Environmentally relevant mixture of S-metolachlor and its two metabolites affects thyroid metabolism in zebrafish embryos

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HIGHLIGHTS (3-5, max 85 characters per highlight**)**

- Herbicide toxicity analysis on embryo-larval stages of zebrafish
- Reduced spontaneous movements in embryos exposed to S-metolachlor alone and in mixture
- S-metolachlor caused no effect on molecular level, unlike its metabolites
- Low concentration of pesticide mixture overexpressed thyroid genes *dio2*, *thra*, *thrb*
- Larvae locomotion, heart rate not affected; minor developmental malformations found

KEYWORDS (6 max)

- Zebrafish embryo, embryotoxicity, pesticide mixture, environmental concentration, sublethal effect, metabolite

Abstract **<400 words**

Herbicides and their metabolites are found in water bodies where they may harm non-target organisms. Their effects at environmentally relevant concentrations are often unclear, especially concerning mixture of pesticides. This study thus investigated the impacts of one of the most used herbicides: S-metolachlor and its two metabolites, metolachlor oxanilic acid (MOA) and metolachlor ethanesulfonic acid (MESA) on the development of zebrafish embryos (*Danio rerio)*. Embryos were exposed to the pure substances and their environmentally relevant mixture until 120 hpf (hours post-fertilization). The focus was set on sublethal endpoints such as malformations, hatching success, length of fish, spontaneous movements, heart rate and locomotion of zebrafish larvae. Moreover, gene expression levels of eight genes linked to the thyroid system disruption, oxidative stress defense, mitochondrial metabolism, regulation of the cell cycle and retinoic acid signaling pathway were analyzed.

Exposure to the S-metolachlor (1 µg/L) and to the pesticide mixture (1 µg/L of each substance) significantly reduced spontaneous tail movements of 21 hpf embryos. Few rare developmental malformations were observed, but only in larvae exposed to more than 100 µg/L of pure substances (craniofacial deformation, non-inflated gas bladder, yolk sac malabsorption) and to 30 µg/L of each substance in the pesticide mixture (spine deformation). However, no effect on hatching success, length of larvae, heart rate or larvae locomotion were found. Strong responses were detected at the molecular level, especially on the *p53* gene regulating the cell cycle, caused by the pesticide mixture (1 µg/L of each substance), MESA (30 µg/L) and MOA (100 µg/L), as well as on the *cyp26a1* – gene encoding for cytochrome P450 after exposure to the pesticide mixture (1 µg/L of each substance). Genes implicated in the thyroid system disruption (*dio2*, *thra*, *thrb*) were all overexpressed following exposure to the environmentally relevant concentrations of the pesticide mixture (1 µg/L of each substance) and to the metabolite MESA (1 µg/L). In conclusion, the zebrafish thyroid system disruption was revealed not only by the overexpressed genes, but also by some of the developmental malformations considered as markers of this type of disruption (mainly gas bladder and yolk sac abnormalities) and spontaneous tail movements linked to the T4 hormone influencing its neurodevelopment. Thus, the thyroid system disruption represents a likely hypothesis behind the effects caused by the low environmental concentrations of S-metolachlor, its two metabolites and their mixture.

Introduction

S-metolachlor is one of the most commonly used pesticides in the world (Atwood and Paisley-Jones, 2017). This selective pre-emergent herbicide from the chloroacetanilides family was first registered in 1997 (Heydens et al., 2010). It is an S-enantiomer of 2-chloro-N-(2-ethyl-6-methylphenyl)-N-(1-methoxypropan-2-yl)acetamide. It replaced the racemic metolachlor mixture due to its higher herbicidal activity (Müller et al., 2001; Thomas Poiger, 2002), thus reducing the quantity of pesticide needed by 40% (Blaser and Spindler, 1997). S-metolachlor inhibits the biosynthesis of very-long-chain fatty acids (Götz and Böger, 2004) and interferes with gibberellin synthesis enzymes (Rose et al., 2016), thereby inhibiting the growth of target plants such as grass weeds in corn fields (Heydens et al., 2010). However, S-metolachlor can easily contaminate surface water by agricultural runoff (Zemolin et al., 2014) and it is thus often detected in surface or coastal waters (Accinelli et al., 2002; De Liguoro et al., 2014; Fauvelle et al., 2018; Glinski et al., 2018; Kapsi et al., 2019; Meffe and de Bustamante, 2014; Ryberg et al., 2014). In soil, S-metolachlor is microbially degraded to two principal metabolites (Zemolin et al., 2014): metolachlor oxanilic acid (MOA) and metolachlor ethanesulfonic acid (MESA), which are mobile and persistent in the environment (Sidoli et al., 2016). As a result, these degradation products are often detected in higher concentrations than the parent compound itself in water samples (Elliott and VanderMeulen, 2017; Hladik et al., 2005). However, there is a general lack of information about the toxicity of individual pesticide metabolites and their mixtures occurring in the environment. Only recently, some studies highlighted the importance of aquatic toxicity of environmentally relevant pesticide mixtures (Gustavsson et al., 2017; Tian et al., 2018). Furthermore, the importance of evaluating mixture toxicity is emphasized by the European Chemicals Agency (2014).

The contamination of aquatic environments by herbicides may adversely affect non-target organisms such as fish. Fish are among the most sensitive organisms in aquatic ecosystems and are used as standard testing species in the hazard assessment of chemicals and their mixtures (Hayes et al., 2019). To the best of our knowledge, studies on the effects of S-metolachlor or its metabolites on fish are scarce. Most use high concentrations (mg/L) to obtain LC_{50} or EC_{50} values, and these are far from being environmentally relevant (Dobšíková et al., 2011; Munn et al., 2006; Quintaneiro et al., 2017). Unlike these studies, Jin et al. (2011) observed that lower concentrations (µg/L) of metolachlor (mixture of both R- and S-enantiomers) altered the thyroid system in Japanese medaka. Thyroid system disruption was

also caused by low concentrations of acetochlor (µg/L), another member of the chloroacetanilide family (Yang et al., 2015). In other non-target aquatic organisms, S-metolachlor and its metabolites MOA and MESA induced developmental abnormalities in Pacific oyster larvae and sperm at low environmental concentrations (Mai et al., 2014). Moreover, the deleterious effects of environmental concentrations of S-metolachlor and MOA on crayfish were also reported (Velisek et al., 2018a, 2018b). Although several studies pointed out the toxicity of high concentrations of these substances on non-target aquatic organisms (Gutiérrez et al., 2019; Munn et al., 2006; Quintaneiro et al., 2018, 2017), comprehensive information on sublethal effects caused by environmental concentrations is still missing.

Early life stage zebrafish (*Danio rerio*) are widely used as sensitive and reliable alternative toxicity models with multiple advantages including high-throughput screening, embryo transparency, quick breeding and development, a sequenced genome (Love et al., 2004) and are in compliance with the 3Rs principle (Russell et al., 1959). Zebrafish development is highly influenced by its thyroid metabolism (Jarque and Piña, 2014). Moreover, the early development of the zebrafish thyroid system is comparable to its development in humans (Alt et al., 2006), complete with a similar hypothalamus–pituitary–thyroid (HPT) axis (Porazzi et al., 2009). This similarity makes the zebrafish a suitable model for evaluating thyroid gland disruption (Raldúa et al., 2012). In a recent review, Spaan et al. (2018) write that the complexity of thyroid disruption assessment calls for the evaluation of multiple endpoints. Morphological effects, transcription level of several genes interfering with the HPT axis, hatching, heart rate, behavior and swim bladder inflation were cited as the most common.

Although the toxicity of high concentrations of S-metolachlor on non-target aquatic organisms has been previously studied, the effects posed by low environmentally relevant concentrations of this compound and its metabolites have yet to be examined. The present study investigated the sublethal toxicity of environmentally relevant concentrations of S-metolachlor, its two metabolites MOA and MESA and their mixture on embryo-larval stages of *Danio rerio*. We evaluated apical endpoints of zebrafish embryos, conducted a neurobehavioral study measuring swimming activity to assess potential neurotoxicity and assessed biochemical responses by measuring the transcription changes of selected genes. This integrative approach allowed for detailed examination of sublethal toxicity, including potential thyroid metabolism disruption.

2. Materials and methods

2.1 Chemicals

S-metolachlor (S-M, CAS 87392-12-9, Pestanal, purity ≥ 98.0%), metolachlor oxanilic acid (MOA, CAS 152019-73-3, Pestanal, purity ≥ 98.0%), metolachlor ethanesulfonic acid (MESA, CAS 947601-85-6, Pestanal, purity ≥ 95.0%) were purchased from Sigma-Aldrich. Their stock solutions (50 mg/L) were prepared in Milli-Q water and were stored at 4 °C. The hormone triiodothyronine (T3, dissolved in methanol, CAS 6893-02-3, purity ≥ 95%, purchased from Sigma-Aldrich, used immediately) was used as positive control in the gene modulation experiments. Ethanol absolute (CAS 64-17-5, purity ≥ 99.8%, purchased from VWR Chemicals) was used as positive control in the spontaneous movement analysis and for the PCR analysis. ISO medium (ISO, 1996) ($CaCl_2$*$2H_20$ (294 mg/L), $MgSO_4$*$7H_20$ (123.3 mg/L), $NaHCO_3$ (63 mg/L), KCl (5.5 mg/L) in Milli-Q water) was used to prepare the final dilutions for the tests. RNA*later* ® (Ambion) was purchased from Sigma-Aldrich.

2.2 Test organism

Zebrafish (*Danio rerio*) embryos were collected from an unspecified wild type zebrafish strain maintained at RECETOX, Masaryk University (Czech Republic). Adult fish are held in aquariums with tap water at 26 ± 1 °C, at 14:10h light:dark photoperiod and fed 3 times a day with commercially available feeds (of which at least once with live brine shrimp *Artemia salina*). About 10-14 hours before the experimentation a box for collecting the embryos is placed in the aquarium and the spawning is induced in the morning by turning the light on. The eggs are collected 30 minutes after, rinsed and transferred to the ISO medium. Only fertilized and normally developing eggs are picked for the tests using a stereomicroscope (OLYMPUS, Japan) and exposed at 3-4 hpf (hours post fertilization). The embryos were then maintained at 26 °C, at 14:10h light:dark photoperiod till 120 hpf without feeding or renewal of test solutions.

2.3 FET (Fish embryo toxicity) test

The experiments were conducted in accordance with the fish early-life stage toxicity guideline (OECD, 2013). Selected eggs were exposed to the chosen herbicide S-metolachlor and its two metabolites MOA and MESA. The experiments were done with individual compounds and then with the mixture of the three. Chosen concentrations are based on environmental

concentrations detected in the rivers in the Czech Republic and in the Arcachon Bay in France during 2010-2014 (Table 1).

Table 1

Maximum concentrations of metolachlor and its metabolites MOA and MESA detected in Czech rivers (CHMU (Czech Hydrometeorological Institute), 2018) and Arcachon Bay in France (source) during 2010-2014. Number of samples: N=703 values for every substance in Arcachon Bay; N=13 175, 7 503 and 7 502 for metolachlor, MOA and MESA respectively in surface water)

	Metolachlor	MOA	MESA	Location
Maximum concentration (ng/L)	5800	4200	4200	Surface water (CZ)
	1696	1609	1059	Arcachon Bay (FR)
% of values > 100 ng/L	2.5%	4.1%	19.1%	Surface water (CZ)
	36.0%	6.1%	36.6%	Arcachon Bay (FR)

The lowest used test concentration (1 µg/L) reflects the actual environmental concentration, and three higher (non-lethal) concentrations were also tested (30, 100 and 300 µg/L). Corresponding concentrations in nmol/L are showed in Supplementary Table S2. The mixture exposure included two concentration levels: 1 µg/L of each substance and 30 µg/L of each substance. ISO medium was used as negative control. The tests were conducted in glass crystallization dishes with transparent lids containing 20 ml of media and 20 embryos in three replicates. Every test was repeated independently three times with eggs from different spawning. Mortality, hatching success and malformations were controlled daily using a stereomicroscope. Eventual dead embryos/larvae were immediately removed from dishes to prevent contamination. Length of larvae (without the caudal fin) was measured on the last, fifth, day of the experiment on photos of larvae mounted in mixture of cellulose and anesthetics MS-222. Fifteen fish per replicate in 3 independent tests were measured. Photos were analyzed using the software QuickPhoto Micro 2.3.

"Morphological score" endpoint was assessed in order to evaluate global state of individual fish using slightly modified approach as previously published (Panzica-Kelly et al., 2010). Every fish embryo is classified using 0-3 point scores (description is in Supplementary Figure S1).

2.4 Light: dark locomotor test

Embryos for the locomotor activity analysis were handled as described above (2.3). The evening before measurements (to allow for acclimation), larvae were transferred to transparent 96-well microplates (Gama, Czech Republic), one larva per well. The distribution was random, 32 fish per exposure condition were used. Only morphologically normal, i.e.

non-malformed embryos were used. The experimentations were performed during the 5 dpf between 9 am and 16 pm. Prior to the experiment, the microplates were kept for 1 hour next to the ZEBRABOX (Viewpoint Life Sciences) at 26 °C for acclimation after the transport. Measurement done in the ZEBRABOX consisted of four 15 minutes alternating cycles (white light, i.e. 100% stimulus / dark, i.e. no stimulus / white light / dark). Total distances swam (mm) by individual fish were measured with integration period of 60 seconds and processed in real time using the software Zebralab (Viewpoint Life Sciences). The threshold between "inactive" fish and "active" fish was set on 0.3 cm/sec. The sensitivity threshold of ZEBRABOX was set on 120.

2.5 Spontaneous movement and heartbeat

Embryos were handled as described above (2.2) and exposed to concentrations of 1 and 100 µg/L of substances alone and to the mixtures at two concentration levels (1 and 30 µg/L of each substance in the mixture). They were maintained at 26 °C until 21 hpf when randomized capture of videos was realized till 23 hpf. Crystallization dish containing 20 embryos (= one replicate) was acclimatized under the stereomicroscope for 30 sec and then 2 min video was captured. Positive control (1% v/v ethanol) was used to enhance the spontaneous movements (tail coils). Videos were then analyzed by DanioScope software (Noldus, Netherlands) and reported as spontaneous movements per minute.

The same embryos were used for the heart rate activity measurement during the 3 dpf starting at 72 hpf. Hatched larvae were first put in solution of 2.5% methylcellulose containing 50 mg/L of anesthetics MS-222 and placed in Petri dish. Larvae were acclimatized for 10 seconds under the stereomicroscope and 20 second videos were captured. Videos were analyzed using the DanioScope software and reported as beat per minute.

2.6 Zebrafish exposures for analysis of gene expressions

Embryos were handled as described above (2.3) and exposed to 1, 30 and 100 µg/L of pesticides assessed alone and two concentrations of the mixture of the three pesticides (1 and 30 µg/L, respectively of each compound). Hormone T3 (triiodothyronine) was used as positive control to assess the expression of thyroid-related genes. Dead or heavily malformed embryos/larvae were removed daily. Whole larvae were collected at 120 hpf into pools of 20 larvae per Eppendorf tube containing 500 µl RNA*later* ® and stored at -20 °C upon RNA extraction.

2.6.1 RNA isolation

The total RNAs were extracted using the SV Total RNA Isolation System Kit (Promega). Whole larvae were homogenized in 500 µl of RNA Lysis Buffer using glass beads (Sigma) in the FastPrep®-24 (M.P. Biomedicals, USA). Lysed samples were collected, centrifuged and mixed with 450 µl of 75% v/v ethanol and transferred on the spin column. Handling with spin columns was performed according to the manufacturer's instructions. Before the DNase incubation step, an additional centrifuge step was added (13500 rpm, 2 minutes) to dry the column. All RNA samples were treated with DNase I mixture (40 µl of Yellow core buffer, 5 µl of $MnCl_2$ and 5 µl of DNase I) for 15 minutes in water bath at 37 °C. The rest of the isolation was performed according to the manufacturer's instructions and purified RNAs were collected in 50 µl of Nuclease-Free water. The concentration and purity of collected RNA samples were verified spectrophotometrically at 260/280 and 260/230 nm wavelength with Nanodrop™. Purity of all the samples was between 2.0-2.1 for the ratio 260/280 and 1.8-2.2 for the ratio 260/230.

2.6.2 Reverse transcription

Reverse transcription phase was performed with GoScript™ Reverse Transcription System kit (Promega) according to the manufacturer's instructions using TProfessional Thermocycler (Biometra, Analytik Jena). Purified RNA (1 µg) was reversely transcribed in order to get final volume of 20 µl of cDNA, and it was stocked at -20 °C upon qPCR analysis.

2.6.3 Quantitative PCR

8 genes were selected to evaluate the effects of chosen pesticides and their mixture on the thyroid hormone system, mitochondrial metabolism, regulation of the cell cycle and oxidative stress defense. Three reference zebrafish genes were used in the analysis (*β-actin*, *ef1a* and *rpl13*). Functioning and references of used primers are shown in Table 2. Sequences, efficiencies, and accession numbers are presented in Supplementary Table S1. Primers were purchased from Elisabeth Pharmacon (Czech Republic).

QPCR was carried out with Brillant III Ultra-fast SYBR Green QPCR Master Mix kit (Stratagene-Agilent) on the LightCycler® 480 (Roche). Each qPCR mix (20 µl) contained 5 µl of cDNA (10 times diluted), 2 µl of reverse and forward primer (2 µM) and 10 µl of SYBR from the kit and 3 µl of nuclease free water.

LightCycler was set according to the instructions of the SYBR kit manufacturer (Stratagene-Agilent). Briefly, the pre-incubation lasted 3 min at 95 °C, then the amplification consisted of

45 cycles with 1 cycle at 95 °C for 5 sec and 60 °C for 10 sec. The melting curve continued at 95 °C for 5 sec and at 65 °C for 1 min. Finally, the cooling step was performed at 40 °C for 30 sec. Melting curves of every reaction were analyzed to assess reaction specificity. The data were normalized to the mean of the Ct values for three reference genes, *β-act*, *elf1a* and *rpl13* and analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Results are shown as fold changes of exposed to control group.

Table 2
Primers used for qPCR analysis in zebrafish (*Danio rerio*) and their references.

Gene	Function	References
β-act	Reference gene	Dedeh *et al.*, 2015
ef1a	Reference gene	Gentès *et al.*, 2015
rpl13	Reference gene	Gentès *et al.*, 2015
12S	Mitochondrial metabolism	Arini *et al.*, 2015
p53	Regulation of the cell cycle/apoptose	Gentès *et al.*, 2015
cat	Oxidative stress defense	Lerebours *et al.*, 2009
sod1	Oxidative stress defense	Gentès *et al.*, 2015
cyp26a1	RA (retinoic acid) signaling pathway	Oliveira *et al.*, 2013
dio2	Thyroid hormone system	Yan *et al.*, 2012
thra	Thyroid hormone system	Yan *et al.*, 2012
thrb	Thyroid hormone system	Yan *et al.*, 2012

2.7 Chemical analysis and water quality

At the beginning and at end of the experiments, pH, dissolved oxygen and conductivity were controlled. Saturation by oxygen was always higher than 98%. pH varied between 7.6-7.9. Conductivity varied between 500 and 700 µS/cm with no difference between ISO medium and pesticide solutions. Stability of substances during the five-day experiment was verified by measuring their concentrations by LC-MS/MS analysis as described in detail in Supplementary Material S1. In brief, LC-MS/MS was performed with a Waters LC chromatograph (Waters, Manchester, U.K.) using Acquity BEH C18 column and gradient elution. Detection was performed on a Xevo TQ-S quadrupole mass spectrometer (Waters Manchester, U.K.) after ESI ionisation in a positive ion mode The analytes were quantified using external calibration of metolachlor, MOA and MESA (0.1 – 500 µg/L in 20% of methanol) and the limit of detection (LOD, signal to noise ratio S/N>3) and quantification (LOQ, S/N>10) were 0.05 and 0.1 µg/L, respectively for each analyte.

2.8 Data analysis

Length of larvae was assessed with one-way ANOVA test. Sum of different observed malformations in all replicates of the three independent experiments were compared with

control animals using Fisher's exact test in software GraphPad Prism (Version 5, GraphPad Software).

Different independent locomotion tests (independent experiments) were all assessed statistically apart because of high variability in control fish. Final results are presented as mean of fold changes with control of the three independent experiments.

Data of locomotion tests, spontaneous movements and heart rate were verified for normality (Shapiro-Wilk test; $p > 0.01$) and homoscedasticity (Levene test; $p > 0.05$), and if confirmed, ANOVA followed by Dunnet post-hoc test was used. In the other case, non-parametric Kruskall-Wallis test with Mann-Withney post-hoc test was used.

All data for the gene expression analysis were treated as described above (Shapiro-Wilk test; $p > 0.01$; Levene test; $p > 0.05$). Eventual values higher or smaller than 3 standard deviations were discarded. Data were log normalized prior to the analysis. ANOVA followed by Dunnet post-hoc test was used. If normality or homoscedasticity were not confirmed, non-parametric Kruskall-Wallis test with Mann-Whitney post-hoc tests were performed. All analyses were performed using software Statistica 13.3 (StatSoft, version 13.2, USA).

3. Results

3.1. Exposure and chemical analysis

Stability of all three compounds during the five-day experiment was verified. Nominal and measured concentrations, as well as the stability are shown in Supplementary Table S2. The concentrations of compounds were stable with the maximum decline of 13,3% at 30 µg/L of S-metolachlor. Measured values corresponded to nominal ones in case of MOA and were slightly higher at S-metolachlor and MESA. To simplify the presentation, nominal concentrations are shown and discussed in the results and discussions sections.

3.2 Mortality, hatching success, length of fish and malformation results

No significant effects on mortality were observed in any concentration tested of S-metolachlor, MOA, MESA or mixture of the three compounds. All controls had mortality < 10% as preconized by OECD guideline (OECD, 2013). Survival in all tested conditions was between 97.2% and 100%. Similarly, no significant effects on hatching success were found in all conditions tested. Hatching success values were between 95% and 100% in all variants.

Observed developmental abnormalities and length of fish are presented in Table 3. Generally, no patterns or dose-response effects were observed after exposure to S-metolachlor, its two metabolites MOA and MESA or in the mixture. No effects on the length of fish were observed in any condition tested. The most frequently observed malformations were at swim bladder (non-inflation) and yolk sac (malabsorption). No statistically significant effects were detected after exposures to the lower concentrations (1 and 30 µg/L) of S-metolachlor, MOA and MESA or after exposure to the low concentration mixture (1 µg/L of each substance). Following statistically significant effects were observed: S-metolachlor induced non-inflation of the swim bladder (100 µg/L) and malabsorption of the yolk sac (300 µg/L) and MESA (300 µg/L) induced craniofacial malformations. In addition, mixture of the three substances at concentration of 30 µg/L per each substance induced spinal deformations. Morphological score endpoint which allows assessment of each fish individually revealed as most problematic concentrations of 100 and 300 µg/L of S-metolachlor, 100 µg/L of MESA and 30 µg/L of mixture.

Table 3 Frequencies (%) of different types of developmental malformations, length of larvae (µm) and morphological score of zebrafish after 120 hours exposure to different concentrations of pesticide, metabolites and their mixtures. Each value represents mean ± standard deviation from 3 independent experiments each based on N=60 embryos. *P < 0.05; **P < 0.01

		µm	%								Absolute value
Endpoint		Body length	Edemas	Craniofacial deformation	Spine deformation	Non-inflated gas bladder	Tail deformation	Necrosis	Yolk sac malabsorption	Cardiovascular toxicity	**Morphological score**
Concentration µg/L											
S-metolachlor	*Control*	3768 ± 103	1.1 ± 1.6	1.1 ± 1.6	0 ± 0	0.6 ± 0.8	0 ± 0	0.6 ± 0.8	1.1 ± 0.8	1.1 ± 1.6	**0.9 ± 1.0**
	1	3772 ± 118	0.6 ± 0.8	1.7 ± 1.4	1.1 ± 0.8	2.8 ± 2.8	0 ± 0	0.6 ± 0.8	3.9 ± 2.8	0 ± 0	**1.6 ± 1.1**
	30	3767 ± 131	1.7 ± 2.4	2.8 ± 3.9	1.1 ± 1.6	3.3 ± 1.4	0 ± 0	0 ± 0	3.3 ± 3.6	0 ± 0	**1.8 ± 1.8**
	100	3763 ± 123	1.7 ± 1.4	2.3 ± 3.2	0.6 ± 0.8	**4.6 ± 1.6***	0 ± 0	0.6 ± 0.8	4.0 ± 0.8	0 ± 0	**2.0 ± 1.1***
	300	3738 ± 131	1.7 ± 1.4	2.8 ± 1.6	1.7 ± 1.4	2.3 ± 0.8	0 ± 0	0.6 ± 0.8	**5.8 ± 2.2***	0 ± 0	**2.2 ± 0.8***
MOA	*Control*	3714 ± 136	1.1 ± 0.8	2.3 ± 2.1	0.6 ± 0.8	3.4 ± 2.4	0 ± 0	2.8 ± 4.0	2.3 ± 2.1	0 ± 0	**1.9 ± 1.5**
	1	3712 ± 130	2.3 ± 2.2	2.3 ± 2.2	1.8 ± 1.5	1.8 ± 2.5	0 ± 0	0.6 ± 0.8	1.8 ± 2.5	1.2 ± 1.7	**1.8 ± 1.9**
	30	3719 ± 160	1.1 ± 1.6	0.6 ± 0.8	0 ± 0	2.9 ± 1.6	0 ± 0	0.6 ± 0.8	1.7 ± 0	1.7 ± 2.4	**1.3 ± 0.8**
	100	3678 ± 169	3.5 ± 2.4	2.3 ± 1.6	2.9 ± 0.7	5.1 ± 2.4	0 ± 0	1.7 ± 2.4	2.8 ± 0.8	3.4 ± 4.8	**3.0 ± 1.4**
	300	3694 ± 163	1.1 ± 1.6	1.1 ± 1.6	1.7 ± 1.4	3.9 ± 0.7	0 ± 0	2.3 ± 3.2	2.9 ± 0.8	0.6 ± 0.8	**2.1 ± 1.6**
MESA	*Control*	3661 ± 140	0.6 ± 0.8	0.6 ± 0.8	1.1 ± 0.8	2.2 ± 2.1	0 ± 0	0 ± 0	1.7 ± 1.4	0 ± 0	**0.7 ± 0.5**
	1	3708 ± 112	0 ± 0	1.1 ± 1.6	0 ± 0	2.2 ± 2.1	0 ± 0	0 ± 0	0.6 ± 0.8	0 ± 0	**0.6 ± 0.2**
	30	3700 ± 146	0 ± 0	1.1 ± 1.6	0 ± 0	1.1 ± 1.6	0 ± 0	0.6 ± 0.8	0.6 ± 0.8	0 ± 0	**0.6 ± 0.4**
	100	3683 ± 118	1.1 ± 0.8	**4.4 ± 5.2***	1.7 ± 1.4	3.3 ± 1.4	0 ± 0	1.1 ± 1.6	2.8 ± 0.8	0 ± 0	**2.0 ± 1.7****
	300	3628 ± 127	0 ± 0	2.3 ± 2.1	0.6 ± 0.8	1.1 ± 0.8	0 ± 0	0 ± 0	1.7 ± 0	0 ± 0	**0.9 ± 0.4**
MIXTURE	*Control*	3755 ± 149	0.7 ± 1.0	4.3 ± 2.6	0.6 ± 0.8	6.3 ± 4.4	0.6 ± 0.8	2.3 ± 1.7	6.9 ± 6.6	0.6 ± 0.8	**3.2 ± 1.7**
	1 (of each)	3763 ± 163	2.8 ± 0.8	2.9 ± 1.6	3.4 ± 2.8	4.5 ± 0.8	0.6 ± 0.8	1.1 ± 0.8	5.1 ± 2.4	0 ± 0	**3.1 ± 1.1**
	30 (of each)	3740 ± 193	3.1 ± 2.5	8.0 ± 1.0	**4.6 ± 3.5***	6.7 ± 5.2	0 ± 0	3.0 ± 2.3	12.2 ± 8.6	1.9 ± 1.6	**5.9 ± 2.0****

3.2 Light:dark locomotion test and heartbeat

None of the studied compounds or their mixtures caused any statistically significant effects on the swimming behavior. Detailed results are shown in Supplementary Material S2. Similarly, there were no effects observed at the heart rate of zebrafish larvae, detailed results are shown in Supplementary Material S3.

3.3 Spontaneous movements

Results of the spontaneous movements of the zebrafish embryos are presented in Figure 1. Environmental concentrations of S-metolachlor (1 µg/L) and of the mixture of the three substances (1 µg/L of each substance) significantly (P=0.001 and 0.022 respectively) reduced frequency of the tail movements. 0.5% solution of ethanol, used as positive control, significantly enhanced tail movements (P=0.009).

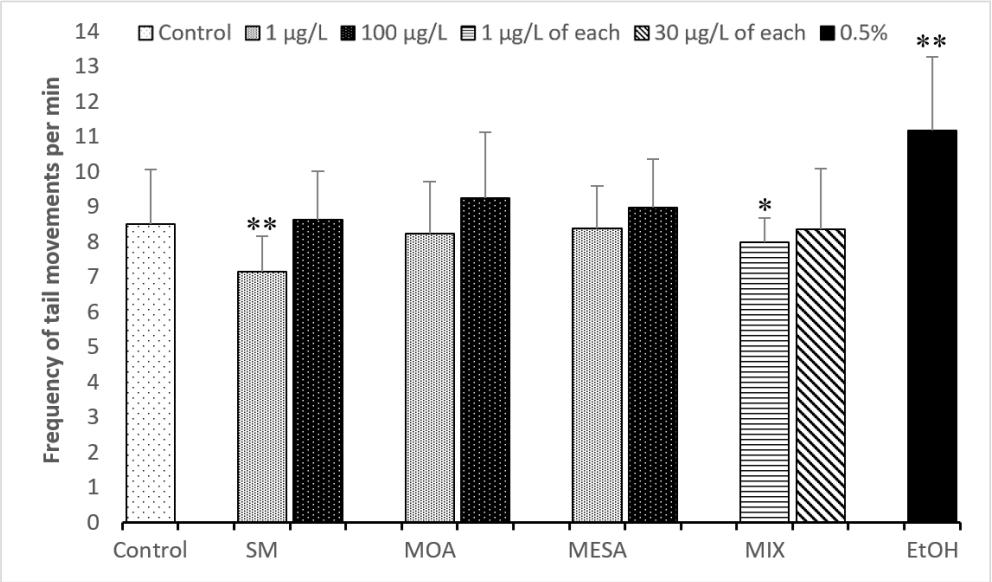

Figure 1. Spontaneous tail movements frequency per minute in zebrafish embryos exposed SM: S-metolachlor; MOA: Metolachlor oxanilic acid; MESA: Metolachlor ethanesulfonic acid; MIX: mixture of SM, MOA and MESA (concentration of 1 et 30 µg/L of each substance in the mixture); EtOH: ethanol; *P < 0.05, **P < 0.01

3.5 Analysis of gene expression

Gene expression of eight pre-selected genes was analyzed. Results are shown in Figure 2 as fold changes of ratio between selected genes and housekeeping genes. Exposures to T3 was used as positive control for thyroid-related genes.

The expression of mitochondrially encoded 12S rRNA (*12S*) gene was not significantly upregulated or downregulated in comparison with the control for all the concentrations of all the pesticides tested (Fig.3a). *P53,* gene related to the regulation of the cell cycle and cell apoptosis, was significantly upregulated at 100 µg/L of MOA (1.8-fold, P=0.005) and 30 µg/L of MESA (2-fold, P=0.022). The mixture of SM, MOA and MESA in concentration of 1 µg/L of each pesticide (2-fold, P<0.001) induced significant upregulation of *p53* (Fig.3b). Oxidative stress defense associated genes catalase - *cat* (Fig.3c) and copper/zinc superoxide dismutase - *sod1* (Fig.3d) were not affected by any of the pesticides tested.

We also studied the genes associated to RA signaling pathway: gene coding for cytochrome P450, family 26, subfamily A, polypeptide 1 - *cyp26a1* (Fig.3e) and genes associated to thyroid metabolism disruption: iodothyronine deiodinase 2 - *dio2* (Fig.3f), thyroid hormone nuclear receptor αa - *thra* (Fig.3g), and thyroid hormone nuclear receptor beta - *thrb* (Fig.3h). None of the four genes was deregulated after exposure to S-metolachlor. On the contrary, the metabolites and the mixture affected gene transcriptions. MOA significantly increased the expression of *dio2* (3.5-fold, P=0.015) and *thrb* (1.8-fold, P=0.034) in the highest concentration tested 100 µg/L. MESA significantly increased the mRNA expression level of *dio2* (5.6-fold, P=0.002), *thra* (2.1-fold, P=0.004) and *thrb* (1.9-fold, P=0.003) in the concentration of 1 µg/L. Upregulation of *thra* was also observed in the MESA concentration of 30 µg/L (2-fold, P=0.027). The mixture of the three substances (at low level - 1 µg/L of each substance) significantly upregulated the gene expression in all four genes - *cyp26a1* (2.3-fold, P<0.001), *dio2* (2.5-fold, P=0.007), *thra* (2.5-fold, P=0.001), *thrb* (2.2-fold, P=0.001). The T3 hormone significantly increased the expression of the mRNA transcription level of *cyp26a1* (4.2-fold, P=0.001) and *thra* (4.7-fold, P<0.001).

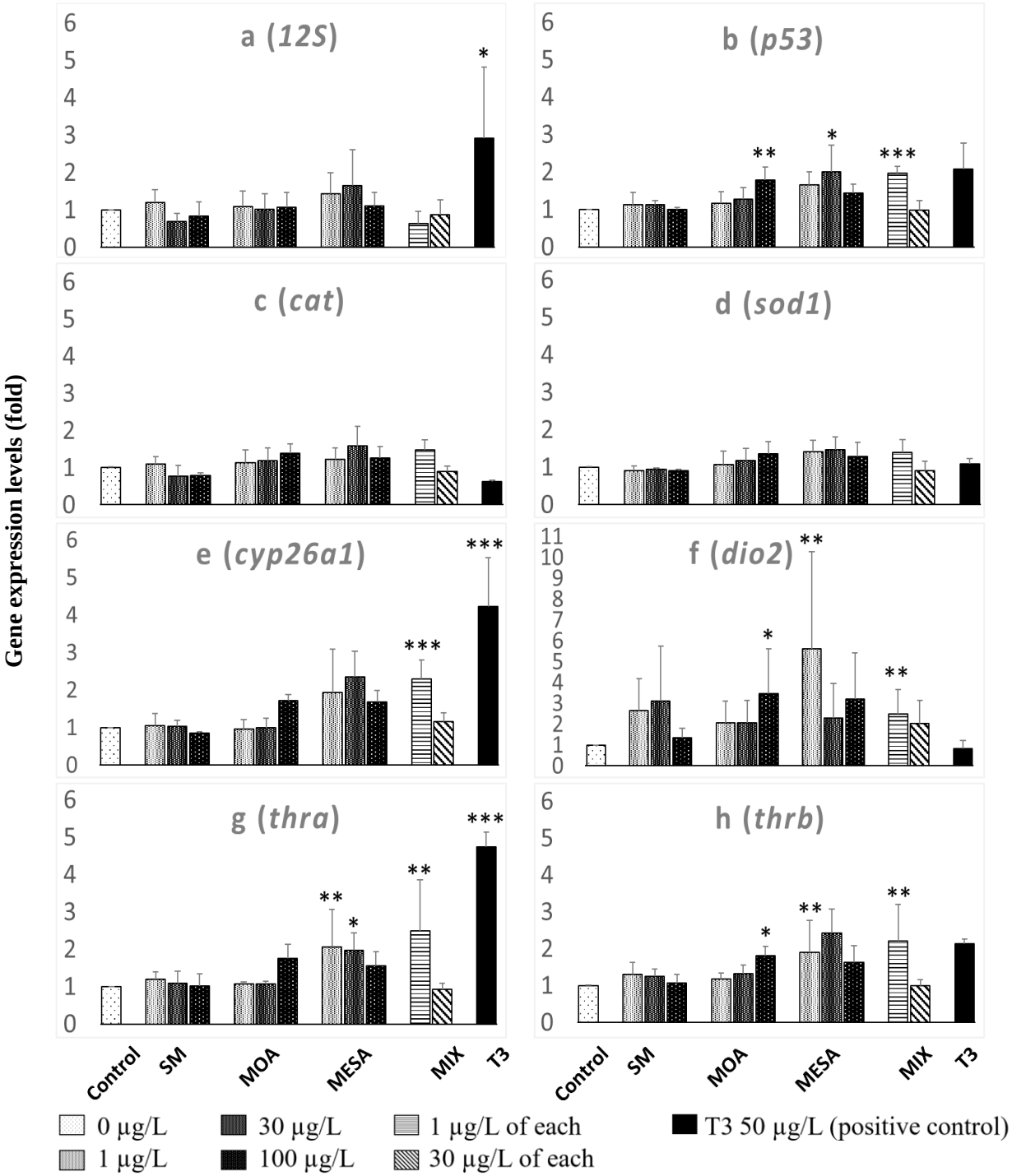

Figure 2. Expression levels of selected genes involved in mitochondrial metabolism (a), regulation of the cell cycle (b), oxidative stress defense (c, d), and thyroid metabolism disruption (e,f,g,h) associated genes in zebrafish larvae exposed for 5 days to SM: S-metolachlor; MOA: Metolachlor oxanilic acid; MESA: Metolachlor ethanesulfonic acid; MIX: mixture of SM, MOA and MESA; T3: triiodothyronine hormone; $P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

4. Discussion

S-metolachlor and its metabolites are among the most commonly reported herbicides occurring in relatively high concentrations in European waterbodies (Accinelli et al., 2002; Cerejeira et al., 2003; Farlin et al., 2018; Kapsi et al., 2019; Meffe and de Bustamante, 2014; Vryzas et al., 2009). Despite of the overall importance, the toxicity of S-metolachlor on fish was commonly evaluated at environmentally non-relevant conditions and high concentrations (Quintaneiro et al., 2017) or using only the racemic mixture of metolachlor (Jin et al., 2011). More recently, toxicity of one of the metabolites - MOA, was assessed by Velisek et al. (2018a) on crayfish but the effects of S-metolachlor and its metabolites on fish remain poorly characterized. Our interest was mainly focused on thyroid disruption, due to findings of Jin et al. (2011) and Yang et al. (2015) who linked this type of disruption with some chloroacetanilide pesticides. Regarding the complexity of the thyroid system, an integrative approach combining multiple endpoints was used.

4.1 Effects on embryo-larval development

Few statistically significant induced malformations were detected in the present study but only at higher exposure levels. Spine deformations were induced in fish exposed to mixture of substances (30 µg/L of each of the three compounds, ~ 90 µg/L sum concentration) but, interestingly, no such effects were observed at any of the substances alone up to 300 µg/L. Apparently, concentration addition (CA) effects in the mixture of different compounds was a major driver beyond this observation (European Chemicals Agency, 2014). Correspondingly, Lydy *et al.*, (2004) discuss that, generally, pesticides of the same chemical class exert the CA effects. This is supported also by findings of Junghans et al. (2003) who reported the CA effects of the mixture of eight chloroacetanilide herbicides in the study with susceptible organism - algae. It should be noted that despite of the statistical significance, the frequency of spine malformation (4.6%) is rather low and might be of minor biological significance. Nevertheless, further experiments could eventually further explore the mechanism behind this observation.

Other significant malformations (craniofacial deformations, non-inflated swim bladder, yolk sac malabsorption) were induced by individual substances at higher concentrations (100 or 300 µg/L). The observed malformations could be linked to the disruption of thyroid signaling. Liu and Chan (2002) showed that thyroid disruption lead to retarded inflation of the swim bladder, retarded absorption of the yolk sac and retarded maturation of the gastrointestinal

system. Quintaneiro et al. (2017) described impairment of yolk sac absorption of 96 hpf zebrafish larvae exposed to high concentrations of S-metolachlor (45 mg/L).

In our study, no statistically significant mortality was observed for tested concentrations of all substances and the mixture. According to U.S. EPA Ecotox database the NOEL of S-metolachlor for bluegill is 1.5 mg/L and for rainbow trout 2.5 mg/L (measured at 96 hpf). Both concentrations are higher than our highest tested concentration of 300 µg/L. To our knowledge, no data are available for the effects of metabolites on fish. Recently, Velisek et al. (2018a) found no mortality of embryo-larval stages of crayfish exposed for 45 days up to 420 µg/L of MOA. No effect was found on the length of larvae or the hatching success of fish exposed to tested substances and the mixture. This finding is in concordance with 100 times higher LOEC (29.0 mg/L) of S-metolachlor for zebrafish embryos (96 hpf) for hatching success established by Quintaneiro et al. (2017). Thus, as it was expected, low environmentally relevant concentrations used in this study elicited no significant effects on traditional apical endpoints such as mortality, hatching success and length of zebrafish larvae and we further investigated other sublethal endpoints.

4.3 Effects on behavior

Use of zebrafish larvae in behavioral studies have multiple advantages for evaluation of neurotoxicity of pesticides as discussed by Pittman (2017). To our knowledge, this is the first study that investigated the effects of S-metolachlor and its metabolites on fish behavior although some studies suggested S-metolachlor effects on neurobehavior. Quintaneiro et al. (2017) exposed zebrafish embryos for 4 days to 25 mg/L of S-metolachlor and observed inhibited acetylcholinesterase activity. Villa et al. (2018) described decreased speed and distance travelled by Chironomous larvae upon metolachlor exposures (27.4 mg/L). Cook & Moore (2008) found that sublethal concentration of metolachlor (80 µg/L) altered the fighting behavior of crayfish. On the contrary, available study with MOA revealed no effect on the behavior of crayfish (activity, distance, speed) exposed up to 420 µg/L (Velisek et al., 2018a). Moreover, some pesticides of the same chemical class of chloroacetanilides were also reported to influence fish locomotion. Pretilachlor (1 mg/L) decreased feeding attempts and increased burst swimming reactions and buccal movements in adult fish *Clarias batrachus* (Soni and Verma, 2018). Acetochlor at 5 mg/L decreased spontaneous tail movements in zebrafish embryos (24 hpf) as well as total distance travelled, average speed and time of movement of larvae 6 dpf (Wang et al., 2019).

In the present study, S-metolachlor decreased spontaneous tail movements in zebrafish embryos, although this observation was recorded only in the lowest concentration (1 µg/L). Corresponding decrease was also observed after exposure to the lower concentration mixture (all three compounds, 1 µg/L each). The observed effects on spontaneous movements may be caused by S-metolachlor interfering with maternal T4 stock in yolk sac or its signaling via an αVβ3 integrin as shown by Yonkers and Ribera (2008). The thyroid follicles start to secrete T4 hormone at 72 hpf (Porazzi et al., 2009), and nongenomic mechanism with maternal T4 influencing the neurodevelopment of zebrafish was previously reported (Yonkers and Ribera, 2008).

On the contrary, light:dark locomotion test didn't reveal any abnormal behavior. High biological variability in larvae movement could be linked to relatively wide time frame within the day (9 am – 4 pm), when the measurements were carried out. This was discussed by some authors who recommend specific more narrow frames like 11 am – 3 pm (Colwill and Creton, 2011) or 1 pm – 3:30 pm (MacPhail et al., 2009).

4.4 Effects on gene expression

Exposures to the metabolites appeared to have more pronounced effects on zebrafish larvae than the exposure to the parent compound. The highest tested concentration (100 µg/L) of MOA caused overexpression of genes *dio2, thrb* and *p53*. MESA induced overexpression of *dio2, thra* and *thrb* in fish exposed to low 1 µg/L, and *thra* and *p53* in fish exposed to 30 µg/L. The induction of *p53* indicates an activation of apoptotic processes or cell cycle arrest to protect the cells from additional detrimental effects (Ko and Prives, 1996). Genes *dio2, thra, thrb*, were proposed by Jin et al., (2011) as suitable biomarkers for thyroid system disruption in fish. Spaan et al., (2019) describe them as important genes (together with other deiodinases *dio1* and *dio3* and genes *hhex* and *NIS*) in the HPT axis. Deiodinase 2 is a selenoprotein that catalyzes the transformation of T4 to T3, thyroid hormones regulating the neurodevelopment. It is the most important deiodinase in zebrafish embryonic development as it controls the quantity of T3 hormone in the tissues (Walpita et al., 2009). Furthermore, deiodinases were found to affect the eye development of zebrafish (Houbrechts et al., 2016) and the overexpression of *dio2* has been linked with hypothyroidism (Orozco and Valverde-R, 2005). Thyroid hormone receptors αa and β act as transcription factors for thyroid hormones. According to Walter et al. (2019), mRNA levels of deiodinases and thyroid hormone receptors vary throughout the zebrafish development, with expression peak of *dio2* and *thrb* around 72-96 hpf and following decrease at 120 hpf. Expression of *thra* is stable from 24 hpf

to 120 hpf. Furthermore, as described by Porazzi et al., (2009) and Raldúa et al., (2012), thyroid system in zebrafish is fully developed during 5 dpf.

Although MESA and MOA affected expressions of several genes, no changes were observed after exposure to S-metolachlor. Contradictory observations for S-metolachlor were found by Jin et al. (2011), where 10 µg/L caused overexpression of *dio2*, *thra* and *thrb* in juvenile medaka, only in females. These differences may be related to multiple factors such as exposure duration (14 days in Jin et al. (2011) in comparison to 5 days in our study) or use of racemic mixture of metolachlor by Jin et al. (2011), which has been found more toxic to non-target organisms than pure S-metolachlor (Liu et al., 2006; Ye et al., 2010). Use of different fish species in different life stage (1-month-old juveniles used by Jin et al. (2011) in comparison with embryos in the present study) is also of importance; differences in sensitivity of medaka and zebrafish have already been documented (Perrichon et al., 2014; Vignet et al., 2019).

The mixture of S-metolachlor and its two metabolites appeared to be most hazardous, and low environmental concentration (1 µg/L of each substance) altered transcription of several genes like *p53*, *dio2*, *thra*, *thrb* and *cyp26a1*. Gene *cyp26a1*, encoding for enzyme degrading retinoic acid, is very important in embryogenesis, and its overexpression can disturb all the retinoic acid signalling pathway and may lead to developmental malformations (Hu et al., 2008). Effects on gene expression observed at low concentration of the mixture were not confirmed at the high concentration. This mixture "low-dose effects" mechanism has been discussed in the field of thyroid, and generally endocrine, system disruption, where nonmonotonic responses and U-shaped dose-response curves have been documented (Melnick et al., 2002; Vandenberg et al., 2012).

The most known pesticides to exert thyroid dysfunction are organochlorine pesticides (Calsolaro et al., 2017). However, several chloroacetanilides have also been shown to disrupt the thyroid system including the above discussed metolachlor in adult and juvenile medaka (Jin et al., 2011), acetochlor (Jiang et al., 2015; Yang et al., 2015) and pretilachlor (Jiang et al., 2016) in zebrafish embryos, acetochlor in larval rare minnow (Li et al., 2009) or butachlor in adult rare minnow (Zhu et al., 2014). Similarly to the present work, the above-mentioned studies documented changes in gene expressions of deiodinases and thyroid nuclear receptors and used lower concentrations below hundreds of micrograms. Comparatively, Li et al., (2009) and Yang et al., (2015) also mentioned a non-sigmoidal dose response curve.

No changes were observed in the expression of the next three genes: *12S*, *sod1* and *cat*. Gene *12S* is associated with mitochondrial metabolism thus suggesting no effect on metabolic homeostasis (Arini et al., 2015). *Sod1* and *cat* are important genes managing some parts of the chain of the reactive oxygen species (ROS) in cell, scavenging respectively the superoxide radicals and hydrogen peroxide (Seifried et al., 2007). From these findings we conclude that S-metolachlor, its two metabolites and their mixture did not induce any oxidative stress detectable by *sod1* and *cat* in the zebrafish embryos. Quintaneiro et al. (2017) did not found either an effect of S-metolachlor on the catalase activity in zebrafish embryos exposed up to 25 mg/L. In contrast, MOA seems to have an inhibiting effect on catalase activity of a water crustacean: crayfish exposed to low concentrations (4.2 µg/L) (Velisek et al., 2018a).

5. Conclusion

Our work brings important findings about the effects of environmentally relevant mixture of one of the most used herbicides S-metolachlor and its two metabolites on early life stages of zebrafish. We have observed specific malformations that could be linked to thyroid disruption as well as decreased spontaneous movements of zebrafish larvae. Interestingly, metabolites were more harmful that S-metolachlor to zebrafish larvae at the level of gene expression with apparently strong effects on genes involved in cell cycle regulation and thyroid-related signaling. Further detailed analyses are needed to fully elucidate the dose-response relationship. The present study brings new information highlighting the importance of metabolites and the pesticides mixtures as emphasized in the European Environmental Action Programme (European Parliament and Council, 2013).

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