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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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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

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 LC₅₀ or EC₅₀ 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) (CaCl₂*2H₂O (294 mg/L),
131 MgSO₄*7H₂O (123.3 mg/L), NaHCO₃ (63 mg/L), KCl (5.5 mg/L) in Milli-Q water) was used
132 to prepare the final dilutions for the tests. RNA^{later}® (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)

	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)

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 MnCl₂ 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 (μm) 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

		μm									Absolute value
Endpoint											
Concentration $\mu\text{g/L}$		Body length	Edemas	Craniofacial deformation	Spine deformation	Non-inflated gas bladder	Tail deformation	Necrosis	Yolk sac malabsorption	Cardiovascular toxicity	Morphologica I score
S-metolachlor	<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	0.9 \pm 1.0
	<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	1.6 \pm 1.1
	<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	1.8 \pm 1.8
	<i>100</i>	3763 \pm 123	1.7 \pm 1.4	2.3 \pm 3.2	0.6 \pm 0.8	4.6 \pm 1.6*	0 \pm 0	0.6 \pm 0.8	4.0 \pm 0.8	0 \pm 0	2.0 \pm 1.1*
	<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	5.8 \pm 2.2*	0 \pm 0	2.2 \pm 0.8*
MOA	<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	1.9 \pm 1.5
	<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	1.8 \pm 1.9
	<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	1.3 \pm 0.8
	<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	3.0 \pm 1.4
	<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	2.1 \pm 1.6
MESA	<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	0.7 \pm 0.5
	<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	0.6 \pm 0.2
	<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	0.6 \pm 0.4
	<i>100</i>	3683 \pm 118	1.1 \pm 0.8	4.4 \pm 5.2*	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	2.0 \pm 1.7**
	<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	0.9 \pm 0.4
MIXTU RE	<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	3.2 \pm 1.7
	<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	3.1 \pm 1.1
	<i>30 (of each)</i>	3740 \pm 193	3.1 \pm 2.5	8.0 \pm 1.0	4.6 \pm 3.5*	6.7 \pm 5.2	0 \pm 0	3.0 \pm 2.3	12.2 \pm 8.6	1.9 \pm 1.6	5.9 \pm 2.0**

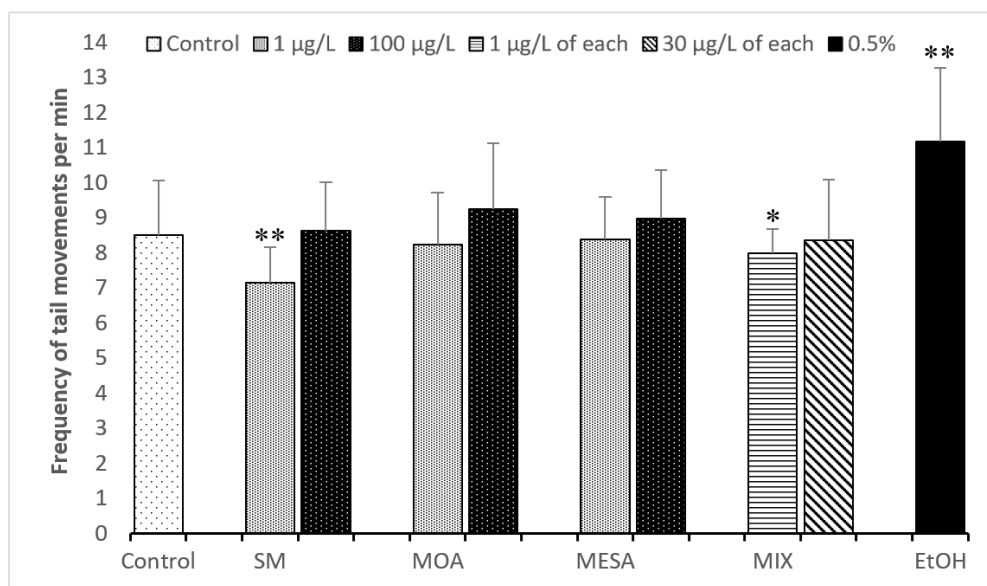
321 3.2 Light:dark locomotion test and heartbeat

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

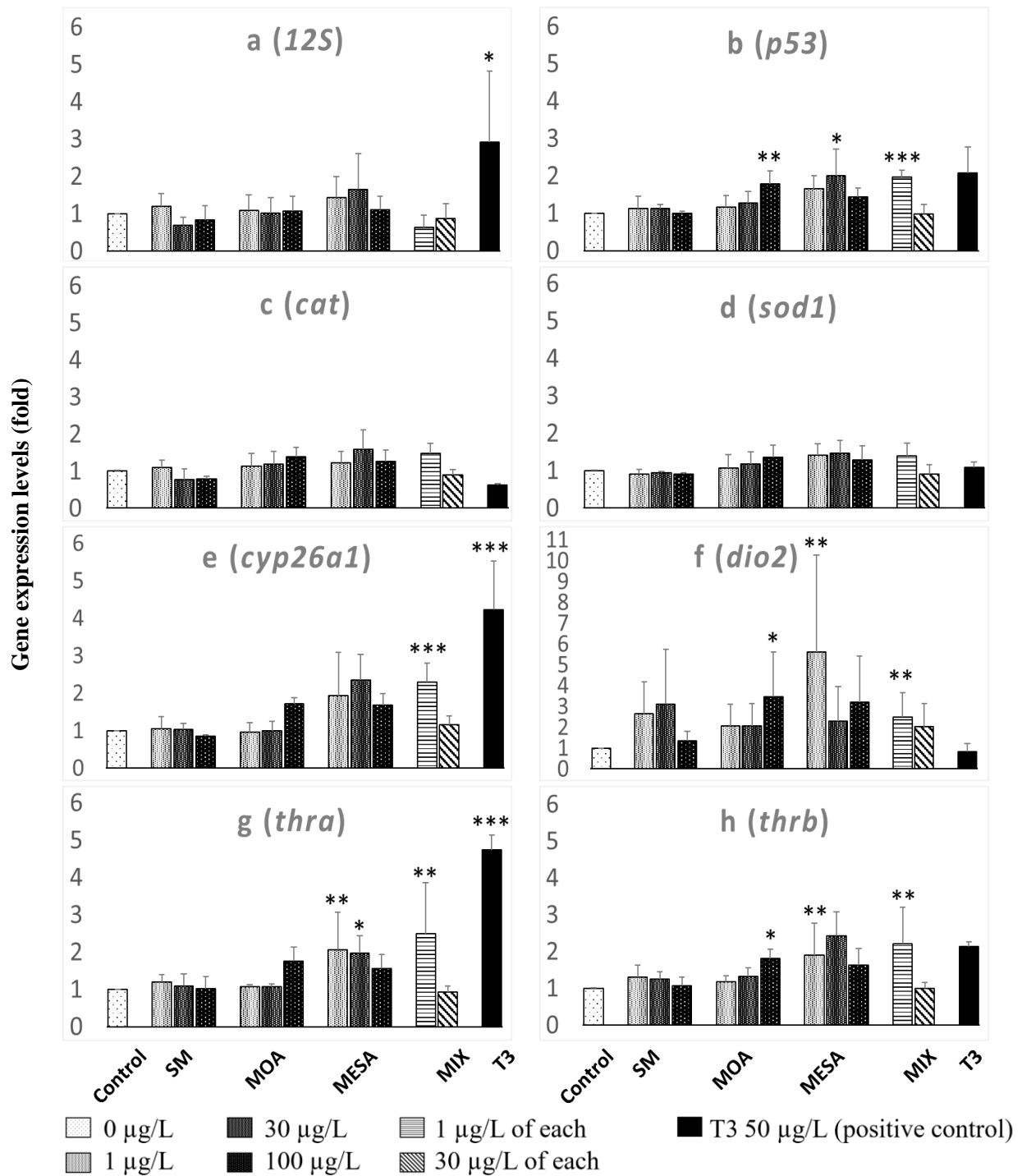
338

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 α and β 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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