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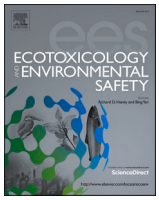

Embryonic zebrafish response to a commercial formulation of azoxystrobin at environmental concentrations

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ABSTRACT

Azoxystrobin is a broad-spectrum strobilurin fungicide for use on a wide range of crops available to end-users as formulated products. Due to its extensive application, it has been detected in aquatic ecosystems, raising concerns about its environmental impact, which is still poorly explored. The objective of this work was to study the effects of a commercial formulation of azoxystrobin in the zebrafish embryo model. Sublethal and lethal effects were monitored during the exposure period from 2 h post fertilisation (hpf) to 96 hpf after exposure to azoxystrobin concentrations (1, 10 and 100 μg L^{-1}). The responses of antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR)) as well as detoxifying enzymes (glutathione-s-transferase (GST) and carboxylesterase (CarE)) were evaluated at 96 hpf. Similarly, glutathione levels (reduced (GSH) and oxidised (GSSG) glutathione), neurotransmission (acetylcholinesterase (AChE)) and anaerobic respiration (lactate dehydrogenase (LDH)) -related enzymes were assayed. At 120 hpf, larvae from each group were used for behaviour analysis. Results from this study showed concentration-dependent teratogenic effects, particularly by increasing the number of malformations (yolk and eye), with a higher prevalence at the highest concentration. However, it was found that the lowest concentration induced a high generation of reactive oxygen species (ROS) and increased activity of SOD, GST, and CarE. In addition, GR and GSSG levels were decreased by the lowest concentration, suggesting an adaptive response to oxidative stress, which is also supported by the increased AChE activity and absence of behavioural changes. These findings advance the knowledge of the azoxystrobin developmental and environmental impacts, which may impose ecotoxicological risks to non-target species.

1. Introduction

Azoxystrobin is a highly effective systemic and broad-spectrum fungicide developed by Syngenta in 1992. It belongs to the class of methoxyacrylates compounds, which are derived from the naturally-occurring strobilurins compounds (Bartlett et al., 2002). Although it has been developed as a fungicide against a variety of plant pathogenic fungi, its wide and extensive use mean its residues found in amounts above the maximum residue limits in different environmental matrices. In this context, environmental concentrations as high as 4.55 μg L^{-1} have been described in an estuary (Smalling and Orlando, 2011), while concentrations around 30 μg L^{-1} have been described in runoff water and river systems (Battaglin et al., 2011; Jiang et al., 2018). Currently, its impact on non-target species is not fully known although recent studies have shown it to contribute to endocrine disruption (Jiang et al., 2018), associated with the impairment of zebrafish fertility and reproduction (Cao et al., 2016). In addition, azoxystrobin has also been shown to affect embryonic development (Mu et al., 2017; Jiang et al., 2018) by inducing mitochondrial dysfunction and activating apoptosis signalling pathways by reactive oxygen species (ROS) (Cao et al., 2018; Jia et al., 2018; Kumar et al., 2020). In general, the findings point to oxidative stress-induced apoptosis as the possible mechanisms underlying azoxystrobin toxicity, as observed in other aquatic models (Olsvik et al., 2010; Liu et al., 2013). ROS are regulators of several physiological

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processes during nervous system development (Olguin-Albuerne and Moran, 2018; Wilson et al., 2018), which is especially sensitive to the action of developmental toxicants (Bjorling-Poulsen et al., 2008; Grandjean and Landrigan, 2014). In this regard, behavioural endpoints have been considered one of the earliest signals of sublethal changes at the individual level, coupling both biochemical, physiological and ecological processes (Scott and Sloman, 2004). In the last years, the developmental neurobehavioral toxicity of pollutants in the early-life stages has become the basis for studying the mechanisms and outcomes of drug- and toxicant-induced toxicity (Weichert et al., 2017; Gauthier and Vijayan, 2020; Lanzarin et al., 2020). In this regard, a recent study has shown a decreased swimming behaviour after 5 days exposure to azoxystrobin at concentrations of 1000 μg L^{-1} (Kumar et al., 2020). However, the study of the embryotoxic effects resulting from the embryonic exposure to commercial formulations of azoxystrobin, which may simulate a real environmental exposure scenario, is scarce.

To address this knowledge gap, the goal of this study was to understand the toxicological outcomes of compromised embryo development due to the exposure to a commercial formulation of azoxystrobin with a multiple embryonic, oxidative and behavioural biomarkers approach using the zebrafish (*Danio rerio*) model. The embryonic and larval forms of zebrafish have been increasingly used for toxicity testing of pesticides (Goncalves et al., 2020). Taking this into account, early embryos were acutely (96 h) exposed to a series of concentrations based on the mean lethal concentration (96 h LC_{50}) to investigate embryo developmental effects, behavioural profiles, and its relationship with oxidative biomarkers. The findings of this study will be useful to understand the physiological and biological effects of azoxystrobin on the embryonic development of zebrafish which may have translational environmental impacts.

2. Material and methods

2.1. Chemicals

The commercial azoxystrobin formulation (Quadris, 250 g azoxystrobin (active ingredient - a.i.) L^{-1}, CAS number: 131860-33-8) was purchased from Syngenta Crop Protection (Lisboa, Portugal) and a stock solution was prepared based on the a.i. at 100 mg L^{-1} in freshly prepared embryo water (28.0 ± 0.5 °C, 200 mg L^{-1} Instant Ocean Salt and 100 mg L^{-1} sodium bicarbonate; UV sterilised from City of Vila Real filtered-tap water) and stored at 4 °C until further dilution. This concentration was selected based on the application rate of this commercial formulation for crop protection (75–100 mL hL^{-1} – 188–250 mg L^{-1} a.i.). Instant Ocean Salt was obtained from Aquarium Systems Inc. (Sarrebourg, France). The reagents for the chromatographic measurements were of HPLC grade and obtained from Fisher Scientific (Porto Salvo, Portugal). Azoxystrobin chromatographic standard was purchased from HelloBio (Bristol, United Kingdom). All other chemicals were of analytical grade or higher and obtained from standard commercial suppliers. Except when specified, solutions were prepared with ultra-pure water purified by a Milli-Q Gradient system (Millipore, Bedford, USA).

2.2. Zebrafish husbandry and embryo collection

The wild-type (AB strain) zebrafish were maintained in an open water system supplied with aerated, dechlorinated, charcoal-filtered and UV-sterilised City of Vila Real tap water (pH 7.3–7.5) at 28.0 ± 0.5 °C in a 14:10 h light–dark cycle at the University of Trás-os-Montes and Alto Douro (Vila Real, Portugal). Animals were fed twice a day with a commercial diet (Sera, Heinsberg, Germany) supplemented with *Artemia* sp. Nauplii. The reproduction was performed by overnight crossing of male and female zebrafish (2:1 ratio) in breeding tanks with grids placed at the bottom to prevent eggs from being predated by progenitors as previously described (Felix et al., 2014; Vieira et al., 2020). The newly fertilised eggs were collected, washed in embryo water and bleached before being selected for the subsequent tests under a SMZ 445 stereomicroscope (Nikon, Japan). The ethical principles and other requirements on the use of laboratory animals of the EU directive (2010/63/EU) and National legislation for animal experimentation and welfare (Decreto-Lei 113/2013) were carried out in strict accordance in the realised experiments.

2.3. Determination of median lethal concentration 50 (LC_{50})

The determination of the lethal concentration that cause 50% mortality (LC_{50}) in the zebrafish embryos was based on the OECD test guideline (TG) 236 with minor modifications. At the early blastula stage (~2.0 h post-fertilisation – hpf), embryos were exposed in 50 mL beakers to different concentrations of azoxystrobin: 2.5, 25, 250, 2500 and 25,000 μg L^{-1} based on the active ingredient concentration of azoxystrobin. These concentrations were chosen according to the recommended field application of this azoxystrobin formulation (0.2–0.5 g L^{-1}) and according to a previous trial on zebrafish embryos (data not shown). For each concentration, three independent replicates of 20 embryos were used and exposed for 96 h under the standard conditions previously referred with exposure solutions being renewed every 24 h. Embryo water was used as the blank control. Embryo-larval mortality was recorded daily (identified by the white or opaque colour and by the missing heartbeat), and the percentage of mortality corrected to that of the control group. The 96 h LC_{50} value was derived through probit analysis with the 96 h LC_{50} being calculated as 1150 ± 320 μg L^{-1} (Fig. S1). Based on this, three sub-lethal concentrations of azoxystrobin (1, 10 and 100 μg L^{-1}) were selected for the subsequent exposure experiments, based on the active concentration of the compound.

2.4. Developmental toxicity testing

For the embryo toxicity tests, newly fertilised eggs were randomly distributed into single beakers (50 mL) according to methods delineated before (Felix et al., 2014; Vieira et al., 2020). Each random 100 embryos with ~2 hpf (blastula stage) were exposed to three different concentrations of azoxystrobin (1, 10 and 100 μg L^{-1}), each with five replicates, for the evaluation of different observable lethal, sub-lethal and teratogenic parameters. The mortalities were recorded at 24, 48, 72 and 96 hpf with dead individuals being removed and solutions renewed daily. Parameters such as failure of somites, eyes and otoliths development, missing heartbeat, nondetached tail and head, and pigmentation formation were evaluated as present or absent at 24, 48, 72 and 96 hpf. Sublethal endpoints, such as spontaneous movements (quantified over 20 s period), hatching rate, and heart rate (quantified over 15 s period) were analysed at 24, 48, 72 and 96 hpf. These parameters were analysed or quantified in 10 random animals removed from each group and under a SMZ800 stereomicroscope. Deformities of larvae were perceived at 96 hpf by immobilising them in 3% methylcellulose under an inverted microscope (IX 51, Olympus, Antwerp, Belgium) coupled to a colour digital CCD camera (Color View III, Olympus, Hamburg, Germany). Images were further combined, merged, and processed with Adobe Photoshop CS6 (Adobe Systems, San Jose, USA). Malformations (oedema, spinal and notochord abnormalities) as well as to body length, yolk sac, heart and eye area were taken using a digital image analysis software (Digimizer version 4.1.1.0, MedCalc Software, Mariakerke, Belgium). After termination of the exposure, embryos were collected for subsequent biochemical analysis or washed three times with embryo water to remove superficial chemicals and allowed to develop until 120 hpf for behavioural analysis.

2.5. Chemical analysis of exposure solutions

The analysis of exposure solutions was conducted in triplicate samples to confirm the stability and concentrations of azoxystrobin in the embryo water at the initial time (t = 0 h) and after 24 h (t = 24 h), before

the solution renewal. The method used was based on the protocol described by Jia et al. (2018) with slight modifications. Briefly, 5 mL of collected azoxystrobin sample and 5 mL of acetonitrile were added to 10 mL centrifuge tube followed by vortex shaken for 30 s. Then 2 g of NaCl and 3 g of $MgSO_4$ were added and vortexed for another 1 min. The mixture was then centrifuged at 4000 rpm for 5 min with the organic phase being collected in new tubes and analysed by high performance liquid chromatography (HPLC). Separations were carried out on a Hichrom ACE Ultracore 5 Super C18 (150 × 4.6 mm, 5 μm) using a Dionex UltiMate 3000 HPLC system (Dionex, Olten, Switzerland) coupled with a diode array detector (Dionex PDA 100 photodiode array, Dionex, Olten, Switzerland) linked to a PC computer running the Dionex Chromeleon Software 6.70 Build 1820. A mixture of acetonitrile/water (70:30 v/v) was maintained for 20 min allowing column stabilisation for another 10 min. Samples (20 μL) were injected into the column thermostatised at 30 °C with a constant flowrate of 0.9 mL min^{-1} with detector wavelength set at 255 nm. Standard solutions (up to 1250 μg L^{-1}) were prepared to establish a calibration curve and quantification was achieved by comparing the UV spectra and retention time with the respective standard. The limit of detection (LOD, 3.3σ/S) and limit of quantification (LOQ, 10σ/S) were calculated based on the standard deviation of the responses (S) and the slope (σ) (Chandran and Singh, 2007).

2.6. Biochemical assays

For the biochemical assays, five new experimental replicate exposures were performed as described before. On the completion of the exposure period, at 96 hpf, surviving larvae were collected and homogenised in cold buffer (0.32 mM of sucrose, 20 mM of HEPES, 1 mM of $MgCl_2$, and 0.5 mM of phenylmethyl sulfonylfluoride (PMSF), pH 7.4) (Deng et al., 2009). Homogenisation was achieved using steel beads in a Tissuelyser II (30 Hz for 30 s - Qiagen, Hilden, Germany), before being centrifuged at 15,000*g* for 20 min in a refrigerated centrifuge (4 °C, Sigma 3K30). The supernatant from each replicate was used to measure different biomarkers as previously described (Felix et al., 2018; Vieira et al., 2020). Briefly, determination of total ROS was performed at 485 nm (excitation) and 530 nm (emission) wavelengths, using the fluorescent probe DCFH-DA. The activities of superoxide dismutase (SOD) and catalase (CAT) were evaluated based on the inhibition of the photochemical reduction of nitrobluetetrazolium (NBT) at 560 nm and on the catalytic decomposition of hydrogen peroxide at 240 nm, respectively. The oxidation and reduction of NADPH at 340 nm was used to measure the glutathione peroxidase (GPx) and the glutathione reductase (GR) activity, respectively.The glutathione-s-transferase (GST) activity was measured by observing the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione (GSH) at 340 nm. The carboxylesterase (CarE) activity was evaluated by monitoring the reaction product of *p*-nitrophenol at 405 nm. The reduced (GSH) and oxidised glutathione (GSSG) were derivatized with ortho-phthalaldehyde (OPA) and measured at 320 nm and 420 nm for excitation and emission wavelengths, respectively. The oxidative stress index (OSI) was calculated as the ratio GSH:GSSG. The extent of lipid peroxidation was measured by the quantification of thiobarbituric acid reactive substances (TBARS) at 535 nm (excitation) and 550 nm (emission) wavelengths. The acetylcholinesterase (AChE) activity was determined by the Ellman's method adapted to microplates while for lactate dehydrogenase (LDH) activity, the oxidation of NADH at 340 nm was used. The protein concentration of samples was determined by the Bradford assay at 595 nm and used to normalise activities which were further normalised to the control group values. All samples were analysed in duplicate against an appropriate reagent blank at 30 °C in a PowerWave XS2 microplate scanning spectrophotometer (Bio-Tek Instruments, USA) or a Cary Eclipse fluorescence spectrophotometer (Varian, Palo Alto, USA).

2.7. Larval behaviour

At 120 hpf, the locomotor activity of normal exposed larvae was monitored during the light period as previously described (Felix et al., 2017; Vieira et al., 2020). Briefly, zebrafish larvae were placed into 6-well agarose-coated plates (1 randomly picked larva per well) in a dark room at 27 ± 1 °C. A 14.2 megapixels Sony Nex-5 digital camera (30 fps, APS-C CMOS sensor, Sony International) with a zoom lens (Sony SEL1855, E 18–55 mm, F3.5–5.6 OSS zoom) placed above a 15.6″ laptop LCD screen (1366 × 768 pixel resolution) showing a white Microsoft PowerPoint (Microsoft Corp., Washington, USA) presentation was used to monitor the plates. Following a 5 min adaptation period, the swimming behaviour was evaluated for 10 min by measuring the mean speed, total distance moved, percentage of time spent in each zone, mean distance to centre zone (5 mm radius circle) of the well, mean absolute turn angle (mean of all the angular differences in orientation between consecutive frames across the interval of observation) and the percentage of time active. The larval avoidance response was tested on a cycle of alternating periods (5 min) of a visual stimulus (a red bouncing ball present at the upper half of the well and moving from left to right) provided by the presentation in the Microsoft PowerPoint (Microsoft Corp., Washington, USA). The time spent in the bottom area with and without stimulus presentation was evaluated. Additionally, the anxiety-like behaviour of larval zebrafish was tracked during a cycle of two alternating periods of 10 min light or dark (940 nm LED illumination environment using an in-house constructed light box. An infrared-capable camera (GENIUSPY, GS-NQ140CML) with a 3.6 mm lenses was used to record the agarose-coated plates from above at 30 fps. A total of five replicates (5 larvae per treatment) were used and behavioural metrics were computed through the X,Y coordinates using a video-tracking system (TheRealFishTracker) (Buske and Gerlai, 2014).

2.8. Data analysis

The statistical analyses were performed on the averaged values from each independent exposure (considered as n = 1). The LC_{50} values and their respective confidence intervals were calculated using the GraphPad Prism software (version 7). The normality of data was assessed using the Shapiro-Wilk normality test and the Levene's test was employed to test the homogeneity of variances before ANOVA. When data followed the normal distribution, differences among groups were assessed by one-way analysis of variance (ANOVA) followed by the Tukey multiple comparison test and data expressed as mean ± standard deviation. When followed a non-normal distribution, the data treatment was performed using the non-parametric Kruskal-Wallis analysis of variance followed by Dunn's test with a Bonferroni correction for multiple comparisons and data expressed as medians and interquartile range (25th; 75th percentiles). For the aversive behavioural responses, the student's *t*-test was used to evaluate differences within the group in the different situation (presence or absence of stimulus). The statistical analysis was performed using the statistical package SPSS 20.0 for Windows (SPSS Inc., Chicago, USA) and the significance level was set to $p < 0.05$.

3. Results

3.1. Chemical analysis of exposure solutions

The chemical concentrations of the exposure solutions at 0 and 24 h are shown in Table 1. The method used was found to be linear over the range of concentrations tested ($r^2 = 0.999$) and the retention time for azoxystrobin was 5.89 ± 0.16 min. The amounts detected in the exposure medium for the lowest concentration at 0 and 24 h were respectively 45% and 58% higher than the nominal concentration. For the middle concentration, the rates were 16% and 42% higher than the prepared concentration. Relative to the highest concentration, the values retrieved were respectively 69.5% and 64% below the nominal

Table 1
Quantification of azoxystrobin (μg L^{-1}) in exposure medium using the reported HPLC-PDA method.

Nominal concentration	0 h		24 h		% of variation (0–24 h)
	Measured concentration	% of nominal concentration	Measured concentration	% of nominal concentration	
1	1.45 ± 0.48	+ 45.0	1.59 ± 0.59	+ 59.0	+ 10
10	11.6 ± 2.96	+ 16.0	16.5 ± 2.42	+ 65.0	+ 42
100	30.5 ± 4.18	- 69.5	36.0 ± 4.83	- 64.0	+ 18

Values represent the mean±standard deviation of three experiments (n = 3). (detection limits: LOD = 0.25 μg L^{-1}, LOQ = 0.75 μg L^{-1}).

concentration. After 24 h of exposure, increases of 10%, 42% and 18% for 1, 10 and 100 μg L^{-1} solutions, respectively, were observed in relation to the concentrations at the beginning of the experiment (0 h). Other compounds (impurities, adjuvants, or degradation products) were detected in the chromatogram (Fig. S2) which were not possible to identify. Therefore, the following results are presented and discussed based on the nominal concentrations instead of the measured concentrations.

3.2. Concentration-dependent increase in malformations

The effects of azoxystrobin on zebrafish embryo-larval development were observed from 3 to 96 hpf and the results are shown in Table 2 and in Table 3. The maximum mortality observed for the control group was around 10% during the entire test period and no significant mortality was observed between the experimental groups and the control group, at the same time of exposure. At 24 hpf, tail detachment, head detachment and development of the somites were visible in all treatment groups and the same was observed for the developmental parameters evaluated at 48 hpf (i.e., eyes and otoliths development and blood circulation). The embryo spontaneous tail movements at 24 hpf were not affected by the exposure to azoxystrobin. At 48 hpf, the heart rate was visually measured for all groups and statistical differences were observed, particularly between azoxystrobin concentrations (1 vs 10 μg L^{-1}: $p = 0.006$ and 1 vs 100 μg L^{-1}: $p = 0.007$). The hatching rate, evaluated at 72 hpf, showed that more than 50% of the embryos hatched for all the concentrations evaluated. However, the 10 ($p = 0.027$) and 100 μg L^{-1} ($p = 0.003$) groups showed a decrease in the hatching rate while no differences were observed for the 1 μg L^{-1} ($p = 0.128$) in relation to the control group. Relative to the oedema presence at 72 hpf, a concentration-dependent increase was observed with 1, 10 and 100 μg L^{-1} showing a higher incidence of oedema in relation to the control group ($p = 0.046$, 0.040 and < 0.0001, respectively for 1, 10 and 100 μg L^{-1}). At 96 hpf, the total malformations (Fig. 1A and B) observed at the animals exposed to azoxystrobin were below 20% although significant differences were observed between groups ($X^2(3) = 14.180$, $p = 0.003$). In this regard, the control group showed no apparent malformations while in azoxystrobin-exposed embryos, a higher percentage of yolk sac oedema, eye changes or tail deviations were observed. Though, exposure to the highest concentration of azoxystrobin induced a number of malformations compared to the control group ($p = 0.001$), 1 ($p = 0.001$) and 10 ($p = 0.041$) μg L^{-1} groups. After 96 h exposure, changes in the yolk (Fig. 1D, $F(3,15) = 24.49$, $p < 0.0001$) and eye area (Fig. 1F, $F(3,16) = 12.80$, $p = 0.0002$) were observed. In this regard, an increased yolk area was observed in the larvae exposed to 10 ($p = 0.022$) and 100 ($p = 0.0004$) μg L^{-1} groups in relation to the control group. The observed increase was also statistically different from the lowest concentration ($p < 0.001$). A smaller eye area was also observed in the middle ($p = 0.037$) and highest ($p = 0.002$) groups in comparison to the control group.

3.3. Azoxystrobin led to changes in enzymatic activities

Following exposure for 96 h to azoxystrobin, different ROS-related parameters were evaluated and normalised to control values (Fig. 2, original data is shown in Table S1). The data showed that azoxystrobin exposure induced changes in the overall ROS ($X^2(3) = 16.750$, $p = 0.001$) with the lowest group showing a significant increase relative to the control ($p < 0.001$) and to the 100 μg L^{-1} ($p = 0.011$) groups. The activity of SOD was also affected by azoxystrobin exposure ($F(3,20) = 43.228$, $p < 0.001$) with an increased activity observed in the 1 μg L^{-1} group in relation to the remaining groups ($p < 0.0001$). The activity of CAT ($X^2(3) = 13.187$, $p = 0.004$) showed a smaller activity in the 1 μg L^{-1} group in relation to 10 ($p = 0.008$) and 100 ($p = 0.013$) μg L^{-1}

Table 2
Lethal and sublethal responses observed after exposure to azoxystrobin for 96 h during zebrafish development.

Time (hpf)	Response	Concentration (μg L^{-1})				Statistical test	p-value
		0	1	10	100		
24	Mortality (%)	6 (6–10)	6 (4–7)	8 (5–15)	9 (8–19)	$X^2(3) = 6.106$	0.107
	Tail not detached (%)[1]	ND	ND	ND	ND	NA	NA
	Head not detached (%)[1]	ND	ND	ND	ND	NA	NA
	Somite not formed (%)[1]	ND	ND	ND	ND	NA	NA
	Spont. movement (mpm)	3 (2–3)	1 (1–2)	3 (2–3)	3 (2–3)	$X^2(3) = 5.534$	0.137
48	Cumulative mortality (%)	8 (7–10)	6 (5–8)	8 (5–15)	10 (8–19)	$X^2(3) = 4.741$	0.192
	Eye not developed (%)[1]	ND	ND	ND	ND	NA	NA
	Otoliths not developed (%)[1]	ND	ND	ND	ND	NA	NA
	Blood circ. not present (%)[1]	ND	ND	ND	ND	NA	NA
	Heart beat (bpm)	**127 (122 – 128)^{ab,}**	**122 (120 – 123)^{a}**	**134 (131 – 134)^{b}**	**130 (129 – 142)^{b}**	**$X^2(3) = 10.680$**	**0.014**
72	Cumulative mortality (%)	10 (8–12)	8 (7–10)	9 (7–16)	10 (8–19)	$X^2(3) = 2.118$	0.548
	Hatching rate (%)	**69 ± 3^{a}**	**63 ± 2^{ab,}**	**60 ± 6^{b}**	**57 ± 5^{b}**	**$F(3,20) = 6.701$**	**0.004**
	Oedema presence (%)[1,2]	**0 (0–0)^{a}**	**27 (18–35)^{b}**	**25 (11–50)^{b}**	**67 (61–75)^{c}**	**$X^2(3) = 16.357$**	**0.001**
96	Cumulative mortality (%)	10 (8–12)	11 (7–10)	10 (7–16)	10 (8–19)	$X^2(3) = 0.851$	0.837

[1] Parameter quantified as present/absent.
[2] Yolk sac and cardiac oedema were quantified as one. Data from at least 5 independent replicates of 10 random animals each, is expressed as mean ± SD for parametric data distribution or median (25th–75th quartile) for non-parametric data. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test or Kruskal-Wallis followed by Dunn's test. Different lowercase letters indicate significant differences between groups ($p < 0.05$). mpm: movements per minute; bpm: beats per minute; ND: not detected; NA: not applicable.

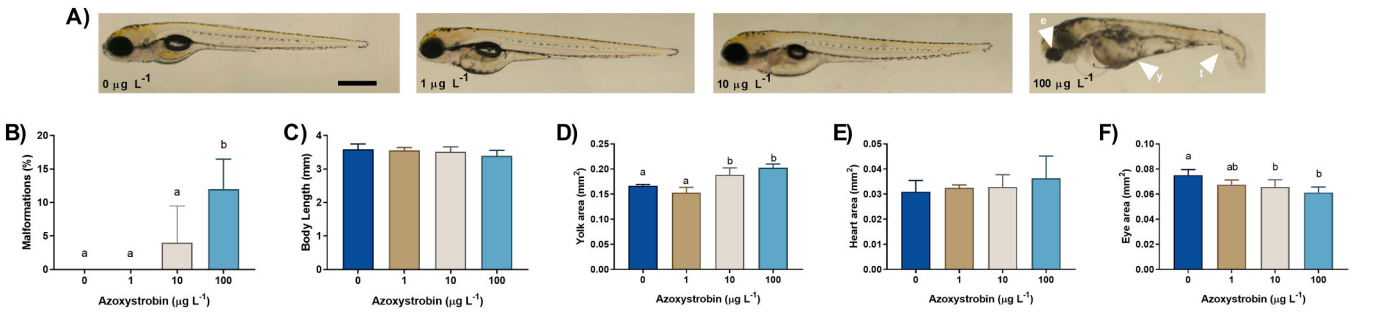

Fig. 1. Malformations observed after 96 h exposure of zebrafish embryos to azoxystrobin commercial formulation. A) Representative optical images of the zebrafish larvae at 96 hpf. Animals exposed to the concentration of 100 μg L^{-1} showed eye changes (e), yolk sac oedema (y) and tail deviations (t). The scale bar represents 500 μm. B) Malformation (%), C) Body length (mm), D) Yolk sac area (mm^2), E) Heart area (mm^2) and F) Eye area (mm^2) of animals at 96 hpf. Data from at least five independent replicate exposures (n = 10/each replicate). Parametric data were expressed as mean ± SD and statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test. Different lowercase letters indicate significant differences between groups ($p < 0.05$).

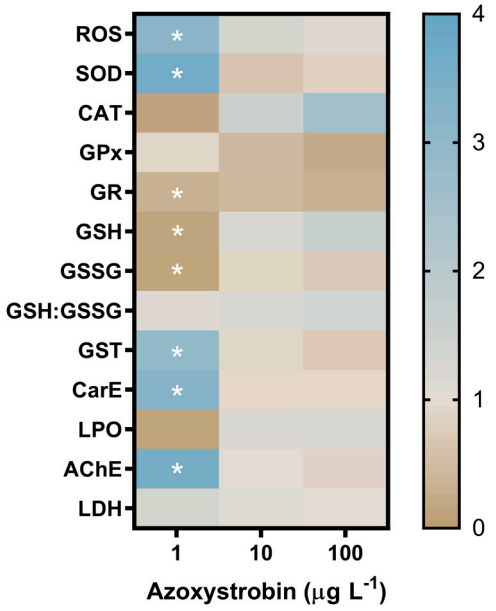

Fig. 2. Heatmap with biochemical parameters evaluated in zebrafish larvae after 96 h exposure to azoxystrobin. Data from at least five independent samples (n = 100/each). The data used for the evaluation of the biochemical parameters were normalised as a function of the control. Parametric data were expressed as mean ± SD and statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test. The * indicate significant differences relative to the control group ($p < 0.05$).

groups while no changes were determined in the activity of GPx ($F(3,15) = 2.483$, $p = 0.101$). The exposure to axozystrobin also affected the GR activity ($X^2(3) = 9.160$, $p = 0.027$) with a decrease in its activity induced by exposure to 1 μg L^{-1} in comparison to the control group ($p = 0.045$). The reduced and oxidised glutathione contents (GSH and GSSG, respectively) were also changed by azoxystrobin exposure. The GSH content ($X^2(3) = 12.085$, $p = 0.007$) showed a significative decrease in the 1 μg L^{-1} relative to the 10 ($p = 0.003$) and 100 ($p = 0.002$) μg L^{-1} and to the control ($p = 0.011$) groups while the GSSG content ($X^2(3) = 12.351$, $p = 0.006$) presented a statistical decrease for the lowest concentration in comparison to the control ($p = 0.006$) and 10 μg L^{-1} ($p = 0.023$) groups. No differences were detected in the OSI ($X^2(3) = 2.609$, $p = 0.456$). The GST ($F(3,18) = 32.625$, $p < 0.0001$) and CarE ($X^2(3) = 11.391$, $p = 0.010$) enzymes, responsible for xenobiotic degradation, were increased by the exposure to 1 μg L^{-1} in comparison to the remaining groups ($p < 0.0001$ and $p = 0.031$ for GST and CarE, respectively). For the CarE, the lowest exposed group also showed differences to the highest treated group ($p = 0.016$). Significant differences ($X^2(3) = 13,261$ $p = 0.004$) were observed in the lipid peroxidation with changes between the lowest and the other azoxystrobin-exposed groups ($p = 0.015$ and 0.007, respectively for 10 and 100 μg L^{-1}). The exposure to 1 μg L^{-1} significantly elevated AChE activity ($X^2(3) = 11.255$, $p = 0.004$) in zebrafish larvae, as compared to the control ($p = 0.016$) and 100 μg L^{-1} group ($p = 0.033$). No significant changes were perceived for the activity of LDH ($X^2(3) = 5.907$, $p = 0.116$).

3.4. *Azoxystrobin induced no behavioural toxicity in zebrafish larvae*

The behavioural responses measured at 120 hpf after a 96-h exposure to azoxystrobin are shown in Fig. 3. The locomotor activity tests showed no significant effects for the average speed ($X^2(3) = 4.291$, $p = 0.232$) and total distance travelled ($F(3,15) = 2.002$, $p = 0.168$). Similarly, no differences were observed for the percentage of time active ($F(3,15) = 0.944$, $p = 0.444$), for the absolute turn angle ($F(3,16) = 1.711$, $p = 0.205$) nor for the distance to the centre of the well ($F(3,16) = 0.616$, $p = 0.615$). When larvae were tested for the escape response (Fig. 3F), azoxystrobin-treated larvae showed no deficit in escaping the bouncing aversive ball (Student's *t*-test, $p < 0.001$). Larval zebrafish behaviour in response to alternating light- and dark-periods (Fig. 3G) was also assessed at 120 hpf but no significant differences in comparison to the control group under the two light ($F(3,16) = 1.446$, $p = 0.267$ and $F(3,16) = 2.785$, $p = 0.075$, respectively for the first and second light period) and dark states ($F(3,15) = 2.808$, $p = 0.078$ and $F(3,16) = 0.674$, $p = 0.581$, respectively for the first and second dark period) were observed.

4. Discussion

The objective of the present study was to evaluate the developmental effects of a commercial formulation of azoxystrobin on zebrafish embryos following exposure to sublethal concentrations below the calculated 96 h LC_{50}. In general, the observed LC_{50} value was in line with the previously proposed for early life stages of zebrafish (Jia et al., 2018; Jiang et al., 2018, 2019). Thus, embryos were exposed for 96 h to 1, 10 and 100 μg L^{-1}, based on the active concentration of azoxystrobin in the commercial formulation. The results showed azoxystrobin as a toxic compound to zebrafish resulting in concentration-dependent teratogenic effects, such as reduced hatching rate and increased malformations. However, exposure to the lowest concentration induced different responses towards oxidative stress, while no behavioural effects were evident following exposure to any of the azoxystrobin concentrations.

The process of embryonic development is a very complex process, consisting of the interplay of several pathways that relate to each other (Kimmel et al., 1995). In this context, azoxystrobin has been reported to

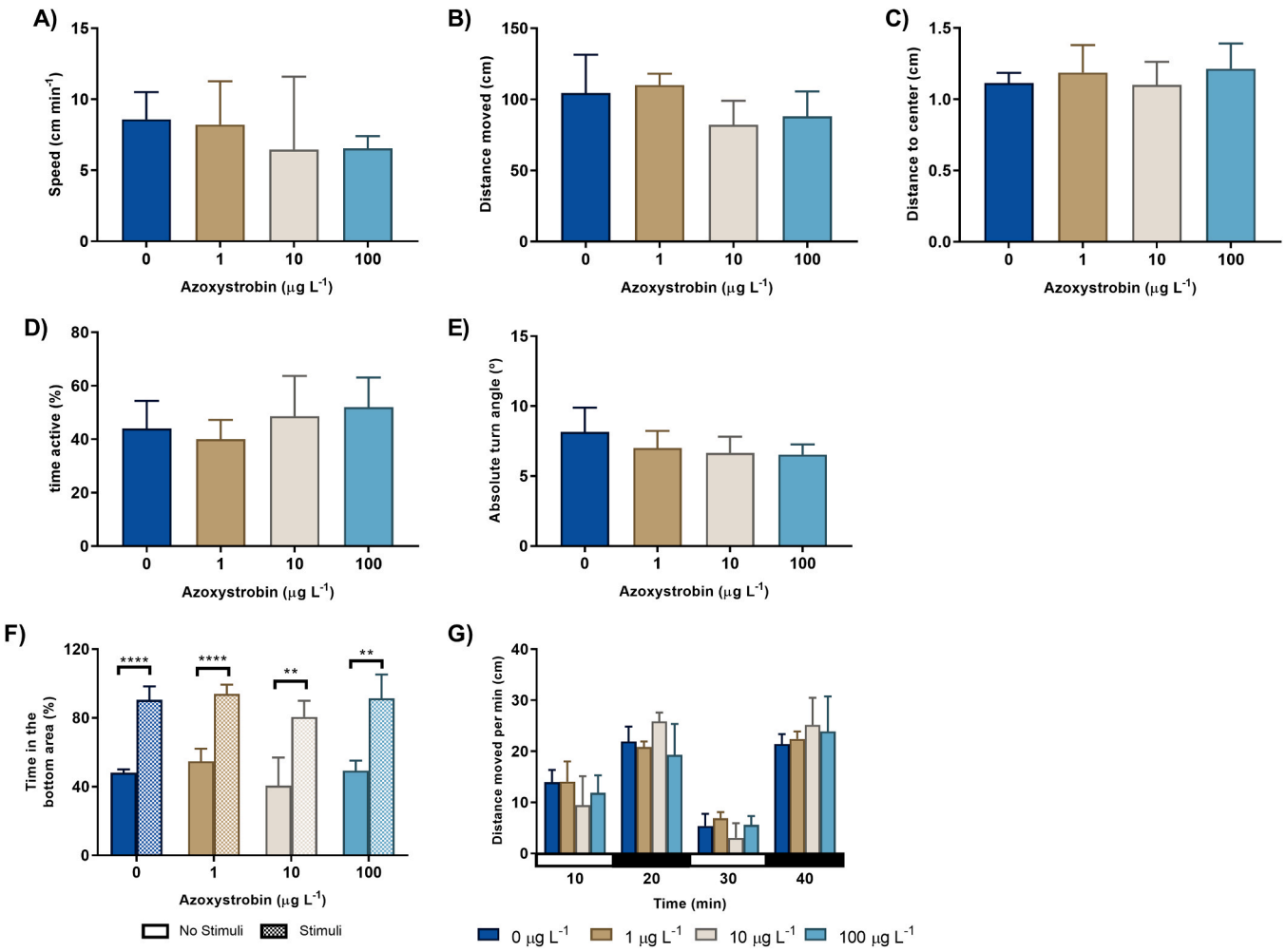

Fig. 3. Larvae behavioural responses measured at 120 hpf and following a 96-h exposure to azoxystrobin. A) Swim speed, B) distance moved, C) distance to centre, D) percentage of time active, E) absolute turn angle, F) nearest neighbour distance (NND), G) inter-individual distance (IID), H) the larval avoidance response evaluated by the time spent in the bottom area with and without stimulus presentation, and I) anxiety-like behaviour of larval zebrafish measured as distance moved per minute in two alternating periods of 10 min light or dark. Data from at least five independent replicate exposures (n = 5/each replicate). Parametric data were expressed as mean ± SD and statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test. Non-parametric data (A) is presented as median and interquartile ranges and statistical analysis was performed using the Kruskal-Wallis test followed by Dunn's test. The Student's *t*-test was used to compare the behaviour with and without the presence of the aversive stimulus (bouncing ball). Different letters indicate statistical differences between groups/responses ($p < 0.05$).

interfere in the development of zebrafish by inducing hatching rate changes and causing several malformations (Cao et al., 2018; Jia et al., 2018; Jiang et al., 2018). Although these results are associated with higher concentrations of azoxystrobin (above 1000 µg L^{-1}), the effects were verified at lower concentrations in the current study. In fact, a decreased hatching was observed similarly to previous studies (Jia et al., 2018), being described as a potential sensitive biomarker for azoxystrobin exposure. Overall, damage on the biosynthesis of the hatching enzyme or its abnormal distribution has been described as the main cause of hatching inhibition (De la Paz et al., 2017), followed by the diminished activity of the embryo and inability of the emerging larvae to break through the chorion (Papiya and Kanamadi, 2000). Despite this, the malformations observed in the animals can also limit the fish's ability to break the chorion and acquire normal swimming movements, as previously described for fungicidal compounds of the triazole class (Cao et al., 2019). Still, to what extent this situation represents the outcome of azoxystrobin exposure deserves further investigation. Moreover, as changes were observed between nominal and measured concentrations, a more detailed pharmacokinetic study which may give further indications as to the possible absorption of the compound during zebrafish development is required.

Notwithstanding, and although the developmental effects were more evident for the highest concentration tested, the biochemical parameters were more affected by the lowest concentration. A higher oxidative environment was depicted after exposure to the lowest concentration (1 µg L^{-1}), similar to what has been described for strobilurin fungicides in other organisms (Špalková et al., 2012). Studies suggest that the toxicity of azoxystrobin is associated with mitochondrial dysfunction and consequent oxidative damage (Gao et al., 2014; Kumar et al., 2020). It has been documented an increase in the activity of SOD, GST, low ATP level (Cao et al., 2018; Li et al., 2018), ROS, lipid peroxidation and other oxidative stress enzymes (Jiang et al., 2018) while different transcriptional responses have been reported (Jiang et al., 2018). In this context, in this study, increased SOD activity was observed following exposure to the lowest concentration, which has already been associated with an increase in ROS following exposure to azoxystrobin (Cao et al., 2018; Jia et al., 2018) while concentration-dependent variations in the transcriptional levels of *cz-sod* have been described (Jiang et al., 2018). Additionally, the GR activity, responsible for the reduction of GSSG to GSH, as well as the content of GSSG were lower following exposure to 1 µg L^{-1}, which may be associated with a strategy to cope with the increased oxidative status of the animals as the ratio of GSH to GSSG,

often used as an indicator of intracellular redox status (Timme-Laragy et al., 2013), was not affected. In fact, the glutathione system and related enzymes are considered a second line of defence against oxidative damage being critical during embryo development (Timme-Laragy et al., 2013; Massarsky et al., 2017). In accordance with this, different fish studies have proposed changes in the antioxidant system as an adaptive or protective response against compound-induced toxicity (Brandao et al., 2013; Gandar et al., 2017). In addition, GST, a family of enzymes with a central role in the biotransformation (phase II) of xenobiotics and endogenic compounds (Glisic et al., 2015), might contribute to the elimination of superoxide radicals caused by oxidative stress (Jiang et al., 2018). This may explain the increase in its activity since there is a very clear production of ROS following exposure to 1 μg L^{-1}. Also, CarE is an important pathway of detoxification (Kuster and Altenburger, 2006) which has been reported to be affected by azoxystrobin in adult zebrafish (Jia et al., 2018) and that was increased in the current study after exposure to 1 μg L^{-1} azoxystrobin. These results may represent an attempt of GST and CarE to increase the elimination of reactive radicals thereby supporting the referred protective response, as previously observed with other pesticides (diazinon and diuron) in zebrafish (Velki et al., 2017).

Moreover, it has been shown that oxidative stress plays a role in the regulation and activity of AChE (Rodriguez-Fuentes et al., 2015), which was increased after exposure to 1 μg L^{-1}. AChE is an useful biomarker of the biological effect of pollutants in the aquatic environment (Lionetto et al., 2005) being important in the neurotransmission process (Olsen et al., 2001). Although most studies suggest that pesticide exposure cause AChE inhibition in aquatic species, exposure to azoxystrobin induced an increase in the activity of AChE. Although further studies are required, a previous study has suggested the release of hippocampal acetylcholine and the activation of a regulatory overcompensation mechanism by increasing AChE activity or the *de novo* synthesis of the enzyme induced by the release of AChE (Badiou and Belzunces, 2008) as possible mechanisms for the increased activity of AChE after exposure to pesticides. In addition, the disturbance of the synthesis and activity of important enzymes or the transcription of important genes associated with oxidative stress responses and apoptosis has been previously reported for the short-term exposure of zebrafish larvae to azoxystrobin at concentrations ranging from 0.1 to 100 μg L^{-1} (Zhou et al., 2009; Jiang et al., 2018) to dampen excessive neurotransmission (Kaufer et al., 1998). However, further neurophysiological studies are required to gain a better understanding of the observed impacts of azoxystrobin on zebrafish development. Notwithstanding, there is evidence of its involvement in different embryonic developmental processes such as the nervous system function and behaviour outcomes (Picciotto et al., 2012). The behaviour evaluation is, in fact, an important sensitive endpoint for toxicological evaluation in zebrafish (Sloman and McNeil, 2012) and, in the present study, exposure to azoxystrobin induced no behavioural changes contrary to the mitochondrial bioenergetics-induced hypoactivity described in the literature at concentrations of 100 μg L^{-1} azoxystrobin in its pure form (Kumar et al., 2020). However, the complexity of the involved interactions and the effects of toxicant compounds is limited demanding further investigation to refine mechanistic targets. Still, the toxicity of commercial formulations is either over- or under-estimated in relation to the corresponding active ingredient (Mesnage et al., 2014; Basopo and Naik, 2015; Stevanovic et al., 2017), which may justify the discrepancies observed and indicates the need for further research on this topic.

In summary, this study provides further evidence of the deleterious effects of the exposure of zebrafish embryo to a commercial formulation of azoxystrobin through interference with developmental and oxidative stress sensitive pathways although not interfering with behavioural outcomes. Interestingly, the effects were more pronounced following exposure to the lowest concentration tested, suggesting an adaptive mechanism against azoxystrobin toxicity. Furthermore, these results provide valuable information on the risk assessment of azoxystrobin and raise safety concerns for environmental health. Yet, further studies are required towards elucidating the neurochemical and molecular mechanisms underlying azoxystrobin-induced developmental effects.

CRediT authorship contribution statement

Raquel Vieira: Investigation, Visualization, Writing - Original Draft. **Carlos Venâncio**: Conceptualization, Resources, Writing - Review & Editing, Supervision. **Luís Félix**: Conceptualization, Investigation, Resources, Writing - Review & Editing, Supervision.

Declaration of Competing Interest

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2021.111920.

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