Supplementary Information

DNA damage and molecular level effects induced by polystyrene (PS) nanoplastics (NPs) after *Chironomus riparius* (Diptera) larvae.

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Material and methods

RNA isolation

Total RNA was extracted from five fourth instar larvae exposed to PS NPs and from the control by a guanidine isothiocyanate-based method performed with a commercial kit (TRIzol, Invitrogen) according to the manufacturer's instructions. The five frozen larvae were homogenized in 300µl TRIzol. Next, 0.2 vol. chloroform was added to each sample, mixed and left for 5 min at room temperature. Subsequently, the samples were centrifuged for 15 min at 4 °C and 15000g. The aqueous phase was then transferred, and the RNA was precipitated with isopropyl alcohol (0.5 v/v), washed with 70% ethanol and resuspended in DEPC water. The RNA was treated with RNase-free DNase (Roche) (Martínez-Paz et al., 2017). The quality and quantity of total RNA were determined by agarose electrophoresis and absorbance spectrophotometry (Biophotomer Eppendorf). Finally, the RNA was stored at -80 °C until the complementary DNA synthesis.

Complementary DNA Synthesis

Complementary DNA (cDNA) was synthesized from 500 ng total RNA, 500 ng oligonucleotide (polyT) (Invitrogen, Waltham, MA, USA), 1 µL dNTP (10 mM) (Biotools, Madrid, Spain) and DEPC water in a final volume of 20 µL and incubated for 5 min at 65 C, cooled on ice. Subsequently, samples were centrifuged and 4 µL of 5X first strand buffer (Invitrogen, Waltham, MA, USA), 2 µL of DTT (Invitrogen, Waltham, MA, USA) and 0.5 µL of M-MLV enzyme (Invitrogen, Waltham, MA, USA) were added and the samples were incubated 50 min at 37 C. The reaction was inactivated for 15 min at -70 °C. The cDNA was stored at -20 °C until the PCR.

Real-time polymerase chain reaction (RT-PCR)

From cDNA, Real-time polymerase chain reaction (PCR) is performed to analyze mRNA expression. For this purpose, SsoFast EvaGreen Supermix (BioRad, Hercules, CA, USA) was used in a CFX96 thermal cycler (BioRad, CA, USA). The reaction used 50 ng of cDNA, forward and reverse oligonucleotides (2.5 µM) and 2X solution of dNTPs and reaction buffer (Morales et al., 2020). The oligonucleotides used are shown in Table 1. The reference genes used in this study were glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and ribosomal protein L13 (rpL13). Samples were analyzed in duplicate, and two replicates were made from each plate. Amplifications consisted of 30 s at 95 °C followed by 39 cycles of 95 °C for 5 s, 58 °C for 15 s and 65 °C for 10 s, including plate readout and a denaturation curve consisting of 0.5 °C increments from 65 to 95 °C for 5 s, each with plate readout.

List of Figure and Table

Table S1

Table S1. Oligonucleotides sequences.

Gene name	Gene symbol	Function	Efficiency	Sequence	Reference
Glyceraldehyde-3-phosphate dehydrogenase	*GAPDH*	Production of energy	94,1%	F: GGTATTTCATTGAATGATCACTTTG R: TAATCCTTGGATTGCATGTACTT	(Martínez-Paz et al., 2012)
Ribosomal protein L13	*rpL13*	Ribosomal subunit	97,3%	F: ACCAGCTAGAAAGCACCGTC R: ATGGGCATCTGACGATTGGG	(Morales et al., 2011)
Heat shock protein	*hsp90*	Stress response	108,1%	F: AGGCTGAAGCTGACAAGAATG R: TCATGCGATAAATGCGAGCAG	(Carrasco-Navarro et al., 2021)
Heat shock protein	*hsp70*	Stress response	102,8%	F: ACTTGAACCAGTTGAGCGT R: TTGCCACAGAAGAAATCTTG	(Morales et al., 2013)
Heat shock protein	*hsp27*	Stress response	94,4%	F: TCAACACACAGGACCG R: ATCCTTTATTGGTGATTAATTATG	(Martínez-Paz et al., 2014
Heat shock protein	*hsp60*	Stress response	105%	F: TGCTGTCCTTAAAGTCGGTGG R: TCCACCACCAGCAACGATTC	(Martín-Folgar et al., 2017)
X-ray repair cross complementing 1	*xrcc1*	DNA repair. SSB/DSB	109,7%	F: GACGATTTGCATTGGATAGT R: ATCAACATATCGCCATCAG	(Aquilino et al., 2018)
Ataxia-telangiectasia mutated	*ATM*	DNA repair. SSB	102%	F: ACATTTGGCGTAGATCAGGCA R: ACGAGATGCATCAAATCATGC	(Aquilino et al., 2018)
NEMO-like kinase	*NLK*	DNA repair. SSB	98,3%	F: CATCTCACCAGATCGTCTCT R: GAATTTATTTGATTATGCGGC	(Aquilino et al., 2018)
Death executioner caspase related to Apopain/Yama	*DECAY*	DNA repair. Apoptosis	97,9%	F: AAAGTGTTCCGATTATGGC R: TTCACACCAGTTAAAATCCAC	(Aquilino et al., 2018)
Super oxide dismutase	*SODMn*	Oxidative stress	107,2%	F: AAGTCGCTGCTGTTGGAGTT R: TGGAACTAAGCCGGTTGTGG	(Carrasco-Navarro et al., 2021)
FK506-binding protein of 39 kDa	*FKBP39*	Oxidative stress	100,2%	F: AGGCTGGGATATCGGACTCAT R: GTAAGCAAATGCAGGCGGG	(Carrasco-Navarro et al., 2021)
Death regulator Nedd2-like caspase	*DRONC*	Endrocrine system (20-E)	99,3%	F: GAAATGTCACAGATTTCAGTGCC R: GTGAATATCGTAAGCATGTTCTG	(Martín-Folgar and Martínez-Guitarte, 2019)
Chironomus riparius ecdysone receptor	*EcR*	Endrocrine system (20-E)	102,9%	F: TCTTCTCACGGCCATCGTCA R: GCTGCATCTTGTTTCGCCAC	(Planelló et al. 2008)
Ecdysteroid-regulated gene E74	*E74*	Endrocrine system (20-E)	93,5%	F: TCTTACTGAAACTTCTTCAAGATCG R: GCTTTGAGACAGCTTTGGAATCG	(Morales et al., 2013)
Ecdysone-induced protein 93F	*E93*	Endrocrine system (20-E)	107,1%	F: CGAGAACCGAAACCACAGCC R: GCGCTGCCATTGATGGATGATC	(Muñiz-González et al., 2021)
Hemoglobin C	*Hb C*	Hemoglobin production	109,25%	F: AACGTGGCTTTGGAGGTATG R: GTTGGCAAGGATCTCGACTC	(Ha and Choi 2008)
Prophenoloxidase	*ProPO*	Immune system	108%	F: CTCGAACAGCACCTTTGTCTG R: CCATGAATCGTTCACGCCATC	(Muñiz-González and Martínez-Guitarte 2020)

Table S2

Table S2. Genotoxic effects of PS NPs on the cells of *C. riparius* larvae measured by two comet assay parameters. The different concentrations of the nanomaterial and the exposure time is indicated. Significant differences from the corresponding control exposed groups (Control-SE) *p < 0.05; **p < 0.01; ***p < 0.001.

Time (h)	Concentration of PS NPs	Olive moment	% DNA in tail
24	Control-SE	1 ± 0,24	1 ± 0,57
	0,1ppm	25,53 ± 4,98***	8,01 ± 3,41***
	0,5ppm	36,45 ± 2,11***	8,50 ± 4,27***
	3ppm	88,80 ± 10,13***	15,60 ± 5,72***