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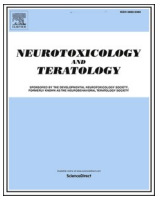

Full length article

Acute toxicity of methomyl commercial formulation induces morphological and behavioral changes in larval zebrafish (*Danio rerio*)

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A R T I C L E I N F O

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A B S T R A C T

The use of pesticides has continue grown over recent years, leading to several environmental and health concerns, such as the contamination of surface and groundwater resources and associated biota, potentially affecting populations that are not primary targets of these complex chemical mixtures. In this work, we investigate lethal and sublethal effects of acute exposure of methomyl commercial formulation in zebrafish embryo and larvae. Methomyl is a broad-spectrum carbamate insecticide and acaricide that acts primarily in acetylcholinesterase inhibition (AChE). Methomyl formulation 96 h-LC_{50} was determined through the *Fish Embryo Acute Toxicity Test* (FET) and resulted in 1.2 g/L ± 0.04. Sublethal 6-day exposure was performed in six methomyl formulation concentrations (0.5; 1.0; 2.2; 4.8; 10.6; 23.3 mg/L) to evaluate developmental, physiological, morphological, behavioral, biochemical, and molecular endpoints of zebrafish early-development. Methomyl affected embryo hatching and larva morphology and behavior, especially in higher concentrations; resulting in smaller body and eyes size, failure in swimming bladder inflation, hypolocomotor activity, and concentration-dependent reduction of AChE activity; demonstrating methomyl strong acute toxicity and neurotoxic effect.

1. Introduction

The use of pesticides has grown in recent years with a direct effect on increasing food production. If, on the one hand, their use increase efficiency in the field, it also generates concerns due to potential non-target environmental and health harms. Methomyl (*S*-methyl-N – [(methyl-carbamoxyl) oxy] thioacetimidate) is a carbamate first synthesized in 1966, and since has been used worldwide as an broad spectrum insecticide and acaricide of contact and ingestion, particularly to control invading organisms in agricultural crops (Seleem, 2019). As a carbamate, methomyl acts primarily in the reversible inhibition of acetylcholinesterase (AChE), the main enzyme of the cholinergic system. Its essential function is the degradation of acetylcholine, which acts as a neurotransmitter in the autonomic nervous system and neuromuscular junctions (Yoon et al., 2016). When AChE is inhibited, acetylcholine accumulates in the synaptic clefts, leading to continuous and uncontrolled transmission of nerve impulses (hyperexcitability), resulting in muscle paralysis, impaired breathing, and ultimately, death, due to lack of oxygen in the brain (Yoon et al., 2016).

Despite pesticide application aims to (economically and efficiently)

Abbreviations: a.i., active ingredient; AChE, acetylcholinesterase; Dpf, days post-fertilization; FET, fish embryo acute toxicity test; Hpf, hours post-fertilization; LC_{50}, median lethal concentration; M1, 0.5 mg/L a.i. of methomyl; M2, 1.0 mg/L a.i.; M3, 2.2 mg/L a.i.; M4, 4.8 mg/L a.i.; M5, 10.6 mg/L a.i.; M6, 23.3 mg/L a.i.; MSDS, Material Safety Data Sheet; STC, spontaneous tail coiling.

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apply the correct amount of the active ingredient to the desired target (Rojo Baio et al., 2019), they may ultimately reach natural surface and groundwater habitats, through run-off, spray drift, leaching, and subsurface drainage (Mojiri et al., 2020). The presence of methomyl in natural environments is mostly described at low levels, in the order of some tens of μg/L (Van Scoy et al., 2012), however, significant higher concentrations have also been identified, as reported in a northern Brazil study, that detected methomyl at up to 32 mg/L in waters from Branco river, in Roraima state (Farias Filho et al., 2013).

Pesticides residues detrimentally affect non-target organisms, such as fish, plants, amphibians, invertebrates, or microorganisms (Mojiri et al., 2020). Toxic sublethal effects of methomyl exposure have been observed in adult fishes including oxidative stress, tissue damage, increased apoptosis rate, dysregulation of sex hormones (Meng et al., 2019, 2017, 2014) and altered immune response (Mohamed et al., 2021). Yet, their potential toxic effects to fish (or even vertebrates) developmental stages are mostly unknown. In tadpoles models, locomotor, morphological, histopathological and immunohistochemical changes were observed upon methomyl exposures (Seleem, 2019; Trachantong et al., 2016), while oxidative stress, mitochondrial impairment, increased apoptosis and autophagy has been recently reported in mice embryos exposed to methomyl (He et al., 2021).

Pesticides active ingredients are not used in its pure form, since commercial formulations include co-formulants to improve its dissolution, stability, absorption and pesticidal action (Nagy et al., 2020). To consider how and how much pesticide product is applied in the field is utmost relevant because such complex, and mostly undisclosed, chemical mixtures are the ones reaching the environment. Its toxicological hazards should not be underestimated in both environmental- and health-relevant scenarios. Therefore, this work objective was to determine lethal concentration (LC_{50}) of methomyl commercial formulation and its sublethal effects in zebrafish embryos and larvae. For that, we evaluated developmental toxicity in terms of physiological, morphological, behavioral, biochemical, and molecular aspects of zebrafish early-development.

2. Material and methods

2.1. Methomyl

The commercial methomyl formulation (BrilhanteBR®; Ourofino Química LTDA, Brazil), contained 21.5% of methomyl, as active ingredient (a.i.) (CAS 16752-77-5), 42% of ethanol (CAS 64-17-5) and 32.4% of unspecified ingredients, with field application recommended between 215 mg/L and 2800 mg/L (a.i.), according to the manufacturer. For in vivo experiments, the liquid product was easily diluted and manually mixed by agitation (for 10 s) in recirculating system water sample (Center for Experimental Biological Models, Pontifical Catholic University of Rio Grande do Sul – CeMBE/PUCRS). Exposure solutions were freshly prepared and substituted every 24 h, as semi-static system, to ensure stability.

2.2. LC-MS/MS

Exposure solutions, with different concentration of methomyl formulation, were analyzed by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) to analytically confirm nominal concentrations, following a modified method described elsewhere (Rübensam et al., 2013). LC-MS/MS system consisted by an ultra-high-performance chromatographer model UPLC Acquity 1 Class Plus, and a mass spectrometer model Xevo TQ-S micro (Waters Waters, Milford, MA, USA). Chromatographic separations were performed on a Zorbax Eclipse Plus C18 RRHD (5 × 2.1 mm, 1.8 μm, Agilent Technologies, USA) column with a pre-column of the same packing, using a mobile phase consisted of (A) 10 mM ammonium acetate with 0.1% formic acid and (B) 10 mM ammonium acetate with 0.1% formic acid in acetonitrile. The gradient started with 10% of B, passing to 50% in 2.4 min, and programmed to reach 95% of B at 2.5 min. This composition was maintained for 1 min before returning to the start condition. The total chromatographic run was 6 min, using a mobile phase flow of 0.2 mL/min, column temperature of 50 °C, and an injection volume of 2 μL. The spectrometer was operated in MRM mode to monitor Methomyl (m/z 163 > 88 for quantification; m/z 163 > 106 for confirmation) and D3-Methomyl (IS, m/z 166 > 88). Quantification was performed by internal standardization, using Methomyl/IS ratio, with calibration curve at concentrations of 1.0, 5.0, 10.0, 50.0, 100.0 ng/mL.

2.3. Zebrafish husbandry, reproduction, and embryo collection

Adult zebrafish (AB line) were maintained in continuous water flow tanks (Zebtec, Techniplast), with automatically controlled optimal physical-chemical parameters (27 ± 1 °C; pH = 7–8; conductivity = 500–800 μS; 14:10 light:dark cycle), in addition to being fed twice a day with flocked feed (TetraMin Tropical Flake, Tetra) and *Arthemia* sp. in the night feed. For breeding, males and females were separated in a ratio of 2:1, respectively, in small breeding tanks, at the end of the day. Within the first hours of the following morning, the physical barrier was removed for mating, for around one hour. Embryos were cleaned and collected for further experiments. All the methodologies here described were previously approved by Institutional Animal Use Ethics Committee (CEUA/PUCRS No. 9593).

2.4. Determination of LC_{50}

Methomyl formulation median lethal concentration (LC_{50}) was determinate through the fish embryo acute toxicity test (FET), according to OECD Guidelines No. 236 (OECD, 2013). Based on previous concentration-response curve pilot test, methomyl formulation was directly dissolved in recirculation system water samples into five nominal concentrations, represented by mg/L of the active ingredient (mg/L a.i.) and spaced by a 1.5 constant factor (OECD, 2013): 373.12; 559.68; 839.52; 1259.28 and 1888.92 mg/L a.i. Nominal concentrations were analytically confirmed through LC-MS/MS (Table S1) and are within recommended concentration range for field application (Section 2.1). Zebrafish fertilized eggs of up to 1.5 hpf were selected and individually placed in 24-well plates containing 2 mL of exposure solution each, plus control groups: negative control (recirculating system water), solvent control (vehicle, at 0.004% ethanol *v*/v – equivalent concentration of ethanol present in the highest concentration of methomyl formulation) and positive control (4 mg of 3,4-dichloroaniline). Embryos were exposed for 96 h (n = 60/group), in an incubator with controlled temperature (26 ± 1 °C) and 14:10 photoperiod under a semi-static regime renewed every 24 h. Embryos were observed daily, using a stereomicroscope (Nikon®, SMZ 1500), registering four apical observations as lethality indicators (OECD, 2013): coagulation of fertilized eggs, lack of somites formation, absence of tail detachment and absence of heartbeat. The LC_{50} was calculated using the *Toxicity Relationship Analysis Program* (*TRAP*/U.S.EPA; version 1.30a), based in three independent experiments.

2.5. Sublethal exposures: concentrations and experimental design

Six concentrations of methomyl formulation were established for sublethal tests: 0.5 (M1); 1.0 (M2); 2.2 (M3); 4.8 (M4); 10.6 (M5); 23.3 (M6) mg/L a.i. Additionally, two control groups were included: a negative control and vehicle group (0.00004% *v*/v of ethanol – equivalent ethanol concentration present in M6 group). Nominal concentrations range was defined through concentration-response curve pilot test based on up to 1/3 of LC_{50} and ideal mortality rate lower than 10% (Beekhuijzen et al., 2015), spaced by a 2.2 constant factor (OECD, 2013), and analytically confirmed through LC-MS/MS (Table S2).

All sublethal exposure experiments were performed in acrylic Petri

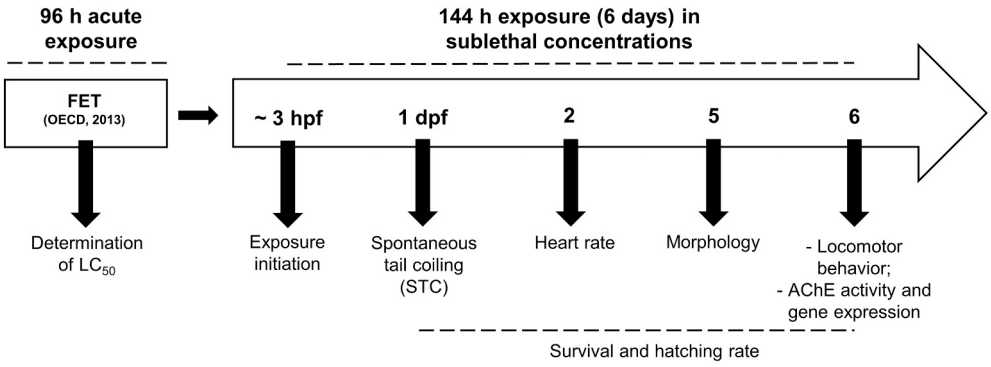

Fig. 1. Experimental design of exposure to methomyl formulation. Healthy embryos (i.e., verified through a stereomicroscope for normal cleavages) were exposed to methomyl formulation diluted in system water. Freshly prepared solutions were exchanged daily, as semi-static exposure, in both FET (≤ 1.5–96 h) and sublethal exposure (~3 h – 6 dpf) protocols. Spontaneous tail coiling, heart rate, morphology, locomotor behavior, AChE activity, and gene expression were evaluated at designated time points. Survival and hatching rates were analyzed daily. [FET: Fish Embryo Acute Toxicity; hpf: hours post-fertilization; dpf: days post-fertilization; LC_{50}: median lethal concentration; AChE: acetylcholinesterase.]

dishes with ≤2 embryos/mL and kept in incubator with stable temperature (28,5 °C ± 1 °C) and controlled light:dark cycle (14:10), with daily fresh solutions and both pre- and post-exposure measurements of water physicochemical parameters (temperature, conductivity and pH). Embryos with ~3 hpf were individually observed under a stereomicroscope to ensure their viability and were arbitrarily distributed among all experimental groups (Control, Vehicle, M1, M2, M3, M4, M5, and M6) to a 6-day exposure protocol, based on previous study (Pereira et al., 2020). In vivo parameters were analyzed at specific animal developmental stage, as follows: at 1 dpf, spontaneous tail coiling (STC); at 2 dpf, heart rate; at 5 dpf, morphology; and at 6 dpf, the locomotor behavior. Survival and hatching rate were analyzed daily throughout the experiment (n = 460). Biochemical and molecular samples were collected after hypothermal shock euthanasia of 6 dpf larvae. The experimental design is summarized in Fig. 1.

2.5.1. Embryonic endpoints: Spontaneous tail coiling (STC) and heart rate

At 1 dpf, the embryos were removed from the incubator and acclimatized for 1 min at room temperature. Under a stereomicroscope (Nikon®, SMZ 1500), STC events were registered during 1 min (by two independent observers (n = 8/group/experiment; 4 independent experiments), as previously described (Pereira et al., 2020). At 2 dpf, and after 1 min acclimatation at room temperature, embryo heartbeats were quantified in triplicates of 10 s each, per animal, by two independent observers, under a stereomicroscope (Nikon®, SMZ 1500) (n = 8/group/experiment; 4 independent experiments). Finally, data was converted and presented as beats per min (bpm) (Lanzarin et al., 2019).

2.5.2. Morphological evaluation

At 5 dpf, larvae without visual morphological and locomotor deformities were selected for image capture (3× magnification) using a stereomicroscope with attached digital camera (Nikon®, SMZ 1500). Morphological analysis was performed based on the following parameters: body length (mm), distance between the eyes (mm) and total eye area (mm^2), at dorsal view (Pereira et al., 2020); and body surface area (mm^2), swimming bladder surface area (mm^2) (Yang et al., 2021), and the yolk sac surface area (mm^2) (Cheng et al., 2020), measured at side view ($n \geq 21$/group; ≥ 3 independent experiments). All parameters were measured using calibrated dorsal and lateral images through *Image J* software (*https://imagej.nih.gov/ij/download.html*) by two independent observers.

2.5.3. Locomotor behavior

At 6 dpf, the larvae were tested for their locomotor behavior (n = 48). Larvae without visual morphological and locomotor deformities were pre-selected and acclimated individually in 24-well plate, for 1 min. Locomotor behavior was recorded for 5 min, at 25 frames/s, by a high-resolution digital camera (AltaVision®, Basler ace, model scA1300-60gc) coupled with *EthoVisionXT* software (Noldus Information Technology) (Costa et al., 2019). The following parameters were analyzed: total distance moved (cm), average speed (cm/s), maximum acceleration (cm/s^2), total time on the periphery of the arena (s), total time in the center of the arena (s), total entries in the center of the arena (f), total entries in the periphery of the arena (f), latency to enter the center (s), latency to enter the periphery (s), absolute turn angle (°), total time of high mobility (s) and absolute meander (°/cm).

2.5.4. Determination of acetylcholinesterase activity

Acetylthiocholine, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), Trizma Base, ethylenedioxy-diethylene-dinitrilo-tetraacetic acid (EDTA), ethylene glycol bis (beta-aminoethyl ether)-N,N,N′,N′-tetra-acetic acid (EGTA), sodium citrate, Coomassie blue, bovine serum albumin, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents used were from analytical grade.

Samples containing a pool of 10 larvae (6 dpf) were homogenized on ice bath with an Ultra-Turrax (T10 basic IKA®) in 60 vol. (*v/w*) of 50 mM Tris-HCl, pH 8.0. Protein concentration was measured by the Coomassie blue method (Bradford, 1976), using bovine serum albumin as standard. AChE activity was determined according to Ellman et al. (1961) with minor modifications. Briefly, the activity in the homogenate was measured by determining the hydrolysis rate of acetylthiocholine iodide (ACSCh, 0.88 mM) in a final volume of 300 µL, with 33 µL of 100 mM phosphate buffer, pH 7.5 mixed to 33 µL of 2.0 mM DTNB (5,50-dithionitrobis2-nitrobenzoic acid). In this solution, 5 µg of protein of each sample were added. The reaction was initiated with the addition of the substrate acetylthiocholine, followed by the immediate analyses of hydrolysis and dianion of DTNB formation at 420 nm, for 6 min (in intervals of 1 min) using a microplate reader. AChE activity was expressed as micromole of thiocholine (SCh) released per hour per milligram of protein. All enzyme assays were performed in six different experiments, each one performed in triplicate.

2.5.5. RT-qPCR

Molecular analysis of *ache* gene expression was performed following the *Minimum Information Guidelines for Publication of Quantitative Real-Time PCR Experiments (MIQE)* (Bustin et al., 2009). The total RNA was isolated from 6 dpf larva pool (n = 25/sample; 4 biological and 4 technical replicates per group) with TRIzol® reagent (Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to product instructions. RNA purity (Abs 260/280 nm ~2.0) and concentration were determined using NanoDrop Lite (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and then treated with Deoxyribonuclease I – Amplification grid (Sigma-Aldrich Inc., St Louis, Missouri, USA) to eliminate genomic DNA contamination, following product instructions. The cDNA was synthesized with ImProm-II ™ Reverse Transcription System (Promega, Madison, Wisconsin, USA) from 1 µg of total RNA,

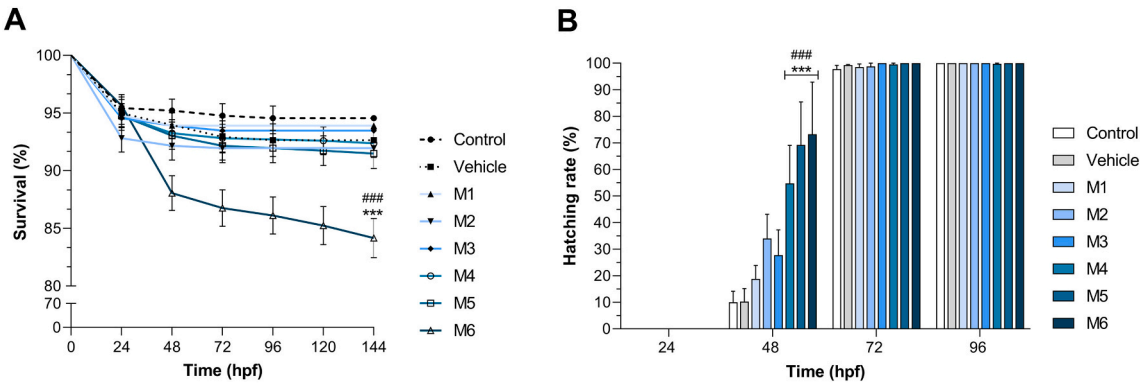

Fig. 2. Survival and hatching rate of zebrafish during sublethal exposure to the methomyl formulation ($n = 460$/group of 4 independents experiments). Survival (A) and hatching rate (B) during 6-day exposure protocol. The survival rate was estimated according to the Log-rank (Mantel-Cox) test. The hatching rate analyses employed two-way ANOVA followed by Tukey's post-test. "*" represents differences against control or "#" vehicle group. (*** = $p \leq 0.001$, vs. control group; $^{\#\#\#}$ = $p \leq 0.001$, vs. vehicle group). [Control: system water; Vehicle: 0.00004% of ethanol; M1: active ingredient (a.i.) methomyl – 0.5 mg/L; M2: 1 mg/L a.i.; M3: 2.2 mg/L a.i.; M4: 4.8 mg/L a.i.; M5: 10.6 mg/L a.i.; M6: 23.3 mg/L a.i.]

Quantitative PCR was performed using SYBR® Green I (Thermo Fisher Scientific, Waltham, Massachusetts, USA) to detect cDNA double-strand synthesis in a 7500 Real-Time PCR system (Applied Biosystems, Foster City, California, USA). PCR cycling conditions followed an initial polymerization activation step for 5 min at 95 °C and 40 cycles of 15 s at 95 °C for denaturation, 35 s at 60 °C for annealing and 15 s at 72 °C for elongation. At the end, a melting curve analysis was included, and fluorescence measured from 60 to 99 °C to confirm primers specificity. The amplification efficiency was calculated for each well using *LinRegPCR* software (http://LinRegPCR.nl). The *ache* mRNA relative expression (Pereira et al., 2012) were determined through the $2^{-\Delta\Delta Cq}$ method (Bustin et al., 2009; Pfaffl, 2001), using *rpl13α* and *β-actin* as reference genes (Tang et al., 2007).

2.6. Statistical analyses

The Shapiro-Wilk normality test was performed for all in vivo parameters. The Log-Rank (Mantel-Cox) test was applied to the survival curve, and two-way ANOVA followed by Tukey's post-hoc test for hatching rate. Nonparametric results were analyzed with Kruskal-Wallis test followed by Dunn's post-hoc test, while parametric data were evaluated by one-way ANOVA followed by the Tukey's post-hoc test. $p \leq 0.05$ was considered statistically significant.

3. Results

3.1. LC_{50}

Based on nominal concentrations, calculated 96 h-LC_{50} value for zebrafish embryos exposed to methomyl formulation was 1.2 ± 0.04 g/L, while based on analytically measured concentrations it resulted in 1.3 ± 0.04 g/L, a difference that is statistically contained within confidence interval range (95% C.I.: 1.2–1.4 g/L). LC_{50}, survival curve and hatching rate results are presented in Fig. S1. Methomyl formulation showed lower lethality for embryos than reported for adult zebrafish with 96 h-LC_{50} of 28.28 mg/L (Material Safety Data Sheet MSDS BrilhanteBR®, 2018).

3.2. Survival and hatching rate

All groups remained within the expected 90% survival range, except the M6 group, that reached around 85% survival, which is statistically different then control and vehicle survival curves (Fig. 2A). Regarding hatching, most animals had completely hatched by 3 dpf (72 hpf), as is expected (Kimmel et al., 1995). At 2 dpf (48 hpf), however, groups exposed to higher doses of methomyl formulation (M4, M5, M6) showed increased hatching of around 55%, 70% and 73%, respectively, while control and vehicle showed ≈10% hatching (Fig. 2B), showing a significant difference with both control groups ($F_{(7, 96)} = 4.910$; $p \leq 0.001$).

3.3. Spontaneous tail coiling (STC) and heart rate

The STC frequencies and heart rate analyses presented no significant differences for groups exposed to methomyl formulation, when compared with control and vehicle groups, as shown Fig. 3A–B ($p > 0.05$).

3.4. Zebrafish larvae morphology

Morphological effects of methomyl formulation exposure on zebrafish larvae are presented in Fig. 4A, as representative images, in both dorsal and lateral views. Regarding body length, ≈9,7% decrease was observed in exposed groups, when compared to control and vehicle ($F_{(7, 176)} = 35.54$; $p \leq 0.001$) (Fig. 4B). The distance between the eyes presented significant reduction only among the highest methomyl formulation concentrations (respectively, 16.1% and 22.3% for M5 and M6 ($F_{(7, 179)} = 11.30$; $p \leq 0.001$) (Fig. 4C) and ocular surface area showed ≈18% reduction ($F_{(7, 184)} = 10.65$; $p \leq 0.001$) in all groups exposed to methomyl formulation (Fig. 4D). Lateral body surface area (Fig. 4E) showed significant reduction in M3 and M4 groups vs. both controls ($F_{(7, 184)} = 5.299$; $p \leq 0.05$), although significant reduction was also seen in M2 and M5 groups, when compared to vehicle only ($F_{(7, 184)} = 5299$; $p \leq 0.05$). No significant differences were identified for yolk sac surface area ($F_{(7, 182)} = 1.973$; $p = 0.06$) (Fig. 4F). The swimming bladder surface area was significantly smaller in all exposed groups when compared to both control and vehicle groups ($F_{(7, 184)} = 97.82$; $p \leq 0.001$); specially for M4, M5 and M6 groups, with ≈70% reduction (Fig. 4G). Beyond all quantifiable parameters mentioned, morphological deformations (e.g., spine deviations) were also observed in M4, M5, and M6 groups, as presented in Fig. S2.

3.5. Locomotor behavior

Locomotor behavior analyses showed decreased distance travelled (Fig. 5A), acceleration (Fig. 5B), average speed (Fig. 5C), and low-mobility state period (Fig. 5F) for all groups exposed to methomyl formulation when compared to both control and vehicle groups ($p \leq 0.05$). While no differences were identified in latency to enter the center among all groups (Fig. 5D), only M5 and M6 groups presented an

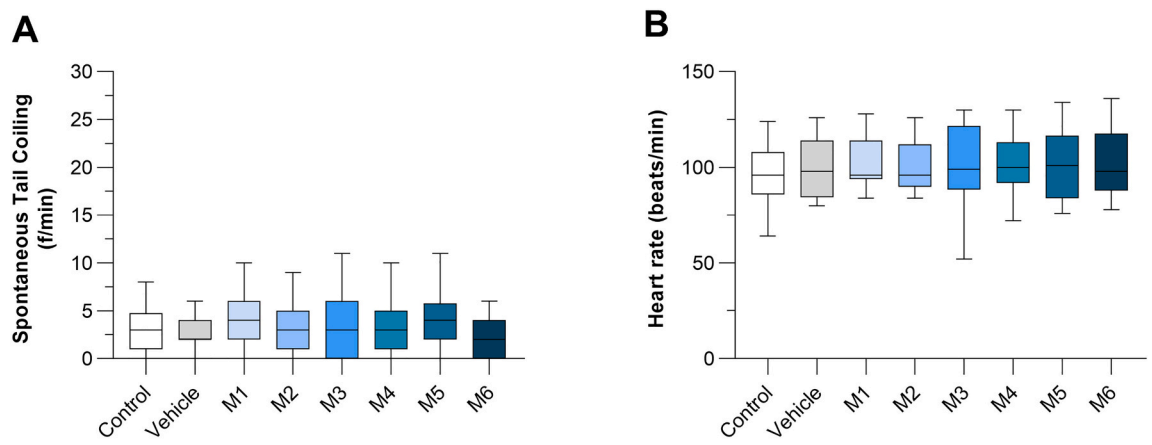

Fig. 3. Methomyl formulation effects on the functional aspects of zebrafish embryos and larvae. (n = 32/group of 4 independents experiments). Spontaneous tail coiling (A) was analyzed at 1 dpf and heart rate (B) at 2 dpf. The boxplot bars represent data min to max range and median. Statistical analyses employed Kruskal-Wallis test followed by Dunn's post-test. [Control: system water; Vehicle: 0.00004% of ethanol; M1: active ingredient (a.i.) methomyl – 0.5 mg/L; M2: 1 mg/L a.i.; M3: 2.2 mg/L a.i.; M4: 4.8 mg/L a.i.; M5: 10.6 mg/L a.i.; M6: 23.3 mg/L a.i.]

increased latency to enter the periphery (Fig. 5E) ($p \leq 0.001$). Regarding frequency of entries in each zone, exposed animals showed decreased central ($p \leq 0.001$) and peripherical ($p \leq 0.001$) zone entries, when compared to at least the control group (Fig. 5G–H). Concerning the time spent in the center (Fig. 5J) or periphery (Fig. 5K), no significant differences were identified against controls ($p > 0.05$). The absolute turn angle (Fig. 5I) and absolute meander (Fig. 5L) increased within the groups exposed to methomyl formulation when compared to both control and vehicle ($p \leq 0.001$). Fig. 5M shows representative heatmaps for average distance travelled in each experimental group.

3.6. AChE activity and gene expression

Animals exposed to methomyl formulation showed AChE activity reduction of 35.2%, 42.7%, 49.4% 58.2%, 63.9%, and 67% for M1, M2, M3, M4, M5, and M6, respectively, when compared to control ($F_{(7, 40)} = 22.08$; $p \leq 0.001$). Similarly, a 29%, 37.3%, 44.9%, 54.2%, 60.5% and 63.8% decrease in AChE activity was observed for M1, M2, M3, M4, M5, and M6, respectively, when compared to vehicle ($F_{(7, 50)} = 0.6974$; $p = 0.6739$). No significant differences were observed for *ache* gene expression among all experimental groups (Fig. 6B).

4. Discussion

Pesticides' migration into waterways is a function of their mobility in soil and air as well as their rate of degradation, and once in the aquatic environment, the effect is a function of their solubility (Mojiri et al., 2020). Thus, environmental concentrations of pesticide residue are expected to be variable, as consequence of application, climatic factors, soil characteristics and pesticide chemical properties, such as persistence, volatility, and solubility (Materu et al., 2021). Although the concentrations used in this study are higher than those generally found in the environment, methomyl has a great soil mobility, presenting a risk of water contamination, through runoff and leaching processes (Van Scoy et al., 2012). Conversely, it is known that methomyl has a fast degradation in the environment (≈15 days) in superficial soils (Kahl et al., 2007). In that sense, Wang et al. (2021) reported that methomyl was not constantly detected in the environment during the whole year, but mainly in dry seasons. Together, it might suggest that while high chronic methomyl exposures would be less likely, acute high exposure seems more environmentally relevant.

Methomyl formulation acute lethal toxicity was determined by the FET test and surprisingly reached a greatly higher LC_{50} value for zebrafish embryos/larva than reported for adult zebrafish (presented within the product's MSDS). FET is a reliable and validated guideline, shown to predict toxic effects of contaminants on juvenile zebrafish (Lammer et al., 2009), yet, recent studies suggest that zebrafish embryonic stage might be less sensitive for neurotoxic substances than its juvenile stage, and therefore, might failure to predict toxicity to other developmental stages (Glaberman et al., 2017). According to Klüver et al. (2015), neurotoxicants act by failure to breathe, that is, the body's inability to supply adequate levels of oxygen for essentials vital functions. For AChE inhibitors specifically, dysregulation in cholinergic signaling, with symptoms like spasms associated with blood vessels bleeding, decreased heart rate and vasoconstriction in gill lamellae have been identified in adult zebrafish (Klüver et al., 2015). In contrast, in embryos and larvae of up to ≈14 dpf, oxygen supply is not dependent on cardiovascular system function and is mainly supplied through skin diffusion (Klüver et al., 2015), suggesting a reduced impact on lethality. Nevertheless, and beyond the observed differences in experimental design conditions, a preprint data by Ahmad et al. (2020) showed a LC_{50} of 59.7 ± 0.39 mg/L for zebrafish larvae exposed to methomyl, as pure active ingredient, among other pesticides tested. That is much lower than observed here, using a commercial methomyl formulation, which reinforces that co-formulants might be important players in such mixtures; and only experiments designed to compare exposures to pure active ingredients versus different pesticide product formulations will help to elucidate their role. That is especially interesting because it has been reported for other pesticides both decreased and increased toxicity of pesticide product formulation over its active ingredient counterpart, as recently reviewed elsewhere for glyphosate (Nagy et al., 2020).

Accumulating evidence indicates that including analysis of sublethal endpoints significantly increase toxicity tests sensibility in >30% (Stelzer et al., 2018). Here, physiological, morphological, and behavioral parameters were evaluated at embryonic and/or larval stages. Among embryonic endpoints for developmental toxicity are the evaluation of spontaneous tail colling and heart rate. Zebrafish heart is already formed, with an atrium and a ventricle, by 2 dpf (Pathak and Barresi, 2019) and can be easily visualized due to embryo transparency, allowing non-invasive assessments (Bournele and Beis, 2016). Our results demonstrated that methomyl formulation did not affect heart rate in any of the exposed groups (Fig. 3B). The STC event is the first motor activity generated by neural network development, and occurs as a result of muscle innervation through axons that begin to proliferate in embryonic somites (Bachour et al., 2020; Ogungbemi et al., 2019). It consists of a single or alternating tail movements observed as early as 19 hpf, with an activity peak at 23–26 hpf; the ideal time-point for assessing it as neurotoxicity marker (Bachour et al., 2020; Zindler et al., 2019).

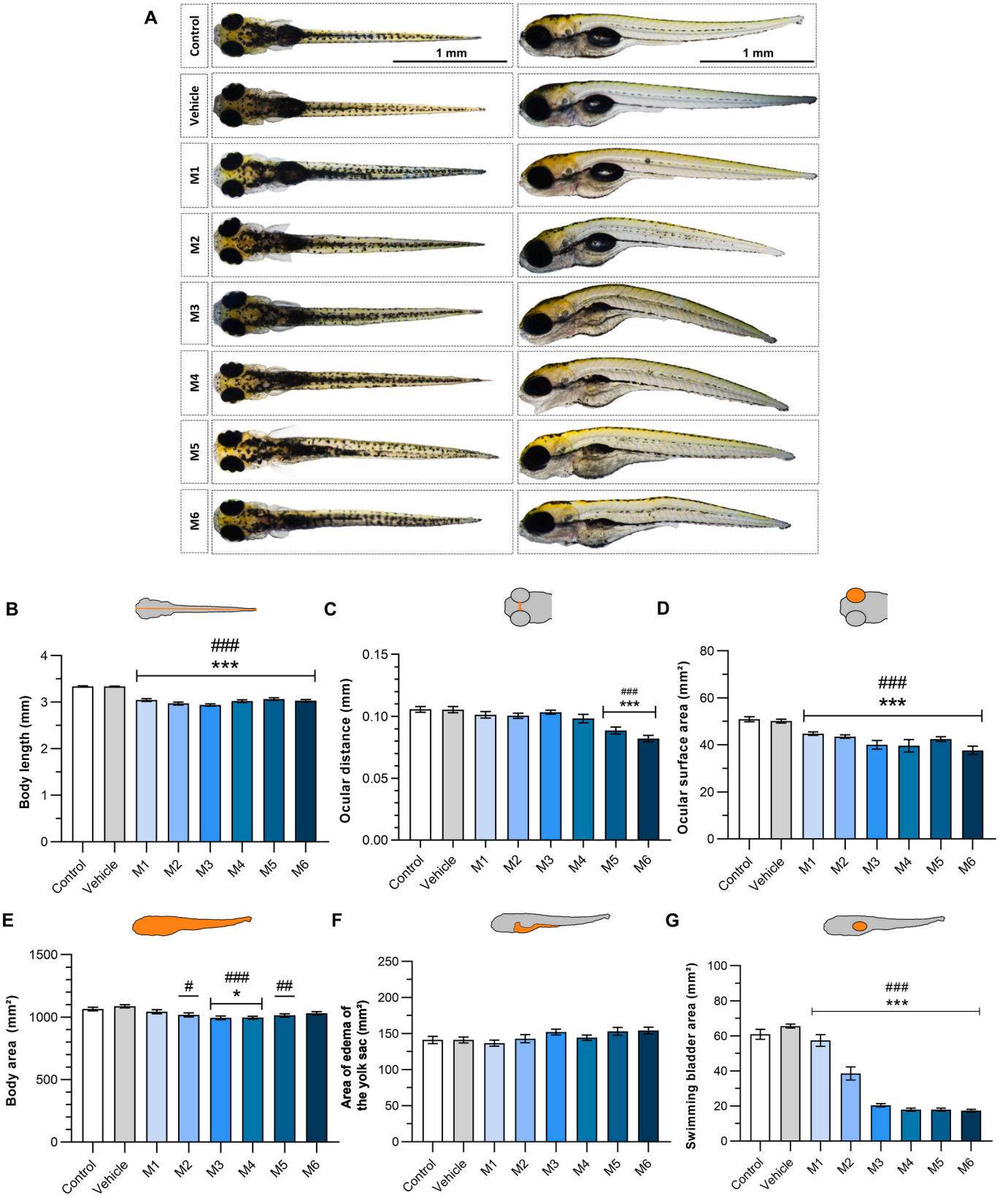

Fig. 4. Methomyl formulation effects on zebrafish larvae morphology ($n \geq 21$/group of 4 independents experiments). Body length (B), ocular distance (C), ocular surface area (D), body area (E), yolk sac edema area (F), and swimming bladder area (G) were measured at 5 dpf, using ImageJ software. Error bars represent the standard error. Statistical analyses employed one-way ANOVA followed by Tukey's post-test. "*" represents differences against control or "#" vehicle group. (*** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$; ### $p \leq 0.001$, ## $p \leq 0.01$, # $p \leq 0.05$). [Control: system water; Vehicle: 0.00004% of ethanol; M1: active ingredient (a.i.) methomyl – 0.5 mg/L; M2: 1 mg/L a.i.; M3: 2.2 mg/L a.i.; M4: 4.8 mg/L a.i.; M5: 10.6 mg/L a.i.; M6: 23.3 mg/L a.i.]

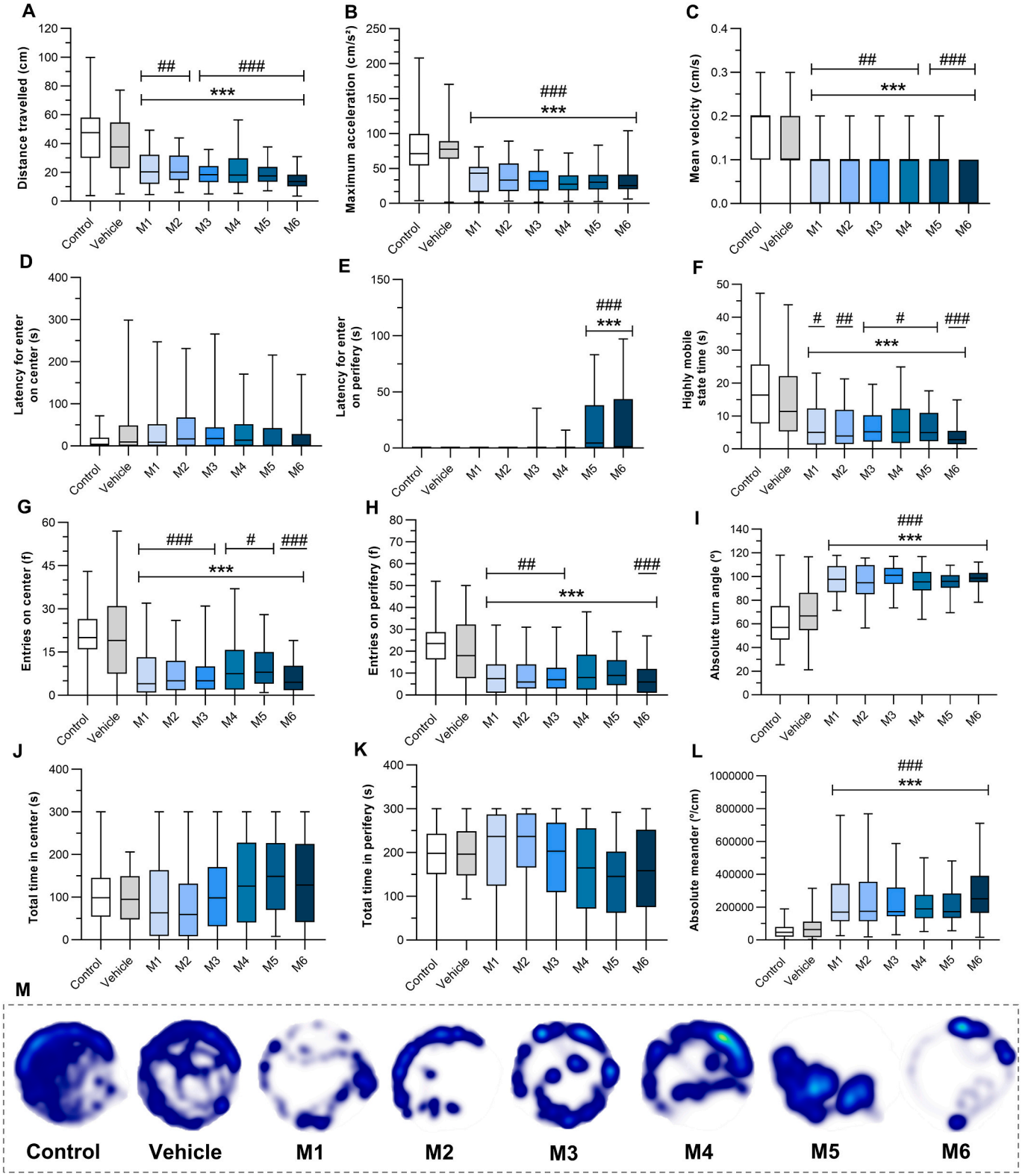

Fig. 5. Methomyl formulation effects on locomotor behavior of 6 dpf zebrafish larvae ($n \geq 46$/group of 4 independent experiments). Distance travelled (A), maximum acceleration (B), mean velocity (C), latency for enter on center (D), latency for enter on periphery (E), time in high-mobility state (F), entries on center (G), entries on periphery (H), absolute turn angle (I), total time in center (J), total time in periphery (K) and absolute meander (L) parameters were evaluated in 5-min recorded behavioral trials. The heatmap (M) represents average distance travelled for each group. The boxplots bars represent data range variation from minimum to maximum and median. Statistical analyses employed Kruskal-Wallis test followed by Dunn's post-test. "*" represents differences against control or "#" vehicle group. (*** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$; ### $p \leq 0.001$, ## $p \leq 0.01$, # $p \leq 0.05$). [Control: system water; Vehicle: 0.00004% of ethanol; M1: active ingredient (a.i.) methomyl – 0.5 mg/L; M2: 1 mg/L a.i.; M3: 2.2 mg/L a.i.; M4: 4.8 mg/L a.i.; M5: 10.6 mg/L a.i.; M6: 23.3 mg/L a.i.]

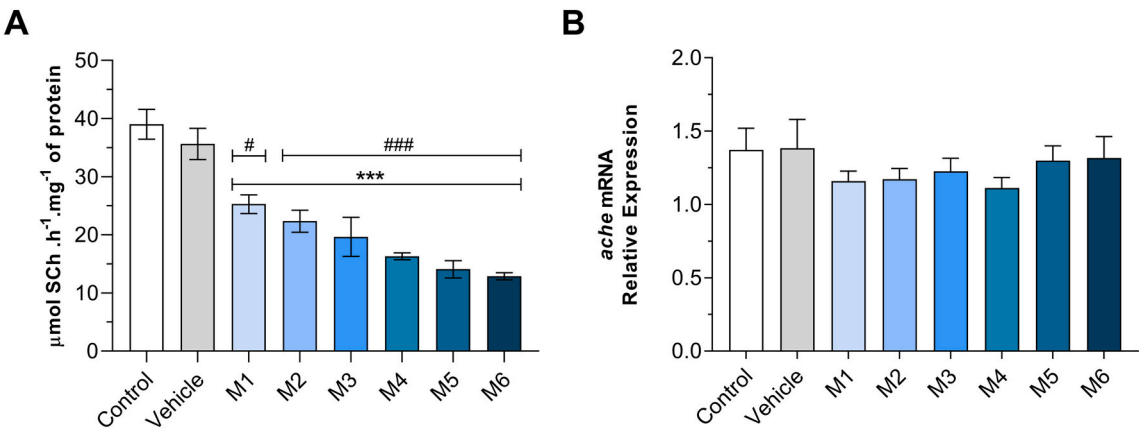

Fig. 6. Effects of zebrafish 6-day exposure on AChE. Results for AChE activity (A) (n = 10/sample) and gene expression (B) (n = 25/sample) at different concentrations of methomyl formulation. Statistical analyses employed one-way ANOVA followed by Tukey's post-test. Error bars represent the standard error. "*" represents differences against control or "#" vehicle group. (*** $p \leq 0.001$; $^{\#\#\#}$ $p \leq 0.001$, $^{\#}$ $p \leq 0.05$). [Control: system water; Vehicle: 0.00004% of ethanol; M1: active ingredient (a.i.) methomyl – 0.5 mg/L; M2: 1 mg/L a.i.; M3: 2.2 mg/L a.i.; M4: 4.8 mg/L a.i.; M5: 10.6 mg/L a.i.; M6: 23.3 mg/L a.i.]

Some studies showed an increase in STC frequency after neurotoxicants exposure, such as chlorpyrifos, an organophosphate AChE inhibitor (Ogungbemi et al., 2020). This was not the case here, where no significant differences were observed among all experimental groups (Fig. 3A).

Hatching is an important point in fish life cycle, and several physical and biochemical factors interfere in this process (Jin et al., 2009). Our data showed that most embryos had hatched by 3 dpf, which is the expected for zebrafish raised at 28.5 °C (Westerfield, 2000). However, methomyl formulation seemed to induce an anticipation in zebrafish hatching at 2 dpf, specially at higher concentrations (M4, M5 and M6 groups), when compared to both controls (Fig. 2B); which was also observed with pure methomyl exposure (Ahmad et al., 2020). Zhang et al. (2017) also had similar result evaluating glyphosate commercial formulation exposure. Higher concentrations of glyphosate increased hatching rate at 2 dpf. They hypothesized that glyphosate may decrease chorion surface tension, which could lead to rapid chorion degradation, affecting the hatching. Interestingly, while sublethal exposure presented a concentration-dependent increase in hatching anticipation, in FET test, performed with much higher concentrations, a significant reduction in hatching rate was observed, particularly in highest concentrations of methomyl formulation. At 4 dpf, almost all animals from control, vehicle, and lowest methomyl formulation concentration had hatched, against 40% and 5% hatching observed in higher concentrations (Fig. S1B). These results might suggest that at sublethal concentrations, methomyl formulation accelerates zebrafish chorion outbreak, but in very high concentrations, embryos show stronger signs of acute toxicity, likely with delays in morphological development, preventing hatching and ultimately, leading to death (Blahova et al., 2020).

Quantifiable growth parameters are an easily and useful tool, that may reflect altered molecular and cellular responses of an organism exposed to xenobiotics (Cook et al., 2005). Our results demonstrated that methomyl formulation affect zebrafish larvae morphology in terms of smaller body and eye size and lack of swimming bladder inflation (Fig. 4B, D, G). In addition, several animals exposed to higher concentrations of methomyl formulation (M4-M6) showed spinal cord deformations (Fig. S2). Mu et al. (2016) reported decreased larval body length and spinal column deformation after exposure to the fungicide difeconazole. They related these events to a possible interference in Insulin-like growth factor 1 (IGF-1) and Growth Hormone (GH), which are two important proteins for healthy growth and development and were under expressed in exposed groups. It was observed that exposed groups also showed alterations in swimming bladder; which is responsible for fish movement across the water column, by decrease or increase of their body density, helping in food search, predators escape, and swimming (Robertson et al., 2007). Similar to our work, Yue et al. (2015) evaluated zebrafish exposure to dioxin, a persistent and bioaccumulative hydrocarbon environmental contaminant. They observed altered swimming bladder inflation at 5 dpf and identified that this phenotype was only maintained if dioxin exposure occurred between 65 and 96 hpf. The authors speculate that this period might be more sensitive, as swimming bladder mesenchymal and mesothelial layers are still forming, and so, signal interference between these layers and the epithelial layer play an important role in its organization and growth. Vision has also a critical role in individual survival and organism populations maintenance, where vital activities significantly depend on visual system development and function, such as the search for food, avoiding predators, reproduction and selection of habitats (Chen, 2020). Our data showed that methomyl formulation induced a decrease in eyes size in all exposed groups (Fig. 4D), and M5 and M6 groups also presented a smaller distance between the eyes (Fig. 4B). Other studies have reported reduction in zebrafish larvae eye and head exposed to different pesticides (Cook et al., 2005; Liu et al., 2018) and correlated such changes with hindbrain segmentation signaling and brain development impairments (Liu et al., 2018). Underlying molecular mechanisms for methomyl formulation impacts in zebrafish morphology, however, are still unexplored.

AChE activity is used as a biomarker for assessing neurotoxicity in aquatic organisms (Zhu et al., 2020). As expected, AChE activity was decreased in larvae exposed to methomyl formulation, in a concentration-dependent fashion (Fig. 6A). Similarly, a study with the carbamate insecticide fenucab (Zhu et al., 2020) reported concentration-dependent reduction in AChE activity, mainly among highest concentrations. They suggested that changes in AChE activity affects developmental and locomotor behavior by direct dysfunction of the cholinergic nervous system. To test whether AChE activity reduction here observed had a transcriptional regulation contribution, *ache* gene expression was evaluated by RT-qPCR, and no significant difference was observed in exposed animals when compared to controls (Fig. 6B).

In recent years, locomotor behavior studies in zebrafish larval stages have significantly increased. Zebrafish behavior analysis is a simple and inexpensive way to discover changes in the nervous system caused by xenobiotics (Gerlai, 2020). Together, our behavior analyses showed a significant hypolocomotor activity of larvae exposed to methomyl formulation (Fig. 5). Corroborating our findings, reduced travelled distance has also been observed in 5 dpf larvae after pure methomyl exposure (Ahmad et al., 2020). Similarly, a study by Cheng et al. (2020) presented that larva exposed to fungicide famoxadone-cymoxanil displayed decreased distance travelled, mean velocity and movement time, and increased turn angle and meander, indicating larval abnormal

behaviors due to toxicity. Here, exposure to higher concentrations of methomyl formulation (M5 and M6) also showed increased latency to enter the arena peripheral zone and no differences in the latency for the larvae to enter the center (Fig. 5D), which most likely is attributed to the fact that larvae were place at the center of the arena, at the beginning of the experiment, and in these groups, had very limited locomotion during the experiment, as clearly illustrated in representative heatmaps (Fig. 5M). Methomyl formulation exposure also altered zebrafish swimming trajectory, as indicated by increased absolute turn angle (that measures how many times the animal changes direction in its trajectory in relation to the point of the body, between one sample and the next) (Fig. 5I) and increased absolute meander (which measures the change in movement direction in relation to distance moved) (Fig. 5L). Huang et al. (2021) also observed a hypolocomotor behavior of zebrafish larvae when exposed to the fungicide pyraclostrobin and associated it to cellular energy deficit. Furthermore, energy deficit might also cause developmental delay, which could contribute to both morphological and behavioral alterations observed, and ultimately, preventing larvae water column stability and impairing swimming (Huang et al., 2021; Yang et al., 2021). Nevertheless, considering AChE vital role for the functioning of nervous system and neuromuscular junctions of organisms, the concentration-dependent decrease in AChE activity must also be directly contributing to the hypolocomotor activity, corroborating with the neurotoxic effect attributed to carbamates (Zhu et al., 2020). Future studies, aiming to evaluate the molecular and biochemical mechanisms involved in such toxic effects, will certainly contribute to understand these results more deeply.

5. Conclusions

Sublethal exposure to xenobiotics can help to predict the impacts of a given substance, or mixture, in living organisms. To the best of our knowledge, this is the very first work to analyze methomyl formulation developmental toxicity, using a robust and broad set of physiological, morphological, and behavioral endpoints during both embryonic and larval zebrafish stages. Methomyl formulation alters survival and hatching rates, morphological, behavioral, and biochemical aspects of zebrafish embryo and larvae, altering development. Morphological analysis showed that methomyl formulation exposure impacted body length, eye size, distance between eyes, and swimming bladder inflation, which may be crucial to ensure fish development, and even survival. Such morphological impairments most likely reflected in locomotor activity and pattern changes observed in larval behavior. Furthermore, biochemical analysis showed expected decrease in AChE activity, validating methomyl formulation (neuro)toxic effect, in a concentration-dependent manner. Our data showed that it can trigger other undesirable and harmful effects in non-targeted organism's health and further studies will be imperative to understand molecular and biochemical mechanisms (beyond cholinergic system) underlying the developmental changes observed. Furthermore, considering that pesticides, as complex mixtures with 'co-formulants', might reach non-target habitats and biota, to investigate the exposure effect to such mixtures has significant ecotoxicological relevance.

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. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ntt.2021.107058.

References

Ahmad, F., Ali, S., Richardson, M.K., 2020. Effect of pesticides and metals on zebrafish embryo development and larval locomotor activity. bioRxiv. https://www.biorxiv.org/content/10.1101/2020.10.05.326066v1.full, 2020. (Accessed 21 December 2021).

Bachour, R.L., Golovko, O., Kellner, M., Pohl, J., 2020. Behavioral effects of citalopram, tramadol, and binary mixture in zebrafish (*Danio rerio*) larvae. Chemosphere 238, 124587. https://doi.org/10.1016/j.chemosphere.2019.124587.

Beekhuijzen, M., de Koning, C., Flores-Guillén, M.E., de Vries-Buitenweg, S., Tobor-Kaplon, M., van de Waart, B., Emmen, H., 2015. From cutting edge to guideline: a first step in harmonization of the zebrafish embryotoxicity test (ZET) by describing the most optimal test conditions and morphology scoring system. Reprod. Toxicol. 56, 64–76. https://doi.org/10.1016/j.reprotox.2015.06.050.

Blahova, J., Cocilovo, C., Plhalova, L., Svobodova, Z., Faggio, C., 2020. Embryotoxicity of atrazine and its degradation products to early life stages of zebrafish (*Danio rerio*). Environ. Toxicol. Pharmacol. 77, 103370 https://doi.org/10.1016/j.etap.2020.103370.

Bournele, D., Beis, D., 2016. Zebrafish models of cardiovascular disease. Heart Fail. Rev. 21, 803–813. https://doi.org/10.1007/s10741-016-9579-y.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55, 611–622. https://doi.org/10.1373/clinchem.2008.112797.

Chen, L., 2020. Visual system: an understudied target of aquatic toxicology. Aquat. Toxicol. 225, 105542 https://doi.org/10.1016/j.aquatox.2020.105542.

Cheng, B., Zhang, H., Hu, J., Peng, Y., Yang, J., Liao, X., Liu, F., Guo, J., Hu, C., Lu, H., 2020. The immunotoxicity and neurobehavioral toxicity of zebrafish induced by famoxadone-cymoxanil. Chemosphere 247, 125870. https://doi.org/10.1016/j.chemosphere.2020.125870.

Cook, L.W., Paradise, C.J., Lom, B., 2005. The pesticide malathion reduces survival and growth in developing zebrafish. Environ. Toxicol. Chem. 24, 1745–1750. https://doi.org/10.1897/04-331R.1.

Costa, K.M., Pereira, T.C.B., Valente, C.A., Pissinate, K., Soares, J.C., Cruz, F.F., Corte, T. W.F., Machado, P., Basso, N.R.S., Bogo, M.R., 2019. Adverse effects of p-TSA-doped polypyrrole particulate exposure during zebrafish (*Danio rerio*) development. Colloids Surf. B: Biointerfaces 177, 58–67. https://doi.org/10.1016/j.colsurfb.2019.01.041.

Ellman, G.L., Courtney, K.D., Andres, V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88–95. https://doi.org/10.1016/0006-2952(61)90145-9.

Farias Filho, L. de H., da Silva, H.E.B., Vital, M.J.S., 2013. Investigação de agrotóxicos carbamatos em águas superficiais bruta e tratada do Rio Branco em Roraima. Programa Pós-Graduação em Recur. Nat. Universidade Federal de Roraima, Boa Vista, Roraima. http://repositorio.ufrr.br:8080/jspui/handle/prefix/327. (Accessed 21 December 2021).

Gerlai, R.T., 2020. The design of behavioral screening in zebrafish. Behav. Neural Genet. Zebrafish 513–526. https://doi.org/10.1016/b978-0-12-817528-6.00029-2.

Glaberman, S., Padilla, S., Barron, M.G., 2017. Evaluating the zebrafish embryo toxicity test for pesticide hazard screening. Environ. Toxicol. Chem. 36, 1221–1226. https://doi.org/10.1002/etc.3641.

He, D., Han, G., Zhang, X., Sun, J., Xu, Y., Jin, Q., Gao, Q., 2021. Oxidative stress induced by methomyl exposure reduces the quality of early embryo development in mice. Zygote 1–8. https://doi.org/10.1017/S0967199421000277.

Huang, T., Souders, 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.

Jin, M., Zhang, X., Wang, L., Huang, C., Zhang, Y., Zhao, M., 2009. Developmental toxicity of bifenthrin in embryo-larval stages of zebrafish. Aquat. Toxicol. 95, 347–354. https://doi.org/10.1016/j.aquatox.2009.10.003.

Kahl, G., Ingwersen, J., Nutniyom, P., Totrakool, S., Pansombat, K., Thavornyutikarn, P., Streck, T., 2007. Micro-trench experiments on interflow and lateral pesticide transport in a sloped soil in northern Thailand. J. Environ. Qual. 36, 1205–1216. https://doi.org/10.2134/jeq2006.0241.

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, 253–310. https://doi.org/10.1002/aja.1002030302.

Klüver, N., König, M., Ortmann, J., Massei, R., Paschke, A., Kühne, R., Scholz, S., 2015. Fish embryo toxicity test: identification of compounds with weak toxicity and analysis of behavioral effects to improve prediction of acute toxicity for neurotoxic compounds. Environ. Sci. Technol. 49, 7002–7011. https://doi.org/10.1021/acs.est.5b01910.

Lammer, E., Carr, G.J., Wendler, K., Rawlings, J.M., Belanger, S.E., Braunbeck, T., 2009. Is the fish embryo toxicity test (FET) with the zebrafish (*Danio rerio*) a potential alternative for the fish acute toxicity test? Comp. Biochem. Physiol. C Toxicol. Pharmacol. 149, 196–209. https://doi.org/10.1016/j.cbpc.2008.11.006.

Lanzarin, G.A.B., Félix, L.M., Santos, D., Venâncio, C.A.S., Monteiro, S.M., 2019. Dose-dependent effects of a glyphosate commercial formulation – Roundup ® UltraMax – on the early zebrafish embryogenesis. Chemosphere 223, 514–522. https://doi.org/10.1016/j.chemosphere.2019.02.071.

Liu, X.Y., Zhang, Q.P., Li, S.B., Mi, P., Chen, D.Y., Zhao, X., Feng, X.Z., 2018. Developmental toxicity and neurotoxicity of synthetic organic insecticides in zebrafish (*Danio rerio*): a comparative study of deltamethrin, acephate, and thiamethoxam. Chemosphere 199, 16–25. https://doi.org/10.1016/j.chemosphere.2018.01.176.

Material Safety Data Sheet (MSDS) BrilhanteBR®, 2013. OuroFino Química S.A.. In: Uberaba. Minas Gerais, Brazil.

Materu, S.F., Heise, S., Urban, B., 2021. Seasonal and spatial detection of pesticide residues under various weather conditions of agricultural areas of the Kilombero Valley Ramsar Site, Tanzania. Front. Environ. Sci. 9 https://doi.org/10.3389/fenvs.2021.599814.

Meng, S.L., Chen, J.Z., Hu, G.D., Song, C., Fan, L.M., Qiu, L.P., Xu, P., 2014. Effects of chronic exposure of methomyl on the antioxidant system in liver of Nile tilapia (*Oreochromis niloticus*). Ecotoxicol. Environ. Saf. 101, 1–6. https://doi.org/10.1016/j.ecoenv.2013.10.020.

Meng, S.L., Qiu, L.P., Hu, G.D., Fan, L.M., Song, C., Zheng, Y., Wu, W., Qu, J.H., Li, D.D., Chen, J.Z., Xu, P., 2017. Effect of methomyl on sex steroid hormone and vitellogenin levels in serum of male tilapia (*Oreochromis niloticus*) and recovery pattern. Environ. Toxicol. 32, 1869–1877. https://doi.org/10.1002/tox.22409.

Meng, S.L., Liu, T., Chen, X., Qiu, L.P., Hu, G.D., Song, C., Fan, L.M., Zheng, Y., Chen, J. Z., Xu, P., 2019. Effect of chronic exposure to Methomyl on tissue damage and apoptosis in testis of Tilapia (*Oreochromis niloticus*) and recovery pattern. Bull. Environ. Contam. Toxicol. 102, 371–376. https://doi.org/10.1007/s00128-018-2522-2.

Mohamed, A.A.R., Rahman, A.N.A., Salem, G.A., El Deib, M.M., Nassan, M.A., Rhouma, N.R., Khater, S.I., 2021. The antioxidant role of a taurine-enriched diet in combating the immunotoxic and inflammatory effects of pyrethroids and/or carbamates in oreochromis niloticus. Animals 11. https://doi.org/10.3390/ani11051318.

Mojiri, A., Zhou, J.L., Robinson, B., Ohashi, A., Ozaki, N., Kindaichi, T., Farraji, H., Vakili, M., 2020. Pesticides in aquatic environments and their removal by adsorption methods. Chemosphere 253, 126646. https://doi.org/10.1016/j.chemosphere.2020.126646.

Mu, X., Chai, T., Wang, K., Zhu, L., Huang, Y., Shen, G., Li, Y., Li, X., Wang, C., 2016. The developmental effect of difenoconazole on zebrafish embryos: a mechanism research. Environ. Pollut. 212, 18–26. https://doi.org/10.1016/j.envpol.2016.01.035.

Nagy, K., Duca, R.C., Lovas, S., Creta, M., Scheepers, P.T.J., Godderis, L., Ádám, B., 2020. Systematic review of comparative studies assessing the toxicity of pesticide active ingredients and their product formulations. Environ. Res. 181, 108926 https://doi.org/10.1016/j.envres.2019.108926.

OECD, 2013. Test No. 236: Fish Embryo Acute Toxicity (FET) test. OECD Guidel. Test. Chem. Sect. 2 https://doi.org/10.1787/9789264203709-en. OECD Publ. 1–22.

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 https://doi.org/10.1186/s12302-019-0270-5.

Ogungbemi, A.O., Teixido, E., Massei, R., Scholz, S., Küster, E., 2020. Optimization of the spontaneous tail coiling test for fast assessment of neurotoxic effects in the zebrafish embryo using an automated workflow in KNIME®. Neurotoxicol. Teratol. 81 https://doi.org/10.1016/j.ntt.2020.106918.

Pathak, N.H., Barresi, M.J.F., 2019. Zebrafish as a model to understand vertebrate development. In: The Zebrafish in Biomedical Research: Biology, Husbandry, Diseases, and Research Applications. Elsevier. https://doi.org/10.1016/B978-0-12-812431-4.00045-2.

Pereira, V.M., Bortolotto, J.W., Kist, L.W., de Azevedo, M.B., Fritsch, R.S., Oliveira, R. da L., Pereira, T.C.B., Bonan, C.D., Vianna, M.R., Bogo, M.R., 2012. Endosulfan exposure inhibits brain AChE activity and impairs swimming performance in adult zebrafish (*Danio rerio*). Neurotoxicology 33, 469–475. https://doi.org/10.1016/j.neuro.2012.03.005.

Pereira, T.C.B., Batista dos Santos, K., Lautert-Dutra, W., de Souza Teodoro, L., de Almeida, V.O., Weiler, J., Homrich Schneider, I.A., Reis Bogo, M., 2020. Acid mine drainage (AMD) treatment by neutralization: evaluation of physical-chemical performance and ecotoxicological effects on zebrafish (*Danio rerio*) development. Chemosphere 253, 1–9. https://doi.org/10.1016/j.chemosphere.2020.126665.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Res. 29, e45. https://doi.org/10.1093/nar/29.9.e45.

Robertson, G.N., McGee, C.A.S., Dumbarton, T.C., Croll, R.P., Smith, F.M., 2007. Development of the swimbladder and its innervation in the zebrafish, *Danio rerio*. J. Morphol. 268, 967–985. https://doi.org/10.1002/jmor.10558.

Rojo Baio, F.H., Antuniassi, U.R., Castilho, B.R., Teodoro, P.E., da Silva, E.E., 2019. Factors affecting aerial spray drift in the Brazilian Cerrado. PLoS One 14. https://doi.org/10.1371/journal.pone.0212289.

Rübensam, G., Barreto, F., Hoff, R.B., Pizzolato, T.M., 2013. Determination of avermectin and milbemycin residues in bovine muscle by liquid chromatography-tandem mass spectrometry and fluorescence detection using solvent extraction and low temperature cleanup. Food Control 29, 55–60. https://doi.org/10.1016/j.foodcont.2012.05.075.

Seleem, A.A., 2019. Teratogenicity and neurotoxicity effects induced by methomyl insecticide on the developmental stages of *Bufo arabicus*. Neurotoxicol. Teratol. 72, 1–9. https://doi.org/10.1016/j.ntt.2018.12.002.

Stelzer, J.A.A., Rosin, C.K., Bauer, L.H., Hartmann, M., Pulgati, F.H., Arenzon, A., 2018. Is fish embryo test (FET) according to OECD 236 sensible enough for delivering quality data for effluent risk assessment? Environ. Toxicol. Chem. 37, 2925–2932. https://doi.org/10.1002/etc.4215.

Tang, R., Dodd, A., Lai, D., McNabb, W.C., Love, D.R., 2007. Validation of zebrafish (*Danio rerio*) reference genes for quantitative real-time RT-PCR normalization. Acta Biochim. Biophys. Sin. Shanghai 39, 384–390. https://doi.org/10.1111/j.1745-7270.2007.00283.x.

Trachantong, W., Saenphet, S., Saenphet, K., Chaiyapo, M., 2016. Lethal and sublethal effects of a methomyl-based insecticide in *Hoplobatrachus rugulosus*. J. Toxicol. Pathol. 30, 15–24. https://doi.org/10.1293/tox.2016-0039.

Van Scoy, A.R., Yue, M., Deng, X., Tjeerdema, R.S., 2012. Environmental fate and toxicology of methomyl. In: de Voogt, P. (Ed.), Reviews of Environmental Contamination and Toxicology, Reviews of Environmental Contamination and Toxicology. Springer International Publishing, Cham, pp. 93–109. https://doi.org/10.1007/978-1-4614-4717-7_3.

Wang, T., Zhong, M., Lu, M., Xu, D., Xue, Y., Huang, J., Blaney, L., Yu, G., 2021. Occurrence, spatiotemporal distribution, and risk assessment of current-use pesticides in surface water: a case study near Taihu Lake, China. Sci. Total Environ. 782, 146826 https://doi.org/10.1016/j.scitotenv.2021.146826.

Westerfield, M., 2000. The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio rerio). University of Oregon Press, Eugene. https://zfin.org/zf_info/zfbook/zfbk.html.

Yang, L., Ivantsova, E., Souders, C.L., Martyniuk, C.J., 2021. The agrochemical S-metolachlor disrupts molecular mediators and morphology of the swim bladder: implications for locomotor activity in zebrafish (*Danio rerio*). Ecotoxicol. Environ. Saf. 208, 111641 https://doi.org/10.1016/j.ecoenv.2020.111641.

Yoon, D., Kim, Siwon, Lee, M., Yoon, C., Kim, Suhkmann, 2016. 1H-NMR-based metabolomic study on toxicity of methomyl and methidathion in fish. J. Environ. Sci. Heal. – Part B Pestic. Food Contam. Agric. Wastes 51, 824–831. https://doi.org/10.1080/03601234.2016.1208460.

Yue, M.S., Peterson, R.E., Heideman, W., 2015. Dioxin inhibition of swim bladder development in zebrafish: is it secondary to heart failure? Aquat. Toxicol. 162, 10–17. https://doi.org/10.1016/j.aquatox.2015.02.016.

Zhang, S., Xu, J., Kuang, X., Li, S., Li, X., Chen, D., Zhao, X., Feng, X., 2017. Biological impacts of glyphosate on morphology, embryo biomechanics and larval behavior in zebrafish (*Danio rerio*). Chemosphere 181, 270–280. https://doi.org/10.1016/j.chemosphere.2017.04.094.

Zhu, X.Y., Wu, Y.Y., Xia, B., Dai, M.Z., Huang, Y.F., Yang, H., Li, C.Q., Li, P., 2020. Fenobucarb-induced developmental neurotoxicity and mechanisms in zebrafish. Neurotoxicology 79, 11–19. https://doi.org/10.1016/j.neuro.2020.03.013.

Zindler, F., Beedgen, F., Braunbeck, T., 2019. Time-course of coiling activity in zebrafish (*Danio rerio*) embryos exposed to ethanol as an endpoint for developmental neurotoxicity (DNT) – hidden potential and underestimated challenges. Chemosphere 235, 12–20. https://doi.org/10.1016/j.chemosphere.2019.06.154.