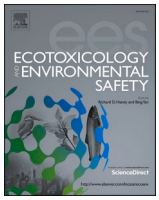
Ecotoxicology and Environmental Safety 279 (2024) 116493

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv



Effect of short-term exposure to the strobilurin fungicide dimoxystrobin: Morphofunctional, behavioural and mitochondrial alterations in *Danio rerio* embryos and larvae

Abdalmoiz I.M. Ahmed [a,1], Rachele Macirella [a,1], Federica Talarico [a], Mariarosaria F. Muoio [a], Marcello Mezzasalma [a], Valentina Tronci [b], Pradeep Lal [b], Naouel Gharbi [b,*], Elvira Brunelli [a,**]

[a] Department of Biology, Ecology and Earth Science (DiBEST) - University of Calabria, Via P. Bucci 4/B, Rende, Cosenza 87036, Italy
[b] Fish Biology and Aquaculture Group, Ocean and Environment Department, NORCE Norwegian Research Center, Bergen 5006, Norway

ARTICLE INFO

Edited by Dr. Caterina Faggio

Keywords:
Strobilurins
Dimoxystrobin
Development toxicity
Locomotor activity
Swim bladder inflation
Mitochondrial function

ABSTRACT

Strobilurins, among the most used fungicides worldwide, are considered non-toxic to mammals and birds, but there is growing evidence that these compounds are highly toxic to aquatic species. Dimoxystrobin has been included in the 3rd Watch List of the European Commission, and it has been classified as very toxic to aquatic life. However, previous studies focused on acute toxicity and only two reports are available on its impact on fish, and none on its effects during the early life stages. Here, we evaluated for the first time the effects induced on zebrafish embryos and larvae by two dimoxystrobin sublethal concentrations (6.56 and 13.13 μg/L) falling in the range of predicted environmental concentrations. We demonstrated that short-term exposure to dimoxystrobin may exert adverse effects on multiple targets, inducing severe morphological alterations. Moreover, we showed enhanced mRNA levels of genes related to the mitochondrial respiratory chain and ATP production. Impairment of the swim bladder inflation has also been recorded, which may be related to the observed swimming performance alterations.

1. Introduction

Dimoxystrobin, is a synthetic strobilurin, released onto the market nearly 20 years ago as a plant protection product (PPP) and authorized in 16 EU Member States (Regulation (EC) No 1107/2009). Although originally expiring in September 2016, the approval period of dimoxystrobin has been extended by the European Commission several times without adequate evaluation. In 2020, this fungicide was also included in the 3rd Watch List (WL) under the Water Framework Directive since it fulfils two of the five criteria adopted to identify the substances for the WL update (Gomez et al., 2020). In 2023, the European Commission decided not to renew the authorization (Regulation (EU) 2023/1436) based on the high potential for groundwater contamination reported by the European Food Safety Authority (EFSA). The Member States are granted a grace period that shall expire by 31 July 2024, during which they are allowed to submit another application for approval within the European Union. This fungicide is still commercialised in other countries, such as the United Kingdom and Brazil (Lewis et al., 2016). As with all strobilurins, dimoxystrobin can enter the water environment through air spraying or indirectly via soil runoff and drain flow (Li et al., 2018a). Accurate values of dimoxystrobin environmental concentrations in water bodies are very scant, and only one report is available on dimoxystrobin concentration in a southern China river (Zhao et al., 2024). Indeed, its chemical properties indicate that this fungicide has a long aqueous photolysis and hydrolysis time and can be persistent in both soil and water systems (Lewis et al., 2016).

Dimoxystrobin has been classified as very toxic to aquatic life with long-lasting effects (CLH report, 2019); however, available data are limited and mainly focused on acute toxicity in a few aquatic invertebrates and fish. To date, there is a lack of information on the sub-lethal effects of this fungicide on aquatic biota (European Food Safety Authority, 2023), with only two reports available on its impact on fish. It has been recently demonstrated that short-term exposure to two sublethal concentrations of dimoxystrobin can affect gill morphology

* Corresponding author.
** Correspondence to: Department of Biology, Ecology and Earth Science (DiBEST) - University of Calabria, Via P. Bucci, Rende, Cosenza 87036, Italy.
E-mail addresses: nagh@norceresearch.no (N. Gharbi), elvira.brunelli@unical.it (E. Brunelli).
[1] Abdalmoiz I.M. Ahmed and Rachele Macirella contributed equally to this work and are the co-first authors.

https://doi.org/10.1016/j.ecoenv.2024.116493
Received 21 March 2024; Received in revised form 16 May 2024; Accepted 20 May 2024
Available online 27 May 2024

and ultrastructure in adult zebrafish (Ahmed et al., 2023). Moreover, a preliminary investigation on strobilurin bioaccumulation tendency revealed that dimoxystrobin could be considered bioaccumulative (from considerable to highly bioaccumulative) in 21 of 28 analysed species according to bioconcentration factor (BCF) values measured in muscle (Zhao et al., 2024).

Several taxa can serve as model organisms in most fields of biology, including genetics, comparative morphology, invasion and conservation biology (Mezzasalma et al., 2019; Petraccioli et al., 2019). In recent years, zebrafish (*Danio rerio*) have gained increasing popularity as a valuable model for assessing the effects of pollutants, including pesticides and emerging contaminants (Plhalova et al., 2018; Cahova et al., 2023; Rashidian et al., 2023; de Arruda Leite et al., 2024). Many advantages, such as easy husbandry and maintenance, transparent embryo, and rapid growth (fully developed organ system reached within 96 h post-fertilization), make zebrafish a popular vertebrate model for ecotoxicological studies.

In fish, differences in sensitivity to waterborne chemicals have been widely documented across life stages, the early being the most susceptible (Mohammed, 2013; Pereira et al., 2023). Despite putative differences in pathway toxicity among fungicides, it seems to be now established that the larval stages represent the most sensitive phase to the action of these compounds, including strobilurins (Cao et al., 2018; Huang et al., 2021; Jiang et al., 2019a; b; Li et al., 2021; Wang et al., 2021). It must also be emphasized that any detrimental effects arising during larval development might also lead to carryover effects on survival, juvenile recruitment, fecundity and adult population dynamics.

Starting from this background, we evaluated for the first time the effects induced by exposure to two dimoxystrobin sublethal concentrations (6.56 and 13.13 μg/L) on zebrafish embryos and larvae. The selected doses are environmentally relevant and fall within the range of dimoxystrobin concentrations in surface waters worldwide (0.10 ng/L-16.42 μg/L) (Gomez et al., 2020; Zhao et al., 2024). Multiple toxicity targets on which dimoxystrobin exerts its harmful effects were investigated using an integrated approach to obtain a comprehensive overview of its toxicity potential.

Exposure to pollutants may elicit multiple effects at different levels of the biological organization, representing the integrated organisms' response to the perturbation of the internal milieu. The most obvious outcome, particularly during the delicate phases of development, is the emergence of body malformations, which are acknowledged as a powerful and highly effective biomarker of contamination in fish (Curcio et al., 2022; Sun et al., 2009). Considering the complete lack of literature data on dimoxystrobin-induced effects in embryonic and larval fish, in this study, we first assessed morphological progression and incidence of alterations after 24, 48, and 96 hours of exposure by applying a grading system that allows an unbiased comparison between groups.

It is well known that strobilurins' mechanism of action lies in their ability to disrupt mitochondrial bioenergetics by blocking electron transfer in the mitochondrial respiratory chain (Cao et al., 2018; Huang et al., 2021; Jiang et al.,2019a; 2019b; Kumar et al., 2020; Li et al., 2021; Luz et al., 2018; Nicodemo et al., 2018; Wang et al., 2021). Therefore, in a second step we evaluated mitochondrial dysfunction in exposed and non-exposed zebrafish by directly measuring various bioenergetic parameters (i.e., basal respiration, ATP production-linked oxygen consumption, and non-mitochondrial respiration) and the expression of some genes involved in mitochondrial performance (NADH dehydrogenase, ubiquinol-cytochrome c reductase, cytochrome c oxidase, ATP synthase F0 subunit 6). It has been recently suggested that the decreased mitochondrial bioenergetics induced by other strobilurins may be responsible for the behavioural disorders of zebrafish larvae (Huang et al., 2021; Kumar et al., 2020; Li et al., 2021). To determine if this hypothesis also applies to other strobilurins, we finally investigated behavioural changes using a well-described dark photokinesis assay.

To the best of our knowledge, this is the first report on dimoxystrobin effects in fish embryos and larvae. By integrating measurements of different types of responses to this fungicide, our findings fill a gap in the research literature, and they will also definitively contribute to the determination of safe field concentrations for strobilurin fungicides.

2. Materials and methods

2.1. Fish maintenance and egg production

Adult wild-type AB-strain zebrafish (*Danio rerio*) were maintained in a recirculating system at the Zebrafish Facility of the University of Bergen (Norway). Animals were maintained under a 14-hour light/10-hour dark period and fed twice per day with brine shrimp (*Artemia salina*). Water parameters were maintained as follows: a pH value of 7.5 ±0.5, a conductivity value of 600±100 $\mu S \cdot cm^{-1}$, dissolved oxygen concentration > 80 % of air saturation, and a temperature of 28±0.5 °C.

Embryos were obtained from spontaneous spawning of adult fish following the standard spawning protocol (www.zfin.org). Briefly, two adult males and two adult females were randomly selected and placed in spawning boxes. The following morning, eggs were collected 1 hour after light stimulation and rinsed in dechlorinated tap water to remove debris. All embryos were carefully checked under a stereoscope to remove dead and unfertilized embryos. Fertilized embryos were incubated at 28±0.5 °C in Petri dishes (density=1 embryo/mL) until the beginning of the exposure.

This study was approved by the Norwegian Food Safety Authority (Permit number FOTS ID 29916), and the experiments were conducted using the non-protected embryonic zebrafish stages (before the free-feeding stage - 5 days post fertilization) (Council Directive 2010/63/EU, 2010) and following the OECD Guidelines (OECD, 2013; 2019). Animal care and killing were performed according to the zebrafish facility rules and the European Convention for the Protection of Vertebrates used for Experimental and Other Scientific Purposes (Council of Europe, 1986).

2.2. Test substance

A stock solution was prepared by dissolving 1000 μg of dimoxystrobin (purity ≥ 98.0%, Sigma-Aldrich Chemical Co., Gillingham, UK) in 100 μL of acetone and then diluting in 1000 mL of water coming from the zebrafish facility systems. The stock solution (1 mg/L) was finally diluted in facility systems water to obtain the two selected concentrations of dimoxystrobin: 6.56 and 13.13 μg/L, also referred to as low and high concentrations, respectively. The selected doses correspond to 40 % and 80 % of the Predicted Environmental Concentration of dimoxystrobin in freshwater (PEC_{fw}=16.42 μg/L), respectively (Gomez et al., 2020). The analytic determination of dimoxystrobin in water samples was performed using a Varian Cary 50 Scan UV Visible Spectrophotometer as previously described (Ahmed et al., 2023). Briefly, using a quartz micro-cuvette (thickness=1 cm; volume=1 mL), the absorbance values were measured at 220 nm. A calibration curve for dimoxystrobin was created to calculate the concentration of the two samples: L (low concentration) and H (high concentration). Data acquisition was carried out through a computer connected to the instrument. The concentrations of dimoxystrobin standard solutions were 1.63 ÷ 26.37 ppm using a water/acetonitrile (5:2, v-v) solution. The curve was created using five data points (acquired in triplicate). A 250 mL volume of each sample (L and H) was dried and recovered in 600 μL for the measurement. The measured concentrations of dimoxystrobin were 6.44±0.12 for the sample L (low concentration) and 13.26±0.11 for the sample H (high concentration).

2.3. Exposure conditions

Embryos' developmental stage was determined based on distinctive morphological features (Curcio et al., 2021; Kimmel et al., 1995), and

when embryos reached the gastrula stage (6 hpf), they were randomly distributed in Petri dishes (n=18; density=1 embryo/mL) containing the appropriate concentrations of dimoxystrobin or un-treated tap water (control group). The Petri dishes were placed in a temperature and light-controlled incubator (28±0.5 °C; 14 h light/10 h dark period). For each experiment, three independent biological replicates were conducted. Water parameters were maintained constant during the whole experiment with the daily changing of the water system solution.

2.3.1. Mortality and hatchability

Embryo mortality and hatchability were evaluated daily, using a sample of 30 embryos for each exposed group, including the control group. Embryos/larvae were scored as 'dead' if they did not show a heartbeat and locomotor activity and had an opaque appearance. The mortality (%) was determined as follows: the number of dead animals/total number of individuals×100.

The number of hatched embryos was also recorded, and the hatching rate (%) was calculated for each experimental group as the number of hatched individuals/total number of individuals×100.

2.3.2. Morphological and morphometrical evaluation

Morphological evaluation was conducted at three time points: 24, 48, and 96 hours after the beginning of the exposure (30, 54, and 102 hpf, respectively). Ten embryos/larvae for each experimental group, including the control, were anaesthetized using the buffered tricaine methanesulphonate (0.20 mg/mL). All individuals were observed and photographed using a stereomicroscope Zeiss Lumar.V12 (Zeiss, Oberkochen, Germany). The severity of the detected alterations was determined by applying a scoring system (Curcio et al., 2021, 2022; Herrmann, 1995) that allowed a morphological objective comparison between the exposed and unexposed groups. Briefly, observing the images of each individual of both treated and no treated groups, the severity of each observed malformation was quantified by assigning the following numerical value: 0 = no effect (-); 1 = weak (+); 2 = medium (++); 4 = strong (+++). The overall severity of deformities in each group was expressed as the sum of the obtained values of all detected malformations, and the value of the resulting score was statistically compared.

After 96 hours of exposure (102 hpf), swim bladder measurements were also performed using the image analysis program ImageJ (software version 1.53a, National Institutes of Health, Bethesda, MD) according to the method reported in the literature (Lindsey et al., 2010; Venuto et al., 2023). The major and minor axes of the lateral view of the swim bladder were measured for each larva of the treated and untreated group using a specific program measurement tool. The swim bladder volume was determined using the following formula $V=4/3\pi ab^2$, where a=0.5 major axis and b=0.5 minor axis.

2.4. Mitochondrial bioenergetics

The oxygen consumption rate (OCR) of whole embryos was determined after 48 hours of exposure (54 hpf) using the XFe24 Extracellular Flux Analyzer (Seahorse Bioscience, Agilent Technologies, Massachusetts, USA). After calibration of the 24-well Islet Plate overnight at 28 °C, a single embryo was inserted in each well (five wells per treatment, including the control).

OCR measurements were conducted in accordance with the method reported by Liang et al., (2017). The modulators of the respiration were added in each well in the following order: oligomycin, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone-FCCP, and sodium azide to reach the final concentrations of 9.4 μM, 6 μM, and 20 mM, respectively. The basal oxygen consumption rate was measured over 10 cycles of data collection, whereas 18 cycles were run for oligomycin (inhibiting ATP production of embryos), and 8 cycles were run for FCCP injection to calculate the maximum respiration rate. Finally, sodium azide was injected for 24 cycles to completely inhibit the embryos' mitochondrial respiration (allowing the calculation of non-mitochondrial respiration). Data on oxygen consumption rates were exported to GraphPad using the Wave Desktop 2.6 Software (Agilent Technologies).

2.5. Zebrafish larval behaviour test

Locomotor behavioural analysis was conducted after 96 hours of exposure (102 hpf) using the AD-Zantiks LT Z2S Unit (Zantiks, Cambridge, UK); room temperature was maintained at 28±1 °C during the whole experiment. Eight samples from each experimental group (including the control) were individually transferred into 24-well plates. Each well contained 400 μl of exposure solutions or dechlorinated tap water (control group). After being acclimated in the instrument for 1 hour, larvae were tracked during a standard 50-minute "white light routine" by alternating light/dark periods of 10 min each, beginning with a dark period. At the end of the analysis, data from each period were independently collected and binned into an average value for each minute to calculate the distance moved. Then, the total distance travelled and velocity were calculated, considering two light/dark cycles.

2.6. RNA extraction, cDNA synthesis, and quantitative real-time PCR

After 96 hours of exposure, larvae (102 hpf) were pooled into one centrifuge tube (n=10/treatment group) for RNA extraction performed using 1 mL TRIzol® Reagent (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions. The purity and concentration of extracted RNA were determined by a Nanodrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA samples were assessed for quality using the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Using the SuperScript III kit (Invitrogen, Carlsbad, CA, USA), 800 ng of total purified RNA was employed for cDNA synthesis according to the manufacturer's instructions. Relative gene expression was measured using the SYBR Green Master Mix reagent (Applied Biosystems, Foster City, CA, USA) on the CFX-384 Real-Time system (Bio-Rad Laboratories, Inc., CA, USA). The specific primers set for zebrafish larvae were obtained from the literature (Li et al., 2018b, 2021) and listed in Table S1.

Each qRT-PCR reaction contained 3 μL of cDNA (diluted 20-fold before real-time analysis), 0.25 μL of each forward and reverse primer (10 μmoll-1), 3.25 μL of diethylpyrocarbonate (DEPC)-treated dH_2O, and 6.25 μL of SYBR Green Master Mix. The thermal cycles are reported below: denaturation for 2 min at 95 °C, followed by 40 amplification cycles at 95 °C for 15 s and 60 °C for 25 s. Two housekeeping genes (Table S1), ribosomal subunit 18 (*rps18*) and beta-actin (*β-actin*), were assessed for stability and normalization. Target genes were normalized to the geometric mean of the two housekeeping genes using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.7. Statistical analyses

All statistical analyses were performed using GraphPad Prism 8.00 (GraphPad Software Inc., San Diego, CA, USA) with a significance level 0.05. Data from biological replicates were statistically compared using the Mann-Whitney test. As no significant differences were found among the three replicates, data were pooled for statistical analyses. For all statistical analyses, the normality assumption and the homoscedasticity were tested using the Shapiro-Wilk or Kolmogorov-Smirnov tests and the Brown-Forsythe test, respectively. The log-rank (Mantel-Cox) test was performed to compare survival rates between treated and untreated groups; the Kaplan-Meier survival graph was applied to display cumulative survival. The Fisher's exact and Chi-square tests were used to compare the deformity incidence and the cumulative deformity between the exposed and unexposed groups. The one-way ANOVA followed by Tukey's multiple comparisons tests were used to evaluate the mitochondrial bioenergetics, the locomotor behaviour, the gene transcript levels, and the swim bladder volume between the dimoxystrobin-treated

and control groups. Moreover, the correlation between swim bladder volume and total distance moved was carried out using the Pearson correlation analysis. The two-way ANOVA (followed by Tukey's multiple comparison test) was applied to evaluate significant differences in the scoring system between treated and untreated groups.

3. Results

3.1. Survival and hatching rate

After 24 hours of exposure to dimoxystrobin, the survival rate of embryos from the high-concentration group was significantly lower than the control and the low-concentration groups (Figure S1a). The low-concentration group also showed a decrease in survival rate, which is still not significant. Later than 24 h, the survival percentage remained constant as no mortality was detected in both treated and non-treated groups (Figure S1a).

As regards the hatching rate, at 30 hpf (24 h from the start of the experiment), embryos from all experimental groups, including control, had not yet hatched (Figure S1b). As expected from the literature data, the hatching of the embryos began at 54 hpf (48 h from the start of the experiment), when the hatching rate was 50 %. All embryos hatched at 102 hpf (96 h from the beginning of the experiment). No differences were seen among the control and exposed groups (Figure S1b).

3.2. Morphological abnormalities

3.2.1. Control group

In the control group, embryonic and larval growth proceeded according to literature data, and no morphological deformities were observed during the experiment.

At 30 hpf, it is possible to appreciate a prominent yolk sac, big pigmentated eyes and a well-developed pericardial cavity of the embryos, which are still enclosed in the chorion (Fig. 1a). Along the developing tail, some chromatophores can be noted, which appear larger and more conspicuous in the dorsal part of the embryos.

At 54 hpf, most of the larvae were out of the chorion, allowing the detection of more anatomical structures. Sensory organs, including eyes and otoliths, are well developed, and the spinal cord and the notochord are visible. The yolk sac is still prominent, and the heart is well recognizable; moreover, the caudal fin is formed, and the somites can be easily distinguishable. An increasing number of black melanophores can be detected in the ventral part of the larvae and yellow xanthophores on the dorsal side (Fig. 1b).

At 102 hpf, the yolk sac is less prominent but not completely reabsorbed, while the caudal fin, otoliths and eyes were further developed. The swim bladder was inflated in all samples and showed a dark appearance due to melanin accumulation. The black pigmentation on the ventral part of the larvae is less evident, while the yellow pattern in the dorsal portion due to xanthophores accumulation is more accentuated. The notochord and somites are clearly evident, and the cloaca can also be noted (Fig. 1c).

3.2.2. Exposed groups

Exposure to both dimoxystrobin concentrations induces numerous morphological abnormalities, whose severity (score value) and frequency (i.e., cumulative deformity) increased with exposure time (**** $p < 0.0001$; Table 1).

After 24 hours of exposure (30 hpf) to the low dimoxystrobin concentration, the first detected morphological alterations were the spinal deformities and the swelling of the yolk sac that were observed in 30 % and 40% of individuals, respectively (Fig. 2a,b, Table 1). After 48 h (54 hpf), the frequency of spinal deformities and yolk sac swelling did not further increase (Fig. 2c, Table 1). In contrast, it was possible to detect the appearance of tail deformities and pericardial edema, affecting 20 and 10% of embryos, respectively (Fig. 2d, Table 1). After 96 h (102 hpf), there was an increase in the incidence of yolk sac swelling (50 %) (Fig. 2e, Table 1) and a dramatic rise in pericardial edema, which reached a frequency of 60 % (Fig. 2f, Table 1). The swim bladder was uninflated or underinflated in all individuals (Fig. 2e,f, Table 1).

The incidence of deformities in the high-concentration group was massive, and both deformity rate and score value were significantly higher in the high-concentration group (**** $p < 0.0001$; Table 1). Moreover, the onset was early, and all alterations observed in the low-concentration group were already detected after 24 h.

After 24 h (30 hpf), the swelling of the yolk sac was the most frequent alteration (80 %), followed by spinal deformity (40 %) (Fig. 3a, Table 1). Pericardial edema and tail deformity were also frequently detected (20;%) (Fig. 3b, Table 1). After 48 h of exposure (54 hpf), both of the latter showed an increasing incidence (70 and 40 % of embryos, respectively) (Fig. 3c,d, Table 1). At this time point, the incidence of spinal deformities and yolk sac swelling was not further enhanced (Table 1). After 96 h (102 hpf), the frequency of pericardial edema and tail deformity further increased, becoming significantly higher

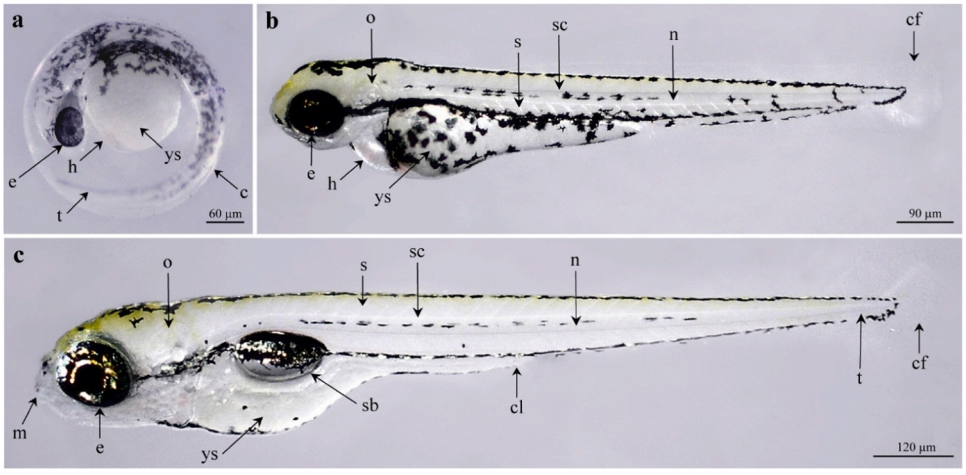


Fig. 1. Gross morphology of zebrafish embryos and larvae under basal conditions. (a) At 30 hpf, embryos within the chorion display a prominent yolk sac and high-pigmented eyes. Note the pericardial cavity and the developing tail. (b) At 54 hpf, most of the larvae are out of the chorion and show a prominent yolk sac. The eyes, otoliths, heart, spinal cord, and notochord are visible. The somites are well distinguishable, and the caudal fin is formed. (c) At 102 hpf, all larvae have an inflated swim bladder. The caudal fin, otoliths and eyes are further developed. c=chorion, ys=yolk sac, e=eyes, h=heart, t=tail, o=otolith, cf=caudal fin, sc=spinal cord, n=notochord, s=somites, m=mouth, sb=swim bladder, cl=cloaca.

Table 1
Frequency (%), cumulative deformity (%) and severity of morphological abnormalities in zebrafish embryos and larvae after exposure to dimoxystrobin.

	6.56 µg/L			13.13 µg/L		
	24 h	**48 h**	**96 h**	**24 h**	**48 h**	**96 h**
Spinal deformity	30[a, **]	30[a, **]	30[a, **]	40[a, **]	40[a, ***]	50[a, ****]
Tail deformity	0	20[a, *]	20[a, *]	20[a, b, *]	40[a, ***][b, *]	60[a, ****][b, **]
Yolk sac swelling	40[a, ***]	40[a, ***]	50[a, ****]	80[a, ****][b, **]	80[a, ****][b, **]	80[a, ****][b, *]
Pericardial edema	0	10	60[a, ****]	20[a, b, *]	70[a, b, ****]	80[a, ****][b, *]
Swim bladder alteration	-	-	100[a, ****]	-	-	100[a, ****]
Cumulative deformity	60[a, ****]	60[a, ****]	100[a, ****]	90[a, ***][b, *]	100[a, ****][b, ***]	100[a, ****]
Score value	11 ±2[a, ****]	26 ±3[a, ****]	64 ±2[a, ****]	45 ±3[a, b, ****]	59 ±2[a, b, ****]	121 ±4[a, b, ****]

[a] =significant difference between the treated and control group;
[b] = significant difference be-tween high concentration and low concentration group;
[*] $p<0.05$;
[**] $p<0.01$;
[***] $p<0.01$;
[****] $p<0.0001$.

compared to the control and the low-concentration group (Fig. 3e-g, Table 1). Yolk sac swelling and spinal deformity were detected in 80 and 50 % of the group, respectively (Fig. 3e-g, Table 1). Moreover, at this time point all individuals showed uninflated or severely underinflated swim bladder (Fig. 3e-g, Table 1).

Comparing swim bladder volume in animals exposed to both low and high dimoxystrobin concentrations, we revealed a highly significant reduction compared to the control (Figure S2).

3.3. Mitochondrial bioenergetics

The oxygen consumption rate (OCR) was measured after 48 h of exposure to dimoxystrobin in zebrafish embryos at 54 hpf (Fig. 4a).

The analysis of samples exposed to the low concentration demonstrated no significant differences in non-mitochondrial respiration (Fig. 4b), basal respiration (Fig. 4c), maximal respiration (Fig. 4d) and ATP production (Fig. 4e) compared to control. Instead, a decrease, although not significant, in spare respiratory capacity (Fig. 4f) was seen. The high dose did not affect non-mitochondrial respiration (Fig. 4b), basal respiration (Fig. 4c), and ATP production (Fig. 4e) but induces a significant decrease in maximal respiration (Fig. 4d) and spare respiratory capacity compared to control (Fig. 4f).

3.4. Locomotion behavior

We analysed the locomotion behaviour of larvae (102 hpf) after 96 h of exposure to dimoxystrobin (Fig. 5a). We revealed a significant increase in the total distance moved (Fig. 5b) and velocity (Fig. 5c) of larvae in both dimoxystrobin concentration groups compared to the control.

Besides, Pearson's correlation of total distance moved versus the volume of the swim bladder was analyzed. The Pearson correlation coefficient ($r= -0.9999$; $p < 0.008$) revealed a strong negative relationship between the total distance moved and swim bladder volume (Fig. 5d) in both exposed groups.

3.5. Molecular analyses

The transcription levels of genes related to mitochondrial activity were significantly modulated after exposure to both dimoxystrobin concentrations (Fig. 6).

NADH dehydrogenase subunit (*ndi*) was significantly upregulated in all exposed samples compared to the control (Fig. 6a). The expression levels of the ubiquinol-cytochrome c reductase core protein (*uqcrc*), the cytochrome c oxidase subunit I (*coxI*) and ATP synthase F0 subunit 6 (*atp6*) showed a highly significant upregulation in all exposed samples compared to the control (Fig. 6).

4. Discussion

While strobilurins are considered relatively non-toxic to mammals and birds, there is growing evidence that these compounds are highly toxic to aquatic species (Ahmed et al., 2023; Wang et al., 2021 and references therein; Zhao et al., 2024). Within strobilurins, dimoxystrobin has quickly attracted the attention of international governmental organizations since it has been classified as very toxic to aquatic life with long-lasting effects. Despite this, reports on dimoxystrobin-induced injuries in fish are extremely scarce compared to other strobilurins, amounting to just two reports currently available (Ahmed et al., 2023; Zhao et al., 2024) thus leaving the toxicity potential of this fungicide widely undisclosed. Here, we provide for the first time clear evidence that dimoxystrobin, even at low concentrations, exerts its harmful potential on the early life stages of zebrafish.

4.1. Morphological modifications

All samples exposed to dimoxystrobin exhibited morphological deformities, with the severity and frequency increasing over time. It is worth noting that although the type of alterations was consistent in both experimental groups, some substantial differences were detectable since the onset time was different in the specimens exposed to the two concentrations. Indeed, some severe alterations, such as pericardial edema and tail deformity, precociously appeared in animals exposed to the high dimoxystrobin concentration. Moreover, the incidence of deformities in the high-concentration group was massive, involving 100 % of individuals starting from 48 h of exposure. Also, the severity of the lesions progressed more intensely through the exposure period, as shown by the score values. In agreement with some previous reports on teratogenic effects concentration-dependent in zebrafish exposed to other fungicides, including strobilurins (Vieira et al., 2021; Li et al., 2020; Sun et al., 2020), our results support the strong correlation between fungicide dose and both the occurrence and severity of morphological alterations.

A direct comparison with our results is difficult since no studies have examined dimoxystrobin-induced alterations in fish embryos and larvae. Besides, according to literature evidence, strobilurins can trigger distinct and specific effects on developing fish (Yang et al., 2021). Despite this, our morphological observations agree with the available literature data showing similar malformations and injuries in zebrafish embryos and larvae exposed to other strobilurins and fungicides (Huang et al., 2021; Jiang et al., 2019a; 2019b,; Li et al., 2018a, 2019, 2020, 2021; Mao et al., 2020; Sun et al., 2020; Vieira et al., 2021; Wang et al., 2018; Yao et al., 2018; Zhang et al., 2020, 2024).

4.2. Mitochondrial dysfunctions

Most studies on the toxicity of strobilurins have been focused on adverse outcome pathways related to mitochondrial dysfunction (Cao et al., 2018; Huang et al., 2021; Jiang et al., 2019b, 2019b; Kumar et al., 2020; Li et al., 2021; Luz et al., 2018; Nicodemo et al., 2018; Wang et al., 2021Yang et al., 2021).

Since the mode of action of strobilurins lies in their ability to block electron transfer in the mitochondrial respiratory chain, here we

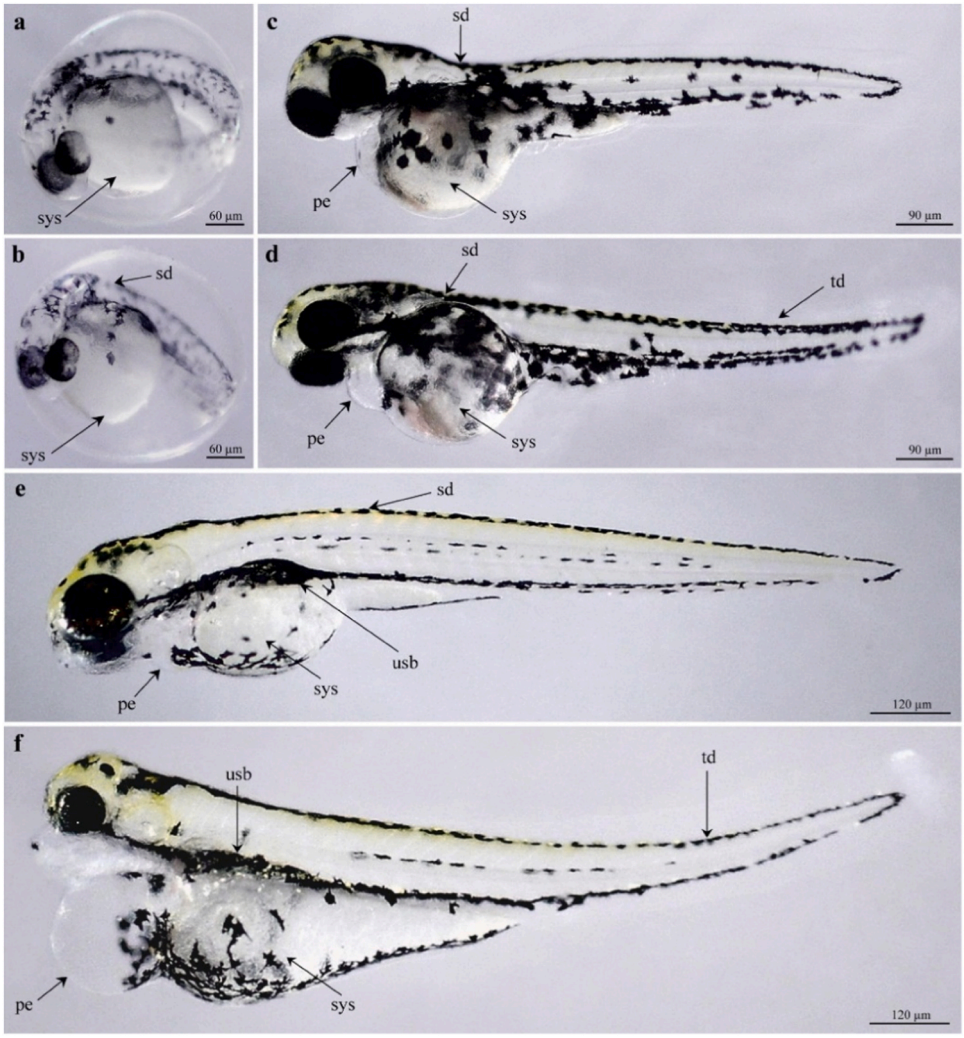


Fig. 2. Gross morphology of zebrafish embryos and larvae exposed to 6.56 µg/L of dimoxystrobin. (a,b) After 24 h of exposure, embryos exhibit yolk sac swelling and spinal deformity. (c,d) After 48 h of exposure, tail deformity and pericardial edema appear. (e,f) After 96 hours of exposure, yolk sac swelling and pericardial edema are more frequent and severe. Note the alteration of the swim bladder. sys=swelling of yolk sac, sd=spinal deformity, td=tail deformity, pe=pericardial edema, usb=uninflated swim bladder.

evaluated the mRNA levels of *ndi*, *uqcrc*, *coxI* and *atp6*, which are essential genes encoding mitochondrial complex I, III, IV and V, respectively. Our results clearly showed that zebrafish larvae exposed for 96 h to both dimoxystrobin concentrations underwent a significant upregulation of all considered genes, thus supporting the hypothesis of a compensatory response mounted to counterbalance the impairment of the electron transport chain as reported in larvae of the same species after exposure to other strobilurins (Cao et al., 2018; Li et al., 2018b, 2021).

In principle, it is usually accepted that exposure to strobilurins may also result in the alteration of mitochondrial bioenergetics (Huang et al., 2021; Jiang et al., 2018; Yang et al., 2021), and the mitochondrial stress test has been successfully used to evaluate chemical-induced OCR alteration in zebrafish. Nonetheless, the assays on zebrafish exposed to different strobilurins and other fungicides led to uneven findings, and both an increase and a decrease of each OCR parameter have been reported (Huang et al., 2021; Kumar et al., 2020; Li et al., 2021; Qin et al., 2022; Wang et al., 2018; Yang et al., 2021).

Here, we showed that dimoxystrobin exposure induced mitochondrial dysfunction in zebrafish embryos by reducing maximal respiration and spare respiratory capacity. Such a decrease was detected only after exposure to the high concentration, thus suggesting that it is necessary to reach a threshold dose before the mitochondrial function is compromised. Our results are in agreement with previous reports, showing significantly reduced maximal respiration and spare capability after exposure to the highest tested concentrations of three strobilurins (Yang et al., 2021). Similarly, other fungicides (fluazinam and fenbuconazole) induced a reduction in basal respiration and ATP production only when administered at higher doses (Qin et al., 2022; Wang et al., 2018). We suggest that both compound-specific differences and experimental design might explain these discrepancies.

4.3. Locomotor behaviour and swim bladder inflation

Behavioural tests on the zebrafish embryo are claimed as a sensitive tool for chemical exposure (Sloman and McNeil, 2012; Wang et al., 2018) and have been proposed as an alternative test model for toxicity tests (Ogungbemi et al., 2019). To disclose the potential locomotor impairment induced by dimoxystrobin in zebrafish, we conducted behavioural assays after 96 hours of exposure (102 hpf) to both tested concentrations, revealing a significant increase in the larvae's total distance moved and velocity compared to the control.

A recent review of behavioural assays conducted in zebrafish embryos exposed to neuroactive substances highlights the role of the experimental setup in determining reproducible and comparable outcomes (Ogungbemi et al., 2019). Experiments conducted on the

Fig. 3. Gross morphology of zebrafish embryos and larvae exposed to 13.13 µg/l of dimoxystrobin. (a,b) After 24 h, embryos show severe yolk sac swelling. Spinal deformity, tail deformity, and pericardial edema can be frequently detected. (c,d) After 48 h of exposure, the frequency of pericardial edema and tail deformity increases. (e-f) After 96 h of exposure, larvae show severe pericardial edema, yolk sac swelling, and spinal and tail deformities. The alteration of the swim bladder is visible in all larvae. sys=swelling of yolk sac, sd=spinal deformity, td=tail deformity, pe=pericardial edema, usb=uninflated swim bladder.

locomotor behaviour of zebrafish larvae focused on different strobilurins and used various exposure periods and concentrations. As a consequence, test results can be significantly different and no change in behavioural activities, hyperactivity, and hypoactivity have all been reported after exposure to strobilurins (Kumar et al., 2020; Vieira et al., 2021; Yang et al., 2021; Zhang et al., 2020).

The assessment of published results on behaviours in zebrafish larvae (Ogungbemi et al., 2019) revealed that several parameters influence the direction of behavioural change (hypo or hyperactivity), including developmental stage, endpoint, exposure concentration and duration. Long exposure time and high concentrations during development may affect axonal morphology and/or neuromuscular physiology, thus resulting in hypoactivity (Stehr et al., 2006; Yang et al., 2011). Therefore, the potential shift from a hyperactive behaviour at low doses to a hypoactive behaviour at high doses cannot be excluded also for dimoxystrobin. The uneven experimental setup and diagnostic tools make it overwhelming to interpret available literature data and even harder to compare with our results. Despite this, our observations agree with those conducted by Zhu et al. (2015) in another cyprinid species (*G. rarus*) exposed to low doses of the strobilurin trifloxystrobin.

Interestingly, our morphological investigation revealed that the anticipated swim bladder inflation at 102 hpf was not witnessed in exposed samples. The swim bladder plays an essential role in the movement balance of zebrafish (Huang et al., 2021), and the missed or impaired inflation of this organ may alter swimming performance (Stinckens et al., 2020).

Initial swim bladder inflation is a critical phase of zebrafish development as it can only occur during a narrow time window (Lindsey et al., 2010). Fish with uninflated swim bladders must swim at higher speeds to maintain hydrostatic equilibrium, which in turn results in less favourable feed conversion ratios and slower growth (Schwebel et al., 2018). Moreover, the failure of swim bladder inflation has been linked

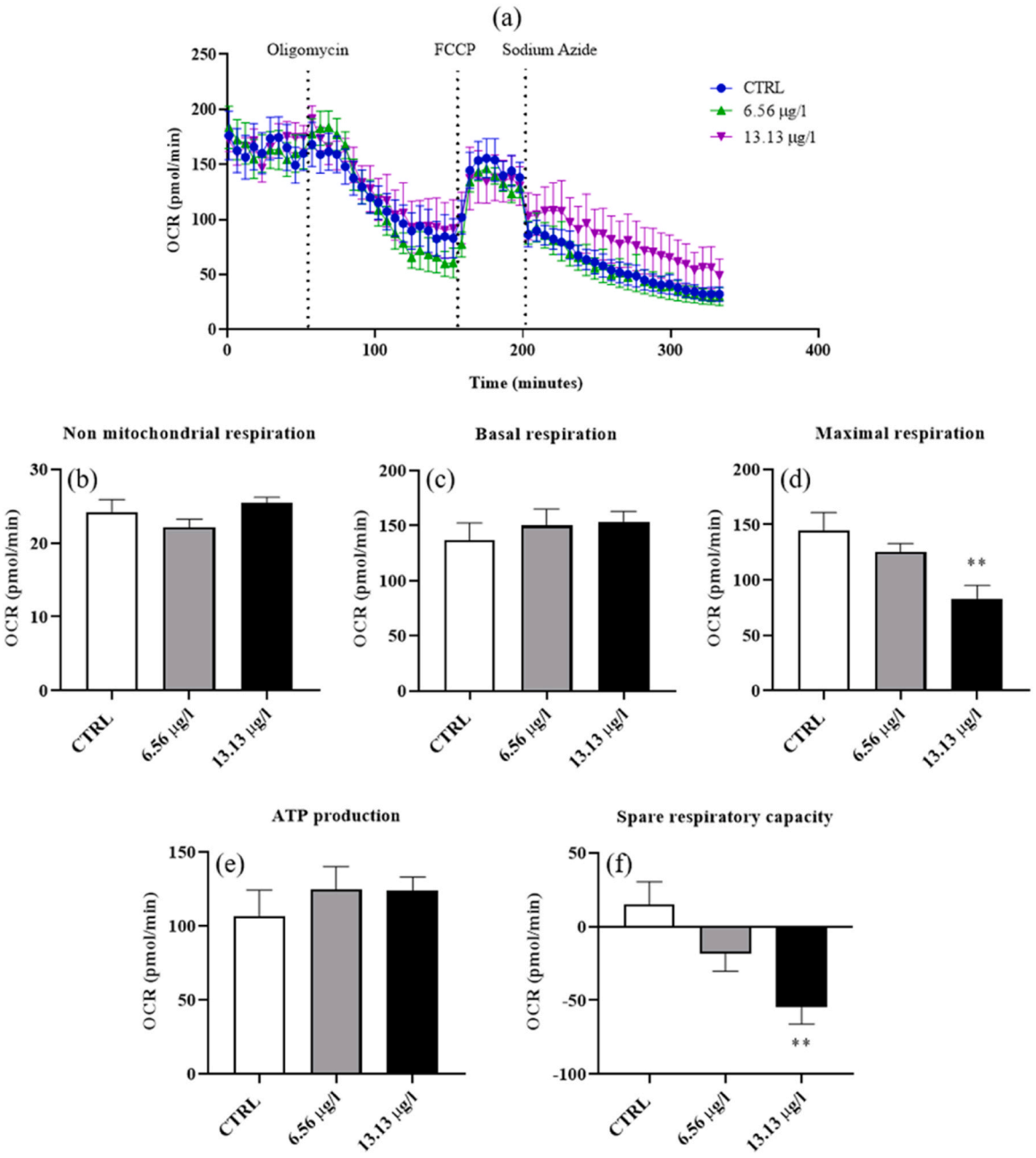


Fig. 4. (a) Oxygen consumption rate (OCR) of zebrafish embryos after 48 hours of exposure to 6.56 and 13.13 µg/L of dimoxystrobin. (b-f) Mitochondrial parameters. (b) non-mitochondrial respiration, (c) basal respiration, (d) maximal respiration, (e) ATP production, (f) spare respiratory capacity. Asterisks indicate significant differences between the exposed and unexposed groups (**$p<0.01$). Data are presented as mean±standard error of the mean (SEM).

with developmental abnormalities, including lordosis (Lindsey et al., 2010; Schwebel et al., 2018).

Accordingly, our analysis also unravelled a strong negative relationship between the total distance moved by exposed and unexposed larvae and swim bladder volume. This latter result fits with previous data showing similar effects on zebrafish larvae exposed to two strobilurins (Yang et al., 2021). Such findings suggest that the impairment of the swim bladder inflation is an adverse outcome of dimoxystrobin exposure at sublethal dose, which in turn affects the swimming performance of larvae. Although the molecular mechanisms of zebrafish swim bladder inflation are poorly understood, Cao et al. (2023) have recently demonstrated that the abnormal development and guidance of axons in motoneuron, altering the swim-up behaviour in zebrafish, resulted in the failure of the swim bladder's inflation. As a whole, our results identify the abnormal development of the nervous system as an important outcome of dimoxystrobin toxicity, which warrants further investigation.

Interestingly, our morphological investigation revealed that the anticipated swim bladder inflation at 102 hpf was not witnessed in exposed samples. The swim bladder plays an essential role in the movement balance of zebrafish (Huang et al., 2021), and the missed or impaired inflation of this organ may alter swimming performance (Stinckens et al., 2020). Accordingly, our analysis also unravelled a strong negative relationship between the total distance moved by exposed and unexposed larvae and swim bladder volume. This latter result fits with previous data showing similar effects on zebrafish larvae exposed to two strobilurins (Yang et al., 2021). Such findings suggest that the impairment of the swim bladder inflation is an adverse outcome of dimoxystrobin exposure at sublethal dose, which in turn affects the swimming performance of larvae. Initial swim bladder inflation is a

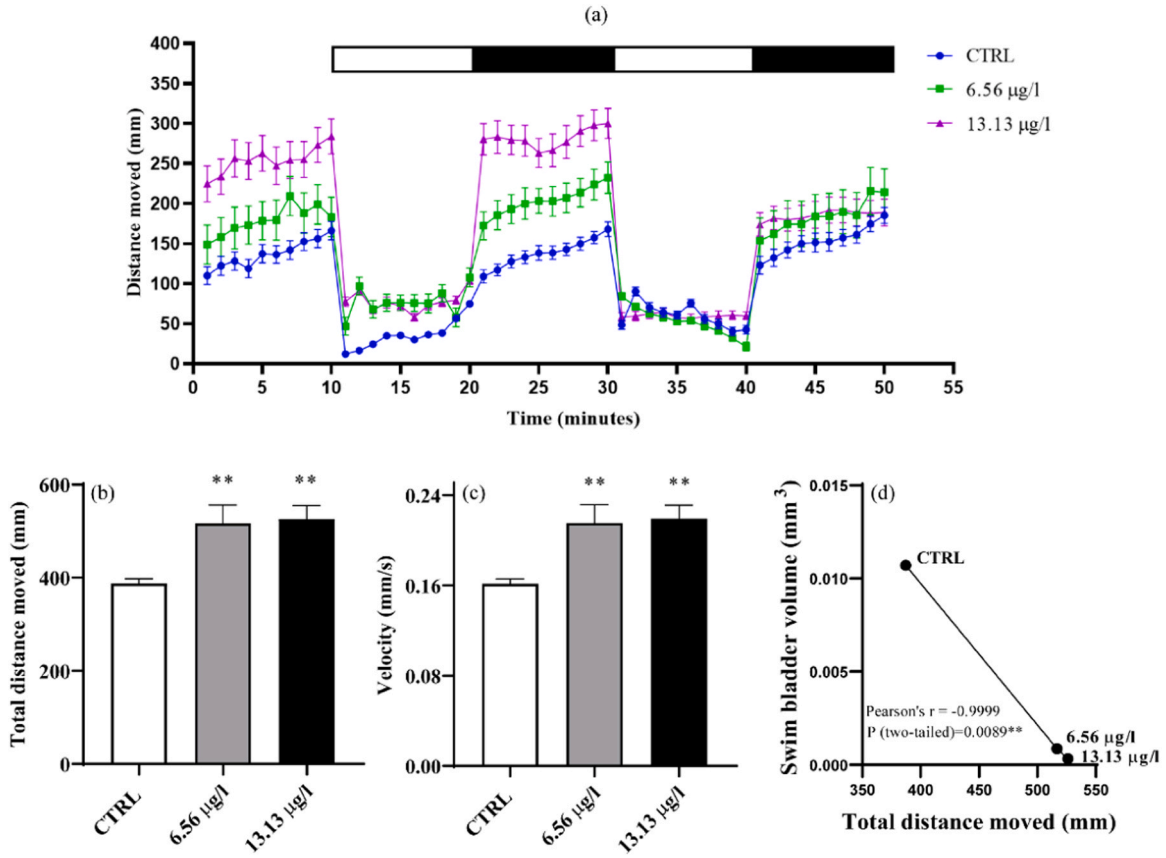


Fig. 5. (a-c) Locomotor activity of zebrafish larvae after 96 hours of exposure to 6.56 and 13.13 µg/L of dimoxystrobin. (a) Swimming distance of zebrafish larvae in 1 min, (b) total distance moved and (c) velocity. Asterisks indicate significant differences between the exposed and unexposed groups (**p<0.01). (d) Total distance moved vs. swim bladder volume; Pearson correlation coefficient (r=-0.9999; p=0.0089**). Data are presented as mean±standard error of the mean (SEM).

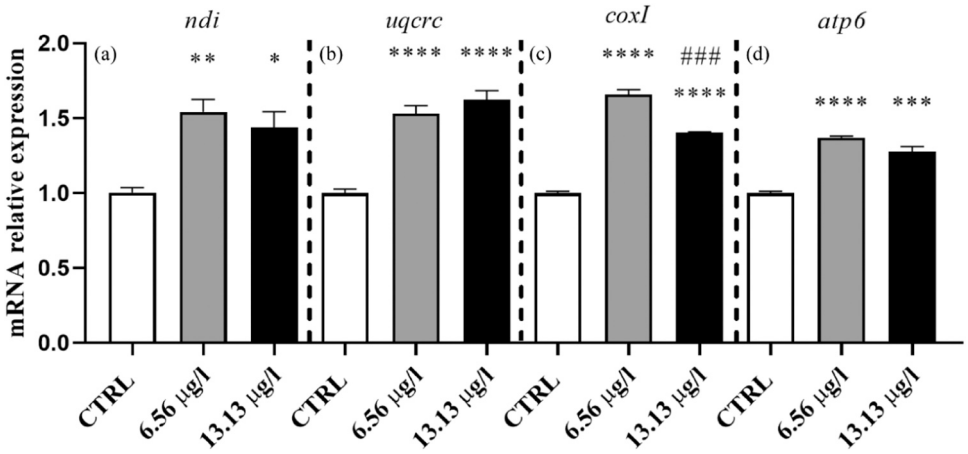


Fig. 6. Gene expression in zebrafish larvae after 96 hours of exposure to 6.56 and 13.13 µg/l of dimoxystrobin. Relative mRNA expression of NADH dehydrogenase (a), ubiquinol-cytochrome c reductase (b), cytochrome c oxidase (c), ATP synthase F0 subunit 6 (d). Asterisks indicate significant differences between the exposed and unexposed groups (*p<0.05; **p<0.01; ****p<0.0001). Hashtags indicate significant differences between high-concentration and low-concentration groups (###p<0.001). Data are presented as mean±standard error of the mean (SEM).

critical phase of zebrafish development as it can only occur during a narrow time window (Lindsey et al., 2010). Fish with uninflated swim bladders must swim at higher speeds to maintain hydrostatic equilibrium, which in turn results in less favourable feed conversion ratios and slower growth (Schwebel et al., 2018). Moreover, the failure of swim bladder inflation has been linked with developmental abnormalities, including lordosis (Lindsey et al., 2010; Schwebel et al., 2018).

5. Conclusion

When evaluating the potential toxicity of a substance, it is crucial to focus attention on the most sensitive stage to ensure reliable and ecologically relevant results. This is the first report on dimoxystrobin, effects during the early life stages of zebrafish. Available studies on strobilurins envisaged various endpoints to disclose the underlying toxicity mechanisms, thus evidencing that these compounds may exert

their adverse effects on multiple targets. According to the strobilurin mechanism of action, we showed enhanced mRNA levels of genes related to the mitochondrial respiratory chain, thus supporting the hypothesis of a compensatory response mounted to balance the impairment of the electron transport chain in exposed samples. In our study, developmental disorders are hallmarked by the emergence of severe morphological deformities whose severity and frequency increase over time. Among other alterations, the failure of swim bladder inflation was recorded. By interpolating these results with the behavioural analysis, we concluded that impairment of the swim bladder inflation is an adverse outcome of dimoxystrobin exposure at the sublethal dose, which is able to induce alteration of the swimming performance.

Funding

Part of this work was supported by funding from the Norwegian Research Center, Bergen, Norway (NORCE). The behavioural method has been developed in the context of the Project "Pigghå FRI – Hvordan unngå problemer med pigghå?" (The Norwegian Seafood Research Fund Project code: 901704).

CRediT authorship contribution statement

Naouel Gharbi: Resources, Methodology, Investigation. **Elvira Brunelli:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Conceptualization. **Pradeep Lal:** Resources, Methodology. **Marcello Mezzasalma:** Visualization, Methodology, Investigation. **Valentina Tronci:** Resources, Methodology. **Federica Talarico:** Software, Investigation, Formal analysis. **Mariarosaria F. Muoio:** Formal analysis. **Abdalmoiz I.M. Ahmed:** Writing – original draft, Investigation. **Rachele Macirella:** Writing – original draft, Investigation, Conceptualization.

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.

Data availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2024.116493.

References

Ahmed, A.I.M., Macirella, R., Talarico, F., Curcio, V., Trotta, G., Aiello, D., Gharbi, N., Mezzasalma, M., Brunelli, E., 2023. Short-term effects of the strobilurin fungicide dimoxystrobin on zebrafish gills: a morpho-functional study. Chemosphere 333, 138914. https://doi.org/10.1016/j.chemosphere.2023.138914.

Cahova, J., Blahova, J., Plhalova, L., Marsalek, P., Doubkova, V., Hostovsky, M., Divisova, L., Mares, J., Faggio, C., Svobodova, Z., 2023. Long-term exposure to polycyclic musk tonalide - A potential threat to juvenile zebrafish (*Danio rerio*)? Vet Med-Czech 68, 218–224.

Cao, S., Dong, Z., Dong, X., Jia, W., Zhou, F., Zhao, Q., 2023. Zebrafish sox2 is required for the swim bladder inflation by controlling the swim-up behavior. Zebrafish 20 (1), 10–18. https://doi.org/10.1089/zeb.2022.0043.

Cao, F., Wu, P., Huang, L., Li, H., Qian, L., Pang, S., Qiu, L., 2018. Short-term developmental effects and potential mechanisms of azoxystrobin in larval and adult zebrafish (*Danio rerio*). Aquat. Toxicol. 198, 129–140. https://doi.org/10.1016/j.aquatox.2018.02.023.

CLH Report, 2019. Proposal for Harmonised Classification and Labelling Based on Regulation (EC) No 1272/2008 (CLP Regulation), Annex VI, Part 2 Substance Name: Dimoxystrobin. [(accessed on 20 Aprile 2024)]. Available online: https://echa.europa.eu/documents/10162/71711b06-bc63-d700-6553-dd633d93ea8b.

Council of Europe, 1986. European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes. European Treaty Series n° 123 [(accessed on 20 Aprile 2024)]. Available online: https://rm.coe.int/168007a67b.

Curcio, V., Macirella, R., Sesti, S., Pellegrino, D., Ahmed, A.I., Brunelli, E., 2021. Morphological and molecular alterations induced by lead in embryos and larvae of *Danio rerio*. Appl. Sci. 11 (16), 7464. https://doi.org/10.3390/app11167464.

Curcio, V., Macirella, R., Sesti, S., Ahmed, A.I., Talarico, F., Pizzolotto, R., Tagarelli, A., Mezzasalma, M., Brunelli, E., 2022. The role of exposure window and dose in determining lead toxicity in developing Zebrafish. Chemosphere 307, 136095. https://doi.org/10.1016/j.chemosphere.2022.136095.

de Arruda Leite, B., Meireles, G., Renata Abe, F., Gravato, C., Dorta, D.J., de Oliveira, D. P., 2024. Do zebrafish become blind or is it too much red dye in water? Distinguishing the embryo-larval development and physiology effects of DR 60, 73, and 78. Sci. Total Environ. 908, 168062 https://doi.org/10.1016/j.scitotenv.2023.168062.

Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the Protection of Animals Used for Scientific Purposes. [(accessed on 20 Aprile 2024)]. Off. J. Eur. Union. 2010 276:33–79. Available online: https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2010:276:0033:0079:en:PDF.

European Food Safety Authority, 2023. Peer review of the pesticide risk assessment of the active substance dimoxystrobin. EFSA J. 21 (10), e08329 https://doi.org/10.2903/j.efsa.2023.8329.

Gomez, C.L., Marinov, D., Sanseverino, I., Navarro, C.A., Niegowska, M., Porcel, R.E., Lettieri, T., 2020. Selection of substances for the 3rd watch list under the water framework directive, EUR 30297 EN, 978-92-76-19426-2. Publications Office of the European Union, Luxembourg, p. JRC121346. ⟨https://doi.org/10.2760/194067⟩.

Herrmann, K., 1995. Teratogenic effects of retinoic acid and related substances on the early development of the zebrafish (*BrachyDanio rerio*) as assessed by a novel scoring system. Toxicol. Vitr. 9 (3), 267–283. https://doi.org/10.1016/0887-2333(95)00012-W.

Huang, T., Souders II, C.L., Wang, S., Ganter, J., He, J., Zhao, Y.H., Cheng, H., Martyniuk, C.J., 2021. Behavioral and developmental toxicity assessment of the strobilurin fungicide fenamidone in zebrafish embryos/larvae (*Danio rerio*). Ecotoxicol. Environ. Saf. 228, 112966 https://doi.org/10.1016/j.ecoenv.2021.112966.

Jiang, J., Lv, L., Wu, S., An, X., Wang, F., Liu, X., Zhao, X., 2019a. Developmental toxicity of kresoxim-methyl during zebrafish (*Danio rerio*) larval development. Chemosphere 219, 517–525. https://doi.org/10.1016/j.chemosphere.2019.12.061.

Jiang, J., Shi, Y., Yu, R., Chen, L., Zhao, X., 2018. Biological response of zebrafish after short-term exposure to azoxystrobin. Chemosphere 202, 56–64. https://doi.org/10.1016/j.chemosphere.2018.03.055.

Jiang, J., Wu, S., Lv, L., Liu, X., Chen, L., Zhao, X., Wang, Q., 2019b. Mitochondrial dysfunction, apoptosis and transcriptomic alterations induced by four strobilurins in zebrafish (*Danio rerio*) early life stages. Environ. Pollut. 253, 722–730. https://doi.org/10.1016/j.envpol.2019.07.081.

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. Dev. Dyn. 203 (3), 253–310. https://doi.org/10.1002/aja.1002030302.

Kumar, N., Willis, A., Satbhai, K., Ramalingam, L., Schmitt, C., Moustaid-Moussa, N., Crago, J., 2020. Developmental toxicity in embryo-larval zebrafish (*Danio rerio*) exposed to strobilurin fungicides (azoxystrobin and pyraclostrobin). Chemosphere 241, 124980. https://doi.org/10.1016/j.chemosphere.2019.124980.

Lewis, K.A., Tzilivakis, J., Warner, D., Green, A., 2016. An international database for pesticide risk assessments and management. Hum. Ecol. Risk Assess. 22 (4), 1050–1064. https://doi.org/10.1080/10807039.2015.1133242.

Li, H., Cao, F., Zhao, F., Yang, Y., Teng, M., Wang, C., Qiu, L., 2018a. Developmental toxicity, oxidative stress and immunotoxicity induced by three strobilurins (pyraclostrobin, trifloxystrobin and picoxystrobin) in zebrafish embryos. Chemosphere 207, 781–790. https://doi.org/10.1016/j.chemosphere.2018.05.146.

Li, X.Y., Qin, Y.J., Wang, Y., Huang, T., Zhao, Y.H., Wang, X.H., Martyniuk, C.J., Yan, B., 2021. Relative comparison of strobilurin fungicides at environmental levels: Focus on mitochondrial function and larval activity in early staged zebrafish (*Danio rerio*). Toxicology 452, 152706. https://doi.org/10.1016/j.tox.2021.152706.

Li, H., Yu, S., Cao, F., Wang, C., Zheng, M., Li, X., Qiu, L., 2018b. Developmental toxicity and potential mechanisms of pyraoxystrobin to zebrafish (*Danio rerio*). Ecotoxicol. Environ. Saf. 151, 1–9. https://doi.org/10.1016/j.ecoenv.2017.12.061.

Li, W., Yuan, M., Wu, Y., Liu, X., 2020. Bixafen exposure induces developmental toxicity in zebrafish (*Danio rerio*) embryos. Environ. Res. 189, 109923 https://doi.org/10.1016/j.envres.2020.109923.

Li, H., Zhao, F., Cao, F., Teng, M., Yang, Y., Qiu, L., 2019. Mitochondrial dysfunction-based cardiotoxicity and neurotoxicity induced by pyraclostrobin in zebrafish larvae. Environ. Pollut. 251, 203–211. https://doi.org/10.1016/j.envpol.2019.04.122.

Liang, X., Souders II, C.L., Zhang, J., Martyniuk, C.J., 2017. Tributyltin induces premature hatching and reduces locomotor activity in zebrafish (*Danio rerio*) embryos/larvae at environmentally relevant levels. Chemosphere 189, 498–506. https://doi.org/10.1016/j.chemosphere.2017.09.093.

Lindsey, B.W., Smith, F.M., Croll, R.P., 2010. From inflation to flotation: contribution of the swimbladder to whole-body density and swimming depth during development of the zebrafish (Danio rerio). Zebrafish 7 (1), 85–96. https://doi.org/10.1089/zeb.2009.0616.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}_{T}$ method. Methods 25, 402–408. https://doi.org/10.1006/meth.2001.1262.

Luz, A.L., Kassotis, C.D., Stapleton, H.M., Meyer, J.N., 2018. The high-production volume fungicide pyraclostrobin induces triglyceride accumulation associated with mitochondrial dysfunction, and promotes adipocyte differentiation independent of

PPARγ activation, in 3T3-L1 cells. Toxicology 393, 150–159. https://doi.org/10.1016/j.tox.2017.11.010.
Mao, L., Jia, W., Zhang, L., Zhang, Y., Zhu, L., Sial, M.U., Jiang, H., 2020. Embryonic development and oxidative stress effects in the larvae and adult fish livers of zebrafish (*Danio rerio*) exposed to the strobilurin fungicides, kresoxim-methyl and pyraclostrobin. Sci. Total Environ. 729, 139031 https://doi.org/10.1016/j.scitotenv.2020.139031.
Mezzasalma, M., Andreone, F., Glaw, F., Guarino, F.M., Odierna, G., Petraccioli, A., Picariello, O., 2019. Changes in heterochromatin content and ancient chromosome fusion in the endemic Malagasy boid snakes *Sanzinia* and *Acrantophis* (Squamata: Serpentes). Salamandra 55 (2), 140–144. ⟨https://hdl.handle.net/11588/769017⟩.
Mohammed, A., 2013. Why are early life stages of aquatic organisms more sensitive to toxicants than adults? In: Gowder, S.J.T. (Ed.), New Insights into Toxicity and Drug Testing. InTech, pp. 50–62. https://doi.org/10.5772/55187.
Nicodemo, D., Mingatto, F.E., Carvalho, A.D., Bizerra, P.F.V., Tavares, M.A., Balieira, K. V.B., Bellini, W.C., 2018. Pyraclostrobin impairs energetic mitochondrial metabolism and productive performance of silkworm (*Lepidoptera: Bombycidae*) caterpillars. J. Econ. Entomol. 111 (3), 1369–1375. https://doi.org/10.1093/jee/toy060.
OECD, 2013. OECD guidelines for the testing of chemicals, fish embryo acute toxicity (FET) Test. Section 2, OECD Publishing, Paris. ⟨https://doi.org/10.1787/9789264203709-en⟩.
OECD, 2019. OECD Guidance document on aqueous-phase aquatic toxicity testing of difficult test chemicals. No. 23 (Second Edition), OECD Publishing, Paris. ⟨https://one.oecd.org/document/ENV/JM/MONO(2000)6/REV1/en/pdf⟩.
Ogungbemi, A., Leuthold, D., Scholz, S., Küster, E., 2019. Hypo-or hyperactivity of zebrafish embryos provoked by neuroactive substances: a review on how experimental parameters impact the predictability of behavior changes. Environ. Sci. Eur. 31 (1), 1–26. https://doi.org/10.1186/s12302-019-0270-5.
Pereira, S.P., Boyle, D., Nogueira, A.J., Handy, R.D., 2023. Comparison of toxicity of silver nanomaterials and silver nitrate on developing zebrafish embryos: Bioavailability, osmoregulatory and oxidative stress. Chemosphere, 139236. https://doi.org/10.1016/j.chemosphere.2023.139236.
Petraccioli, A., Guarino, F.M., Kupriyanova, L., Mezzasalma, M., Odierna, G., Picariello, O., Capriglione, T., 2019. Isolation and characterization of interspersed repeated sequences in the common lizard *Zootoca vivipara* and their conservation in Squamata. Cytogenet. Genome Res. 157, 65–76. https://doi.org/10.1159/000497304.
Plhalova, L., Blahova, J., Divisova, L., Enevova, V., Casuscelli di Tocco, F., Faggio, C., Vecerek, V., Tichy, F., Svobodova, Z., 2018. The effects of subchronic exposure to NeemAzal T/S on zebrafish (Danio rerio). Chem. Ecol. 34, 199–210.
Qin, Y., Wang, X., Yan, X., Zhu, D., Wang, J., Chen, S., Wang, S., Wen, Y., Martyniuk, C. J., Zhao, Y., 2022. Developmental toxicity of fenbuconazole in zebrafish: Effects on mitochondrial respiration and locomotor behavior. Toxicology 470, 153137. https://doi.org/10.1016/j.tox.2022.153137.
Rashidian, G., Mohammadi-Aloucheh, R., Hosseinzadeh-Otaghvari, F., Chupani, L., Stejskal, V., Samadikhah, H., Zamanlui, S., Multisanti, C.R., Faggio, C., 2023. Long-term exposure to small-sized silica nanoparticles (SiO2-NPs) induces oxidative stress and impairs reproductive performance in adult zebrafish (Danio rerio). Comp. Biochem. Phys. C. 273, 109715.
Schwebel, L.N., Stuart, K., Lowery, M.S., Wegner, N.C., 2018. Swim bladder inflation failure affects energy allocation, growth, and feed conversion of California Yellowtail (*Seriola dorsalis*) in aquaculture. Aquaculture 497, 117–124. https://doi.org/10.1016/j.aquaculture.2018.07.050.
Sloman, K.A., McNeil, P.L., 2012. Using physiology and behaviour to understand the responses of fish early life stages to toxicants. J. Fish. Biol. 81 (7), 2175–2198. https://doi.org/10.1111/j.1095-8649.2012.03435.x.
Stehr, C.M., Linbo, T.L., Incardona, J.P., Scholz, N.L., 2006. The developmental neurotoxicity of fipronil: notochord degeneration and locomotor defects in zebrafish embryos and larvae. Toxicol. Sci. 92 (1), 270–278. https://doi.org/10.1093/toxsci/kfj185.
Stinckens, E., Vergauwen, L., Blackwell, B.R., Ankley, G.T., Villeneuve, D.L., Knapen, D., 2020. Effect of thyroperoxidase and deiodinase inhibition on anterior swim bladder inflation in the zebrafish. Environ. Sci. Technol. 54 (10), 6213–6223. https://doi.org/10.1021/acs.est.9b07204.
Sun, Y., Cao, Y., Tong, L., Tao, F., Wang, X., Wu, H., Wang, M., 2020. Exposure to prothioconazole induces developmental toxicity and cardiovascular effects on zebrafish embryo. Chemosphere 251, 126418. https://doi.org/10.1016/j.chemosphere.2020.126418.
Sun, P.L., Hawkins, W.E., Overstreet, R.M., Brown-Peterson, N.J., 2009. Morphological deformities as biomarkers in fish from contaminated rivers in Taiwan. Int. J. Environ. Res. Public Health 6 (8), 2307–2331. https://doi.org/10.3390/ijerph6082307.
Venuto, A., Thibodeau-Beganny, S., Trapani, J.G., Erickson, T., 2023. A sensation for inflation: initial swim bladder inflation in larval zebrafish is mediated by the mechanosensory lateral line. J. Exp. Biol. 226 (11), jeb245635 https://doi.org/10.1242/jeb.245635.
Vieira, R.S., Venâncio, C.A., Félix, L.M., 2021. Embryonic zebrafish response to a commercial formulation of azoxystrobin at environmental concentrations. Ecotoxicol. Environ. Saf. 211, 111920 https://doi.org/10.1016/j.ecoenv.2021.111920.
Wang, X., Li, X., Wang, Y., Qin, Y., Yan, B., Martyniuk, C.J., 2021. A comprehensive review of strobilurin fungicide toxicity in aquatic species: emphasis on mode of action from the zebrafish model. Environ. Pollut. 275, 116671 https://doi.org/10.1016/j.envpol.2021.116671.
Wang, X.H., Zheng, S.S., Huang, T., Su, L.M., Zhao, Y.H., Souders II, C.L., Martyniuk, C. J., 2018. Fluazinam impairs oxidative phosphorylation and induces hyper/hypo-activity in a dose specific manner in zebrafish larvae. Chemosphere 210, 633–644. https://doi.org/10.1016/j.chemosphere.2018.07.056.
Yang, L., Huang, T., Li, R., Souders II, C.L., Rheingold, S., Tischuk, C., Li, N., Zhou, B., Martyniuk, C.J., 2021. Evaluation and comparison of the mitochondrial and developmental toxicity of three strobilurins in zebrafish embryo/larvae. Environ. Pollut. 270, 116277 https://doi.org/10.1016/j.envpol.2020.116277.
Yang, D., Lauridsen, H., Buels, K., Chi, L.H., La Du, J., Bruun, D.A., Olson, J.R., Tanguay, R.L., Lein, P.J., 2011. Chlorpyrifos-Oxon disrupts zebrafish axonal growth and motor behavior. Toxicol. Sci. 121 (1), 146–159. https://doi.org/10.1093/toxsci/kfr028.
Yao, H., Xu, X., Zhou, Y., Xu, C., 2018. Impacts of isopyrazam exposure on the development of early-life zebrafish (*Danio rerio*). Environ. Sci. Pollut. Res. 25, 23799–23808. https://doi.org/10.1007/s11356-018-2449-5.
Zhang, T., Yuan, J., Guo, Y., Wang, X., Li, Q.X., Zhang, J., Xie, J., Miao, W., Fan, Y., 2024. Combined toxicity of trifloxystrobin and fluopyram to zebrafish embryos and the effect on bone development. Aquat. Toxicol. 268, 106834 https://doi.org/10.1016/j.aquatox.2024.106834.
Zhang, C., Zhang, J., Zhu, L., Du, Z., Wang, J., Wang, J., Li, B., Yang, Y., 2020. Fluoxastrobin-induced effects on acute toxicity, development toxicity, oxidative stress, and DNA damage in *Danio rerio* embryos. Sci. Total Environ. 715, 137069 https://doi.org/10.1016/j.scitotenv.2020.137069.
Zhao, Y., Zhang, H., Liu, Y., Lan, Y., Zhu, J., Cai, Y., Gou, F., Li, F., Zhang, Y., Zhang, T., Kannan, K., Xue, J., Yang, Z., 2024. Evidence of strobilurin fungicides and their metabolites in Dongjiang River ecosystem, southern China: Bioaccumulation and ecological risks. Sci. Total Environ. 908, 168427 https://doi.org/10.1016/j.scitotenv.2023.168427.
Zhu, B., Liu, G.L., Liu, L., Ling, F., Wang, G.X., 2015. Assessment of trifloxystrobin uptake kinetics, developmental toxicity and mRNA expression in rare minnow embryos. Chemosphere 120, 447–455. https://doi.org/10.1016/j.chemosphere.2014.07.100.