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

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1           **Environmentally relevant mixture of S-metolachlor and its two**  
2           **metabolites affects thyroid metabolism in zebrafish embryos**  
3

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11  
12   **HIGHLIGHTS (3-5, max 85 characters per highlight)**

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

19  
20   **KEYWORDS (6 max)**

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

23   **Abstract <400 words**

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

35 Exposure to the S-metolachlor (1 µg/L) and to the pesticide mixture (1 µg/L of each  
36 substance) significantly reduced spontaneous tail movements of 21 hpf embryos. Few rare  
37 developmental malformations were observed, but only in larvae exposed to more than  
38 100 µg/L of pure substances (craniofacial deformation, non-inflated gas bladder, yolk sac  
39 malabsorption) and to 30 µg/L of each substance in the pesticide mixture (spine deformation).  
40 However, no effect on hatching success, length of larvae, heart rate or larvae locomotion were  
41 found. Strong responses were detected at the molecular level, especially on the *p53* gene  
42 regulating the cell cycle, caused by the pesticide mixture (1 µg/L of each substance), MESA  
43 (30 µg/L) and MOA (100 µg/L), as well as on the *cyp26a1* – gene encoding for cytochrome  
44 P450 after exposure to the pesticide mixture (1 µg/L of each substance). Genes implicated in  
45 the thyroid system disruption (*dio2*, *thra*, *thrb*) were all overexpressed following exposure to  
46 the environmentally relevant concentrations of the pesticide mixture (1 µg/L of each  
47 substance) and to the metabolite MESA (1 µg/L). In conclusion, the zebrafish thyroid system  
48 disruption was revealed not only by the overexpressed genes, but also by some of the  
49 developmental malformations considered as markers of this type of disruption (mainly gas  
50 bladder and yolk sac abnormalities) and spontaneous tail movements linked to the T4  
51 hormone influencing its neurodevelopment. Thus, the thyroid system disruption represents a  
52 likely hypothesis behind the effects caused by the low environmental concentrations of  
53 S-metolachlor, its two metabolites and their mixture.

## 54 **Introduction**

55 S-metolachlor is one of the most commonly used pesticides in the world (Atwood and  
56 Paisley-Jones, 2017). This selective pre-emergent herbicide from the chloroacetanilides  
57 family was first registered in 1997 (Heydens et al., 2010). It is an S-enantiomer of 2-chloro-  
58 N-(2-ethyl-6-methylphenyl)-N-(1-methoxypropan-2-yl)acetamide. It replaced the racemic  
59 metolachlor mixture due to its higher herbicidal activity (Müller et al., 2001; Thomas Poiger,  
60 2002), thus reducing the quantity of pesticide needed by 40% (Blaser and Spindler, 1997).  
61 S-metolachlor inhibits the biosynthesis of very-long-chain fatty acids (Götz and Böger, 2004)  
62 and interferes with gibberellin synthesis enzymes (Rose et al., 2016), thereby inhibiting the  
63 growth of target plants such as grass weeds in corn fields (Heydens et al., 2010). However,  
64 S-metolachlor can easily contaminate surface water by agricultural runoff (Zemolin et al.,  
65 2014) and it is thus often detected in surface or coastal waters (Accinelli et al., 2002; De  
66 Liguoro et al., 2014; Fauvelle et al., 2018; Glinski et al., 2018; Kapsi et al., 2019; Meffe and  
67 de Bustamante, 2014; Ryberg et al., 2014). In soil, S-metolachlor is microbially degraded to  
68 two principal metabolites (Zemolin et al., 2014): metolachlor oxanilic acid (MOA) and  
69 metolachlor ethanesulfonic acid (MESA), which are mobile and persistent in the environment  
70 (Sidoli et al., 2016). As a result, these degradation products are often detected in higher  
71 concentrations than the parent compound itself in water samples (Elliott and VanderMeulen,  
72 2017; Hladik et al., 2005). However, there is a general lack of information about the toxicity  
73 of individual pesticide metabolites and their mixtures occurring in the environment. Only  
74 recently, some studies highlighted the importance of aquatic toxicity of environmentally  
75 relevant pesticide mixtures (Gustavsson et al., 2017; Tian et al., 2018). Furthermore, the  
76 importance of evaluating mixture toxicity is emphasized by the European Chemicals Agency  
77 (2014).

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

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

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

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

119

## 120 **2. Materials and methods**

### 121 **2.1 Chemicals**

122 S-metolachlor (S-M, CAS 87392-12-9, Pestanal, purity  $\geq$  98.0%), metolachlor oxanilic acid  
123 (MOA, CAS 152019-73-3, Pestanal, purity  $\geq$  98.0%), metolachlor ethanesulfonic acid  
124 (MESA, CAS 947601-85-6, Pestanal, purity  $\geq$  95.0%) were purchased from Sigma-Aldrich.  
125 Their stock solutions (50 mg/L) were prepared in Milli-Q water and were stored at 4 °C. The  
126 hormone triiodothyronine (T3, dissolved in methanol, CAS 6893-02-3, purity  $\geq$  95%,  
127 purchased from Sigma-Aldrich, used immediately) was used as positive control in the gene  
128 modulation experiments. Ethanol absolute (CAS 64-17-5, purity  $\geq$  99.8%, purchased from  
129 VWR Chemicals) was used as positive control in the spontaneous movement analysis and for  
130 the PCR analysis. ISO medium (ISO, 1996) (CaCl<sub>2</sub>\*2H<sub>2</sub>O (294 mg/L),  
131 MgSO<sub>4</sub>\*7H<sub>2</sub>O (123.3 mg/L), NaHCO<sub>3</sub> (63 mg/L), KCl (5.5 mg/L) in Milli-Q water) was used  
132 to prepare the final dilutions for the tests. RNA<sup>later</sup>® (Ambion) was purchased from Sigma-  
133 Aldrich.

### 134 **2.2 Test organism**

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

### 146 **2.3 FET (Fish embryo toxicity) test**

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

151 concentrations detected in the rivers in the Czech Republic and in the Arcachon Bay in France  
152 during 2010-2014 (Table 1).  
153

154 **Table 1**

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

	<b>Metolachlor</b>	<b>MOA</b>	<b>MESA</b>	<b>Location</b>
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)

160  
 161 The lowest used test concentration (1 µg/L) reflects the actual environmental concentration,  
 162 and three higher (non-lethal) concentrations were also tested (30, 100 and 300 µg/L).  
 163 Corresponding concentrations in nmol/L are showed in Supplementary Table S2. The mixture  
 164 exposure included two concentration levels: 1 µg/L of each substance and 30 µg/L of each  
 165 substance. ISO medium was used as negative control. The tests were conducted in glass  
 166 crystallization dishes with transparent lids containing 20 ml of media and 20 embryos in three  
 167 replicates. Every test was repeated independently three times with eggs from different  
 168 spawning. Mortality, hatching success and malformations were controlled daily using a  
 169 stereomicroscope. Eventual dead embryos/larvae were immediately removed from dishes to  
 170 prevent contamination. Length of larvae (without the caudal fin) was measured on the last,  
 171 fifth, day of the experiment on photos of larvae mounted in mixture of cellulose and  
 172 anesthetics MS-222. Fifteen fish per replicate in 3 independent tests were measured. Photos  
 173 were analyzed using the software QuickPhoto Micro 2.3.  
 174 “Morphological score” endpoint was assessed in order to evaluate global state of individual  
 175 fish using slightly modified approach as previously published (Panzica-Kelly et al., 2010).  
 176 Every fish embryo is classified using 0-3 point scores (description is in Supplementary Figure  
 177 S1).

178 **2.4 Light: dark locomotor test**

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



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

## 192 **2.5 Spontaneous movement and heartbeat**

193 Embryos were handled as described above (2.2) and exposed to concentrations of 1 and  
194 100 µg/L of substances alone and to the mixtures at two concentration levels (1 and 30 µg/L  
195 of each substance in the mixture). They were maintained at 26 °C until 21 hpf when  
196 randomized capture of videos was realized till 23 hpf. Crystallization dish containing  
197 20 embryos (= one replicate) was acclimatized under the stereomicroscope for 30 sec and then  
198 2 min video was captured. Positive control (1% v/v ethanol) was used to enhance the  
199 spontaneous movements (tail coils). Videos were then analyzed by DanioScope software  
200 (Noldus, Netherlands) and reported as spontaneous movements per minute.

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

## 206 **2.6 Zebrafish exposures for analysis of gene expressions**

207 Embryos were handled as described above (2.3) and exposed to 1, 30 and 100 µg/L of  
208 pesticides assessed alone and two concentrations of the mixture of the three pesticides (1 and  
209 30 µg/L, respectively of each compound). Hormone T3 (triiodothyronine) was used as  
210 positive control to assess the expression of thyroid-related genes. Dead or heavily malformed  
211 embryos/larvae were removed daily. Whole larvae were collected at 120 hpf into pools of  
212 20 larvae per Eppendorf tube containing 500 µl RNAlater® and stored at -20 °C upon RNA  
213 extraction.

### 214 **2.6.1 RNA isolation**

215 The total RNAs were extracted using the SV Total RNA Isolation System Kit (Promega).  
216 Whole larvae were homogenized in 500 µl of RNA Lysis Buffer using glass beads (Sigma) in  
217 the FastPrep®-24 (M.P. Biomedicals, USA). Lysed samples were collected, centrifuged and  
218 mixed with 450 µl of 75% v/v ethanol and transferred on the spin column. Handling with spin  
219 columns was performed according to the manufacturer's instructions. Before the DNase  
220 incubation step, an additional centrifuge step was added (13500 rpm, 2 minutes) to dry the  
221 column. All RNA samples were treated with DNase I mixture (40 µl of Yellow core buffer,  
222 5 µl of MnCl<sub>2</sub> and 5 µl of DNase I) for 15 minutes in water bath at 37 °C. The rest of the  
223 isolation was performed according to the manufacturer's instructions and purified RNAs were  
224 collected in 50 µl of Nuclease-Free water. The concentration and purity of collected RNA  
225 samples were verified spectrophotometrically at 260/280 and 260/230 nm wavelength with  
226 Nanodrop™. Purity of all the samples was between 2.0-2.1 for the ratio 260/280 and 1.8-2.2  
227 for the ratio 260/230.

### 228 **2.6.2 Reverse transcription**

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

### 233 **2.6.3 Quantitative PCR**

234 8 genes were selected to evaluate the effects of chosen pesticides and their mixture on the  
235 thyroid hormone system, mitochondrial metabolism, regulation of the cell cycle and oxidative  
236 stress defense. Three reference zebrafish genes were used in the analysis (*β-actin*, *ef1a* and  
237 *rpl13*). Functioning and references of used primers are shown in Table 2. Sequences,  
238 efficiencies, and accession numbers are presented in Supplementary Table S1. Primers were  
239 purchased from Elisabeth Pharmacon (Czech Republic).

240 QPCR was carried out with Brilliant III Ultra-fast SYBR Green QPCR Master Mix kit  
241 (Stratagene-Agilent) on the LightCycler® 480 (Roche). Each qPCR mix (20 µl) contained  
242 5 µl of cDNA (10 times diluted), 2 µl of reverse and forward primer (2 µM) and 10 µl of  
243 SYBR from the kit and 3 µl of nuclease free water.

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

246 45 cycles with 1 cycle at 95 °C for 5 sec and 60 °C for 10 sec. The melting curve continued at  
 247 95 °C for 5 sec and at 65 °C for 1 min. Finally, the cooling step was performed at 40 °C for  
 248 30 sec. Melting curves of every reaction were analyzed to assess reaction specificity. The data  
 249 were normalized to the mean of the Ct values for three reference genes, *β-act*, *elf1a* and *rpl13*  
 250 and analyzed using the  $2^{-\Delta\Delta CT}$  method (Livak and Schmittgen, 2001). Results are shown as  
 251 fold changes of exposed to control group.

252 **Table 2**

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

Gene	Function	References
<i>β-act</i>	Reference gene	Dedeh <i>et al.</i> , 2015
<i>elf1a</i>	Reference gene	Gentès <i>et al.</i> , 2015
<i>rpl13</i>	Reference gene	Gentès <i>et al.</i> , 2015
<i>12S</i>	Mitochondrial metabolism	Arini <i>et al.</i> , 2015
<i>p53</i>	Regulation of the cell cycle/apoptose	Gentès <i>et al.</i> , 2015
<i>cat</i>	Oxidative stress defense	Lerebours <i>et al.</i> , 2009
<i>sod1</i>	Oxidative stress defense	Gentès <i>et al.</i> , 2015
<i>cyp26a1</i>	RA (retinoic acid) signaling pathway	Oliveira <i>et al.</i> , 2013
<i>dio2</i>	Thyroid hormone system	Yan <i>et al.</i> , 2012
<i>thra</i>	Thyroid hormone system	Yan <i>et al.</i> , 2012
<i>thrb</i>	Thyroid hormone system	Yan <i>et al.</i> , 2012

254

255 **2.7 Chemical analysis and water quality**

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

268 **2.8 Data analysis**

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

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

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

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

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

286

287

## 288 **3. Results**

### 289 **3.1. Exposure and chemical analysis**

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

### 296 **3.2 Mortality, hatching success, length of fish and malformation results**

297 No significant effects on mortality were observed in any concentration tested of  
298 S-metolachlor, MOA, MESA or mixture of the three compounds. All controls had  
299 mortality < 10% as preconized by OECD guideline (OECD, 2013). Survival in all tested  
300 conditions was between 97.2% and 100%. Similarly, no significant effects on hatching  
301 success were found in all conditions tested. Hatching success values were between 95% and  
302 100% in all variants.

303 Observed developmental abnormalities and length of fish are presented in Table 3. Generally,  
304 no patterns or dose-response effects were observed after exposure to S-metolachlor, its two  
305 metabolites MOA and MESA or in the mixture. No effects on the length of fish were  
306 observed in any condition tested. The most frequently observed malformations were at swim  
307 bladder (non-inflation) and yolk sac (malabsorption). No statistically significant effects were  
308 detected after exposures to the lower concentrations (1 and 30 µg/L) of S-metolachlor, MOA  
309 and MESA or after exposure to the low concentration mixture (1 µg/L of each substance).  
310 Following statistically significant effects were observed: S-metolachlor induced non-inflation  
311 of the swim bladder (100 µg/L) and malabsorption of the yolk sac (300 µg/L) and MESA  
312 (300 µg/L) induced craniofacial malformations. In addition, mixture of the three substances at  
313 concentration of 30 µg/L per each substance induced spinal deformations. Morphological  
314 score endpoint which allows assessment of each fish individually revealed as most  
315 problematic concentrations of 100 and 300 µg/L of S-metolachlor, 100 µg/L of MESA and  
316 30 µg/L of mixture.

317 **Table 3** Frequencies (%) of different types of developmental malformations, length of larvae ( $\mu\text{m}$ ) and morphological score of zebrafish after  
 318 120 hours exposure to different concentrations of pesticide, metabolites and their mixtures. Each value represents mean  $\pm$  standard deviation  
 319 from 3 independent experiments each based on N=60 embryos. \*P < 0.05; \*\*P < 0.01

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

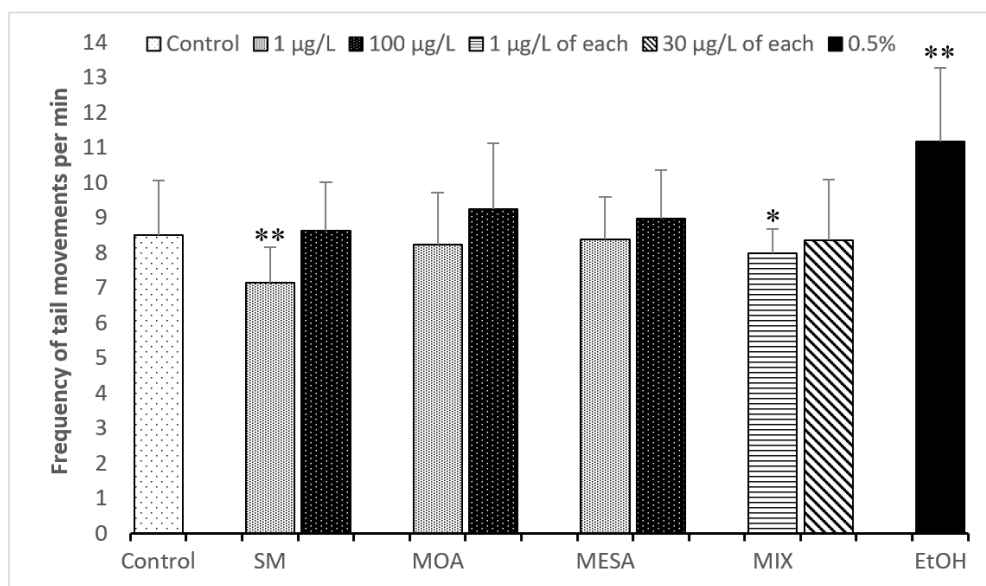
### 321 3.2 Light:dark locomotion test and heartbeat

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

326

### 327 3.3 Spontaneous movements

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



333

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

338

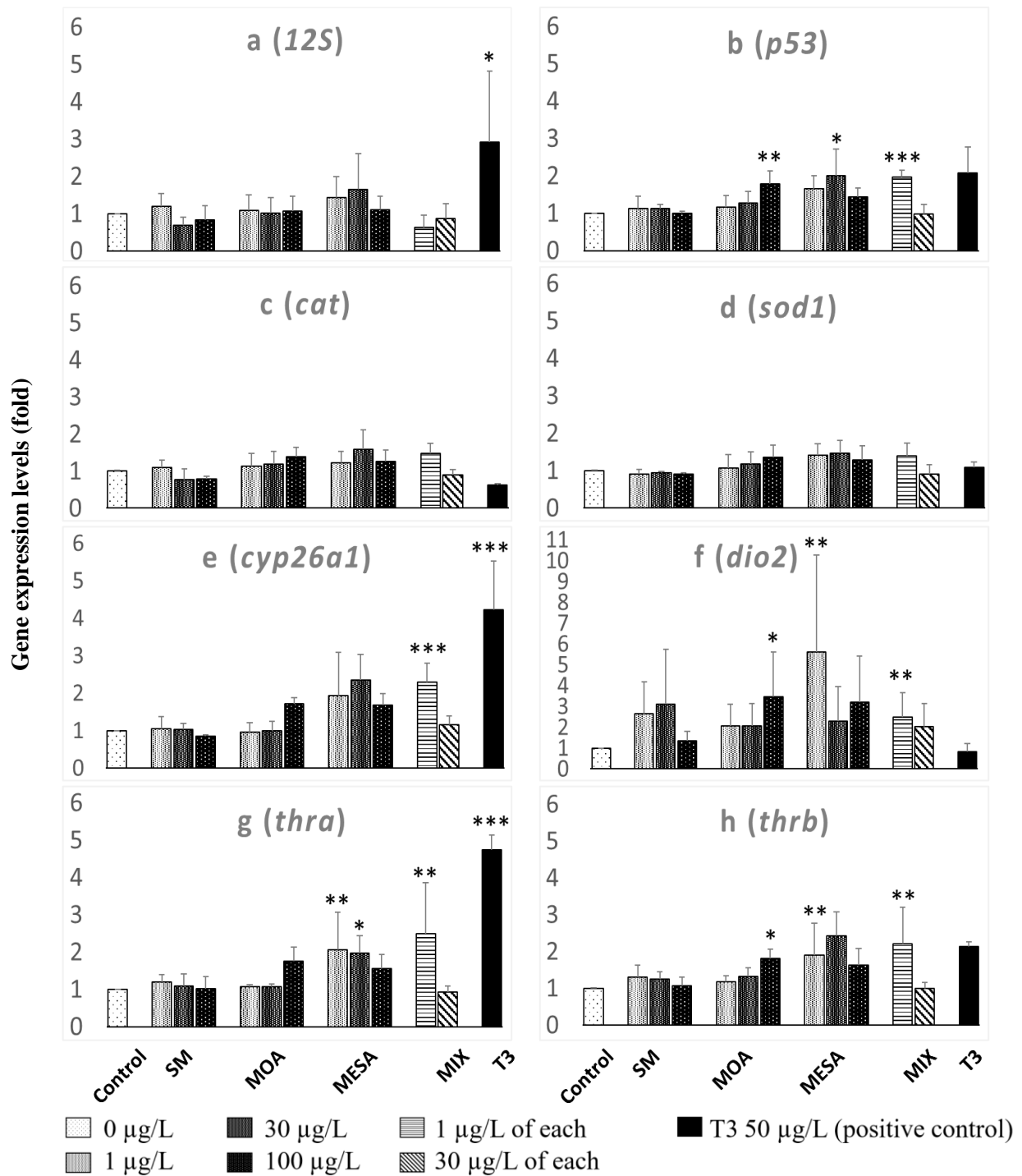
### 339 **3.5 Analysis of gene expression**

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

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

351 We also studied the genes associated to RA signaling pathway: gene coding for cytochrome  
352 P450, family 26, subfamily A, polypeptide 1 - *cyp26a1* (Fig.3e) and genes associated to  
353 thyroid metabolism disruption: iodothyronine deiodinase 2 - *dio2* (Fig.3f), thyroid hormone  
354 nuclear receptor *α* - *thra* (Fig.3g), and thyroid hormone nuclear receptor beta - *thrb* (Fig.3h).  
355 None of the four genes was deregulated after exposure to S-metolachlor. On the contrary, the  
356 metabolites and the mixture affected gene transcriptions. MOA significantly increased the  
357 expression of *dio2* (3.5-fold, P=0.015) and *thrb* (1.8-fold, P=0.034) in the highest  
358 concentration tested 100 µg/L. MESA significantly increased the mRNA expression level of  
359 *dio2* (5.6-fold, P=0.002), *thra* (2.1-fold, P=0.004) and *thrb* (1.9-fold, P=0.003) in the  
360 concentration of 1 µg/L. Upregulation of *thra* was also observed in the MESA concentration  
361 of 30 µg/L (2-fold, P=0.027). The mixture of the three substances (at low level - 1 µg/L of  
362 each substance) significantly upregulated the gene expression in all four genes - *cyp26a1*  
363 (2.3-fold, P<0.001), *dio2* (2.5-fold, P=0.007), *thra* (2.5-fold, P=0.001), *thrb* (2.2-fold,  
364 P=0.001). The T3 hormone significantly increased the expression of the mRNA transcription  
365 level of *cyp26a1* (4.2-fold, P=0.001) and *thra* (4.7-fold, P<0.001).





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**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

## 372 **4. Discussion**

373 S-metolachlor and its metabolites are among the most commonly reported herbicides  
374 occurring in relatively high concentrations in European waterbodies (Accinelli et al., 2002;  
375 Cerejeira et al., 2003; Farlin et al., 2018; Kapsi et al., 2019; Meffe and de Bustamante, 2014;  
376 Vryzas et al., 2009). Despite of the overall importance, the toxicity of S-metolachlor on fish  
377 was commonly evaluated at environmentally non-relevant conditions and high concentrations  
378 (Quintaneiro et al., 2017) or using only the racemic mixture of metolachlor (Jin et al., 2011).  
379 More recently, toxicity of one of the metabolites - MOA, was assessed by Velisek et al.  
380 (2018a) on crayfish but the effects of S-metolachlor and its metabolites on fish remain poorly  
381 characterized. Our interest was mainly focused on thyroid disruption, due to findings of Jin et  
382 al. (2011) and Yang et al. (2015) who linked this type of disruption with some  
383 chloroacetanilide pesticides. Regarding the complexity of the thyroid system, an integrative  
384 approach combining multiple endpoints was used.

### 385 **4.1 Effects on embryo-larval development**

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

399 Other significant malformations (craniofacial deformations, non-inflated swim bladder, yolk  
400 sac malabsorption) were induced by individual substances at higher concentrations (100 or  
401 300 µg/L). The observed malformations could be linked to the disruption of thyroid signaling.  
402 Liu and Chan (2002) showed that thyroid disruption lead to retarded inflation of the swim  
403 bladder, retarded absorption of the yolk sac and retarded maturation of the gastrointestinal

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

406 In our study, no statistically significant mortality was observed for tested concentrations of all  
407 substances and the mixture. According to U.S. EPA Ecotox database the NOEL of  
408 S-metolachlor for bluegill is 1.5 mg/L and for rainbow trout 2.5 mg/L (measured at 96 hpf).  
409 Both concentrations are higher than our highest tested concentration of 300 µg/L. To our  
410 knowledge, no data are available for the effects of metabolites on fish. Recently, Velisek et al.  
411 (2018a) found no mortality of embryo-larval stages of crayfish exposed for 45 days up to  
412 420 µg/L of MOA. No effect was found on the length of larvae or the hatching success of fish  
413 exposed to tested substances and the mixture. This finding is in concordance with 100 times  
414 higher LOEC (29.0 mg/L) of S-metolachlor for zebrafish embryos (96 hpf) for hatching  
415 success established by Quintaneiro et al. (2017). Thus, as it was expected, low  
416 environmentally relevant concentrations used in this study elicited no significant effects on  
417 traditional apical endpoints such as mortality, hatching success and length of zebrafish larvae  
418 and we further investigated other sublethal endpoints.

#### 419 **4.3 Effects on behavior**

420 Use of zebrafish larvae in behavioral studies have multiple advantages for evaluation of  
421 neurotoxicity of pesticides as discussed by Pittman (2017). To our knowledge, this is the first  
422 study that investigated the effects of S-metolachlor and its metabolites on fish behavior  
423 although some studies suggested S-metolachlor effects on neurobehavior. Quintaneiro et al.  
424 (2017) exposed zebrafish embryos for 4 days to 25 mg/L of S-metolachlor and observed  
425 inhibited acetylcholinesterase activity. Villa et al. (2018) described decreased speed and  
426 distance travelled by Chironomous larvae upon metolachlor exposures (27.4 mg/L). Cook &  
427 Moore (2008) found that sublethal concentration of metolachlor (80 µg/L) altered the fighting  
428 behavior of crayfish. On the contrary, available study with MOA revealed no effect on the  
429 behavior of crayfish (activity, distance, speed) exposed up to 420 µg/L (Velisek et al.,  
430 2018a). Moreover, some pesticides of the same chemical class of chloroacetanilides were also  
431 reported to influence fish locomotion. Pretilachlor (1 mg/L) decreased feeding attempts and  
432 increased burst swimming reactions and buccal movements in adult fish *Clarias batrachus*  
433 (Soni and Verma, 2018). Acetochlor at 5 mg/L decreased spontaneous tail movements in  
434 zebrafish embryos (24 hpf) as well as total distance travelled, average speed and time of  
435 movement of larvae 6 dpf (Wang et al., 2019).

436 In the present study, S-metolachlor decreased spontaneous tail movements in zebrafish  
437 embryos, although this observation was recorded only in the lowest concentration (1 µg/L).  
438 Corresponding decrease was also observed after exposure to the lower concentration mixture  
439 (all three compounds, 1 µg/L each). The observed effects on spontaneous movements may be  
440 caused by S-metolachlor interfering with maternal T4 stock in yolk sac or its signaling via an  
441  $\alpha V\beta 3$  integrin as shown by Yonkers and Ribera (2008). The thyroid follicles start to secrete  
442 T4 hormone at 72 hpf (Porazzi et al., 2009), and nongenomic mechanism with maternal T4  
443 influencing the neurodevelopment of zebrafish was previously reported (Yonkers and Ribera,  
444 2008).

445 On the contrary, light:dark locomotion test didn't reveal any abnormal behavior. High  
446 biological variability in larvae movement could be linked to relatively wide time frame within  
447 the day (9 am – 4 pm), when the measurements were carried out. This was discussed by some  
448 authors who recommend specific more narrow frames like 11 am – 3 pm (Colwill and Creton,  
449 2011) or 1 pm – 3:30 pm (MacPhail et al., 2009).

#### 450 **4.4 Effects on gene expression**

451 Exposures to the metabolites appeared to have more pronounced effects on zebrafish larvae  
452 than the exposure to the parent compound. The highest tested concentration (100 µg/L) of  
453 MOA caused overexpression of genes *dio2*, *thrb* and *p53*. MESA induced overexpression of  
454 *dio2*, *thra* and *thrb* in fish exposed to low 1 µg/L, and *thra* and *p53* in fish exposed to  
455 30 µg/L. The induction of *p53* indicates an activation of apoptotic processes or cell cycle  
456 arrest to protect the cells from additional detrimental effects (Ko and Prives, 1996). Genes  
457 *dio2*, *thra*, *thrb*, were proposed by Jin et al., (2011) as suitable biomarkers for thyroid system  
458 disruption in fish. Spaan et al., (2019) describe them as important genes (together with other  
459 deiodinases *dio1* and *dio3* and genes *hhex* and *NIS*) in the HPT axis. Deiodinase 2 is a  
460 selenoprotein that catalyzes the transformation of T4 to T3, thyroid hormones regulating the  
461 neurodevelopment. It is the most important deiodinase in zebrafish embryonic development as  
462 it controls the quantity of T3 hormone in the tissues (Walpita et al., 2009). Furthermore,  
463 deiodinases were found to affect the eye development of zebrafish (Houbrechts et al., 2016)  
464 and the overexpression of *dio2* has been linked with hypothyroidism (Orozco and Valverde-R,  
465 2005). Thyroid hormone receptors  $\alpha$  and  $\beta$  act as transcription factors for thyroid hormones.  
466 According to Walter et al. (2019), mRNA levels of deiodinases and thyroid hormone  
467 receptors vary throughout the zebrafish development, with expression peak of *dio2* and *thrb*  
468 around 72-96 hpf and following decrease at 120 hpf. Expression of *thra* is stable from 24 hpf

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

471 Although MESA and MOA affected expressions of several genes, no changes were observed  
472 after exposure to S-metolachlor. Contradictory observations for S-metolachlor were found by  
473 Jin et al. (2011), where 10 µg/L caused overexpression of *dio2*, *thra* and *thrb* in juvenile  
474 medaka, only in females. These differences may be related to multiple factors such as  
475 exposure duration (14 days in Jin et al. (2011) in comparison to 5 days in our study) or use of  
476 racemic mixture of metolachlor by Jin et al. (2011), which has been found more toxic to non-  
477 target organisms than pure S-metolachlor (Liu et al., 2006; Ye et al., 2010). Use of different  
478 fish species in different life stage (1-month-old juveniles used by Jin et al. (2011) in  
479 comparison with embryos in the present study) is also of importance; differences in sensitivity  
480 of medaka and zebrafish have already been documented (Perrichon et al., 2014; Vignet et al.,  
481 2019).

482 The mixture of S-metolachlor and its two metabolites appeared to be most hazardous, and low  
483 environmental concentration (1 µg/L of each substance) altered transcription of several genes  
484 like *p53*, *dio2*, *thra*, *thrb* and *cyp26a1*. Gene *cyp26a1*, encoding for enzyme degrading  
485 retinoic acid, is very important in embryogenesis, and its overexpression can disturb all the  
486 retinoic acid signalling pathway and may lead to developmental malformations (Hu et al.,  
487 2008). Effects on gene expression observed at low concentration of the mixture were not  
488 confirmed at the high concentration. This mixture “low-dose effects” mechanism has been  
489 discussed in the field of thyroid, and generally endocrine, system disruption, where  
490 nonmonotonic responses and U-shaped dose-response curves have been documented (Melnick  
491 et al., 2002; Vandenberg et al., 2012).

492 The most known pesticides to exert thyroid dysfunction are organochlorine pesticides  
493 (Calsolaro et al., 2017). However, several chloroacetanilides have also been shown to disrupt  
494 the thyroid system including the above discussed metolachlor in adult and juvenile medaka  
495 (Jin et al., 2011), acetochlor (Jiang et al., 2015; Yang et al., 2015) and pretilachlor (Jiang et  
496 al., 2016) in zebrafish embryos, acetochlor in larval rare minnow (Li et al., 2009) or butachlor  
497 in adult rare minnow (Zhu et al., 2014). Similarly to the present work, the above-mentioned  
498 studies documented changes in gene expressions of deiodinases and thyroid nuclear receptors  
499 and used lower concentrations below hundreds of micrograms. Comparatively, Li et al.,  
500 (2009) and Yang et al., (2015) also mentioned a non-sigmoidal dose response curve.

501 No changes were observed in the expression of the next three genes: *12S*, *sod1* and *cat*. Gene  
502 *12S* is associated with mitochondrial metabolism thus suggesting no effect on metabolic  
503 homeostasis (Arini et al., 2015). *Sod1* and *cat* are important genes managing some parts of the  
504 chain of the reactive oxygen species (ROS) in cell, scavenging respectively the superoxide  
505 radicals and hydrogen peroxide (Seifried et al., 2007). From these findings we conclude that  
506 S-metolachlor, its two metabolites and their mixture did not induce any oxidative stress  
507 detectable by *sod1* and *cat* in the zebrafish embryos. Quintaneiro et al. (2017) did not found  
508 either an effect of S-metolachlor on the catalase activity in zebrafish embryos exposed up to  
509 25 mg/L. In contrast, MOA seems to have an inhibiting effect on catalase activity of a water  
510 crustacean: crayfish exposed to low concentrations (4.2 µg/L) (Velisek et al., 2018a).

511

512 **5. Conclusion**

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

523

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