Proteotoxicity and Apical Toxicity of Nicosulfuron to *Danio rerio* Embryos: A Comprehensive Assessment at Different Temperatures and pH

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Abstract: In the present study, the toxicity of nicosulfuron to *Danio rerio* embryos was evaluated in three experiments through standardized toxicity tests according to OECD TG236 guidelines. In the first experiment, six concentrations of nicosulfuron (0, 0.1, 1, 10, 100, 1000 mg/L) were tested under optimal conditions (26 °C, pH 7.0) to assess the general sensitivity of zebrafish embryos to nicosulfuron. The second and third experiment examined the effects of different pH levels (5.0 and 9.0) and temperatures (21 °C and 31 °C) on the toxicity at four nicosulfuron concentrations (0, 10, 100, 1000 mg/L). Additionally, the sub-organismic effects of nicosulfuron on stress protein levels (Hsp70) of fish embryos were analyzed. Throughout the embryo experiments, no malformations were observed in all experiments. The survival rate exceeded 80% in all groups except for the 21 °C (pH 7.0) treatment groups. No significant effect of nicosulfuron on the survival rate was found at the same temperature or pH (p > 0.05). No significant difference in the heart rate was found among all nicosulfuron groups (p > 0.05) at 21 °C. The heart rate of fish embryos at 31 °C, pH 5.0 and pH 9.0 increased with nicosulfuron concentrations. Except for the pH 5.0 (26 °C) and 21 °C (pH 7.0) treatment groups, nicosulfuron was found to increase the hatching rate of embryos in other treatments; however, the corresponding times of action were different. At 21 °C (pH 7.0), the embryos did not hatch until 144 h post-fertilization. In terms of proteotoxicity, nicosulfuron was found to be more toxic to zebrafish embryos in the 21 °C, pH 5.0 and pH 9.0 treatment groups. However, at 31 °C, no significant difference in Hsp70 levels was found among all the different nicosulfuron concentrations (p > 0.05). Our results show that nicosulfuron exerts a weak toxicity to zebrafish embryos; however, this toxicity is amplified by inappropriate pH or temperature conditions.

Keywords: herbicide; fish; stress protein; confounding factor

1. Introduction

Pesticides have become an important issue of concern in terms of their negative impact on terrestrial and aquatic ecosystems [1–3]. Notably, herbicides and fungicides are the most economically significant [4,5]. Nicosulfuron is a sulfonylurea class herbicide used globally as a post-emergence herbicide to protect, e.g., maize crops from weeds [6]. It inhibits the activity of acetolactate synthase, a key enzyme in the biosynthesis of branched-chain amino acids [7–9], resulting in the inhibition of plant growth. According to the Joint Research Centre, nicosulfuron is considered a high-risk chemical for aquatic wildlife [10]. Environmental concentrations of nicosulfuron found in various surface waters from Canada, the United States and Europe average 0.03–0.5 µg/L [10–13], with the highest detected amount peaking at 16 µg/L [10].

At present, knowledge on the toxicity of nicosulfuron to non-target organisms is scarce and debated. Nicosulfuron has been shown to induce embryotoxic and oxidative stress in amphibian (*Bufo spinosus*) embryos [14]. It also causes abnormal behavior in goldfish and increases the acetylcholinesterase activity in goldfish and earthworms [7,15,16]. On the
contrary, it has been suggested that nicosulfuron has little effect on mammals, birds or amphibians [9]. Therefore, it is necessary to evaluate the toxicity of nicosulfuron on non-target organisms in order to obtain more data to grasp the full toxic potential of nicosulfuron. In addition, we would like to give more consideration to the influence of environmental factors on the toxicity of nicosulfuron, as the interaction between environmental factors and toxicants directly or indirectly affects the bioavailability and bio-susceptibility of the chemical, and thus its toxicity [17–19]. For chemical hazards to aquatic organisms, temperature and pH are two of the most important environmental factors [17]. However, the importance of environmental factors is often overlooked in the derivation of environmental quality standards or in toxicity assessments [20,21]. Therefore, a comprehensive evaluation of nicosulfuron toxicity encompassing the effects of pH and temperature is necessary.

Zebrafish (Danio rerio) is a widely used model species in biological research [22–24] and tests with its early life stages are well established as tools in environmental risk assessment. These Early Life Stage (ELS) tests are cost-effective, quick to perform and are not classified as animal experiments under the Animal Welfare Act [24,25]. In addition, the transparency of the eggshell allows the assessment of embryotoxic effects, such as malformations [24,26]. The tests comply with the requirements of the Animal Welfare Act with regard to the principles of replacement, reduction and refinement [24,25]. In 2005, Germany replaced the traditional adult fish toxicity test with the standardized zebrafish embryo test [24,27], and in 2013, the zebrafish embryo test was adopted by the OECD to replace the acute fish toxicity test [28].

Therefore, to assess the potential toxicity of nicosulfuron to non-target organisms and the effect of environmental factors on the toxicity of nicosulfuron, we investigated the sensitivity of zebrafish embryos to 0–1000 mg/L nicosulfuron and compared the results with those of tests conducted at different temperatures (21 °C and 31 °C) and pH levels (5.0 and 9.0). In addition to apical endpoints, we also investigated the stress protein Hsp70 in exposed zebrafish embryos as a sublethal endpoint of toxicity and as a biomarker for proteotoxicity.

2. Materials and Methods
2.1. Test Compound

Nicosulfuron is a rather hydrophilic organic ionizable compound, the hydrophilicity of which is increased at pH 5.0 and pH 9.0. Relevant physicochemical information is given in Table 1.

**Table 1.** Physicochemical properties of nicosulfuron.

<table>
<thead>
<tr>
<th>Properties</th>
<th>C_{15}H_{18}N_{6}O_{6}S</th>
<th>410.4 g/mol</th>
<th>7400 mg/L</th>
<th>0.35</th>
<th>1.8</th>
<th>-2.0</th>
<th>4.60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Formula</td>
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<td></td>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Solubility in water</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>log P_{ow} (at pH 5)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>log P_{ow} (at pH 7)</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>log P_{ow} (at pH 9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>pK_a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: Data are obtained according to Pubchem (NIH, National library of Medicine).

Nicosulfuron (CAS111991-09-4, purity > 98.0%) purchased from Sigma-Aldrich (Merck, Darmstadt, Germany) was dissolved in reconstituted water consisting of 25 mL each of 0.23 g/L KCl, 2.59 g/L NaHCO_3, 4.93 g/L MgSO_4·7 H_2O and 11.76 g/L CaCl_2·2 H_2O and 900 mL double distilled water.

An initial stock solution (1000 mg/L, pH 7.0) was prepared and then other concentrations of nicosulfuron were obtained using the stepwise dilution of the stock solution. To obtain solutions of pH 5.0, pH 7.0 and pH 9.0, the pH was adjusted by adding 1M HCl.
or NaOH. The pH was measured using a pH meter (SevenCompactDuo; Mettler Toledo, Gießen, Germany).

Nicosulfuron concentrations used in the present study were selected based on toxicity data provided by EFSA (2007) [9].

### 2.2. Fish Maintenance

Adult zebrafish (USA Westaquarium strain) were kept at the Animal Physiological Ecology, University of Tübingen in 200 L tanks filled with filtered water at a dark/light regime of 12 h/12 h. Fish were fed dry flake food (TertraMin®; Tetra GmbH, Melle, Germany) three times a day. Feces were removed daily before feeding and 30–50% of the water was changed every two weeks. All animal procedures were approved by the Animal Welfare Committee of the Regional Council of Tuebingen, Germany (approval number ZO 2/16 and ZO 02/21 G).

Water parameters were maintained consistently: water temperature 26 ± 1 °C, pH 7.4 ± 0.2, total hardness 8–12° dH and oxygen saturation 100% ± 5%. Nitrate and nitrite concentrations never exceeded critical levels of 0.025–1 mg/L and 1–5 mg/L, respectively.

### 2.3. Fish Embryo Test

Three tests were carried out to achieve our objective (Figure 1). Test 1: Six concentrations of nicosulfuron (0, 0.1, 1, 10, 100, 1000 mg nicosulfuron/L) were set at 26 °C, pH 7.0, to investigate the sensitivity of embryos to nicosulfuron. As there was no apparent effect of 0.1 and 1 mg/L nicosulfuron in test 1, we decided to investigate only the other concentrations of nicosulfuron (0, 10, 100, 1000 mg/L) in subsequent tests. Test 2: This test was carried out to determine the effect of nicosulfuron on embryos at pH 5.0 and pH 9.0 (with a maintained temperature at 26 °C). Test 3: This test was performed to determine the effect of nicosulfuron on embryos at 21 °C and 31 °C (with a maintained pH at 7.0).

![Figure 1. Flow chart of the embryo test. Note: The different exposure times depended on the hatching of zebrafish embryos. For those that remained unhatched for more than 96 h, the exposure time was extended by 48 h.](image-url)
The ELS tests were performed based on OECD 236, modified according to the protocol of Schweizer et al. (2019, 2022) [23,29]. The day before collecting eggs, the spawning device was set up and the test dish was soaked. To induce spawning activity, a day before the start of the experiments, the breeding boxes covered with a grid (mesh size of 1.5 mm) and artificial sea grass was placed at the bottom of the aquaria. For the concentrations of nicosulfuron per test, eight small Petri dishes (30 mm) were filled with 3 mL and one large Petri dish (90 mm) with 30 mL of the respective test solutions, in order to guarantee the saturation of the Petri dishes with the test substance. Before the start of each experiment, these test solutions were replaced with fresh test solutions.

Spawning occurred in the time just after the onset of light. Freshly laid eggs were collected 1 h after onset light the following morning. To ensure uniform exposure from the beginning of embryonic development, the eggs were quickly transferred to the large Petri dishes for the pre-exposure of the embryos. The embryos were kept in the pre-exposure Petri dishes at 26 °C for 2 h (in test 3, the embryos were kept at the appropriate test temperature). Well-developed eggs (128–256 cell stage, according to Kimmel et al., 1995) were then randomly selected and transferred into small Petri dishes (8 eggs per dish, a total of 32 individuals per group). The embryos in the small Petri dishes were kept at 26 °C in a heating cabinet for 96 h (in test 3, the embryos were kept at the appropriate temperature).

Embryotoxicity endpoints, i.e., survival rate, developmental delays, heart rate, hatching success and malformations were examined under a stereomicroscope at 12, 24, 48, 60, 72 and 96 h post-fertilization (hpf). The specific endpoints observed at each time point are listed in Table 2. During the study, coagulated embryos were recorded and subsequently removed. The heart rate of the embryos was counted for 20 s and extrapolated to 1 min. The hatching rate was recorded between 60 and 96 hpf. Considering the effect of temperature on hatching rate, the observation time of hatching rate at 21 °C was extended to 144 hpf, and the observation time at 31 °C was advanced to 48 hpf.

<table>
<thead>
<tr>
<th>Endpoints</th>
<th>12 hpf</th>
<th>24 hpf</th>
<th>48 hpf</th>
<th>60 hpf</th>
<th>72 hpf</th>
<th>96 hpf</th>
</tr>
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<tr>
<td>Mortality</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
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<tr>
<td>Developmental Delays¹</td>
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<td></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>Heart rate</td>
<td></td>
<td></td>
<td></td>
<td>✓</td>
<td></td>
<td></td>
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<tr>
<td>Hatching success</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
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<tr>
<td>Malformations²</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>✓</td>
</tr>
</tbody>
</table>

Note: ¹ No somites, non-detachment of the tail and no development of the eyes; ² edema, eye defects, deformation of the spine and light pigmentation.

2.4. Hsp70 Quantification in Zebrafish Embryos

For Hsp70 quantification, 50 freshly laid zebrafish eggs per group were placed in a dish. The embryos were incubated in a heating cabinet and 10 individuals were pooled into one Eppendorf tube after incubating for 96 h, and the water that had accumulated at the bottom of the tube was removed as thoroughly as possible. The samples were immediately frozen in liquid nitrogen and stored at −80 °C for further testing.

Hsp70 quantification was performed according to the method of Vincze et al., 2014 [30]. In brief, pooled larvae from each tube were homogenized in 20 µL extraction buffer (80 mM potassium acetate, 4 mM magnesium acetate, 20 mM Hepes pH 7.5 (Sigma Aldrich, Deisenhofen, Germany)). After centrifugation (10 min, 20,000 × g, 4 °C), the supernatant was collected. The total protein concentration was determined according to Bradford 1976 [31]. For each sample, 20 µg total protein was subjected to SDS-PAGE for protein separation. Electrophoresis was performed at 80 V for 30 min followed by 120 V for approximately 90 min. Immunostaining with an antibody complex was then performed. After the digitalization of the filters, the gray value intensity of the Hsp70 bands was measured using densitometric image analysis (Herolab E.A.S.Y., Wiesloch, Germany). Sample Hsp70 levels
were normalized to the mean of the two internal standards (made from adult Danio) of the corresponding filters.

2.5. Statistic Analysis

Statistical analysis was performed using SAS JMP program version 16.0. The results were presented as means ± standard error. The data were assessed for normal distribution and homogeneity of variances. ANOVA was then performed using Tukey’s HSD. Data with non-normal distributions or non-homogeneous variances were analyzed using the non-parametric Steel–Dwass method.

3. Results

No malformations were observed during embryonic development in all tests and all groups.

There was no significant difference ($p > 0.05$) in the survival rate among the different nicosulfuron groups at the same pH (Figure S1).

All the 21 °C groups showed a significantly lower survival rate compared to the 26 °C and 31 °C groups ($p < 0.05$). There was no significant difference ($p > 0.05$) in survival rate among the different nicosulfuron groups at the same temperature (Figure S2).

Figures 2 and 3 show the heart rate of zebrafish embryos at 48 hpf. At both pH 5.0 and pH 9.0, the heart rate of zebrafish embryos increased with nicosulfuron concentration. The heart rate of the 1000 mg/L group was significantly higher ($p < 0.05$) than that of the 0 and 10 mg/L groups at both pH 5.0 and pH 9.0 (Figure 2). At pH 7.0, there was no significant difference in heart rate among any of the groups ($p > 0.05$).

![Heart rate graph](image)

**Figure 2.** The effect of nicosulfuron on the heart rate of zebrafish embryos at different pH. Note: Values are mean ± SE. Different letters indicate a significant difference ($p < 0.05$).

The heart rate of zebrafish embryos increased with nicosulfuron concentration at 31 °C, and the 1000 mg/L group had a significantly higher ($p < 0.05$) heart rate than the 0 and 10 mg/L groups of that treatment (Figure 3). At both 21 °C and 26 °C, no significant difference was found among all different nicosulfuron concentration groups ($p > 0.05$). The heart rate was significantly higher in all groups at 31 °C compared to both 26 °C and 21 °C ($p < 0.05$).
The hatching rate of the embryos is shown in Figures 4–6. In the 26 °C, pH 7.0 group, no significant difference \((p > 0.05)\) was found among all groups at 72 hpf (Figure 4). At 96 hpf, the hatching rate in the 1000 mg/L group was significantly higher \((p < 0.05)\) than that of all the other groups, except for the 100 mg/L group.

The effect of nicosulfuron on the heart rate of zebrafish embryos at different temperatures. Note: Values are mean ± SE. Different letters indicate a significant difference \((p < 0.05)\).

Figure 3. The effect of nicosulfuron on the heart rate of zebrafish embryos at different temperatures. Note: Values are mean ± SE. Different letters indicate a significant difference \((p < 0.05)\).

Figure 4. The effect of nicosulfuron on the hatching rate of the zebrafish embryo (26 °C, pH 7.0). Note: Values are mean ± SE. Different letters indicate a significant difference \((p < 0.05)\).
Figure 4. The effect of nicosulfuron on the hatching rate of the zebrafish... (Figure 8). At 21 °C, the Hsp70 level was significantly higher in the 10 mg/L group.

Figure 5. The effect of nicosulfuron on the hatching rate of the zebrafish embryos at pH 5.0 (A) and pH 9.0 (B). Note: Values are mean ± SE. Different letters indicate a significant difference (p < 0.05). The plot is darker, indicating a higher concentration of nicosulfuron. From low to high, 0, 10, 100 and 1000 mg/L.

Figure 6. The effect of nicosulfuron on the hatching rate of the zebrafish embryos at 31 °C (A) and 21 °C (B). Note: Values are mean ± SE. Different letters indicate a significant difference (p < 0.05). The plot is darker, indicating a higher concentration of nicosulfuron. From low to high, 0, 10, 100 and 1000 mg/L.

At pH 5.0, there was no significant difference (p > 0.05) in hatching rate among all concentrations at both 72 hpf and 96 hpf (Figure 5). At pH 9.0, the hatching rate of zebrafish embryos increased with nicosulfuron concentrations at 72 hpf, with the 1000 mg/L group showing a significantly higher hatching rate than the 0 mg/L group (p < 0.05). No significant difference in hatching rate was found at 96 hpf at pH 9.0 (p > 0.05).
At 31 °C, a significant difference ($p < 0.05$) was found only at 48 hpf, with the hatching rate increasing with nicosulfuron concentration (Figure 6). At 21 °C, no hatching embryos were found until 144 hpf, and there was no significant difference among all groups at 144 hpf ($p > 0.05$).

The Hsp70 level of fish embryos is shown in Figures 7 and 8. At 26 °C, pH 7.0, the lowest Hsp70 level was found in the 0 mg/L group (Figure 7). The Hsp70 levels in the 10 mg/L and 1000 mg/L groups were significantly higher than those in the 0 mg/L group ($p < 0.05$).

![Figure 7](image7.png)

**Figure 7.** Relative Hsp70 level of zebrafish embryos at 26 °C, pH 7.0. Note: Values are mean ± SE. Different letters indicate a significant difference ($p < 0.05$).

![Figure 8](image8.png)

**Figure 8.** Relative Hsp70 level of zebrafish embryos at 21 °C (A), 31 °C (B), pH 5.0 (C) and pH 9.0 (D). Note: Values are mean ± SE. Different letters indicate a significant difference ($p < 0.05$).

At 31 °C, there was no significant difference ($p > 0.05$) in the Hsp70 level among all groups (Figure 8). At 21 °C, the Hsp70 level was significantly higher in the 10 mg/L group than in the 0 mg/L group ($p < 0.05$). At pH 5.0 and 9.0, the Hsp70 levels showed the same significance and trend as observed at 21 °C.
4. Discussion

To our knowledge, the present work is the first to report the effect of nicosulfuron on *Danio rerio* embryos. Survival rates indicate that nicosulfuron does not impact the survival of zebrafish embryos at different pH levels or temperatures. Zebrafish are tropical fish and their optimum temperature for embryonic development is 26 °C, so it is normal for zebrafish embryo survival to decrease when the temperature decreases (21 °C). This is only the effect of temperature on embryo survival, and there was no significant difference in survival among the concentration groups at 21 °C, so no effect of nicosulfuron on the survival rate was found at this temperature. In addition, no malformations were observed during the embryo development in all tests and all groups. While nicosulfuron has been reported to be extremely toxic to aquatic organisms, the most sensitive non-target aquatic organisms to nicosulfuron, based on the very limited data currently available, are aquatic plants (*Lemna gibba, Lemna paucicostata*) [32]. The reason for no or very low embryotoxicity of nicosulfuron to *Danio rerio* embryos (less than 1000 mg/L) may be as follows: Tan et al. 2021 identified a typical butyrylcholinesterase-like protein in zebrafish which may have the potential to degrade nicosulfuron and protect zebrafish embryos from the harmful influence of nicosulfuron [33].

The toxicity data for nicosulfuron formations in fish and aquatic invertebrates are as follows: *Oncorhyncus mykiss* with a 96 h LC50 of 55.6–100 mg formulation/L (2.2–4.0 mg nicosulfuron/L) and a 28-day NOEC (No Observed Effect Concentration) of 10 mg nicosulfuron/L [9]. The acute toxicity data for nicosulfuron to aquatic invertebrates are available for *Daphnia magna* with 48 h EC50 of 82.3 mg formulation/L (3.3 mg nicosulfuron/L) and a 21-day NOEC of 5.2 mg nicosulfuron/L [9]. It is possible that the above results differ from the present study because (1) the authors did not use the active ingredient nicosulfuron but different formulations of it, and (2) the exposure time was longer than in the present study.

Heart rate is a reliable sublethal endpoint in the zebrafish embryo test as an early warning parameter [23,34]. In this experiment, nicosulfuron was not able to induce profound heart rate changes in zebrafish embryos at 26 °C, pH 7.0, whereas nicosulfuron increased the heart rate of zebrafish embryos at pH 5.0, pH 9.0 and 31 °C. In general, an increase in heart rate at sub-lethal exposures usually represents a biological response to toxicity by increasing metabolism [19,23]. Thus, these findings suggest that low pH, high pH, and high temperature may exacerbate nicosulfuron toxicity, but that these environmental parameters do not potentiate nicosulfuron toxicity to the extent that metabolic balance is disrupted and heart failure occurs. Low temperature appears to have little effect on nicosulfuron toxicity, which may be due to the already low heart rate of embryos under such conditions, so therefore significant differences are not detectable.

Similarly to heart rate, few significant differences were found in the hatching rate. However, the increase in hatching rate using nicosulfuron was also observed at 96 hpf at pH 7.0, 26 °C. Nicosulfuron had no significant effect on the final hatching success at any of the temperatures or pH treatments, but the significant difference in hatching rates at different time points suggests that nicosulfuron probably shortened the hatching time. This premature hatching is most likely due to the disturbance of the chorioallantoic membrane by nicosulfuron [35]. Moreover, we did not observe any differences in the early development (early development of the heart at any time point during the experiment, including early appearance of melanin, early formation of the swim bladder, etc.) of the embryos between the nicosulfuron groups at different concentrations of nicosulfuron, neither at the same temperature nor pH treatments. Thus, early hatching is not a result of spontaneous embryonic development accelerated by nicosulfuron. Instead, it can be attributed to the toxic stimulation induced by nicosulfuron exposure.

The possible effects of pH and high temperature may include an increase in metabolism, as fish metabolism increases with temperature, resulting in increased oxygen demand and increased heart rate [19,29]. Additionally, the bioavailability of substances increases with increasing temperature, further contributing to the toxicity of substances such as nicosulfuron.
This is in part due to the increase intermolecular movement with higher temperatures, which increases the diffusion or active absorption rate of the substance [17,19].

With regard to the mechanism by which pH may affect toxicity, it is generally accepted that pH affects the efficiency of biological uptake of a chemical mainly by influencing the form in which it exists in water [20,21,36]. Nicosulfuron becomes charged in an aqueous environment and releases hydrogen ions, so it exists in a more neutral state in an acidic environment compared to an alkaline environment. This suggests that nicosulfuron would be more likely to enter cells by passive diffusion in an acidic environment, and therefore should exhibit increased toxicity under such conditions.

However, this assumption is not consistent with the results of this experiment, as the effect of pH 5.0 on the hatching rate is not as pronounced as that of pH 9.0. At pH 5.0, nicosulfuron only showed a trend towards earlier hatching, which was not statistically significant. This may be attributed to the fact that the pH can have additional impacts, such as affecting the energy budget to maintain intracellular pH levels, or disrupting osmoregulation of essential ions, ammonia excretion or acid-base regulation in alkaline environments [37–39].

Although apical endpoints such as mortality or embryo development appear to be less affected by nicosulfuron, our Hsp70 analyses showed that this herbicide induces sub-organismal stress. The Hsp70 family is an important and highly conserved class of stress proteins. Their responses are known as suitable biomarkers for indicating cytotoxic hazards for both cells and proteins [40–42]. In developing zebrafish embryos, Hsp70 has been shown to respond rapidly to chemical stressors [30,43]. In this study, the Hsp70 levels of all nicosulfuron-treated embryos were elevated to varying degrees compared to the negative controls, indicating that nicosulfuron is proteotoxic to zebrafish embryos, with the exception of the 31 °C treatment groups.

In order to interpret the Hsp70 results, the response kinetics of this biomarker have to be considered. The general course of the Hsp70 response to increasing toxicity can be divided into three stages (Figure 9) [44]. In the first phase of the response, the cells remain in equilibrium and the Hsp70 response is at most marginally elevated. In the second phase, the stress response of the cells causes Hsp70 levels to rise sharply, reaching a maximum expression level. If the toxicity of the chemicals exceeds the maximum tolerance of cells, pathological damage to the protein synthesis apparatus is likely, resulting in a decrease in Hsp70 levels [44].

![Figure 9. Response kinetics of Hsp70 with rising toxicity according to [44].](image)

At 26 °C, pH 7.0, the Hsp70 level was expected of all groups to be located between the first and second stages of response. There was no trend towards a decrease in Hsp70 levels, suggesting that exposure to 1000 mg/L nicosulfuron did not exceed the ability of zebrafish embryos to produce Hsp70 as a protective mechanism. Furthermore, a decrease in cellular heat shock protein levels can be interpreted as a sign of a very strong stress response,
which is generally accompanied by physiological breakdown and destruction [30,44]. However, there was no indication of physiological collapse or other anomalies in the embryo test and, on the contrary, zebrafish embryos in the 1000 mg/L group were in good condition. Therefore, it can be concluded that nicosulfuron induced some proteotoxicity in zebrafish embryos, but its effects were very limited and did not exceed the tolerance range. The third phase of the Hsp70 response kinetics (destruction phase) was not reached by 1000 mg/L nicosulfuron.

The Hsp70 levels in embryos were concentration-dependent at 21 °C, pH 5.0 and pH 9.0 with a tendency to increase and then decrease with a higher nicosulfuron concentration. Based on the above-mentioned response kinetics of Hsp70, it could be implied that the lower Hsp70 values resulting from exposure to the higher concentrations may be allocated in the beginning of the third stage of the response kinetics. Compared with the first and second stages in the 26 °C, pH 7.0 groups, the proteotoxic effects of nicosulfuron may have been tentatively enhanced by low temperature, low pH and high pH effects, despite lacking significance between the 10, 100 and 1000 mg/L groups, and lacking mortality. Alternatively, our data can be explained by the fact that they represent the level of Hsp70 protein relative to the total amount of protein in the sample. Thus, a decrease in Hsp70 protein levels in the high-dose nicosulfuron treatment group could be the result of an increased expression of other proteins, resulting in a decrease in the relative proportion of Hsp70 within the total protein fraction. For example, this could be due to elevated levels of candidates for nicosulfuron-induced non-Hsp proteins, such as cytochrome P450 monoxygenases (P450) which are thought to be associated with resistance to this pesticide [45,46]. In the 31 °C groups, the lack of significant difference in Hsp70 between the different exposure groups is most probably due to the fact that the embryos were in a general state of heat shock because of the initial high temperature of the treatment; temperature seemed to play a dominant role for the expression of Hsp70 compared to the minor effect of nicosulfuron.

Overall, we found that nicosulfuron was weakly toxic to zebrafish embryos at different temperatures and pH levels. Although the concentrations of nicosulfuron tested in this experiment were high, the large and widespread use of nicosulfuron in practice is a reason for concern. In addition, this experiment also showed that changing pH and temperature increases the sublethal toxicity of nicosulfuron. Therefore, the potential threat of this herbicide in the environment merits concern and should not be disregarded.

5. Conclusions

This comprehensive study of the toxicity of nicosulfuron to zebrafish embryos at different temperatures and pH led to three main conclusions:

(1) Data for the survival of zebrafish embryos exposed to nicosulfuron at different temperatures and pH indicate that nicosulfuron has a low acute toxicity to zebrafish embryos.

(2) Environmental factors (pH and temperature) increased the sublethal toxic effects of nicosulfuron, as evidenced by earlier hatching, elevated heart rate and elevated Hsp70 levels. Although there were no significant differences in survival rates in this experiment, the role of environmental factors is still noteworthy.

(3) Hsp70 responses can be regarded as a sensitive biomarker for sublethal chemical stress but lose their indicative power when the organism’s physiology is dominated by a concomitant heat shock.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pollutants4030025/s1, Figure S1: The effect of nicosulfuron on the survival rate of zebrafish embryos 96 hpf at different pH; Figure S2: The effect of nicosulfuron on the survival rate of zebrafish embryos 96 hpf at different temperature.

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References

9. European Food Safety Authority. Conclusion Regarding the Peer Review of the Pesticide Risk Assessment of the Active Substance Nicosulfuron. EFSA 2007, 120, 1–91. [CrossRef]


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