

Interacting Effects of Polystyrene Microplastics and the Antidepressant Amitriptyline on Early Life Stages of Brown Trout (*Salmo trutta f. fario*)

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Received: 21 July 2020; Accepted: 20 August 2020; Published: date

Table S1. Size ranges (in μm) and counted particle numbers (per mL) of polystyrene particles in the stock suspension.

Size Range	Particle Number (per mL)
2–3	9067
3–4	6488
4–5	8872
5–6	4832
6–7	3770
7–8	3484
8–9	3076
9–10	2412
10–12	3259
12–14	2330
14–16	1628
16–18	1195
18–20	1081
20–25	1698
25–30	1041
30–35	643
35–40	473
40–50	511
50–60	220
60–70	89
70–80	43
80–90	18

90–100	6
100–150	5
150–200	0
200–250	0
250–300	0
300–350	0
350–400	0

Table 2. Volume of changed test medium in each Petri dish/tank during experiment 1 and experiment 2.

Experiment 1 part one 50 mL	Experiment 1 part one 100 mL	Experiment 1 part one 100 mL	Experiment 1 part two 2.5 L	Experiment 2 2.5 L
11.12.2016	09.12.2016	10.03.2017	12.05.2017	08.01.2018
13.12.2016	16.12.2016	12.03.2017	16.05.2017	12.01.2018
22.12.2016	18.12.2016	16.03.2017	19.05.2017	17.01.2018
28.12.2016	20.12.2016	18.03.2017	23.05.2017	31.01.2018
30.12.2016	24.12.2016	20.03.2017	26.05.2017	06.02.2018
01.01.2017	26.12.2016	22.03.2017	29.05.2017	09.02.2018
03.01.2017	09.01.2017	24.03.2017	01.06.2017	14.02.2018
05.01.2017	11.01.2017	26.03.2017		21.02.2018
07.01.2017	13.01.2017	28.03.2017		
15.01.2017	17.01.2017	30.03.2017		
19.01.2017	21.01.2017	01.04.2017		
23.01.2017	25.01.2017	03.04.2017		
27.01.2017	29.01.2017	05.04.2017		
31.01.2017	02.02.2017	07.04.2017		
04.02.2017	06.02.2017	09.04.2017		
08.02.2017	10.02.2017	11.04.2017		
12.02.2017	14.02.2017	13.04.2017		
16.02.2017	18.02.2017	15.04.2017		
20.02.2017	22.02.2017	17.04.2017		
14.03.2017	24.02.2017	19.04.2017		
23.04.2017	26.02.2017	21.04.2017		
	28.02.2017	25.04.2017		
	02.03.2017	27.04.2017		
	04.03.2017	29.04.2017		
	06.03.2017	01.05.2017		
	08.03.2017	03.05.2017		

Table 3. Average measured water parameters of the experiment 1b. Nitrate, NH₄-N, NH₄⁺ and NH₃ were only measured in mixed samples of all three blocks at the end of the experiment.

	pH	Temperature	Conductivity (μS/cm)	O ₂ (%)	O ₂ (mg/L)	Nitrate	NH ₄ -N [mg/L]	NH ₄ ⁺ [mg/L]	NH ₃ [mg/L]
Control	8.40 ± 0.14	6.37 ± 0.42	489.33 ± 9.55	107.60 ± 2.63	12.66 ± 0.29	1.47	0.99	1.27	1.20
100 particles/L	8.47 ± 0.11	6.55 ± 0.48	493.17 ± 4.10	107.07 ± 1.18	12.61 ± 0.26	-	0.93	1.20	1.13
10 ⁴ particles/L	8.51	6.30	496.67	107.83	12.81	-	1.05	1.35	1.28

	± 0.08	± 0.41	± 6.29	± 0.42	± 0.09				
10⁵ particles/L	8.52	6.32	494.17	107.53	12.76	-	0.93	1.19	1.13
	± 0.07	± 0.43	± 6.15	± 0.41	± 0.02				

Table S4. Average measured water parameters of the experiment 2. Nitrite, $\text{NH}_4^- \text{N}$, NH_4^+ and NH_3 were only measured at the end of the experiment. $\text{NH}_4^- \text{N}$, NH_4^+ and NH_3 were analyzed in mixed samples of all three blocks of each treatment.

	pH	Temperature	Conductivity ($\mu\text{S}/\text{cm}$)	O_2 (%)	O_2 (mg/L)	Nitrite	$\text{NH}_4^- \text{N}$ (mg/L)	NH_4^+ (mg/L)	NH_3 (mg/L)
Control	8.15 $\pm$ 0.14	7.19 $\pm$ 0.16	434.67 $\pm$ 21.56	94.90 $\pm$ 0.86	10.85 $\pm$ 0.13	0.05 $\pm$ 0.00	1.23	1.58	1.50
10^5 particles/L	8.26 $\pm$ 0.10	7.06 $\pm$ 0.20	433.67 $\pm$ 22.54	95.14 $\pm$ 0.79	10.94 $\pm$ 0.09	0.05 $\pm$ 0.00	1.50	1.92	1.82
10^6 particles/L	8.33 $\pm$ 0.05	7.01 $\pm$ 0.16	430.22 $\pm$ 21.15	95.28 $\pm$ 0.80	10.96 $\pm$ 0.09	0.05 $\pm$ 0.00	1.32	1.69	1.60
Amitriptyline (AMI2)	8.33 $\pm$ 0.05	7.14 $\pm$ 0.17	430.56 $\pm$ 12.75	95.08 $\pm$ 0.53	10.89 $\pm$ 0.04	0.05 $\pm$ 0.00	1.24	1.60	1.51
Amitriptyline + 10^5 particles/L	8.37 $\pm$ 0.02	7.07 $\pm$ 0.18	430.00 $\pm$ 14.02	94.96 $\pm$ 0.95	10.90 $\pm$ 0.11	0.05 $\pm$ 0.00	1.64	2.10	1.99
Amitriptyline + 10^6 particles/L	8.39 $\pm$ 0.03	6.87 $\pm$ 0.23	425.89 $\pm$ 13.13	95.00 $\pm$ 0.94	10.97 $\pm$ 0.11	0.05 $\pm$ 0.00	1.37	1.77	1.68

Table S5. Operating parameters of the triple quadrupole MS (Agilent 6490 QqQ) in positive mode.

Parameter	Set point
Gas temperature	250 °C
Gas flow	14 L/min
Nebulizer gas pressure	45 psi
Sheath gas heater	300 °C
Sheath gas flow	12 L/min
Capillary voltage	3500 V
Nozzle voltage	0 V

Table S6. Specific measurement parameters for amitriptyline with LC-QqQ in water samples. Intraday variations (RSD) is calculated with 1 $\mu\text{g}/\text{L}$ standard (10 μL injection volume and 4 replicates (n)). Limit of quantification = LOQ.

Precursor	Product ion (Quan/Qual) (m/z)	Collision Energy (setpoint in V)	Retention Time (min)	RSD (%) n = 4	LOQ (ng/L)
278.2	117.1/91	21/25	5.84	2.2	10

Table S7. Parameters for the determination of the lipid peroxide content in the different experiments.

Parameter	Experiment 1 – Part I	Experiment 1– Part II	Experiment 2
Dilution Factor	15	15	5
Sample Volume	25 μL	20 μL	25 μL
Incubation Time	180 min	90 min	240 min

Table 8. Used data transformations for the statistical analysis.

Experiment	Endpoint	Transformation
Experiment 1 – part I	body mass	x5
	heart rate	1/x
Experiment 1 – part II	body mass	x2
	length	x8
	FOX	sqrt(x)
	SOD	sqrt(x)
Experiment 2	no movement	x7
	body mass	1/x1/3
	FOX	1/x
	SOD	log(x)
	AChE	log(x)
	Cbe-pnpv	x2

Table 9. Summary of all p-values of the single comparisons of the different endpoints.

Endpoint	Group 1	Group 2	p-value	Endpoint	Group 1	Group 2	P-value
Time to hatch	C2	MP2ht	0.785709	Larvae resting on their side	C2	MP2ht	0.4048
	C2	MP2mio	0.580022		C2	MP2mio	0.01816
	C2	AMI2	<0.001		C2	AMI2	<0.001
	C2	MIX2ht	<0.001		C2	MIX2ht	<0.001
	C2	MIX2mio	<0.001		C2	MIX2mio	<0.001
	MP2ht	MP2mio	0.999439		MP2ht	MP2mio	0.2108
	MP2ht	MIX2ht	<0.001		MP2ht	MIX2ht	<0.001
	MP2mio	MIX2mio	<0.001		MP2mio	MIX2mio	<0.001
	AMI2	MIX2ht	0.995093		AMI2	MIX2ht	1.000
	AMI2	MIX2mio	0.998782		AMI2	MIX2mio	0.4972
	MIX2ht	MIX2mio	0.941511		MIX2ht	MIX2mio	1.000
Endpoint	Group 1	Group 2	p-value	Endpoint	Group 1	Group 2	P-value
Total distance moved	C2	MP2ht	0.385	Body contact	C2	MP2ht	1.00000
	C2	MP2mio	0.756		C2	MP2mio	0.99930
	C2	AMI2	< 0.001		C2	AMI2	0.08204
	C2	MIX2ht	< 0.001		C2	MIX2ht	0.02260
	C2	MIX2mio	< 0.001		C2	MIX2mio	0.06591
	MP2ht	MP2mio	0.993		MP2ht	MP2mio	0.99891
	MP2ht	MIX2ht	< 0.001		MP2ht	MIX2ht	0.02522
	MP2mio	MIX2mio	< 0.001		MP2mio	MIX2mio	0.02350
	AMI2	MIX2ht	1.000		AMI2	MIX2ht	0.99775
	AMI2	MIX2mio	0.998		AMI2	MIX2mio	1.00000
	MIX2ht	MIX2mio	0.999		MIX2ht	MIX2mio	0.99917
Endpoint	Group 1	Group 2	p-value	Endpoint	Group 1	Group 2	P-value
Mean velocity	C2	MP2ht	0.129	No movement	C2	MP2ht	0.628
	C2	MP2mio	0.505		C2	MP2mio	0.902
	C2	AMI2	< 0.001		C2	AMI2	<0.001
	C2	MIX2ht	< 0.001		C2	MIX2ht	<0.001
	C2	MIX2mio	< 0.001		C2	MIX2mio	<0.001
	MP2ht	MP2mio	0.976		MP2ht	MP2mio	0.996
	MP2ht	MIX2ht	< 0.001		MP2ht	MIX2ht	<0.001

	MP2mio	MIX2mio	< 0.001		MP2mio	MIX2mio	<0.001
	AMI2	MIX2ht	1.000		AMI2	MIX2ht	1.000
	AMI2	MIX2mio	0.992		AMI2	MIX2mio	0.781
	MIX2ht	MIX2mio	0.998		MIX2ht	MIX2mio	0.648
Endpoint	Group 1	Group 2	p-value	Endpoint	Group 1	Group 2	P-value
Length	C2	MP2ht	0.603	Body mass	C2	MP2ht	1.000
	C2	MP2mio	1.000		C2	MP2mio	0.963
	C2	AMI2	< 0.001		C2	AMI2	<0.001
	C2	MIX2ht	< 0.001		C2	MIX2ht	<0.001
	C2	MIX2mio	< 0.001		C2	MIX2mio	<0.001
	MP2ht	MP2mio	0.749		MP2ht	MP2mio	0.972
	MP2ht	MIX2ht	< 0.001		MP2ht	MIX2ht	<0.001
	MP2mio	MIX2mio	< 0.001		MP2mio	MIX2mio	<0.001
	AMI2	MIX2ht	0.664		AMI2	MIX2ht	0.895
	AMI2	MIX2mio	0.528		AMI2	MIX2mio	0.179
	MIX2ht	MIX2mio	1.000		MIX2ht	MIX2mio	0.795
Endpoint	Group 1	Group 2	p-value	Endpoint	Group 1	Group 2	P-value
AChE activity	C2	MP2ht	0.99698	CbE-pnpa activity	C2	MP2ht	0.91530
	C2	MP2mio	0.99944		C2	MP2mio	0.91530
	C2	AMI2	0.00776		C2	AMI2	0.00126
	C2	MIX2ht	0.00785		C2	MIX2ht	0.00126
	C2	MIX2mio	0.00746		C2	MIX2mio	0.00080
	MP2ht	MP2mio	0.99999		MP2ht	MP2mio	0.85775
	MP2ht	MIX2ht	0.03637		MP2ht	MIX2ht	0.00126
	MP2mio	MIX2mio	0.02260		MP2mio	MIX2mio	0.00042
	AMI2	MIX2ht	1.00000		AMI2	MIX2ht	0.88001
	AMI2	MIX2mio	1.00000		AMI2	MIX2mio	0.91473
	MIX2ht	MIX2mio	1.00000		MIX2ht	MIX2mio	0.91530
Endpoint	Group 1	Group 2	p-value				
CbE-pnpv activity	C2	MP2ht	0.859				
	C2	MP2mio	1.000				
	C2	AMI2	<0.001				
	C2	MIX2ht	<0.001				
	C2	MIX2mio	<0.001				
	MP2ht	MP2mio	0.936				
	MP2ht	MIX2ht	<0.001				
	MP2mio	MIX2mio	<0.001				
	AMI2	MIX2ht	1.000				
	AMI2	MIX2mio	0.796				
	MIX2ht	MIX2mio	0.760				

CRED Reporting

1. General information

a) Purpose of study

Evaluation of effects of polystyrene microplastic particles, the antidepressant amitriptyline, and the mixture of both on

b) Description of endpoints

Mortality, time until eyed ova stage and hatch, heart rate, swimming and resting behavior, length, weight, level of lipid peroxides (FOX), SOD activity, AChE activity, CbE activity

2. Test design

a) standard/modified standard

1. Experiment – part I on basis of OECD guideline 212 all other parts not performed according to standard procedures

b) Good Laboratory Practices (GLP)

Not GLP-accredited

c) Controls

negative control (90 fish in 3 replicate tanks)

d) Validity

Overall control mortality did not exceed 2%, oxygen concentration was over 60% and the temperature difference between the aquaria in each test was smaller than 1.5 °C.

3.1. Test compound – amitriptyline

a) Identification

Amitriptyline hydrochloride (CAS number: 549-18-8; Molecular Formula: $C_{20}H_{23}N \cdot HCl$)

b) Physico-chemical characteristics

Octanol/water partition coefficient: log Pow 5.0; melting point; 198 -200 °C

c) Source

Sigma-Aldrich, Lot: BCBV1175

d) Purity in %

 $\geq 98\%$;

e) Formulation

No formulation, no impurities

3.2. Test compound – polystyrene

a) Identification

Polystyrene pellets (Polystyrol 158 K)

b) Physico-chemical characteristics

Transparent, density: 1.05 g/mL

c) Source

BASF, Germany

4. Test organism

a) Scientific name

Salmo trutta f. fario

b) Body weight/length

Experiment 1 part I: 2.72 ± 0.18 cm and 0.15 ± 0.03 g; Experiment 1 part II: 2.92 ± 0.21 cm and 0.19 ± 0.04 g Experiment 2: 2.67 ± 0.13 cm and 0.14 ± 0.03 g

c) Age/life stage

Embryos, larvae and fry

d) Reproductive condition

Not in reproductive condition

e) Sex

Not determinable

f) Strain/clone	No defined clone
g) Source	Commercial trout farm (Forellenzucht Lohmühle, D-72275 Alprischbach-Ehlenbogen)
h) Acclimatization	No acclimatization

5. Exposure conditions

a) Schedule	Semi-static system for details see Table S1
b) System	Closed
c) Test medium	Experiment 1– part I artificial water (294 mg/L $\text{CaCl}_2 \times 2 \text{H}_2\text{O}$, 123.25 mg/L $\text{MgSO}_4 \times 7 \text{H}_2\text{O}$, 64.75 mg/L NaHCO_3 , 5.75 mg/L KCl); experiment 1 – part II and experiment 2 (tap water, filtered (iron filter, active charcoal filter, particle filter) cooled and aerated
d) Temperature	Climate chamber set to 7 °C, Experiment 1 – part I: 6.7 ± 0.2 °C, Experiment 1 - part II: 6.38 ± 0.45 °C, Experiment 2: 7.06 ± 0.20 °C
e) pH	Experiment 1 – part II: average pH= 8.47 ± 0.12 (measured 01.06.2017 and 07.06.2017); experiment 2: average pH= 8.31 ± 0.22 (measured: 29.12.2017, 21.02.2020 and 27.02.2020)
f) Hardness	Not measured
g) Conductivity	Experiment 1 – part II: average conductivity = 493.33 ± 7.30 $\mu\text{S}/\text{cm}$ (measured 01.06.2017 and 07.06.2017); experiment 2: average conductivity= 430.83 ± 17.24 $\mu\text{S}/\text{cm}$ (measured: 29.12.2017, 21.02.2020 and 27.02.2020)
h) Dissolved oxygen	Experiment 1 – part II: 12.71 ± 0.22 mg/L, $107.33 \pm 1.60\%$ (measured 07.06.2017); experiment 2: 10.92 ± 0.10 mg/L, $95.06 \pm 0.79\%$ (measured: 29.12.2017, 21.02.2020 and 27.02.2020)
i) Light intensity/quality	10 h:14 h light/dark cycle; In experiment 1 until eyed ova stages eggs were kept in complete darkness afterwards and in the second experiment tanks were shaded from direct light with cardboard or black plastic foil
j) Feeding	After consumption of the yolk sac fish were fed daily (3% of body weight) of

	commercially available trout feed (0.8 mm, Biomar, Brande, Denmark)
k) Aquaria	Experiment 1—part 1: Petri dishes filled with 200 mL test medium; experiment 1—part II and experiment 2: 12 L glass aquaria filled with 5 L of medium, covered with glass plane, silicone tubing and glass pipette for aeration
l) Sand/sediment	No sediment tested
m) Stock solutions	8.5 mg amitriptyline hydrochloride (7.5 mg amitriptyline) was solved in 1 L bidestilled water. For each aquarium containing amitriptyline 100 mL freshly prepared stock solution was used per 2.5 L water For polystyrene: 56,240 particles/mL
n) Nominal concentrations	C1: 0 particles/L; MP1 _h : 100 particles/L; MP1 _{ht} : 10 ⁴ particles/L; MP1 _{ht} : 10 ⁵ particles/L; C2: 0 particles/L and 0 µg/L amitriptyline; MP2 _{ht} : 10 ⁵ particles/L and 0 µg/L amitriptyline; MP2 _{mio} : 10 ⁶ particles/L and 0 µg/L amitriptyline; AMI2: 0 particles/L and 300 µg/L amitriptyline; MIX2 _{ht} : 10 ⁵ particles/L and 300 µg/L amitriptyline ; MIX2 _{mio} : 10 ⁶ particles/L and 300 µg/L amitriptyline
o) Measured concentrations	See Table 5
p) Method	LC-MS
q) Duration	Experiment 1- part 1: 150 d (07.12.2016–05.05.2017). Experiment 1 – part two: 33 d (05.05.2017–06.06.2017). Experiment 2: 60 d (29.12.2017–26/27.02.2018).
r) Observations	See material and methods
s) Results	See Table 3, Table 4, and Table 6 in the manuscript
t) Biomass loading	At the end of the experiments: experiment 1 part one: 0.75 g/L experiment 1 part two: 0.038 g/L experiment 2: 0.026 g/L

a) Replicates	Three replicate Petri dish/aquaria per test concentration
b) Number of organisms	30 individual fish per replicate
c) Design	Three blocks, one replicate per treatment present in each block, placed randomized within each block
d) Statistical methods	Mortality, time until eyed ova stage and hatch: mixed effects Cox models; length (experiment 1- part I) and CbE-pnpa (experiment 2) activity: Kruskal-Wallis test; resting behavior: likelihood-ratio test followed by fisher`s exact tests; body weight, length, SOD-activity, AChE activity, CbE activity, lipid peroxide level, heart rate, body contact total distance swum, mean velocity and time of no movement: linear mixed model
e) Biological response	Resting behavior: increased resting in ventral position of fish exposed to 10^6 particles/L reduced resting in ventral position in AMI2, MIX2 _{ht} , MIX2 _{mio} . Reduced time until hatch in AMI2 and mixture treatments. Reduced velocity and travelled distance as well as increased time of no movement and body contact in AMI2, MIX2 _{ht} , MIX2 _{mio} treatment groups. AChE-activity was increased and CbE-activity as well as body weight and length were reduced in amitriptyline and mixture treatments; No effect of any treatment group on heart rate, mortality, SOD-activity, lipid peroxide level.
f) Dose-response	Not tested
g) Statistical significances	Not determined
h) Significance level	$\alpha = 0.05$, in case of multiple comparisons adjusted via the method of Benjamini and Hochberg
i) Variability	Not estimated
j) Raw data	Provided on request