

Supplementary Materials

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

AUTHORS

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Supplementary Figure S1: Morphological score endpoints



Supplementary Figure S1. Assessment of the “morphological score” endpoint on zebrafish embryos (120 hpf). To assess morphological changes, simple scoring system, modified from (Panzica-Kelly et al., 2010) was used. No malformations, 0 point (A); Mild craniofacial malformation, 1 point (B); Non-inflated swimming bladder and mild malabsorption of yolk sac, 2 points (C); Severe craniofacial malformation, malabsorption of yolk sac, no swimming bladder, 3 points (D); Severe craniofacial malformation, malabsorption of yolk sac, no swimming bladder, heart edema, 3 points (E); Severe craniofacial malformation, malabsorption of yolk sac, non-inflated swimming bladder, spinal and tail deformation, 3 points (F);

33 **Supplementary Table S1: Sequences, accession numbers, references and efficiency of**
 34 **used primers for qPCR analysis in zebrafish (*Danio rerio*)**

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Gene	Sequence (5'-3')	Accession number	Efficiency (amplification factor)	References
<i>β-act</i>	F: AAGTGCACGTGGACA R: GTTTAGGTTGGTCGTTTCGTTTGA	NM_131031	1.89	Dedeh <i>et al.</i> , 2015
<i>ef1a</i>	F: GGGAAAGGAAAAGACCCAC R: CGCTCGGCCTTCAGTT	NM_131263	1.90	Gentès <i>et al.</i> , 2015
<i>rpl13</i>	F: ACCCTTCCCCTGGATCA R: GTGGGTTTCAGACGCACA	NM_198143	1.92	Gentès <i>et al.</i> , 2015
<i>12S</i>	F: ACTTTCTACCCAGAAATATAC R: GCACCTTCCGGTACAC	NC_002333.2	1.91	Arini <i>et al.</i> , 2015
<i>p53</i>	F: GGGCAATCAGCGAGCAAA R: ACTGACCTTCTGAGTCTCCA	NM_131327	1.87	Gentès <i>et al.</i> , 2015
<i>cat</i>	F: CCTGTGGGGCGTTTTG R: CGGTACGGGCAGTTGAC	NM_130912	1.90	Lerebours <i>et al.</i> , 2009
<i>sod1</i>	F: TGAGACACGTCCGAGACC R: TGCCGATCACTCCACAGG	NM_131294	1.87	Gentès <i>et al.</i> , 2015
<i>cyp26a1</i>	F: AACTACATCCCCTTCGGAGGA R: TTGCAATGCTGCGTTAACTCA	NM_131146	1.95	Oliveira <i>et al.</i> , 2013
<i>dio2</i>	F: GCATAGGCAGTCGCTCATTT R: TGTGGTCTCTCATCCAACCA	NM_212789	1.24	Yan <i>et al.</i> , 2012
<i>thra</i>	F: CTATGAACAGCACATCCGACAAGAG R: CACACCACACACGGCTCATC	NM_131396	1.90	Yan <i>et al.</i> , 2012
<i>thrb</i>	F: TGGGAGATGATACGGGTTGT R: ATAGGTGCCGATCCAATGTC	NM_131340	1.98	Yan <i>et al.</i> , 2012

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37 **Supplementary Material S1:**

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39 **Supplementary Table S2:**

40 Expected nominal and measured concentrations of S-metolachlor and its two metabolites
 41 (MOA and MESA) on the beginning (0 dpf) and at the end (5 dpf) of the test. Stability (%) of
 42 pesticides during the five-day test is shown.

43 Details on the analytical method are provided below the table.

Pesticide	<i>Nominal</i> ($\mu\text{g/L}$)	<i>Nominal</i> (<i>nM</i>)	Measured (0 dpf) ($\mu\text{g/L}$)	Measured (5 dpf) ($\mu\text{g/L}$)	Stability (%)
S-metolachlor	<i>1</i>	<i>3.52</i>	1.46	1.35	91.9
	<i>30</i>	<i>106</i>	39.1	33.9	86.7
	<i>100</i>	<i>352</i>	116	112	96.1
	<i>300</i>	<i>1057</i>	296	267	90.0
MOA	<i>1</i>	<i>3.58</i>	1.02	0.97	95.5
	<i>30</i>	<i>107</i>	30.2	30.8	102
	<i>100</i>	<i>358</i>	105	107	102
	<i>300</i>	<i>1074</i>	321	303	94.4
MESA	<i>1</i>	<i>2.85</i>	1.74	1.76	102
	<i>30</i>	<i>85,4</i>	51.1	49.9	97.6
	<i>100</i>	<i>285</i>	162	162	100
	<i>300</i>	<i>854</i>	459	453	98.6

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45 **Analytical method details:** Concentrations were measured by LC-MS/MS. Samples of
 46 medium were first filtered using centrifuge filter (cellulose acetate; 0.22 μm) to purify them
 47 from chorion and other debris and filtrates were stored in glass vials at 20 °C upon the
 48 analysis. Analysis by LC-MS/MS was performed with a Waters LC chromatograph (Waters,
 49 Manchester, U.K.) and the chromatographic separation was achieved using a column Acquity
 50 BEH C18 of 100 x 2.1 mm ID and 1.7 μm particle size. A gradient elution method was set
 51 with a phase A (0.1% formic acid in water) and phase B (0.1% formic acid in methanol). The
 52 gradient elution started with 20% B, increased to 90% B over 9 min, held (90% B) to 11 min,
 53 then decreased to 20% B in 11 min and held for 4 min to equilibrate the system before the
 54 next injection. The flow rate was set at 0.3 mL/min and the injection volume at 5 μL . The
 55 column and sample temperatures were set at 35 °C and 10 °C, respectively.

56 Detection was performed on a Xevo TQ-S quadrupole mass spectrometer (Waters
 57 Manchester, U.K.) and analytes after ESI ionisation were detected in positive ion mode using
 58 tandem mass spectrometry. The following m/z transitions were monitored, for metolachlor:

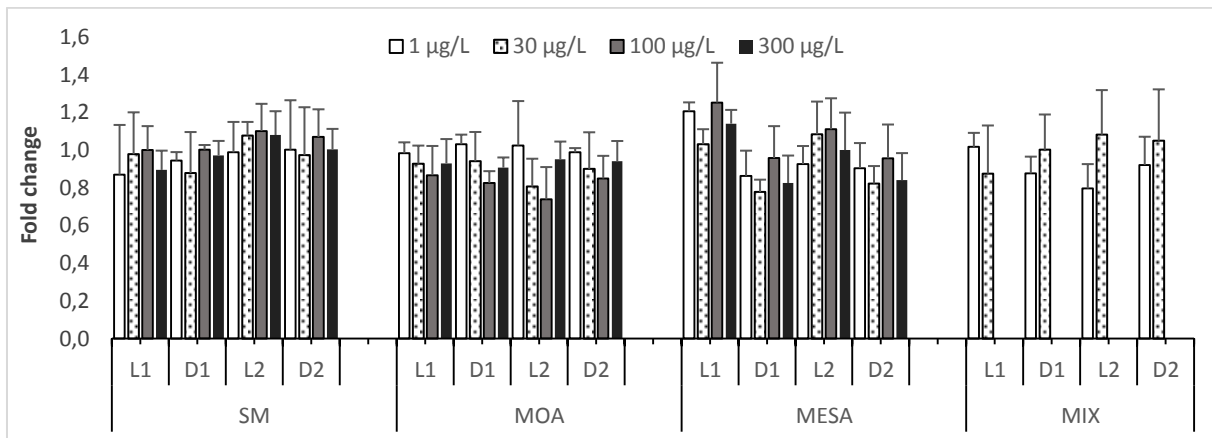
59 m/z 284.1 > 176.1, (quant.: cone voltage 30V; collision energy 25V) and m/z 284.1 > 252.1
60 (qual.: cone voltage 30V; collision energy 15V); for metabolite MOA: m/z 280.3 > 146.2,
61 (quant.: cone voltage 20V; collision energy 25V) and m/z 280.3 > 131.0 (qual.: cone voltage
62 20V; collision energy 30V) and m/z 280.3 > 119.0 (qual.: cone voltage 20V; collision energy
63 30V); for metabolite MESA: m/z 330.4 > 160.2, (quant.: cone voltage 25V; collision energy
64 30V) and m/z 330.4 > 145.5 (qual.: cone voltage 25V; collision energy 50V) and m/z 330.4 >
65 132.1 (qual.: cone voltage 25V; collision energy 50V). The capillary voltage was set at 3 kV
66 and the cone, desolvation and collision gas flows were set at 150 (L/h), 700 (L/h) and 0.15
67 (mL/min), respectively. The source and desolvation temperature were set at 150 °C and 400
68 °C, respectively. Data were processed by MassLynx™ software (Manchester, U.K.). The
69 analytes were quantified using external calibration of metolachlor, MOA and MESA (0.1 –
70 500 µg/L in 20% of methanol) and the limit of detection (LOD, signal to noise ratio S/N>3)
71 and quantification (LOQ, S/N>10) were 0.05 and 0.1 µg/L, respectively for each analyte.

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73 **Supplementary Material S2: Results of the locomotion analysis**

74 Total distance swam by the zebrafish larvae after five days of exposure to pesticides was
75 measured. High variability among tested organisms (32 fish per condition) was observed and
76 mean values of distance swam by control fish were different in the independent experiments.
77 The final values are reported as fold changes compared to control (Fold-change=1).

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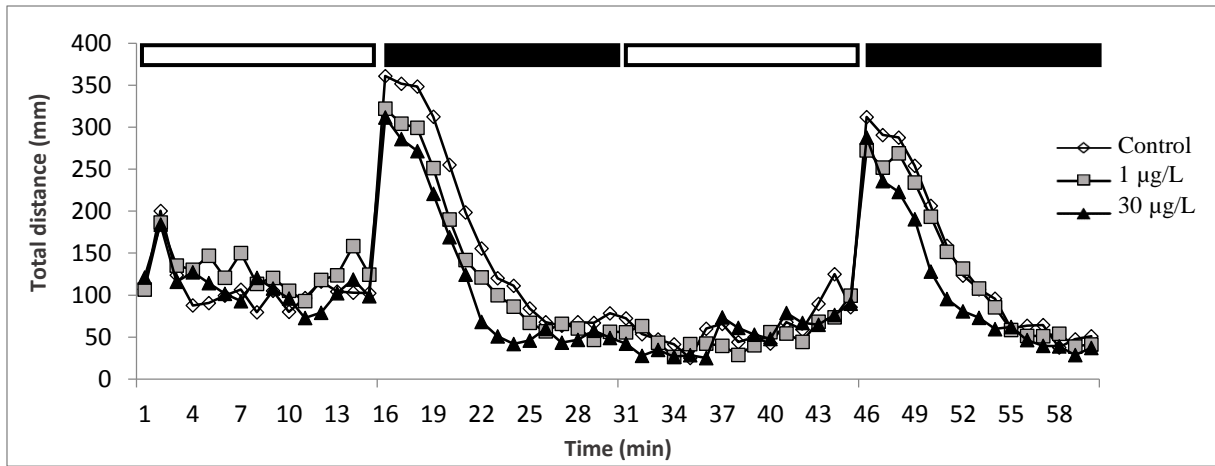


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80 **Supplementary Figure 2.** Total distance swam by zebrafish larvae exposed for five days to
81 S-metolachlor (SM), metolachlor oxanilic acid (MOA), metolachlor ethanesulfonic acid
82 (MESA) and mixture of the three substances (MIX)* presented as fold changes of the exposed
83 group vs control group. Each bar represents mean \pm SEM of three independent experiments.
84 One trial consisted of four 15 minutes light stimulation periods (L1 1st light period, D1 1st
85 dark period, L2 2nd light period, D2 2nd dark period). No significant difference was observed.
86 *The concentration in the mixture is 1 and 30 μ g/L of each substance.

87 No significant differences were observed in any of the conditions tested. Exposed fish swam
88 similar distances as control fish (the fold changes values are all situated around 1) with the
89 minimal and maximal value respectively 0.74 (MOA 100 μ g/L, L2 phase) and 1.25
90 (MESA 100 μ g/L, L1 phase).

91 Statistical differences from control were observed within some of the independent
92 experiments (Supplementary Figure S3) but they were not repeated during independently run
93 repetitions. For example, Figure 2 shows the results of a single experiment - the total distance
94 swam by zebrafish larvae exposed to two concentrations of MESA and subjected to four 15
95 minutes alternating trials of light-dark-light-dark. Zebrafish is triggered by switching off the
96 light and instantly become more active (more than 3 times fold) and gradually calms down
97 during the dark period. Statistical difference (Kruskall-Wallis test with Mann-Whitney post-
98 hoc with Bonferroni correction $p < 0.025$) is observed between control and 30 μ g/L of MESA
99 in the dark 1 phase (16-30 minutes of the trial).



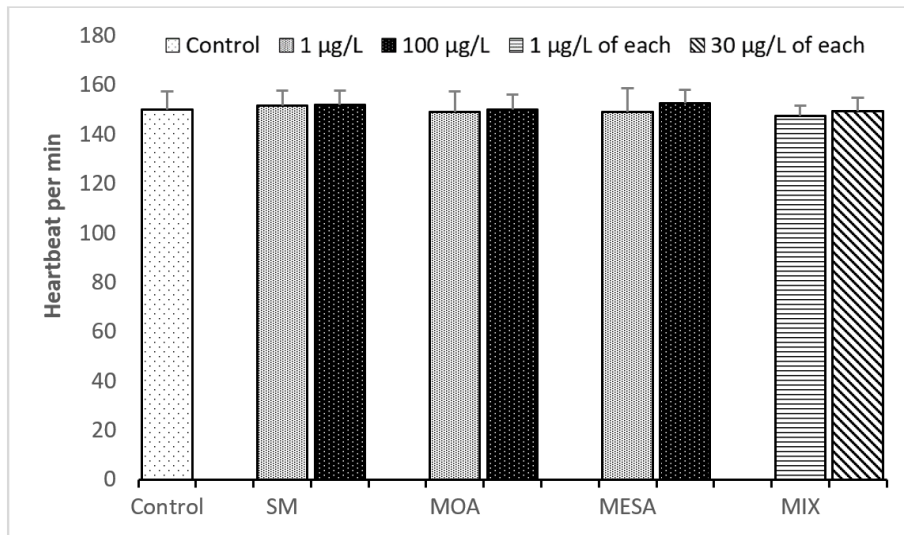
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101 **Supplementary Figure 3.** Total distance swam (mm) by zebrafish *larvae* exposed for five days to two
 102 concentrations of MESA and subjected to light-dark-light-dark alternating periods of 15 minutes.
 103 Values represent sum of distance of 32 fish per condition.

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105 **Supplementary Material S3: Results of the heart rate analysis**

106 No significant effects were found on the heart rate of zebrafish larvae. The heartbeat of non-
107 exposed larvae was 150.2 ± 7.2 bpm. The lowest (147.5 ± 4.3) and the highest (152.7 ± 5.4)
108 heartbeat was measured respectively in larvae exposed to environmental concentration of
109 MIX (1 $\mu\text{g/L}$ of each compound) and to the concentration of 100 $\mu\text{g/L}$ of MESA.



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111 **Supplementary Figure 4.** Heartbeat per minute in 3 dpf zebrafish larvae exposed to
112 SM: S-metolachlor; MOA: Metolachlor oxanilic acid; MESA: Metolachlor ethanesulfonic
113 acid; MIX: mixture of SM, MOA and MESA (concentration of 1 et 30 $\mu\text{g/L}$ of each substance
114 in the mixture)

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