 36 CS, on zebrafish embryos. These products are designed to be dissolved in water before application. Generally, EC formulations contain active ingredients which are dissolved in an organic solvent, and surfactants are added to ensure good emulsification after dilution with water. The active ingredient in CS formulations is located inside microcapsules (with wall made of polymeric material), which are suspended in water with the help of surfactants that ensure product stabilization and easy formation of suspension after dilution with water. The rate of release of active ingredients from microcapsules is a diffusion-controlled process, which can be regulated by adjusting the capsule size, thickness and porosity of capsule wall (Knowles, 2008). Both types of formulation are in use and have a similar grade of effectiveness on target organisms. A significant difference between them is that the formulation GAT Cenit 36 CS releases the active ingredient gradually, while the active ingredient in Rampa® EC formulation is completely available immediately after application. It results in a lower exposure concentration of the active ingredient in the case of GAT Cenit 36 CS than the product Rampa® EC. Our major goal was to characterize the embryotoxicity of clomazone-based formulations and reveal potential differences in the toxicological properties of the formulated products and active ingredient itself. Mortality, neurological and cardiac disorders and different morphological deformations were evaluated. The phenotypes provoked by the same (or approximately equivalent) applied concentrations of technical ingredient and formulations were compared and differences assessed.

2. Materials and methods

2.1. Chemicals

The technical ingredient, clomazone (CAS 81777-89-1) with ≥95% declared purity, was purchased from Shenzhen Yancheng Chemicals Co., China. Two commercial formulations (products) used in this study, Rampa® EC (emulsifiable concentrate, Galenika fitofarmacija, Belgrade, Serbia) and GAT Cenit 36 CS (aqueous capsule suspension, GAT Microencapsulation AG, Ebenfurth, Austria), contained nominally 360 g/l clomazone (363.2 g/l and 358.4 g/l, respectively) as their active ingredient. The exact composition of commercial pesticide products (formulations) is a trade secret and only general information about their content other than the active ingredient is available. Prior to starting the experiments, stock solutions of the technical ingredient and formulations were prepared by diluting them in standard test medium (100–200 mg/l). The prepared stock solutions were then diluted to the final concentrations in standard test medium (ISO, 1996). All concentrations are expressed as mg a.i. clomazone per liter of standard test medium. At the beginning of the experimental procedure, dissolved oxygen was measured and found to be > 98%, while pH varied between 8 and 8.3.

Concentrations of the active ingredient in all stock solutions were analyzed using an HPLC-DAD Agilent 1100 Series coupled with PDA detector (Agilent Technologies, Germany) on a ZORBAX Eclipse XDB-C18 column, 150 × 4.6 mm, 5 µm (Agilent) at 30 °C. The analyses were performed at the beginning of experiments using a method described by Niell et al. (2010) with some modifications. The binary gradient of the mobile phase consisted of (A) acetonitrile and (B) water/0.1% formic acid (48% B at 0–9 min; linear increase from 48% B to 80% B at 9–11 min; 80% B at 11–15 min); the flow rate was 1 ml min^{-1}; the injection volume was 20 µl. Chromatograms were recorded at 220 nm. The analyte was identified by comparing the UV spectra and retention time with the standard (Sigma-Aldrich) and quantified using external calibration (0.5–10 mg/l).

2.2. Test organism

Zebrafish (*Danio rerio*) embryos were obtained from the Research Center for Toxic Compounds in the Environment, Masaryk University (Czech Republic). The fish were reared in tanks with local tap water ($CaCO_3$ 100–180 mg/l, NO_3^- 10–25 mg/l, pH 7.0–7.2), at 26 ± 1 °C and 14/10 h light/dark photoperiod. The fish were fed on a combination of live brine shrimp (*Artemia salina*) and a mixture of commercially available dried feeds (Spirulina, Sera; Gammarus, Dajana; Tubifex, Easyfish; Flake food, Sera). The evening before spawning, traps for collecting embryos were placed on the bottom of spawning aquaria and removed in the morning immediately after spawning. Fertilized eggs were rinsed and then transferred to standard test medium. Embryos were examined under a stereomicroscope (OLYMPUS, Japan), viable eggs were selected, and the test was initiated not later than 3 h post-fertilization (hpf).

2.3. Test design

Tests were conducted following the fish embryo toxicity test guidelines (OECD, 2013) with a few modifications. Fertilized, viable eggs were exposed to the herbicide clomazone (technical), and two commercial formulations of the ingredient − Rampa® EC and GAT Cenit 36 CS. Preliminary tests were carried out in order to determine definitive concentrations to be evaluated (Fig. S1, supplementary material). Four concentrations of technical ingredient were preliminary tested (6.3, 25, 50, 100 mg a.i./l), and based on the mortality after treatment with the lowest tested concentration the final range was extended (definitive concentrations: 3.1, 6.3, 12.5, 25, 50, 100 mg a.i./l). The preliminary range of concentrations for the formulation Rampa® EC was the same as the definite concentrations chosen for the technical ingredient. All embryos treated with concentrations ≥25 mg a.i./l died, and preliminary testing was repeated with adjusted concentrations (1.6, 3.1, 6.2, 9.4, 12.5, 18.8 mg a.i./l, nominally). As no mortality occurred in treatments with the two lowest concentrations, and 100% mortality was observed in treatments with concentrations ≥12.5 mg a.i./l, a definite range of nominal concentrations for the formulation Rampa® EC was determined (3.1, 6.3, 7.3, 8.3, 9.4 and 12.5 mg a.i./l). The preliminary tested nominal concentrations of the formulation GAT Cenit 36 CS did not differ from the final concentration range (25, 50, 75, 100 mg a.i./l). In determination of final concentration test ranges, 100 mg a.i./l was established as a limit concentration (a concentration so high that it is unlikely to be found in the environment). All treatments and controls were tested in triplicate. Each exposure dish (80 ml crystallization dishes) contained 20 embryos and was filled with 40 ml of an appropriate dilution. Exposure was static and terminated at 120 hpf. Both the technical ingredient and the formulations were tested in two independent experiments at different times.

2.4. Toxicity evaluation

Observations of morphological endpoints, mortality and teratogenicity (all deviations from normal development) were recorded daily using a stereomicroscope. Spontaneous movements of the tail were measured in 22 hpf embryos. Each embryo movement was recorded and the number of movements over a period of 2 min counted. Embryos with delayed development of ≥4 h were marked as underdeveloped and their frequency of occurrence was recorded. At the same time, immobile embryos of regular developmental stage were counted. At the stage of 48 hpf, after the heart with regular beat had formed, the heart rate was measured. This endpoint was recorded by direct observation for 20 s. The number of hatched embryos was recorded daily, starting from 48 hpf, and hatching percentage was calculated for each replicate. The mean hatching time (HT_{50}) for each concentration was determined. Edema occurrence was recorded in 96 and 120 hpf embryos. Spine, tail tip and craniofacial deformations, as well as absence of gas bladder were evaluated in 120 hpf embryos. At the end of the experiment embryos were anesthetized using MS-222 (150 mg/l), fish were positioned on the lateral side and photographed (PROMICRA, Czech Republic). The length of each fish without the caudal fin was measured using the software QuickPhoto Micro 2.3.

2.5. Statistical analysis

Statistical analysis was conducted using Graph Pad Prism version 5 (Graph Pad Software, USA). The LC_{50} and EC_{50} were calculated from nonlinear logarithmic regression of the concentration-response curves, based on measured concentrations. In order to compare the effects of treatments on the heart rate, spontaneous tail movements and embryo length, one-way ANOVA was performed. When statistical significance was found, post hoc multiple comparisons by Dunnett's method were used to evaluate further the differences between the tested concentrations and controls. Whenever data had irregular distribution, Kruskal-Wallis ANOVA was performed, followed by Dunn's method when significant differences were found.

Fisher's exact chi-square test was used to assess the significance of teratogenic effects and mortality. Median hatching time (HT_{50}) was calculated using Graph Pad Prism software with the Hill slope model.

Two-way ANOVA (Statistica verson 7, StatSoft, USA) was performed in order to compare the effects of approximately equivalent concentrations of technical ingredient on the one hand, and formulations on the other. Transformation of data for mortality and instances of edemas, spine, tail tip and craniofacial deformations, absence of gas bladder and length reduction was performed according to formula:

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

where: X – transformed data, Xi – % of effect normalized by control and b – correction factor.

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

3. Results

Chemical analysis showed that the measured concentrations of technical clomazone and the formulation GAT Cenit 36 CS in exposure media did not differ greatly from nominal ones (< 20%), while the measured content of the active ingredient clomazone was about 25% higher in the formulation Rampa® EC. Effective concentrations and the results of LC_{50} and EC_{50} are shown in this paper as derived from the measured concentrations of active ingredient, and Tables of Results contain information on both the nominal and measured concentrations.

3.1. Clomazone technical ingredient

In embryos exposed to clomazone technical ingredient, statistically significant ($p \leq 0.01$) mortality was observed and it gradually increased with time, starting from 22 hpf (Table S1, supplementary material). The LC_{50} at the end of experiment (120 hpf, Fig. 1) was 61.4 ± 6.5 mg/l, while NOEC and LOEC for mortality were 3.4 and 6.7 mg/l, respectively (Table 1). An EC_{50} (120 hpf) of 12.1 ± 6.9 mg/l and corresponding teratogenic index (LC_{50}/EC_{50}) of 5.1 were calculated based on malformations in 120 hpf embryos (edemas, spine and tail tip deformations and gas bladder absence).

Tail movements in 22 hpf embryos are considered spontaneous since they result from development of motoneurons with no control from the central nervous system (Kimmel et al., 1995). Exposure to the clomazone technical ingredient provoked a significant reduction in spontaneous movements (Fig. 2) and increase in the number of embryos with complete absence of movements. Besides, a concentration-dependent underdevelopment was observed in immobile embryos, with peak at the highest tested concentration (95% of the surviving 22 hpf embryos) (Fig. 3). The EC_{50} for reduction in spontaneous movements and underdevelopment caused by exposure to the technical ingredient clomazone was 23.1 ± 6.2 and 46.4 ± 6.2 mg/l, respectively (Table 2). Hatching started at 48 hpf and almost all exposed embryos hatched by the end of the experiment. Significant differences from the control in hatching percentage were observed at 48, 72 and 96 hpf, and a comparison of linear regression curves revealed significantly delayed hatching after treatment with most of the tested concentrations (Table S1, supplementary material).

Clomazone technical ingredient induced bradycardia (low heart rate) in 48 hpf embryos with an observed reduction in heart rate of 22.5% at 53.5 mg/l (Fig. 4) and estimated median effective concentration (EC_{50}) of 65.3 ± 6 mg/l (Table 2).

By the end of the experiment significant instances of diverse teratogenic effects were documented (Table 3; Fig. 5). A significant

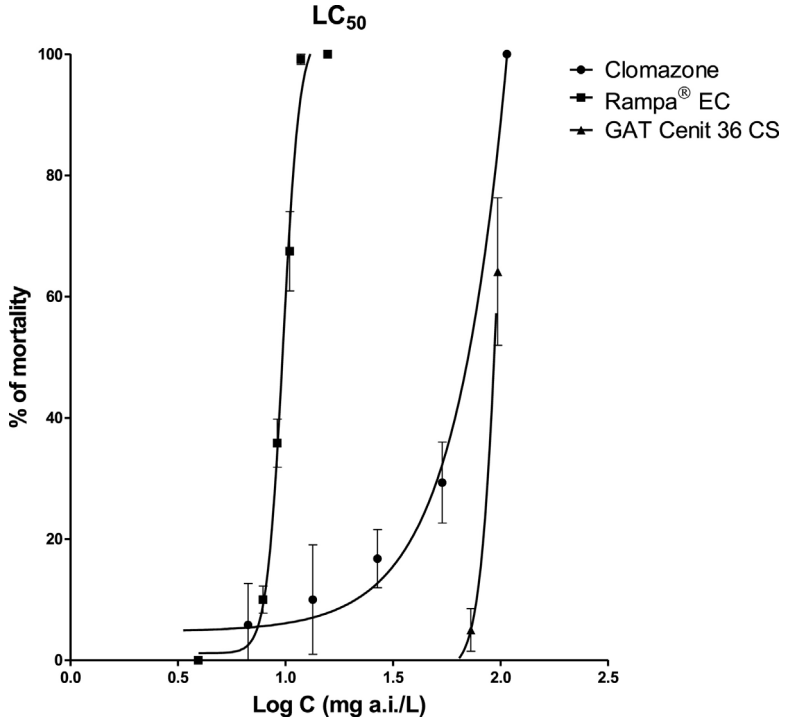

Fig. 1. Regression curves of mortality for technical clomazone and formulations Rampa® EC and GAT Cenit 36 CS. Each value represents mean and standard error.

Table 1
Median lethal concentrations (LC_{50}), median effective concentrations (EC_{50}) and their 95% confidence intervals in parenthesis, teratogenicity index (TI), no observed effect concentrations (NOEC) and lowest observed effect concentrations (LOEC) for mortality and malformations of zebrafish embryos exposed to technical clomazone and formulations Rampa® EC and GAT Cenit 36 CS.

mg a.i./l	Clomazone	Rampa® EC	GAT Cenit 36 CS
LC_{50}[a]	61.4 ± 6.5 (52.6–71.6)	9.6 ± 6.1 ** (9.5–9.8)	92.5 ± 5.0 ** (89.8–97.4)
EC_{50}[a]	12.1 ± 6.9 (9.1–16)	10.1 ± 6.1 (9.6–10.6)	24.1 ± 5.5 * (20.9–27.9)
TI	5.1	1	3.8
NOEC	3.4[a] (3.1[b])	3.9[a] (3.1[b])	72.8[a] (75[b])
LOEC	6.7[a] (6.3[b])	7.9[a] (6.3[b])	97[a] (100[b])

LC_{50}, EC_{50}, NOEC and LOEC are from two replicate experiments with standard deviation. Statistically significant difference from technical ingredient clomazone – *($p < 0.05$), **($p < 0.01$).

[a] Measured concentration.

[b] Nominal concentration.

($p < 0.01$) frequency of craniofacial disorders (head and jaw deformations) and failed formation or inflation of gas bladder was recorded starting from the lowest tested concentration with the EC_{50} of 12.8 ± 6.0 and 15.2 ± 5.9 mg/l, respectively (Table 2). Significant spine deformations were observed in 7.5% of the embryos exposed to the lowest concentration ($p < 0.05$). Increase in the number of embryos with spine deformations was notable for concentrations ≥26.8 mg/l (Table 3; $p < 0.01$). A significant ($p < 0.01$) frequency of edema (pericardial and yolk) and decrease in length was observed starting from the same treatment (26.8 mg/l). Tail tip deformations were statistically significant only after exposure to the concentration of 53.5 mg/l (Table 3).

3.2. Rampa® EC

Significant mortality was recorded starting from 22 hpf, it increased gradually with time and by the end of the experiment it was only the lowest tested concentration that caused no mortality (Table S2, supplementary material). Based on the results of nonlinear regression (Fig. 1), the LC_{50} was determined to be 9.6 ± 6.1 mg/l, while NOEC and LOEC for mortality were 3.9 and 7.9 mg/l, respectively (Table 1). A comparison of mortalities caused by comparable concentrations of the technical ingredient and formulation Rampa® EC, and LC_{50} nonlinear regression curves, revealed that the formulation led to a significantly greater mortality of embryos (Table 4) and was more toxic than the active compound alone. The EC_{50} for malformations frequency in 120 hpf embryos was 10.1 ± 6.1 mg/l (95% confidence interval 9.6–10.6) and corresponding teratogenic index (TI) 1 (Table 1). A comparison of EC_{50} nonlinear regression curves for the technical ingredient and formulation Rampa® EC, based on $LogEC_{50}$, revealed statistically insignificant differences.

The formulation Rampa® EC led to significant decrease in spontaneous movements and to underdevelopment in 22 hpf embryos (Fig. 2 and 3; Table S2, supplementary material). Comparing the exposures to technical ingredient and formulation Rampa® EC, a significantly higher impact of the formulation was revealed at comparable concentrations (Table 4). Significant differences were noted also regarding EC_{50} (Table 2), the formulation being the more toxic.

Hatching percentage was disturbed starting from 72 hpf (Table S2, supplementary material). The average heart rate was lower by about 9.3–26% for all tested concentrations (Fig. 4). The formulation Rampa® EC induced a significantly stronger reduction of the heart rate, in comparison to the active ingredient alone (Table 4; $p < 0.01$).

Edema occurrence, craniofacial disorders and lack of formation or inflation of gas bladder were noted at the end of the experiment (Table 5; Fig. 6), starting from treatment with 7.9 mg/l. After comparing the effects to those caused by technical ingredient, the formulation exhibited greater effects, except for the inflation of gas bladder at the lowest tested concentration (Table 4). Also, statistically significant tail tip and spine deformations, and reduced length were observed in 120 hpf embryos (Table 5). A comparison of median effective concentrations for teratogenic effects, relative to the technical ingredient, revealed significant differences, the formulation Rampa® EC being more toxic (Table 2).

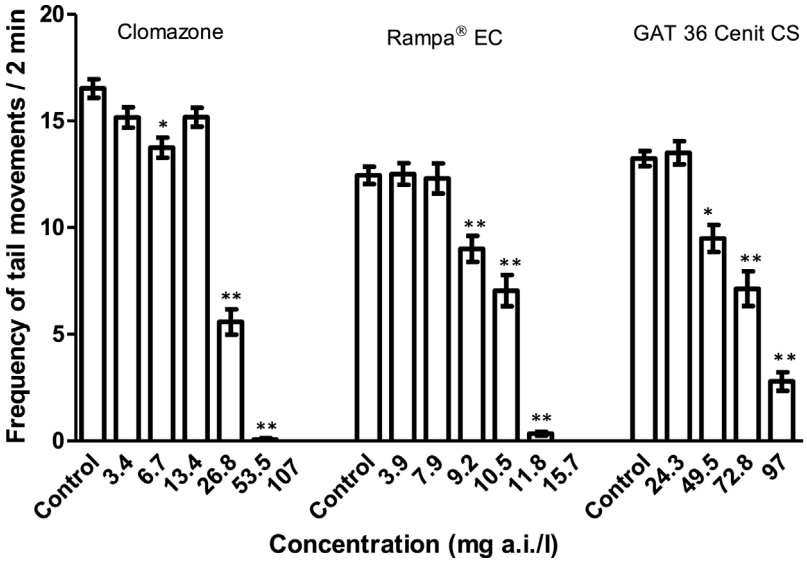

Fig. 2. Effects of different concentrations of active ingredient clomazone and formulations Rampa® EC and GAT Cenit 36 CS on spontaneous tail movements (mean and standard error) counted for 2 min in zebrafish embryos at 22 hpf.Statistically significant difference from control – $^{*}(p < 0.05)$, $^{**}(p < 0.01)$.

3.3. GAT cenit 36 CS

The formulation GAT Cenit 36 CS caused no mortality prior to 96 hpf, when a significant mortality was observed only in exposure to the highest tested concentration (Table S3, supplementary material). The LC_{50} calculated at the end of the experiment was 92.5 ± 5 mg/l (Table 1, Fig. 1), NOEC was 72.8 mg/l and LOEC 97 mg/l. The LOEC and LC_{50} were comparable, since the lower tested concentrations did not cause a statistically significant effect. The formulation elicited a lower toxicity at comparable concentrations than the active ingredient alone (Tables 1 and 4). The EC_{50} for all malformations in 120 hpf embryos was 24.1 mg/l and the corresponding TI was 3.8.

Embryos exposed to GAT Cenit 36 CS manifested a significant decrease in spontaneous movements, even complete immobility of a number of embryos, but no underdevelopment (Figs. 2 and 3; Table S3, supplementary material). The formulation also caused a significantly lower effect on the frequency of spontaneous movements than the technical ingredient (Table 4; $p < 0.01$). A significant delay in hatching was observed, but by the end of the experiment all surviving embryos have hatched (Table S3, supplementary material).

Heart rate was affected in 48 hpf embryos (Fig. 4), the observed decrease being 7.4–16.8%, but compared to the effect reported for technical ingredient the decrease was less pronounced (Table 4).

Edema frequency was significant for all tested concentrations at the end of the exposure (Table 6). The effect was generally lower in embryos exposed to GAT Cenit 36 CS in comparison to technical ingredient (Table 4).

Exposure to GAT Cenit 36 CS led to a significant frequency in craniofacial deformations and absence of gas bladder in 120 hpf embryos (Table 6; Fig. 6). Also, spine deformation was noted, and the

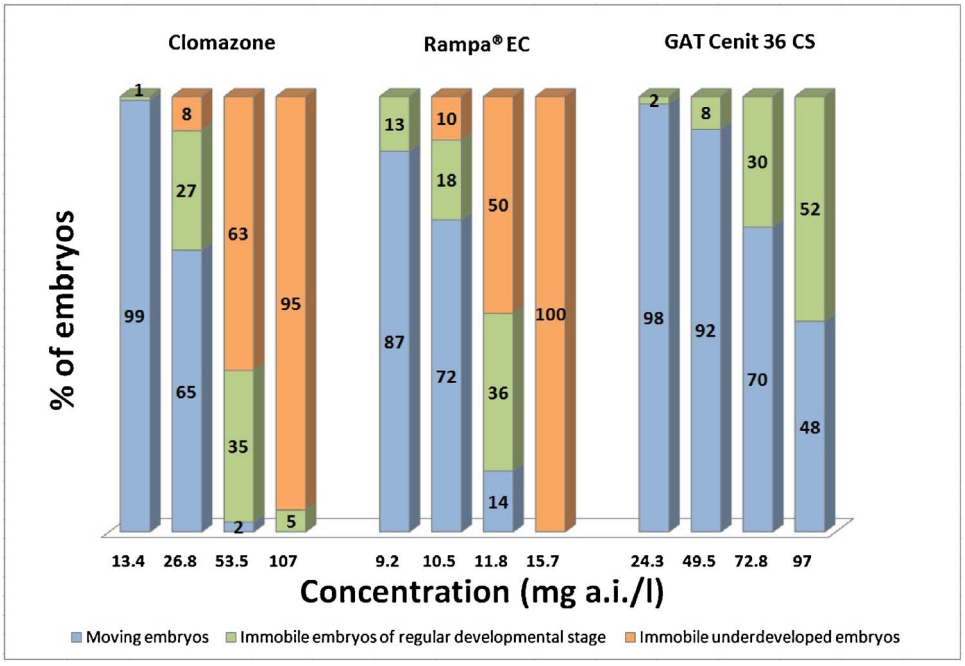

Fig. 3. Distribution (%) of 22 hpf embryos of: moving embryos of normal development, immobile embryos of regular developmental stage and immobile underdeveloped embryos, exposed to different concentrations of active ingredient clomazone and formulations Rampa® EC and GAT Cenit 36 CS.

Table 2
Medium effective concentrations (EC_{50} with standard deviation) and their 95% confidence intervals of technical ingredient clomazone and formulations Rampa® EC and GAT Cenit 36 CS on zebrafish embryos.

EC_{50} (mg a.i./l)	Technical ingredient clomazone	Formulation Rampa® EC	Formulation GAT Cenit 36 CS
Spontaneous movements	23.1 ± 6.2 (20.4–26.1)	10.3 ± 6.1* (9.9–10.7)	71.9 ± 5.2* (63.7–81.2)
Underdevelopment	46.4 ± 6.2 (41.3–52.2)	11.8 ± 6.0* (11.6–11.9)	> 100*
Heart beat	65.3 ± 6.0 (59.8–71.2)	10.7 ± 5.7* (10.3–11.1)	> 100* (67.5–726.0)
Edemas	27.2 ± 5.6 (25.8–28.7)	9.5 ± 5.1* (9.0–10.0)	38.4 ± 5.1* (35.5–41.4)
Spine deformation	38.9 ± 5.8 (34.1–44.3)	10.8 ± 5.2* (9.9–11.8)	90.7 ± 5.0* (86.0–95.7)
Craniofacial deformation	12.8 ± 6.0 (10.7–15.4)	8.2 ± 5.1* (7.8–8.7)	53.0 ± 5.1* (49.3–56.9)
Absence of gas bladder	15.2 ± 5.9 (12.9–18.1)	10.4 ± 5.1* (9.8–11.0)	24.2 (not covered)

Statistically significant difference from technical ingredient – *($p < 0.01$).

length of surviving embryos was significantly reduced (Table 6; $p < 0.01$). A comparison of teratogenic effects revealed lower toxicity of formulation GAT Cenit 36 CS (Tables 2 and 4).

4. Discussion

Studies on clomazone toxicity to fish have been reported since 2004 (Dos Santos Miron et al., 2004, 2005; Crestani et al., 2007; Cattaneo et al., 2012; Pereira et al., 2013), but they are limited only to the effects of clomazone on adult fish. There are neither data on embryotoxicity of the active ingredient nor on the toxicity of formulated products. The novelty of this investigation is also in comparing the toxicity, both mortality and sublethal effects, of different types of formulations and the technical substance.

In our experiments, we noted that the technical clomazone 120 h LC_{50} in zebrafish embryos was in a similar range as the values in previous studies. The differences could be attributed to interspecies variations and different life stages (adults vs. embryos). Although early life stages are usually the most sensitive in fish development, some findings suggest that the chorion might reduce incorporation of some toxicants into the embryo (Strmac and Braunbeck, 1999; Pamanji et al., 2015). Interestingly, both types of formulations tested in this study showed toxicological properties that differed from the technical ingredient. The formulation Rampa® EC exhibited higher and GAT Cenit 36 CS lower toxicity. Although the applied concentrations of active ingredient in both formulations were the same, lower toxicity of GAT Cenit 36 CS can mainly be attributed to the specificity of that type of formulation. Namely, gradual-release formulations are not usually less effective to target organisms, but could notably reduce negative impacts on non-target organisms by lowering exposure concentration. It is important to emphasize that the LOECs for mortality for the technical clomazone and formulation Rampa® EC observed in our study were within the same order of magnitude as the environmentally detected levels (Hug et al., 2014).

A developing nervous system can be affected during chemical exposure. Significant reduction in the number of spontaneous tail movements of zebrafish has been observed in investigations involving different herbicides (Lin et al., 2013; Wiegand et al., 2001). A similar trend of decrease in locomotor activity was noticed in our study with clomazone, and a difference was revealed in the intensity of effect between the technical ingredient and the formulated products. In contrast to the effects on zebrafish caused by insecticides that interfere with the nervous system (Fraysse et al., 2006; Jin et al., 2009), the mode of action of clomazone on spontaneous movements of 22 hpf zebrafish embryos is unclear. However, some investigators have discovered that clomazone interferes with AChE by lowering its activity in brain tissue (dos Santos Miron et al., 2005; Crestani et al., 2007; Moraes et al., 2007; dos Santos Miron et al., 2008; Pereira et al., 2013; Murussi et al., 2015), and these findings are consistent with the interference with motoneurons development and reduction in spontaneous movements that was observed in our study.

Underdevelopment was observed in embryos exposed to technical clomazone and the formulation Rampa® EC, while no underdevelopment was noted under exposure to GAT Cenit 36 CS. These differences are assumed to stem from the specificity of microencapsulation formulation. It is not clear why the underdevelopment occurred but certain assumptions should be considered. Namely, clomazone inhibits the synthesis of isopentenyl pyrophosphate (IPP), a substance that is precursor for a great number of biologically active substances, such as cholesterol, terpenoids, steroid hormones, coenzyme Q, etc. Inhibition of IPP synthesis by clomazone in plants is provoked in the methylerythrol 4-phosphate (MEP) pathway (non-mevalonate), while mevalonate pathway is undisturbed (Ferhatoglu and Barrett, 2006). The MEP pathway does not exist in animal cells, so a potential disruption of isoprenoids in fish is unlikely to be the target point. On the other hand, clomazone inhibits deoxyxylulose 5-phosphate (DXP) synthase in the MEP pathway, preventing a reaction between pyruvate and glyceraldehyde 3-phosphate (G3P). Both pyruvate and G3P are intermediates in several metabolic pathways, engaged in the citric acid cycle (cell respiration) and pentose phosphate pathway. Pyruvate is also essential

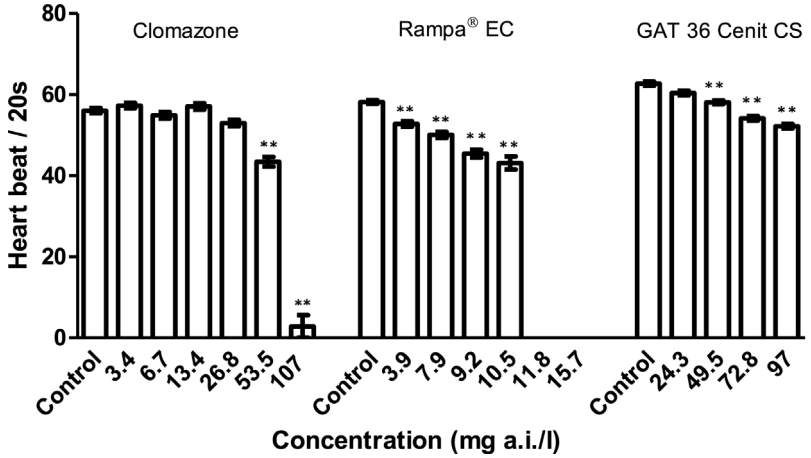

Fig. 4. Heart beat rate of 48 h old zebrafish embryos (mean number of heart beats per 20 s with corresponding standard error) exposed to different concentrations of active ingredient clomazone and formulations Rampa® EC and GAT Cenit 36 CS.Statistically significant difference from control – *($p < 0.05$), **($p < 0.01$)

Table 3
Frequency of toxic effects (in %) observed in 120 hpf zebrafish embryos after exposure to technical ingredient clomazone.

Endpoint	Control	Concentration (mg ai/l)					
		3.1[a] 3.4[b]	6.3[a] 6.7[b]	12.5[a] 13.4[b]	25[a] 26.8[b]	50[a] 53.5[b]	100[a] 107[b]
edemas	1.7 ± 3	3.3 ± 3.8	1.9 ± 1.7	5.6 ± 7.6	48.4 ± 3.2**	95.2 ± 2.3**	–
spine deformation	0.8 ± 1.5	7.5 ± 9*	1.9 ± 1.7	2.9 ± 0.6	24.2 ± 10.7**	71.4 ± 18.6**	–
craniofacial deformation	1.7 ± 1.5	12.7 ± 6.8**	27.7 ± 15.4**	48.9 ± 11.2**	76.9 ± 9**	96.4 ± 3.7**	–
absence of gas bladder	2.5 ± 2.5	27.9 ± 21.9**	23.5 ± 7.3**	34.4 ± 14**	97 ± 0.2**	96.4 ± 3.7**	–
tail tip deformation	0 ± 0	0 ± 0	0.8 ± 1.4	0 ± 0	2 ± 3.5	14.4 ± 6.8**	–
length (μm)[c]	3836 ± 194	3862 ± 168	3861 ± 87	3803 ± 185	3646 ± 365**	3387 ± 297**	–

Frequency of effects for malformations from two replicate experiments (60 embryos each) with standard deviation.
Statistically significant difference from control – *($p < 0.05$), **($p < 0.01$) and – not assessed due to mortality.
[a] Nominally applied concentration.
[b] Measured applied concentration.
[c] Length of embryos (μm) shown as an average length and standard deviation from two independent experiments.

in alanine, fatty acid and carbohydrate metabolism. Under aerobic conditions, pyruvate is converted either to acetyl-coenzyme A or to oxaloacetate, but under anaerobic conditions in fermentation processes it can be converted to lactate (Dashty, 2013). Becker et al. (2009) found that pesticide mixtures containing clomazone affected the metabolism of silver catfish (*Rhamdia quelen*), resulting, among other effects, in elevated lactate levels in muscles. This could be an indicator that our hypothesis of clomazone targeting and disrupting pyruvate metabolism is plausible. If pyruvate metabolism is disrupted, a major metabolic pathway would be disrupted, and embryo development consequently delayed or interrupted.

Diverse teratogenic effects (gas bladder, craniofacial, spine and tail tip deformations) observed at the end of our study, after exposure to technical clomazone and two formulations, could be the result of the same potential mode of action in zebrafish. The swim bladder is necessary for normal locomotor activity and buoyancy (Robertson et al., 2007). Defects in gas bladder formation may affect food capture and predator escape abilities (Li et al., 2011). The combination of uninflated swim bladder and craniofacial malformations can indirectly cause death of zebrafish embryos from starvation or predators.

Developing organisms normally require a fast heart rate in order to support metabolic activity. Interference with the cardiovascular system by lowering heart rate can lead to many health problems (Singleman and Holtzman, 2012; Watson et al., 2014). The reduction in heart rate caused by organophosphate insecticides (chlorpyrifos, dichlorvos and monocrotophos) was considered a consequence of possible continuous signals from the acetylcholine receptor (Pamanji et al., 2015; Watson et al., 2014). Clomazone interference with AChE activity in muscle tissues has been reported (dos Santos Miron et al., 2005; Crestani et al., 2007; Moraes et al., 2007), and the reduction in heart rate observed in our study could be potentially a consequence of that obstruction. Yamauchi et al. (2005) reported that malformations of the heart and pericardium may affect cardiac function, resulting in abnormal heart beat and blood circulation failure.

Besides the effects on heart rate, additional measurements of cardiovascular toxicity include morphological abnormalities and edemas (Watson et al., 2014). Edemas were observed also in our study, and the data are consistent with our general observation that negative impacts gradually increased in the following order: GAT Cenit 36 CS – technical ingredient – Rampa® EC.

Exposure to technical clomazone and the formulations caused delays in hatching when compared to the controls. As indicated by other authors, a delay in hatching could occur due to an impairment of the enzyme responsible for digestion of the chorion in the normal hatching process. Disturbance in proteolytic enzyme activity resulting from interference with osmosis could lead to the weakening of spontaneous movements, which may then further delay hatching (Strmac et al., 2002; Haendel et al., 2004; Pandey and Guo, 2014). The developmental progress in embryos is not conditioned by hatching time, and embryos hatched earlier are not by default more developed than those remaining in the chorion (Kimmel et al., 1995). Even though the impact of clomazone on hatching percentage was not significant at the end of the experiment, a delay in hatching makes embryos unhatched for a longer period a more accessible pray than hatched

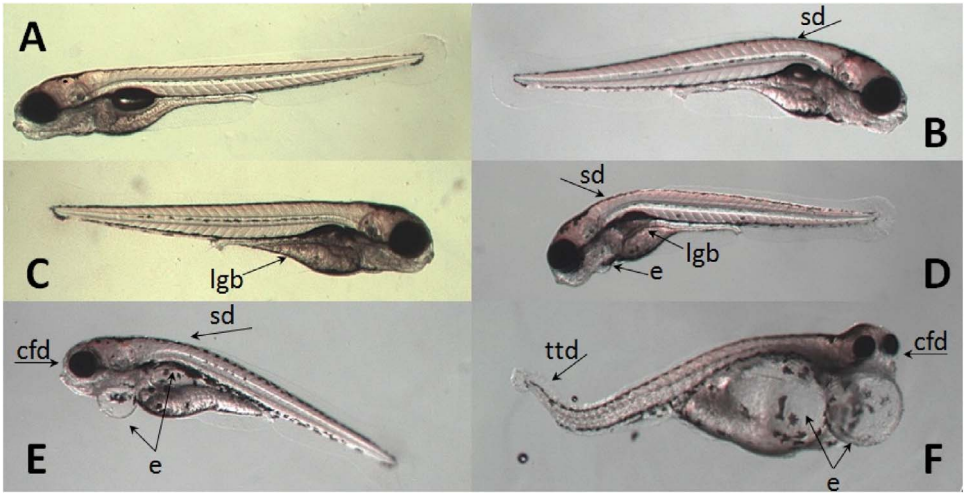

Fig. 5. Comparison of zebrafish embryos (120 hpf) from control (A) and exposure to active ingredient clomazone: 3.4 mg/l (B), 6.7 mg/l (C), 13.4 mg/l (D), 26.8 mg/l (E) and 53.5 mg/l (F). Arrows and abbreviations indicate edema (e), lack in gas bladder formation (lgb), craniofacial deformations (cfd), tail tip (ttd) and spine deformations (sd).

Table 4
Comparison of differences in the effects of comparable applied concentrations of clomazone technical ingredient and formulations Rampa® EC and GAT Cenit 36 CS on zebrafish embryos.

Concentration (mg a.i./l)		Technical ingredient clomazone					
	Nominal	3.1	6.3	12.5	25	50	100
	Measured	3.4	6.7	13.4	26.8	53.5	107
		Formulation Rampa® EC			Formulation GAT Cenit 36 CS		
	Nominal	3.1	6.3	9.4	25	50	100
	Measured	3.9	7.9	11.8	24.6	49.5	97
Endpoint	**Time (hpf)**						
Mortality	22	ns	ns	ns	↓ (**)	↓ (**)	↓ (**)
	48	ns	ns	↑ (*)	↓ (**)	↓ (**)	↓ (**)
	72	ns	ns	↑ (**)	↓ (**)	↓ (**)	↓ (**)
	96	ns	ns	↑ (**)	↓ (**)	↓ (**)	↓ (**)
	120	ns	ns	↑ (**)	↓ (**)	↓ (**)	↓ (**)
Underdeveloped	22	ns	ns	↑ (**)	↓ (*)	↓ (**)	↓ (**)
Spontaneous movements	22	ns	ns	↑ (**)	↓ (**)	↓ (**)	ns
Heart beat	48	↑ (**)	↑ (**)	↑ (**)	ns	↓ (**)	↓ (**)
Edemas	96	ns	ns	↑ (**)	↓ (**)	↓ (**)	-
	120	ns	↑ (**)	↑ (**)	↓ (**)	ns	-
Spine deformation	120	ns	ns	↑ (**)	↓ (**)	↓ (**)	-
Craniofacial deformation	120	ns	ns	↑ (*)	↓ (**)	↓ (**)	-
Absence of gas bladder	120	↓ (*)	ns	↑ (**)	↓ (**)	ns	-
Tail tip deformations	120	ns	ns	ns	ns	↓ (**)	-
Length	120	ns	ns	-	↓ (**)	↓ (**)	-

↑ – statistically significantly greater effect of formulation compared with corresponding concentration of technical ingredient – *($p < 0.05$), **($p < 0.01$), ↓ – statistically significantly lower effect of formulation, compared with corresponding concentration of technical ingredient, ns – statistically not significant difference, compared with corresponding concentration of technical ingredient, and "–" not assessed due to mortality.

Table 5
Frequency of toxic effects (in %) observed in different developmental stages of zebrafish embryos during the exposure to formulation – Rampa® EC.

Endpoint	Control	Concentration (mg ai/l)					
		3.1[a] 3.9[b]	6.3[a] 7.9[b]	7.3[a] 9.2[b]	8.3[a] 10.5[b]	9.4[a] 11.8[b]	12.5[a] 15.7[b]
edemas	1.7 ± 1.4	0.8 ± 1.4	16.6 ± 7**	50.1 ± 9.5**	64.6 ± 27.3**	100 ± 0*	–
spine deformation	0.8 ± 1.4	0 ± 0	0 ± 0	10.3 ± 4**	17.4 ± 6.5**	100 ± 0*	–
craniofacial deformation	14.2 ± 6.3	22.5 ± 6.6	47 ± 14**	81.6 ± 6.3**	90 ± 10**	100 ± 0	–
absence of gas bladder	1.7 ± 1.4	0.8 ± 1.4	12.9 ± 3.9**	35.9 ± 9.2**	45.2 ± 13.4**	100 ± 0*	–
tail tip deformation	0 ± 0	0 ± 0	0 ± 0	5.2 ± 2*	6.7 ± 11.5	0 ± 0	–
length (μm)[c]	3819 ± 19	3800 ± 105	3793 ± 151	3709 ± 250**	3697 ± 327	–	–

Frequency of effects for malformations from two replicate experiments (60 embryos each) with standard deviation.
Statistically significant difference from control – *($p < 0.05$), **($p < 0.01$) and – not assessed due to mortality.
[a] Nominally applied concentration of technical ingredient clomazone.
[b] Measured applied concentration of technical ingredient clomazone.
[c] Length of embryos (μm) shown as an average length and standard deviation from two independent experiments.

embryos.

Shorter embryo length was observed at higher concentrations of technical clomazone and the formulations. Some authors emphasize growth as an easily measured endpoint that can reflect many molecular and cellular responses. Also, decrease in body size at certain developmental stages may indicate a reduction in individual fitness (Newman and Unger, 2003; Cook et al., 2005). Decrease of length of the exposed embryos could be the result of potentially disrupted metabolic processes, interference with amino acid synthesis or carbohydrate metabolism.

To summarize the results of this study, the formulated products Rampa® EC and GAT Cenit 36 CS showed toxicological properties that differed from the technical ingredient clomazone. Overall, the formulation Rampa® EC exhibited higher acute toxicity, while GAT Cenit 36 CS had lower acute toxicity to zebrafish embryos than technical clomazone. Differences in the toxicological properties of formulated products had been reported in some earlier studies. Beggel et al. (2010) found that formulated products exhibited higher toxicity than technical bifenthrin and fipronil on fathead minnow (*Pimephales promelas*). Also, Jarvinen and Tanner (1982) highlighted that encapsulated formulations of methyl parathion and diazinone had less acute toxicity to fathead minnow, but caused chronic exposure problems. In a study with green frog (*Rana clamitans*), Howe et al. (2004) examined the toxicity of technical glyphosate, polyethoxylated tallowamine surfactant (POEA) and 5 formulations containing glyphosate. Their results indicated the greatest toxic potency of POEA, followed by two formulations, while the technical ingredient and three formulations showed no signs of acute toxicity. Puglis and Boone (2011) confirmed a lower toxicity of the technical ingredient glyphosate than Roundup formulation for *R. clamitans*. In a study with three human cell lines, 8 out of 9 formulations were 1000-times more toxic than their corresponding active substances (glyphosate, isoproturon, fluroxypyr, pirimicarb, imidacloprid, acetamiprid, tebuconazole, epoxiconazole and prochloraz) (Mesnage et al., 2014). All authors emphasized the importance of determining the

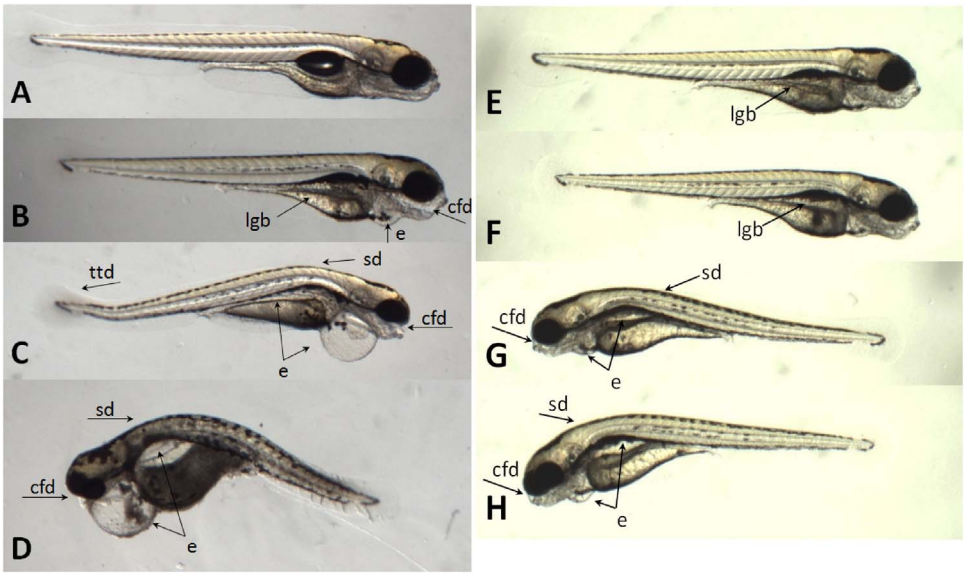

Fig. 6. Comparison of zebrafish embryos (120 hpf) from control (A) and exposure to clomazone formulation Rampa® EC: 7.9 mg/l (B), 9.2 mg/l (C) and 10.5 mg/l (D) and GAT Cenit 36 CS: 24.3 mg/l (E), 49.5 mg/l (F), 72.8 mg/l (G) and 97 mg/l (H). Arrows and abbreviations indicate edema (e), lack in gas bladder formation (lgb), craniofacial deformations (cfd), tail tip (ttd) and spine deformations (sd).

Table 6
Frequency of toxic effects (in %) observed in different developmental stages of zebrafish embryos during the exposure to formulation − GAT Cenit 36 CS.

Endpoint	Control	Concentration (mg ai/L)			
		25[a] 24.3[b]	50[a] 49.5[b]	75[a] 72.8[b]	100[a] 97[b]
edemas	0 ± 0	8.3 ± 8.8**	79.2 ± 8**	96.3 ± 1.8**	100 ± 0**
spine deformation	0 ± 0	0.8 ± 1.4	0 ± 0	15.3 ± 1**	61.9 ± 16.9**
craniofacial deformation	6.8 ± 3	19.2 ± 8.8**	36.7 ± 9.5**	97.3 ± 0.1**	100 ± 0**
absence of gas bladder	1.7 ± 2.9	52.5 ± 17.3**	100 ± 0**	100 ± 0**	100 ± 0**
tail tip deformation	0 ± 0	2.5 ± 2.5	0 ± 0	0.9 ± 1.5	0 ± 0
length (μm)[c]	3813 ± 188.9	3743 ± 211	3725 ± 133**	3534 ± 148**	3346 ± 127**

Frequency of effects for malformations from two replicate experiments (60 embryos each) with standard deviation.
Statistically significant difference from control – *($p < 0.05$), **($p < 0.01$) and – not assessed due to mortality.
[a] Nominally applied concentration of technical ingredient clomazone.
[b] Measured applied concentration of technical ingredient clomazone.
[c] Length of embryos (μm) shown as an average length and standard deviation from two independent experiments.

toxicity of formulated products or inert ingredients in those products.

5. Conclusion

In conclusion, our investigation is the first report on clomazone embryotoxicity to zebrafish embryos, and also on its sublethal effects, i.e. morphological and developmental impairments. Spontaneous movements, heart rate, hatching, length and the incidence of deformities were found to be sensitive indicators of the toxicity of clomazone and two formulations during early stages of zebrafish development. We demonstrated that toxicity, both mortality and sublethal effects, differs between the technical ingredient and formulated products, and those differences occur at environmentally relevant levels. The higher toxicity of the EC formulation may be a result of enhanced uptake of the active ingredient because the solvent and surfactant (from the product) can enhance its mobility in the aquatic environment, while it was lower in the case of CS formulation due to the controlled release of the active ingredient from it. These facts mean that careful choice of formulations is very important for environmental protection purposes. In the end, the present study shows that risk assessment based only on active (technical) ingredient toxicity might not be accurate and revealing the true risk that formulated products pose to the environment and non-target organisms.

Further research is needed in order to characterize possible differences in the uptake of technical ingredient and formulations during embryonic development of zebrafish. Since the mode of action of clomazone in fish is unknown, an effort should be made to reveal possible sites of action, and an investigation of interference with pyruvate metabolism or cellular respiration enzymes could be a starting point.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2017.04.007.

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