Influence of Temperature and pH on the Toxicity of Nicosulfuron and Azoxystrobin on Early Life Stage of Zebrafish (*Danio rerio*): A Comprehensive Assessment

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Statement on the Use of Artificial Intelligence and Language Tools

This thesis was prepared with the support of digital tools for language improvement and programming assistance. DeepL was used for English translation and language refinement in selected parts of the manuscript. ChatGPT was used as a technical aid to support R programming, especially in the development of code for graphical data presentation.

All outputs generated with these tools were carefully reviewed, edited, and verified by the author. The responsibility for the accuracy of the text, the correctness of the analyses, the interpretation of the data, and the scientific conclusions lies solely with the author.

Abstract

Pesticides are widely used in modern agriculture and are frequently detected in aquatic environments. However, ecotoxicological risk assessments are commonly performed under standardized laboratory conditions that do not adequately reflect environmentally relevant variations. This thesis investigates how temperature and pH influence the toxicity of two widely used pesticides, nicosulfuron (an herbicide) and azoxystrobin (a fungicide), on the early life stages of zebrafish (*Danio rerio*).

To achieve this purpose, I used zebrafish in their early life stages as experimental subjects to test the developmental toxicity, proteotoxicity and behavior toxicity of nicosulfuron and azoxystrobin at different pH and temperature. The results demonstrated that nicosulfuron exhibits relatively low toxicity to zebrafish embryos under optimal conditions; however, sub-organismic responses, particularly stress protein (Hsp70) expression, revealed the changes in sensitivity under environmentally stressful conditions.

Azoxystrobin induces developmental abnormalities, delayed development, and altered physiological responses at higher concentrations. The results further demonstrated that environmental factors significantly modulate the toxicity of azoxystrobin, with complex interactions between chemical exposure and abiotic stressors influencing survival, hatching, heart rate, and stress protein expression.

In the final part of this thesis, the findings revealed that environmental factors strongly influence pesticide-induced behavioral responses. Nicouslfuron has a neuroexcitotoxic effect that enhances swimming activity in zebrafish larvae, while azoxystrobin inhibits swimming activity. Furthermore, pesticide effects were shown to be context-dependent. Light conditions significantly altered locomotor activity and enhanced the detection of behavioral toxicity. In contrast, azoxystrobin elicited detectable behavioral toxicity only under acidic conditions in darkness

Overall, abiotic environmental factors such as temperature, pH, and light play a crucial role in shaping toxicological responses during early developmental stages. These findings highlight the importance of incorporating environmentally relevant conditions into ecotoxicological risk assessments to improve the ecological relevance and predictive power of pesticide hazard evaluation.

Zusammenfassung

Pestizide werden in der modernen Landwirtschaft weit verbreitet eingesetzt und häufig in aquatischen Ökosystemen nachgewiesen. Allerdings werden ökotoxikologische Risikobewertungen meist unter standardisierten Laborbedingungen durchgeführt, die die umweltrelevanten Variationen nicht ausreichend berücksichtigen. Diese Dissertation untersucht, wie Temperatur und pH-Wert die Toxizität von zwei weit verbreiteten Pestiziden – Nicosulfuron (Herbizid) und Azoxystrobin (Fungizid) – auf die frühen Entwicklungsstadien von Zebrabärblingen (*Danio rerio*) beeinflussen.

Zu diesem Zweck wurden Zebrabärblinge in frühen Entwicklungsstadien als Versuchstiere verwendet, um die entwicklungsbezogene Toxizität, Proteotoxizität und Verhaltenstoxizität von Nicosulfuron und Azoxystrobin unter unterschiedlichen pH- und Temperaturbedingungen zu untersuchen. Die Ergebnisse zeigten, dass Nicosulfuron unter optimalen Bedingungen eine relativ geringe Toxizität für Zebrabärblingsembryonen aufweist; jedoch zeigten suborganismische Reaktionen, insbesondere die Expression des Stressproteins Hsp70, veränderte Sensitivitäten unter umweltbedingt stressigen Bedingungen.

Azoxystrobin verursachte Entwicklungsanomalien, Entwicklungsverzögerungen und veränderte physiologische Reaktionen bei höheren Konzentrationen. Die Ergebnisse zeigten weiterhin, dass Umweltfaktoren die Toxizität von Azoxystrobin signifikant modulieren, wobei komplexe Wechselwirkungen zwischen chemischer Exposition und abiotischen Stressoren Überleben, Schlupfrate, Herzfrequenz und Stressproteinexpression beeinflussen.

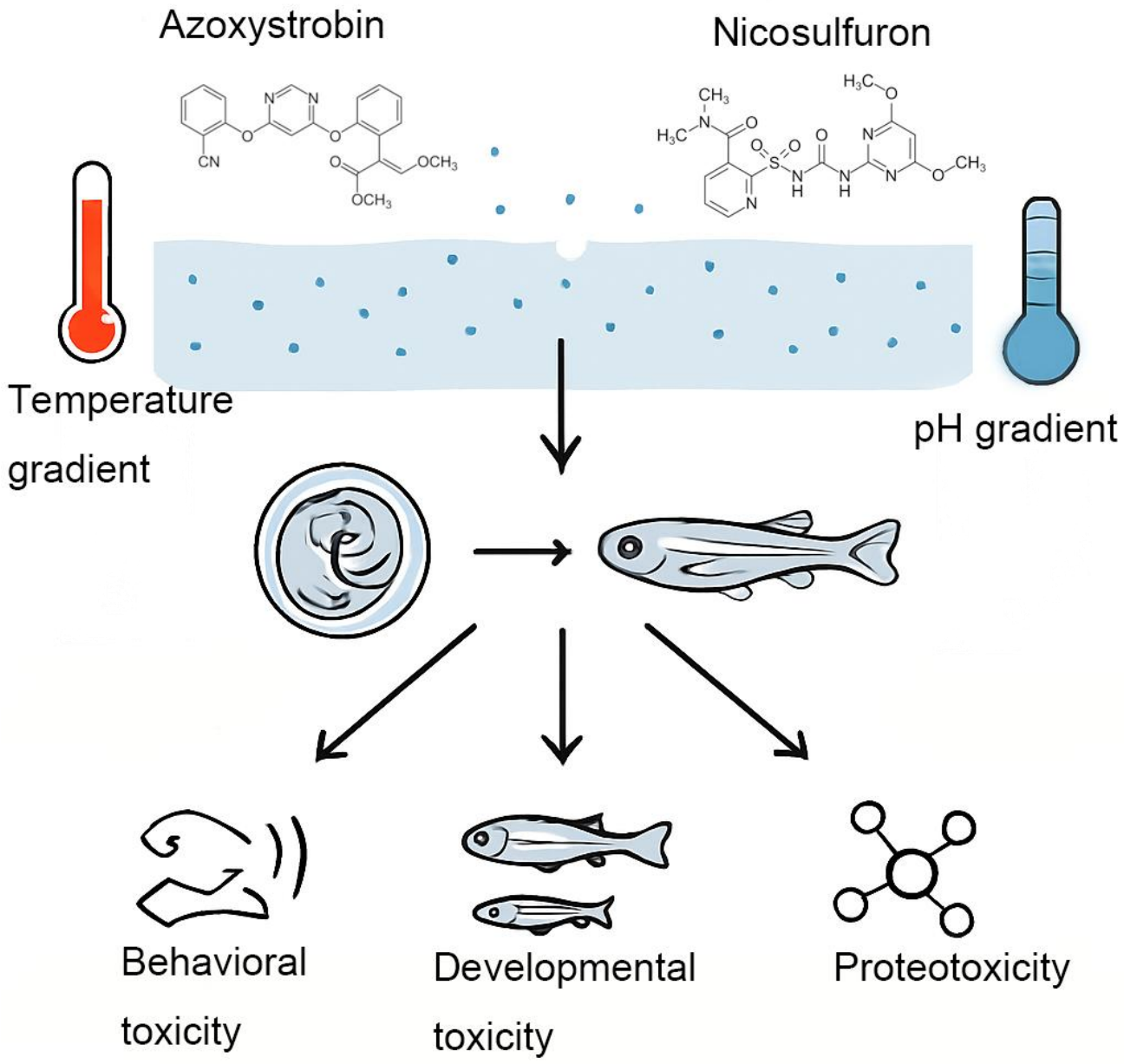
Im letzten Teil der Arbeit wurde gezeigt, dass Umweltfaktoren die durch Pestizide induzierten Verhaltensreaktionen stark beeinflussen. Nicosulfuron wirkt neuroexzitotoxisch und steigert die Schwimmaktivität der Larven, während Azoxystrobin die Schwimmaktivität hemmt. Die Effekte der Pestizide waren zudem kontextabhängig: Lichtverhältnisse veränderten die Bewegungsaktivität erheblich und erleichterten die Erkennung der Verhaltenstoxizität. Azoxystrobin zeigte hingegen nur unter sauren Bedingungen bei Dunkelheit eine nachweisbare Verhaltenstoxizität.

Insgesamt spielen abiotische Umweltfaktoren wie Temperatur, pH-Wert und Licht eine entscheidende Rolle bei der Formung toxikologischer Reaktionen in frühen Entwicklungsstadien.

Diese Ergebnisse unterstreichen die Bedeutung der Einbeziehung umweltrelevanter Bedingungen in ökotoxikologische Risikobewertungen, um deren ökologische Relevanz und Vorhersagekraft für die Bewertung der Pestizidgefahr zu verbessern

Part I: Summary

Graphical abstract



1. Introduction

Pesticides are chemical or biological agents used to prevent, repel, or control agricultural pests, including insects, weeds, pathogenic microorganisms, and rodents (Janardhan Namdeo Nehul, 2025;

Zhou et al., 2025). Their primary mode of action is to interfere with key physiological or metabolic processes in target organisms, thereby causing mortality, reducing reproductive capacity, or preventing further damage to crops (Janardhan Namdeo Nehul, 2025; Zhou et al., 2025).

Undoubtedly, pesticides have played a crucial role in increasing crop yields and safeguarding food supplies. However, their large-scale application has also generated a range of environmental problems, including acute and chronic toxicity to non-target organisms, bioaccumulation and biomagnification through food webs, contamination of soil and water bodies, and the development of resistance in target pests (Karaoğlan et al., 2024; Wan et al., 2025; Zhou et al., 2025; Köninger et al., 2026). Moreover, pesticide contamination is not confined to application sites. Environmental transport processes such as surface runoff, soil leaching, spray drift, and atmospheric deposition can carry pesticides far beyond treated areas, resulting in widespread contamination of natural ecosystems (De Souza et al., 2020; Baćmaga et al., 2024). Among these, aquatic ecosystems often act as the ultimate sinks for pesticide pollutants due to their geographical position (Farah et al., 2024). Once pesticides enter water bodies, they can exert persistent adverse effects on plankton, invertebrates, fish, amphibians, and other aquatic organisms (De Souza et al., 2020; Ahmed et al., 2025). These impacts include growth inhibition, developmental abnormalities, reduced reproductive capacity, increased oxidative stress, behavioral alterations, a nd elevated mortality (Cook et al., 2005; Cao et al., 2016; Crall et al., 2018; Abdel-Tawwab et al., 2025). Given the complex trophic interactions within aquatic food webs, such adverse effects may propagate across trophic levels, ultimately disrupting ecosystem structure and functional stability (Köhler and Triebskorn, 2013; Farah et al., 2024).

Beyond local and regional ecological risks, pesticide pollution should also be considered within the broader framework of the planetary boundaries concept proposed by Johan Rockström and colleagues in 2009 (Rockström et al., 2009; Richardson et al., 2023). The planetary boundaries framework defines a “safe operating space for humanity” by identifying critical Earth system processes that regulate planetary stability. Among the nine identified boundaries, the “novel entities” boundary explicitly includes synthetic chemicals such as pesticides, while the biosphere integrity and freshwater change boundaries are closely linked to ecological impacts observed in contaminated

environments. Recent assessments suggest that the boundary for novel entities has already been transgressed, largely due to the rapid production and environmental release of synthetic chemicals exceeding global regulatory and monitoring capacities (Richardson et al., 2023).

Despite growing awareness among both the public and the scientific community regarding the adverse effects of pesticides, their overall use has not shown a clear declining trend over the past decades (Köhler and Triebskorn, 2013; Ahmed et al., 2025; Köninger et al., 2026). According to statistics from the Food and Agriculture Organization, global agricultural use of pesticide active ingredients reached 3.73 million tonnes in 2023, representing a 14% increase compared to a decade earlier and a doubling relative to 1990 (FAO 2025). This trend reflects the continued reliance of modern agriculture on chemical control measures to ensure food security and enhance production efficiency.

In this context, banning or reducing high-risk pesticides is meaningful for controlling pesticide pollution. Many regions worldwide have established stringent regulatory frameworks. For example, the Water Framework Directive of the European Union sets out a comprehensive strategy for water protection (Besse et al., 2012). Within this framework, the Environmental Quality Standard (EQS) serves as a core indicator for assessing the chemical and ecological status of water bodies. The EQS refers to the environmental threshold concentration in water, sediment, or biota that should not be exceeded in order to achieve a good chemical and ecological status (Rodrigues et al., 2017). The derivation of EQS requires both environmental monitoring data and robust toxicological evidence.

Currently, a substantial body of research is conducted under standardized and simplified laboratory conditions, typically maintaining constant temperature, pH, and other physicochemical parameters to ensure reproducibility (Andrade et al., 2017; Abdel-Tawwab et al., 2025). However, natural aquatic environments are highly dynamic. Temperature and pH can fluctuate significantly due to seasonal changes, climatic variability, biological activity, and anthropogenic influences (Andrade et al., 2017; Castro et al., 2025; Janardhan Namdeo Nehul, 2025). These abiotic factors not only influence the physicochemical behavior of contaminants but also modulate the physiological responses of organisms. Failure to adequately consider environmental complexity may compromise the ecological relevance of toxicological data.

Temperature affects metabolic rate, enzyme activity, membrane permeability, and the kinetics of chemical uptake in organisms (Alfonso et al., 2021; Schnurr et al., 2013). Elevated temperatures may increase metabolic demands, enhance organismal sensitivity to toxicants, and accelerate chemical degradation, whereas lower temperatures may reduce detoxification capacity and prolong exposure duration (Osterauer, 2008). The pH, in turn, influences the chemical speciation, solubility, stability, and ionization state of pesticides, thereby altering their bioavailability (Besse et al., 2012; Köhler et al., 2023; Kroll et al., 2024). Changes in pH can also affect ion regulation and acid–base balance in aquatic organisms, further modifying their sensitivity to toxicants (Andrade et al., 2017; Kroll et al., 2024).

Therefore, interactions between environmental factors and contaminants are of critical importance. Temperature and pH are not merely background conditions; they may directly or indirectly regulate pesticide toxicity. Ignoring such environmental variability may lead to underestimation or overestimation of environmental risks, thereby weakening the ecological relevance of laboratory toxicity data. Nevertheless, the role of environmental factors is often overlooked in the derivation of environmental quality standards and routine ecotoxicological assessments. In the context of global climate change and intensifying human activities, fluctuations in temperature and pH in freshwater ecosystems are expected to become more pronounced (Alfonso et al., 2021; Kroll et al., 2024). Consequently, evaluating pesticide toxicity under environmentally realistic conditions is increasingly necessary.

In terms of usage categories, herbicides account for the largest share of global pesticide consumption, followed by insecticides and fungicides (FAO 2025). Nicosulfuron, a sulfonylurea herbicide, is widely used worldwide as a post-emergence herbicide to protect crops such as maize from weed infestation (Feng et al., 2017). Its mode of action involves inhibition of acetolactate synthase, a key enzyme in the biosynthesis of branched-chain amino acids, by blocking amino acid synthesis, nicosulfuron effectively suppresses weed growth (Carles et al., 2018; Cheron et al., 2022).

Although the Joint Research Centre has identified nicosulfuron as posing a high risk to aquatic wildlife, current knowledge regarding its toxicity to non-target organisms remains limited and somewhat controversial (Carvalho et al., 2016). Some studies have shown that nicosulfuron induces

embryotoxicity and oxidative stress in embryos of the spiny toad, *Bufo spinosus*, and causes behavioral abnormalities and alterations in acetylcholinesterase activity in goldfish (Bretaud et al., 2000; Cheron et al., 2022, 2023). In contrast, other findings suggest minimal effects on mammals, birds, and amphibians (EFSA, 2007). Reported average concentrations of nicosulfuron in surface waters in Canada, the United States, and Europe range from 0.03 to 0.5 μg/L, with peak levels reaching 16 μg/L (Battaglin et al., 2000; Moschet et al., 2014; Carvalho et al., 2016; Cheron et al., 2023). To comprehensively understand its toxic potential, more in-depth assessments in non-target species are urgently needed.

Azoxystrobin is a methoxyacrylate (strobilurin) fungicide synthesized based on natural antifungal compounds produced by forest fungi (Cao et al., 2016). As a broad-spectrum, systemic fungicide applied to both soil and foliage, it is extensively used worldwide to control fungal diseases in a variety of crops (Amador et al., 2024). Azoxystrobin inhibits the fungal respiratory chain by blocking electron transfer from coenzyme Q (quinol) to cytochrome c1, thereby reducing adenosine triphosphate (ATP) production (Bartlett et al., 2002; Kumar et al., 2020). Although designed to target fungi, substantial evidence indicates that it also exerts significant toxicity on various non-target aquatic organisms. Studies have reported reduced survival, delayed growth, and impaired reproduction in *Daphnia magna* (Cao et al., 2016; Cui et al., 2016); developmental inhibition in amphibian tadpoles (Rodrigues et al., 2017); and oxidative stress induction and behavioral alterations in zebrafish (Gao et al., 2014; Guo et al., 2024). Surface water concentrations of azoxystrobin have been reported to range from 0.06 to 11.10 μg/L in countries including the United States, France, Germany, China, and Brazil, with peak values reaching 29.7 μg/L. Detected concentrations in groundwater typically range between 0.01 and 0.1 μg/L (Berenzen et al., 2005; Filho, 2010; Rodrigues et al., 2017; Peluso et al., 2022; Guo et al., 2024).

In summary, investigating the regulatory mechanisms by which environmental factors influence pesticide toxicity holds significant scientific and practical importance. The present thesis aims to systematically evaluate the effects of temperature and pH on the toxicity of two widely used pesticides, nicosulfuron and azoxystrobin, in aquatic ecosystems. To achieve this purpose, I used zebrafish in their early life stages as experimental subjects to test the developmental toxicity,

proteotoxicity and behavior toxicity of nicosulfuron and azoxystrobin at different pH and temperature. By integrating the interactive effects of abiotic factors, this research seeks not only to enrich the toxicological database for these pesticides under environmentally variable conditions, but also to provide a theoretical foundation for improving existing ecological risk assessment frameworks and deriving environmental quality standards with greater scientific robustness and ecological relevance.

2. Material and methods

2.1 Test substances

Nicosulfuron (CAS 111991-09-4, purity >98.0%) and azoxystrobin (CAS 131860-33-8, purity ≥98.0%) were obtained from Sigma-Aldrich (Merck, Darmstadt, Germany). Azoxystrobin is a lipophilic organic compound that can be ionized and maintains relatively stable lipophilicity within a pH range of 4–14. In contrast, nicosulfuron is a hydrophilic ionizable organic compound, and its hydrophilicity becomes more pronounced at pH 5.0 and 9.0. Physicochemical properties of these two chemicals were displayed in Table 1 and Table 2.

Stock solutions of nicosulfuron (1000 mg/L) and azoxystrobin (100 μg/L) were prepared using reconstituted water. The reconstituted water was produced by adding 25 mL of each of four salt stock solutions (0.23 g/L KCl, 2.59 g/L $NaHCO_3$, 4.93 g/L $MgSO_4 \cdot 7H_2O$, and 11.76 g/L $CaCl_2 \cdot 2H_2O$) to 900 mL of double-distilled water. Working concentrations were generated through stepwise dilution of the stock solutions. The pH of the experimental solutions (5.0, 7.0, and 9.0) was adjusted with 1 M HCl or 1 M NaOH and measured using a SevenCompactDuo pH meter (Mettler Toledo, Gießen, Germany).

Table 1: Physicochemical properties of nicosulfuron

Properties	
Molecular formula	$C_{15}H_{18}N_6O_6S$
Molecular weight	410.4 g/mol
Solubility in water	7400 mg/L (at pH 7, 25°C)
log P_{ow} (at pH 5)	0.35
log P_{ow} (at pH 7)	1.8
log P_{ow} (at pH 9)	-2.0
pK_a	4.60

Data obtained according to Pubchem (NIH, National library of Medicine)

Table 2: Physicochemical properties of azoxystrobin

Properties	
Molecular formula	$C_{22}H_{17}N_3O_5$
Molecular weight	403.4 g/mol
Solubility in water	6 mg/L (at 20 °C)
log P_{ow} (at 20 °C)	2.5
pK_a	not applicable

Data obtained according to Pubchem (NIH, National library of Medicine)

2.2 Test animal

The zebrafish *Danio rerio* (Hamilton 1822) belongs to the family Cyprinidae and is native to South Asia, particularly the river basins of India, Bangladesh, and Nepal (Esmail et al., 2015). It is a small tropical freshwater fish that typically inhabits slow-moving streams, rice paddies, and floodplain waters. Adult zebrafish usually reach 3–5 cm in length and are characterized by a slender body with distinctive horizontal blue and silver stripes extending from the operculum to the caudal fin (Kimmel et al., 1995; Spence et al., 2008). Sexual maturity is generally attained within three to four months under laboratory conditions. Spawning occurs year-round in captivity, with females capable of producing several hundred eggs per clutch. Fertilization is external, and embryonic development proceeds rapidly, with major organ systems forming within 24–72 hours post-fertilization (Kimmel et al., 1995).

Fish Maintenance

Adult zebrafish (*Danio rerio*, USA Westaquarium strain) were housed in 200 L aquaria at the Animal Physiological Ecology unit of the University of Tübingen, Germany. Fish were maintained under a controlled photoperiod of 12 h light and 12 h dark and were fed commercial dry flake food (TetraMin®, Tetra GmbH, Melle, Germany) three times per day. Fecal material was removed daily prior to feeding to maintain water quality. Partial water renewal (30–50% of the tank volume) was conducted on a regular basis.

Water conditions were carefully controlled throughout the maintenance period. The temperature was kept at 26 ± 1 °C, with a pH of 7.4 ± 0.2 and a total hardness of 8–12 °dH. Dissolved oxygen saturation was maintained at approximately 100 ± 5%. Concentrations of nitrate and nitrite were

regularly monitored and remained below critical thresholds (<1 mg/L and <5 mg/L, respectively). All animal handling and experimental procedures were conducted in accordance with the approval of the Animal Welfare Committee of the Regional Council of Tübingen, Germany (approval numbers ZO 2/16 and ZO 02/21 G).

Figure 1. Adult Zebrafish

2.3 Developmental toxicity assessment—Early life stages (ELS) test

ELS of zebrafish are widely applied in ecotoxicology and environmental risk assessment. Zebrafish is a well-established vertebrate model species in biological research, and ELS tests using embryos have become standard tools for evaluating chemical toxicity (Nagel, 2002; Schweizer et al., 2022; Guo et al., 2024). Importantly, ELS tests comply with the principles of replacement, reduction, and refinement (3Rs) and have gained regulatory acceptance (Yang et al., 2009; Su et al., 2021). In many regulatory frameworks embryos up to five days post-fertilization are not classified as protected laboratory animals, meaning that ELS tests are generally not considered animal experiments under animal welfare legislation. The transparency of the chorion further enables direct assessment of embryotoxic endpoints such as developmental delay, cardiac dysfunction, edema formation, and morphological malformations without invasive procedures (Yang et al., 2009; Kumar et al., 2020).

In 2005, Germany tried to replace the traditional acute adult fish toxicity test with the standardized zebrafish embryo test (Braunbeck et al., 2005). Subsequently, in 2013, the zebrafish embryo test was formally adopted by the Organisation for Economic Co-operation and Development (OECD) as Test Guideline 236 (Fish Embryo Acute Toxicity Test (OECD 236)), providing an internationally harmonized alternative to conventional acute fish toxicity assays.

Methodology

ELS toxicity tests were conducted using zebrafish (*Danio rerio*) embryos in accordance with OECD Test Guideline 236 (Fish Embryo Acute Toxicity Test), with minor modifications based on our previous studies (Schweizer et al., 2017, 2022).

Spawning and test setups were prepared one day prior to the start of the experiment. Breeding boxes were equipped with mesh grids (1.5 mm mesh size) and artificial seagrass placed at the bottom of the aquaria to stimulate spawning behavior. For each test concentration, eight small Petri dishes (30 mm diameter) were filled with 3 mL of the respective test solution, and one larger Petri dish (90 mm diameter) was filled with 30 mL of the same solution. This preconditioning step ensured saturation of the glassware with the test substance, thereby minimizing potential adsorption losses. Immediately before the start of exposure, all solutions were replaced with freshly prepared test solutions.

Spawning occurred shortly after the onset of light. Fertilized eggs were collected one hour after light onset the following morning. To ensure uniform exposure from the earliest developmental stage, freshly fertilized eggs were immediately transferred into the large Petri dishes containing the respective test solutions for pre-exposure. Embryos were pre-exposed for 2 h under respective experimental temperature.

After pre-exposure, embryos were examined under a stereomicroscope, and only normally developing embryos at the 128–256 cell stage (according to Kimmel et al., 1995) were selected. Selected embryos were randomly allocated to the small Petri dishes. Embryos were incubated for 96 h under the designated temperature conditions in an incubator (standard temperature: 26 °C; alternative temperatures in temperature-modified experiments). Embryonic development was monitored at 12, 24, 48, 60, 72, and 96 hours post-fertilization (hpf).

The following endpoints were assessed:

- Survival rate
- Developmental delay
- Heart rate
- Hatching success
- Morphological abnormalities

Survival rate was recorded at every time point. Coagulated embryos were recorded and removed immediately upon observation. The development of eyes, somites and non-detachment of the tail was checked at 24 hpf. Heart rate was determined at 48 hpf, by counting beats over 20 seconds and extrapolating to beats per minute. Hatching success was generally recorded between 60 and 96 hpf. Morphological abnormalities (Oedema, eye defects, tail defects, deformation of the spine and light pigmentation) were observed at 96 hpf.

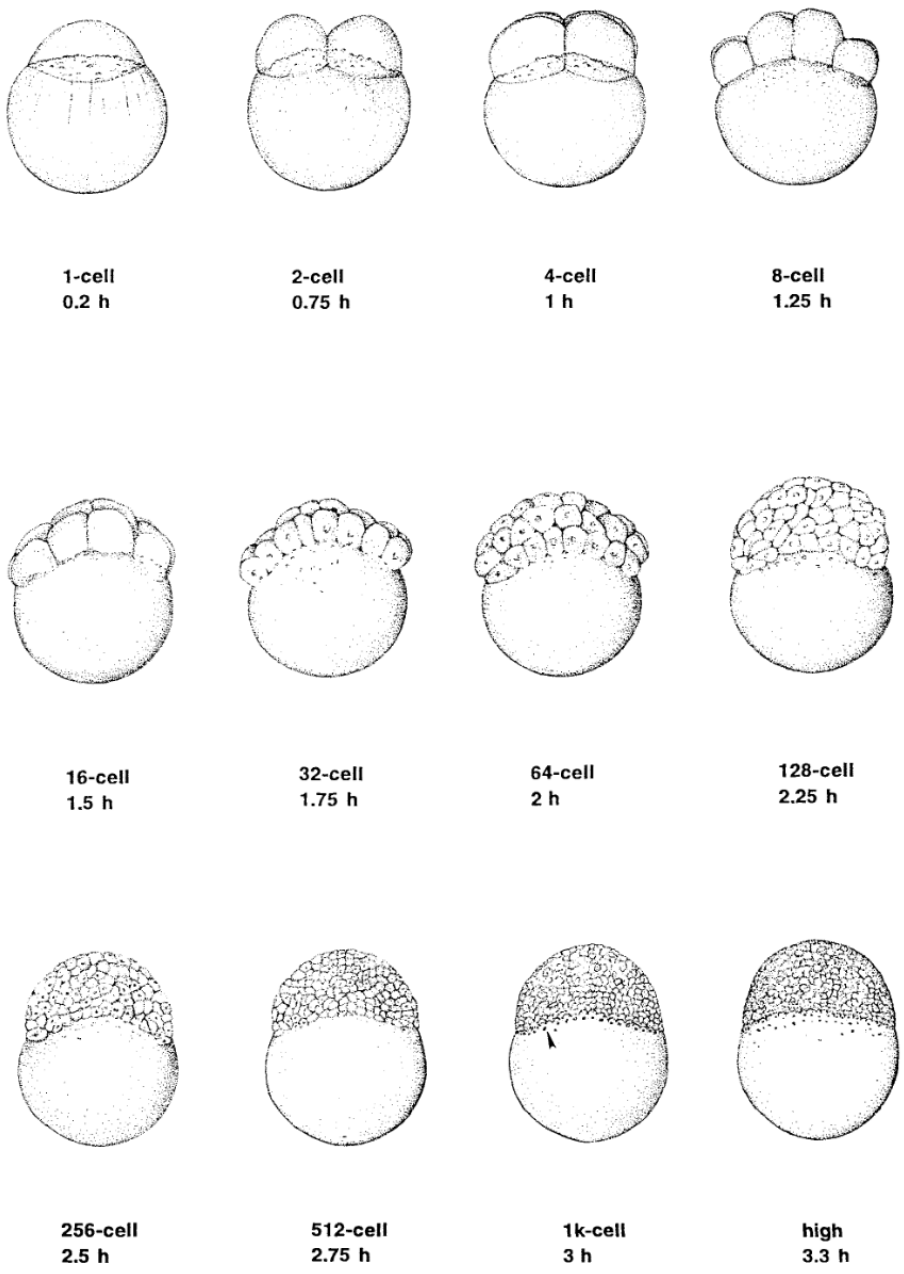


Figure 2. Developmental stages of zebrafish embryo within 3.3 hpf (Kimmel et al., 1995).

2.4 Proteotoxicity assessment—Hsp 70 determination

Hsp70 belongs to a family of evolutionarily conserved stress proteins that occur in virtually all living organisms, including teleost fish (Sanders, 1993). Hsp70 functions as ATP-dependent molecular chaperones that safeguard intracellular protein integrity. Through coordinated cycles of substrate binding and ATP hydrolysis, Hsp70 assists in protein refolding, stabilization, intracellular trafficking, and, when necessary, targeting irreversibly damaged proteins for degradation.

Although Hsp70 initially identified as proteins synthesized in response to elevated temperatures, subsequent research has demonstrated that their induction is not restricted to thermal stress but represents a general cellular defense mechanism against proteotoxic disturbances (Mikulski et al., 2009; Bonomo et al., 2010; Mikulski et al., 2011). Upon exposure to environmental challenges such as pathogen invasion, ultraviolet radiation, temperature shifts, or chemical contaminants, the cellular burden of unfolded or misfolded proteins increases markedly (Sanders, 1993; Mikulski et al., 2009, 2011). This imbalance in protein homeostasis activates stress signaling pathways that enhance Hsp70 synthesis as part of an adaptive response aimed at restoring proteostasis. Importantly, the induction of Hsp70 does not increase indefinitely with rising stress intensity. Instead, it follows a non-linear response pattern: moderate stress levels stimulate elevated expression, whereas excessive or prolonged stress may impair the cellular capacity to sustain Hsp70 production (Eckwert et al., 1997). Under such conditions, expression levels can decline to baseline or even below, reflecting functional exhaustion of the stress response machinery rather than reduced stress exposure.

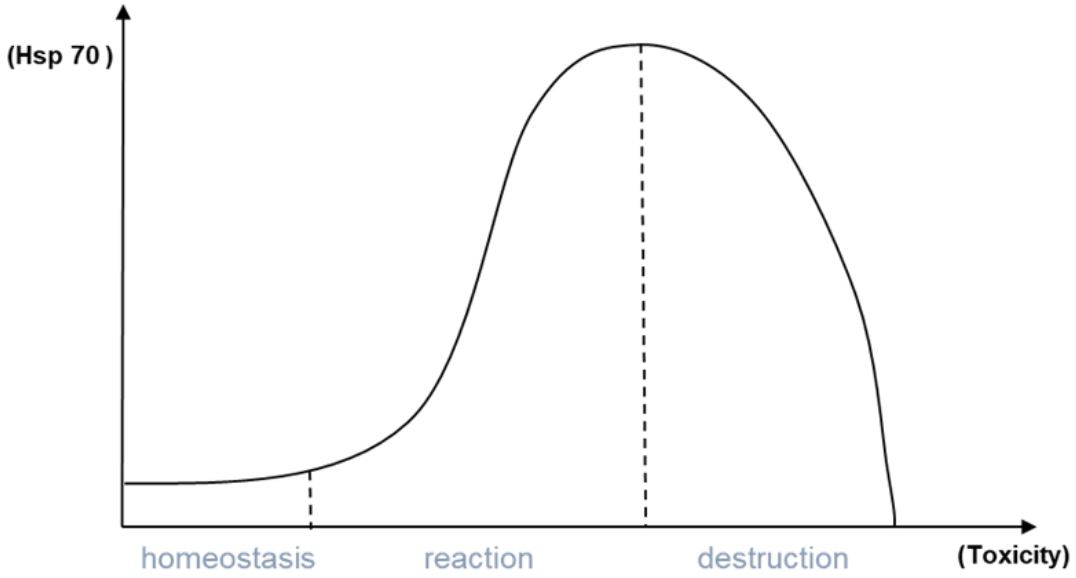


Figure 3. Response kinetics of Hsp70 with rising toxicity according to Eckwert et al. (1997)

Methodology

For the analysis of Hsp70 expression, fertilized zebrafish eggs were randomly assigned to the respective treatment groups immediately after spawning. Each group initially comprised 40–50 embryos, which were maintained under the designated exposure conditions for 96 h. At the end of

the exposure period, embryos were collected with pipette. For protein extraction, 10 individuals were pooled to obtain one biological sample, resulting in three independent replicates per treatment group. Samples were shock-frozen in liquid nitrogen and stored at −80 °C until further processing.

Protein extraction followed a modified protocol based on Vincze et al., (2014). Briefly, pooled embryos were homogenized in 20 µL of ice-cold extraction buffer containing 80 mM potassium acetate, 4 mM magnesium acetate, and 20 mM HEPES (pH 7.5). Homogenization was performed thoroughly to ensure complete tissue disruption. The homogenates were subsequently centrifuged at 20,000 × g for 10 min at 4 °C to separate cellular debris. The supernatants containing soluble proteins were collected for downstream analyses.

Total protein concentrations were quantified using the Bradford assay (Bradford, 1976), and sample concentrations were adjusted accordingly. For electrophoretic separation, 20 µg of total protein per sample was loaded onto SDS–polyacrylamide gels. Proteins were separated by electrophoresis at 80 V for 30 min followed by 120 V for approximately 90 min.

After separation, proteins were subjected to immunodetection using a monoclonal mouse anti-human Hsp70 primary antibody (clone 3A3, Santa Cruz Biotechnology). A horseradish peroxidase-conjugated goat anti-mouse IgG (H+L) secondary antibody (Dianova) was applied for signal detection. Immunoreactive bands were visualized and digitally recorded. Band intensities were quantified by densitometric analysis using the Herolab E.A.S.Y. imaging system (Wiesloch, Germany).

To account for inter-membrane variability and ensure comparability across blots, Hsp70 signals were normalized to two internal reference standards prepared from adult *Danio rerio* tissue and included on each membrane. Relative Hsp70 expression levels were calculated based on these internal controls.

2.5 Behavioral toxicity assessment—Light/Dark transition (LDT) test

The LDT test is a widely applied behavioral paradigm used to evaluate locomotor reactivity and stress responsiveness in zebrafish larvae (Velki et al., 2017; Lovin et al., 2021; Haigis et al., 2022). It is based on the natural photomotor response of zebrafish, which display distinct activity patterns

when exposed to alternating light and dark phases.

Zebrafish larvae typically exhibit relatively low activity during light phases and a marked increase in locomotor activity immediately after transition to darkness (Lovin et al., 2021; Velki et al., 2017). This dark-induced hyperactivity is considered an innate escape-like response and reflects proper sensory processing and neural circuit function (Burgess and Granato, 2007; Tegelenbosch et al., 2012).

In ecotoxicological studies, alterations in LDT responses may indicate neurotoxicity or stress-induced behavioral dysregulation. Reduced responsiveness to light–dark transitions may suggest sensory impairment, metabolic depression, or neural dysfunction, whereas exaggerated responses can indicate hyperexcitability (Tegelenbosch et al., 2012; Orger and De Polavieja, 2017; Velki et al., 2017; Lovin et al., 2021; Haigis et al., 2022). Because temperature, pH, and chemical contaminants can modulate neuronal excitability and metabolic capacity, the LDT test is particularly suitable for investigating interactions between environmental variables and toxicant exposure.

Methodology

The LDT assay was performed using a ZebraBox behavioral tracking system together with ZebraLab® v3 software (Viewpoint, Lyon, France). After incubation, newly hatched larvae were placed into the tracking apparatus and allowed to acclimate for 5 min prior to recording.

The experimental sequence consisted of alternating illumination and darkness periods. The test began with a 5 min light phase followed by 5 min of darkness, and this cycle was repeated twice. During illuminated phases, the light intensity was adjusted to 22,000 lux, which approximates the brightness of outdoor sunlight under clear conditions. Light intensity was measured using an iPhone 13 equipped with the LightMeter application (version 3.1.40).

Experiments were conducted in three independent runs, each including 16 larvae per treatment group. The temperature was maintained within ±1 °C throughout the experiment using a water bath, and the water temperature was checked every 5 min with an electronic thermometer. The behavioral tracking system recorded the following locomotor parameters: distance traveled (distance), number of movement initiations (counts), and total movement duration (duration). Swimming behavior was

further classified into three velocity categories (v): bursting ($v > 1$ cm/s), cruising (0.3 cm/s $< v \leq 1$ cm/s), and freezing ($v \leq 0.3$ cm/s).

2.6 Statistics

All data analyses were performed using SAS JMP Student Version 18. Prior to hypothesis testing, data distribution and variance equality were evaluated using the Shapiro–Wilk test for normality and Levene's test for homoscedasticity. When the assumptions for parametric testing were met, differences among groups were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) test for multiple comparisons. In cases where these assumptions were violated, the non-parametric Steel–Dwass test was employed instead. Figures were generated using R (version 4.1.2) and Origin 2021.

For the behavioral analyses, multiple linear regression (MLR) was selected as the primary statistical approach to simultaneously evaluate the effects of pesticide concentration, pH, and temperature on the response variables 'counts', 'duration' and 'distance' categories of different swimming speeds. Model parameters were estimated using standard least squares regression. Statistical significance was defined at $p \leq 0.05$.

3. Results

3.1 Chapter 1: Proteotoxicity and Apical Toxicity of Nicosulfuron to *Danio rerio* Embryos: A Comprehensive Assessment at Different Temperatures and pH

Pesticides have increasingly raised concerns due to their adverse effects on both terrestrial and aquatic ecosystems. Among them, nicosulfuron, a sulfonylurea herbicide widely applied as a post-emergence treatment to control weeds in crops such as maize, is regarded as potentially hazardous to aquatic organisms. Environmental conditions, particularly temperature and pH, play a crucial role in influencing the toxicity of chemicals in aquatic systems. Nevertheless, these factors are often insufficiently considered in toxicity evaluations and in the establishment of environmental quality standards.

To better understand the risks posed by nicosulfuron to non-target species and to evaluate how environmental variables may alter its toxicity, this study examined the responses of zebrafish embryos exposed to nicosulfuron concentrations ranging from 0 to 1000 mg/L. In addition, toxicity outcomes were compared under varying environmental conditions, including different temperatures (21 °C and 31 °C) and pH levels (5.0 and 9.0).

In this study, the embryotoxic potential of nicosulfuron towards *Danio rerio* was investigated in three independent experiments following the Fish Embryo Acute Toxicity protocol described in OECD Test Guideline 236. In the initial experiment, embryos were exposed to six nominal concentrations of nicosulfuron (0, 0.1, 1, 10, 100, and 1000 mg/L) under standard laboratory conditions (26 °C, pH 7.0) to determine baseline sensitivity. The second and third experiments assessed how altered environmental parameters — specifically pH (5.0 and 9.0) and temperature (21 °C and 31 °C) — influenced toxicity at four concentrations (0, 10, 100, and 1000 mg/L). In addition to apical endpoints, sub-organismal responses were evaluated by quantifying Hsp70 levels as an indicator of proteotoxic stress.

No morphological abnormalities were detected in any treatment. Survival remained above 80% across all groups, except for embryos maintained at 21 °C (pH 7.0). Within identical temperature or pH conditions, nicosulfuron exposure did not significantly affect survival ($p > 0.05$). At 21 °C, heart rate was not significantly altered by herbicide concentration ($p > 0.05$). In contrast, at 31 °C as well as at pH 5.0 and 9.0, cardiac frequency showed an increasing trend with rising nicosulfuron levels.

With the exception of the pH 5.0 (26 °C) and 21 °C (pH 7.0) treatments, exposure generally accelerated hatching, although the onset of this effect varied among environmental conditions. Notably, embryos incubated at 21 °C (pH 7.0) failed to hatch before 144 hours post-fertilization. Regarding molecular stress responses, enhanced Hsp70 expression indicated greater proteotoxicity at 21 °C and under both acidic and alkaline conditions. However, at 31 °C, Hsp70 levels did not differ significantly among exposure concentrations ($p > 0.05$).

Overall, the results indicate that nicosulfuron exhibits relatively low acute toxicity to zebrafish embryos across the tested temperature and pH conditions. Although the exposure concentrations applied in this study were comparatively high, the extensive and widespread application of this

herbicide in agricultural practices still raises environmental concerns. Furthermore, our findings suggest that variations in temperature and pH can enhance its sublethal effects. Therefore, the ecological risks associated with nicosulfuron should be carefully considered and not underestimated.

3.2 Chapter 2: Environmental drivers of pesticide toxicity: temperature and pH shift azoxystrobin's effects on zebrafish (*Danio rerio*) early development

Fungicides, as extensively used agrochemicals with substantial market demand, have attracted increasing attention due to their potential effects on both target and non-target organisms. Azoxystrobin, a representative strobilurin fungicide, is commonly employed to manage fungal infections in a wide range of crops. However, its intensive use has raised concerns about its entry into and persistence within aquatic ecosystems. Although numerous studies have investigated the toxicity of pesticides such as azoxystrobin, most evaluations are performed under standardized laboratory conditions that fail to capture the variability of natural environments. The omission of environmental factors may lead to biased toxicity estimates and limit the ecological relevance of such assessments.

In the present study, we aimed to conduct a hazard-oriented investigation under controlled laboratory settings, with a focus on the developmental toxicity of azoxystrobin in zebrafish embryos and the potential influence of environmental parameters, particularly temperature and pH. To achieve this, both apical endpoints (including survival, hatching success, and morphological abnormalities) and molecular indicators (such as Hsp70 expression) were analyzed under different environmental conditions. This study is intended to explore potential hazards and underlying mechanisms, rather than to provide a comprehensive quantitative risk assessment.

This study evaluated the embryotoxic effects of azoxystrobin on early life stages of *Danio rerio* under varying physicochemical conditions. Fertilized eggs were exposed for 96 hours post-fertilization to concentrations between 0 and 1000 μg/L at three pH levels (5, 7, and 9) and three temperatures (21 °C, 26 °C, and 31 °C). A range of developmental and physiological parameters was monitored, including mortality, hatching success, cardiac frequency, morphological abnormalities, developmental progression, and Hsp70 protein expression.

At the highest tested concentration (1000 µg/L), azoxystrobin consistently caused pronounced teratogenic effects, such as pericardial and yolk sac edema as well as deformities of the eyes, tail, and spine, accompanied by delayed development under all tested environmental settings. No significant developmental toxicity was found at other concentrations. Toxicity was influenced by abiotic factors: higher temperatures and alkaline conditions reduced mortality rates observed at elevated exposure levels. Patterns of Hsp70 expression indicated that chemical stress responses were shaped by the combined influence of azoxystrobin and environmental parameters, suggesting interactive effects.

Although this work was conducted as a hazard-based assessment rather than a quantitative risk evaluation, the sublethal effects observed (delayed development, altered cardiac performance, and disrupted proteostasis) may have important consequences at the population level. These impairments could translate into reduced recruitment, slower growth, and decreased reproductive success. While not immediately fatal, such effects can ultimately influence population dynamics by increasing susceptibility to predation and reducing overall fitness.

In addition, our results demonstrate that the toxicity of azoxystrobin in zebrafish embryos is shaped not only by exposure concentration but also by environmental conditions, particularly temperature and pH. In contrast to conventional toxicity tests performed under standardized laboratory settings, variations in these parameters can substantially modify toxic responses, affecting both their magnitude and manifestation. Together, these findings suggest that current risk assessment frameworks, which often prioritize mortality endpoints and nominal concentrations, may underestimate ecological risks under more realistic environmental scenarios where multiple abiotic factors interact.

3.3 Chapter 3: Modulation of pesticide-induced behavioral effects in early zebrafish larvae by light, temperature and pH

Pesticides are extensively applied in agriculture to enhance crop productivity; however, their unintended environmental impacts have become an increasing focus of both public and scientific concern. Nicosulfuron and azoxystrobin, representing commonly used herbicides and fungicides, are

widely applied in modern farming and are frequently detected in aquatic systems, raising questions about their potential ecological effects.

The objectives of this study were to evaluate the potential hazards of these two pesticides on the behavior of zebrafish larvae under multifactorial exposure conditions. Specifically, variations in light regime, temperature, and pH were incorporated alongside multiple concentration levels. Behavioral responses were quantified using detailed locomotor parameters, including movement frequency, distance traveled, activity duration, swimming speed, and bout-related characteristics across different movement patterns. Aiming to (i) characterize the behavioral effects of nicosulfuron and azoxystrobin on zebrafish larvae, (ii) examine how environmental factors interact with pesticide exposure to modulate behavioral outcomes, and (iii) identify key drivers underlying the combined influence of chemical and environmental stressors.

In this work, the behavioral effects of the herbicide nicosulfuron and the fungicide azoxystrobin were assessed in larval stages of *Danio rerio* under combined environmental stressors. Exposure experiments were designed to include different pesticide concentrations together with variations in photoperiod (light versus dark), temperature, and pH. Locomotor performance was evaluated using several quantitative parameters, including movement frequency, total distance covered, activity duration, swimming velocity, as well as bout duration and bout distance within distinct movement categories.

Behavioral outcomes were markedly influenced by abiotic conditions. Dark phases consistently stimulated higher activity levels and made pesticide-related behavioral changes more apparent. High concentrations of nicosulfuron increased overall locomotion regardless of temperature or pH, whereas responses at moderate concentrations were dependent on the surrounding environmental context. In comparison, azoxystrobin-induced behavioral alterations were observed exclusively under acidic conditions during darkness, indicating a pronounced interaction with specific abiotic factors.

Overall, these findings underscore the limitations of single-factor toxicity assessments. To improve ecological relevance, future research should incorporate multifactorial experimental frameworks that reflect the simultaneous action of chemical and abiotic stressors in natural aquatic environments.

4. Discussion

4.1 Toxicity assessment of nicosulfuron

Our findings indicate that nicosulfuron exerts relatively low toxicity on zebrafish during early developmental stages, with effects largely confined to sublethal endpoints. Under optimal laboratory conditions (26 °C, pH 7), embryonic exposure to nicosulfuron caused minimal toxicity; even at concentrations as high as 1000 mg/L, no overt apical toxicity was detected, and only signs of proteotoxic stress were observed. However, when environmental parameters deviated from these optimal conditions — specifically under suboptimal temperature or pH — the adverse effects became more evident. Changes in pH and temperature were associated with elevated embryonic heart rate and accelerated hatching, suggesting that environmental stressors can amplify the biological impact of nicosulfuron.

Current knowledge regarding the toxicity of nicosulfuron remains limited. Available data are primarily based on formulated products rather than the pure active ingredient. For example, studies on *Oncorhynchus mykiss* reported a 96 h LC_{50} of 55.6–100 mg formulation/L (equivalent to 2.2–4.0 mg nicosulfuron/L) and a 28-day no observed effect concentration (NOEC) of 10 mg nicosulfuron/L (EFSA, 2007). In aquatic invertebrates such as *Daphnia magna*, the 48 h EC_{50} was 82.3 mg formulation/L (3.3 mg nicosulfuron/L), with a 21-day NOEC of 5.2 mg nicosulfuron/L (EFSA, 2007). Discrepancies between these results and our observations may stem from two key factors: the use of commercial formulations rather than analytical-grade nicosulfuron, and longer exposure durations in previous studies.

Behavioral assays further demonstrated that nicosulfuron induces neurobehavioral alterations in zebrafish larvae, characterized by significantly increased locomotor activity and hyperexcitability. Comparable responses have been documented in other aquatic species. In *Carassius auratus*, nicosulfuron exposure enhanced burst swimming and shoaling behavior, while increased swimming velocity was reported in the larvae of amphibian *Bufo spinosus* (Bretaud et al., 2000; Saglio et al., 2003; Cheron et al., 2023). Despite these consistent behavioral patterns across taxa, the underlying molecular and physiological mechanisms responsible for nicosulfuron-induced neurostimulation

remain unresolved.

When assessing the interaction between nicosulfuron concentration and pH, we observed that under both light and dark conditions, most behavioral endpoints in larvae exposed to 0 mg/L or 1000 mg/L were largely unaffected by pH variation, indicating that concentration was the dominant determinant of toxicity at these levels. In contrast, at the intermediate concentration (100 mg/L), behavioral outcomes became more sensitive to pH shifts, displaying patterns that aligned either with control-like or stress-associated responses. This suggests a threshold-dependent effect: once nicosulfuron exceeds a certain concentration, its toxic action predominates and may override environmental modulation by pH.

Temperature-driven behavioral variation followed the same general trend observed in the azoxystrobin–temperature trial. Larvae reared under elevated temperatures were more likely to display short, high-intensity burst movements; however, their sustained swimming performance was reduced, presumably because of increased energetic expenditure. In contrast, individuals maintained at lower temperatures predominantly exhibited low- to moderate-speed swimming, leading to an overall decline in locomotor activity.

This response pattern aligns with the well-established optimal developmental temperature for zebrafish embryos and larvae, which is approximately 26 °C (Schnurr et al., 2013; Schweizer et al., 2017). Exposure to temperatures below these optimum compromises embryonic and larval survival. Although higher temperatures can stimulate metabolic processes and, in some cases, improve larval survival, they simultaneously elevate the energetic costs required to sustain normal physiological functioning (Osterauer, 2008; Schnurr et al., 2013; Wang et al., 2023).

The toxicity of nicosulfuron to zebrafish embryos is mainly reflected in sublethal effects, particularly behavioral toxicity. In this study, the lowest observed effect concentration (LOEC, 100 mg/L) was far higher than the maximum concentrations detected in aquatic environments (16 μg/L), indicating that the acute toxicity of nicosulfuron to zebrafish embryos is relatively low and the associated risk may also be minimal. However, the most sensitive species to nicosulfuron is the aquatic plant Lemna gibba (ETOX database, Umweltbundesamt, accessed 2026), with an EC50 (7 d) of 0.087 μg/L.

Currently, toxicity data for nicosulfuron are available for multiple species. According to the risk quotient (RQ) calculation method under the REACH regulation, using an assessment factor (AF) of 10, the predicted no-effect concentration (PNEC) is 0.0087 μg/L. The highest concentration detected in the environment is 16 μg/L, which results in an RQ value of 1839, far greater than 1, indicating an extremely high risk for this species. Such a high RQ value is mainly due to the very low EC50 of the most sensitive species and the use of the highest detected environmental concentration. These results suggest that the concentrations of nicosulfuron currently observed in surface waters may cause serious adverse effects on primary producers and potentially disrupt aquatic food webs.

Compared with nicosulfuron, other commonly used herbicides such as glyphosate and atrazine show higher toxicity to fish. The toxicity ranges for fish are 0.01–68.79 mg/L for glyphosate (Lopes et al., 2022) and 0.06–71 mg/L for atrazine (ANZECC & ARMCANZ, 2000; Khoshnood, 2024) both lower than that of nicosulfuron (2.2-90 mg/L, EFSA 2007; ETOX database, Umweltbundesamt, accessed 2026), indicating stronger toxicity than nicosulfuron. For aquatic plants, the toxicity ranges are 30 μg/L–360 mg/L for glyphosate (Klátyik et al., 2024) and 100–500 μg/L for atrazine (ANZECC & ARMCANZ, 2000; Ramírez Hernandez et al., 2025), which suggests that aquatic plants are much more sensitive to nicosulfuron (0.087–2.3 μg/L, ETOX database, Umweltbundesamt, accessed 2026).

Nicosulfuron is mainly used in maize and a few tolerant grass crops, but it is toxic to most broadleaf crops and many other plants. Therefore, when using pesticides, greater attention should be given to the composition of aquatic ecosystems near maize fields, especially in areas with abundant aquatic vegetation, where the use of nicosulfuron is not recommended. In areas where fish populations are higher and aquatic vegetation is abundant, nicosulfuron may be considered as a partial alternative to glyphosate and atrazine. Application rates should strictly follow control thresholds and, if possible, remain below the chronic NOEC for sensitive aquatic organisms.

4.2 Toxicity assessment of azoxystrobin

Azoxystrobin exhibited pronounced developmental and proteotoxic effects in zebrafish. At 1000 μg/L, exposure resulted in substantial mortality, severe morphological abnormalities, bradycardia,

and delayed development. Both temperature and pH clearly modified the intensity of these toxic responses, indicating that its hazard profile is strongly influenced by environmental conditions.

Under standard laboratory settings (pH 7, 26 °C), the calculated 96-h LC_{50} was 988.2 μg/L (95% CI: 987.7–988.7 μg/L). This estimate is comparable to previously reported values of 810 μg/L and 1230 μg/L (Jia et al., 2018; Jiang et al., 2019), while another study documented a lower LC_{50} of 488 μg/L (Zhang et al., 2020). The discrepancy likely reflects differences in test materials, as that particular study used a commercial formulation, whereas the others, including ours, applied high-purity (>95%) azoxystrobin. Consistent with earlier findings, concentrations below 200 μg/L did not markedly increase mortality (Cao et al., 2018), and in our experiments, low-concentration treatments (0–100 μg/L) exerted only minor effects on embryonic survival.

Mechanistically, azoxystrobin is known to disrupt mitochondrial respiration, thereby constraining cellular energy production (Rodrigues et al., 2017; Amador et al., 2024). In our study, modest but consistent elevations in zebrafish heart rate were observed at lower concentrations (10–100 μg/L) across all tested temperatures and pH levels. This response may represent a compensatory reaction to mitochondrial stress or a transient stimulation of cardiac metabolic activity (Osterauer, 2008; Schweizer et al., 2017). In contrast, exposure to 1000 μg/L led to a pronounced reduction in heart rate, likely reflecting severe mitochondrial dysfunction, ATP depletion, and oxidative damage in cardiac tissues (Jiang et al., 2019). Comparable cardiac suppression has been reported in other fish species exposed to mitochondrial inhibitors, including decreased embryonic heart rate in *Ctenopharyngodon idella* (Liu et al., 2013; Zhang et al., 2020).

Interestingly, elevated temperature appeared to mitigate azoxystrobin toxicity to some extent. Elevated temperatures are frequently linked to higher chemical toxicity because they can increase passive diffusion, promote active uptake, and raise oxygen demand. Together, these effects intensify metabolic stress and lead to greater accumulation of reactive oxygen species (Eckwert et al., 1997; Osterauer, 2008; Juroszek et al., 2022). In this thesis, the declined toxicity of azoxystrobin at elevated temperatures may be attributed to partially counterbalance azoxystrobin's inhibitory action on mitochondrial respiration, thereby modulating its overall toxic impact.

Behavioral toxicity in zebrafish larvae was highly context-dependent, with acidic conditions acting

as a critical trigger for abnormal responses. High-concentration exposure significantly reduced locomotor activity, consistent with impaired mitochondrial energy supply and diminished metabolic capacity. Notably, these effects were observed exclusively under dark conditions at pH 5, highlighting a strong acid-mediated component of toxicity. Because azoxystrobin remains predominantly non-ionized and maintains a stable logD between pH 5 and 9, the observed pH-related differences are unlikely to result from altered ionization state, hydrophobicity, or membrane permeability (Köhler et al., 2023; Kroll et al., 2024). Instead, they likely reflect physiological sensitivity to acidic stress (Castro et al., 2025).

At pH 5, although overall swimming performance declined with increasing azoxystrobin concentration, both movement distance and bout duration increased across swimming modes. This pattern suggests a strategic reallocation of energy: as mitochondrial inhibition limits ATP availability, larvae may reduce the frequency of movement initiation — thereby lowering the energetic costs associated with repeated start–stop activity — and instead adopt longer, sustained movement bouts (Di Santo and Goerig, 2025).

In this thesis, the LOEC (100 μg/L) was higher than the maximum concentrations detected in aquatic environments (29.7 μg/L), but potential environmental risks for fish should not be underestimated. The extensive and continuous use of pesticides may result in repeated inputs into aquatic ecosystems and increase the likelihood of short-term high-concentration exposure.

The most sensitive group to azoxystrobin is copepods, with a NOEC of 1 μg/L (Van Wijngaarden et al., 2014). Currently, toxicity data for azoxystrobin are available for multiple species. According to the RQ calculation method under the REACH regulation, using an AF of 10, the PNEC is 0.1 μg/L. The highest concentration detected in the environment is 29.7 μg/L, resulting in an RQ value of 297, which is far greater than 1 and indicates an extremely high risk for this species. These values suggest that the concentrations of azoxystrobin currently observed in surface waters may cause serious adverse effects on aquatic invertebrates and potentially disrupt aquatic food webs.

Azoxystrobin is mainly used to control powdery mildew, leaf spot diseases, and rust diseases. Tebuconazole and mancozeb are two fungicides that can be considered competitive alternatives. The toxicity ranges of tebuconazole and mancozeb to fish are 0.012–4.4 mg/L (EPA New Zealand, CCID

database, accessed 2026) and 0.008–13.2 mg/L (Simakani et al., 2018; Paganotto Leandro et al., 2023; Mendes et al., 2024), respectively, which are similar to the effect range of azoxystrobin (0.002–1.5 mg/L). For aquatic invertebrates, the toxicity ranges are 0.01–3.53 mg/L for tebuconazole (Tofan et al., 2023; EPA New Zealand, CCID database, accessed 2026) and 0.058–1.0 mg/L for mancozeb (EPA New Zealand, CCID database, accessed 2026), both of which are lower than that of azoxystrobin (0.001–0.28 mg/L, EPA New Zealand, CCID database, accessed 2026).

At present, most commonly used fungicides show relatively high toxicity to both fish and aquatic invertebrates, making it difficult to find a suitable substitute for azoxystrobin. Therefore, in practical pesticide application, the applied dosage should be kept as low as possible while still meeting crop protection requirements, and the potential effects of chronic toxicity thresholds on aquatic organisms should be considered. In addition, appropriate application timing and methods should be adopted, avoiding periods when rainfall or irrigation may cause runoff into nearby water bodies. Establishing buffer zones between agricultural land and surface waters can also help absorb or reduce pesticide runoff. Furthermore, alternating the use of biological pesticides and chemical pesticides may help reduce the overall pesticide load in the environment.

Summary

The findings reported in this thesis document that intricate interactions between pesticide exposure and abiotic environmental factors regulate toxicity exerted during the early developmental stages of zebrafish. Although the observed outcomes were primarily sublethal, manifestations such as abnormal behavior, altered heart rate, and premature hatching can disrupt the maintenance of normal physiological functions, growth and development, feeding efficiency, and predator avoidance. Over time, these disturbances may translate into sustained survival pressure. Under fluctuating environmental conditions, the risks to organismal fitness may become even more pronounced.

Our results showed that the influence of abiotic factors is multifaceted. Their role extends beyond simply intensifying or mitigating pesticide toxicity. More complex patterns may arise, including toxic responses that are triggered only under specific environmental settings, effects expressed through multi-level regulation (for instance, changes in neural excitability or metabolic thresholds),

and shifts in toxic stress from overt survival endpoints to subtler yet ecologically meaningful physiological imbalances. Moreover, the primary drivers of behavioral responses may vary depending on pesticide concentration. The strength and nature of behavioral effects differ across environmental contexts, and distinct behavioral alterations may ultimately lead to different ecological consequences.

In the context of the continued widespread use of pesticides worldwide and the broader concerns framed by the planetary boundaries concept, incorporating environmental realism into toxicological evaluation becomes imperative. Future risk assessment should combine environmental monitoring with multifactorial experimental designs and mechanistic modeling approaches to better reflect field conditions. Strengthening the ecological relevance of pesticide evaluation is not only scientifically necessary but also fundamental for the effective protection of freshwater ecosystems and the conservation of biodiversity.

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Part II: Own contribution to the work conducted for those in this thesis

Publication 1: Li, Z.; Köhler, H.-R.; Triebskorn, R. Proteotoxicity and apical toxicity of nicosulfuron to *Danio rerio* embryos: a comprehensive assessment at different temperatures and pH. Pollutants 2024, 4, 359-372. https://doi.org/10.3390/pollutants4030025

Rita Triebskorn and I contributed to the overall conceptualization. I was responsible for the research design of this study, and contributed data curation, formal analysis and the creation of figures and tables. I also prepared the original draft of the manuscript on this work. The validation of the results was carried out jointly by me, Rita Triebskorn, and Heinz Köhler to ensure the scientific rigor and reliability of the findings. The manuscript was reviewed and edited collaboratively by all three authors. Rita Triebskorn provided academic supervision throughout the research process, contributing significantly to the refinement of the study framework and the overall quality of the work. I estimate my own contribution to this work at 90%.

Publication 2: Li, Z.; Köhler, H.-R.; Triebskorn, R. Environmental Drivers of Pesticide Toxicity: Temperature and pH Shift Azoxystrobin's Effects on Zebrafish (*Danio rerio*) Early Development. Environments 2025, 12, 334. https://doi.org/10.3390/environments12090334

Rita Triebskorn and I contributed to the overall conceptualization. I was responsible for the research design of this study, and contributed data curation, formal analysis and the creation of figures and tables. I also prepared the original draft of the manuscript on this work. The validation of the results was carried out jointly by me, Rita Triebskorn, and Heinz Köhler to ensure the scientific rigor and reliability of the findings. The manuscript was reviewed and edited collaboratively by all three authors. Rita Triebskorn provided academic supervision throughout the research process, contributing significantly to the refinement of the study framework and the overall quality of the work. I estimate my own contribution to this work at 90%.

Publication 3: Li, Z.; Köhler, H.-R.; Dechent, B.; Triebskorn, R. Modulation of pesticide-induced behavioral effects in early zebrafish larvae by light, temperature and pH

Rita Triebskorn and I contributed to the overall conceptualization. I was responsible for the research design of this study, and contributed data curation, formal analysis and the creation of figures and tables. I also prepared the original draft of the manuscript on this work. The validation of the results was carried out jointly by me and Rita Triebskorn to ensure the scientific rigor and reliability of the findings. The manuscript was reviewed and edited collaboratively by all five authors of the publication. Bianca Dechent provided methodological assistance. Heinz Köhler contributed analysis, tables, and writing related to the multiple linear regression model. Rita Triebskorn provided academic supervision throughout the research process, contributing significantly to the refinement of the study framework and the overall quality of the work. I estimate my own contribution to this work at 85%.

Part III: Publications included in this thesis

Publication 1: 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 branchedchain 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

Properties	
Molecular Formula	$C_{15}H_{18}N_6O_6S$
Molecular Weight	410.4 g/mol
Solubility in water	7400 mg/L (at pH 7, 25°C)
log P_{ow} (at pH 5)	0.35
log P_{ow} (at pH 7)	1.8
log P_{ow} (at pH 9)	-2.0
pK_a	4.60

Note: Data is 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 $MgSO_4 \cdot 7\ H_2O$ and 11.76 g/L $CaCl_2 \cdot 2\ H_2O$ 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).

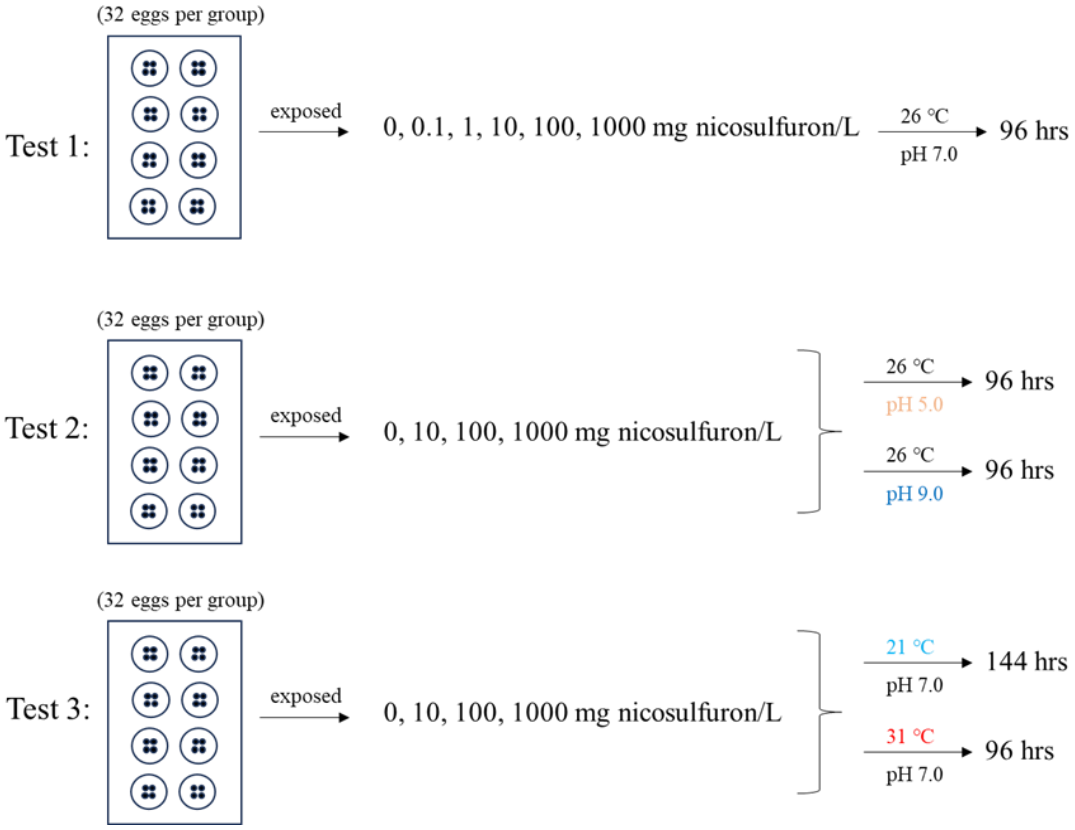

Figure 1. Flow chart of the embryo test. Note: The different exposure times depended on the hatching of zebrafish embryos. For those that remained unhatched for more than 96 h, the exposure was extended by 48 h.

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 2 Endpoints examined at different time points during the 96 hours of fish embryo test

Endpoints	12 hpf	24 hpf	48 hpf	60 hpf	72 hpf	96 hpf
Mortality	✓	✓	✓	✓	✓	✓
Developmental Delays [1]		✓				
Heart rate			✓			
Hatching success				✓	✓	✓
Malformations [2]						✓

Note: 1. No somites, non-detachment of the tail and no development of the eyes
2. Oedema, 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 (Fig. 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 (Fig.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. 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 (Fig. 2). At pH 7.0, there was no significant difference in heart rate among any of the groups ($p > 0.05$).

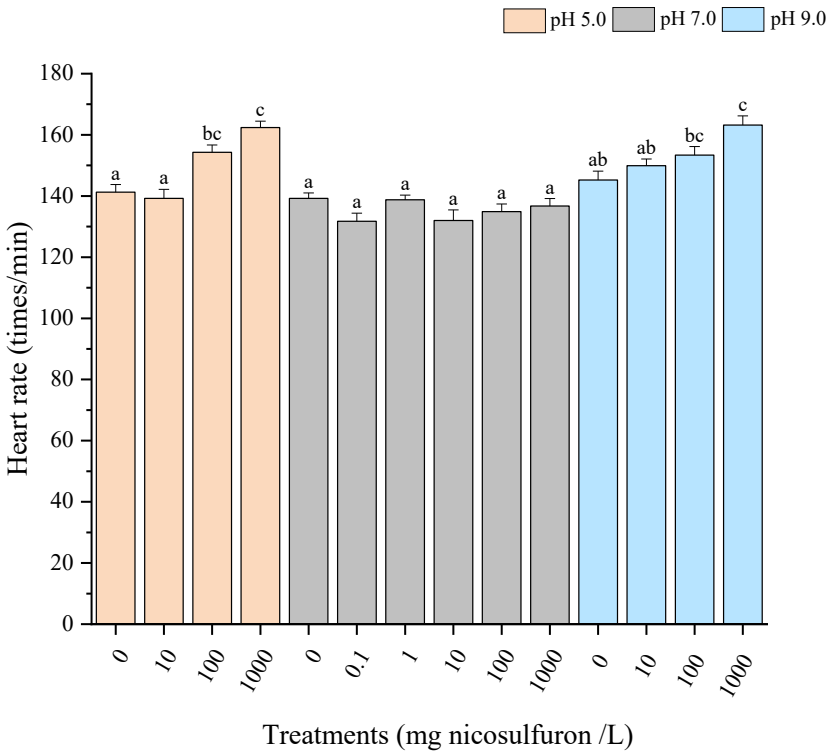

Fig. 2. The effect of nicosulfuron on the heart rate of zebrafish embryos at different pH
Note: Values are mean ± SE. Different letters indicate significant difference ($P < 0.05$)

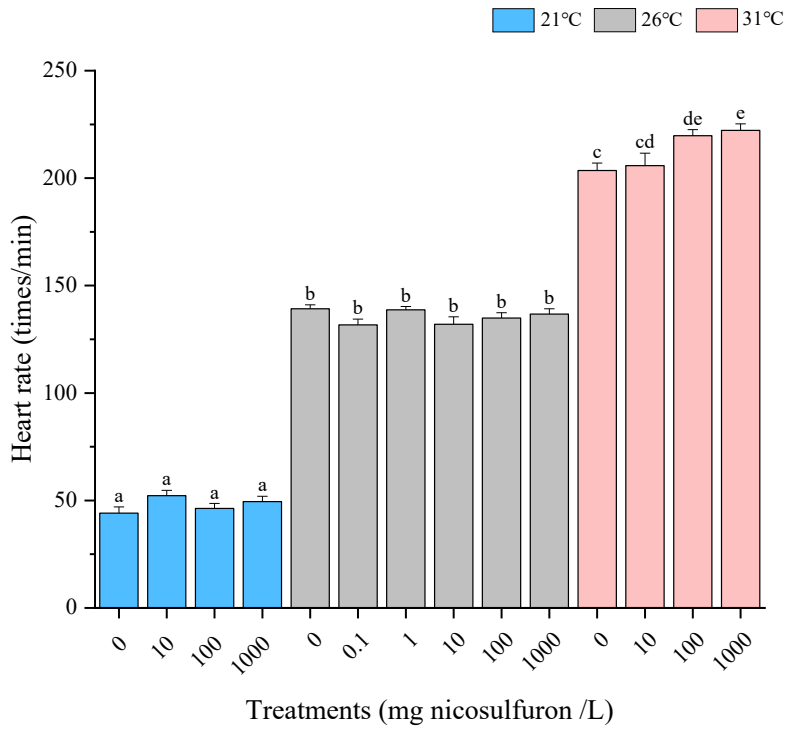

Fig. 3. The effect of nicosulfuron on the heart rate of zebrafish embryos at different temperature
Note: Values are mean ± SE. Different letters indicate significant difference ($P < 0.05$)

The hatching rate of embryos is shown in Fig. 4, Fig. 5 and Fig. 6. In the 26°C, pH 7.0 group, no significant difference ($P > 0.05$) was found among all groups at 72 hpf (Fig. 4). At 96 hpf, the hatching rate in the 1000 mg/L group was significantly higher ($P < 0.05$) than that of all other groups, except for the 100 mg/L group.

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 (Fig. 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 hatching rate increasing with nicosulfuron concentration (Fig. 6). At 21°C, no hatching embryos were found until 144 hpf, and there was no significant difference among all groups at 144 hpf ($P > 0.05$).

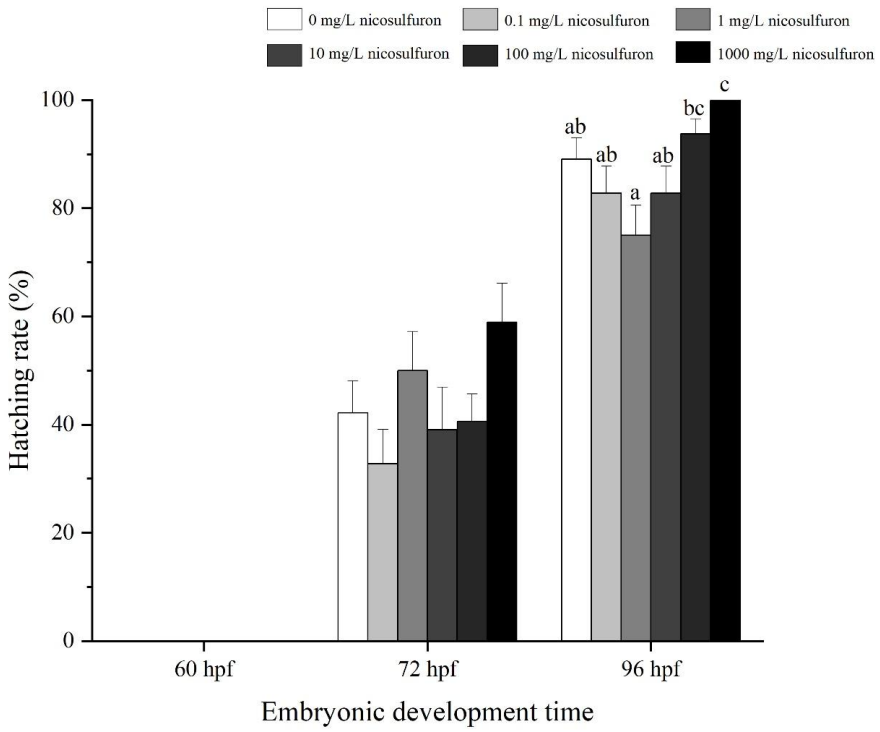

Fig. 4. The effect of nicosulfuron on the hatching rate of zebrafish embryo (26°C, pH 7.0)
Note: Values are mean ± SE. Different letters indicate significant difference ($P < 0.05$)

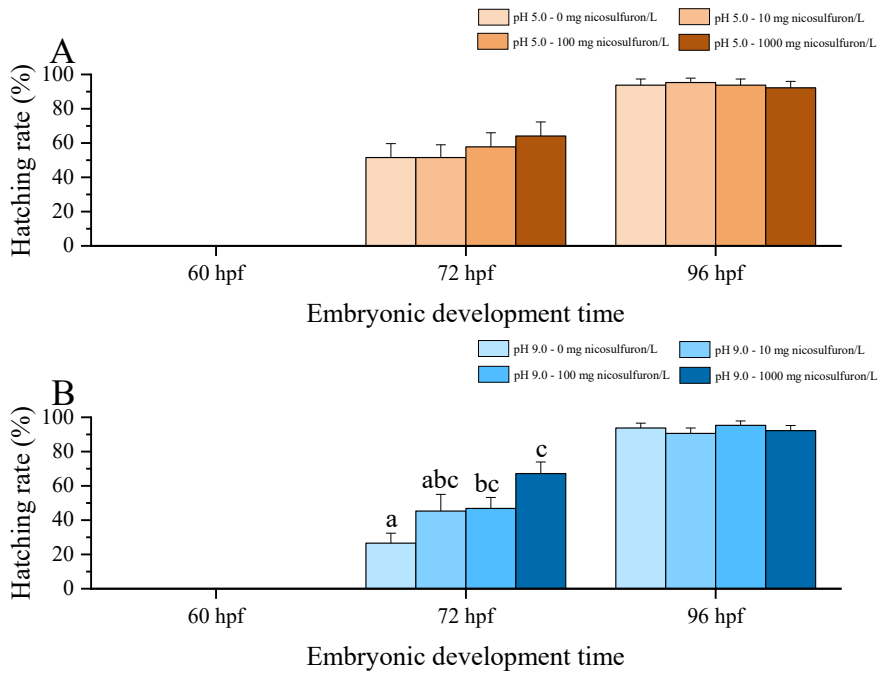

Fig. 5. The effect of nicosulfuron on the hatching rate of zebrafish embryo at pH 5.0 (A) and pH 9.0 (B)
Note: Values are mean ± SE. Different letters indicate significant difference ($P < 0.05$). The plot is darker, indicating a higher concentration of nicosulfuron. From low to high, 0, 10, 100 and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

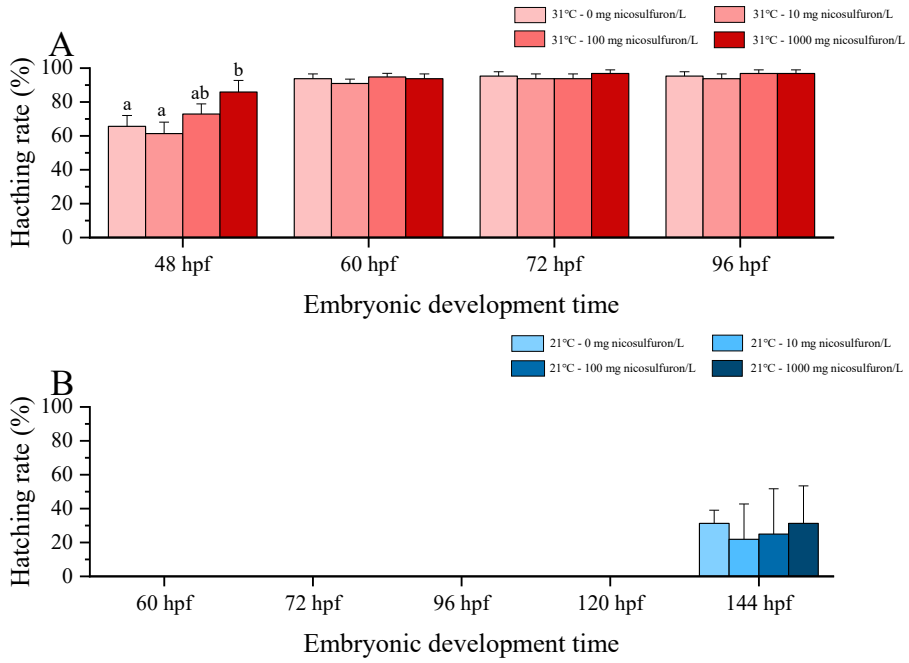

Fig. 6. The effect of nicosulfuron on the hatching rate of zebrafish embryo at 31°C (A) and 21°C (B)
Note: Values are mean ± SE. Different letters indicate significant difference ($P < 0.05$). The plot is darker, indicating a higher concentration of nicosulfuron. From low to high, 0, 10, 100 and 1000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

The Hsp70 level of fish embryos is shown in Fig. 7 and Fig. 8. At 26°C, pH 7.0, the lowest Hsp70 level was found in the 0 mg/L group (Fig. 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 ($P < 0.05$).

At 31°C, there was no significant difference ($P > 0.05$) in Hsp70 level among all groups (Fig. 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, Hsp70 levels showed the same significance and trend as observed at 21°C.

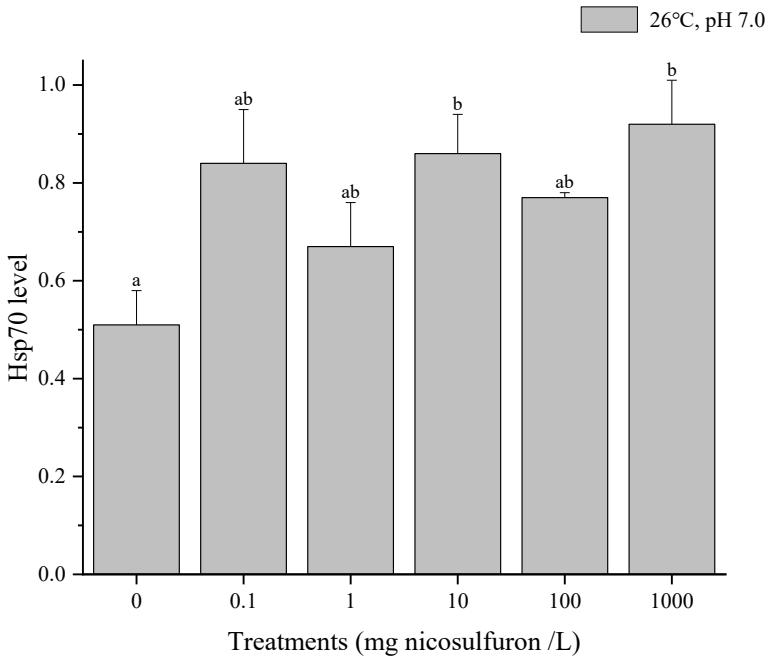

Fig. 7. Relative Hsp70 level of zebrafish embryos at 26°C, pH 7.0.
Note: Values are mean ± SE. Different letters indicate significant difference ($P < 0.05$)

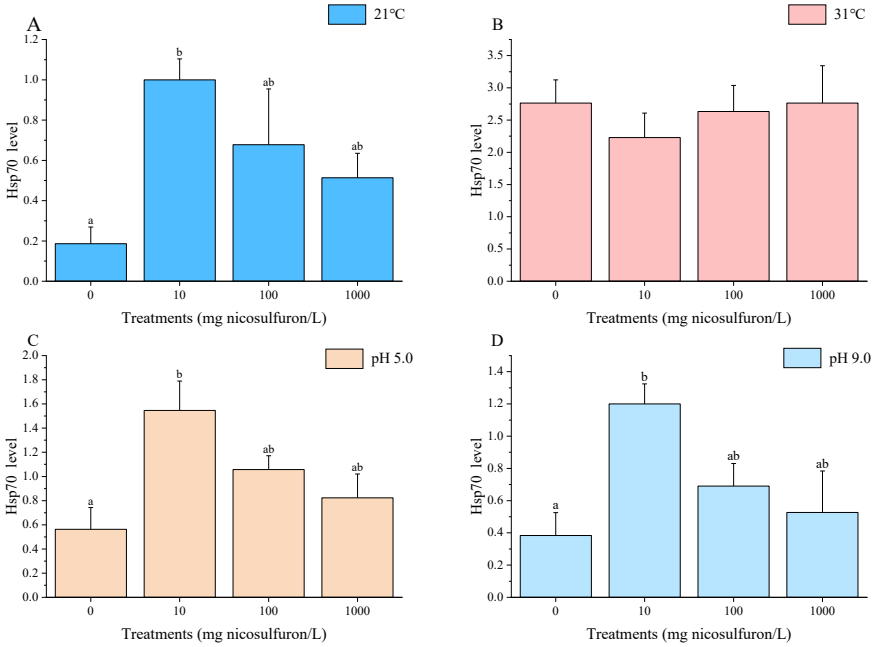

Fig. 8. Relative Hsp70 level of zebrafish embryos at 21°C (A), 31°C (B), pH 5.0 (C) and pH 9.0 (D)
Note: Values are mean ± SE. Different letters indicate significant difference (P < 0.05)

4. Discussion

To our knowledge, the present work is the first to report the effect of nicosulfuron on *Daino rerio* embryos. Survival rates indicate that nicosulfuron does not impact the survival of zebrafish embryos at different pH levels or temperatures. Zebrafish is a tropical fish and its 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 butyrylcholines-terase-like protein in zebrafish which may have the potential to degrade nicosulfuron and protect zebrafish embryos from the harmful influence of nicosulfuron [33].

Toxicity data for nicosulfuron formations in fish and aquatic invertebrates are as follows: *Oncorhyncus mykiss* with a 96 h LC_{50} of 55.6–100 mg formulation/L (2.2–4.0 mg nicosulfuron/L) and 28-day NOEC (No Observed Effect Concentration) of 10 mg nico-sulfuron/L [9]. Acute toxicity data for nicosulfuron to aquatic invertebrates are available for *Daphnia magna* with 48 h EC_{50} 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 nico-sulfuron 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 that significant differences are not detectable.

Similarly to heart rate, few significant differences were found in the hatching rate. However, the increase in hatching rate by 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 temperature 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 ex-periment, 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. In-stead, it can be attributed to the toxic stimulation induced by nicosulfuron exposure.

Possible reasons why 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 existing 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 envi-ronment, 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 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 it into three stages (Fig.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].

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 that 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.

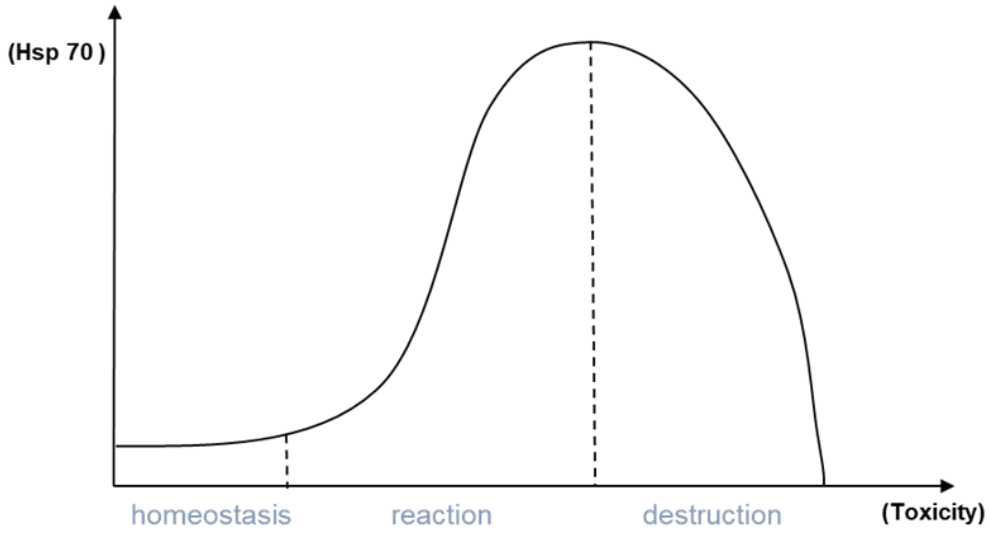

Fig. 9. Response kinetics of Hsp70 with rising toxicity according to Eckwert et al. (1997)

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 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, 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 monooxygenases (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 are 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 was 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 loses its indicative power when the organism´s physiology is dominated by a concomitant heat shock.

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Supplementary Material

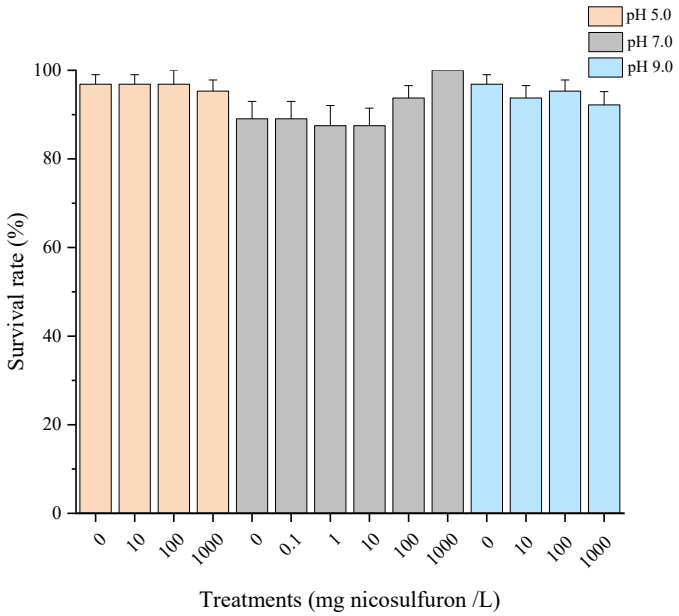

Fig. S1. The effect of nicosulfuron on the survival rate of zebrafish embryos 96hpf at different pH

Note: Values are mean ± SE. No letters indicate no significant difference ($P > 0.05$)

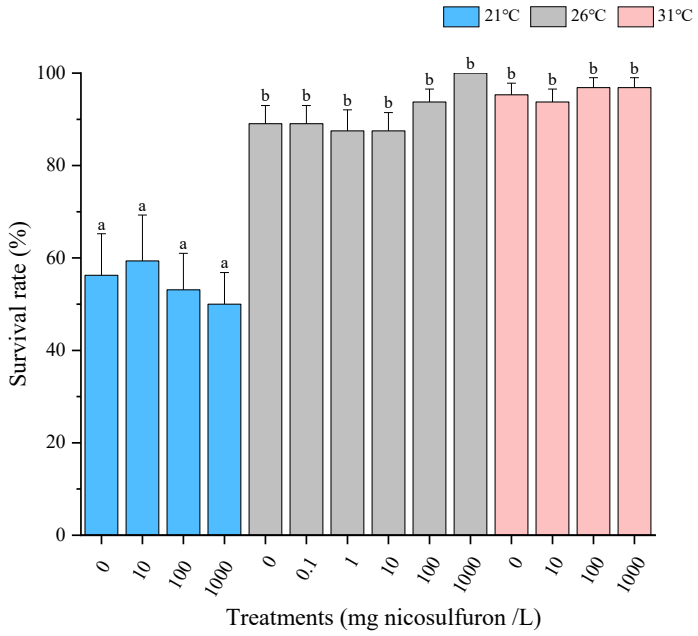

Fig. S2. The effect of nicosulfuron on the survival rate of zebrafish embryos 96 hpf at different temperature

Note: Values are mean ± SE. Different letters indicate significant difference ($P < 0.05$)

Publication 2: Environmental drivers of pesticide toxicity: temperature and pH shift azoxystrobin's effects on zebrafish (*Danio rerio*) early development

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Abstract

Azoxystrobin, a widely used strobilurin fungicide, poses a potential risk to aquatic ecosystems due to its frequent detection in surface waters. Although its toxicity to non-target organisms has been extensively studied under standardized conditions, few investigations have considered how environmental factors can modulate the adverse effects of this chemical. In this study, we examined the toxicity of azoxystrobin to zebrafish (*Danio rerio*) embryos under different pH (5, 7, 9) and temperature (21 °C, 26 °C, 31 °C) conditions. Embryos were exposed to azoxystrobin concentrations ranging from 0 to 1000 μg/L, and endpoints such as survival, hatching rate, heart rate, malformations, developmental delay, and Hsp70 expression were assessed over 96 h post-fertilization. Our results demonstrate that azoxystrobin induces significant malformations (including edema, eye, tail, and spinal defects) and developmental delays at 1000 μg/L across all environmental conditions. Furthermore, both pH and temperature were found to modulate azoxystrobin toxicity: elevated temperature and alkaline pH partly alleviated mortality at high concentrations. The hsp70 expression patterns revealed complex interactions between the effects of the chemical and environmental factors. These findings highlight the importance of incorporating environmental variables into ecotoxicological risk assessments of pesticides to better reflect realistic exposure scenarios and potential ecological impacts.

Keywords: fungicide; fish; confounding factor

1. Introduction

The importance of fungicides as widely used chemicals with high sales volumes and their potential impact on target and non-target organisms was recently addressed in an article by Mohr et al. 2025 [1]. Azoxystrobin is a strobilurin fungicide widely applied to control fungal diseases in crops. As a broad-spectrum, systemic, and soil-applied fungicide, azoxystrobin is extensively used in agriculture worldwide [2,3]. However, its widespread application has raised concerns regarding contamination of the aquatic environment. Due to its persistence, mobility, and the associated relevance for humans and the environment, azoxystrobin was added to the EU Water Framework Directive watch list in 2022 and is therefore regularly monitored in European surface and groundwaters (EU, 2022). Currently, azoxystrobin concentrations in surface water vary from 0.06–11.10 µg/L in industrialized countries such as the United States, France, Germany, Greece, China and Brazil, with peak levels reaching up to 29.7 µg/L [4–7]. In groundwater, concentrations between 0.01 and 0.1 µg/L were found [8,9]. Azoxystrobin inhibits the respiration chain in fungi by blocking the electron transfer from quinol to cytochrome c1, thereby reducing ATP production [10,11]. However, evidence suggests that it can also induce toxicity in a wide range of non-target aquatic animals [5]. For example, azoxystrobin has been reported to reduce survival, to delay growth and to impair reproduction in Daphnia [12,13]; hinder tadpole development [5]; and affect behavior and oxidative stress of zebrafish [7,14].

Despite extensive research that has documented the toxicity of pesticides like azoxystrobin, most chemical toxicity assessments are conducted under standardized laboratory conditions that do not reflect the complexity of natural environments [15,16]. In reality, abiotic factors such as temperature and pH vary considerably across habitats and can modulate both the action of pollutants and the physiological responses of organisms [17,18,19]. For instance, temperature influences metabolic rates and chemical uptake, while pH can affect the chemical speciation and stability of contaminants [17,18,20,21]. Therefore, ignoring environmental variability may result in inaccurate estimates of toxicity, undermining the ecological relevance of laboratory-based risk assessments.

The zebrafish (*Danio rerio*) has emerged as a widely used model organism in biological and environmental research [22–24]. Its early life stages, particularly embryos, are widely used for

toxicity testing because they are cost-efficient, quick, and comply with animal welfare principles as non-protected organisms [25,26]. These tests leverage the transparency of zebrafish eggs, allowing for direct observation of developmental effects like malformations. Since 2013, the OECD has officially recommended zebrafish embryo toxicity testing for environmental risk assessments [27].

In this study, our aim is to perform a hazard assessment under controlled laboratory conditions, focusing on identifying the developmental toxicity of azoxystrobin in zebrafish embryos and assess whether this toxicity is modulated by environmental factors, specifically temperature and pH. To this end, we examined both apical endpoints (e.g., survival, hatching, malformations) and molecular responses (e.g., Hsp70 expression) under varying environmental conditions. This approach is designed to identify potential hazards and mechanistic interactions, rather than to provide a quantitative environmental risk assessment.

2. Materials and Methods

2.1 Test Substance

Azoxystrobin is a lipophilic organic ionizable compound whose lipophilicity re-mains stable between pH 4 and pH 14. Relevant physicochemical information is pro-vided in supplementary Table S1 and Figure S1.

A stock solution of azoxystrobin (CAS131860-33-8, purity ≥ 98%, Merck, Darmstadt, Germany) was prepared by dissolving it in reconstituted water composed of 25 mL stock solutions of 0.23 g/L KCl, 2.59 g/L $NaHCO_3$, 4.93 g/L $MgSO_4 \cdot 7\ H_2O$ and 11.76 g/L $CaCl_2 \cdot 2\ H_2O$ and 900 mL double distilled water. Other test concentrations were obtained by stepwise dilution of the stock solution.

The pH of each test solution was measured using a pH meter (SevenCompactDuo; Mettler Toledo, Gießen, Germany), and adjusted by 1M HCl or 1M NaOH as needed.

2.2 Test organisms

Zebrafish (Danio rerio, USA Westaquarium strain) were maintained in 200 L tanks at the Animal Physiological Ecology section, University of Tübingen, Germany. Fish were fed three times daily with commercial flake food (TertraMin ®, Tetra GmbH, Melle, Germany), and feces were removed

prior to feeding. A water change (change 30%-50%) was performed weekly. All procedures followed permission granted from the Animal Welfare Committee of the Regional Council of Tübingen, Germany (approval number ZO 2/16 and ZO 02/21 G).

The tanks were kept under a controlled 12-hour light/12-hour dark photoperiod. Water quality was regularly monitored, with temperature maintained at 26 ± 1 °C, pH at 7.4 ± 0.2, total hardness ranging from 8 to 12 °dH, and dissolved oxygen at 100 ± 5% saturation. Nitrate and nitrite levels were consistently below 1 mg/L and 5 mg/L, respectively.

2.3 Fish Embryo Test

Three separate experiments were conducted to assess azoxystrobin toxicity under varying pH and temperature conditions (Figure 1). Experiment 1: Embryos were exposed to 0, 0.1, 1, 10, 100, 1000 µg/L azoxystrobin at 26 °C, pH 7. As there was no apparent effect of 0.1 and 1 µg/L azoxystrobin in test 1, we decided to investigate only azoxystrobin concentrations of higher than 1 µg/L (0, 10, 100, 1000 µg/L) in subsequent tests. Experiment 2: With focus on pH effects, embryos were exposed to azoxystrobin at pH 5 and 9, maintaining the temperature at 26 °C. Experiment 3: With focus on tem-perature effects, embryos were exposed to azoxystrobin at 21 °C and 31 °C, maintaining pH constant at 7.

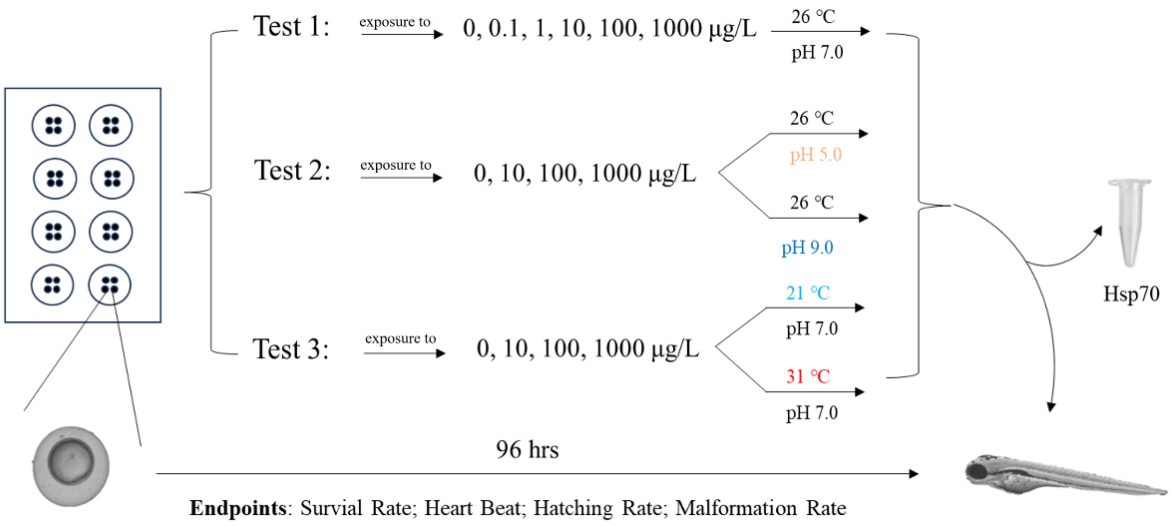

Figure 1. Flow chart of the experimental design

The Early Life Stage (ELS) tests followed our previous work based on OECD 236 [23,24].

Spawning and test setups were prepared one day prior to the start of the experiment. Breeding boxes included 1.5 mm mesh grids and artificial seagrass to stimulate spawning. Eggs were collected one hour after light onset and placed in Petri dishes containing the appropriate test solutions for a 2-hour pre-exposure at the respective experimental temperature.

For each test concentration, eight small Petri dishes (30 mm in diameter) were prepared, each containing 3 mL of the corresponding solution, along with one larger dish (90 mm in diameter) holding 30 mL. Embryos at the 128–256 cell stage were randomly chosen and allocated to the small dishes, with four embryos per dish, resulting in a total of 32 embryos per experimental group. Incubation lasted for 96 h under the designated temperature. Each experiment was conducted in two independent runs, with 32 embryos per treatment group per run. This design follows the OECD Test Guideline 236 and exceeds the recommended minimum sample size, thereby providing sufficient statistical power to detect significant effects.

Embryonic development was monitored using a stereo microscope (Stemi 2000-C; Zeiss, Oberkochen, Germany) at 12, 24, 48, 60, 72, and 96 hours post-fertilization (hpf). Endpoints included survival, developmental delay, heart rates, hatching success, and morphological abnormalities (Table 1). Coagulated embryos were recorded and removed throughout. Heart rates were recorded over 20 seconds and extrapolated to beats per minute. Hatching was monitored at 60 and 96 hpf. To better reflect the teratogenic potential while avoiding bias from high mortality, malformation rates were calculated based on surviving embryos only. This metric is hereafter referred to as the "malformation rates."

Table 1 Endpoints observed at various periods during the 96 hours of fish embryo test

Endpoints	12 hpf	24 hpf	48 hpf	60 hpf	72 hpf	96 hpf
Survival rate	☑	☑	☑	☑	☑	☑
Developmental delays [1]		☑				
Heart rates			☑			
Hatching success				☑		☑
Malformations [2]						☑

[1] No somites, non-detachment of the tail and no development of the eyes

[2] Oedema, eye defects, tail defects, deformation of the spine and light pigmentation

2.4 Hsp70 Determination

For Hsp70 analysis, 40 freshly laid fertilized zebrafish eggs were allocated per group and incubated for 96 hours. After exposure, 10 embryos were pooled per sample, frozen in liquid nitrogen, and stored at −80 °C (3 replicates for each group). Hsp70 levels were measured following the protocol of Vincze et al. (2014) [28]. Briefly, samples were homogenized in 20 µL extraction buffer (80 mM potassium acetate, 4 mM magnesium acetate, 20 mM Hepes, pH 7.5). The homogenates were then centrifuged at 20,000×g for 10 minutes at 4 °C, and the resulting supernatants were collected for analysis. The total protein concentration was determined according to Bradford (1976) [29], and 20 µg of protein per sample was separated via SDS-PAGE (80 V for 30 min, then 120 V for ~90 min). Immunostaining was performed using a monoclonal mouse α human anti-body (Santa Cruz Biotechnology, clone 3A3) and a secondary horse radish peroxidase-coupled goat α mouse IgG (H+L) antibody (Dianova). Bands were visualized, digitized, and analyzed via densitometry (Herolab E.A.S.Y., Wiesloch, Germany). Hsp70 expression was normalized to two internal adult *Danio rerio* standards on the same blotting membrane.

Representative SDS-PAGE images of Hsp70 are provided in Supplementary Figure S2. Although some bands appear diffuse, all quantifications were based on densitometric analysis of raw images to ensure consistency and reproducibility.

2.5 Statistical Analysis

All statistical analyses were conducted using SAS JMP version 16.0. Results are expressed as means ± standard error (SE). Normality and homogeneity of variances were tested before statistical tests. If assumptions were met, one-way ANOVA followed by Tukey's HSD post hoc test was used. If not, the non-parametric Steel–Dwass test was applied. LC_{50} values were determined by non-linear regression analysis using Table-Curve software for azoxystrobin treatments at pH 7 and 26 °C.

3. Results

3.1 Survival Rate

Figures 2 and 3 show the survival rates of zebrafish embryos exposed to azoxystrobin under different

pH (Figure 2) and temperature conditions (Figure 3). At pH 5, pH 7, and pH 9, embryos exposed to 1000 μg/L azoxystrobin exhibited the lowest survival rates, significantly lower than all other treatment groups ($p < 0.05$). At pH 9, the survival rate in the 1000 μg/L group was significantly higher than that at pH 7 ($p < 0.05$).

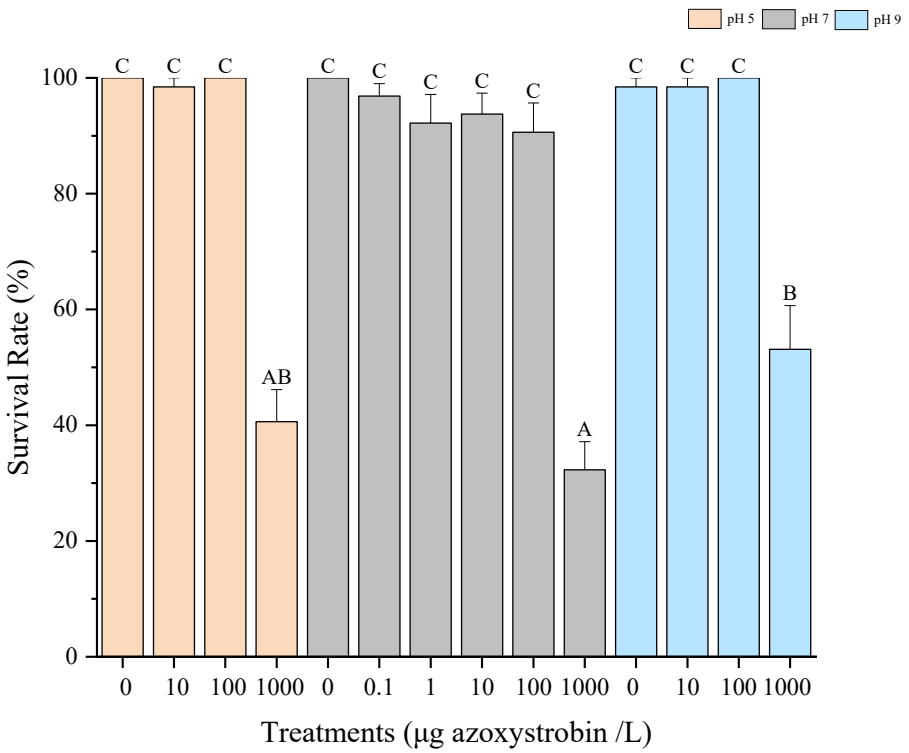

Figure 2. Survival of zebrafish embryos exposed to azoxystrobin at different pH values. Values are means ± SE. Different letters indicate a significant difference ($p < 0.05$)

At 21 °C, the survival rates of all groups were significantly lower than those exposed at 26 °C or 31 °C. In addition, the survival rate in the 1000 μg/L group was significantly lower than in the control group ($p < 0.05$). No significant differences were observed between the 100 and 1000 μg/L groups and the 0 μg/L group ($p > 0.05$).

At 26 °C and 31 °C, the 1000 μg/L treatment groups consistently showed significantly lower survival rates than other treatment groups ($p < 0.05$). The survival rate of the 1000 μg/L group at 31 °C was significantly higher than that at 26 °C ($p < 0.05$).

3.2 Heart Rates

Heart rates responses at different pH levels are shown in Figure 4. At pH 5, pH 7, and pH 9, heart rates followed non-significant non-linear trends, initially increasing and then declining with rising azoxystrobin concentrations (Figure 4). At 1000 µg/L, the heart rates at pH 5 and pH 9 were significantly higher than those at pH 7 ($p < 0.05$).

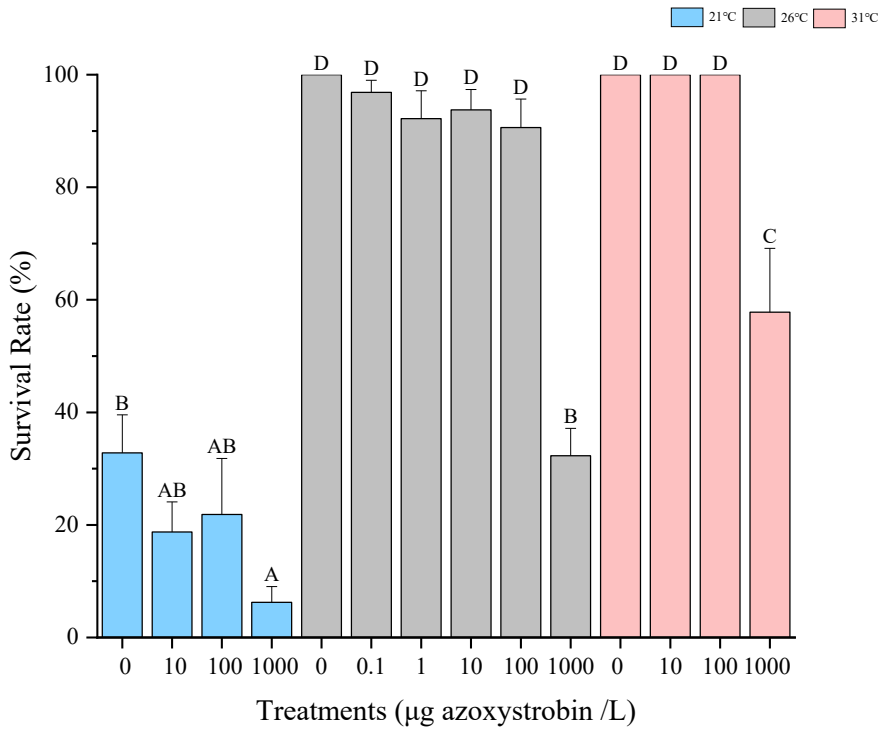

Figure 3. Survival of zebrafish embryos exposed to azoxystrobin at different temperatures. Values are means ± SE. Different letters indicate a significant difference ($p < 0.05$)

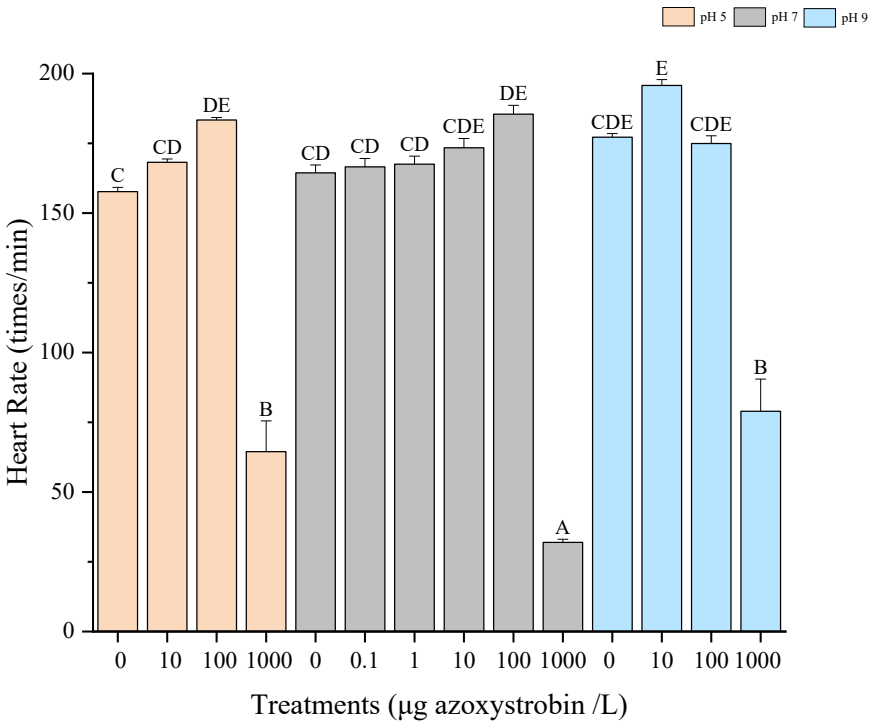

Figure 4. Heart rates of zebrafish embryos exposed to azoxystrobin at different pH values. Values are means ± SE. Different letters indicate a significant difference ($p < 0.05$)

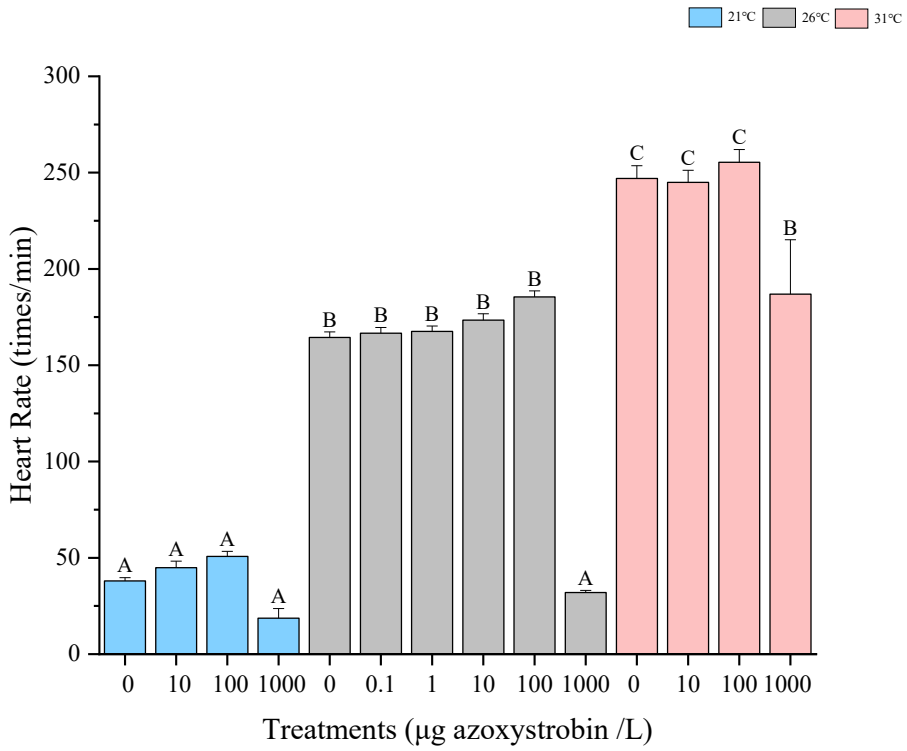

Figure 5. Heart rates of zebrafish embryos exposed to azoxystrobin at different temperatures. Values are means ±

SE. Different letters indicate a significant difference ($p < 0.05$)

Heart rates significantly increased in control group with rising temperature ($p < 0.05$). Across all temperature, the heart rates were significantly lower in embryos exposed to 1000 µg/L compared to the respective other exposure groups at the same temperature (Figure 5). No significant differences were observed between control and exposure groups at 21 °C ($p > 0.05$).

3.3 Hatching Rates

The effect of azoxystrobin on the hatching rates of zebrafish embryos at different pH and temperature are exhibited in Supplementary Figures S3 and S4. Across all temperature and pH conditions, embryonic development in the 1000 µg/L treatment groups was consistently delayed compared to the control at the same time points (Figure S3 and S4) ($p < 0.05$). No embryos hatched within 96 hpf at 21 °C in any treatment group.

3.4 Malformation

As shown in Figure 6, malformation rates were significantly elevated ($p < 0.05$) in the 1000 µg/L treatment groups under all pH and temperature conditions.

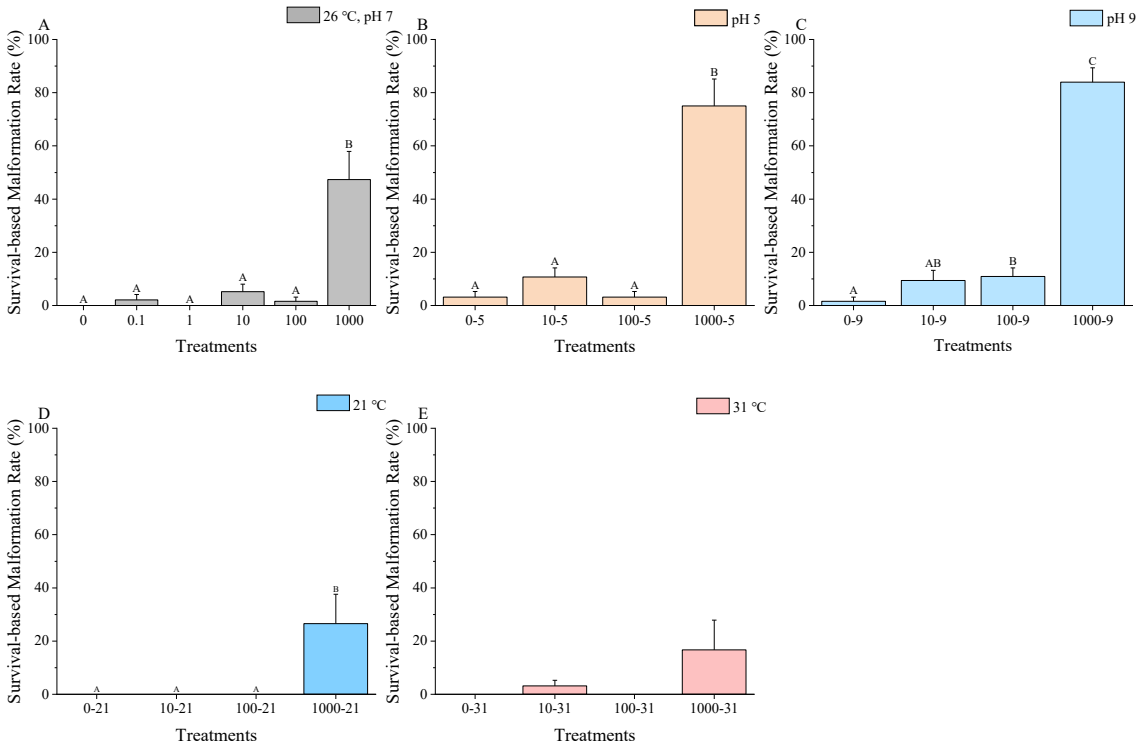

Figure 6. Malformation rate of zebrafish embryos exposed to azoxystrobin at different pH and temperatures. Values are means ± SE. Different letters indicate a significant difference ($p < 0.05$)

Embryonic development from 24 to 96 hpf is shown in Figure 7-8 and Supplementary Figures S5-S9. At 96hpf, all 1000 µg/L groups exhibited varying malformations, including edema, ocular abnormalities, tail deformities, and spinal curvature (Figure 7-8). At 21 °C, embryos in the 1000 µg/L group showed complete developmental arrest within 96 hpf (Figure 7-8 and Figures S5-S9). Images of embryos from the lower concentration groups are not shown, as their morphology appeared similar to that of the control group, with no visible malformations.

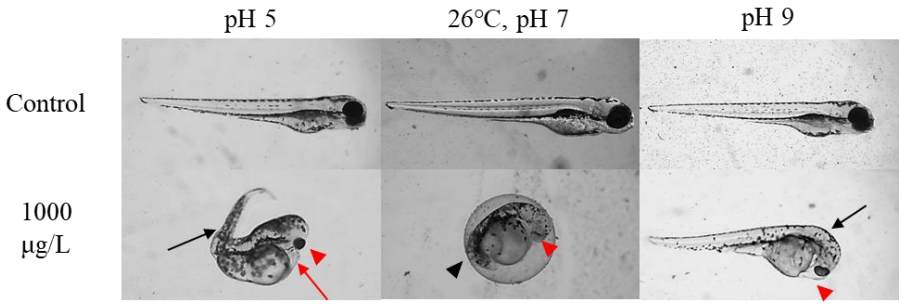

Figure 7. Embryonic development in the 0 and 1000ug/L azoxystrobin treatment groups at 96 hpf at different pH. Red arrows mark edemas, red triangles eye defects, black arrows spinal curvature and black triangles mark tail

defects.

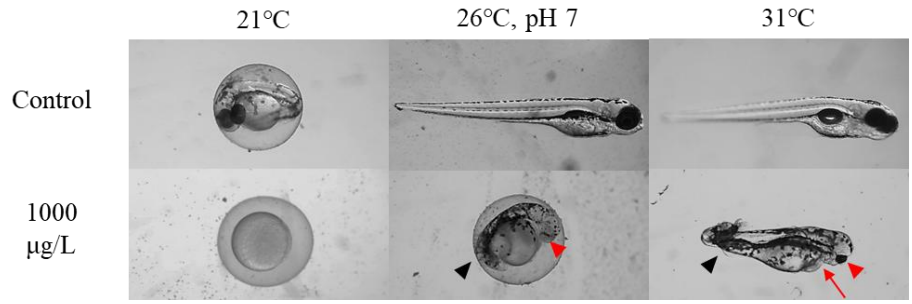

Figure 8. Embryonic development in the 0 and 1000ug/L azoxystrobin treatment groups at 96 hpf at different temperatures. Red arrows mark edemas, red triangles eye defects, and black triangles mark tail defects.

3.5 Hsp70

Figures 9 and 10 show the Hsp70 expression patterns in zebrafish embryos. At 26 °C and pH 7, Hsp70 levels initially increased and then decreased with rising azoxystrobin concentrations. Hsp70 expression in the 100 μg/L and 1000 μg/L groups was significantly lower than in the 0.1 μg/L group ($p < 0.05$). At pH 5, Hsp70 expression remained unchanged across all treatments with a trend to lower values after exposure to 1000 μg/L ($p > 0.05$). At pH 9, Hsp70 levels declined with increasing azoxystrobin concentration, and the 1000 μg/L group exhibited significantly lower expression than the control group ($p < 0.05$). At both 21 °C and 31 °C, there were no significant differences in Hsp70 expression between the treatment groups ($p > 0.05$). This section may be divided by subheadings. It should provide a concise and precise description of the experimental results, their interpretation, as well as the experimental conclusions that can be drawn.

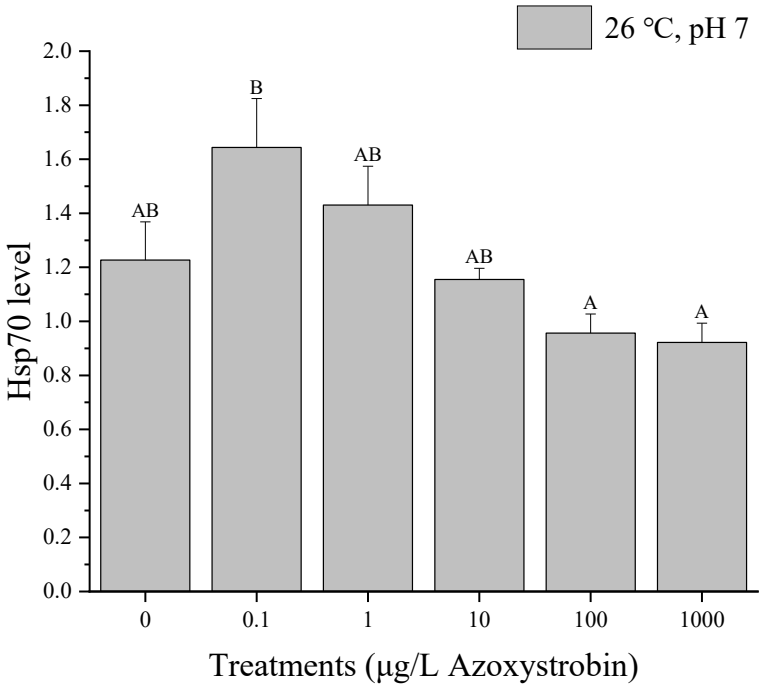

Figure 9. Hsp70 level of zebrafish embryos exposed to azoxystrobin at 26 °C, pH 7. Values are means ± SE. Different letters indicate a significant difference ($p < 0.05$)

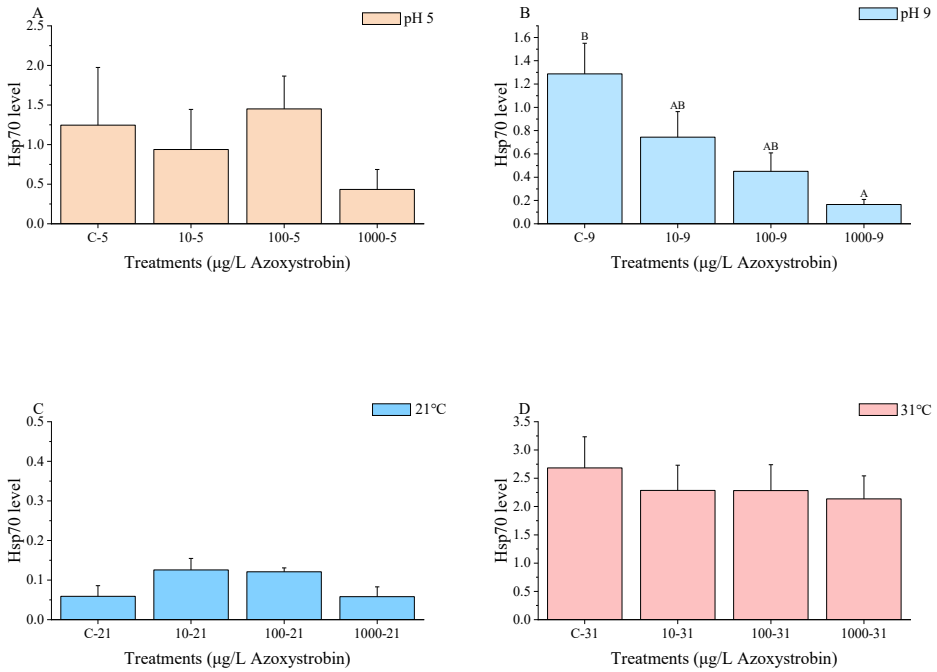

Figure 10. Hsp70 level of zebrafish embryos exposed to azoxystrobin at different pH and temperatures. Values are

means ± SE. Different letters indicate a significant difference ($p < 0.05$)

4. Discussion

The current study investigated the impact of varying temperature and pH conditions on apical toxicity and proteotoxicity of azoxystrobin in zebrafish embryos. Our data resulted in the calculation of a 96 h-LC_{50} value of 988.2 (95% confidence interval from 987.7 to 988.7) µg/L at pH 7 and 26 °C. This value is similar to the findings of Jia et al. (2018) and Jiang et al. (2019) [30-31], who reported the 96 h-LC_{50} of zebrafish embryos exposed to azoxystrobin as 810 µg/L and 1230 µg/L, respectively. Zhang et al. (2020) reported a lower 96 h-LC_{50} of 488 µg/L [32]. This discrepancy may stem from the fact that Zhang et al. (2020) used a commercial product, whereas all other studies, including ours, employed azoxystrobin of ≥ 95% purity. In addition, Cao et al. (2018) reported no significant increase in mortality in groups exposed to less than 200 µg/L azoxystrobin, which aligns with our findings that have shown low concentrations (0-100 µg/L) of azoxystrobin to exert only limited effects on zebrafish embryo survival [33].

Azoxystrobin is known to inhibit mitochondrial respiration, thereby reducing metabolic activity [5,11]. Notably, we observed a biphasic effect of azoxystrobin on heart rates. At the lower concentrations (10–100 µg/L), a moderate increase in heart rates was observed for all temperatures and all pH values tested, possibly due to compensatory mitochondrial stress responses or hormetic stimulation of cardiometabolic activity [18,23]. At the highest concentration (1000 µg/L), however, heart rates were significantly decreased, likely attributable to severe mitochondrial dysfunction, energy depletion, and oxidative damage to cardiac tissues [31]. Similarly, decreased heart rates in embryos and grass carp (*Ctenopharyngodon idella*) caused by cardiac depressants have been ob-served in the studies of Liu et al. (2013) and Zhang et al. (2020) [32,34].

Azoxystrobin has also been shown to inhibit embryonic development, as evidenced by delayed hatching and reduced growth. For instance, Cao et al. (2018) and Kumar et al. (2020) found shortened post-developmental body lengths, while Jiang et al. (2019) and Zhang et al. (2020) reported delayed hatching and decreased hatching success [11,31-33]. Consistently, our study revealed a negative impact of azoxystrobin on the hatching rate and the embryonic development. These developmental impairments are likely related to insufficient energy and reduced metabolism.

Azoxystrobin inhibits the mitochondrial complex III by binding to the Qo site of cytochrome b, disrupting electron transport and reducing proton gradient formation. This leads to impaired oxidative phosphorylation and decreased ATP production [13,32]. Zhang et al. (2020) also provided data which are likely to be related to this mechanism. They observed reduced yolk depletion in exposed embryos, which might also be indicative of decreased energy utilization resulting from impaired ATP production [32].

Even though sublethal concentrations will not cause immediate mortality, they can result in population-relevant effects as e.g. delayed development, reduced mobility, and prolonged vulnerability to predation [34,35]. Moreover, energy deficits during early life stages may compromise reproductive capacity in later life stages [2,14].

Furthermore, our study observed morphological abnormalities in embryos such as oedema, eye defects, spinal curvature, and tail defects, indicating the teratogenic potential of azoxystrobin. These abnormalities are likely associated with the production of excessive reactive oxygen species (ROS) and the induction of apoptosis, which may involve mitochondrial dysfunction among other pathways [13,32]. Jia et al. (2018) also reported azoxystrobin (1500 μg/L) to cause morphological abnormalities to zebrafish embryos including tail defects, edema and spinal curvature [30]. In our study, significant morphological changes were absent at medium to low concentrations (≤ 100 μg/L), which is consistent with Zhang et al. (2020) and Kumar et al. (2020) [11,32]. Similarly, Jiang et al., 2019 found that teratogenic effects appeared only at concentrations ≥ 500 μg/L [31].

The Hsp70 family is a well-known and highly conserved class of stress proteins that plays essential roles in maintaining cellular homeostasis. It assists in the refolding of misfolded proteins, inhibits apoptosis and is indirectly involved in the regulation of ROS levels [36–38]. Since azoxystrobin causes mitochondrial dysfunction, oxidative stress, and apoptosis [13,32], it is reasonable to infer that Hsp70 may be upregulated during embryo exposure to azoxystrobin as part of a cellular defense mechanism. Specifically, Hsp70 likely contributes to preserving proteostasis, protecting cellular structures, and facilitating mitochondrial stress recovery.

The cellular Hsp70 response to increasing chemical toxicity can be broadly divided into three phases. In the initial phase, cells maintain homeostasis, and Hsp70 expression remains at baseline or only

marginally elevated. During the second phase, activation of the stress response leads to a rapid increase in Hsp70 levels, culminating in peak expression. In the final phase, when toxic exposure surpasses the cellular tolerance threshold, pathological impairment of the protein synthesis machinery is likely to occur, resulting in a subsequent decline in Hsp70 expression [24,39]. At 26 °C, pH 7.0, the expression pattern of Hsp70 in response to azoxystrobin exposure aligns well with its typical stress-induced response pattern [24,39]. Hsp70 levels initially increased with rising azoxystrobin concentrations, but declined at higher concentrations. This probably occurs between the reaction and destruction stages [24,39]. Specifically, cells respond to toxicity by sharply upregulating Hsp70 expression. When the toxicity of the azoxystrobin exceeds the cellular tolerance threshold, it may lead to structural and functional damage to the protein synthesis machinery, thereby reducing Hsp70 expression. Notably, in our study, Hsp70 showed a rapid response to azoxystrobin already at a very low concentration (0.1 μg/L), indicating its potential as a highly sensitive and early biomarker for proteotoxicity in aquatic organisms.

Temperature-dependent changes in azoxystrobin toxicity were evident in this study. At elevated temperatures (31 °C), an antagonistic effect was observed compared to 26 °C. Specifically, in the 1000 μg/L group at 31 °C, the survival rate increased significantly, the heart rates were elevated and equal to the normal level, the hatching rate increased, the deformity rate was reduced, and the animals hatched earlier than at 26 °C. No significant differences in Hsp70 levels were observed across the concentration groups at 31 °C. Elevated temperatures are often associated with increased chemical toxicity, as they can enhance passive diffusion, stimulate active uptake, and elevate oxygen demand — factors that collectively promote metabolic stress and ROS accumulation [18,40,24]. Nonetheless, this trend is not consistent across all compounds or organisms. In this study, azoxystrobin toxicity decreased at higher temperatures, suggesting temperature-dependent mitigation. To minimize potential methodological artifacts, a 2-hour acclimation period was included prior to the onset of formal exposure. During this period, embryos were maintained at the respective experimental temperature in the corresponding incubators before selection. Therefore, the temperature-dependent toxicity observed is unlikely to result from abrupt thermal shock. One possible explanation for the alleviation of toxicity at higher temperatures is the result of physiological adaptations. Elevated temperatures can accelerate metabolic processes, potentially

enhancing mitochondrial oxidative capacity and ATP production, which may counteract the respiratory inhibition caused by azoxystrobin [18,24,40]. Cominassi et al. (2022) reported an increased metabolic rate with increased acclimation temperature in the threespine stickleback (*Gasterosteus aculeatus*) [42]. Similarly, Voituron et al. (2022) reported that acclimation to high temperature improved mitochondrial efficiency (on average >15%) of sea bass (*Dicentrarchus labrax*) juveniles, and improved ATP/O efficiency in European sea bass mitochondria at 26 °C, compared to 18–22 °C [43]. In addition, heat acclimation upregulates antioxidant enzymes (e.g., SOD, CAT), heat shock proteins (e.g., Hsp70), and other pathways to enhance ROS scavenging capacity [43]. In contrast, at 21 °C, we observed a marked increase in toxicity across all treatment groups: survival rates declined, Hsp70 levels were suppressed, and embryonic development was largely arrested. Moreover, within both the high (31 °C) and low (21 °C) temperature treatments, there were no significant differences among the groups that had been exposed to different concentrations of azoxystrobin, suggesting that temperature had a more dominant effect than the azoxystrobin concentration under these, for *D. rerio*, extreme conditions.

Compared to temperature, the effect of pH on azoxystrobin toxicity was less pronounced and showed no clear gradient-dependent pattern. In our study, the survival rate in the 1000 μg/L treatment group increased to varying degrees when pH changed from neutral to acidic or alkaline conditions; however, sublethal effects were significantly exacerbated under both conditions, such as increased malformation rates, altered Hsp70 expression, and accelerated heart rate. This indicates a possible toxicity shift from lethal to sublethal effects on embryo development under pH modulation, whereby embryos experience more biologically meaningful developmental and physiological disruptions. This shift is also supported by Bittner et al. (2018) and Schweizer et al. (2019) [41,45]. In their zebrafish embryo acute toxicity experiments, LC_{50} levels increased, and LOECs of heart rates, a sublethal concentration indicator, decreased as pH changed from neutral to acidic. The phenomenon was more pronounced at pH 9 in our study. In addition to the increase in malformation rate, we also observed a faster heart rate response to azoxystrobin and an earlier destructive stage of Hsp70 at pH 9 compared with that at pH 7.

Based on the logD and chemical structure of azoxystrobin (Figure S1), the com-pound remains

non-ionized between pH 5 and pH 9, indicating that pH does not affect its ionization state or hydrophobicity. Therefore, the observed variations in toxicity across different pH conditions are unlikely to be due to altered uptake or membrane permeability [15,16,20]. Interestingly, elevated heart rates were observed at both pH 5 and pH 9. In one of our previous studies, we also found that both acidic and alkaline external environments increased the heart rates of zebrafish embryos exposed to pesticides [24]. These results suggest that pH does not alter azoxystrobin chemistry directly, but rather modifies the physiological state of embryos. However, this compensation may come at the cost of enhanced oxidative stress, as ATP production and ROS generation are tightly coupled. At high azoxystrobin concentrations, the collapse of proteostasis further indicates that the combined stress of chemical exposure and pH imbalance overwhelms cellular defense capacity.

Although this study was designed as a hazard assessment rather than a quantitative risk assessment, the observed sublethal impairments (e.g., delayed development, altered cardiac function, reduced proteostatic capacity) may translate into population-relevant outcomes, including reduced recruitment, impaired growth, and com-promised reproductive success [46,47]. Such effects, while not immediately lethal, can ultimately affect population dynamics by increasing predation vulnerability and lowering lifetime fitness. These findings suggest that current risk assessment frameworks (e.g., OECD fish embryo tests, EU WFD guidelines), which often emphasize lethality and nominal exposure concentrations, may underestimate ecological hazards under realistic environmental conditions where multiple abiotic stressors act simultaneously [46-48].

Moreover, organisms in natural ecosystems are rarely exposed to a single stressor. Factors such as oxygen fluctuations, temperature variability, pH shifts, and co-occurring pesticides act simultaneously and may interact in non-additive ways [49,50]. Our results show that even single abiotic variables like temperature and pH can shift toxicity thresholds, implying that combined stressors in the field may further modulate toxic responses. To better capture such complexity, future assessments could benefit from factorial laboratory designs and mechanistic modeling approaches, integrated with environmental monitoring, to improve the ecological realism of pesticide hazard evaluation [51].

5. Conclusions

This study reveals that the toxicity of azoxystrobin to zebrafish embryos is not only concentration-dependent, but also influenced by confounding environmental factors such as temperature and pH. While traditional assessments typically rely on optimal laboratory conditions, our findings demonstrate that deviations in environmental parameters can significantly reshape the toxicity, altering both the severity and the nature of toxic effects.

Importantly, our findings highlight that environmental parameters, especially temperature and pH, can significantly alter the toxicity of azoxystrobin. Elevated temperature (31 °C) alleviated toxicity, possibly through enhanced mitochondrial efficiency and upregulation of stress-response mechanisms, whereas low temperature (21 °C) aggravated toxic outcomes. pH changes from neutral to acidic or alkaline conditions, despite not affecting the chemical's ionization or uptake, caused a toxicity shift, reducing mortality but intensifying sublethal developmental impairments, particularly at pH 9. This suggests that abiotic factors can redirect toxic pressure from survival endpoints toward more subtle but ecologically significant physiological disruptions. These sub-lethal effects, though frequently overlooked, can have ecologically meaningful consequences by impairing individual fitness and population stability.

From an ecotoxicological perspective, these results provide valuable hazard assessment insights. They highlight that environmental parameters can substantially modulate toxic pressure, underscoring the need to account for realistic abiotic variability in toxicity testing. While our study does not constitute a full risk assessment, it supplies mechanistic and hazard-level evidence that can improve the ecological relevance of future risk evaluations. Incorporating such context-dependent responses into regulatory frameworks (e.g., OECD FET, EU WFD) will enhance predictive accuracy and ensure that pesticide hazards are more reliably characterized under changing environ-mental conditions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/doi/s1, Figure S1: title; Table S1: title; Video S1: title.

Author Contributions: Conceptualization, Z.L.; methodology, Z.L.; software, Z.L.; validation, Z.L.,

R.T. and H.-R.K.; formal analysis, Z.L.; data curation, Z.L.; writing—original draft preparation, Z.L.;

writing—review and editing, Z.L., R.T. and H.-R.K.; supervision, R.T.; funding acquisition, Z.L.

All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data are contained within the article or Supplementary Mate-rials.

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Conflicts of Interest: The authors declare no conflicts of interest.

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Supplementary Material

Table S1: Physicochemical properties of azoxystrobin

Molecular Formula	$C_{22}H_{17}N_3O_5$
Molecular Weight	403.4 g/mol
Solubility in water	6 mg/L (at 20 °C)
log P_{ow} (at 20 °C)	2.5
pK_a	not applicable

Data obtained according to Pubchem (NIH, National library of Medicine)

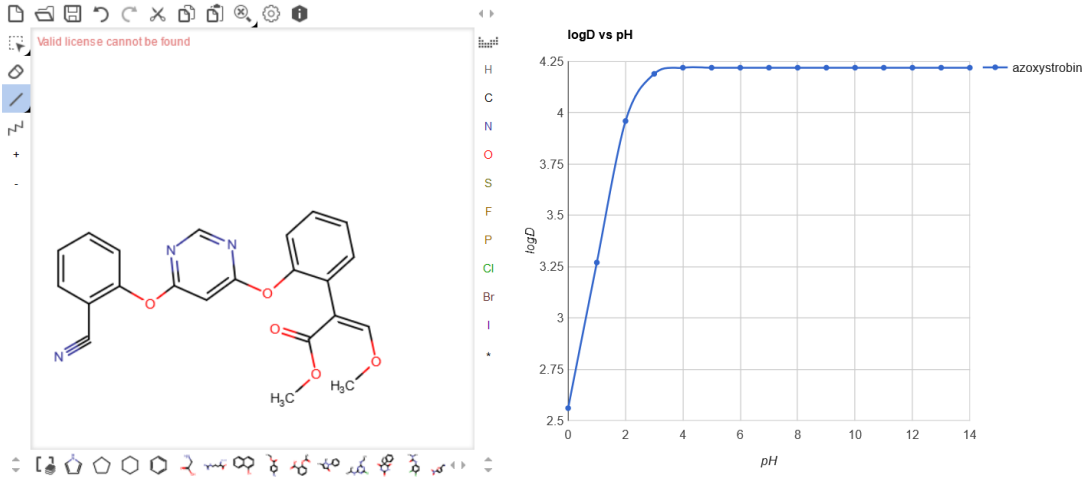

Fig. S1. Chemical structure and logD of azoxystrobin.
Partitioning was calculated by the logD predictor of Chemaxon (open source).

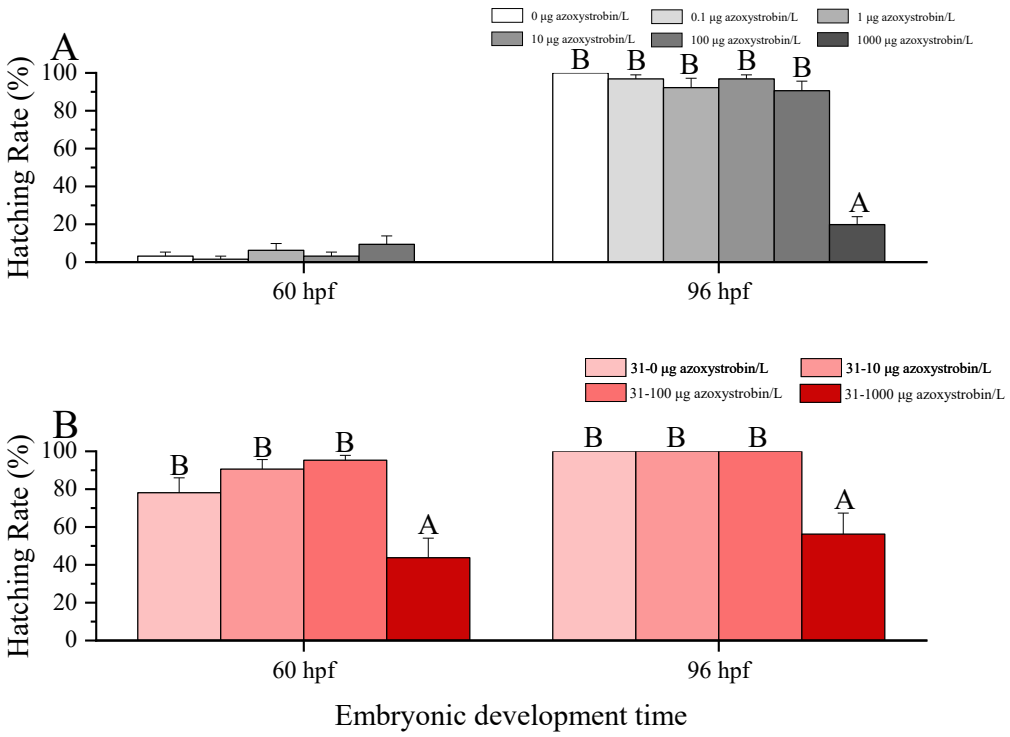

Fig.S2. The effect of azoxystrobin on the hatching rate of zebrafish embryos at 26 ℃ and 31 ℃.

Values are mean ± SE. Different letters indicate a significant difference ($P < 0.05$)

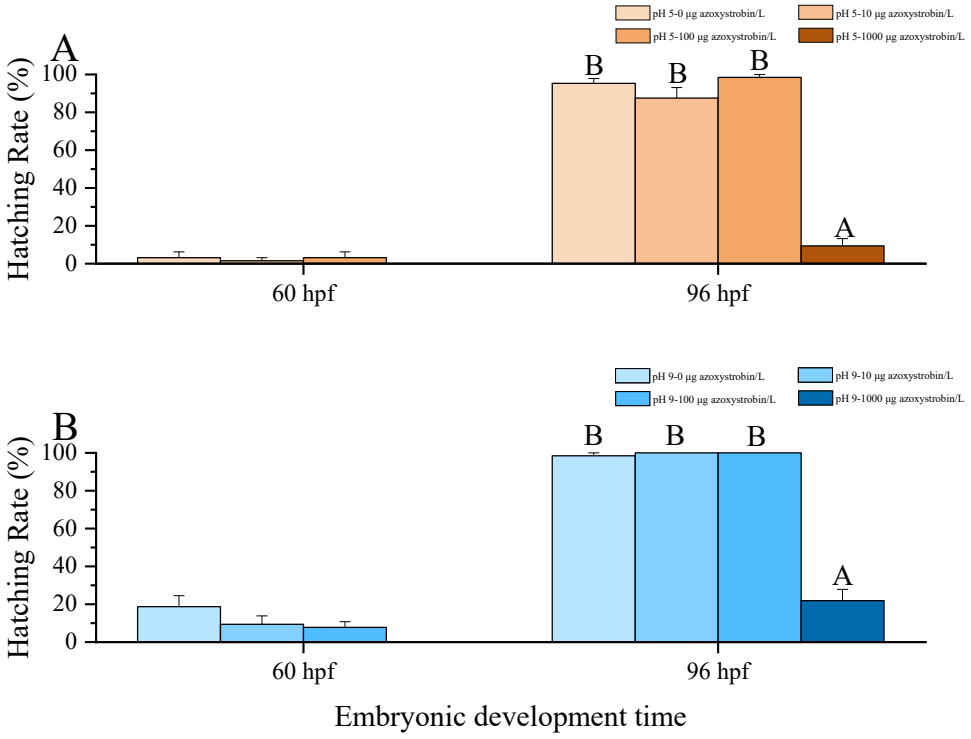

Fig.S3. The effect of azoxystrobin on the hatching rate of zebrafish embryos at different pH. Values are means ± SE. Different letters indicate a significant difference ($P < 0.05$)

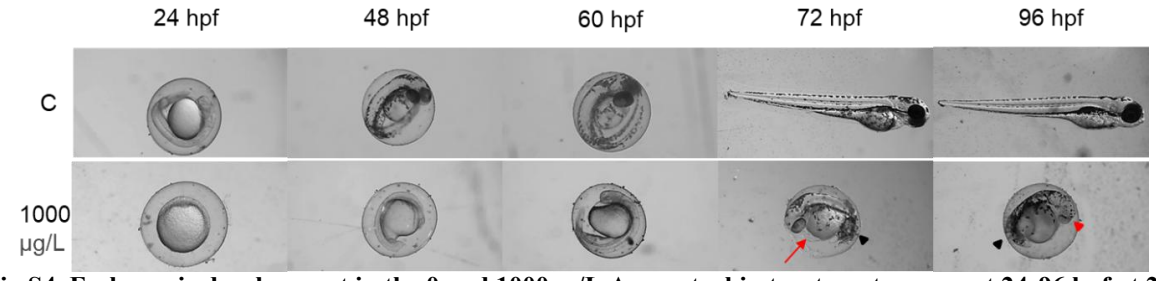

Fig.S4. Embryonic development in the 0 and 1000ug/L Azoxystrobin treatment groups at 24-96 hpf at 26℃, pH 7.
Red arrows mark oedemas, red triangles eye defects, black arrows spinal curvature and black triangles mark tail defects.

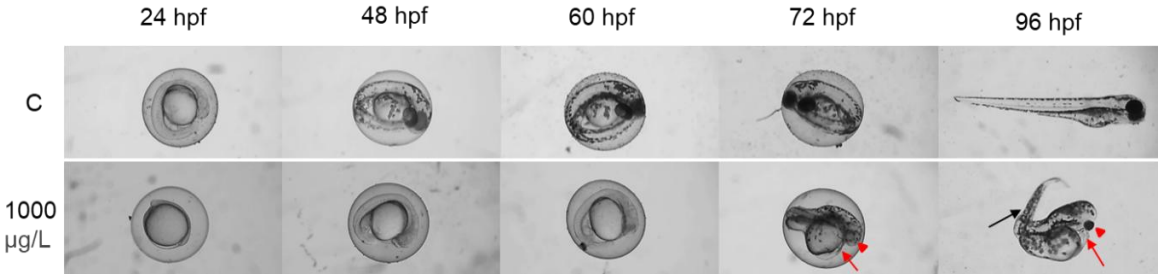

Fig.S5. Embryonic development in the 0 and 1000ug/L Azoxystrobin treatment groups at 24-96 hpf at pH 5.
Red arrows mark oedemas, red triangles eye defects, black arrows spinal curvature and black triangles mark tail defects.

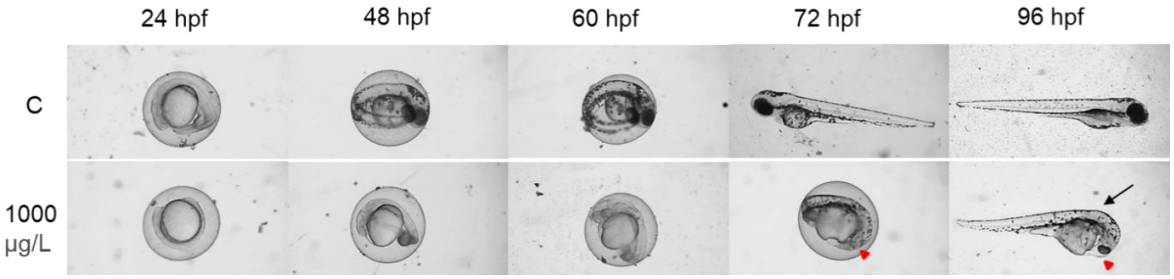

Fig.S6. Embryonic development in the 0 and 1000ug/L Azoxystrobin treatment groups at 24-96 hpf at pH 9.
Note: Red arrows mark oedemas, red triangles eye defects, black arrows spinal curvature and black triangles mark tail defects.

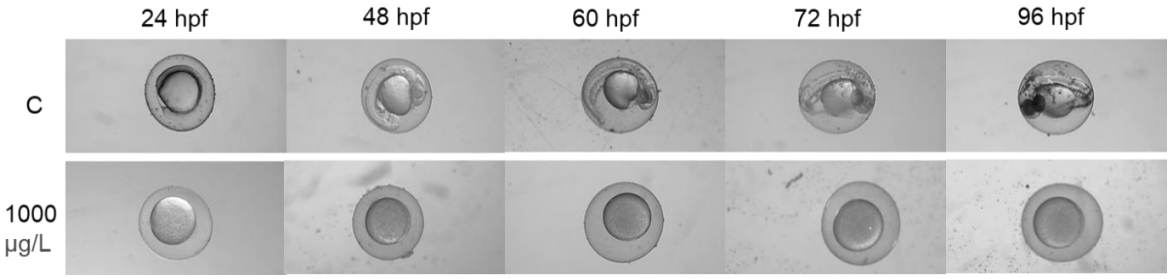

Fig.S7. Embryonic development in the 0 and 1000ug/L Azoxystrobin treatment groups at 24-96 hpf at 21 ℃.
Note: Red arrows mark oedemas, red triangles eye defects, black arrows spinal curvature and black triangles mark tail defects.

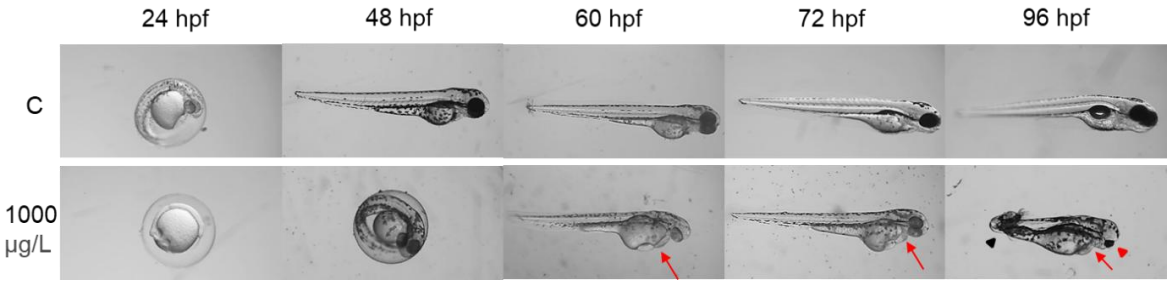

Fig.S8. Embryonic development in the 0 and 1000ug/L Azoxystrobin treatment groups at 24-96 hpf at 31 ℃.
Note: Red arrows mark oedemas, red triangles eye defects, black arrows spinal curvature and black triangles mark tail defects

Publication 3: Modulation of pesticide-induced behavioral effects in early zebrafish larvae by light, temperature and pH

(submitted)

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Abstract

Pesticides are widely used in agriculture and frequently detected in aquatic environments, yet their toxicity is commonly assessed under standardized conditions that neglect environmentally driven variation. In this study, we investigated the behavioral toxicity of the herbicide nicosulfuron and the fungicide azoxystrobin in zebrafish (*Danio rerio*) larvae under multifactorial exposure scenarios incorporating variations in light regime, temperature, and pH across multiple concentrations. Larval locomotor behavior was quantified using multiple endpoints, including movement counts, distance, duration, speed, bout duration and bout distance across different movement modes. Environmental conditions strongly modulated pesticide-induced behavioral responses. Darkness consistently enhanced locomotor activity and revealed more pronounced behavioral alterations. Nicosulfuron elevated overall locomotor activity after exposure to high concentrations independent from temperature and pH. However, environmental factors modulated behavior at intermediate concentrations. In contrast, azoxystrobin elicited detectable behavioral toxicity only under acidic conditions in darkness, highlighting a strong dependence on this environmental factor. The findings demonstrate that abiotic environmental factors can reshape

pesticide toxicity in a non-linear, context-dependent manner, underscoring the importance of incorporating environmental variation into pesticide hazard assessment.

Keywords: behavior; fish; fungicide; herbicide; abiotic factor

1. Introduction

Pesticides are widely used worldwide to support agricultural production, however, their side effects have increasingly raised public and scientific concern (Köhler and Triebskorn, 2013; Hallmann et al., 2014; Crall et al., 2018; Siviter et al., 2021; Wan et al., 2025; Köninger et al., 2026). Despite the UN Global Biodiversity Framework (UN GBF) setting a target to reduce pesticide risks by 50% by 2030, total pesticide application toxicity (TAT) is increasing in most parts of the world, directly offsetting emissions-reduction efforts and threatening global biodiversity (Wolfram et al., n.d.). When present at sufficiently high environmental concentrations, pesticides such as fungicides and herbicides can disrupt metabolic, developmental and behavioral processes in non-target organisms, thereby affecting their growth, aging, survival, maturation and reproduction (Hackenberger et al., 2018; Cao et al., 2019b; Nkontcheu Kenko et al., 2022; Cheron et al., 2023; Karaoğlan et al., 2024; Nanda et al., 2024; Huang et al., n.d.). To better understand the potential risks posed by pesticides to non-target species and to improve regulatory management, numerous studies have assessed pesticide toxicity at molecular, individual, and population levels (Nanda et al., 2024; Guo et al., 2024; Vieira et al., 2021; Song et al., 2021; Schweizer et al., 2019). However, most of these investigations or regulatory assessments have been conducted under the optimal conditions for laboratory animals. While such approaches enhance comparability among studies, they often neglect environmentally driven changes in toxicity or organismal sensitivity, potentially leading to an underestimation of pesticide hazards (Abdel-Tawwab et al., 2025; Andrade et al., 2017; Bittner et al., 2019). Increasing evidence indicates the complexity of how environmental parameters such as temperature, pH, and light conditions influence chemical toxicity (Ahmed et al., 2025; Bittner et al., 2018; Castro et al., 2025; Holmstrup et al., 2010; Juroszek et al., 2022; Schnurr et al., 2013; Wang et al., 2023). When multiple environmental factors act concurrently, these interactions can alter chemical forms and bioavailability, affecting the uptake, distribution, and metabolism of chemicals

by organisms and, thus, the performance of toxicity (Kroll et al., 2024; Li et al., 2025, 2024; Marín-García et al., 2025). To take this into account, a comprehensive assessment of pesticide toxicity should be conducted, including the confounding effects of environmental factors.

Nicosulfuron and azoxystrobin are representative herbicides and fungicides, respectively. They are extensively used in modern agriculture and frequently detected in aquatic environments, raising regulatory concerns regarding their potential ecological risks (Berenzen et al., 2005; Carvalho et al., 2016; De Lafontaine et al., 2014; Moschet et al., 2014; Peluso et al., 2022; Rodrigues et al., 2013). Current knowledge of nicosulfuron toxicity in non-target organisms remains limited and inconsistent. While several studies have reported effects on development, oxidative stress, and behavior of aquatic organisms (Cheron et al., 2022; Saglio, K. H. Olsén, S. Bre, 2001; Saglio et al., 2003), other assessments suggest relatively low toxicity (European Food Safety Authority, 2012; Li et al., 2024), highlighting substantial uncertainty regarding its environmental hazard profile. In contrast, azoxystrobin has been reported high potential toxicity for non-target animals and is widely detected in surface and groundwater worldwide (Peluso et al., 2022; Rodrigues et al., 2013). Numerous studies have documented its adverse effects on non-target aquatic organisms, including impacts on their survival, development, and behavior (Li et al., 2025; Rodrigues et al., 2013; Cao et al., 2019a; Kumar et al., 2020; Amador et al., 2024). However, important gaps remain in understanding the toxicity of these pesticides under multifactorial conditions, necessitating further assessment. Moreover, selecting these two pesticides, which differ markedly in chemical structure and modes of action, provides an opportunity to examine how different classes of pesticides respond to environmental variation.

The early life stage of zebrafish (*Danio rerio*) is a well-established model in ecotoxicological research due to their rapid development, optical transparency, and well-characterized behavioral phenotypes(Guo et al., 2024; Haigis et al., 2022; Kumar et al., 2020; Marín-García et al., 2025). Behavioral responses, particularly locomotor activity, represent one of the most sensitive endpoints for detecting effects possibly related to neurotoxicity during early life stages and are widely used to assess sublethal chemical effects (Haigis et al., 2022; Velki et al., 2017; Lovin et al., 2021; Portugues and Engert, 2011). Compared to conventional apical endpoints such as mortality or severe

malformations, behavioral alterations often occur at lower exposure levels and can reveal subtle disruptions in neural function, energy metabolism, and stress regulation (Haigis et al., 2022; Lovin et al., 2021; Velki et al., 2017). Moreover, pollutant-induced behavioral disruptions in animals can affect population dynamics and may even exert far-reaching impacts on communities and ecosystems (Bertram et al., 2026; Ford et al., 2021). Consequently, zebrafish larval behavioral assays provide a sensitive, mechanistically informative, and ecologically relevant approach for evaluating pesticide toxicity (Bertram et al., 2025).

The aim of this study is to assess the potential hazard of nicosulfuron and azoxystrobin to the behavior of zebrafish larvae under multifactorial exposure scenarios. This will incorporate variations in illumination, temperature, and pH across multiple concentration levels. By quantifying detailed swimming endpoints, including counts of movement, distance, duration, speed, and bout characteristics across distinct movement modes, we aimed to (i) investigate the behavioral impact of nicosulfuron and azoxystrobin on zebrafish larvae (ii) determine the interactions between pesticides and environmental factors that amplify or suppress pesticide-induced behavioral effects, and (iii) identify the main regulatory factors affected by the combined effects of pesticides and environmental factors. This approach is designed to identify potential hazards and mechanistic interactions, rather than to provide a quantitative environmental risk assessment.

2. Method and Material

2.1 Substance

Nicosulfuron (CAS 111991-09-4, purity >98.0%) and azoxystrobin (CAS 131860-33-8, purity ≥98.0%) were purchased from Sigma-Aldrich (Merck, Darmstadt, Germany). Azoxystrobin is a lipophilic organic ionizable compound with stable lipophilicity across the pH range of 4 to 14 (Table S1). Nicosulfuron is a hydrophilic organic ionizable compound, exhibiting increased hydrophilicity at pH 5.0 and 9.0 (Table S2).

Stock solutions of nicosulfuron (1000 mg/L) and azoxystrobin (100 μg/L) were both prepared in reconstituted water (294.0 mg/l $CaCl_2 \cdot 2H_2O$, 123.3 mg/l $MgSO_4 \cdot 7H_2O$, 63.0 mg/l $NaHCO_3$ and 5.5 mg/l KCl). Test concentrations were obtained by stepwise dilution of the stock solutions. The

reconstituted water was prepared one day before use and aerated for at least 1h, the pH value was then adjusted using 1 M HCl or 1 M NaOH and measured with a SevenCompactDuo pH meter (Mettler Toledo, Gießen, Germany).

2.2 Animal Management

Adult wild type zebrafish (WestAquarium strain, Bad Lauterburg, Germany) were maintained at 26 ± 1 °C under a 14:10 light/dark photoperiod. Breeding setups were prepared on the evening prior to egg collection. Spawning containers were placed into aquaria containing approximately 150–200 adult fish at a stocking density of 5–7 animals per liter. Each container was equipped with a mesh cover to prevent adult fish from consuming the eggs and contained artificial grass to provide a suitable substrate that stimulates spawning behavior. Mating was induced the following morning by the onset of light, which served as the environmental cue to initiate spawning. Fertilized eggs were subsequently collected from the bottom of the containers after spawning had occurred. Egg quality was assessed under a stereomicroscope (Motic Microscopy).

To ensure uniform exposure from the beginning of embryonic development, the eggs were quickly transferred to large Petri dishes for the pre-exposure of the embryos. Well-developed eggs at the 128–256 cell stage (according to Kimmel et al., 1995 (Kimmel et al., 1995)) were randomly selected and transferred into 96 well plates containing 250 µL of exposure medium and a single embryo per well. Embryos were incubated in temperature-controlled incubators under the corresponding experimental conditions, with a 14:10 h light/dark photoperiod.

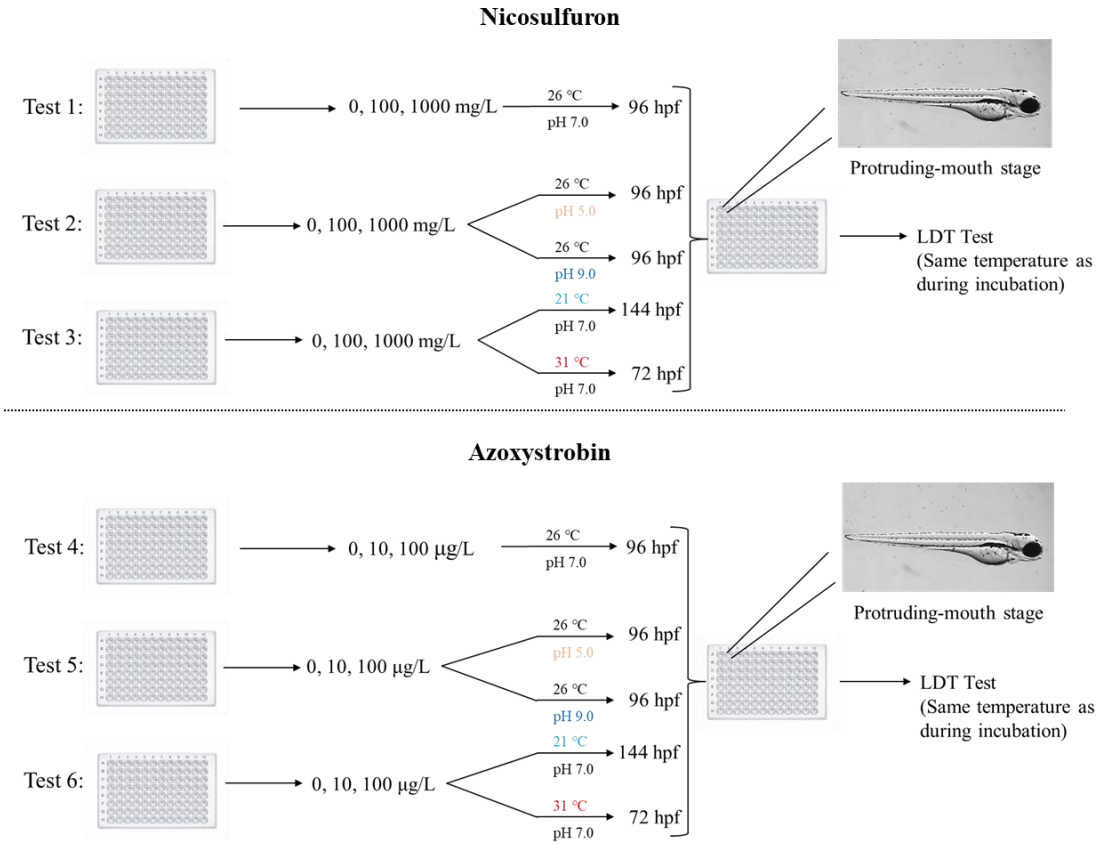

Figure 1. Flowchart of the experimental design. LDT = Light/dark transition, hpf = hours post fertilization

2.3 Experimental design

Six experiments were conducted to assess the toxicity of azoxystrobin and nicosulfuron under varying pH and temperature conditions (Figure 1). Zebrafish embryos are raised to the protruding mouth stage under different pesticides and environmental factors, and then subjected to Light/dark transition (LDT) testing for behavioral readout. Experiments (Exp.) 1–3 focused on nicosulfuron toxicity with exposure concentrations of 0, 100, 1000 mg /L nicosulfuron Exp. 1: Embryos were exposed at 26 °C, pH 7. Exp. 2: With a focus on pH effects, embryos were exposed to nicosulfuron at pH 5 and 9 while maintaining the temperature at 26 °C. Exp. 3: With a focus on temperature effects, embryos were exposed to nicosulfuron at 21 °C and 31 °C, while maintaining pH 7. Exp. 4 – 6 were conducted on azoxystrobin toxicity with exposure concentrations of 0, 10, 100 µg/L. The experimental design reflecting influences of temperature and pH was the same as that of Exp. 1 – 3. All tested concentrations were selected based on non-lethal concentration ranges for zebrafish embryos identified in our previous studies (Li et al., 2025, 2024).

Temperature variations resulted in varying hatching time of the fish larvae across different temperature groups, with a 2-3 day difference between high and low temperature conditions. To minimize the impact of differences in larval swimming ability at different stages, a uniform developmental endpoint (the protruding-mouth stage) was selected for subsequent assessments. At this stage, larvae exhibit distinct mouth protrusion, signifies the maturation of mouthparts but not the onset of active feeding. Based on research by Kimmel et al. (1995) (Kimmel et al., 1995) and Straehle et al. (2012) (Strähle et al., 2012), as well as our previous experimental experience, we set the testing time at 21℃, 26℃, and 31℃ to 72 h, 96 h, and 144 h post fertilization, respectively.

2.4 LDT Testing

The LDT test was performed according to Haigis et al. (2022) (Haigis et al., 2022). Larval locomotor activity was tracked using a ZebraBox system and the corresponding software (ZebraLab® v3, Viewpoint, France). After incubation, the hatched larvae were transferred into the system and acclimated for 5 mins. The treatment protocol comprised alternating light and dark phases, starting with 5 min of light followed by 5 min of darkness, both repeated twice. Light intensity during the light phases was set to 22000 lux (comparable to outdoor sunlight on a sunny day). The illumination was measured using iPhone13 with the LightMeter App V3.1.40. Each experiment was conducted in three runs, with 16 larvae per treatment group per run. During the experiment, the temperature was kept constant within ± 1℃ using a water bath, and the water temperature was monitored every 5 minutes with an electronic thermometer. Distance swam (distance), the number of initiated movements (counts), and duration of movements (duration) across three speed (v) categories (bursting, $v>1$ cm/s; cruising, 0.3 cm/s $<v\leq 1$ cm/s; and freezing, $v\leq 0.3$ cm/s) were recorded.

Data from the first and second phases of darkness were used to analyze larval behavior under dark conditions. Similarly, data from the first and second phases of illumination were used to analyze larval behavior under light conditions.

The counts, distance, duration, and speed in each swimming mode reflect the overall level of swimming performance. In addition, the following metrics were calculated from these data using equations (1) – (3): total distance, bout distance and bout duration.

Total distance = freezing distance + cruising distance + bursting distance (1)

Bout duration in a speed category = duration in this speed category / counts in this speed category (2)

Bout distance in a speed category = distance in this speed category / counts in this speed category (3)

2.5 Data analysis

All statistical analyses were conducted using SAS JMP Student version 18. Results are expressed as means ± standard error (SE). Normality (Shapiro-Wilk test) and homogeneity of variances (Levene´s test) were checked before applying statistical tests for significances of differences. If assumptions were met, one-way ANOVA followed by Tukey's HSD post hoc test was used. If not, the non-parametric Steel–Dwass test was applied. All Figures were created using R version 4.1.2. In order to analyze the relationship between the response variables 'counts', 'duration' and 'distance' in the categories 'freezing', 'cruising' and 'bursting', and the independent explanatory variables 'pesticide concentration', 'pH' and 'temperature' simultaneously, multiple linear regression modelling (MLR) was given preference over other statistical approaches. MLR was conducted with a standard least square fitting of the model. The level of significance was set to $p \leq 0.05$.

3. Results

All data for movement-related parameters in illumination were lower compared to those recorded in darkness, and the number of significant differences in swimming performance among groups was markedly reduced under light conditions (Figures S1-S9). Figures 2 and 3 illustrate this pattern using the swimming distance of zebrafish larvae exposed to nicosulfuron at different pH levels as an example. As statistically significant differences between the experimental groups were observed almost exclusively in darkness, the following results refer only to the dark phases.

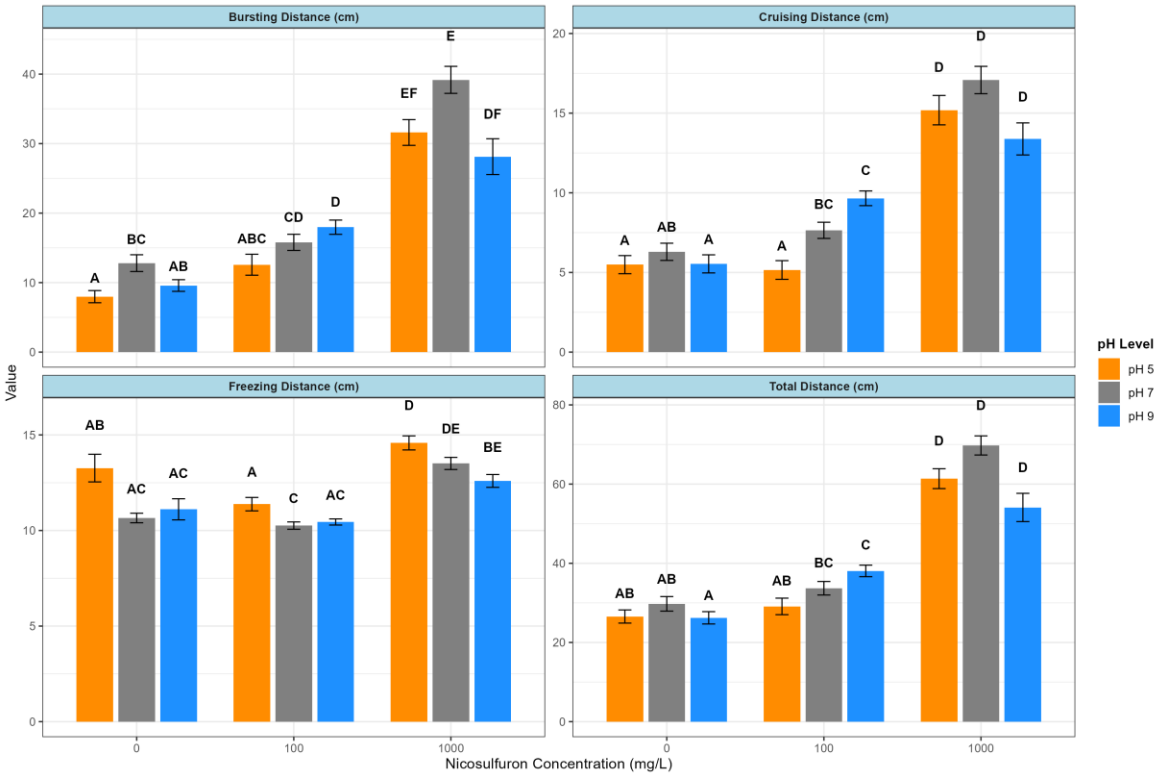

Figure 2. Swimming distance of zebrafish larvae exposed to nicosulfuron at different pH levels in the dark phases.

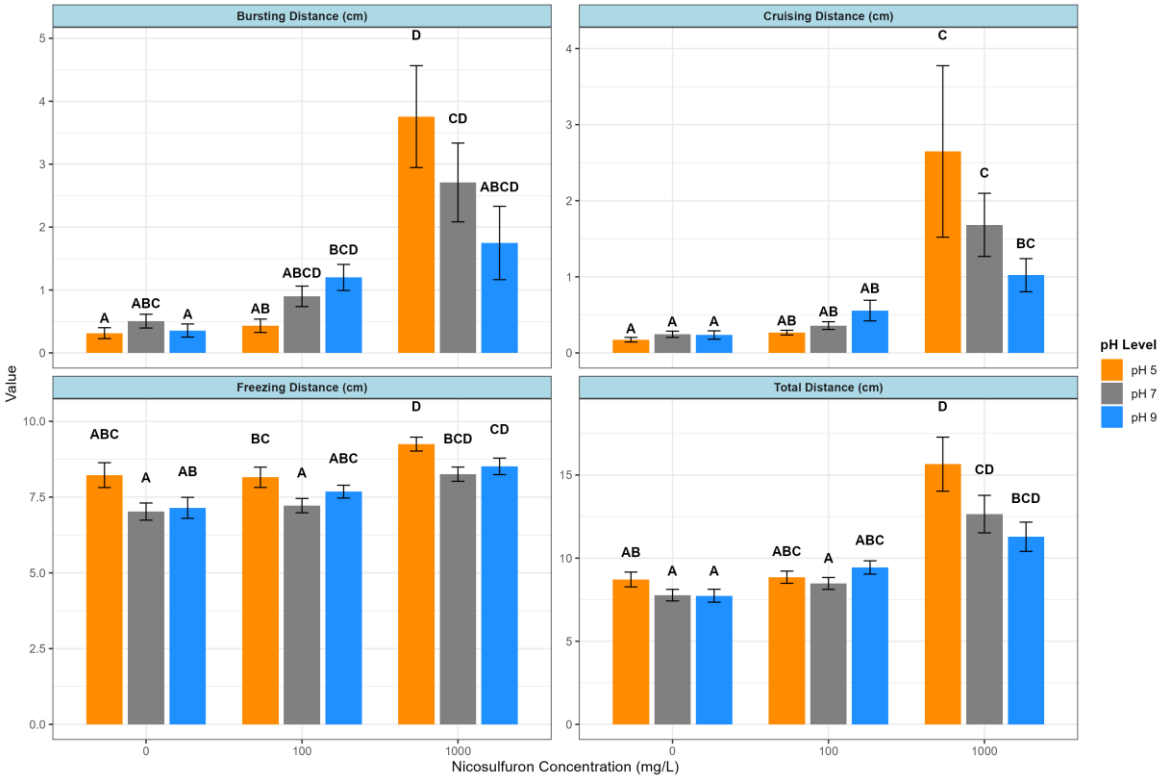

Figure 3. Swimming distance of zebrafish larvae exposed to nicosulfuron at different pH levels in the light phases.

The heatmap shown in Figure 4 illustrates the behavioral patterns of zebrafish larvae after exposure to different azoxystrobin concentrations at different pH conditions. All groups kept at pH 9 and the group exposed to 100 µ/L azoxystrobin at pH 5 showed lower values for most swimming parameters and higher values for bout movement parameters (Figure 4). In contrast, all pH 7 groups showed higher values in most swimming parameters and lower values in bout movement parameters. Thus, in the experiments with azoxystrobin, the pH plays a dominant role at all pesticide concentrations, overriding the effects of azoxystrobin concentration itself (Figure 4 & Table S3). Except for the pH 5 groups, larvae at different concentrations within the same pH level clustered closely together.

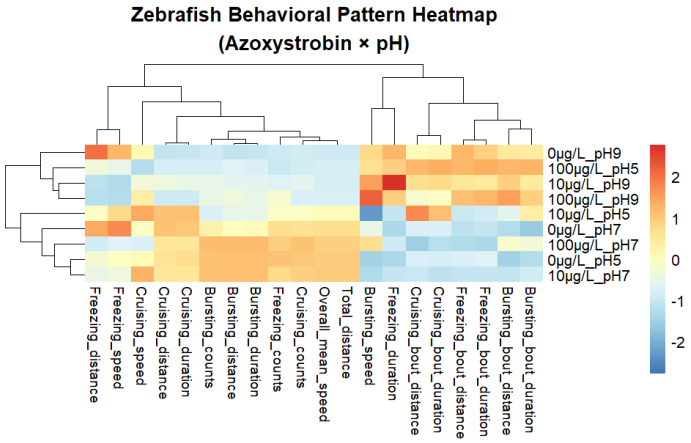

Figure 4. Hierarchical clustering heatmap of behavioral patterns in zebrafish larvae exposed to different azoxystrobin concentrations at different pH levels in the dark phases. Behavioral endpoints were integrated and standardized using Z-scores. The color gradient represents the relative increase (red) or decrease (blue) of Z-scores in each behavioral parameter. Dendrograms are based on Euclidean distance.

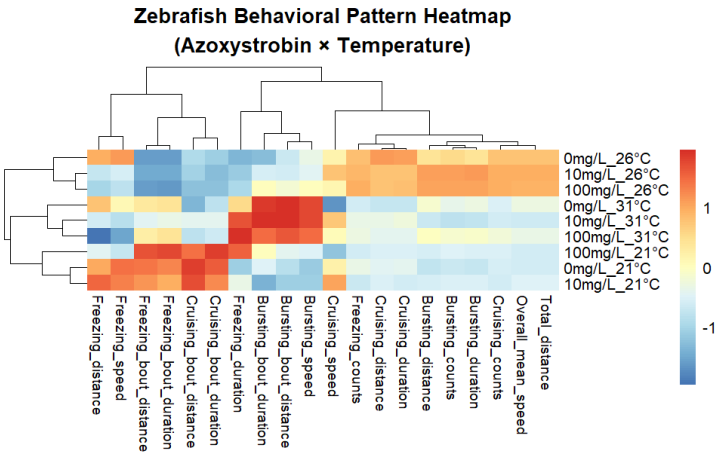

Figure 5. Hierarchical clustering heatmap of behavioral patterns in zebrafish larvae exposed to different azoxystrobin concentrations at different temperature levels in the dark phases. Behavioral endpoints were integrated and standardized using Z-scores. The color gradient represents the relative increase (red) or decrease (blue) of Z-scores in each behavioral parameter. Dendrograms are based on Euclidean distance.

Figure 5 shows the behavioral patterns of zebrafish larvae resulting from combined exposure to different azoxystrobin concentrations and different temperatures. Temperature was the dominant factor shaping the behavioral profiles of zebrafish larvae, also overriding the effects of azoxystrobin concentration (Figure 5 & Table S3), as reported for the pH before. The hierarchical clustering distinctly separated the experimental groups into three primary clusters based on the exposure temperature (21°C, 26°C, and 31°C).

Compared to 26°C, all 21°C groups showed lower levels for most swimming (= 'Cruising' + 'Bursting') parameters and higher values for 'Freezing' related parameters as well as for cruising bout distance and cruising bout duration. All 31°C groups exhibited extremely high values for bursting bout distance, bursting bout duration, bursting speed and freezing duration.

Performance of a multiple linear regression analysis revealed that only a small fraction of the behavioral variance in the experiments with azoxystrobin can be explained by variations in the independent factors concentration, pH, and temperature (Table S3).

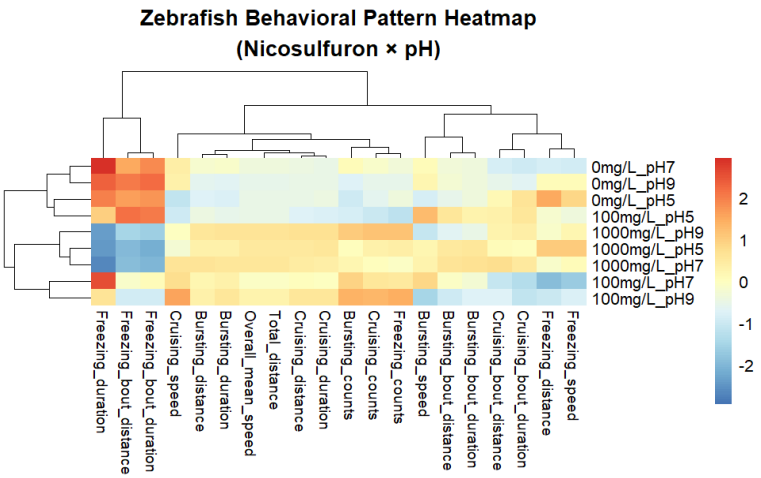

Figure 6. Hierarchical clustering heatmap of behavioral patterns in zebrafish larvae exposed to different nicosulfuron concentrations at different pH levels in the dark phases. Behavioral endpoints were integrated and standardized using Z-scores. The color gradient represents the relative increase (red) or decrease (blue) of Z-scores in each behavioral parameter. Dendrograms are based on Euclidean distance.

Figure 6 displays the behavioral patterns of zebrafish larvae resulting from combined exposure to different nicosulfuron concentrations and different pH conditions. The nicosulfuron concentration was the dominant factor shaping the behavioral profiles of zebrafish larvae at 0 and 1000 mg/L (Figure 6 & Table S4). Also, the 100 mg/L groups at pH 7 and 9 clustered closely together. The pH dominated over the test concentration only for the 100 mg/L group at pH 5, and the resulting movement pattern resembled that of the control group at pH 5. In the 1000 mg/L groups, high positive Z-scores were recorded for most swimming parameters. Conversely, the 0 mg/L groups showed low levels for most swimming parameters at all pH levels.

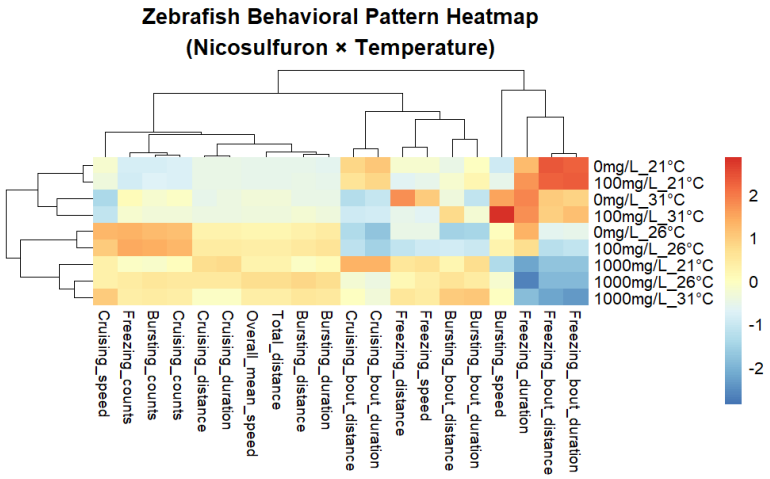

Figure 7. Hierarchical clustering heatmap of behavioral patterns in zebrafish larvae exposed to different nicosulfuron concentrations at different temperature levels in the dark phases. Behavioral endpoints were integrated and standardized using Z-scores. The color gradient represents the relative increase (red) or decrease (blue) of Z-scores in each behavioral parameter. Dendrograms are based on Euclidean distance.

The heatmap analysis reveals a clear concentration-dependent shift in the clustering of behavioral phenotypes (Figure 7 and Table S4). The treatment groups at 1000 mg/L all clustered together and showed higher values for most swimming parameters. However, in groups with lower concentrations (0 and 100 mg/L), temperature was the dominant factor in the movement pattern. All control and 100 mg/L groups (except for 26 °C) exhibited higher levels of freezing bout duration, freezing bout distance and freezing duration.

In contrast to azoxystrobin, multiple regression modelling was able to explain a significant proportion (up to one third) of the variation in behavioral parameters induced by nicosulfuron (Table S4). Here, the pesticide concentration proved to be by far the most dominant factor, particularly in the cruising and bursting categories, accounting for 18.9–32.2% of the observed variation in these categories alone.

Table 1. Significant differences in behavioral parameters among groups subjected to different azoxystrobin concentrations and pH in the dark

		0 μg/L			10 μg/L			100 μg/L				pH 5			pH 7			pH 9		
Swimming parameters	pH	5 *vs.* 7	9 *vs.* 7	9 *vs.* 5	5 *vs.* 7	9 *vs.* 7	9 *vs.* 5	5 *vs.* 7	9 *vs.* 7	9 *vs.* 5	Azoxystrobin (μg/L)	10 *vs.* 0	100 *vs.* 0	100 *vs.* 10	10 *vs.* 0	100 *vs.* 0	100 *vs.* 10	10 *vs.* 0	100 *vs.* 0	100 *vs.* 10
Bursting counts			−	−	−	−		−	−				−							
Cruising counts			−	−		−		−	−											
Freezing counts			−	−		−		−					−							
Bursting distance			−	−		−		−					−	−						
Cruising distance			−	−		−		−					−	−						
Freezing distance																				
Total distance			−	−		−		−					−	−						
Bursting duration			−	−		−		−					−	−						
Cruising duration			−	−		−		−	−				−	−						
Freezing duration			+	+		+		+					+	+						
Bursting speed																				
Cruising speed						−														
Freezing speed																				
overall mean speed			−	−		−		−					−	−						
Bursting bout distance								+					+							
Cruising bout distance					+			+				+	+							
Freezing bout distance			+	+		+		+					+	+						
Bursting bout duration								+					+							
Cruising bout duration					+			+				+	+							
Freezing bout duration			+	+		+		+												

Comparisons of initial variables a *vs.* b: '+' indicates a significantly higher level of the behavioral parameter in the former ($p < 0.05$), '−' indicates a significantly lower level of the behavioral parameter in the former ($p < 0.05$), and blanks indicate no significant difference between groups

Table 1 summarizes the significant differences in swimming behavior among zebrafish larvae exposed to azoxystrobin at different pH. At 0 and 10 μg/L azoxystrobin, the levels of most swimming parameters in the pH 9 group were significantly lower than those in the pH 7 group ($P < 0.05$). As the azoxystrobin concentration increased to 100 μg/L, the significant differences between the two groups were markedly reduced. In contrast, at 0 and 10 μg/L azoxystrobin, most swimming parameters in the pH 5 groups did not show any significant differences compared with those in the pH 7 groups ($P > 0.05$). However, at 100 μg/L azoxystrobin, most swimming parameters in the pH 5 group were at a significantly lower level than those in the pH 7 group ($P < 0.05$).

At pH 7 and pH 9, none of the behavioral parameters showed significant differences among the groups exposed at varying concentrations ($P > 0.05$). At pH 5, most swimming parameters exhibited a decreasing trend with increasing azoxystrobin concentrations (Table 1, Figure S2), and levels at 100 μg/L were significantly lower than those at other concentrations ($P < 0.05$). In contrast, bout distance and bout duration levels increased with increasing azoxystrobin concentrations (Table 1, Figure S2) and were significantly higher in larvae subjected to 100 μg/L than in those exposed to lower concentrations ($P < 0.05$).

Table 2 summarizes the significant differences in swimming behavior among zebrafish larvae exposed to azoxystrobin at different temperatures. At all azoxystrobin concentrations, most swimming parameters in the 21 °C groups were at significantly lower level than those in the 26 °C and 31 °C groups ($P < 0.05$). However, in the freezing and cruising categories of swimming behavior, bout distance and bout duration in the 21 °C groups were significantly higher than those in the 26 °C and 31 °C groups ($P < 0.05$). At all azoxystrobin concentrations, the levels of most swimming parameters in the 31 °C group were significantly lower than those in the 26 °C group ($P < 0.05$). In contrast, in the bursting category, speed, bout distance, and bout duration levels in the 31 °C group were significantly higher than those recorded for the 26 °C and 21 °C groups ($P < 0.05$).

Across all temperature treatments, no effects of azoxystrobin on the swimming behavior of zebrafish larvae were observed, as no significant differences were detected among the groups exposed to different concentrations for any of the analyzed behavioral parameters ($P > 0.05$).

Table 2. Significant differences in behavioral parameters among groups subjected to different azoxystrobin concentrations and temperature in the dark

Comparisons of initial variables a *vs.* b: '+' indicates a significantly higher level of the behavioral parameter in the former ($p < 0.05$), '−' indicates a significantly lower level of the behavioral parameter in the former ($p < 0.05$), and blanks indicate no significant difference between groups.

Swimming parameters	Temperature (℃)	0 μg/L			10 μg/L			100 μg/L			Azoxystrobin	21 ℃			26 ℃			31 ℃		
		21 *vs.* 26	31 *vs.* 26	31 *vs.* 21	21 *vs.* 26	31 *vs.* 26	31 *vs.* 21	21 *vs.* 26	31 *vs.* 26	31 *vs.* 21	(μg/L)	10 *vs.* 0	100 *vs.* 0	100 *vs.* 10	10 *vs.* 0	100 *vs.* 0	100 *vs.* 10	10 *vs.* 0	100 *vs.* 0	100 *vs.* 10
Bursting counts		−	−	+	−	−	+	−	−	+										
Cruising counts		−	−		−	−		−	−											
Freezing counts		−	−		−	−		−	−	+										
Bursting distance		−	−	+	−	−	+	−	−	+										
Cruising distance		−	−		−	−		−	−	+										
Freezing distance																				
Total distance		−	−		−	−		−	−											
Bursting duration		−	−	+	−	−	+	−	−	+										
Cruising duration		−	−		−	−		−	−	+										
Freezing duration			+		+	+		+	+											
Bursting speed		−	+	+	−	+	+	−		+										
Cruising speed			−																	
Freezing speed									−											
overall mean speed		−	−		−	−		−	−											
Bursting bout distance			+	+		+	+			+										
Cruising bout distance		+	−	−	+		−	+		−										
Freezing bout distance		+	−		+	+	−	+	+	−										
Bursting bout duration			+																	
Cruising bout duration		+		−	+		−	+		−										
Freezing bout duration		+	+		+	+		+	+	−										

Table 3. Significant differences in behavioral parameters among groups subjected to different nicosulfuron concentrations and pH in the dark

		0 mg/L			100 mg/L			1000 mg/L				pH 5			pH 7			pH 9		
Swimming parameters	pH	5 *vs.* 7	9 *vs.* 7	9 *vs.* 5	5 *vs.* 7	9 *vs.* 7	9 *vs.* 5	5 *vs.* 7	9 *vs.* 7	9 *vs.* 5	Nicosulfuron (mg/L)	100 *vs.* 0	1000 *vs.* 0	1000 *vs.* 100	100 *vs.* 0	1000 *vs.* 0	1000 *vs.* 100	100 *vs.* 0	1000 *vs.* 0	1000 *vs.* 100
Bursting counts					−		+						+	+		+	+	+	+	
Cruising counts					−		+						+	+		+	+	+	+	
Freezing counts					−		+						+	+		+	+	+	+	
Bursting distance		−					+		−				+	+		+	+	+	+	
Cruising distance					−								+	+		+	+	+	+	+
Freezing distance					+					−			+	+		+	+		+	+
Total distance							+						+	+		+	+	+	+	+
Bursting duration					−		+						+	+		+	+	+	+	
Cruising duration					−		+						+	+		+	+	+	+	+
Freezing duration					+		−						−	−		−	−	−	−	−
Bursting speed							−					+	+			+	+			
Cruising speed							+	−					+	+		+	+			
Freezing speed										−			+	+		+	+		+	+
overall mean speed							+						+	+		+	+	+	+	+
Bursting bout distance					+		−		−	−		+	+	+		+	+		+	+
Cruising bout distance					+		−		−			+	+	+		+	+		+	+
Freezing bout distance					+	−	−						−	−		−			−	
Bursting bout duration					+		−	−		−		+	+	+		+	+		+	+
Cruising bout duration				−	+		−						+			+	+		+	+
Freezing bout duration					+		−						−	−		−	−	−	−	

Comparisons of initial variables a *vs.* b: '+' indicates a significantly higher level of the behavioral parameter in the former ($p < 0.05$), '−' indicates a significantly lower level of the behavioral parameter in the former ($p < 0.05$), and blanks indicate no significant difference between groups.

Table 4. Significant differences in behavioral parameters among groups subjected to different nicosulfuron concentrations and temperature in the dark

Comparisons of initial variables a *vs.* b: '+' indicates a significantly higher level of the behavioral parameter in the former ($p < 0.05$), '−' indicates a significantly lower level of the behavioral parameter in the former ($p < 0.05$), and blanks indicate no significant difference between groups.

Swimming parameters	Temperature (℃)	0 mg/L			100 mg/L			1000 mg/L			Nicosulfuron (mg/L)	21 ℃			26 ℃			31 ℃		
		21 *vs.* 26	31 *vs.* 26	31 *vs.* 21	21 *vs.* 26	31 *vs.* 26	31 *vs.* 21	21 *vs.* 26	31 *vs.* 26	31 *vs.* 21		100 *vs.* 0	1000 *vs.* 0	1000 *vs.* 100	100 *vs.* 0	1000 *vs.* 0	1000 *vs.* 100	100 *vs.* 0	1000 *vs.* 0	1000 *vs.* 100
Bursting counts		−		+	−	−	+	−	−				+	+		+	+		+	+
Cruising counts		−		+	−	−	+	−	−				+	+		+	+		+	+
Freezing counts		−		+	−	−	+	−	−				+	+		+	+		+	+
Bursting distance		−		+	−	−	+	−	−				+	+		+	+		+	+
Cruising distance		−		+	−	−	+		−	−			+	+		+	+		+	+
Freezing distance		−		+	−								+	+		+	+		+	+
Total distance		−		+	−	−	+	−	−				+	+		+	+		+	+
Bursting duration		−		+	−	−	+	−					+	+		+	+		+	+
Cruising duration		−		+	−	−	+		−				+	+		+	+		+	+
Freezing duration		+			+	+	−		+	+			−	−		−	−		−	−
Bursting speed		−			−	+	+	−					+	+		+	+			
Cruising speed			−											+		+	+		+	+
Freezing speed		−		+	−				−	−			+	+		+	+		+	+
overall mean speed		−			−	−	+	−	−				+	+		+	+		+	+
Bursting bout distance				+			+	−	−				+	+		+	+		+	+
Cruising bout distance		+	−	−	+	−	−	+		−			+	+		+	+		+	+
Freezing bout distance		+		−	+	+	−	+					−	−					−	−
Bursting bout duration									−				+	+		+	+		+	+
Cruising bout duration		+		−	+		−	+		−			+	+		+	+		+	+
Freezing bout duration		+		−	+	+	−	+	+				−	−		−	−			−

Table 3 displays the significant differences in swimming behavior among zebrafish larvae exposed to nicosulfuron at different pH. In the 0 and 1000 mg/L nicosulfuron treatment groups, most behavioral parameters showed no significant differences ($P > 0.05$). Across all concentrations, the 100 mg/L treatment group exhibited the most pronounced significant differences in swimming behavior. Following 100 mg/L nicosulfuron exposure, the levels of most parameters decreased with the pH shifting from alkaline to acidic (Table 3 and Figure S6), with values in the pH 5 group being significantly lower than those in the pH 7 group, and values in the pH 9 group being significantly higher than those in the pH 7 group ($P < 0.05$). In contrast, bout distance and bout duration for all categories of swimming increased as the pH changed from alkaline to acidic (Table 3 and Figure S6), with values in the pH 5 group being significantly higher than in the pH 7 group, and values in the pH 9 group being significantly lower than in the pH 7 group ($P < 0.05$).

Across all pH treatments, the levels of most swimming parameters increased with increasing nicosulfuron concentrations (Table 3 and Figure S6). In the 1000 mg/L group, bout distance and bout duration levels were significantly higher in the bursting and cruising categories than those in the other groups ($P < 0.05$), whereas freezing bout distance and freezing bout duration showed the opposite trend (Table 3 and Figure S6).

Table 4 summarizes the significant differences in swimming behavior parameters among zebrafish larvae exposed to nicosulfuron at different temperatures. Across all concentrations, the 100 mg/L treatment group exhibited the most pronounced significant differences in swimming parameters. At all concentrations, the values recorded for most swimming parameters in the 21 °C group were significantly lower than those in the 26 °C group ($P < 0.05$), whereas bout distance and bout duration in the freezing and cruising categories were significantly higher in the 21 °C group compared with the 26 °C group ($P < 0.05$). At 100 and 1000 mg/L, the levels of most swimming parameters were significantly lower in the 31 °C group than those in the 26 °C group ($P < 0.05$). In the 0 and 100 mg/L treatments, bursting bout distance and bursting speed increased with increasing temperature (Table 4 and Figure S8), with values at 31 °C being significantly higher than those at 21 °C ($P < 0.05$). Across all temperature treatments, the levels of most swimming parameters in the 1000 mg/L group were significantly higher than those in the other concentration groups ($P < 0.05$), except for

freezing duration, freezing bout distance, and freezing bout duration.

To sum up, nicosulfuron elevated overall locomotor activity after exposure to high concentrations independent from temperature and pH. However, environmental factors modulated behavior at intermediate concentrations. In contrast, azoxystrobin elicited detectable behavioral toxicity only under acidic conditions in darkness, highlighting a strong dependence on this environmental factor.

4. Discussion

In this study, zebrafish larvae exhibited clearly higher activity in the dark compared to those in the light. The phenomenon of lower activity levels in zebrafish larvae under light and higher activity levels in darkness has been widely reported (Haigis et al., 2022; Velki et al., 2017; Lovin et al., 2021; Portugues and Engert, 2011). It is generally interpreted as an escape-related response to unfamiliar or potentially threatening environmental cues, such as predator shadows (Burgess and Granato, 2007; Tegelenbosch et al., 2012). This suggests that these chemicals may be partially latent under illumination and trigger more pronounced and readily detectable behavioral alterations once switched to a stress state, such as darkness. Given that the principal findings of this study were predominantly observed in darkness, and in order to maintain a focused discussion, the sections below primarily address zebrafish larval behavior under multifactorial exposure conditions in dark environments.

There is limited research on behavioral effects of azoxystrobin in aquatic organisms. One study examining commercial azoxystrobin formulations in zebrafish embryos and larvae did not detect significant changes in motility (Vieira et al., 2021), whereas another study reported increased locomotor activity, including distance and speed, but the observations were based on adult zebrafish (Guo et al., 2024). In the present study, exposure to high concentrations of azoxystrobin significantly reduced larval motility, which may be related to its mode of action in inhibiting mitochondrial respiration, thereby reducing metabolic activity and energy availability (Kumar et al., 2020; Rodrigues et al., 2013) and consequently affecting normal swimming behavior. Notably, these effects were observed only under dark conditions at pH 5, suggesting a strong acid-dependent toxicity. In addition, the limited behavioral response may also be attributed to the experimental concentrations

being below the effective concentrations inducing proteotoxicity, teratogenicity and mortality: In our previous study, azoxystrobin exposure has resulted in high mortality and malformation rates in zebrafish larvae, with changes in Hsp70 protein levels primarily occurring at 1000 μg/L, whereas no significant toxic effects were observed at concentrations ranging from 0 to 100 μg/L (Li et al., 2025).

Across all concentrations (almost all concentrations in the MLR models), the pH had a significant impact on the overall pattern of larval behavior, integrating all recorded behavioral parameters. However, the substantial variation in results across the larvae in the respective exposure groups made it impossible to reliably model the individual behavioral parameters using the three independent variables: concentration, pH and temperature. Since azoxystrobin forms a neutral, non-ionized species with a constant logD between pH 5 and pH 9, the tested pH shifts did not affect the ionization state or hydrophobicity of this compound (Li et al., 2025). Therefore, the differences in toxicity observed under different pH conditions are unlikely to be due to changes in absorption or membrane permeability and thus intracellular uptake (Köhler et al., 2023; Kroll et al., 2024). Under alkaline conditions, larvae exhibited markedly lower overall swimming performance (except for bursting speed), which may reflect the influence of alkaline environments on AChE activity, ion uptake, or ammonia metabolism (Scott et al., 2005; Zhang et al., 2025), thereby indirectly affecting behavior, likely in individually variable intensity. Although at pH 5 the swimming performance of the high-concentration azoxystrobin treatment groups decreased with increasing exposure concentration, the distance and duration per movement bout increased across all swimming modes. This may be associated with energy re-allocation processes (Di Santo and Goerig, 2025). As azoxystrobin inhibits mitochondrial respiration and consequently reduces energy supply, individuals may reduce unnecessary movement initiation to minimize the energetic costs associated with frequent start–stop behavior, thereby adopting a locomotor strategy characterized by longer distance movement and longer duration of movement per bout.

Similar to the observations in the azoxystrobin-pH experiment, temperature exerted a dominant influence on the overall pattern of larval behavior (integrating all behavioral parameters) in the context of azoxystrobin exposure across all concentrations. However, due to high individual variation in the results, the 'temperature' parameter was not significantly included in the MLR models,

with one exception. Even though the general behavioral patterns differed between the exposure groups, these models could only explain a negligible proportion of the variation in each single behavioral parameter. Larvae maintained at 26 °C exhibited the highest overall swimming performance. This pattern is consistent with the fact that 26 °C is close to the optimal developmental temperature for zebrafish embryos and larvae (Osterauer, 2008; Schnurr et al., 2013). Temperatures below this range suppress embryonic and larval viability, whereas higher temperatures, although enhancing metabolic rate and larval viability, impose increased energetic demands to maintain normal physiological functions (Schnurr et al., 2013; Wang et al., 2023; Osterauer, 2008). Consequently, larval activity levels tend to decrease under both low and high temperature conditions. At 31 °C, larvae displayed higher bursting bout distance and bursting bout duration, whereas at 21 °C, larvae showed higher freezing bout distance, freezing bout duration, cruising bout distance, and cruising bout duration. These observations indicate that temperature consistently affects behavior in a similar way as in the nicosulfuron-temperature experiment: high-temperature larvae tended to perform brief, high-intensity bursts but show lower overall swimming performance due to energetic constraints, whereas low-temperature larvae favor low-to-medium speed swimming, resulting in reduced overall activity. Notably, the temperature effect appeared less pronounced in the azoxystrobin experiments, with insignificant differences observed across multiple behavioral indices, and the overall locomotor performance within each temperature group was not substantially altered by azoxystrobin exposure.

In the present study, nicosulfuron exhibited clear behavioral effects in zebrafish larvae. Across all pH and temperature treatments, larvae exposed to high concentrations of nicosulfuron showed elevated overall locomotor levels (counts, distance, duration, and speed). This was also revealed by the MLR models, which, firstly, explained significant portions of the variation in all behavioural parameters and, secondly, were dominated by the 'concentration' parameter. Similar behavioral responses have been reported in previous studies, in which nicosulfuron enhanced the burst swimming reaction and aggregation behavior in gold fish *Carassius auratus* (Saglio, K. H. Olsén, S. Bre, 2001; Saglio et al., 2003), as well as swimming speed and in amphibian *Bufo spinosus* (Cheron et al., 2023). Nevertheless, the molecular or physiological mechanisms underlying nicosulfuron-induced behavioral alterations remain unclear.

The herbicidal mode of action of nicosulfuron involves inhibition of acetolactate synthase (ALS), an enzyme essential for the biosynthesis of branched-chain amino acids (valine, leucine, and isoleucine) in plants. Inhibition of ALS disrupts cell division and growth, ultimately leading to chlorosis, necrosis, and plant death over a period of weeks (Huang et al., 2021). However, animals lack these amino acid synthesis pathways and do not express this synthase. Therefore, the behavioial effects observed in zebrafish larvae are unlikely to be directly attributable to the primary herbicidal mode of action of nicosulfuron. Similar to other herbicides whose intended modes of action are specific to plants, such as glyphosate, nicosulfuron may still exert adverse effects on non-target aquatic organisms through indirect or secondary pathways that are independent of ALS inhibition, for example by altering microbial community composition and subsequently affecting amino acid metabolism, neurotransmitter regulation, and immune-related metabolic processes (Schweizer et al., 2019; Marín-García et al., 2025; Klátyik et al., 2024; Wan et al., 2025a). Consistent with this interpretation, a previous study of us demonstrated that the toxicity of nicosulfuron in zebrafish larvae was predominantly expressed by impairment of protein integrity and other sublethal effects. Specifically, nicosulfuron exposure did not significantly affect larval survival or heart rate, but induced premature hatching (Li et al., 2024). Together, these findings suggest that although nicosulfuron shows a relatively weak acute toxicity in zebrafish larvae, it can elicit pronounced sublethal and behavioral effects at higher concentrations, and they potentially may exert ecological implications.

In experiments examining nicosulfuron and pH as interacting factors, we found that, under both light and dark conditions, most behavioral parameters of zebrafish larvae exposed to 0 mg/L and 1000 mg/L nicosulfuron were relatively insensitive to pH variation. These exposures were clearly dominated by concentration. In contrast, pH-dependent effects were primarily observed at the intermediate concentration (100 mg/L) shifting the behavioral pattern either to the 'non-polluted' or the 'polluted' direction: Once the nicosulfuron concentration exceeds a certain threshold, the toxic effects of nicosulfuron seem to dominate and effectively mask the influence of pH.

The influence of pH was most pronounced under dark conditions at 100 mg/L nicosulfuron. At 100 mg/L, locomotor activity increased progressively from pH 5 to pH 9. Interestingly, under acidic

conditions, nicosulfuron (pKa = 4.5) is expected to exist predominantly in its neutral form, which generally facilitates passive diffusion across biological membranes and, in theory, should increase its bioavailability and toxicity (Kroll et al., 2024; Köhler et al., 2023; Green and Hale, 2005; Carles et al., 2018). However, our results on the behavior contradict this expectation, as reduced locomotor activity was observed under acidic conditions at moderate exposure levels. This discrepancy suggests that pH-dependent changes in nicosulfuron bioavailability alone cannot explain the observed behavioral patterns.

A more plausible explanation is that nicosulfuron and pH may modulate the excitation threshold of the zebrafish larval nervous system. Extracellular pH shifts are known to modulate neuronal excitability by affecting the gating properties of both voltage- and ligand-gated ion channels (Ruusuvuori and Kaila, 2014). Under alkaline conditions, both 100 and 1000 mg/L treatments significantly enhanced swimming activity compared to controls, with no further increase at higher concentrations, suggesting a plateau in behavioral response. In contrast, under acidic conditions, locomotor activity remained low at 0 and 100 mg/L but increased sharply at 1000 mg/L, indicating that a higher excitation threshold must be overcome to elicit behavioral responses. Although no biochemical or electrophysiological endpoints were assessed in the present study, and although the EPA excludes neurotoxic potentials of nicosulfuron in mammals ("EPA-HQ-OPP-2012-0372-0004_content," n.d.), the behavioral metrics observed at 100 mg/L under acidic conditions provide indirect support for this interpretation. Specifically, larvae in the pH 5 treatment group exhibited higher bursting speed, bout distance, and bout duration. Larvae likely were inhibited from initiating locomotion due to a high neural excitation threshold, which requires a strong stimulus exceeding that threshold, resulting in a single, sustained, and high-velocity swimming motion once elicited. At extremely high concentrations of nicosulfuron (1000 mg/L), the influence of excitation-threshold modulation became attenuated, and larvae seemed to enter a state of global overexcitation, characterized by intensive locomotor activity.

Similar to the pH experiment, temperature emerged as a dominant factor modulating the general pattern of behavioral responses under exposure to 0 and 100 mg/L nicosulfuron. The fact that only these two exposure groups, rather than the 1000 mg/L group, were influenced by temperature

probably reduced the percentage of variation explained by this parameter in the MLR analysis. Overall swimming performance was highest in the 26 ℃ group and lowest in the 21 ℃ treatment group, consistent with the results observed in azoxystrobin-temperature experiments.

A similar trend was observed at higher nicosulfuron concentrations; however, with increasing chemical exposure, concentration became the dominant determinant of behavioral outcomes, thereby weakening the influence of temperature on toxicity. Further examination of bursting speed and bursting bout distance indicated that larvae exposed to higher temperatures tended to exhibit high-speed but relatively infrequent and short-duration movements. This behavioral pattern may reflect an energy-conserving strategy to offset the elevated metabolic costs associated with high temperatures. Consistent with this interpretation, our previous study showed that elevated temperatures increased heart rate in zebrafish larvae, and this effect was further amplified by increasing nicosulfuron concentrations, suggesting an interaction between temperature, metabolism, and chemical exposure (Li et al., 2024).

Notably, the behavioral toxicity of nicosulfuron remained evident at low temperatures. Although locomotor activity was markedly reduced in the low-temperature treatment group at lower exposure concentrations, locomotor parameters in the 21 °C group increased significantly under higher concentrations of nicosulfuron, with some parameters even exceeding those observed in the 31 °C treatment group. This finding indicates that low temperature does not mitigate possible behavioral effects of nicosulfuron at high concentrations. In addition, larvae maintained at low temperatures showed higher bout distances and bout durations at low to medium swimming speeds. This indicates that while low temperatures may suppress overall neural responsiveness, they do not impair swimming capacity; rather larvae are less likely to initiate movement, but once initiated, movements tend to be prolonged.

Our findings indicate that environmental factors (light conditions, temperature, and pH) interact with pesticides to exert diverse effects on the swimming behavior of zebrafish larvae. These effects are complex and multifaceted, encompassing not only toxic responses that may be triggered only under specific environmental conditions, but also toxicity manifested through multi-level modulation of e.g. neural excitation or metabolic thresholds. Moreover, the dominant drivers of behavioral responses

varied across pesticide concentrations, indicating that the mechanisms underlying behavioral alterations are also concentration-dependant. The magnitude and nature of behavioral effects differed among environmental contexts, and distinct behavioral outcomes may ultimately lead to different ecological consequences. Collectively, these findings suggest that the combined influence of multiple environmental stressors under field conditions may further modulate toxic responses, underscoring the critical role of the environmental context in toxicity assessment. To better capture this complexity, future evaluations should incorporate factorial experimental designs and mechanistic modeling approaches, in combination with environmental monitoring, to improve the ecological relevance of pesticide risk assessment.

Conclusion

This study elucidates the complex interplay between pesticide exposure and abiotic environmental factors in modulating behavioral responses of zebrafish larvae. A critical methodological finding is the pivotal role of darkness as an environmental stressor. Our results demonstrate that dark conditions unmask latent neurotoxicity that is otherwise undetectable under standard light conditions.

Importantly, both pH and temperature modulated behavioral responses in a non-linear and concentration-dependent manner. Nicosulfuron exerted pronounced behavioral effects, characterized by generalized hyperactivity and altered swimming kinematics. The interaction between nicosulfuron and environmental factors revealed a "multi-layered" toxicity profile dependent on concentration thresholds. At lower concentrations, behavioral responses were predominantly driven by environmental variables (pH and temperature); however, at higher concentrations, chemical toxicity became the dominant driver, masking the effects of the confounding factors. Specifically, acidic conditions and nicosulfuron enhance the behavioral toxicity in zebrafish larvae, while temperature seemed to modulate behavioral strategies through metabolic constraints, forcing a trade-off between movement frequency and intensity at high temperatures (31 °C) and revealing potent, albeit delayed, behavioral toxicity at low temperatures (21 °C). In contrast, azoxystrobin exhibited a more specific toxicity profile, which is highly sensitive to environmental acidification.

These findings highlight the limitations of single-factor toxicity tests. Consequently, to improve the ecological relevance of hazard assessments, future studies must integrate multifactorial designs that account for the coexistence of chemical and abiotic stressors natural aquatic ecosystems.

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Supplementary Material List:

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2. Table S2: Physicochemical properties of nicosulfuron
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Table S1: Physicochemical properties of azoxystrobin

Properties	
Molecular Formula	$C_{22}H_{17}N_3O_5$
Molecular Weight	403.4 g/mol
Solubility in water	6 mg/L (at 20 °C)
log P_{ow} (at 20 °C)	2.5
pK_a	not applicable

Data obtained from Pubchem (NIH, National library of Medicine)

Table S2: Physicochemical properties of nicosulfuron

Properties	
Molecular Formula	$C_{15}H_{18}N_6O_6S$
Molecular Weight	410.4 g/mol
Solubility in water	7400 mg/L (at pH 7, 25°C)
log P_{ow} (at pH 5)	0.35
log P_{ow} (at pH 7)	1.8
log P_{ow} (at pH 9)	-2.0
pK_a	4.60

Data obtained from Pubchem (NIH, National library of Medicine)

Table S3: Multiple regression models for azoxystrobin, percentage explanation of the variance of the target variables and significance of the respective contribution (in parentheses)

Target variable	Independent variables			
	Full model variables[1]	Concentration	pH	Temperature
Azoxystrobin, exposure in the dark				
Freezing, counts	0.8 % (0.0061)	0.4 % (0.0116)	0.4 % (0.0119)	n.s.
Freezing, duration	3.9 % (< 0.0001)	n.s.	n.s.	3.8 % (< 0.0001)
Freezing, distance	1.8 % (< 0.0001)	1.7 % (< 0.0001)	n.s	n.s.
Cruising, counts	1.2 % (0.0005)	0.5 % (0.0077)	0.7 % (0.0008)	n.s.
Cruising, duration	1.3 % (0.0002)	0.6 % (0.0028)	0.7 % (0.0008)	n.s.
Cruising, distance	1.3 % (0.0002)	0.6 % (0.0024)	0.7 % (0.0011)	n.s.
Bursting, counts	0.8 % (0.0078)	n.s.	0.6 % (0.0034)	n.s.
Bursting, duration	0.9 % (0.0034)	n.s.	0.8 % (0.0007)	n.s.
Bursting, distance	0.9 % (0.0037)	n.s.	0.7 % (0.0009)	n.s.
Azoxystrobin, exposure in the light				
Freezing, counts	0.3 % (0.2257)	n.s.	n.s.	n.s.
Freezing, duration	3.5 % (< 0.0001)	n.s.	n.s.	3.3 % (< 0.0001)
Freezing, distance	1.1 % (0.0014)	n.s.	n.s.	1.0 % (< 0.0001)
Cruising, counts	0.1 % (0.8640)	n.s.	n.s.	n.s.
Cruising, duration	2.4 % (0.3114)	n.s.	n.s.	n.s.
Cruising, distance	0.1 % (0.5343)	n.s.	n.s.	n.s.
Bursting, counts	0.1 % (0.7587)	n.s.	n.s.	n.s.
Bursting, duration	0.1 % (0.6918)	n.s.	n.s.	n.s.
Bursting, distance	0.1 % (0.5422)	n.s.	n.s.	n.s.

[1] comprising concentration, pH and temperature. n.s.: not significant.

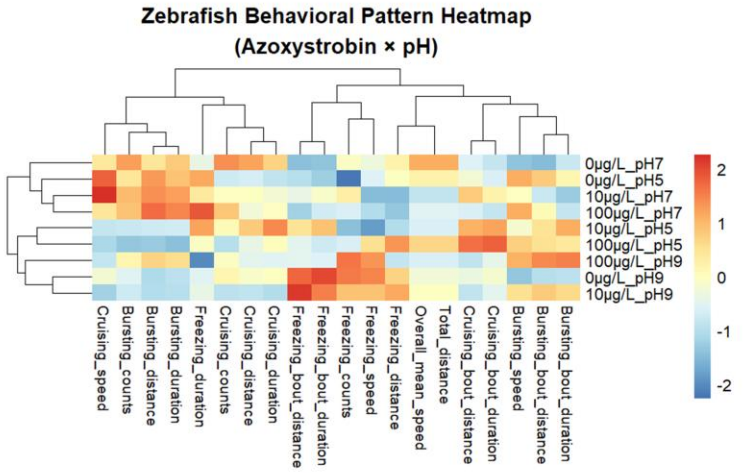
Table S4: Multiple regression models for nicosulfuron, percentage explanation of the variance of the target variables and significance of the respective contribution (in parentheses)

Target variable	Independent variables			
	Full model variables[1]	Concentration	pH	Temperature
Nicosulfuron, exposure in the dark				
Freezing, counts	17.0 % (< 0.0001)	13.6 % (< 0.0001)	0.5 % (0.0003)	2.9 % (< 0.0001)
Freezing, duration	9.5 % (< 0.0001)	9.3 % (< 0.0001)	n.s.	n.s.
Freezing, distance	9.4 % (< 0.0001)	7.1 % (< 0.0001)	1.9 % (< 0.0001)	0.7 % (0.0006)
Cruising, counts	22.1 % (< 0.0001)	18.9 % (< 0.0001)	0.7 % (< 0.0001)	2.5 % (< 0.0001)
Cruising, duration	29.2 % (< 0.0001)	29.0 % (< 0.0001)	n.s.	0.3 % (0.0299)
Cruising, distance	32.5 % (< 0.0001)	32.2 % (< 0.0001)	0.1 % (0.0472)	n.s.
Bursting, counts	22.5 % (< 0.0001)	18.8 % (< 0.0001)	0.8 % (< 0.0001)	2.8 % (< 0.0001)
Bursting, duration	33.3 % (< 0.0001)	31.8 % (< 0.0001)	0.1 % (0.0246)	1.5 % (< 0.0001)
Bursting, distance	33.1 % (< 0.0001)	31.5 % (< 0.0001)	n.s.	1.8 % (< 0.0001)
Nicosulfuron, exposure in the light				
Freezing, counts	8.0 % (< 0.0001)	1.4 % (< 0.0001)	n.s.	n.s.
Freezing, duration	2.2 % (< 0.0001)	0.7 % (0.0006)	n.s.	1.5 % (< 0.0001)
Freezing, distance	5.7 % (< 0.0001)	3.7 % (< 0.0001)	0.6 % (0.0018)	1.6 % (< 0.0001)
Cruising, counts	4.9 % (< 0.0001)	4.6 % (< 0.0001)	n.s.	n.s.
Cruising, duration	4.1 % (< 0.0001)	4.0 % (< 0.0001)	n.s.	n.s.
Cruising, distance	4.1 % (< 0.0001)	4.0 % (< 0.0001)	n.s.	n.s.
Bursting, counts	5.9 % (< 0.0001)	5.8 % (< 0.0001)	n.s	n.s.
Bursting, duration	6.6 % (< 0.0001)	6.6 % (< 0.0001)	n.s.	n.s.
Bursting, distance	7.3 % (< 0.0001)	7.1 % (< 0.0001)	n.s.	n.s.

[1] comprising concentration, pH and temperature. n.s.: not significant.

Figure S1: Hierarchical clustering heatmap of behavioral patterns in zebrafish larvae in the light.

A



B

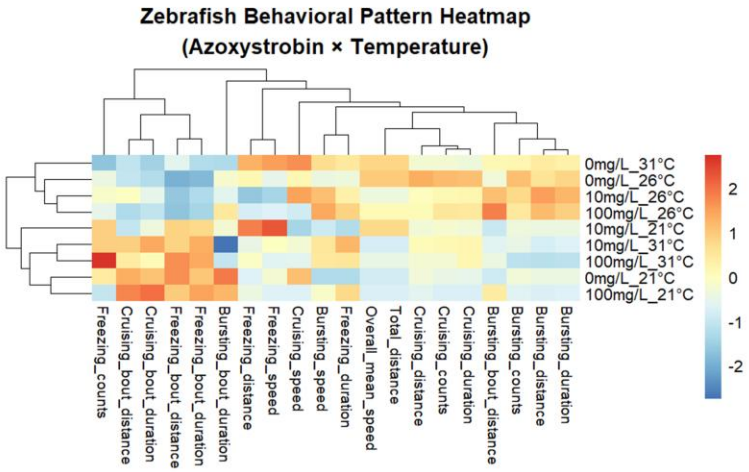


Figure S1 (Continued)

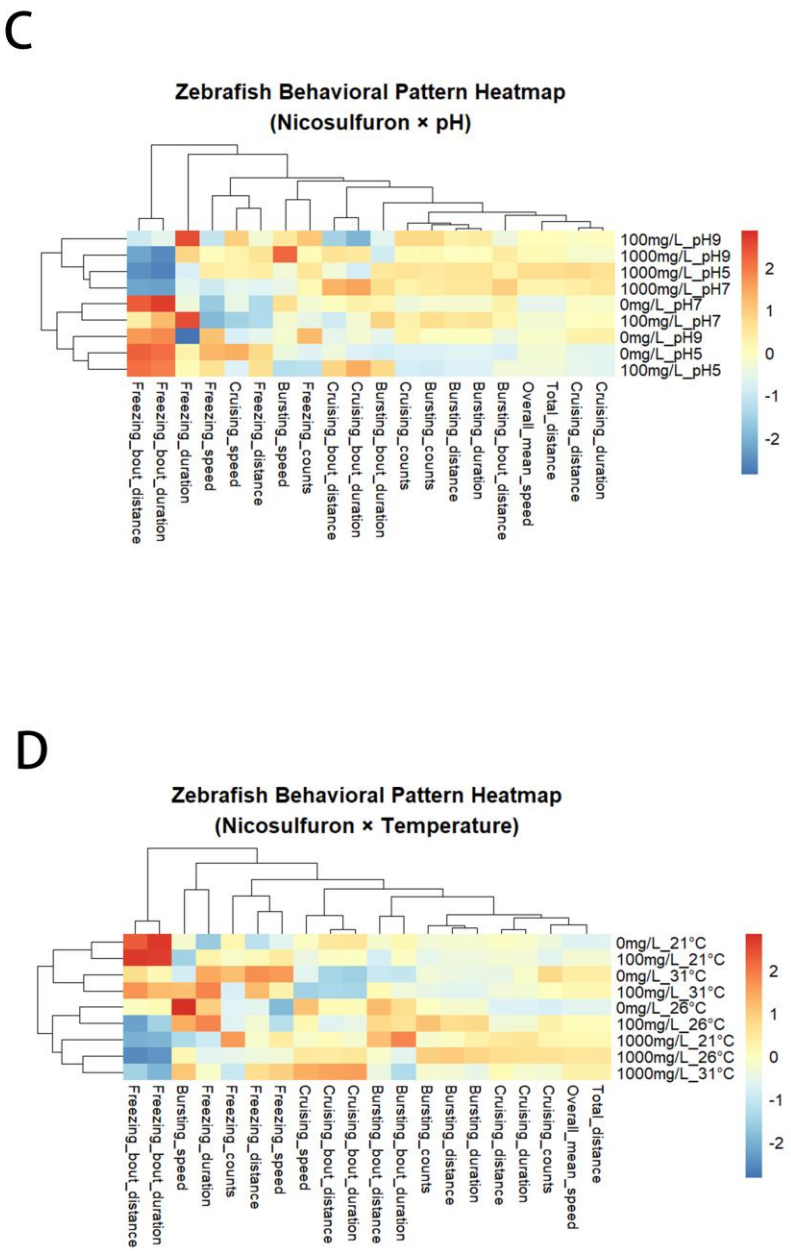


Figure A and B are heatmaps for behavioral patterns in zebrafish larvae exposed to azoxystrobin under different pH and temperature conditions, respectively. Figure C and D are heatmaps for behavioral patterns in zebrafish larvae exposed to nicosulfuron under different pH and temperature conditions, respectively.

Behavioral endpoints were integrated and standardized using Z-scores. The color gradient represents the relative increase (red) or decrease (blue) in each behavioral parameter. Dendrograms based on Euclidean distance.

Figure S2 Swimming parameters of zebrafish larvae exposed to azoxystrobin at different pH levels in the dark.

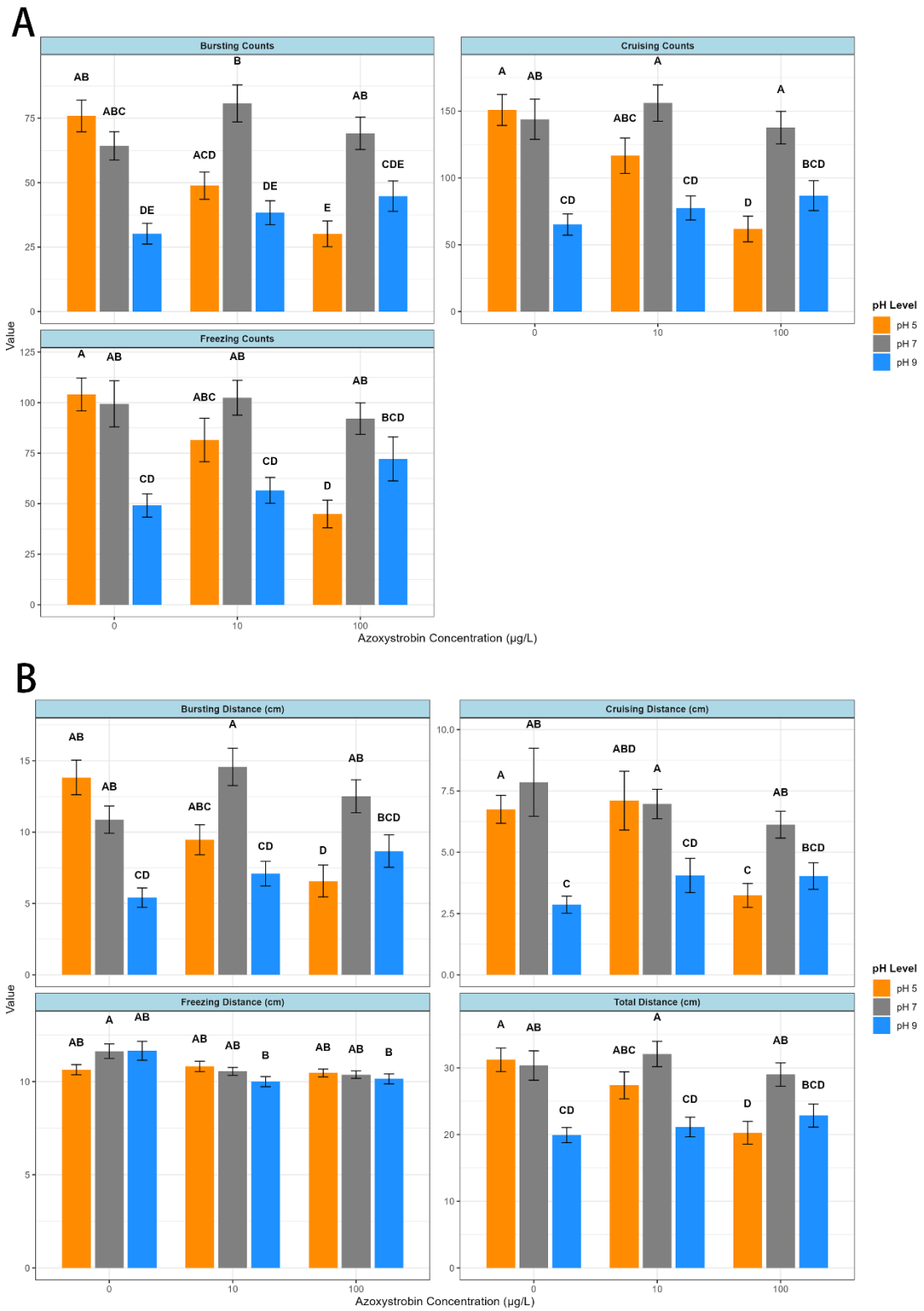
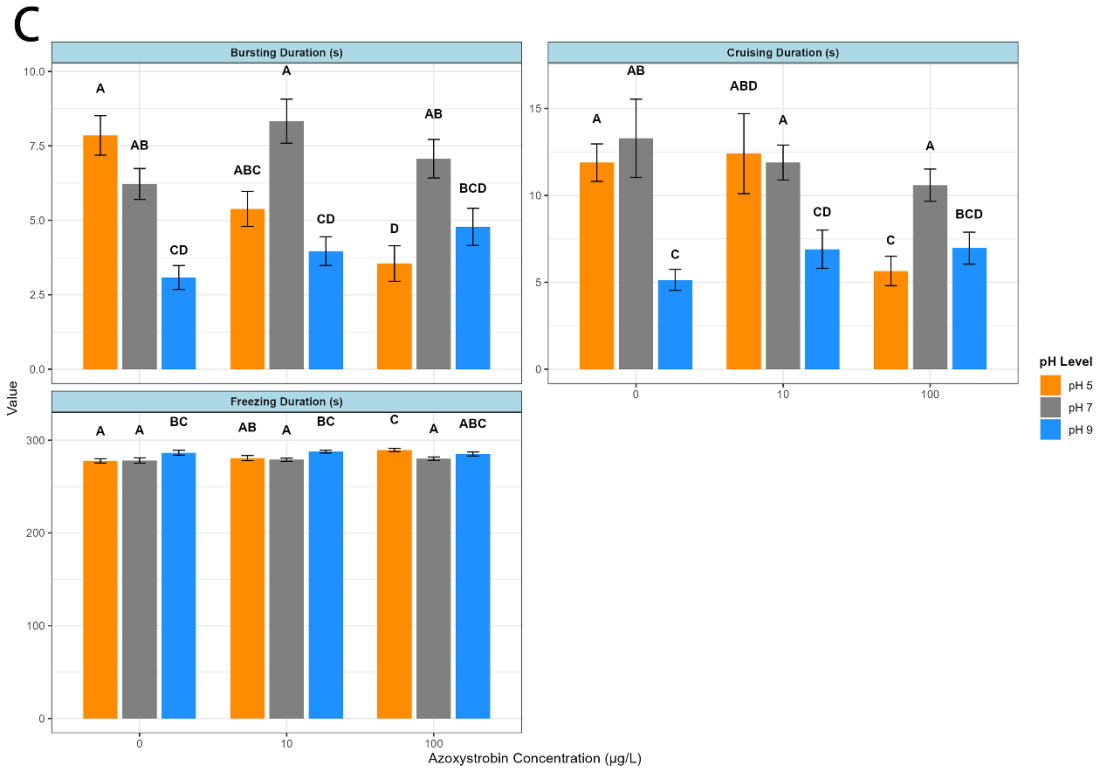


Figure S2 (Continued)



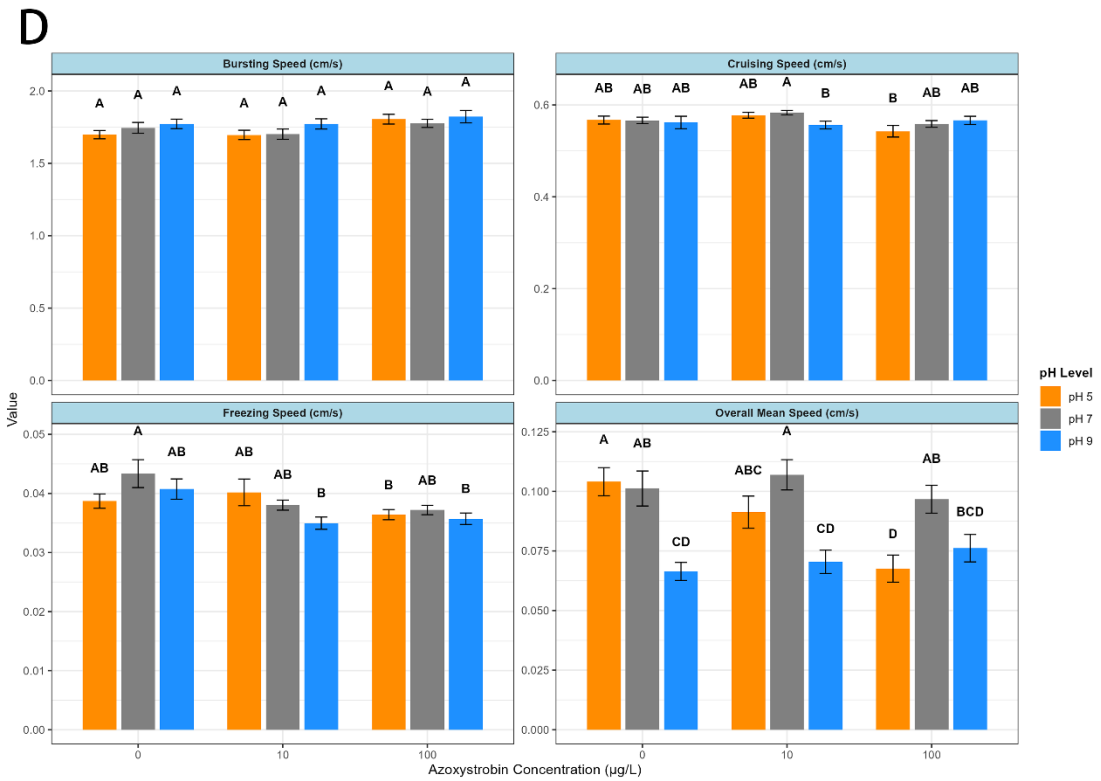
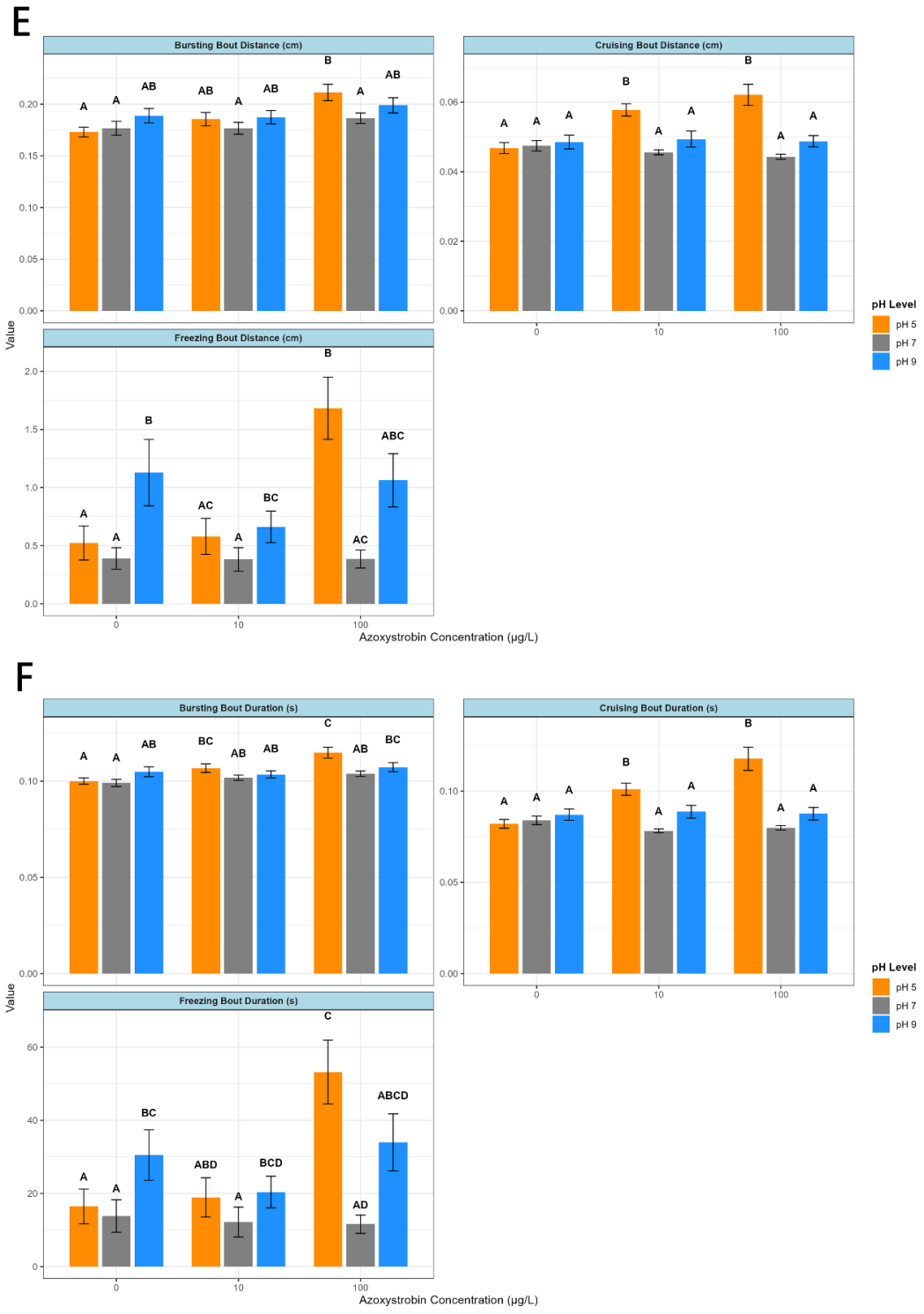


Figure S2 (Continued)



Figures A-F show results for counts, distance, duration, speed, bout distance, and bout duration, respectively. Values are means ± SE. Different letters indicate a significant difference ($p < 0.05$).

Figure S3 Swimming parameters of zebrafish larvae exposed to azoxystrobin at different pH levels in the light.

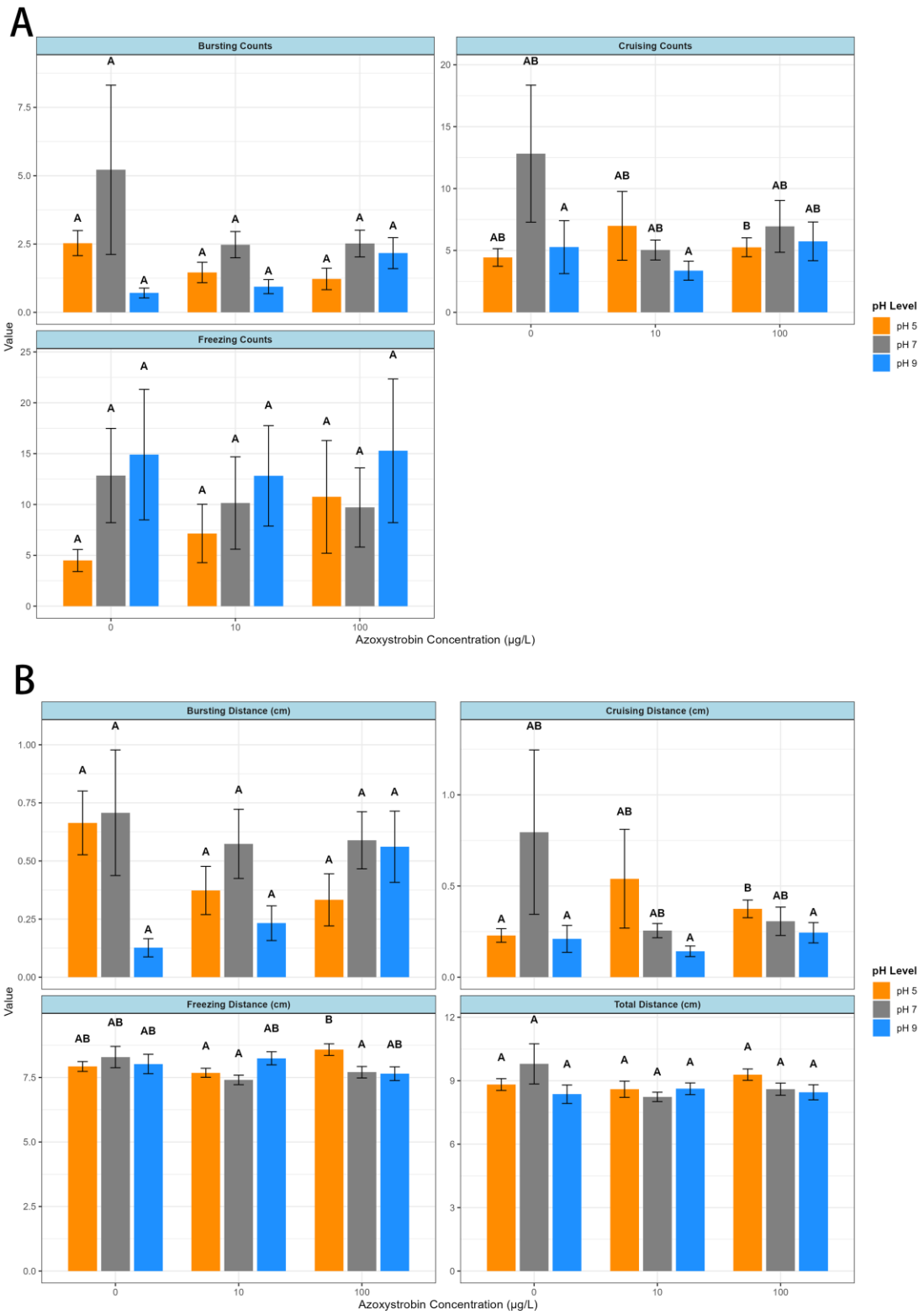
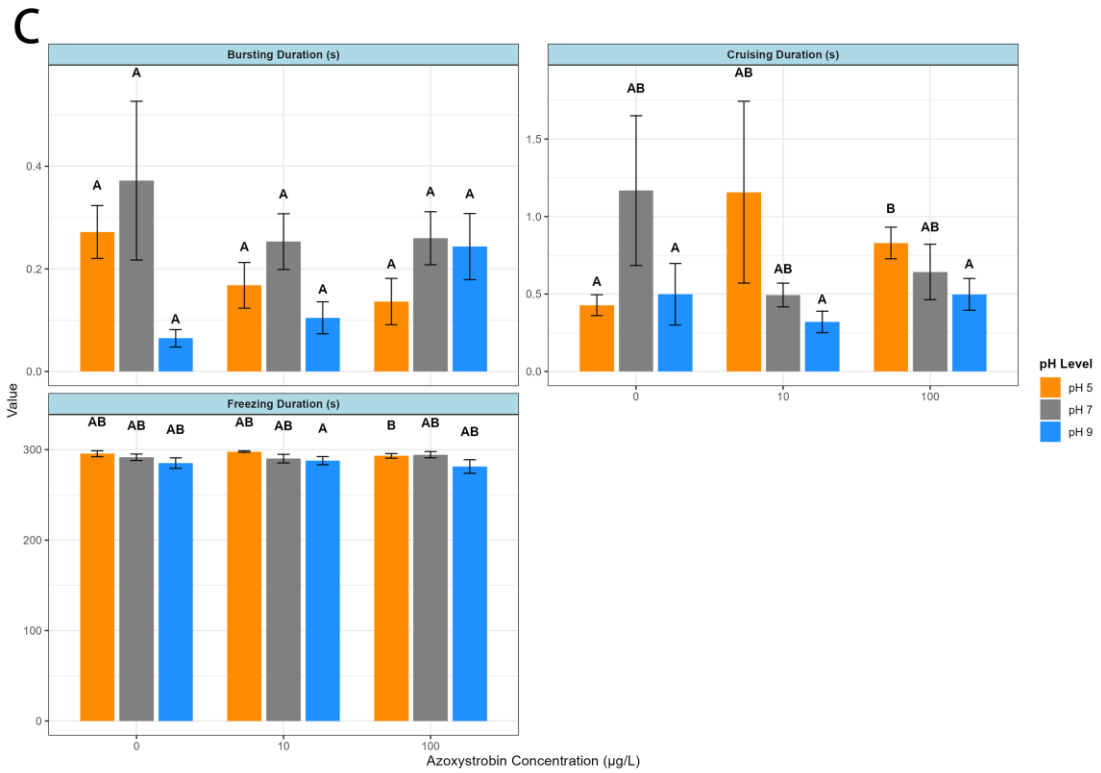
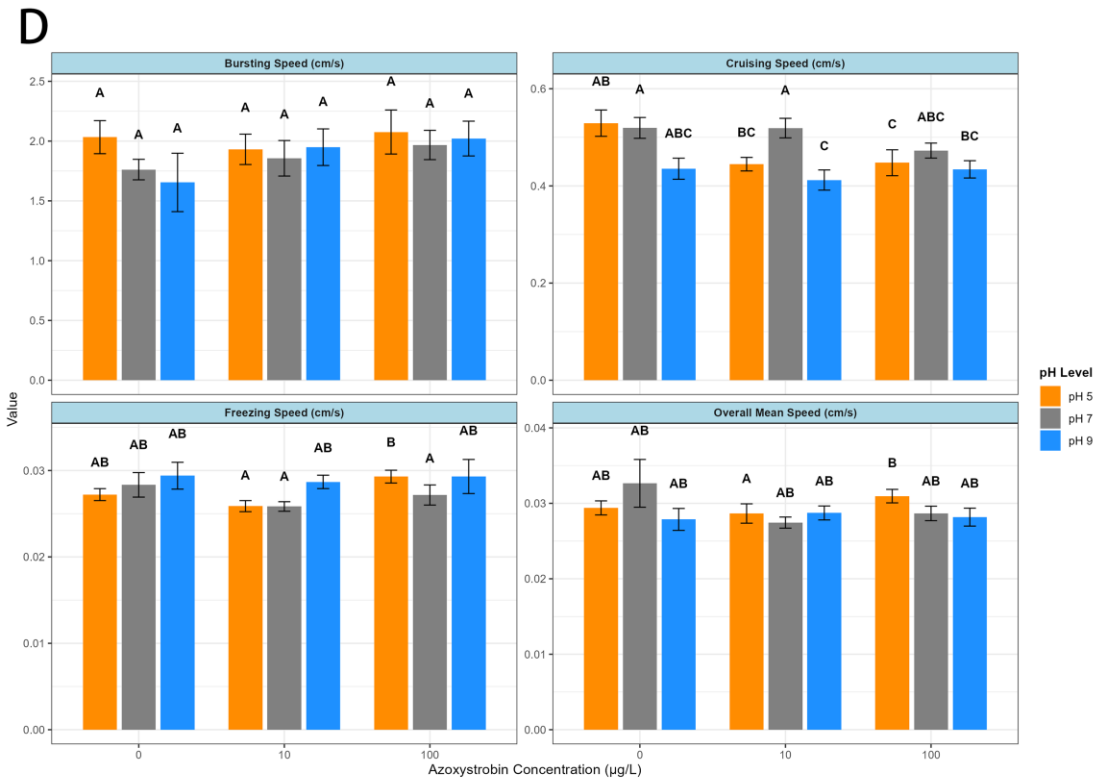


Figure S3 (Continued)





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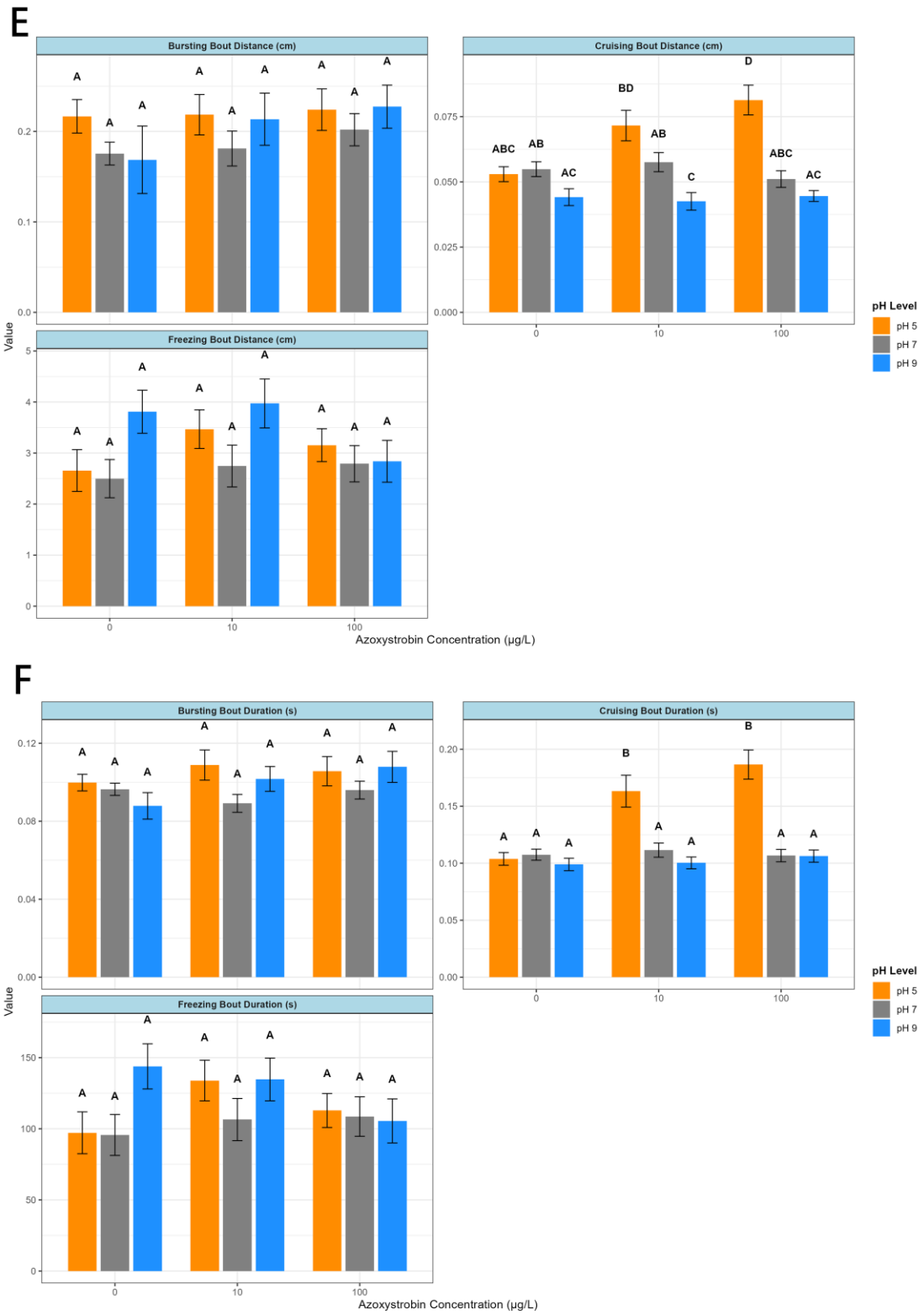
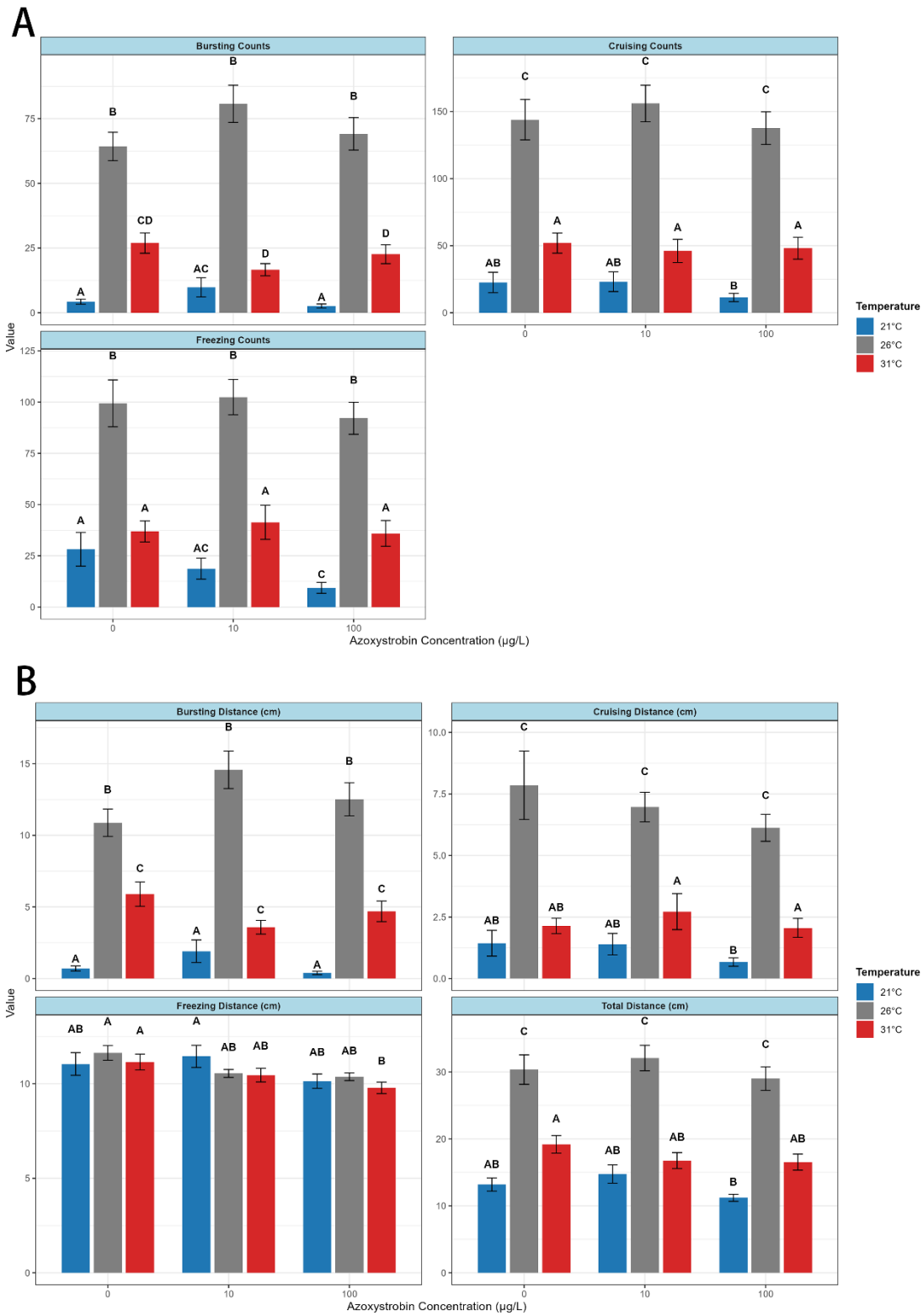


Figures A-F show results for counts, distance, duration, speed, bout distance, and bout duration, respectively. Values are means ± SE. Different letters indicate a significant difference ($p < 0.05$).

Figure S4 Swimming parameters of zebrafish larvae exposed to azoxystrobin at different temperature levels in the dark.



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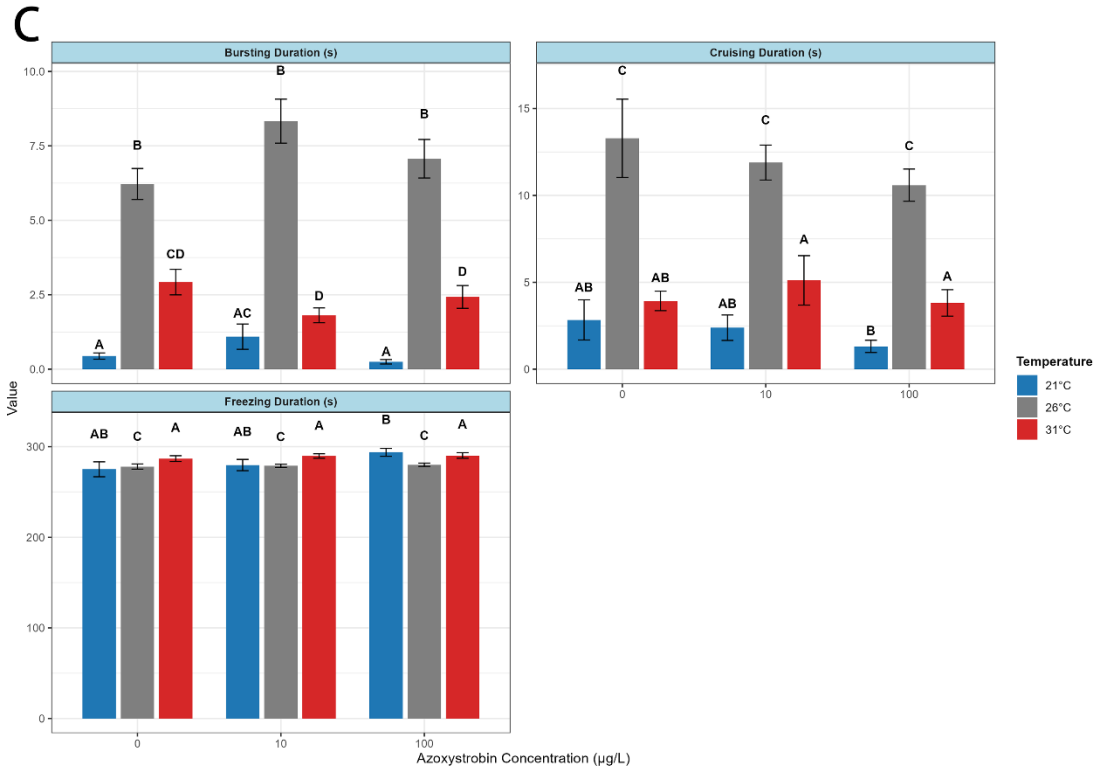


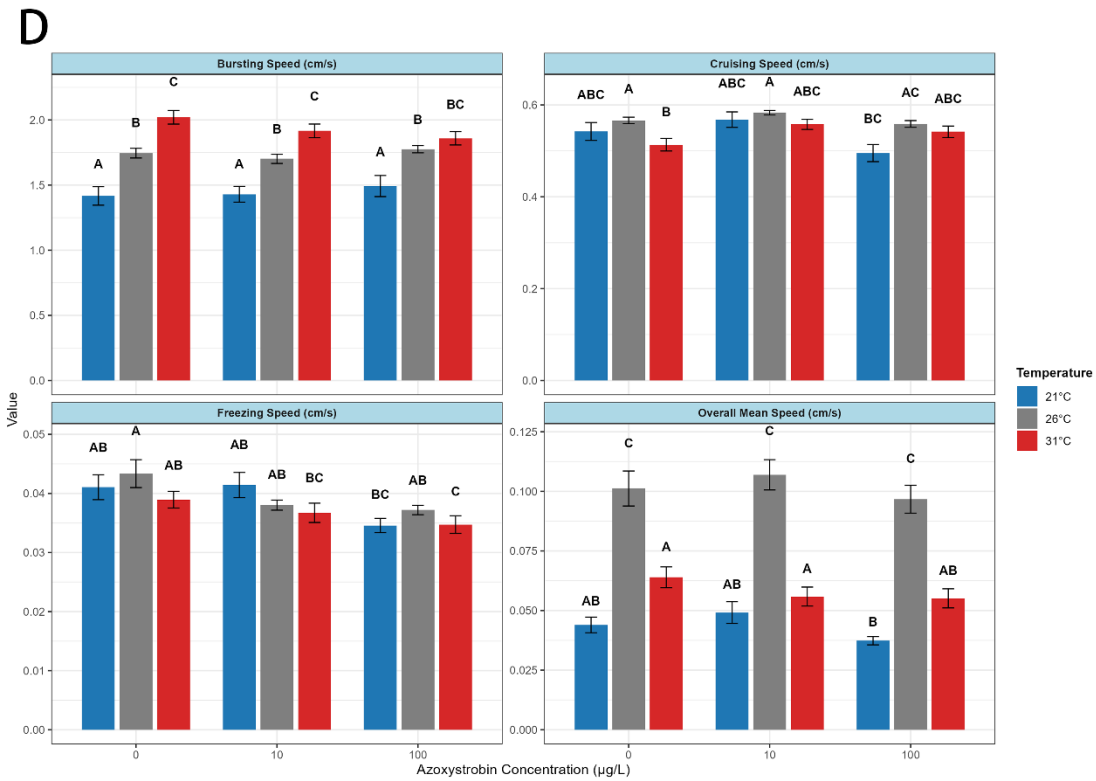
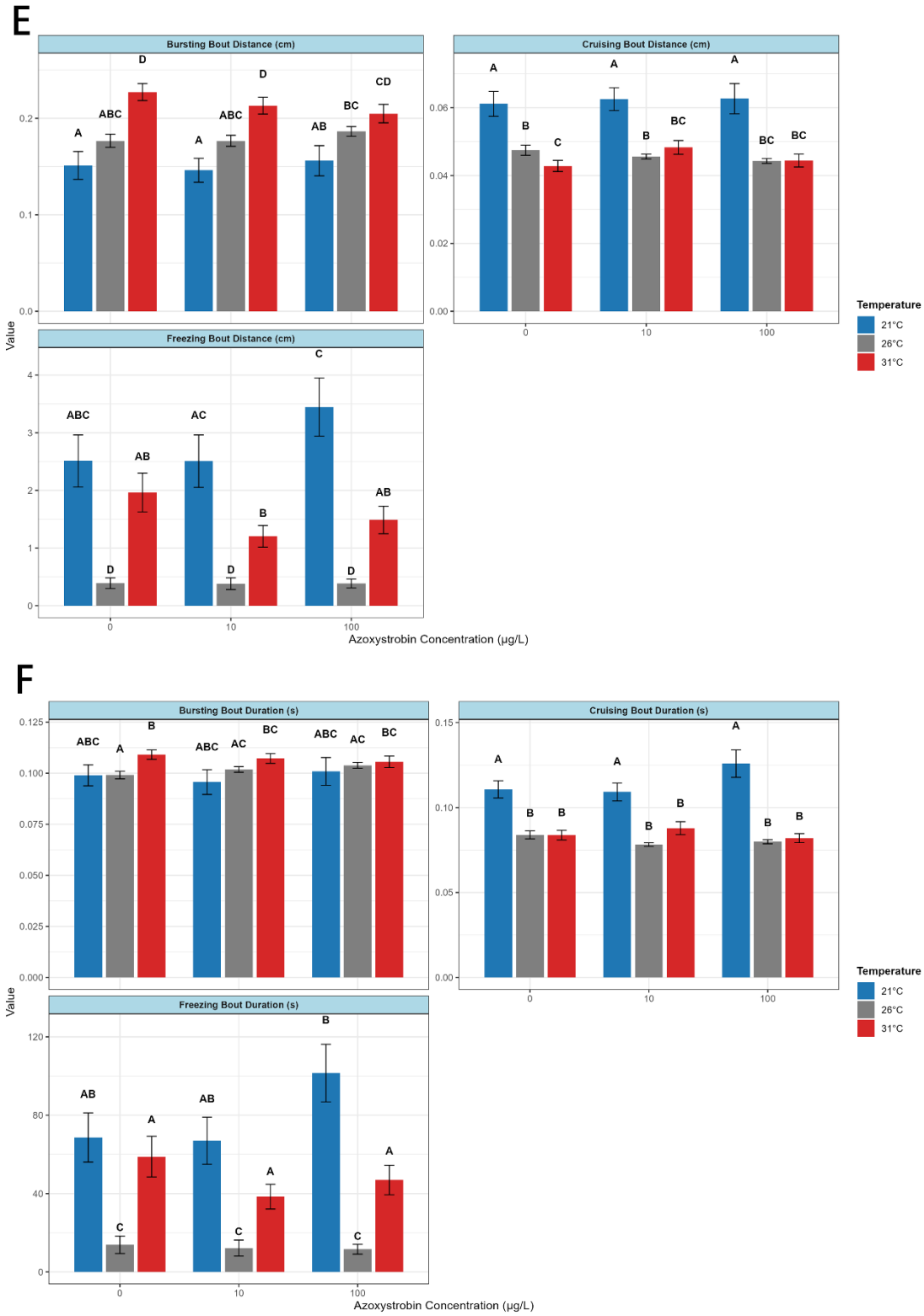
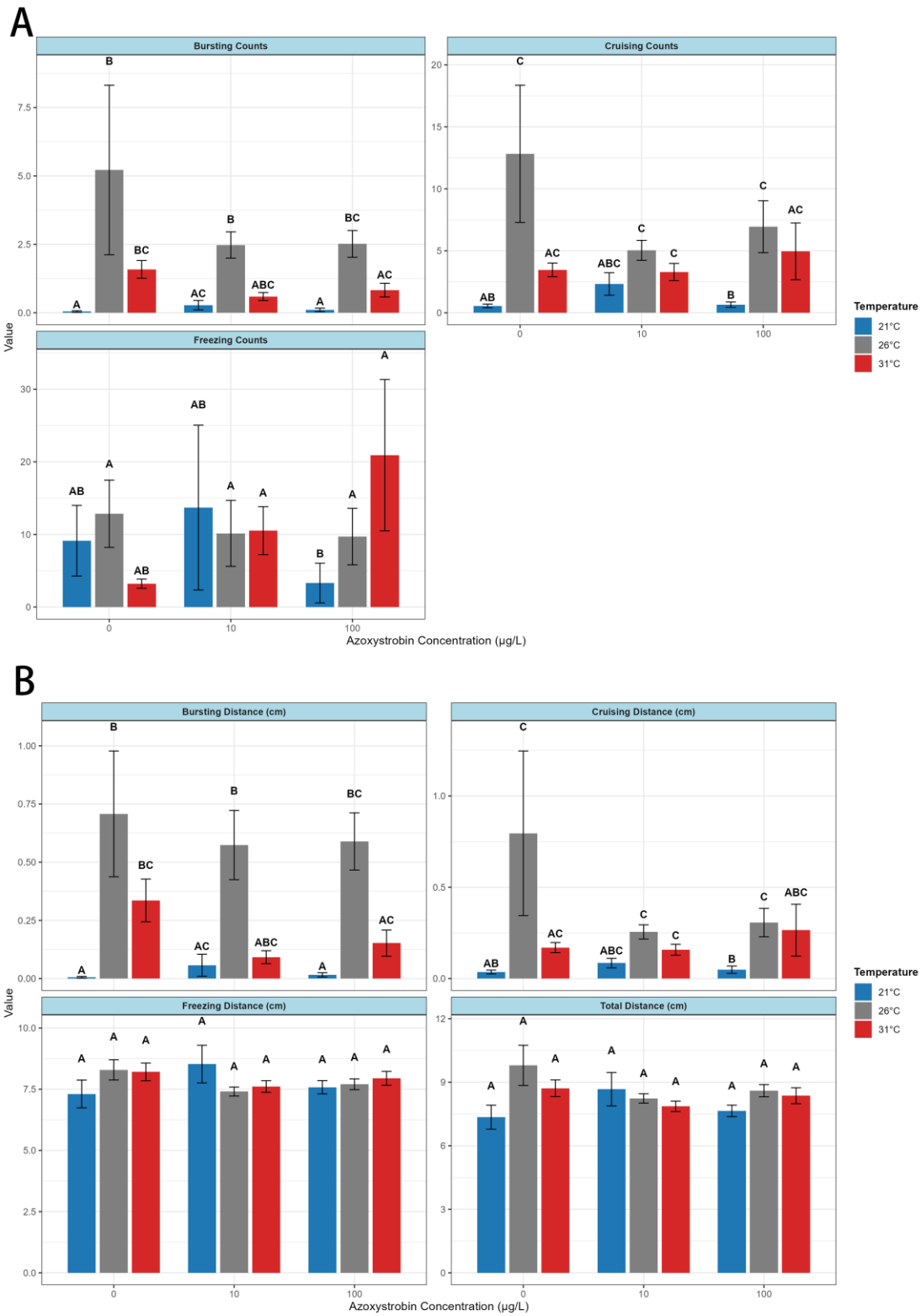


Figure S4 (Continued)



Figures A-F show results for counts, distance, duration, speed, bout distance, and bout duration, respectively. Values are means ± SE. Different letters indicate a significant difference ($p < 0.05$).

Figure S5 Swimming parameters of zebrafish larvae exposed to azoxystrobin at different temperature levels in the light.



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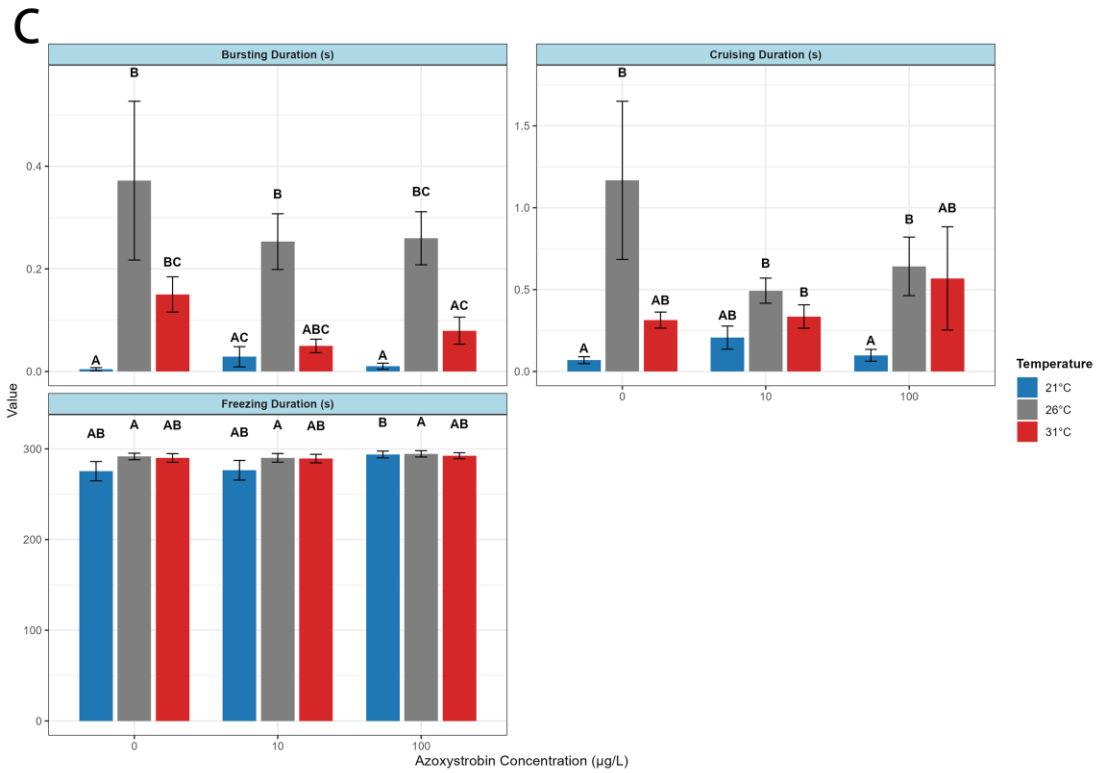


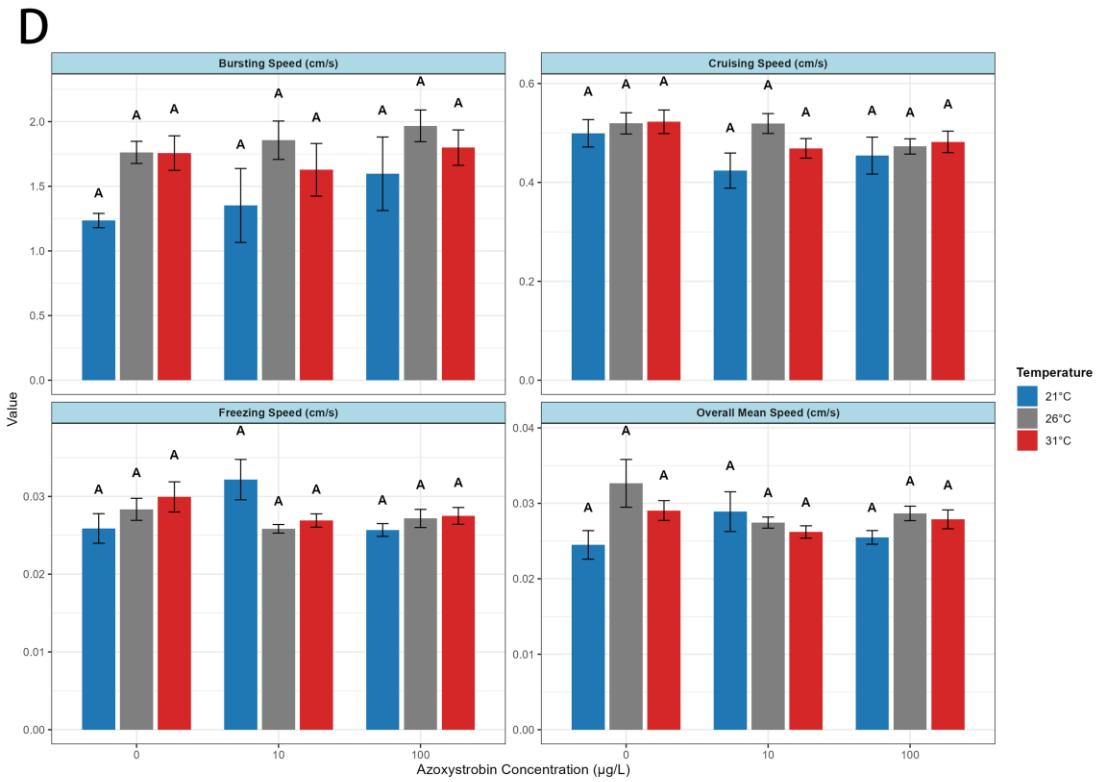
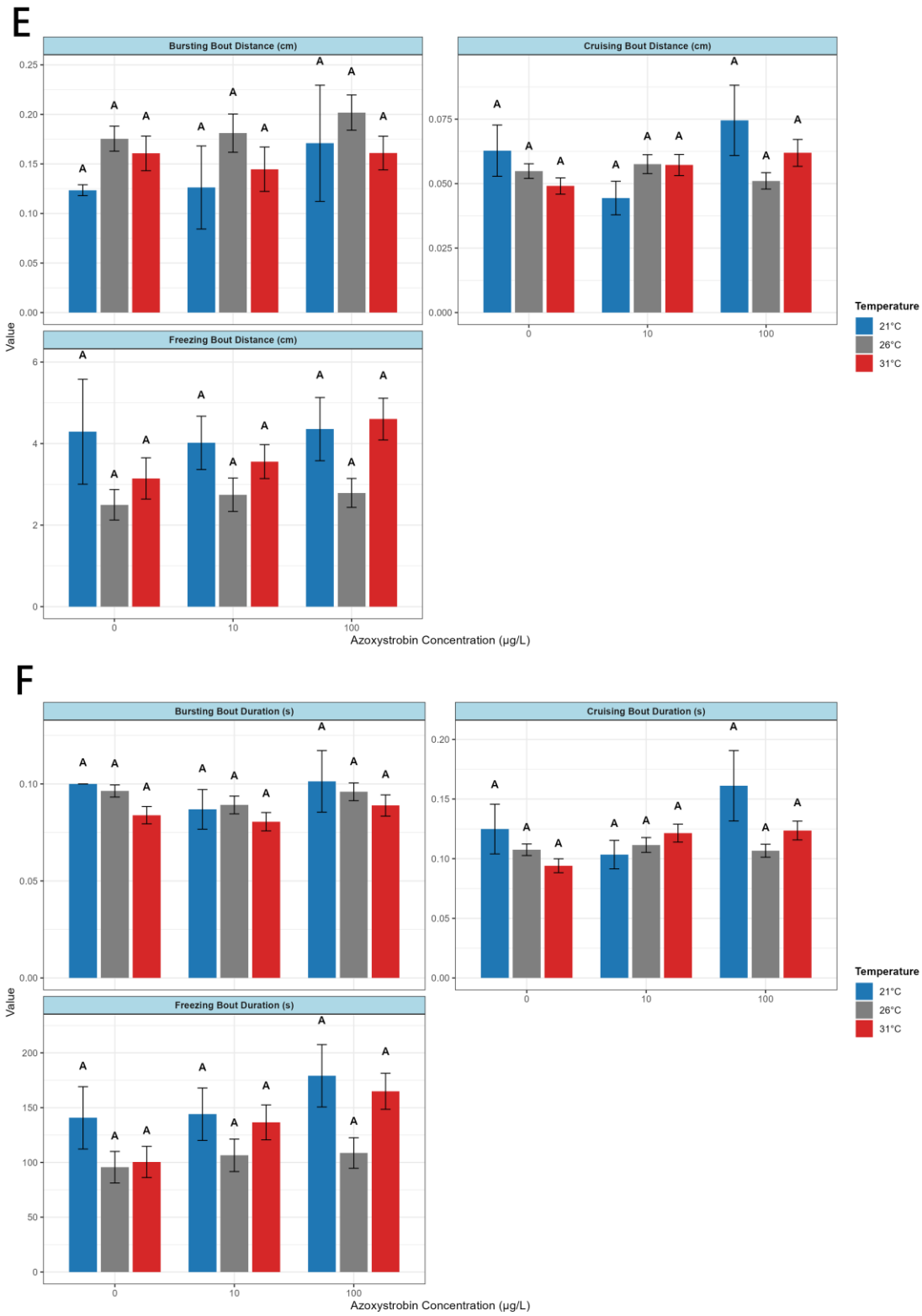
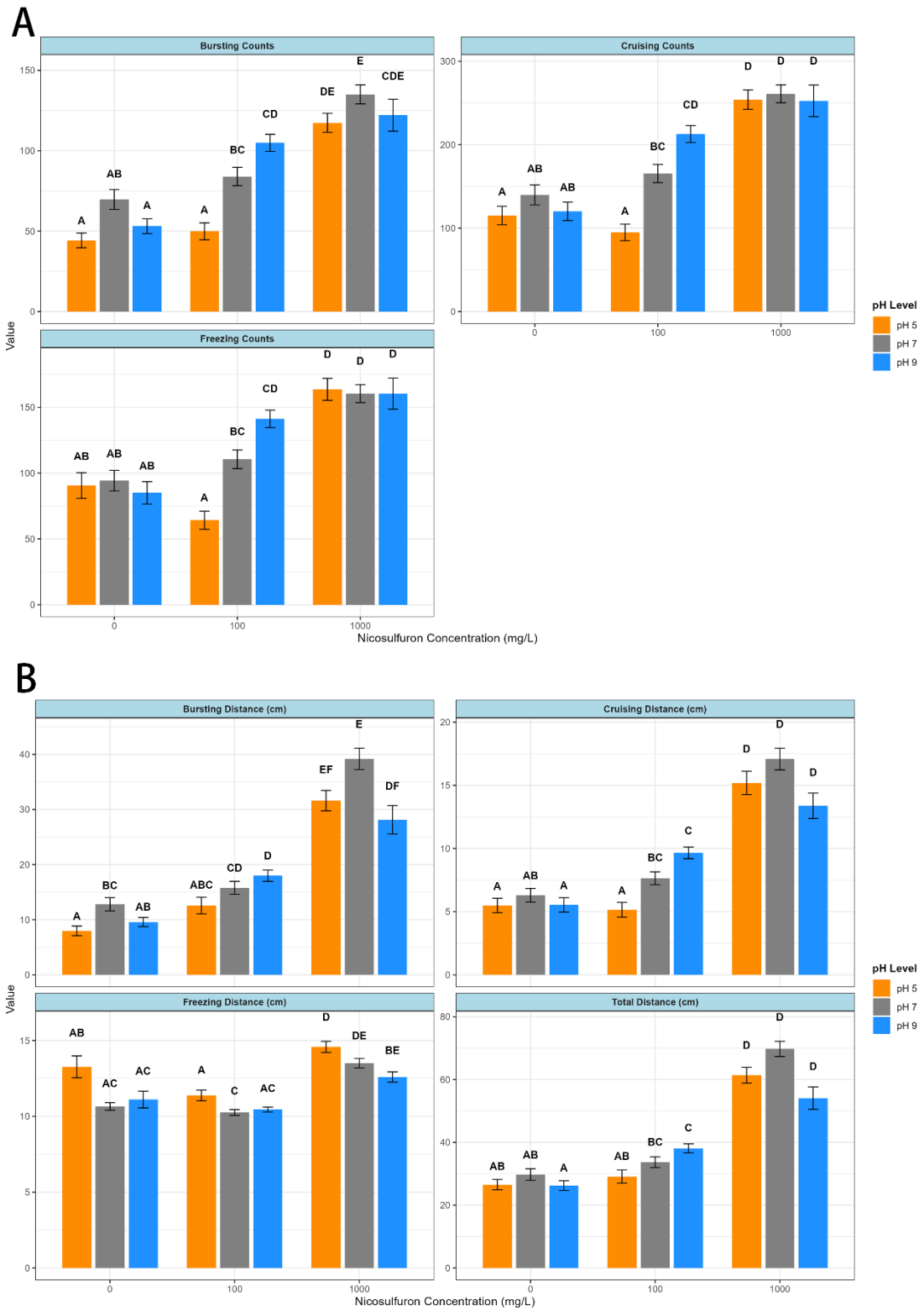


Figure S5 (Continued)



Figures A-F show results for counts, distance, duration, speed, bout distance, and bout duration, respectively. Values are means ± SE. Different letters indicate a significant difference ($p < 0.05$).

Figure S6 Swimming parameters of zebrafish larvae exposed to nicosulfuron at different pH levels in the dark.



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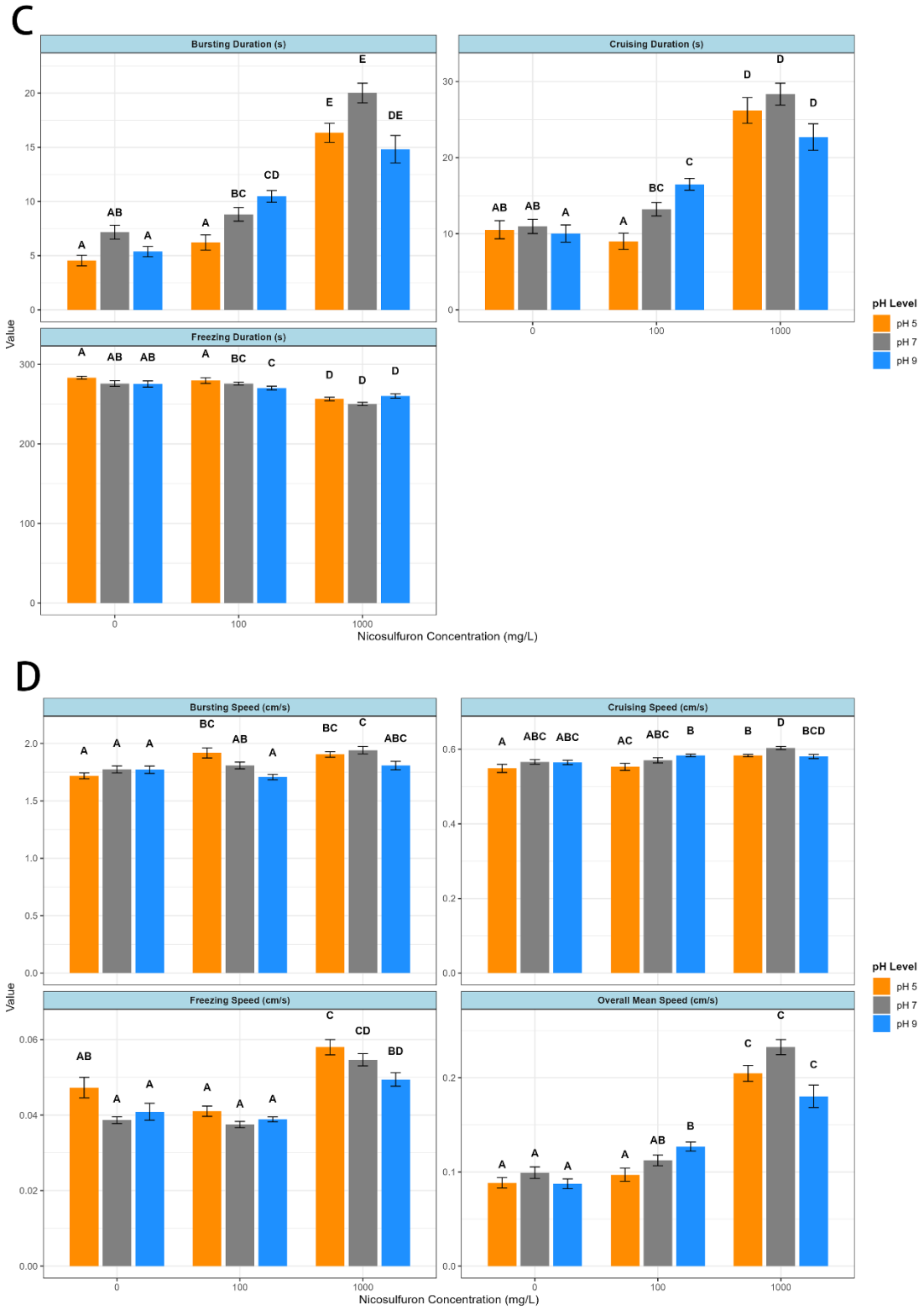

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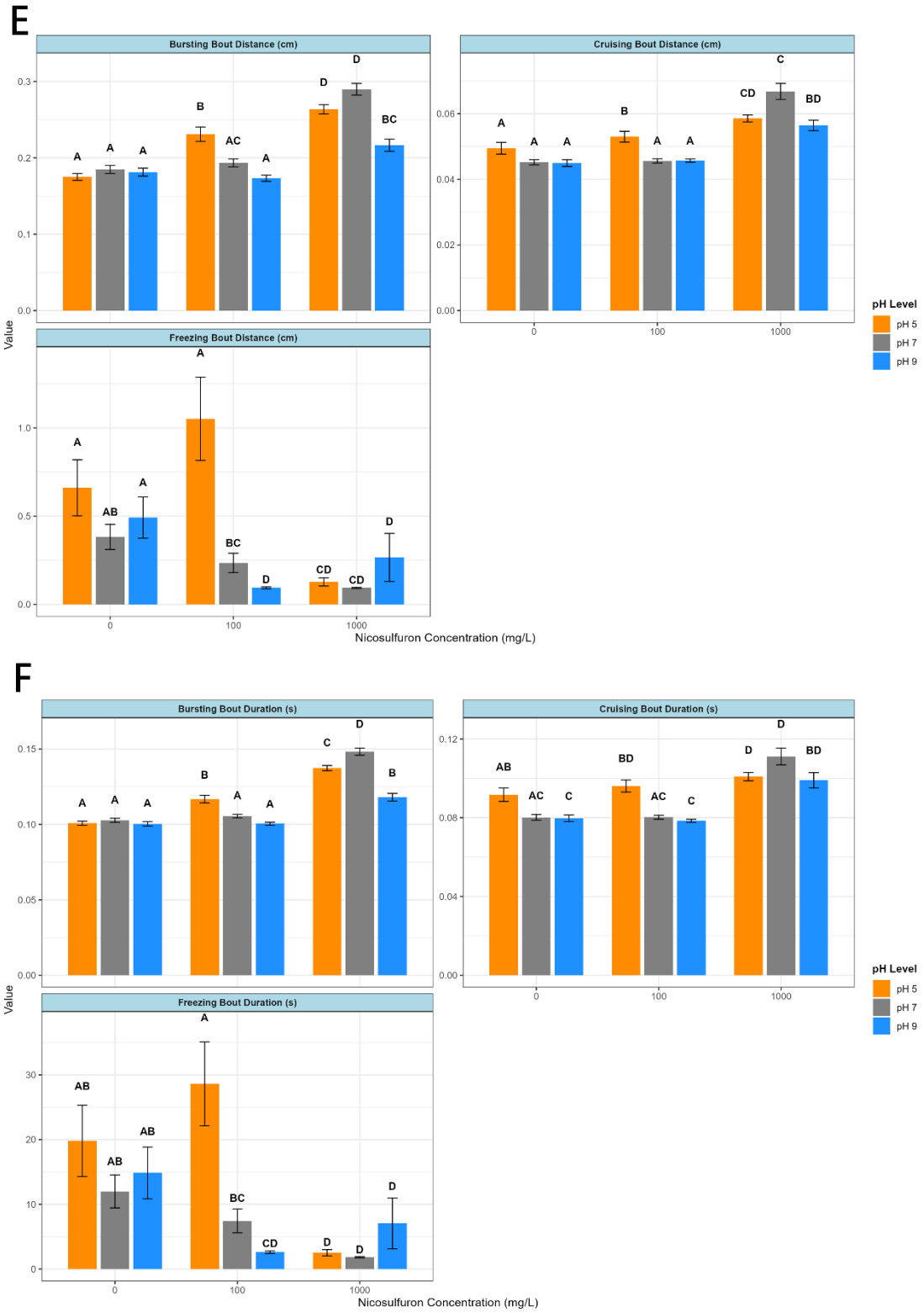
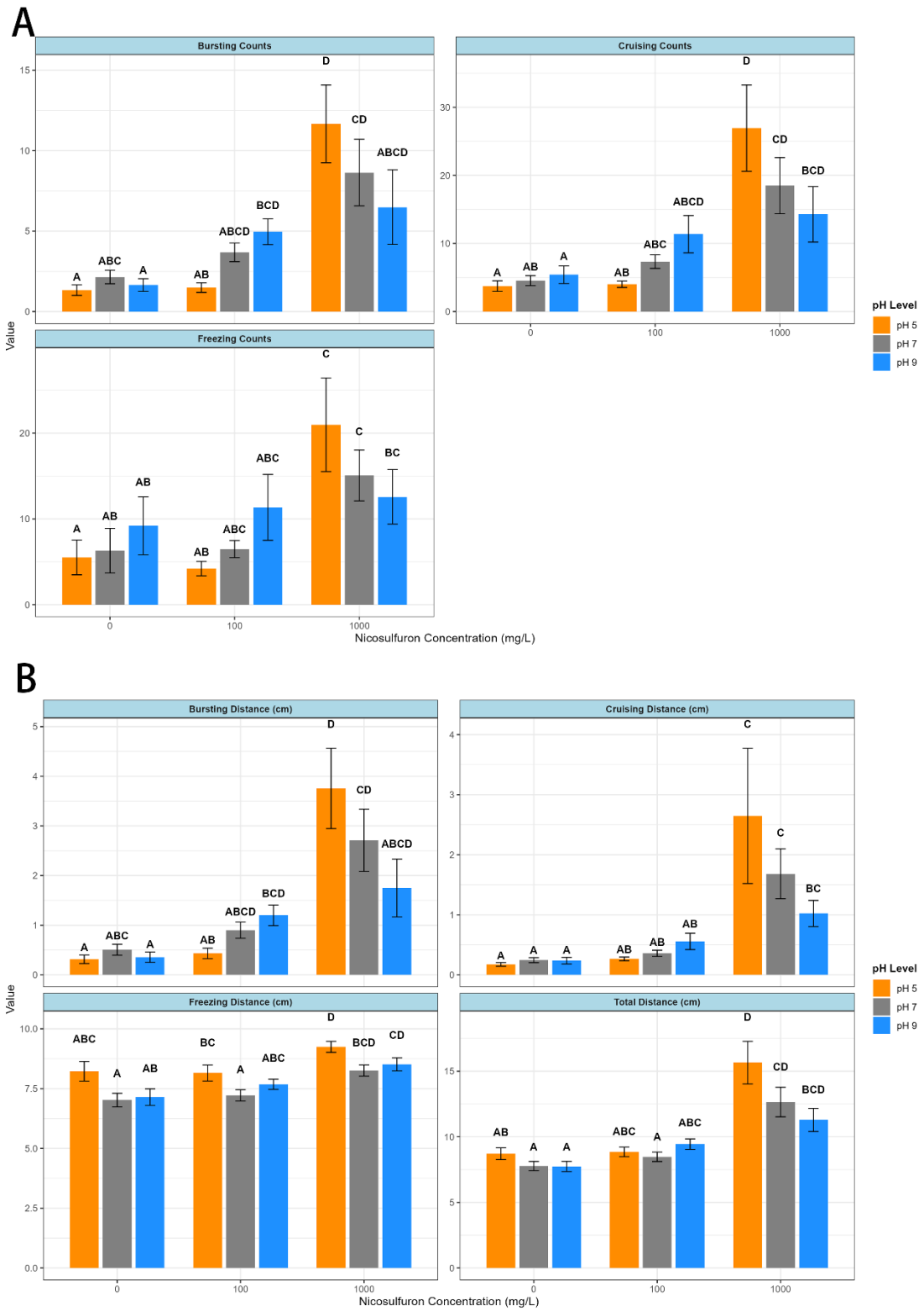


Figures A-F show results for counts, distance, duration, speed, bout distance, and bout duration, respectively. Values are means ± SE. Different letters indicate a significant difference ($p < 0.05$).

Figure S7 Swimming parameters of zebrafish larvae exposed to nicosulfuron at different pH levels in the light.



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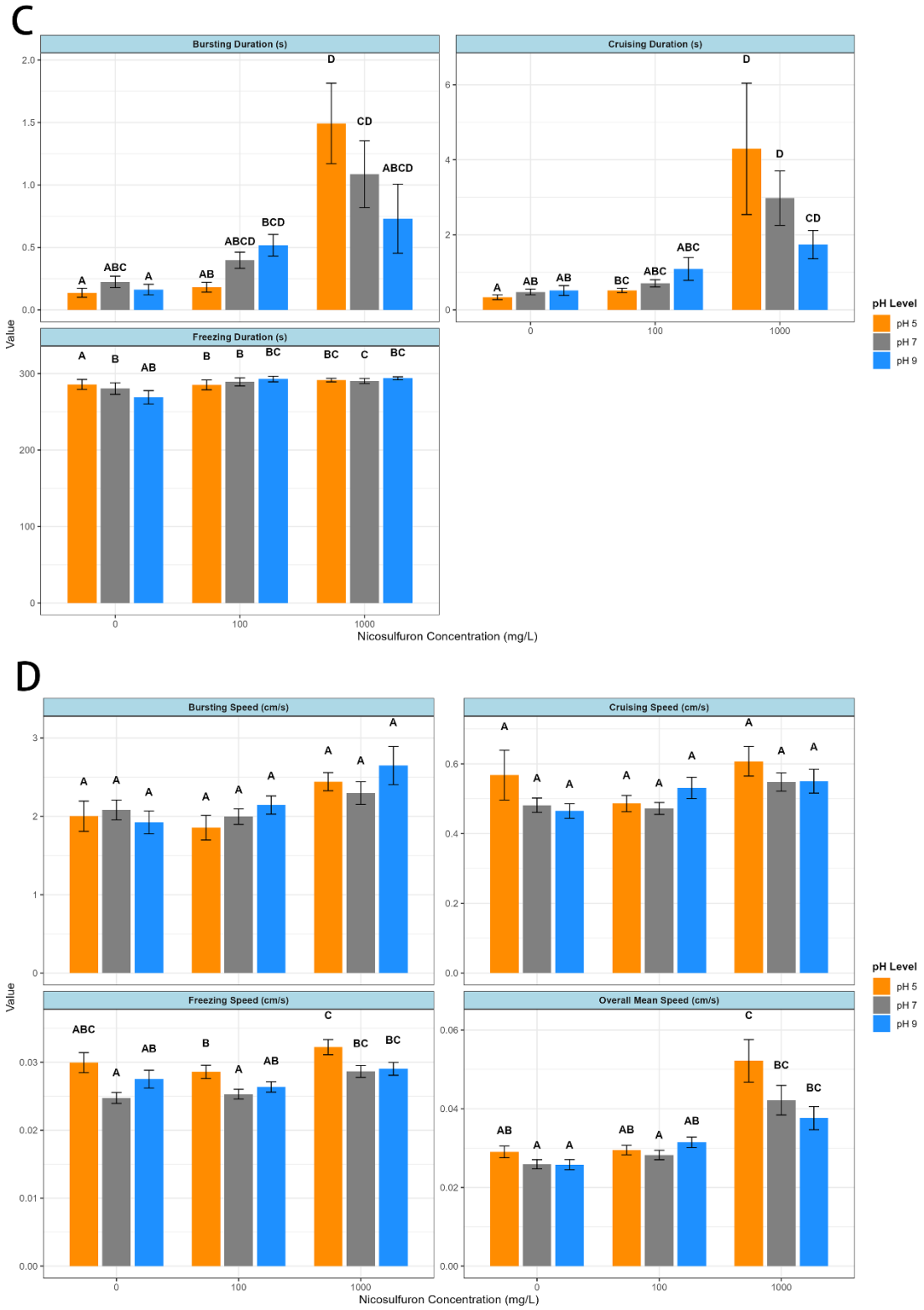
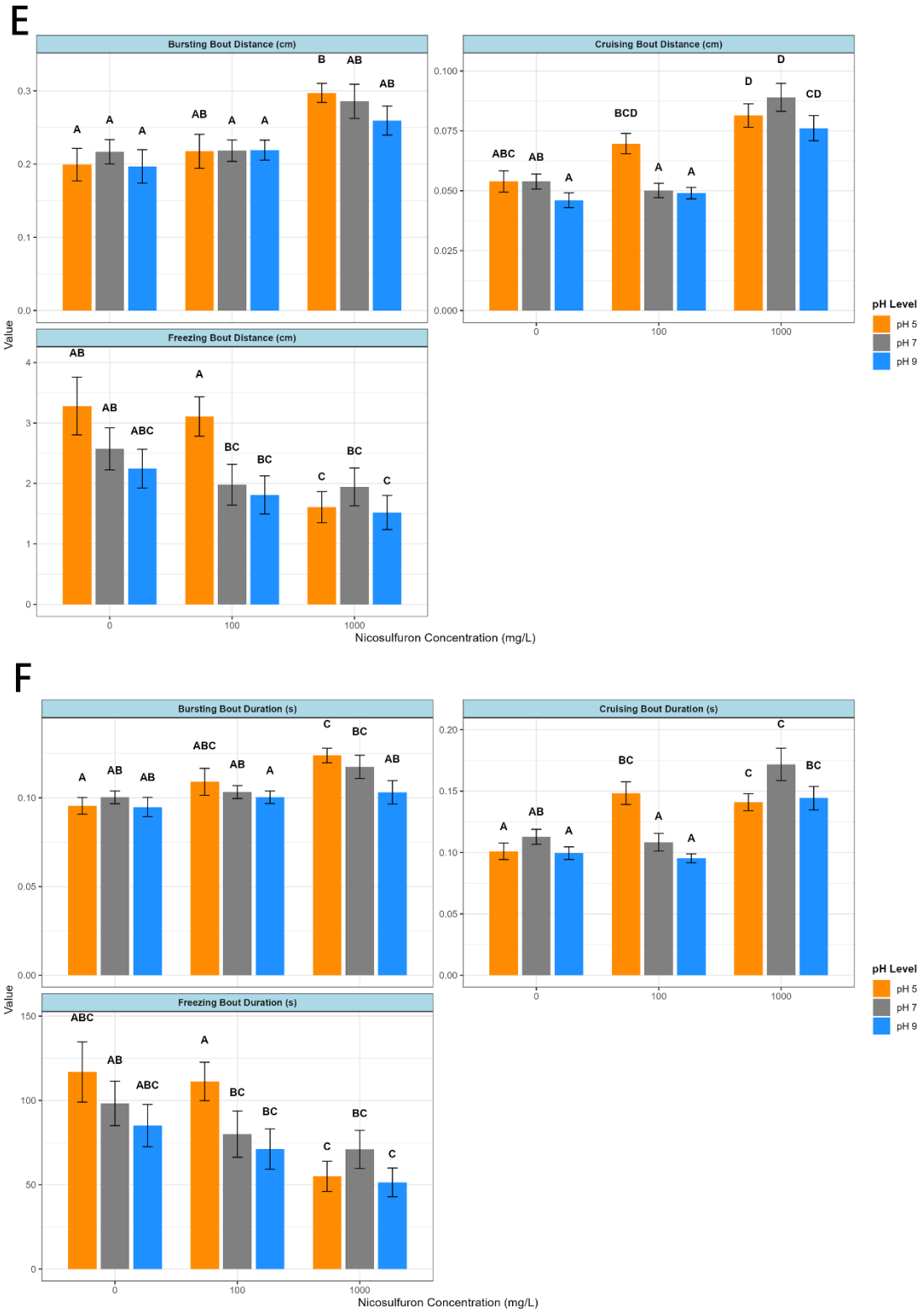
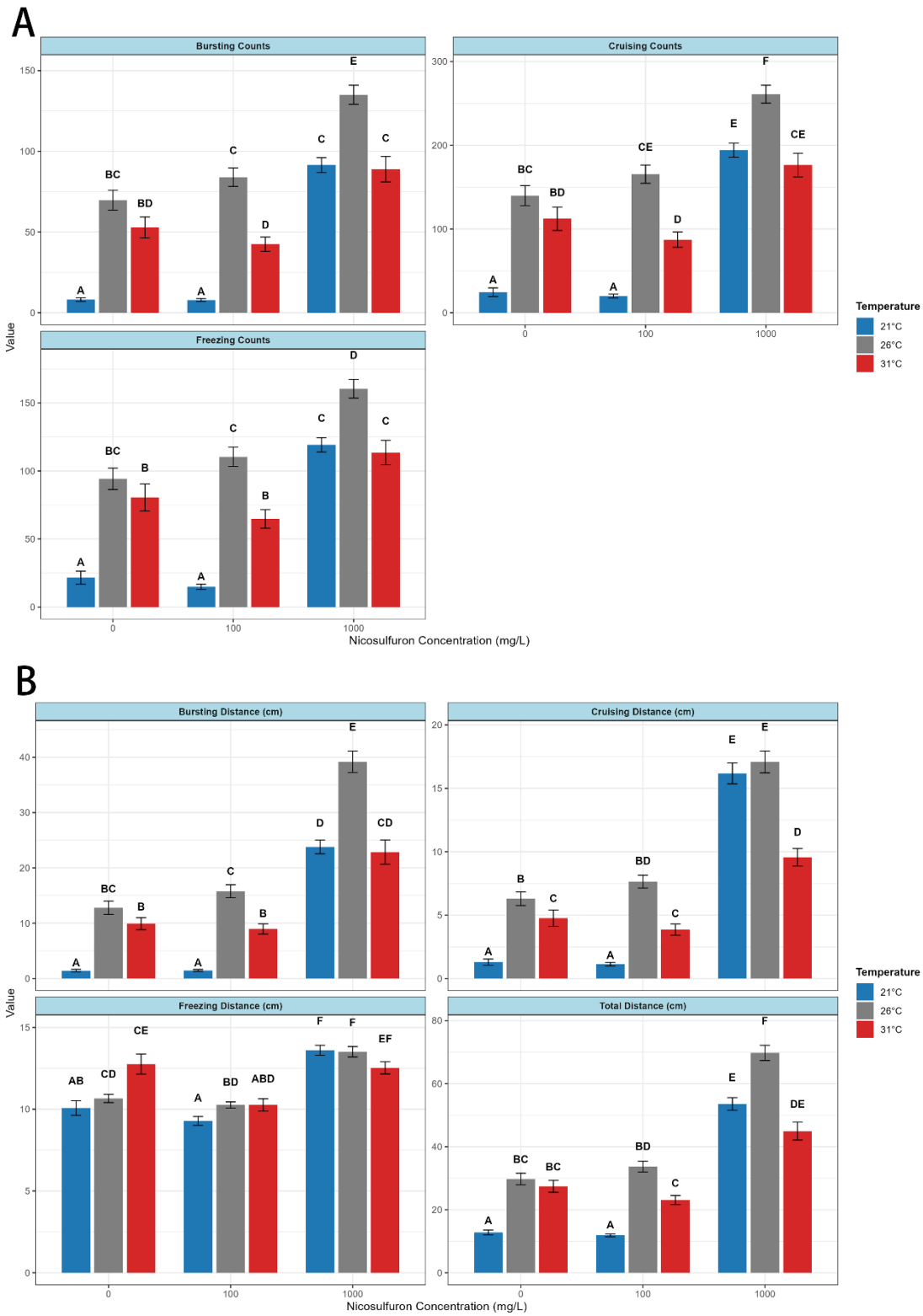


Figure S7 (Continued)



Figures A-F show results for counts, distance, duration, speed, bout distance, and bout duration, respectively. Values are means ± SE. Different letters indicate a significant difference ($p < 0.05$).

Figure S8 Swimming parameters of zebrafish larvae exposed to nicosulfuron at different temperature levels in the dark.



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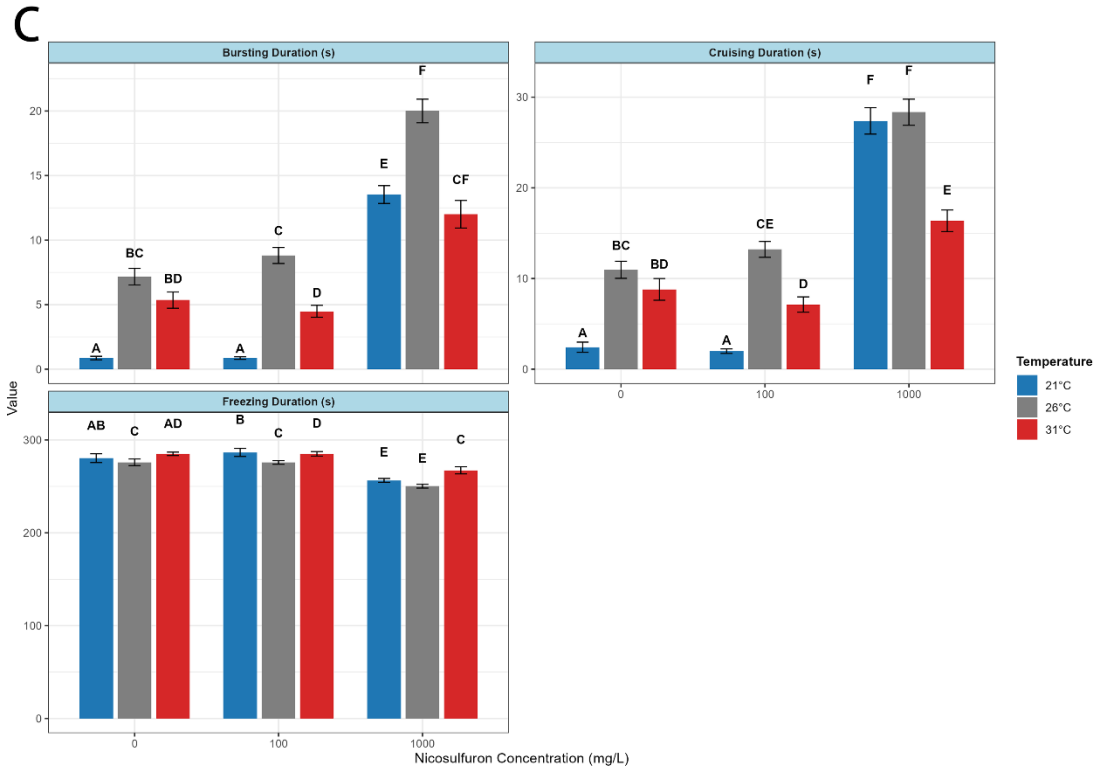

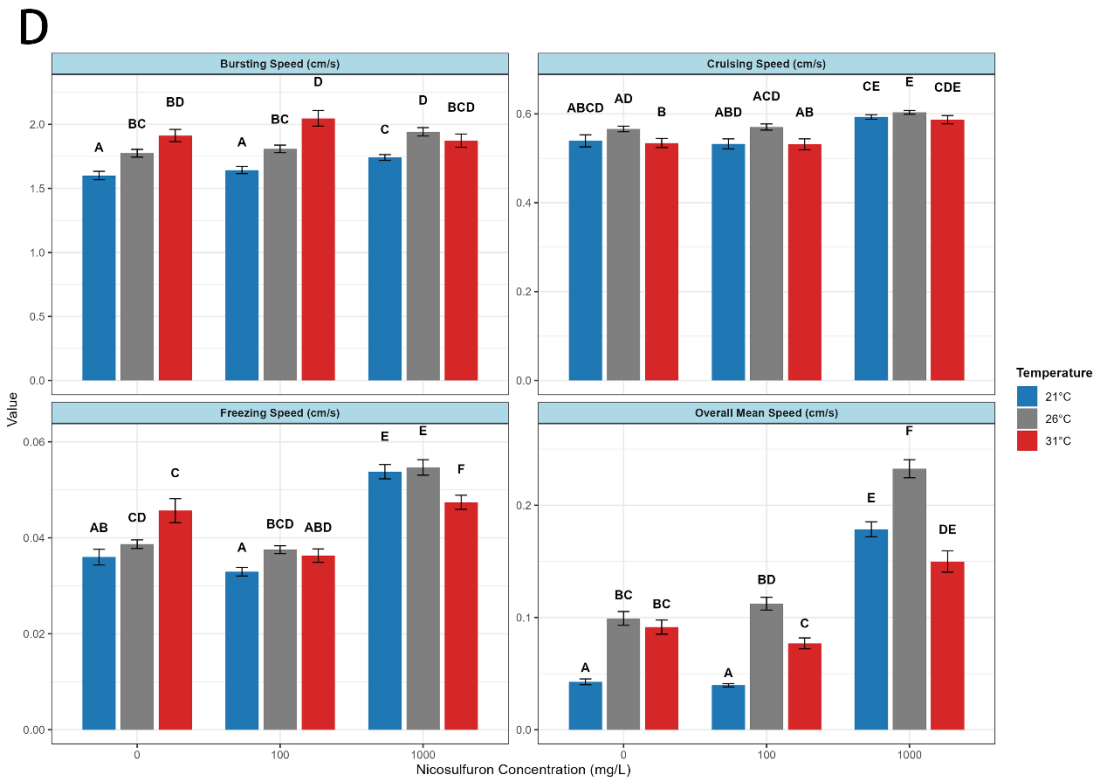

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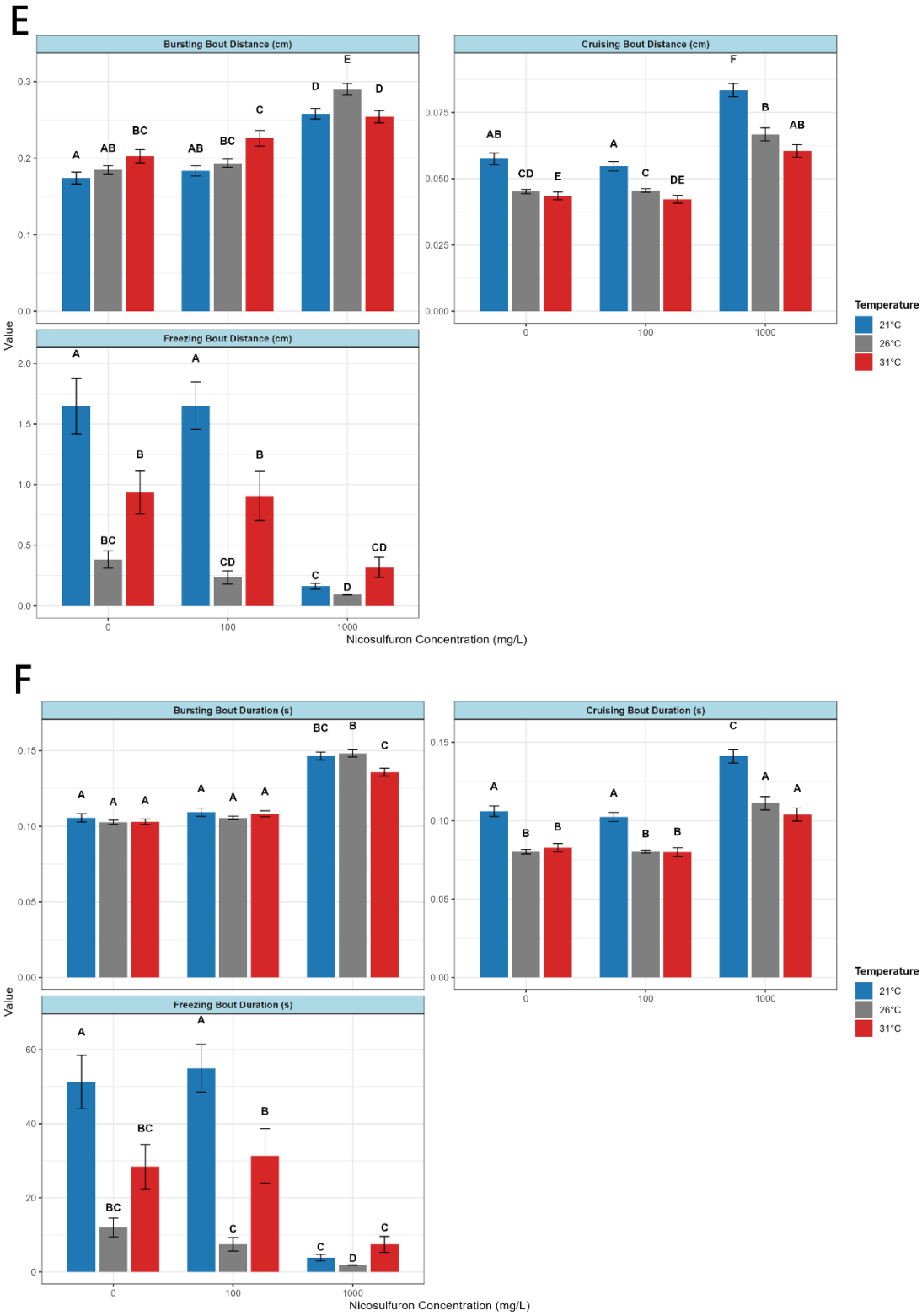
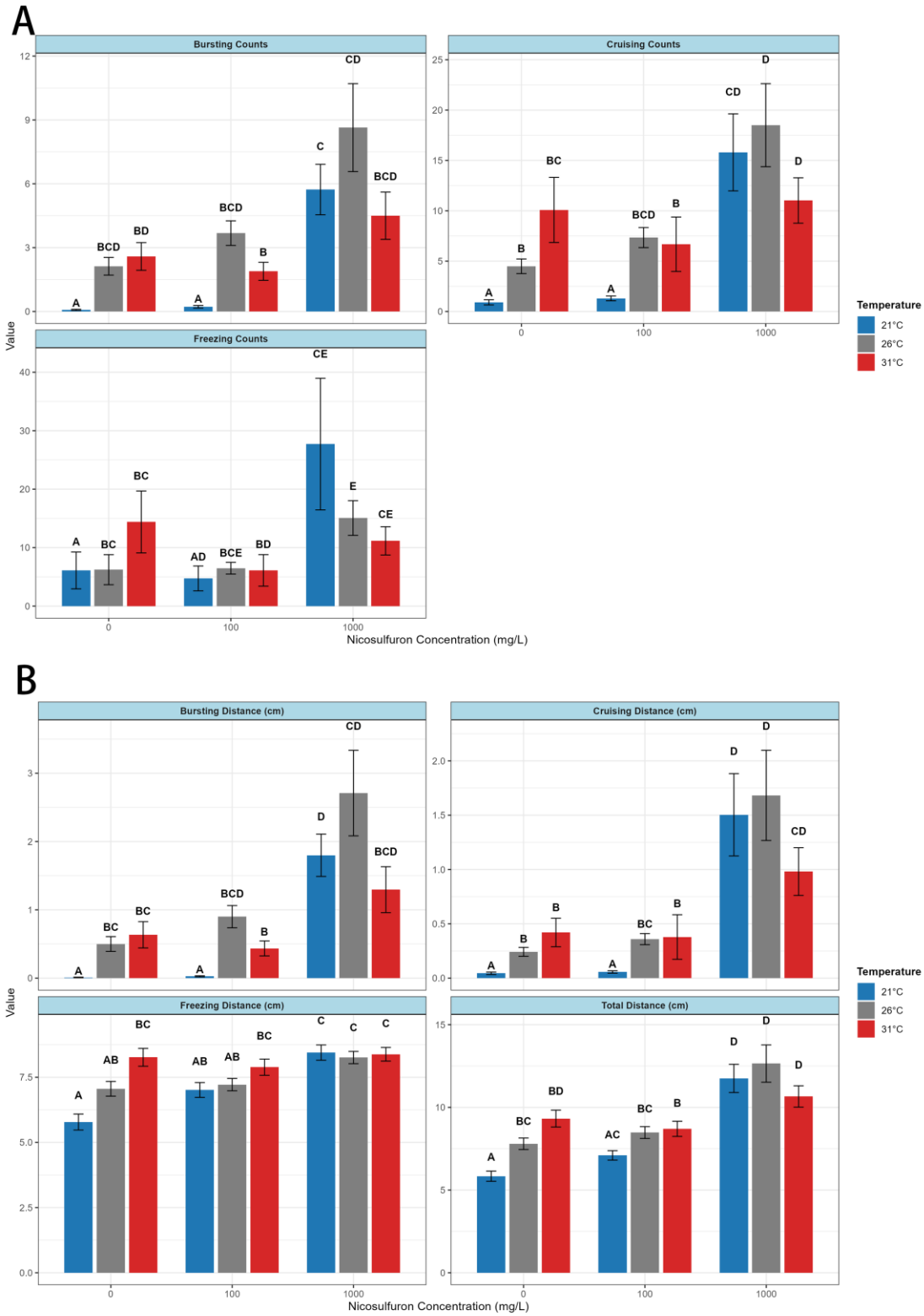


Figures A-F show results for counts, distance, duration, speed, bout distance, and bout duration, respectively. Values are means ± SE. Different letters indicate a significant difference ($p < 0.05$)

Figure S9 Swimming parameters of zebrafish larvae exposed to nicosulfuron at different temperature levels in the light.



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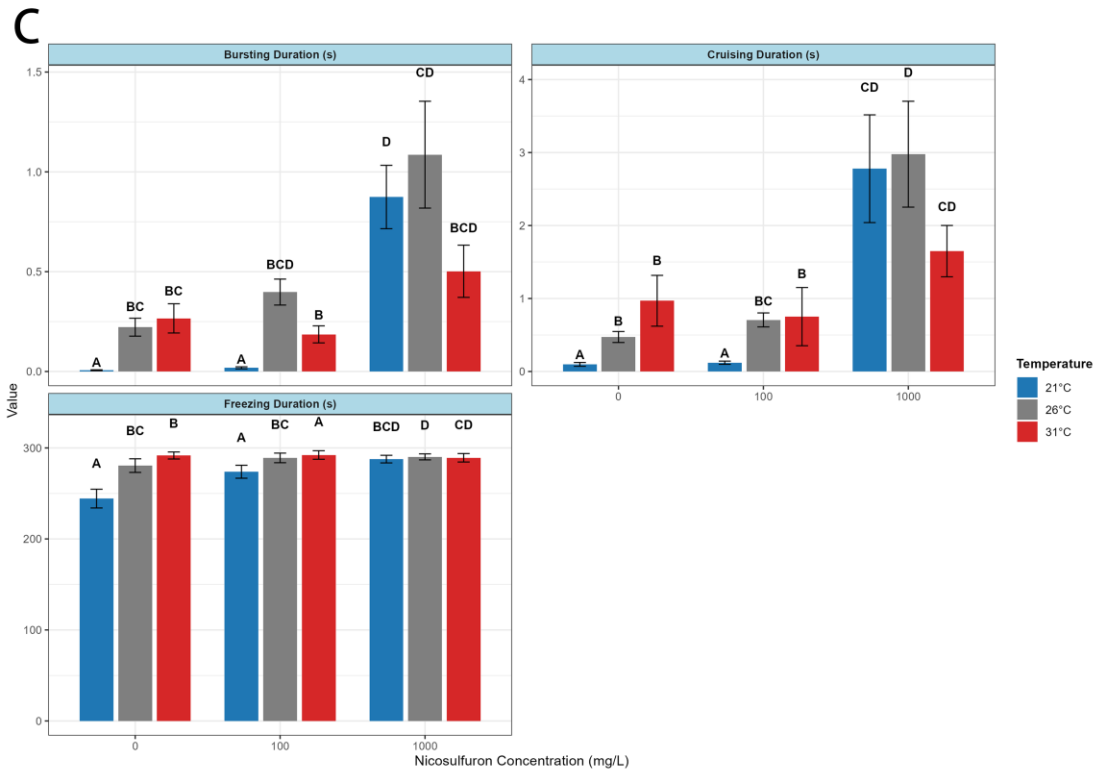


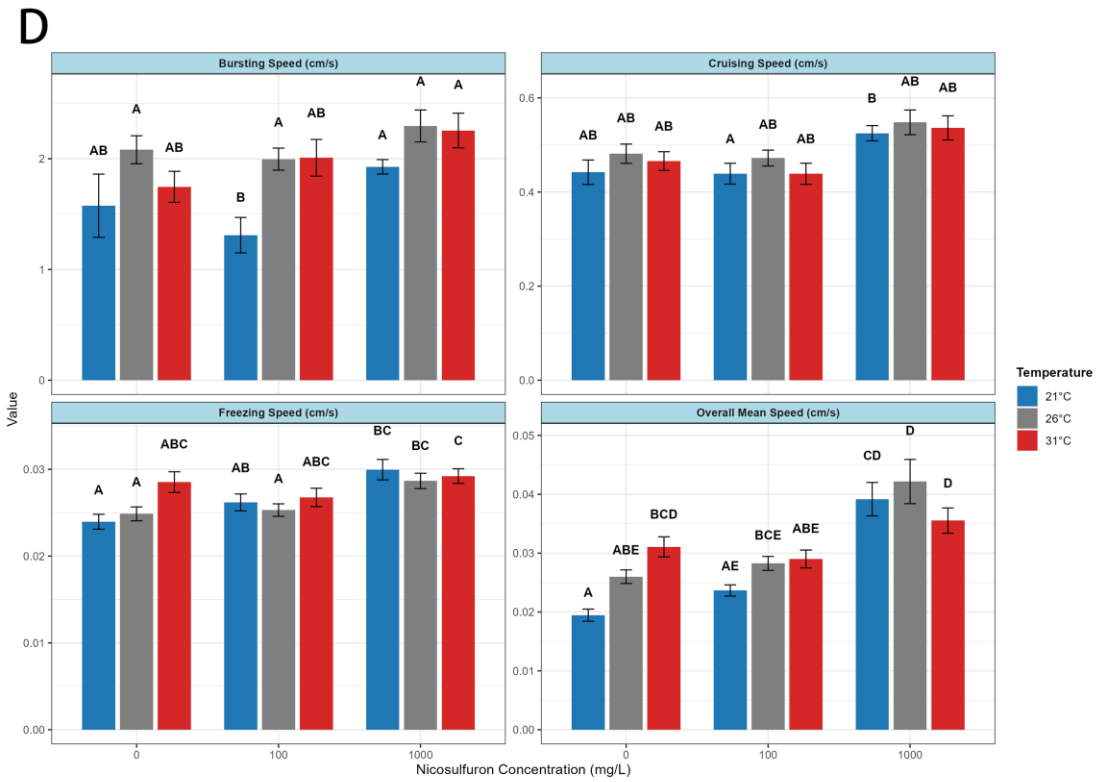
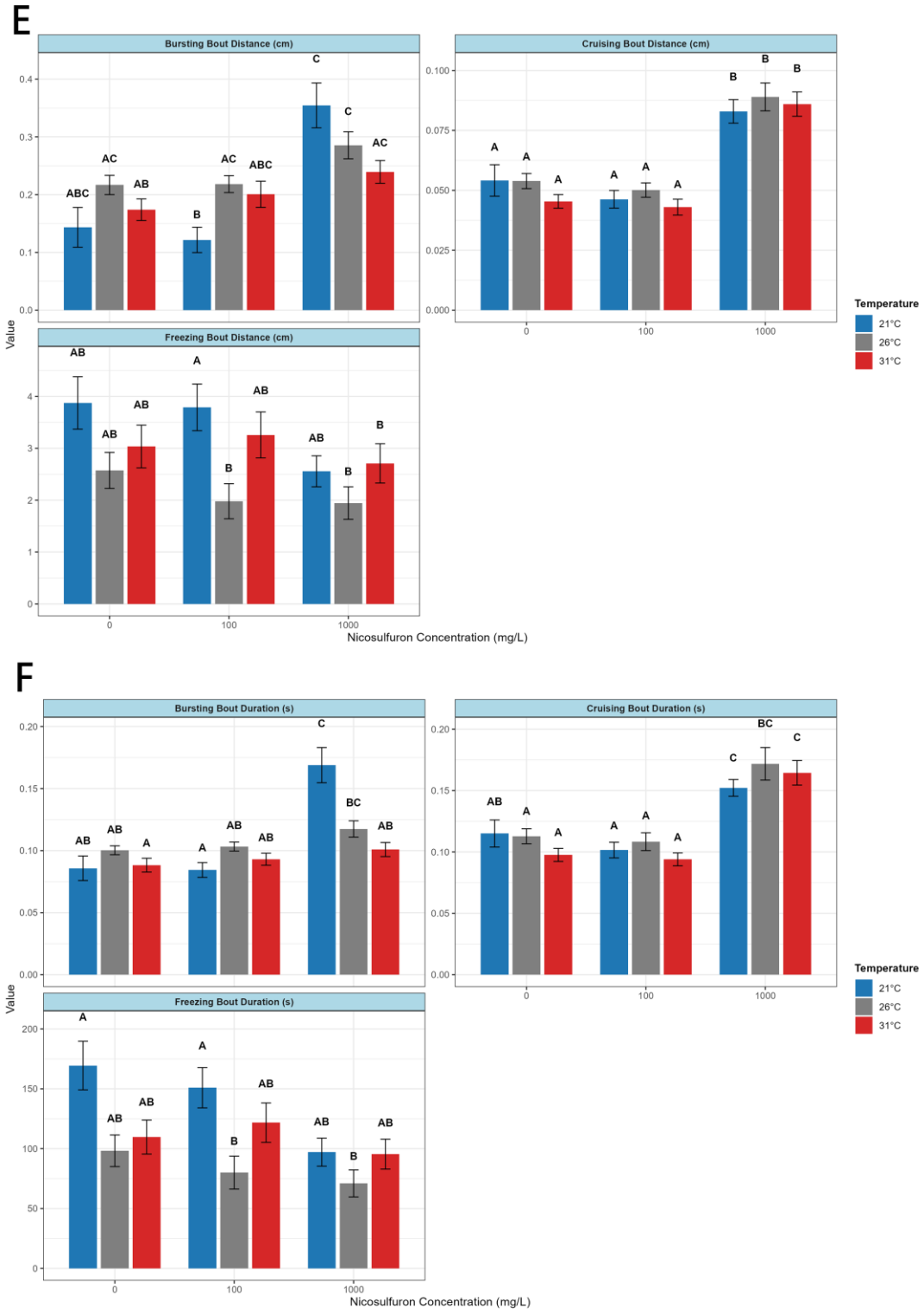


Figure S9 (Continued)



Figures A-F show results for counts, distance, duration, speed, bout distance, and bout duration, respectively. Values are means ± SE. Different letters indicate a significant difference ($p < 0.05$)

Abbreviations

°C	degrees Celsius
µg	microgram
mg	milligram
hsp70	heat shock proteins with a molecular weight of approximately 70 kilodalton
EQS	Environmental Quality Standard
ATP	adenosine triphosphate
ROS	reactive oxygen species
cm	centimeter
ELS	early life stages
OECD	The Organisation for Economic Co-operation and Development
hpf	hours post-fertilization
mM	millimolar
µL	microliter
V	volt
min	minute
LDT	light/dark transition
MLR	multiple linear regression
NOEC	No Observed Effect Concentration
PNEC	predicted no-effect concentration
AF	assessment factor
LC50	Lethal Concentration 50
EC50	Effective Concentration 50
RQ	Risk quotient

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