

The contribution of polymer additives to microplastic toxicity: A long-term study with *Oncorhynchus mykiss* exposed to polystyrene microparticles with different hexabromocyclododecane content

NIKOLA HODKOVICOVA^{1*} , JANA BLAHOVA², KAMIL STASTNY¹,
MICHAELA CHARVATOVA¹, ALES FRANC³, FRANTISEK TICHY⁴,
LUCIE SEDLACKOVA³, JAN MARES⁵, MARTIN FALDYNA¹, ZDENKA SVOBODOVA²

¹Veterinary Research Institute, Brno, Czech Republic

²Faculty of Veterinary Hygiene and Ecology, University of Veterinary Sciences, Brno, Czech Republic

³Faculty of Pharmacy, Masaryk University, Brno, Czech Republic

⁴Faculty of Veterinary Medicine, University of Veterinary Sciences Brno, Brno, Czech Republic

⁵Faculty of AgriSciences, Mendel University in Brno, Brno, Czech Republic

*Corresponding author: nikola.hodkovicova@vri.cz

The authors are fully responsible for both the content and the formal aspects of the electronic supplementary material. No editorial adjustments were made.

Electronic Supplementary Material (ESM)

Table S1. Composition of experimental feeds used throughout the exposure period

Table S2. List of primers used for real-time quantitative polymerase chain reaction

Table S3. Results of HPLC determination of the mass concentration of HBCD in particles

Table S4. Physico-chemical properties of feeds used throughout the adaptation and experiment

Figure S1. Optical microscope images of pure PS particles

Figure S2. Electron microscope images of pure PS particles

Figure S3. SEM images of the feeds used throughout the adaptation and experiment

Figure S4. Representative histological pictures of the liver of *O. mykiss*

Figure S5. Representative histological pictures of the cranial kidney of *O. mykiss*

Table S1. Composition of experimental feeds used throughout the exposure period

Sample (g)	Control I	Control II	PS-HBCD _{free}	PS-HBCD _{low}	PS-HBCD _{high}	HBCD _{only}
Feed pellets	3 200.0	3 104.0	3 136.0	3 136.0	3 136.0	3 103.4
PS-HBCD _{free} particles	–	–	64.0	–	–	–
PS-HBCD _{low} particles	–	–	–	64.0	–	–
PS-HBCD _{high} particles	–	–	–	–	64.0	–
Blend	–	96.0	–	–	–	–
Blend-HBCD	–	–	–	–	–	96.6
Purified water	60.0	160.0	60.0	60.0	60.0	160.0
Total	3 260.0	3 360.0	3 260.0	3 260.0	3 260.0	3 360.0

Commercial feed pellets (EFICO Enviro 921, 3 mm; Biomar, Denmark) were used as cores that were coated by liquid-addition powder coating technology with suitable material (PS microparticles, Blend, Blend-HBCD). The coating material “Blend-HBCD” was prepared by mixing Starch 1500® (Colorcon, USA), Aerosil® 200 (Evonik Industries, Germany) and HBCD, whereas “Blend” contained a mix of Starch 1500® and Aerosil® 200 only. In the end of the coating process, purified water was completely evaporated from all the feed pellets

HBCD = hexabromocyclododecane; HBCD_{only} = feed containing only HBCD in 1.0 mg/g concentration; PS = polystyrene; PS-HBCD_{free} = polystyrene microparticles without HBCD concentration; PS-HBCD_{high} = polystyrene microparticles with 1.0 mg/g concentration of HBCD; PS-HBCD_{low} = polystyrene microparticles with 0.2 mg/g concentration of HBCD

Table S2. List of primers used for real-time quantitative polymerase chain reaction

Primer	Gene name	Function	Sequence (5'-3')	Reference
60S	60S ribosomal protein	house-keeping gene	F: AGCCACCAGTATGCTAACCACT R: TGTGATTGCACATTGACAAAAA	Hodkovicova et al. (2021)
<i>cat</i>	catalase	oxidative stress	F: TGATGTCACACAGGTGCGTA R: GTGGGCTCAGTGTGTGTTGAG	Meiler et al. (2021)
<i>sod1</i>	superoxide dismutase type 1	oxidative stress	F: TGGTCCTGTGAAGCTGATTG R: TTGTCAGCTCCTGCAGTCAC	Meiler et al. (2021)
<i>sod2</i>	superoxide dismutase type 2	oxidative stress	F: TCCCTGACCTGACCTACGAC R: GGCCTCCTCCATTAAACCTC	Meiler et al. (2021)
<i>gst</i>	glutathione S-transferase	oxidative stress	F: TCGCTGACTGGACGAAAGGA R: CGAAGGTCTCTCAAGGCCATC	Meiler et al. (2021)
<i>casp3</i>	caspase 3	apoptosis	F: AGCTGCTTTACACTGTGTCTCAA R: GCTCAGCATCACACACAAAG	Foltz et al. (2009)
<i>cyp1a3</i>	cytochrome P450 family 1 subfamily A member 3	xenobiotic metabolism	F: CCCCTTCCGCCATATTTGTC R: CGGCCGAAGCACATTCC	Skillman et al. (2006)
<i>il1β</i>	interleukin 1 beta	immune response	F: ACATTGCCAACCTCATCATCG R: TTGAGCAGGTCTTGTCTCTG	Hodkovicova et al. (2021)
<i>il2</i>	interleukin 2	immune response	F: ATGCAACACCACATCAGCATGAC R: TGTCCGTTGTGCTGTTCTCTTA	Hodkovicova et al. (2021)
<i>il8</i>	interleukin 8	immune response	F: AGAATGTCAGCCAGCCTTGT R: TCTCAGACTCATCCCCCTCAGT	Hodkovicova et al. (2021)
<i>tnfa</i>	tumour necrosis factor alpha	immune response	F: GGGGACAAACTGTGGACTGA R: GAAGTTCTTGCCCTGCTCTG	Hodkovicova et al. (2021)
<i>thra</i>	thyroid hormone receptor alpha	marker of endocrine disruption	F: TGGGAGCTGATCCGCAATG R: CCTTGCTCCGTCGAC	Quesada-Garcia et al. (2014)
<i>vlg</i>	vitellogenin	marker of endocrine disruption	F: TGTAGCTTTGTAACCCCTGAGA R: TACCCACAACACCCCTTGGCATACT	Giraud et al. (2017)

60S= 60S ribosomal protein; *casp3* = caspase 3; *cat* = catalase; F = forward primer; HBBCD = hexabromocyclododecane; HBBCD_{only} = feed containing only HBBCD in 1.0 mg/g concentration; *il2* = interleukin 2; *il8* = interleukin 8; mRNA = messenger ribonucleic acid; PS = polystyrene; PS-HBBCD_{free} = polystyrene microparticles without HBBCD concentration; PS-HBBCD_{high} = polystyrene microparticles with 1.0 mg/g concentration of HBBCD; PS-HBBCD_{low} = polystyrene microparticles with 0.2 mg/g concentration of HBBCD; Q = quartile; R = reverse primer; *vlg* = vitellogenin

<https://doi.org/10.17221/95/2025-VETMED>

Table S3. Results of HPLC determination of the mass concentration of HBCD in a sample of PS particles without HBCD addition (PS-HBCD_{free}); PS particles with 0.2 mg/g of HBCD (PS-HBCD_{low}); PS particles with 1.0 mg/g of HBCD (PS-HBCD_{high})

Sample	Mass concentration of HBCD in PS (% w/w)	Mass concentration of HBCD in PS (mg/g)
PS-HBCD _{free}	–	–
PS-HBCD _{low}	0.023 7 ± 0.000 9	0.237
PS-HBCD _{high}	0.118 4 ± 0.002 5	1.184

HBCD = hexabromocyclododecane; HBCD_{only} = feed containing only HBCD in 1.0 mg/g concentration; PS = polystyrene; PS-HBCD_{free} = polystyrene microparticles without HBCD concentration; PS-HBCD_{high} = polystyrene microparticles with 1.0 mg/g concentration of HBCD; PS-HBCD_{low} = polystyrene microparticles with 0.2 mg/g concentration of HBCD

Table S4. Physico-chemical properties of feeds used throughout the adaptation (commercial feed pellets EFICO Enviro 921, 3 mm; Biomar, Denmark ~ cores) and the exposure period

Parameter (unit)	Cores	Control I	Control II	PS-HBCD _{free}	PS-HBCD _{low}	PS-HBCD _{high}	HBCD _{only}
Friability (%)	0.52	0.37	1.34	0.63	0.78	0.54	1.24
Bulk density (g/ml)	0.49 ± 0.01	0.56 ± 0.00	0.51 ± 0.02	0.53 ± 0.01	0.54 ± 0.02	0.54 ± 0.01	0.50 ± 0.00
Tapped density (g/ml)	0.52 ± 0.01	0.58 ± 0.01	0.53 ± 0.00	0.56 ± 0.00	0.57 ± 0.00	0.57 ± 0.00	0.52 ± 0.01
Hausner ratio	1.05	1.04	1.04	1.05	1.06	1.07	1.04
Minimal mass (mg)	21.30	24.93	2.20	23.40	24.40	23.00	25.90
Maximal mass (mg)	23.80	28.70	28.80	26.80	28.90	27.60	28.60
Average mass (mg)	22.61 ± 0.81	24.93 ± 1.74	27.21 ± 1.16	25.13 ± 1.12	26.44 ± 1.36	25.52 ± 1.48	27.07 ± 0.87
RSD of mass (%)	3.58	6.98	4.26	4.46	5.14	5.80	3.21
Pycnometric density (g/ml)	1.22 ± 0.01	1.22 ± 0.00	1.21 ± 0.00	1.21 ± 0.00	1.21 ± 0.00	1.23 ± 0.00	1.21 ± 0.00
Charge (nC)	0.76 ± 0.10	0.56 ± 0.09	−4.13 ± 0.26	−0.10 ± 0.04	−1.24 ± 0.21	−0.47 ± 0.10	−5.77 ± 0.36
Moisture content (%)	1.30 ± 0.04	0.59 ± 0.11	0.89 ± 0.11	0.79 ± 0.10	0.72 ± 0.05	0.66 ± 0.06	1.45 ± 0.11
Perimeter (mm)	14.29	13.55	14.47	15.06	14.81	14.92	14.44
Length (mm)	4.82	4.33	4.69	5.14	5.01	5.11	4.78
Width (mm)	2.37	2.65	2.60	2.40	2.42	2.36	2.51
Maximal projection (mm)	4.41	4.35	4.46	4.48	4.44	4.43	4.42
Minimal projection (mm)	3.34	3.48	3.59	3.62	3.60	3.54	3.49

HBCD = hexabromocyclododecane; HBCD_{only} = feed containing only HBCD in 1.0 mg/g concentration; PS = polystyrene; PS-HBCD_{free} = polystyrene microparticles without HBCD concentration; PS-HBCD_{high} = polystyrene microparticles with 1.0 mg/g concentration of HBCD; PS-HBCD_{low} = polystyrene microparticles with 0.2 mg/g concentration of HBCD

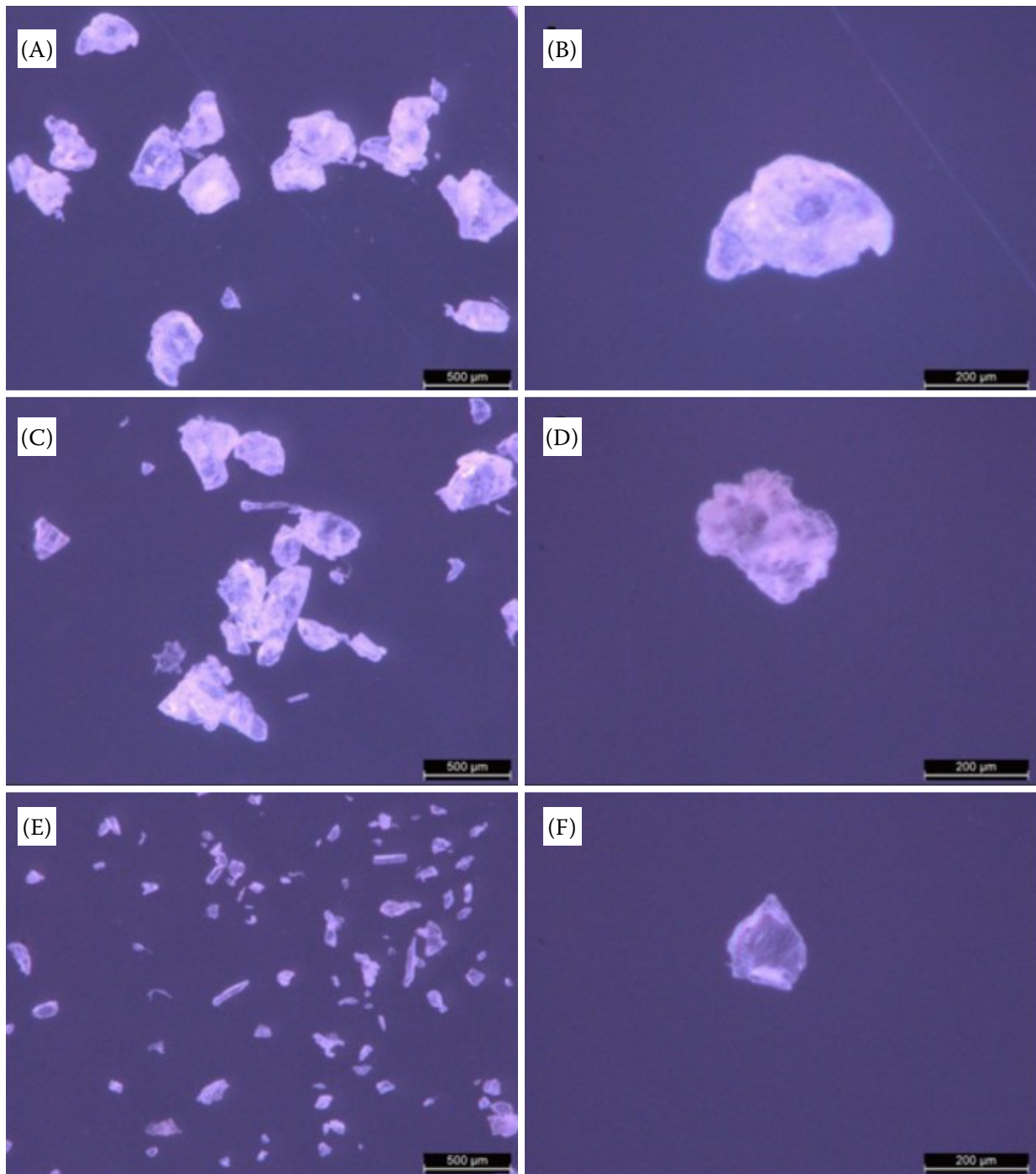


Figure S1. Optical microscope images of pure PS particles without HBCD – PS-HBCD_{free} group (A, B); and images of PS particles containing 0.2 mg/g – PS-HBCD_{low} group (C, D) and 1.0 mg/g HBCD – PS-HBCD_{high} group (E, F). Magnification 100× with a scale of 500 μm (left), and 300× with a scale of 200 μm (right)

HBCD = hexabromocyclododecane; HBCD_{only} = feed containing only HBCD in 1.0 mg/g concentration; PS = polystyrene; PS-HBCD_{free} = polystyrene microparticles without HBCD concentration; PS-HBCD_{high} = polystyrene microparticles with 1.0 mg/g concentration of HBCD; PS-HBCD_{low} = polystyrene microparticles with 0.2 mg/g concentration of HBCD

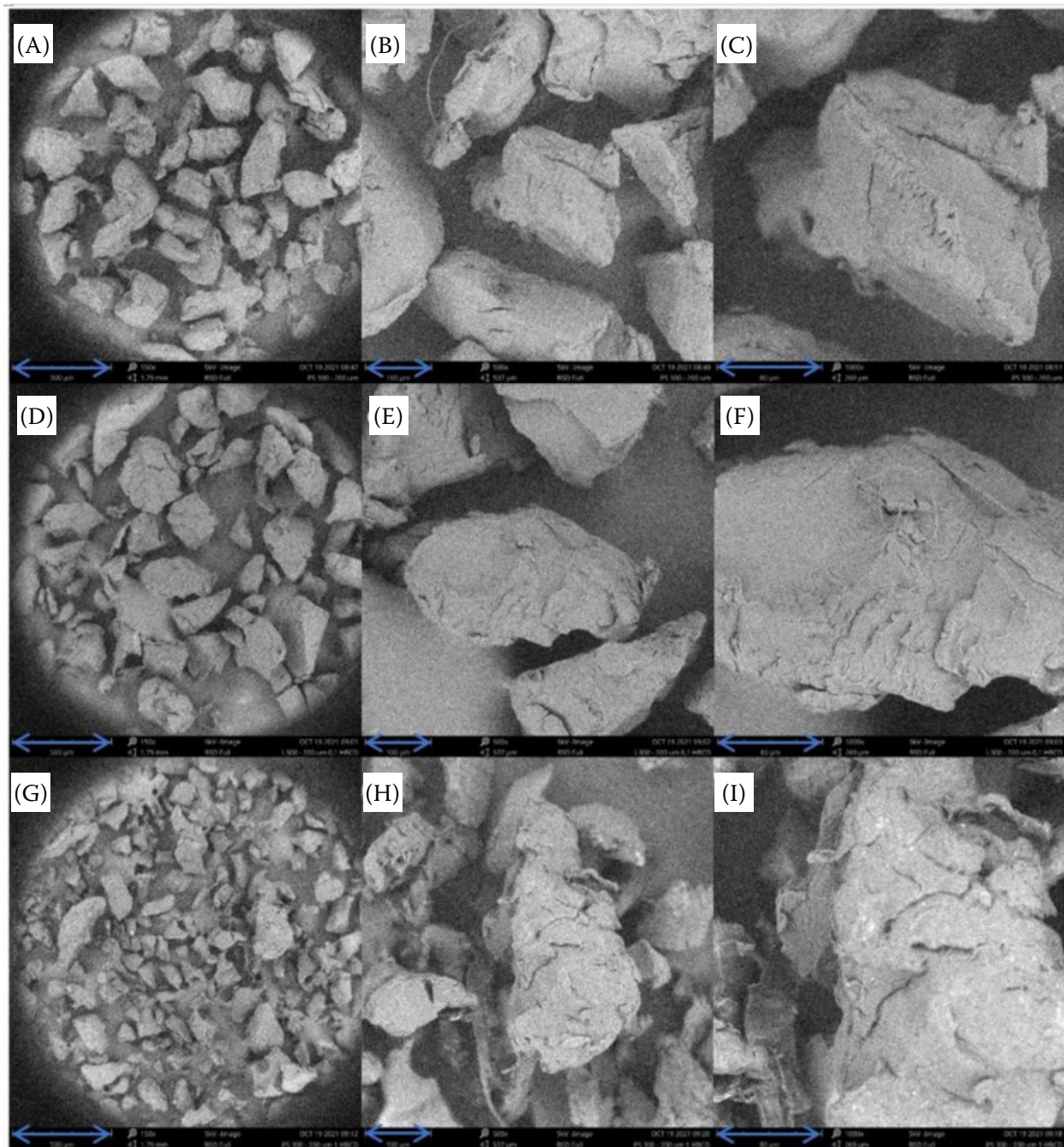


Figure S2. Electron microscope images of pure PS particles without HBCD – PS-HBCD_{free} group (A, B, C); and images of PS particles containing 0.2 mg/g – PS-HBCD_{low} group (D, E, F) and 1.0 mg/g HBCD – PS-HBCD_{high} group (G, H, I). Magnification 150× with a scale of 500 μm (left); magnification 500× with a scale of 100 μm (middle); and magnification 1 000× with a scale of 80 μm (right)

HBCD = hexabromocyclododecane; HBCD_{only} = feed containing only HBCD in 1.0 mg/g concentration; PS = polystyrene; PS-HBCD_{free} = polystyrene microparticles without HBCD concentration; PS-HBCD_{high} = polystyrene microparticles with 1.0 mg/g concentration of HBCD; PS-HBCD_{low} = polystyrene microparticles with 0.2 mg/g concentration of HBCD

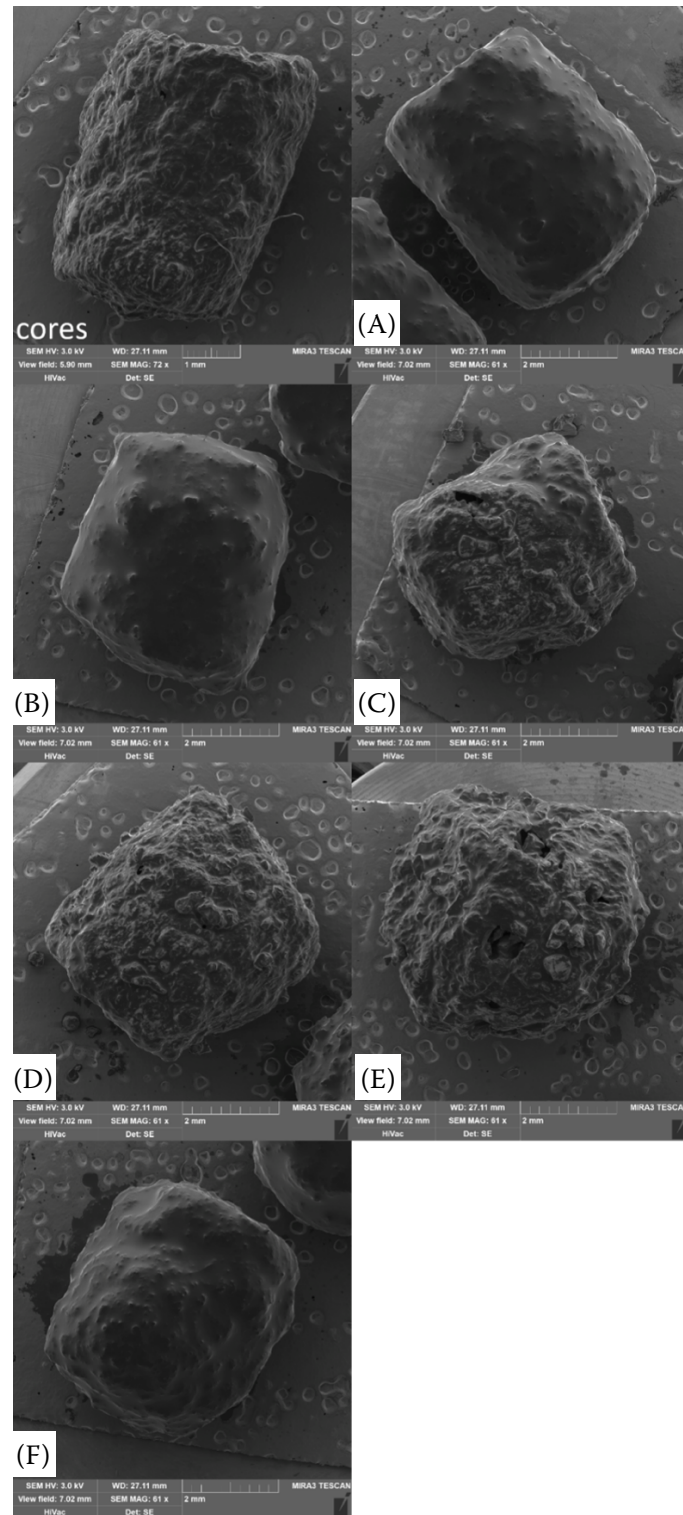


Figure S3. SEM images of the feeds used throughout the adaptation (commercial feed pellets ~ cores) and the exposure period. (A) Control I; (B) Control II; (C) PS-HBCD_{free} (i.e. PS 2 g/kg); (D) PS-HBCD_{low} (i.e. PS 2 g/kg with HBCD 0.2 mg/g); (E) PS-HBCD_{high} (i.e. PS 2 g/kg with HBCD 1.0 mg/g); (F) HBCD_{only} (i.e. HBCD 1.0 mg/g) HBCD = hexabromocyclododecane; HBCD_{only} = feed containing only HBCD in 1.0 mg/g concentration; PS polystyrene; PS-HBCD_{free} = polystyrene microparticles without HBCD concentration; PS-HBCD_{high} = polystyrene microparticles with 1.0 mg/g concentration of HBCD; PS-HBCD_{low} = polystyrene microparticles with 0.2 mg/g concentration of HBCD

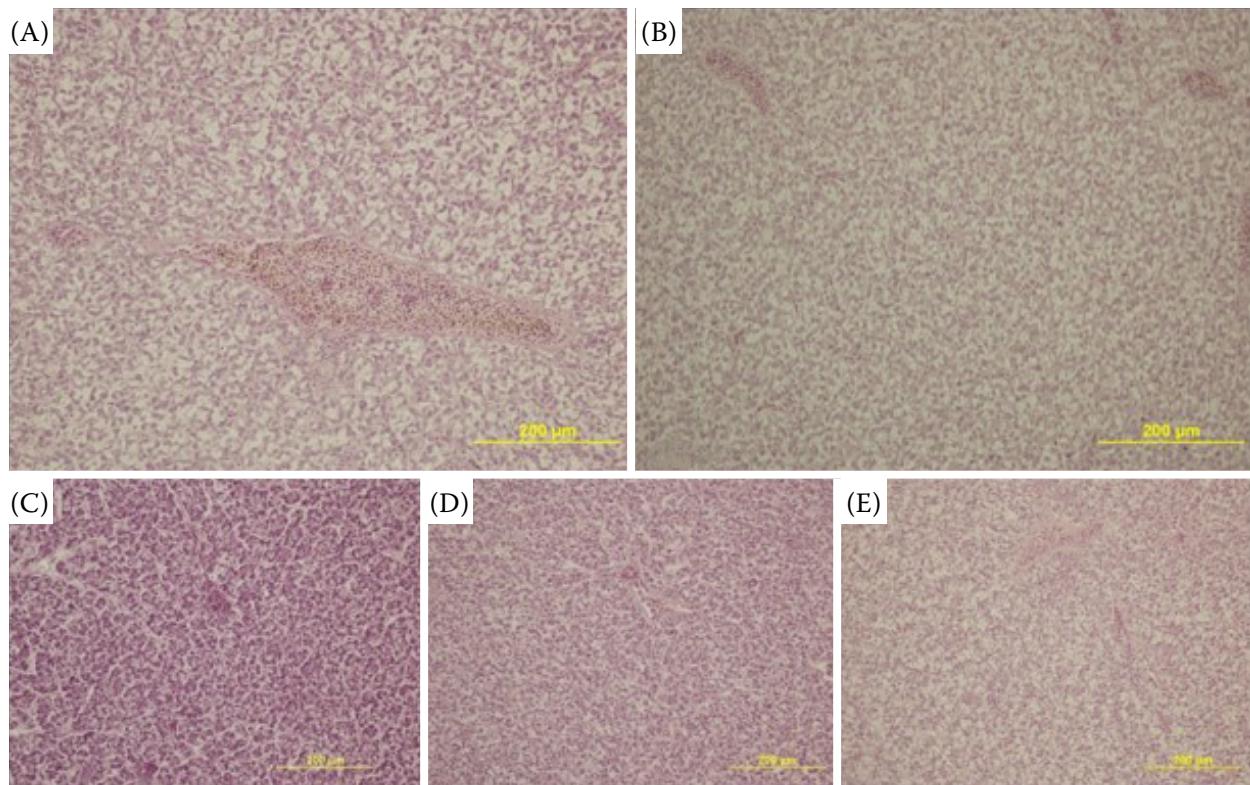


Figure S4. Representative histological pictures of the liver of *O. mykiss* after six weeks of oral exposure to PS particles (PS-HBCD_{free}), HBCD (HBCD_{only}) or their combination – PS-HBCD_{low} (i.e. HBCD in $c = 0.2$ mg/g) and PS-HBCD_{high} (i.e. HBCD in $c = 1.0$ mg/g)

Control group without pathological findings except for mild congestion (A); mild congestion and widespread dystrophy in PS-HBCD_{free} group (B); mild congestion and focal dystrophy in PS-HBCD_{low} group (C); mild congestion and widespread dystrophy in PS-HBCD_{high} group (D); mild congestion and widespread dystrophy in HBCD_{only} group (E). Magnification 100–200 $\times$, $n = 6$ /group

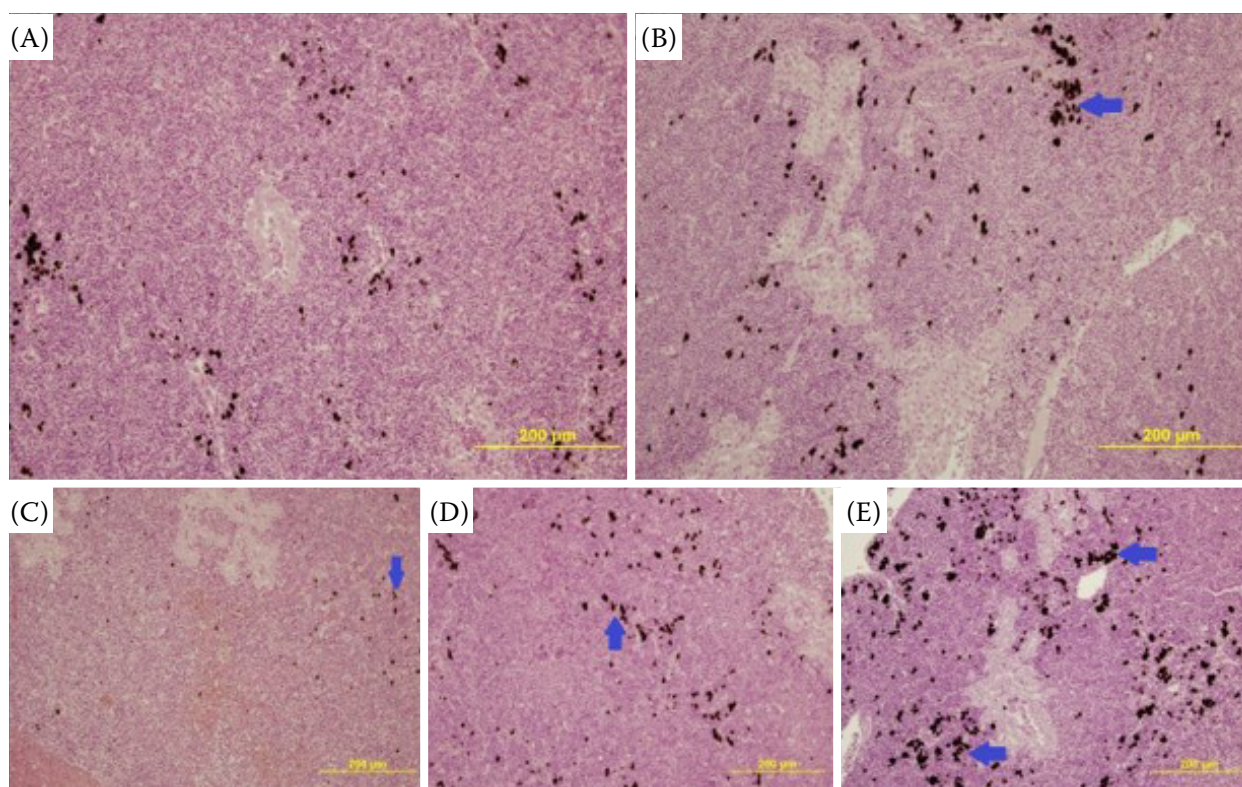


Figure S5. Representative histological pictures of the cranial kidney of *O. mykiss* after six weeks of oral exposure to PS particles (PS-HBCD_{free}), HBCD (HBCD_{only}) or their combination – PS-HBCD_{low} (i.e. HBCD in c = 0.2 mg/g) and PS-HBCD_{high} (i.e. HBCD in c = 1.0 mg/g) Mild tissue congestion and local pigment deposits in control (A); PS-HBCD_{free} (B); and PS-HBCD_{low} (C); higher presence of pigment deposits in PS-HBCD_{high} (D); and especially HBCD_{only} group (E). Magnification 100–200×, *n* = 6/group

REFERENCES

- Foltz JR, Plant KP, Overturf K, Clemens K, Powell MS. Detection of *Nucleospora salmonis* in steelhead trout, *Oncorhynchus mykiss* (Walbaum), using quantitative polymerase chain reaction (qPCR). *J Fish Dis.* 2009; 32(6):551-5.
- Giraud M, Douville M, Letcher RJ, Houde M. Effects of food-borne exposure of juvenile rainbow trout (*Oncorhynchus mykiss*) to emerging brominated flame retardants 1,2-bis(2,4,6-tribromophenoxy) ethane and 2-ethylhexyl-2,3,4,5-tetrabromobenzoate. *Aquat Toxicol.* 2017 May;186:40-9.
- Hodkovicova N, Hollerova A, Caloudova H, Blahova J, Franc A, Garajova M, Lenz J, Tichy F, Faldyna M, Kulich P, Mares J, Machat R, Enevova V, Svobodova Z. Do food-borne polyethylene microparticles affect the health of rainbow trout (*Oncorhynchus mykiss*)? *Sci Total Environ.* 2021 Nov 1;793:148490.
- Meiler KA, Cleveland B, Radler L, Kumar V. Oxidative stress-related gene expression in diploid and triploid rainbow trout (*Oncorhynchus mykiss*) fed diets with organic and inorganic zinc. *Aquaculture.* 2021 Feb 25; 533:736149.
- Quesada-Garcia A, Valdehita A, Kropf C, Casanova-Nakayama A, Segner H, Navas JM. Thyroid signaling in immune organs and cells of the teleost fish rainbow trout (*Oncorhynchus mykiss*). *Fish Shellfish Immunol.* 2014 Jul;38(1):166-174.
- Skillman AD, Nagler JJ, Hook SE, Small JA, Schultz IR. Dynamics of 17alpha-ethynylestradiol exposure in rainbow trout (*Oncorhynchus mykiss*): absorption, tissue distribution, and hepatic gene expression pattern. *Environ Toxicol Chem.* 2006 Nov;25(11):2997-3005.