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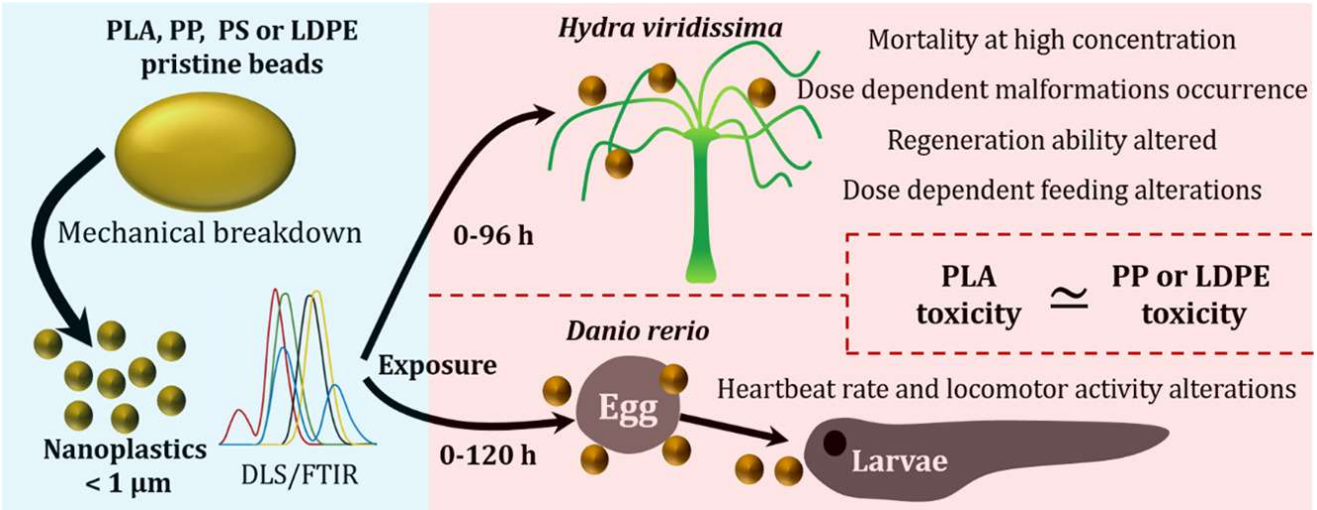

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Effects of petroleum-based and biopolymer-based nanoplastics on aquatic organisms A case study with mechanically degraded pristine polymers

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Abstract

Mismanaged plastic litter submitted to environmental conditions may breakdown into smaller fragments, eventually reaching nano-scale particles (nanoplastics, NPLs). In this study, pristine beads of four different types of polymers, three oil-based (polypropylene, PP; polystyrene, PS; and low-density polyethylene, LDPE) and one bio-based (polylactic acid, PLA) were mechanically broken down to obtain more environmentally realistic NPLs and its toxicity to two freshwater secondary consumers was assessed. Thus, effects on the cnidarian *Hydra viridissima* (mortality, morphology, regeneration ability and feeding behavior) and the fish *Danio rerio* (mortality, morphological alterations, and swimming behavior) were tested at NPLs concentrations in the 0.001 to 100 mg/L range. Mortality and several morphological alterations were observed on hydras exposed to 10 and 100 mg/L PP and 100 mg/L LDPE, whilst regeneration capacity was overall accelerated. The locomotory activity of *D. rerio* larvae was affected by NPLs (decreased swimming time, distance or turning frequency) at environmentally realistic concentrations (as low as 0.001 mg/L). Overall, petroleum- and bio-based NPLs elicited pernicious effects on tested model organisms, especially PP, LDPE, and PLA. Data allowed the estimation of NPLs effective concentrations and showed that biopolymers may also induce relevant toxic effects.

Keywords: Plastic degradation; Nanoplastics; Toxicity; Bioplastics; Freshwater organisms

1. Introduction

Plastic litter submitted to different environmental factors can be fragmented into smaller items know as microplastics (MPs; smaller than 5 mm) and nanoplastics (NPLs; smaller than 1 μm). These particles may pose a risk not only for the environment but also for human health (Hartmann et al., 2019; Mattsson et al., 2018; Mofijur et al., 2021). The behavior and fate of these small plastic particles in the environment and their effects to biota became a subject of intense scientific research (e.g., reviewed by Gangadoo et al., 2020). However, the available studies are clearly biased towards the marine compartment in comparison with its freshwater counterpart (e.g., Gangadoo et al., 2020; Tamayo et al., 2022a). Thus, considering that freshwater systems (e.g., rivers) may be major contributors to marine plastic pollution (Schmidt et al., 2017; van Emmerik and Schwarz, 2020; Gangadoo et al., 2020) and moderate to severely impacted by human activities (Best, 2019), it becomes crucial to understand the effects of small plastic particles on freshwater organisms, to aid the industry to shift towards really sustainable pathways and alert the society regarding the impact of mishandled/environmentally released plastics.

A total mass of nanoplastics ranging from 0.283 to 0.793 μg/L has been reported in a freshwater ecosystem, where polypropylene and polyethylene were the dominant polymers (Xu et al., 2022). Considering such low concentrations, it may be important to study not only relevant but also sensitive endpoints to prevent overlooking the impact of plastics in the environment (e.g., Scherer et al., 2017). Endpoints such as the regeneration ability of some organisms, malformations during ontogenetic development (that may forecast potential teratogenic effects) and altered swimming behavior have been proven very sensitive NPLs (Venâncio et al., 2022, Venâncio et al., 2021). Realistic exposure levels have been suggested to be below 1 mg/L, a concentration range that has been reported to elicit sublethal effects in aquatic biota (Sun et al., 2021). For instance, upon exposure to 15 μg/L of PS-NPLs, alterations on zebrafish swimming behavior have been reported (Santos et al., 2022). The available studies have also demonstrated that the effects of small plastic particles may be polymer-, size-, species-, developmental stage-, and/or endpoint-dependent. For

instance, using a single polymer type (polystyrene, PS) with different size categories, Scherer et al. (2017) reported that *Chironomus riparius*, with developmental stage increase, was able to ingest increasingly larger sized PS beads (1, 10, and 90 μm), whilst *Daphnia magna*, regardless of age, was unable to incorporate the largest PS beads.

Although particle shape may modulate the ecotoxicological profile (Tamayo et al., 2022b), most studies have focused on spherical particles, that are more easily acquirable (Neves et al., 2015; Cerasa et al., 2021; Guimarães et al., 2021a; Guimarães et al., 2021b). For instance, Qiao et al. (2019) that exposed 4-month-old zebrafish (*Danio rerio*), for 24 h, to 10 μg/L of differently shaped MPs (~5400 and ~680 particles/L for beads and fragments, respectively), reported dysbiosis in gut microbiota and shape-dependent accumulation of MPs in the gut. Fibers were the shape with higher accumulation and intestinal toxicity.

Petroleum-based plastics' reported effects have been promoting the reduction and the replacement of those plastics with materials considered more sustainable, such as plastics derived from natural resources (hereinafter biopolymers). There is thus, a strong tendency for biopolymers to be used in a widespread range of industries (Ang et al., 2020). However, their sustainability and use in industry has been questioned (Malafaia et al., 2021; de Oliveira et al., 2021;) since the toxicity of NPLs biopolymers and/or their interaction with biota is poorly studied (Ang et al., 2020; Ribba et al., 2022). For instance, Gonzalez-Pleiter et al. 2019, reported lethal and sublethal effects of secondary NPLs of the biopolymer polyhydroxybutyrate on *D. magna* and on two photosynthetic organisms (Anabaena sp., and *Chlamydomonas reinhardtii*). In the same way, Tamayo et al. (2022) reported sublethal effects on two cyanobacteria, triggered by NPLs and oligomers released from the biodegradable polymer polycaprolactone. Thus, taking into account the precautionary principle, one must use as a starting point the hypothesis that bioplastics may induce toxicity within the same range of magnitude as their traditional fossil-based counterparts.

Thus, the general hypothesis of this study was that NPLs will elicit biological alterations on the freshwater organisms, regardless of the polymer source (i.e., oil- or biobased). Therefore, the effects of NPLs from the three fossil-based polymers: low density polyethylene, LDPE; polypropylene, PP; and polystyrene, PS (selected based on their global relevance in terms of productions which, in 2021, rose up to respectively 19.3%, 26.9% and 5.3% of the plastic production (Plastics Europe, 2022) and a biopolymer, polylactic acid, (PLA; the most produced biodegradable and bio-based plastic, representing 20.9 % of the global production capacity of bioplastics in 2022) were tested on two secondary consumers (*Hydra viridissima*, invertebrate and *D. rerio*, vertebrate). *H. viridissima* plays an important role in freshwater food webs and was selected for this study due to its high sensitivity to a wide range of environmental pollutants (Trottier et al., 1997). The second organism used for the study, *D. rerio*, was selected based on its recognized value as biological model in biomedical studies, the genetic similarities to humans (Bhagat et al. 2020), transparent nature of the embryo and larvae (which provides excellent experimental advantages over other model organisms), as well as ability of perform high-throughput analysis of its locomotor activity. Focus was placed on various sublethal endpoints such as malformations development, regeneration capacity, feeding rate, heartbeat rate, and swimming behavior, as a way to provide more relevant information that can be integrated to derive more realistic risk values. Considering that most of the available studies have been performed with particles of regular shapes, mostly round particles, that are not environmentally relevant, this study assessed the effects of particles of irregular shapes, resulting from plastic mechanical degradation of larger plastics.

2. Materials and methods

2.1. Nanoplastics generation

Secondary NPLs were obtained from commercial pristine plastic materials purchased from Goodfellow Cambridge Ltd (Huntingdon, United Kingdom) in granular shape (~3-5 mm of diameter). Three of the most used plastic polymers (Plastics Europe, 2022) - LDPE (reference: ET31-GL-000100), PP (reference: PP30-SP-000120), PS (reference: ST31-GL-000111) - and one of the most promising biopolymers (Perumal et al. 2019) - PLA (reference: ME34-GL-000110) were selected for this study. As shown in Figure S1 (Supplementary Material, SM), to obtain the nano-sized particles, beads of each polymer were grinded using a stainless-steel blender. Firstly, in a stainless-steel glass, 50 g of beads were immersed in 400 mL of ethanol (96 %) and stored at -20 °C for 1 h. The material was then grinded for 3 min at 10,700 rpm and stored again at -20 °C for 1 h. This cycle was repeated until reaching 30 min of grinding time. Then, the supernatant (with high turbidity) was sequentially filtered through 103 μm and 25 μm stainless steel meshes (to avoid filter clogging), and through 1.6 μm Whatman glass microfiber filters (Grade GF/A), to isolate fragments in the nanometric range (below 1000 nm; Gigault et al., 2018). This filtrate was concentrated using Vivaspin 20 mL centrifugal concentrators with a 50 kDa molecular weight cut-off (MWCO) membrane (Sartorius AG, Goettingen, Germany). The concentrated secondary NPLs, retained by the membrane, were then washed by performing 2 additional centrifugations with clean ethanol (100%). The cleaned secondary NPLs were kept at 60 °C for 24-48 h to evaporate the ethanol. The amount of nanosized material obtained was dried and weighed before stock preparation. The stock

suspensions (1 g/L) of the polymers tested in this study were prepared in the culture media of the two species tested (*H. viridissima* and *D. rerio*), supplemented with 0.0005 % w/w of sodium dodecyl sulphate (SDS), to prevent the aggregation of the nanoplastics. The stocks were mixed in a vortex and ultrasonicated, during 10 min, in an ultrasound bath before use.

2.2. Physicochemical characterization of nanoplastics

The dried nanometric fraction of each plastic polymer was placed over potassium bromide (KBr) discs to obtain the Fourier Transformation Infra-Red (FTIR) spectra, to validate the characteristic peaks of the corresponding polymer. The spectra were obtained using a FTIR BRUKER TENSOR 27 (Billerica, Massachusetts, US), working in transmittance mode and the 4000–50 cm^{-1} range with a resolution of 4 cm^{-1} taken, using 128 scans. Data were analyzed using the software OriginPro 8.5.0. SR1. To study the colloidal status during the assays, particle size distribution was assessed based on the hydrodynamic size (usually slightly higher than the real size) obtained by dynamic light scattering (DLS). ζ-potential was obtained by electrophoretic light scattering (ELS) using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). Measurements were performed at the minimal concentration yielding a reproducible output (10 mg/L), in each culture media used in the toxicity assays (described below), in the absence of organisms, as well as in ultra-pure water (in all cases supplemented with 0.0005 % w/w SDS to prevent rapid NPLs aggregation), at 0 h (after 10 min of bath sonication), 24 h, 48 h, 72 h, and 96 h. The cuvettes were sterilized with ethanol (70 %) before use and kept covered in darkness and static conditions until analyses. Five measurements were conducted for each sample.

2.3. Biological models

2.3.1. Hydra viridissima

The freshwater cnidarian *H. viridissima*, a secondary consumer, was selected for this study, based on previously demonstrated sensitivity to NPLs (Venâncio et al., 2022). This organism allows the study of a variety of endpoints (e.g., regeneration and malformations) that are generally not considered in the ecotoxicological evaluation of NPLs, but that reflect the expression of mechanistic disruptions at molecular and biochemical levels.

Cultures of *H. viridissima* were kept at controlled conditions of temperature and light (20 °C ± 1 and 16L :8D photoperiod), in reconstituted artificial medium, following the description of Trottier et al. (1997). The culture medium was renewed twice a week, immediately after feeding, to avoid degradation of culture medium resulting from uneaten food items and fungi development. *Artemia salina* nauplii (instar II, < 24h, obtained from commercially acquired cysts hatched under brackish optimal conditions (20 g/L of NaCl), under constant light, at 23 °C) were used as fresh live prey.

2.3.2. Danrio rerio

Embryos of the freshwater fish *D. rerio* were also selected as a biological model for this study, based on its rapid embryonic development, chorions' transparency that allows continued monitoring of the different developmental stages under contaminant exposure, and the species' high DNA sequence similarity with humans (approximately 70% of human genes have one clear zebrafish orthologue (Howe et al., 2013)), which may allow to potentially translate effects to humans.

Eggs of *D. rerio* were obtained from mature adults, maintained in a continuous water-carbon filtered and flowing system, at the facility established at the Department of Biology of Aveiro University. The physical and chemical parameters of the water are monitored constantly, and range as follows: temperature of 27 °C ± 1, pH of 7.5 ± 0.5 and electrical conductivity of 750 ± 50 µS cm^{-1}. The dissolved oxygen (DO) saturation level is always above 95%. Daily, fish are fed with a commercial artificial diet (ZM 400 Granular). After natural mating, the eggs are collected (within a 30 to 45-min interval after spawning), rinsed twice with sterilized water from the fish system (to remove debris) and carefully selected under the stereomicroscope (Stereoscopic Zoom Microscope-SMZ 1500, Nikon Corporation), to exclude non-fertilized eggs, embryos with deformities, wounded or with arrested development.

2.4. Ecotoxicological assays

Ecotoxicological assays were performed following the scheme shown in Figure S2 (SM).

2.4.1. Assays for H. viridissima

Two 96 h assays, assembled in 24-well plates, were performed with the freshwater cnidarian, using a standard artificial media commonly utilized for routine lab-cultures. Considering that for the nanometer size range, real environmental concentrations are still unknown, in an attempt to obtain effects that allow establishing toxicity ranking for the tested polymers, the concentrations tested were: 0.001, 0.01, 0.1, 1, 10, and 100 mg/L. A control with organisms exposed to 0.0005 % w/w of SDS (denoted as CTR-SDS) and a negative control (organisms exposed to the culture medium without NPLs) were also tested. Each treatment had six replicates, each with 2 mL of NPLs suspension per well (or only culture media or culture media supplemented with 0.0005 % w/w SDS in case of the CTR-SDS, to assure that the SDS concentration was not detrimental to the organisms), with one organism per replicate. In the first 96-h assay mortality and morphological evaluation was performed, after which post-exposure feeding was tested. In the second 96-h assay, the regenerative capacity was studied, followed

by a post-exposure feeding assay. Both are described in detail below

Assay I: Mortality and morphological evaluation. The morphology of the hydras was evaluated every 24 h, using a stereomicroscope following Wilby's classification as a reference (Wilby and Tesh, 1990). Briefly, the classification reference table has 10 possible score, with a score of 10 indicative of a healthy/normal organism and a score of 0 attributed to a disintegrated organism. A score between 10 and 6 corresponds to reversible and sublethal morphological alterations whereas a score equal or lower than 5 is indicative of an irreversible situation (Blaise and Kusui, 1997). After the 96-h exposure, hydras (with scores equal or higher than 6) were gently washed, and the test media was replaced by 2 mL of clean media (i.e. culture medium without any NPLs), to assess post-exposure effects on feeding. Ten nauplii of *A. salina* (instar II) nauplii were added per replicate and hydras were allowed to feed for 30 min, in the dark. After this period, the remaining nauplii were counted and feeding rate was calculated by subtracting the remaining number of preys to the initial number (n=10).

Assay II: Regenerative capacity. Immediately before the start of the assay, the head of all organisms was cut on ice with the help of a scalpel. After this procedure, each lower part of the body (called columna, constituted by the gastric region, budding region, basal disc, and foot) was exposed to the treatments previously described and its regeneration was evaluated using a stereomicroscope, every 24 h, over a 96-h exposure period time. In this period, a hydra may regenerate a whole specimen (Traversetti et al., 2017; Wilby and Tesh, 1990). After 96 h, a post-exposure feeding assay was performed, with hydras with scores equal or higher than 6, as described in assay I.

2.4.2. Assays with zebrafish

A 96-h zebrafish embryo acute toxicity assay (FET) was carried out for each polymer, following the procedure recommended by the OECD guideline 236 (OECD, 2013). Six concentrations (suspensions of 0.001, 0.01, 0.1, 1, 10 mg/L in sterilized carbon-filtered water, collected from the zebrafish breeding system), a control (0.0005 % w/w of SDS in sterilized carbon-filtered water, collected from the zebrafish breeding system - denoted as CTR-SDS - and a negative control - carbon-filtered water, collected from the zebrafish breeding system, without NPLs) were tested. Briefly, per treatment, ten embryos (4 hours post-fertilization, hpf) were placed individually in 24-well plates. Each well contained 2 mL of the corresponding test medium described above. The test medium was not renewed during the assay, nor the organisms fed since the yolk sac provides enough nutrients at this stage. Assays were carried out at 26 ± 1 °C, with a 16L:8D photoperiod. Every 24 h, organisms were observed under a stereomicroscope (Stereoscopic Zoom Microscope SMZ 1500, Nikon Corporation, Japan) and the following endpoints assessed: a) survival, b) malformations (head, tail, spine), c) tail detachment and d) hatching success (OECD, 2013). The heartbeat rate was assessed after 48 h exposure, using a stereomicroscope as described elsewhere (Andrade et al., 2016). The locomotor activity of the organisms maintained under the experimental conditions described for FET, was assessed at 120 hpf, using the Zebrabox equipment (Viewpoint Life sciences, Lyon, France), with infrared illumination that allows tracking fish movement during both light and dark periods. Before activity recording, 2 min of acclimatation was allowed, followed by 5 min of light period and then 5 min of darkness. Data were automatically collected each 60 s using a video tracking system. Behavioral endpoints, shown in Table 1, were analyzed as described elsewhere (Liu et al., 2020; Ulhaq et al., 2013).

Table 1. Locomotor behavioral endpoints.

Endpoint	Acronym	Description
Activity counts (n)	AC	Number of times the activity of the larvae goes above the minimum threshold level of movement. Average of five periods of 60 s.
Swimming time (s)	TST	Total time the larvae exceeded the minimum threshold level of movement. Average of five periods of 60 s.
Swimming distance (mm)	TSD	Total swimming distance of the larvae when exceeding the minimum threshold level of movement. Average of five periods of 60 s.
Swimming speed (mm/s)	TSS	Swimming distance per total swimming time. Average of five periods of 60 s.
Turning frequency (turns/s)	TF	Total turn number per period of 60 s. Average of five measurements

2.5. Statistical analysis

Data collected from the bioassays were checked for assumptions of normality (ShapiroWilk test) and equality of variance (Levene's test). If assumptions were met, a One-Way ANOVA followed by Dunnett's test was applied to study differences between the negative control (CTR) and the positive control (CTR-SDS). Then, if no differences between controls were verified, the following comparisons were made between CTR and NPLs treatment groups ($p < 0.05$). If assumptions were not met, a non-parametric ANOVA was applied, followed by Dunn's test ($p < 0.05$). Concentration-response curves were obtained using R software (drc package); experimental results were fitted

to the most accurate model from which effective concentrations (mg/L) causing 10, 50, and 90% of effect (EC_{10}, EC_{50}, and EC_{90}, respectively) were obtained also using R software. The median lethal concentrations (LC_{50}) and respective confidence limits at 95% (95% CL) were computed through Probit regression using Pri Probit software (Sakuma, 1998). Principal Component Analysis (PCA) was performed to address the correlation between exposure concentrations by polymer type and *D. rerio* locomotor activity under both lightness and darkness conditions, using the mean values obtained after analyzing the bioassays. The PCA was performed in RStudio software (Version 1.4.1717) where the function "prcomp" was applied with the scaled data to obtain the principal components (PC). Only PCs with an eigenvalue > 1 were considered for the selection of the most important indicators. The parameters used for the analysis were: polymer type (PLA; PP; PS; and LDPE), endpoint (activity counts, swimming time, swimming distance, swimming speed, turning frequency) and the six concentrations tested (from 0.001 to 10 mg/L). In both conditions, lightness and darkness, the two principal component (PC) selected (PC1 and PC2) explained at least the 60 % of the observed variance.

3. Results

3.1. Physicochemical characterization

After the grinding process and the subsequent washes, the nanometric fragments of each polymer were chemically characterized using ATR-FTIR. As show in Figure 1, in PLANPLs, peaks were observed at 2999 and 2949 cm^{-1}, resulting from $-CH_3$ asymmetric and symmetric stretching vibrations, respectively. An intense peak was found at 1751 cm^{-1}, due to C=O vibration. The three peaks at 1456, 1385 and 1363 cm^{-1} can be associated to the $-CH_3$ asymmetric and symmetric bending vibrations. The four peaks at 1046, 1080, 1132 cm^{-1} and 1181 cm^{-1} correspond to C-O stretching vibration and those at 872 and 758 to a CCOO stretching vibration and C-O bending vibration, respectively. The spectrum of PP-NPLs displayed four characteristic peaks in the range 3000–2800 cm^{-1}: at 2960 and 2877 cm^{-1}, attributed to $-CH_3$ asymmetric and symmetric stretching vibrations respectively, while the peaks at 2919 and 2838 cm^{-1} are associated to $-CH_2$

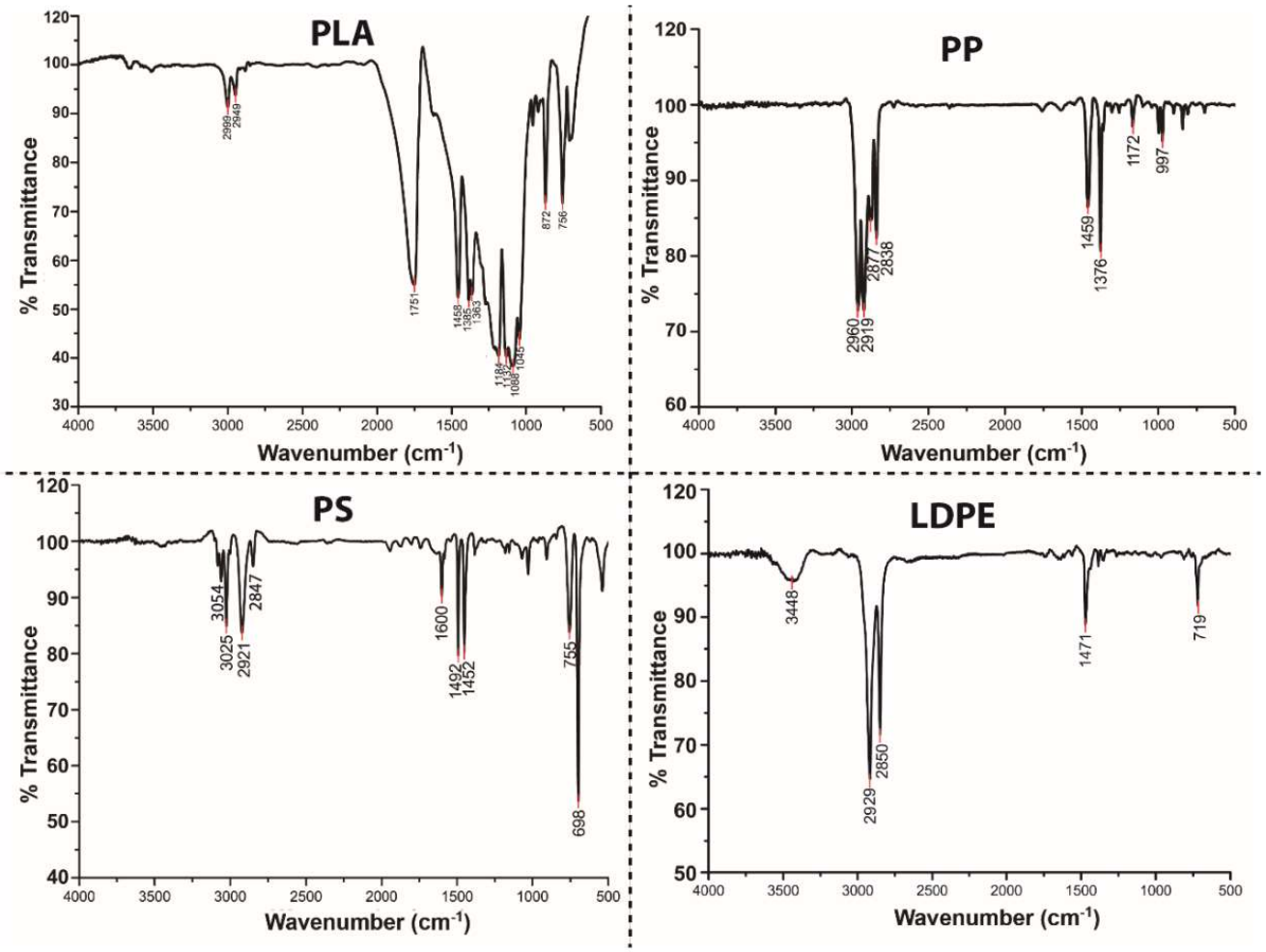

Figure 1. Fourier-transform infrared spectroscopy (FTIR) spectra of NPLs of grinded polylactic acid (PLA), polypropylene (PP), polystyrene (PS) and low-density polyethylene (LDPE). Spectra were obtained in transmittance mode in the 4000-500 cm^{-1} range, with a resolution of 4 cm^{-1} using 128 scans. Characteristic peaks from each polymer are denoted in the corresponding plot.

asymmetric and symmetric stretching vibrations, respectively. Two clear peaks at 1459 and 1376 cm^{-1} are caused by -CH_3 asymmetric deformation vibrations and -CH_2 scissor vibrations. The peak at 1172 cm^{-1} can be attributed to C-C asymmetric stretching and the peak at 997 cm^{-1} to -CH_3 asymmetric rocking vibrations. For PS-NPLs, spectrum peaks observed at 3054 and 3025 cm^{-1} correspond to the aromatic C-H stretching vibrations, while the absorption bands at 2921 and 2847 cm^{-1} are due, respectively, to the asymmetric and symmetric stretching vibrations -CH_2. The C-C stretches in the aromatic ring are observed at 1600, 1492 and 1452 cm^{-1}. This last band may also result from the deformation vibration of –CH_2. The C-H bending bands are also characteristic of the aromatic substitution pattern, being intense at 755 and 698 cm^{-1}. The main peaks presented in the LDPE-NPLs spectrum corresponded to the stretching vibrations of -CH2 at 2929 cm^{-1} and 2850 cm^{-1}, the bending mode of the -CH_2 at 1471 cm^{-1}. The -CH_2 rocking vibration in amorphous domains was clear at 719 cm^{-1}. The colloidal behavior of the four types of NPLs, in the culture media used during the bioassays as well as in ultra-pure water, was analyzed by DLS (Table 2, original data are provided in Figure S3 (SM) and Poly Dispersity Index in Table S1, SM), at 25 °C, in darkness and static conditions, for 96 h. ELS measurements (Table 2) were conducted under the same conditions for 96 h. At 0 h, all NPLs displayed a hydrodynamic size between 1000 and ~30 nm. In ultra-pure water the ζ-potential of the four types of NPLs was between -20 and -30 mV, which corresponds to stable colloids (Table 2). However, NPLs seemed to gradually aggregate with time as denoted by the appearance of peaks of about 5 - 6 μm (Figure S3, SM). Specifically, PLA-NPLs aggregated after 48 h in hydra culture medium (according to the lower ζ-potential values), presenting aggregates larger than 1 μm whilst in zebrafish culture medium the aggregation did not result in particles larger than ~ 1 μm. PP-NPLs in hydra culture medium, at 0 h, showed an intense peak of 68 nm (revealing a considerable amount of NPLs of this size) together with other peaks up to 1000 nm (probably due to a higher monodisperse stability according to the high ζ-potential value of -20 mV (Table 2).

In both hydra and zebrafish culture media, an overall contact time related aggregation process was observed, particularly from 72 h onward, yielding NPLs mostly in

Table 2. DLS hydrodynamic size (by intensity, average of five readings, ten scans/reading) and ζ-potential values of polylactic acid (PLA), polypropylene (PP), polystyrene (PS) and low-density polyethylene (LDPE) NPLs in three different liquid media (ultra-pure water, *H. viridissima* culture medium and *D. rerio* culture medium) at pH 7, with the main peaks observed from 0 to 96 h (mean ± standard error). Raw data are shown in Figure S1 (SM).

	ζ-potential (mV)	DLS size (nm) in ultra-pure water				
	96 h	0 h	24 h	48 h	72 h	96 h
PLA	-22.6 ± 1.3	78 ± 16 142 ± 30	91 ± 26 220 ± 34	220 ± 9 615 ± 10	142 ± 34 396 ± 32	220 ± 18 531 ± 62
PP	-20.0 ± 1.6	142 ± 40 255 ± 26	164 ± 33 459 ± 21	164 ± 10 459 ± 29	106 ± 26 342 ± 31	295 ± 15
PS	-20.0 ± 0.9	122 ± 16 164 ± 23	220 ± 15	220 ± 10	220 ± 11	220 ± 4
LDPE	-28.0 ± 2.8	91 ± 19 220 ± 35	122 ± 8 396 ± 29	142 ± 12 459 ± 36	106 ± 8 396 ± 25	91 ± 4 342 ± 15
	ζ-potential (mV)	DLS size (nm) in *Hydra viridisima* culture medium				
PLA	-14.5 ± 1.1	122 ± 4 342 ± 40	122 ± 30 255 ± 32	396 ± 38	164 ± 35 396 ± 44	712 ± 36
PP	-20.2 ± 2.8	68 ± 9 142 ± 27	91 ± 14 396 ± 32	190 ± 42 459 ± 43	615 ± 35	459 ± 30 712 ± 31
PS	-16.9 ± 1.8	164 ± 10 342 ± 55	106 ± 13 342 ± 25	342 ± 18	122 ± 26 255 ± 35	91 ± 9 615 ± 59
LDPE	-21.3 ± 0.8	60 ± 14 164 ± 26	164 ± 9 712 ± 58	164 ± 24 712 ± 34	122 ± 7 615 ± 18	91 ± 7 531 ± 28
	ζ-potential (mV)	DLS size (nm) in *Danio rerio* culture medium				
PLA	-33.1 ± 1.5 -26.8 ± 1.2	122 ± 30 255 ± 36	255 ± 21 825 ± 51	615 ± 34	615 ± 17	712 ± 32
PP	-26.5 ± 1.7	164 ± 21 531 ± 16	255 ± 9 1110 ± 305	459 ± 27	531 ± 8	220 ± 46 825 ± 60
PS	-35.5 ± 3.4	91 ± 18 190 ± 48	122 ± 11 342 ± 27	220 ± 22 825 ± 29	712 ± 24	825 ± 32
LDPE	-33.1 ± 1.5 -26.8 ± 1.2	164 ± 22 342 ± 29	106 ± 23 342 ± 43	342 ± 13	122 ± 15 255 ± 54	91 ± 9 615 ± 18

the hundreds of nanometers range. This aggregation process occurred earlier in zebrafish media. The size of most PS-NPLs in hydra culture medium ranged between 90 and 300 nm, with a small peak in the tens of nanometers. These NPLs tended to aggregate along the 96 h, reaching a maximum peak of about 615 nm. However, a considerable number of particles of around 100 nm could still be detected at 96h. In zebrafish culture medium, PSNPLs of 20-30 nm as well as an intense peak at 91 nm were detected at 96 h, revealing a higher dispersion in this medium (Figure S3, SM), which was also supported by the more negative ζ-potential observed. Concerning LDPE-NPLs, these particles displayed, at 0 h, a wide distribution with a maximum observed at 164 nm and a small peak detected at 60 nm. LDPE-NPLs were the particles that showed the most negative ζ-potential value in Hydra culture medium. In zebrafish culture medium, the size distribution was considerably different with the majority of the particles remaining between ~200 and ~700 nm over the 96 h period, with some aggregation observed at 24 and 48 h, likely leading to the buoyancy of the aggregates.

3.2. Ecotoxicological assays

3.2.1. Assays with H. viridissima

Assay I: Mortality and morphological evaluation. The data of the 96-h mortality and malformation assay with *H. viridissima* are summarized at Figures 2, 3 and S4 (SM). All assays were valid with controls displaying no mortality or malformations. Since no statistical differences were found between controls (CTR and CTR-SDS), the following statistical analyses compared the results obtained from NPLs exposed organisms to the negative control (containing only culture media).

Overall, PP and LDPE were the polymers most toxic to *H. viridissima*. In PP exposed organisms, at the highest concentrations tested (10 and 100 mg/L), significant mortality rates were found, corresponding to 66.7% and 100%, respectively (Dunn's, $p < 0.05$; Figure 2b). Concerning LDPE, at the highest concentration tested (100 mg /L), 33.3% of the organisms were dead and 16.7% presented irreversible malformations, with a score equal or below 5 according to Wilby's (1990) (Dunn's, $p < 0.05$; Figure 2d). Data obtained for PP and LDPE-NPLs allowed the computation of the LC20,96h and LC_{50},96h (95% CL). Thus, a LC_{20},96h of 7.45 (5.95 – 9.44) mg/L, and a LC_{50},96h of 9.06 (7.21 – 11.6) mg/L were computed for PP, and a LC_{20},96h of 92.1 (75.5 – 114) mg/L and LC_{50},96h of 109 (88.9 - 136) mg/L were computed for LDPE. Organisms exposed to PP and LDPE-NPLs concentrations between 0.1 and 10 mg/L displayed malformations associated with tentacles morphology, like cubed tentacles and short tentacles, as shown in Figure 3 (Wilby and Tesh, 1990).

PLA and PS-NPLs were the least toxic for hydras (Figures 2a and 2c) with no mortality observed in the concentration range tested. Thus, no LC_x was computable for these NPLs. Hydras exposed to PLA-NPLs were at scores 10 or 9 (Figure 2a), with a single malformation detected (Figure 3). Thus, PLA-NPLs exposure elicited no significant effects on hydras morphology (Dunn's, $p > 0.05$). Hydras exposed to PS-NPLs were also in perfect morphological conditions, with the exception of those exposed to the two highest concentrations. Half of the hydras exposed to 10 mg/L of PS-NPLs showed some signs of stress like body or tentacles contraction (score 7 or 8; Figure 2c). All hydras exposed to 100 mg/L of PS-NPLs, were at scores between 6 and 7, and significantly different from controls (Dunn's, $p < 0.05$; Figure 2c). The malformations observed in the organisms exposed to 100 mg/L were all related to tentacles shortening.

Post-exposure feeding rates, measured for 30 minutes after the 96-h exposure period (96 h E + 30 min T), are presented in Figure S4 (SM). Based on the estimated EC_{50},96hE+30minT, NPLs of PS were the least toxic, with an EC_{50}, 96hE+30minT of 6.86 ± 4.99 mg/L, followed by PP and LDPE, with values of 2.12 ± 19.8 mg/L and 2.40 ± 0.91 mg/L, respectively. Significant decreases (Dunn's, $p < 0.05$) in the feeding rate were observed in the organisms that were previously exposed to 10 mg/L and 100 mg/L of PP and LDPE NPLs, when compared to those of previously exposed to control conditions. PLA-NPLs were the particles inducing more effects on the post-exposure feeding rate, as demonstrated by the lowest EC_{50}, 96hE+30minT, 0.55 ± 1.37 mg/L. An analysis of the fitted models for the postexposure feeding rate revealed that PLA and PP fitted models displayed the highest slope, whilst in the other two polymers' curves (PS and LDPE) were more flattened (Figure S4, SM).

Assay II: Regenerative capacity. Hydras regenerated completely after 96 h exposure, in three (PLA, PP, and PS) out of the four polymers tested (Figure S5, SM). Hydras exposed to LDPE-NPLs were also able to fully regenerate, except those at the two highest concentrations. The regeneration scores of organisms exposed to 1 mg/L LDPE-NPLs ranged between 8 and 10, whereas in organisms exposed to 10 mg/L scores ranged between 7 and 10 (with 33.3% of mortality). The observed scores, above the threshold of 5, are indication of reversible regeneration. For a better discrimination between effects induced by the NPLs of the different polymers, the regeneration scores were further explored and data of regeneration scores at 48h are also presented (Figure 4), since at 96 h, organisms exposed to PLA, PP and PS and LDPENPLs were normal with no registered statistical differences (Dunn's, $p > 0.05$; Figure S5, SM). Overall, PLA-NPLs exposed hydras seemed to regenerate faster than control, with the hydras from the highest concentration tested recovering significantly faster (Dunnett's, $p < 0.05$). The same trend, although with no statistical

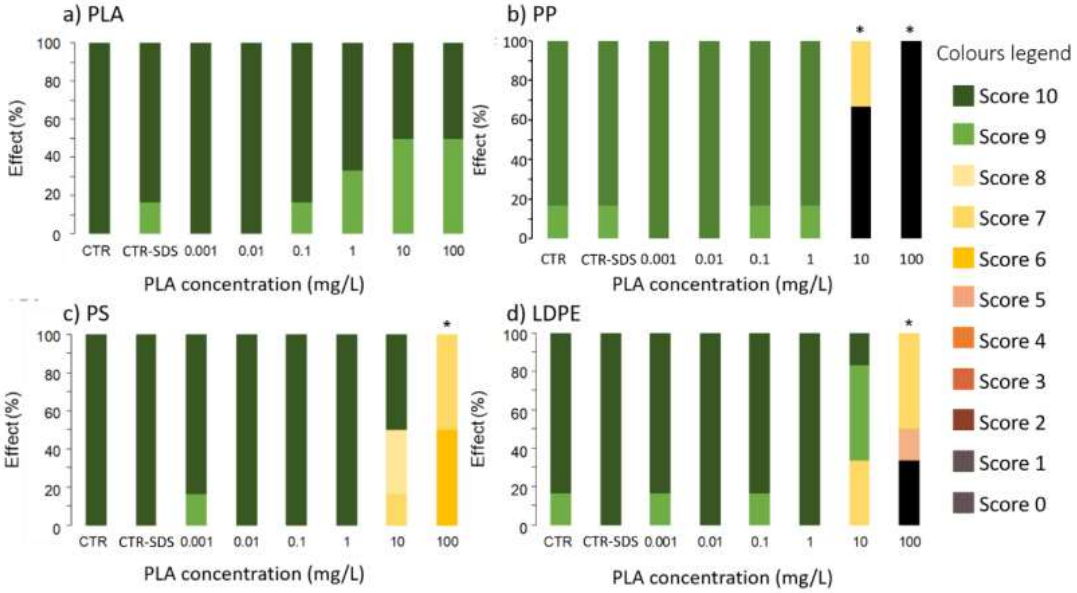

Figure 2. Proportion of hydras in each score of the scale of mortality and malformations at the end of the 96 h exposure to four different NPLs: polylactic acid (PLA), polypropylene (PP), polystyrene (PS) and low-density polyethylene (LDPE) NPLs. A control with organisms exposed to 0.0005 % w/w of SDS (denoted as CTR-SDS) and a negative control (organisms without NPLs, denoted as CTR) were tested. *Indicates average score statistically different from CTR-SDS average score (Dunn's test; $p < 0.05$).

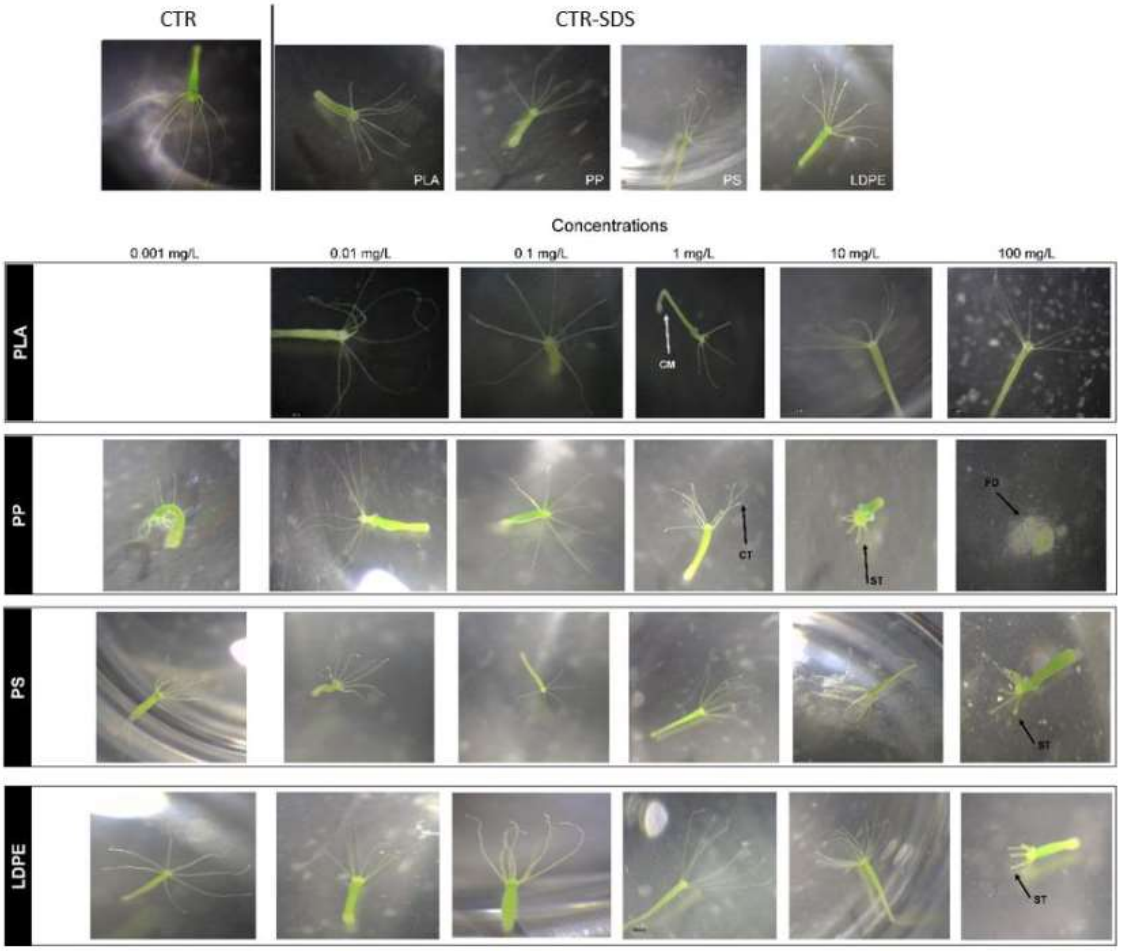

Figure 3. Photographs representing abnormalities on hydras' morphologies (Wilby, 1988) after 96 h of exposure to 0.001, 0.01, 0.1, 1, 10, and 100 mg/L of the four different NPLs (PLA, polylactic acid; PP, polypropylene; PS, polystyrene; and LDPE, low-density polyethylene) and hydras exposed to control conditions (CTR-SDS and CTR)) as reference. The arrows point to malformations observed, whilst photographs without any annotation indicate healthy hydras. Abbreviations stand for: CM - columnae malformation; CT - cubed tentacles; ST - short tentacles; FD - fully disintegrated.

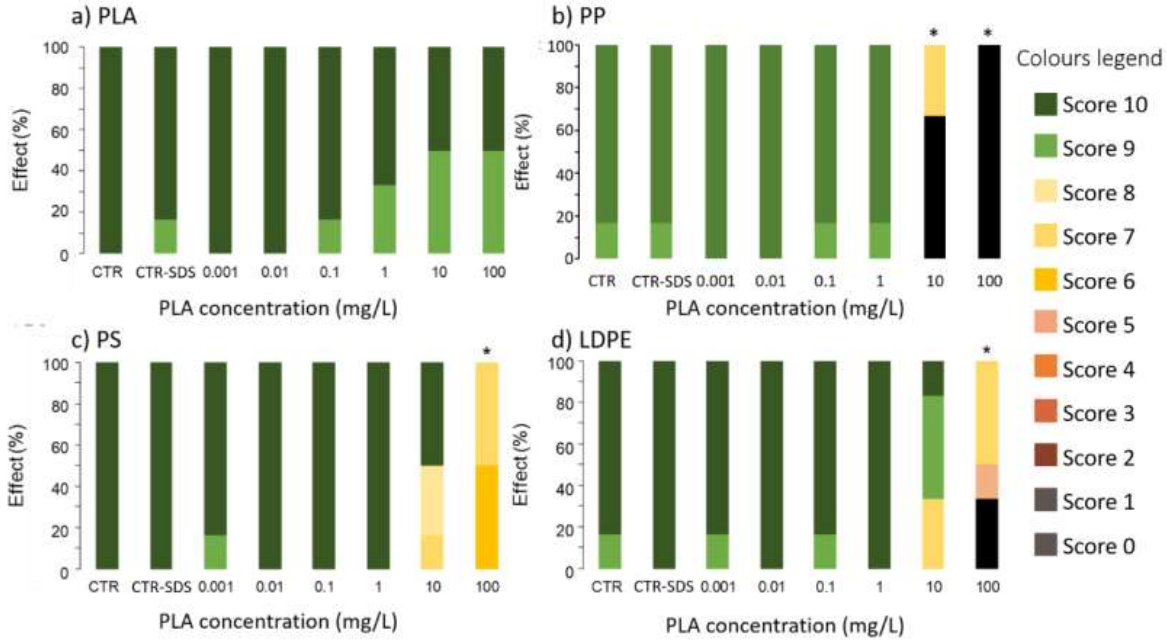

Figure 4. Proportion of hydras in each score of the scale of mortality and malformations at the end of the 96 h exposure to four different NPLs: polylactic acid (PLA), polypropylene (PP), polystyrene (PS) and low-density polyethylene (LDPE) NPLs. A control with organisms exposed to 0.0005 % w/w of SDS (denoted as CTR-SDS) and a negative control (organisms without NPLs, denoted as CTR) were tested. *Indicates average score statistically different from CTR-SDS average score (Dunn's test; $p < 0.05$).

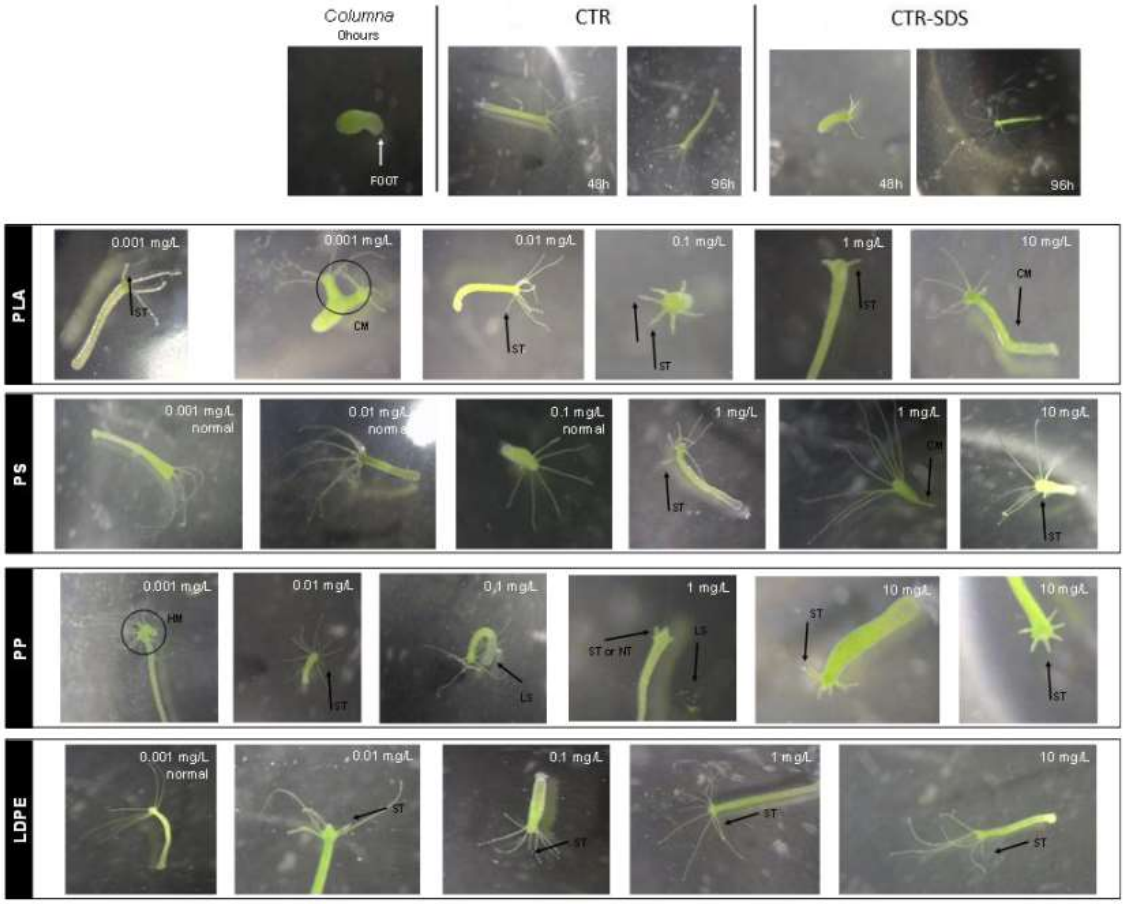

Figure 5. Photographs representing abnormalities of hydras during the regenerative morphology assay (Wilby, 1988), after exposure to NPLs of four different polymers (PLA, polylactic acid; PP, polypropylene; PS, polystyrene; and LDPE, low-density polyethylene) in comparison to a hydra at the beginning of the exposure period (0h, only columna) and hydras exposed to control conditions (CTR-SDS y CTR) as reference. The arrows point to observed malformations whilst photographs without any annotation indicate normal hydras. Abbreviations stand for: CM - columnae malformation; HM – hypostoma malformation; LS – loss of symbiont algae; NT – no tentacles; ST - short tentacles; FD – fully disintegrated.

significance (Dunn's, $p > 0.05$) was observed for LDPE-NPLs, but at intermediate concentrations (0.01 and 0.1 mg/L). Still, it must be highlighted that at the highest tested concentration (10 mg/L), one third of the hydras died. Regarding PP, though not statistically significant, hydras seemed to recover faster than control at 0.001 and 0.01 mg/L. Concerning PS-NPLs, hydras exposed to 0.1 mg/L and higher concentrations displayed significantly delayed regeneration (Dunn's, $p < 0.05$), when compared to the control.

Despite the overall high rate of full regeneration observed in hydras exposed to NPLs of the four polymer types, some malformations were observed at the end of the assay that may be indicative of altered molecular or physiological processes (Figure 5). In their majority, the malformations observed were related to delays in recovering the full length of the tentacles (in NPLs of all polymers), an effect for which there is correspondence in Wilby's regenerative morphology Table. Furthermore, some malformations caused by the NPLs in the hydras are not contemplated in Wilby's regeneration score. These were related to columnae malformations, abnormal division of the columna giving origin to two distinct hypostomes with fully developed and functional tentacles and mouth (e.g., 0.001 mg/L of PLA-NPLs; Figure 5), or the development of a cell mass (without foot or basal area) which seemed like a secondary hypostoma adjacent to the main hydra also fully functional (e.g., 0.001 mg/L of PP-NPLs; Figure 5).

The post-exposure feeding rates of the hydras, measured for 30 minutes after the 96-h regeneration assay are presented in Figure S6, (SM). Up to 0.1 mg/L (with exception of LDPE-NPLs), there was an overall negative correlation between the impact on the postexposure feeding rates and concentrations to which hydras were previously exposed. Thus, the concentration curves were fitted using the part of the curves where the negative correlation was observed (Figure S7, SM). Based on the estimated EC_{50}, 96hE+30minT concerning the post-exposure feeding rate after regeneration, hydras were more sensitive to the lowest concentrations since EC_{50},96hE+30minT values were below 0.5 mg/L.

3.2.2. Assays with D. rerio

In the 96-h assay with *D. rerio*, mortality and malformation percentages were always below 10% in controls (CTR, CTR-SDS) and treatments. No differences in terms of hatching were found between controls and polymers' treatments. In terms of effects on heartbeat rate, presented in Figure S8 (SM), a significant decrease in the heartbeat rate was found in organisms exposed to 1 and 10 mg/L PLA-NPLs and 1 mg/L PP-NPLs. However, 0.01 and 10 mg/L PS-NPLs induced a significant increase in heartbeat rate whereas LDPE-NPLs exposure induced no significant effects.

Despite the slight effects observed during the embryo stage, probably associated with a low permeability of the vitelline membrane, the locomotor activity of organisms exposed to the different NPLs treatments was affected (Figure 6), with more effects during light conditions. PLA-NPLs treatment strongly modified, in a concentration dependent manner, most of the locomotor activity endpoints studied during the light period. AC increased at 0.001 mg/L decreasing at 0.1 and 10 mg/L. TST and TSD decreased to levels 50% below control levels, at the highest concentration (10 mg/L), whilst TSS was only altered at 1 mg/L. TF followed a similar trend, but exposure to the two lowest tested concentrations provoked a slight increase, similarly to AC, that may be related to an hormetic response, before falling at 0.1, 1 and 10 mg/L. During dark period, a reduction of TSD, TST, TSS and TF was also observed in intermediate concentrations.

PP-NPLs were the particles elicited more effects during light period, characterized by a decrease in the majority of the analyzed parameters. However, TSS remained almost unaltered, with only a significant effect (increase) observed in organisms exposed to 1 mg/L. During the dark period, the effects observed in the light period were not found, with the exception of an increase in TSS at 1 mg/L, an increase in TF at 0.1 mg/L and a decrease at the highest concentration tested, 10 mg/L. The exposure of *D. rerio* embryos to PS-NPLs elicited, during light period, effects considerably different than those observed for PLA and PP. Overall, exposure to PS-NPLs resulted in increases on most of the locomotor activity parameters. Interestingly, at 0.001, 0.01 and 1 mg/L, pronounced increases were observed in AC, TST, TSD and TF followed by a drastic fall at the highest concentration. During the dark period, at low concentrations, AC, TST, TSD and TF increased and subsequently decreased after exposure to 0.1 mg/L.

Concerning LDPE-NPLs, in the light period, only slight effects were observed. An increase in AC was observed at 0.001 mg/L and decreases were observed in TST at 0.1 mg/L, TSD at 0.1 and 1 mg/L and TSS at 0.001 mg/L. TF showed higher sensitivity, displaying a significant decrease at 0.001, 0.1 and 1 mg/L. During the dark period, most behavioral alterations disappeared, with the exception of AC, which was significantly higher than control at 0.001 and 0.1 mg/L, and TF that decreased at 0.001 and 0.1 mg/L but increased at the highest tested concentration, 10 mg/L.

The PCA during lightness conditions (Figure S9, SM) indicate a negative correlation between the response to each polymer and concentration. The most negative correlated response was obtained for PP-NPLs followed by PLA-NPLs. This means that the higher the exposure concentration of these polymers the lower value was obtained on the assessed parameters. Furthermore, the PCA revealed a similar response towards the lower

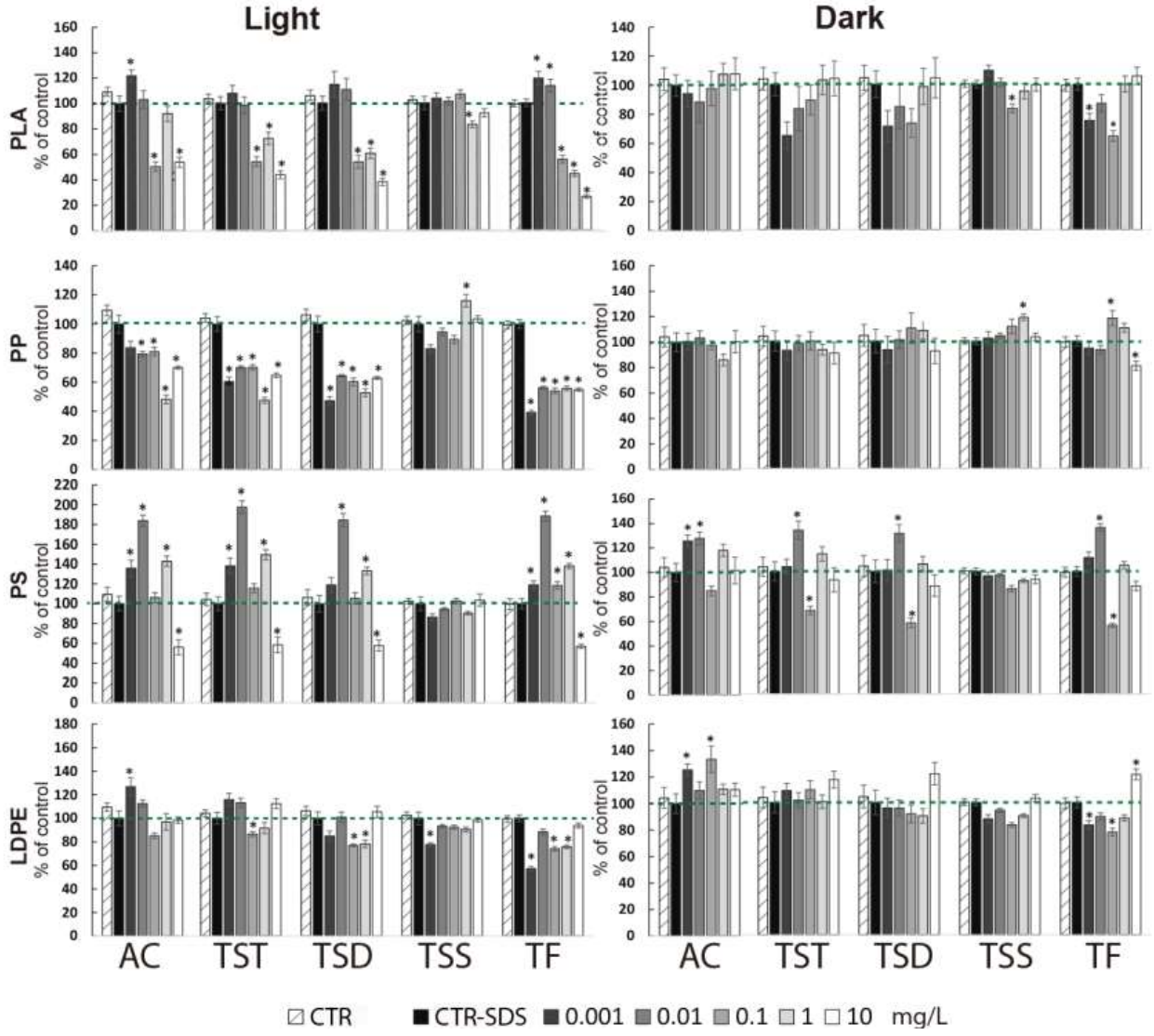

Figure 6. Behavioral endpoints in zebrafish larvae exposed to exposure to NPLs of different polymers: polylactic acid (PLA), polypropylene (PP), polystyrene (PS) and low-density polyethylene (LDPE) NPLs at 120 hours post fertilization. AC (activity counts), TST (swimming time), TSD (swimming distance), TSS (swimming speed), TF (turning frequency). Data are represented as percentage of variation with respect to the control with 0.0005 % w/w of SDS, denoted as CTR-SDS. No significant differences were found between both controls. Negative control (CTR-SDS) was used for the statistical analysis. Raw data are shown in Table SI2 (light) and Table SI3 (dark). * Indicates statistical differences (Dunnett's test; $p < 0.05$)

concentrations while the response towards 1 mg/L and, to a greater extent, 10 mg/L are grouped separately from the response to the lower concentrations. This trend disappeared during darkness conditions. The linear correlation between the response towards increasing NPLs concentrations of each polymer, arranged by assessed parameter, is shown in Table S4 (SM). Here, a highly negative correlation between the parameters AC, TST and TSD, in the case of PP, and TSS and TF, in the case of PLA, and increasing concentrations of NPLs, may be observed. Additionally, these differences regarding the most correlated assessed parameters reveal different ecotoxicological responses depending on the polymer type.

4. Discussion

In this study, PLA, PP, PS and LDPE-NPLs were successfully obtained through mechanical breakdown from pristine beads, with a yield of approximately 0.02% of grinded material within the nanometric range. During this process, the use of surfactants to keep particles well dispersed was avoided for the sake of obtaining more realistic ecotoxicological data, as these compounds may also elicit biological effects that compromise the hazard assessment of NPLs (e.g., Gangadoo et al., 2020). To assess the effects of these NPLs, two secondary consumers (*H. viridissima* and *D. rerio*) were selected based on its previously reported value in toxicity testing, allowing the assessment of the impact of environmental stressors on biochemical

endpoints (e.g., biotransformation), behavior (feeding), morphology (ontogenic development) and reproduction (sexual and asexual) and easy laboratory maintenance. Hydras also possess an ability to regenerate, allowing the assessment of teratogenic effects. Thus, organisms were exposed to a concentration range from 1 μg/L, realistic since it is close to the maximum of 0.79 μg/L reported in freshwater (Xu et al., 2022), to 100 mg/L, a high concentration tested to elucidate potential mechanisms of toxicity.

Overall, results regarding hydra assays, identified PP and LDPE-NPLs as the most toxic polymers, in terms of mortality and malformations. LDPE-NPLs induced toxicity one order of magnitude higher than PP-NPLs. The NPLs of the two other tested polymers, PLA and PS-NPLs, did not induce mortality in hydras. However, when hydras were exposed to the high concentrations of PS-NPLs, signs of stress, like malformations (scores equal or below 6 at the concentration of 100 mg/L) were found, while no differences to control were found in organisms exposed to PLA-NPLs. However, these results may be linked not only to the type of polymer (Zimmermann et al., 2020a), but also to the initial presence of higher proportion of particles of sizes below 100 nm (as shown in Table 2 for hydras culture medium) that, in the case of PP and LDPE-NPLs, was higher than in the other polymers. Toxicity of LDPE-NPLs (also obtained by mechanical breakdown followed by filtration by 0.8 μm), to D. magna was ascribed to the smallest fraction obtained (below ~ 3 nm) (Frankel et al., 2020). Accordingly, data support the idea that NPLs of sizes below 100 nm may be responsible for a higher toxicity than larger ones. Even when aggregation processes occur, they may not necessarily imply a decrease in toxicity, as described elsewhere (Frankel et al., 2020). In this regard, 40 nm polymethylmethacrylate NPLs (PMMA-NPLs) have been reported to affect *H. viridissima* survival at high concentrations (equal or higher than 80 mg/L) and to induce previously unreported morphological alterations at lower concentrations (Venâncio et al., 2022). Hydra attenuata exposure to 50 and 100 nm PSNPLs caused NPLs accumulation in a concentration dependent manner, as well as several physiological alterations such as lipid peroxidation or lipid mobilization (Auclair et al., 2020). It is possible that these effects are due to physical abrasion on the outer layer as well as an ability of incorporated particles to induce oxidative stress. For instance, PP-MPs (12-46 μm) have been reported able to significantly increase catalase activity, an enzyme considered a first line of defense against oxidative stress, in Artemia salina juvenile stage (Jeyavani et al., 2022).

The hydras post-exposure feeding assays, revealed that PLA-NPLs have the lowest EC_x, with effects estimated at concentrations as low as 370 μg/L, an indication that these NPLs may interact with biota, once in the environment. The hypothesis that hydras feeding was decreased as a result of the use of the incorporated particles as a carbon source, is not supported by the findings of Khaldoon et al. (2022) study with *Eudrilus eugeniae* where earthworms underwent a weight reduction when exposed to PLA-MPs with food, in a concentration dependent manner (Khaldoon et al., 2022).

In terms of regeneration ability, hydras exposed to PLA-, PP-, and PS-NPLs were able to fully regenerate, while those exposed to the two highest concentrations LDPE-NPLs were clearly affected by exposure and unable to fully regenerate. It should be highlighted that, organisms exposed to the highest PLA-NPLs concentrations, were able to regenerate significantly faster than controls, a result also reported by Venâncio et al. 2021 with hydras exposed to 40 nm PMMA-NPLs. This effect may also be related to the bio-based characteristic of the PLA-NPLs that could be perceived as available food by the hydras which would increase cell proliferation rate as described elsewhere (Sebestyén et al., 2018). Furthermore, as observed in the post-exposure feeding assay, performed after the mortality assay, PLA-NPLs exposure caused negative impact on hydras prey consumption behavior. These results are supported by other studies that emphasized the need to perform comparative studies between petroleum-based and natural-based polymers (e.g., Zimmerman et al., 2020; Anderson and Shenkar, 2021). The scarce available data suggest that natural-based polymers, although not causing mortality, can induce sublethal effects related to organism fitness that may compromise its long-term survival. It should also be highlighted that some malformations such as the development of two-bodied hydras, in the case of PLA, and two-headed hydras, in the case of PP were found in organisms exposed to the lowest concentrations tested, that may be a sign of altered physiological mechanisms, as previously reported in cnidarians exposed to PMMA-NPLs (Venâncio et al., 2022).

Overall, the approach carried in this work allowed the determination of several effective concentrations of these polymers for lethal and sublethal endpoints for hydra, not previously determined. By recommendation of the Commission Directive 93/67/EEC of 20 July 1993 for standard Risk Assessment (RA) outlines, this work attempted to carry the two first steps of RA, i.e., the assessment of concentration-effect curves, which then allowed the determination of median lethal or effective concentrations (e.g., LC50 or EC50). These values allow a more objective comparison between polymers toxicity, species, and/or studies, largely lacking to date (e.g., Bond et al., 2018), and may also be integrated into risk quotient estimations for these polymers.

In the present study, zebrafish was also exposed to NPLs of the four polymers, at concentrations ranging from 0.001 to 10 mg/L. The heartbeat rate of the zebrafish embryos, assessed after 48 h exposure, suggests that PLA, PP, and PS-NPLs are likely to cause

important alterations to the embryos. A decrease in heartbeat rate, as observed in this study in organisms exposed to PLA and PP-NPLs, was also reported in zebrafish exposed to 10 and 50 mg/L of 20 and 80 nm irregularly shaped polyethylene terephthalate NPLs capped with bovine serum albumin (Ji et al., 2020) and was associated with overproduction of reactive oxygen species. This alteration has also been linked to direct interaction with cardiac sarcomeres affecting normal heart functioning (Pitt et al., 2018). In contrast, PS-NPLs exposure provoked a heartbeat rate acceleration, which was also previously reported in zebrafish exposed to spherical 100 nm PS-NPLs and associated to alteration of chorion permeability due to the pore blockage by the PS-NPLs that did not penetrate the chorion efficiently, inducing a hypoxic microenvironment in the inner space of the chorions (Duan et al., 2020). These findings suggest that different types of NPLs may interact in a dissimilar way with the zebrafish embryos and, consequently, trigger further alterations.

In agreement to the heartbeat rate, the locomotor activity assay, signaled PP and PLANPLs as those inducing more alterations in the larvae as they caused a decrease in all the parameters, during light period, even at the low concentration of 1 μg/L (PP-NPLs). Similar locomotor activity inhibition has been reported throughout a metanalysis of MPs effects towards aquatic biota at concentrations equal or below 1 mg/L (Sun et al., 2021). The increased heartbeat rate observed in organisms exposed to PS-NPLs was associated with an overall increased locomotor activity except at the highest concentration while LDPE-NPLs barely modified the larvae behavior. It is worth noting that NPLs uptake by the larvae has been previously demonstrated with PS-NPLs of several sizes (from 25 to 700 nm) revealing that ingestion followed by biodistribution and eventual accumulation was observed for NPLs of 25 and 50 nm (van Pomeren et al., 2017). Accumulation of PS-NPLs (35 nm) has been also reported in different organs (including the head) at concentrations as low as 0.1 mg/L (Pitt et al., 2018). The authors suggest that PS-NPLs could be located in the brain triggering neurological alterations resulting in the hypoactive locomotor behavior that they observed at 1 mg/L. The low concentration at which statistically significant alterations were observed in this study may be due to the irregular shape of the NPLs generated through mechanical breakdown, as demonstrated for micro-sized PE particles with sharp/smooth surface (Kalčíková et al., 2017). Furthermore, other studies have reported that consistent and direct injection of 20 nm PS-NPLs into the egg yolk may allow particles to reach zebrafish brain, leading to oxidative DNA damage in the specific brain regions where they accumulate (Sökmen et al., 2020). Regarding PLA, 2.3 μm fragments at concentration of 2.5 and 5 mg/L have been reported to accumulate in zebrafish body (liver, brain, gills, and carcass) leading to behavioral, neurotoxic, biochemical, and morphological alterations (Chagas et al., 2021). Thus, the overall hypoactivity observed in the present work, may be related to effects triggered by the smallest fraction (probably below 100 nm) of the PP, PS and LDPE-NPLs. The observed effects may be associated not only with the size or shape of the particles but, especially for the biopolymer, with the chemical nature that, for instance, make them more susceptible to be ingested with the subsequent depolymerization in the digestive tract and lactic acid release (Duan et al., 2022). Furthermore, based on the PCA, there is a clear correlation between the highest exposure concentrations and the assessed parameter depending on the polymer type (Figure S9, SM, and Table S4, SM). Despite no effective concentrations could be delivered for *D. rerio*, results have shown that environmentally relevant concentrations are able to induce effects that may hamper organisms ‘s fitness, which may later interfere with its performance.

Different parameters were analyzed in each species, with malformations (besides mortality) being the only common between them. Malformations may be indicative of potential teratogenic effects. In fish, no malformations were detected upon exposure to NPLs of any of the four polymers, whereas several malformations were recorded in hydras exposed to PS and LDPE-NPLs. Overall, data reinforces the importance of studies that not only include species representing the same trophic level but also allow evaluation of several environmentally relevant parameters. Despite the relevance of this study, some potential limitations may be identified. For instance, although of higher ecological relevance, the obtention of irregularly shaped NPLs as the one here studied, may be rather difficult to replicate, exactly matching in size and shape as highlighted by others (e.g., Cerasa et al., 2021), whilst the follow-up of these particles under laboratorial controlled conditions will certainly not accurately be predictive of the physico-chemical changes that they undergo under realistic scenarios of exposure (e.g., Bond et al., 2018; Cerasa et al., 2021).

5. Conclusions

Through mechanical breakdown, based on the application of freezing/grinding cycles in ethanol, avoiding the use of surfactants, irregularly shaped NPLs of four different types of polymers, both petroleum-based (LDPE, PP, PS) and biopolymer (PLA) were successfully obtained. The obtained data show that for hydra, PP and LDPE-NPLs were the most harmful polymers, inducing several malformations and even mortality at the high concentrations. However, feeding assays revealed clear alterations on prey consumption caused by PLANPLs, with effect concentrations one order of magnitude lower than the petroleum-based polymers. While the EC50 for PP, LDPE and PS-NPLs ranged between 2 to 7 mg/L, for PLANPLs it was 0.55 mg/L. Furthermore, PLA-NPLs induced previously undescribed malformations in the hydra body.

Data obtained with *D. rerio* showed effects at early life stages, during embryo development (heartbeat rate) that correlate with alterations on behavior detected at the larval stage. In this case, PLA and PP-NPLs were the most hazardous as they induced clear alterations at concentrations close to the ones reported in freshwater environments.

Overall, the findings of this study support the hypothesis that bioplastics may induce toxicity within the same magnitude as their traditional fossil-based counterparts, highlighting the need of more studies with these types of polymers. Accordingly, PLA-based fragments may induce biological effects to aquatic biota, that may compromise their fitness, highlighting the need to perform additional studies with biopolymers for a better understanding of its potential use as a safer alternative to synthetic polymers. A straightforward comparison of the biological effects of different types of polymers should be performed, considering relevant sublethal endpoints and exposure conditions.

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Supplementary Materials

Effects of petroleum-based and biopolymer-based nanoplastics on aquatic organisms A case study with mechanically degraded pristine polymers

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Figure S9: Principal component analysis (PCA) of the locomotor activity response of *D. rerio* exposed to 0.001-10 mg/L range of four types of nanoplastics: Polylactic acid (PLA), polypropylene (PP), Polystyrene (PS) and Low-Density Polyethylene (LDPE), under lightness (A) and darkness (B) conditions.

Table S1: Poly Dispersity Index (PDI) of the nanoplastic suspensions. Results are presented as mean ± standard deviation. PLA - polylactic acid; PP - polypropylene; PS - polystyrene; and LDPE - low-density polyethylene).

Table S2: Behavioral endpoints results of *D. rerio* during light period. Control without and with 0.0005 % w/w of SDS are denoted as CTR and CTR-SDS, respectively. Sadistically significant differences were not found between both. The CTR (control organisms not exposed to the NPLs) was used for the statistical analysis. *Indicates statistical differences after Dunnett's test ($p < 0.05$). PLA - polylactic acid; PP - polypropylene; PS - polystyrene; and LDPE - low-density polyethylene).

Table S3: Behavioral endpoints results *D. rerio* during dark period. Control without and with 0.0005 % w/w of SDS are denoted as CTR and CTR-SDS, respectively. Sadistically significant differences were not found between both. The CTR control (organisms not exposed to the NPLs) was used for the statistical analysis. *Indicates statistical differences after Dunnett's test ($p < 0.05$). PLA - polylactic acid; PP - polypropylene; PS - polystyrene; and LDPE - low-density polyethylene).

Table S4: Correlation coefficients between the assessed endpoints (AC: activity counts, TST: swimming time, TSD: swimming distance, TSS: swimming speed, TF: turning frequency) and the exposure concentrations by polymer type (PLA - polylactic acid; PP - polypropylene; PS - polystyrene; and LDPE - low-density polyethylene).

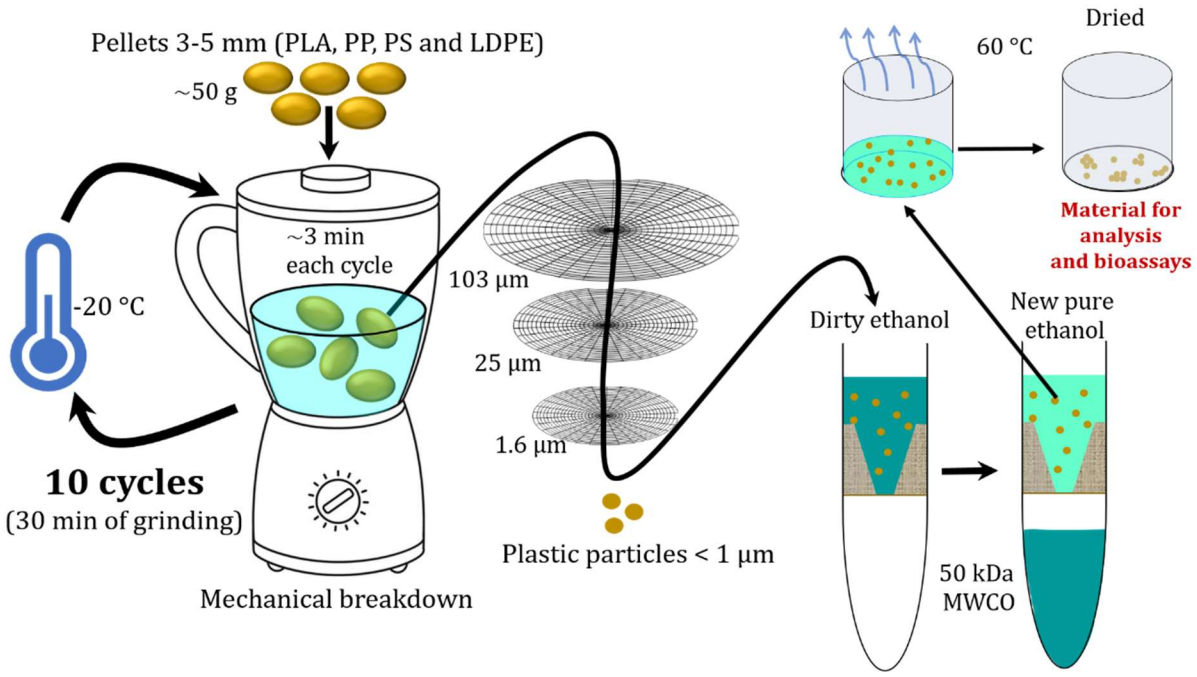

Figure S1: Scheme illustrating the nanoplastics generation methodology.

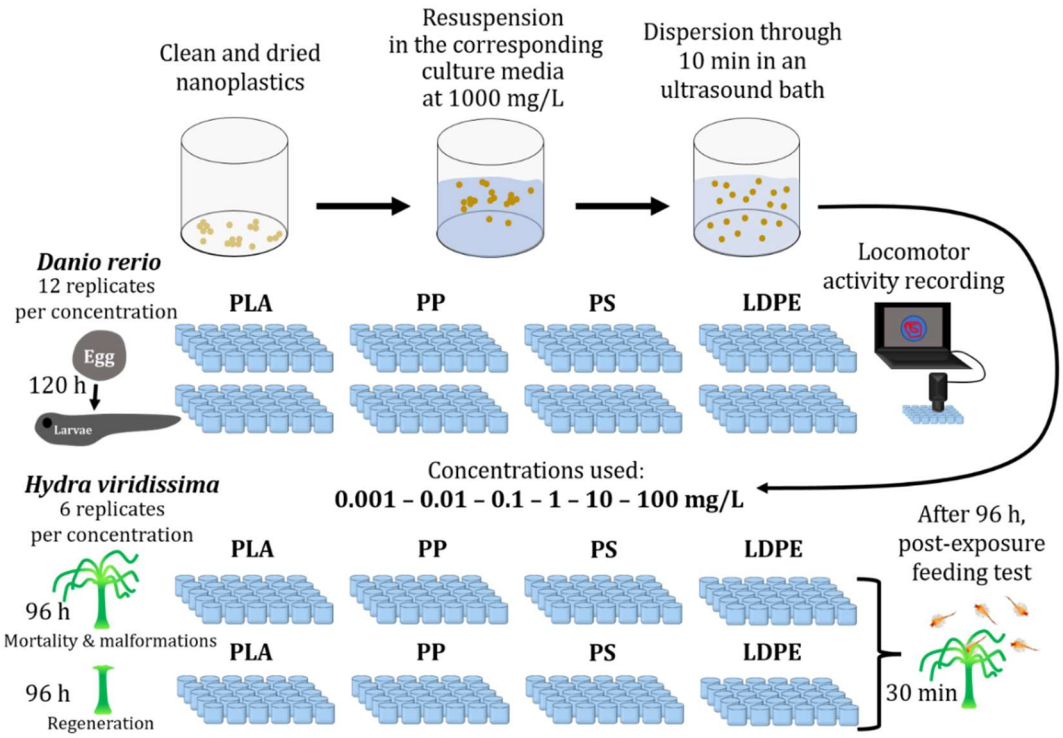

Figure S2: Scheme illustrating the experimental design.

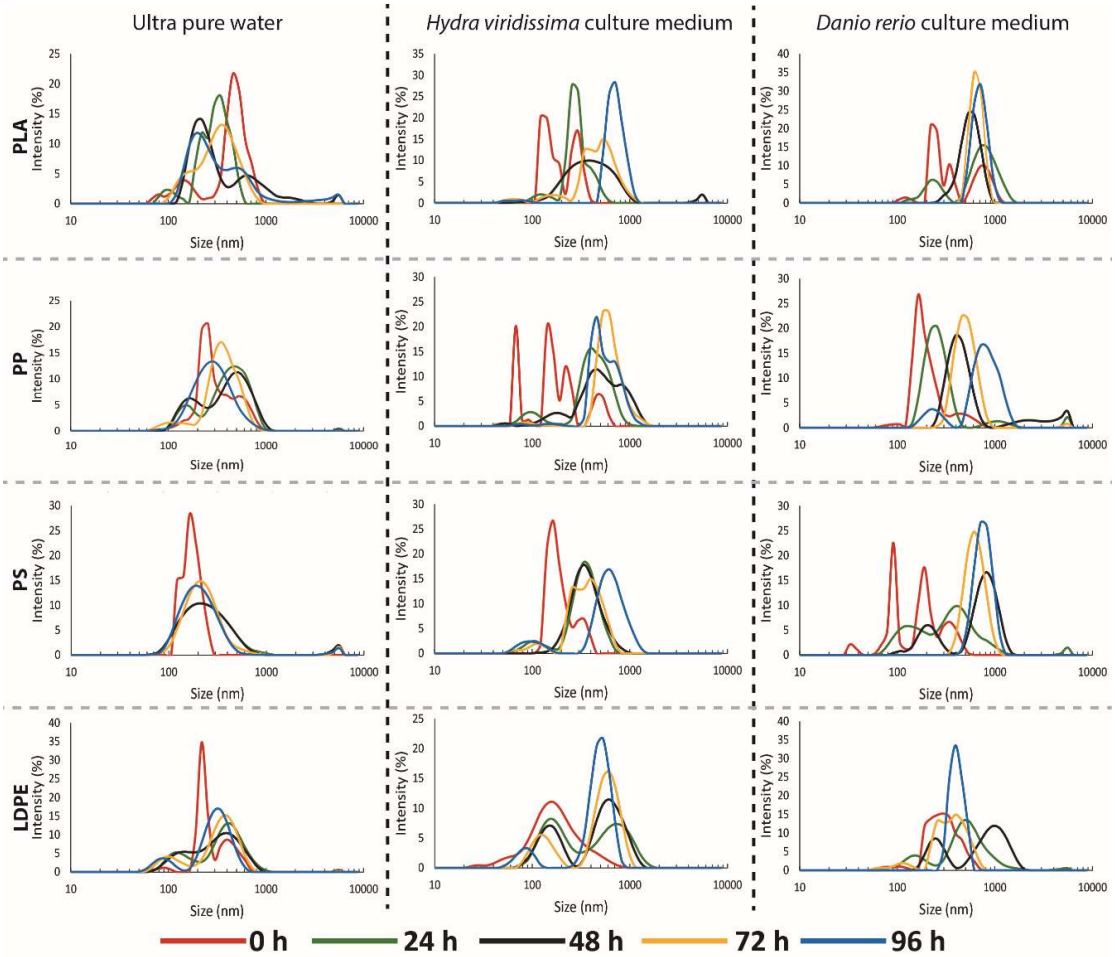

Figure S3: Particle size distribution by intensity of polylactic acid (PLA), polypropylene (PP), polystyrene (PS) and low-density polyethylene (LDPE) NPLs, in three different liquid media (ultra-pure water, *Hydra viridissima* culture medium and *Danio rerio* culture medium) after 0 h, 24 h, 48 h, 72 h and 96 h. Analysis was performed by dynamic light scattering (DLS). Data are shown as the mean of 5 measurements, each one with 10 scans.

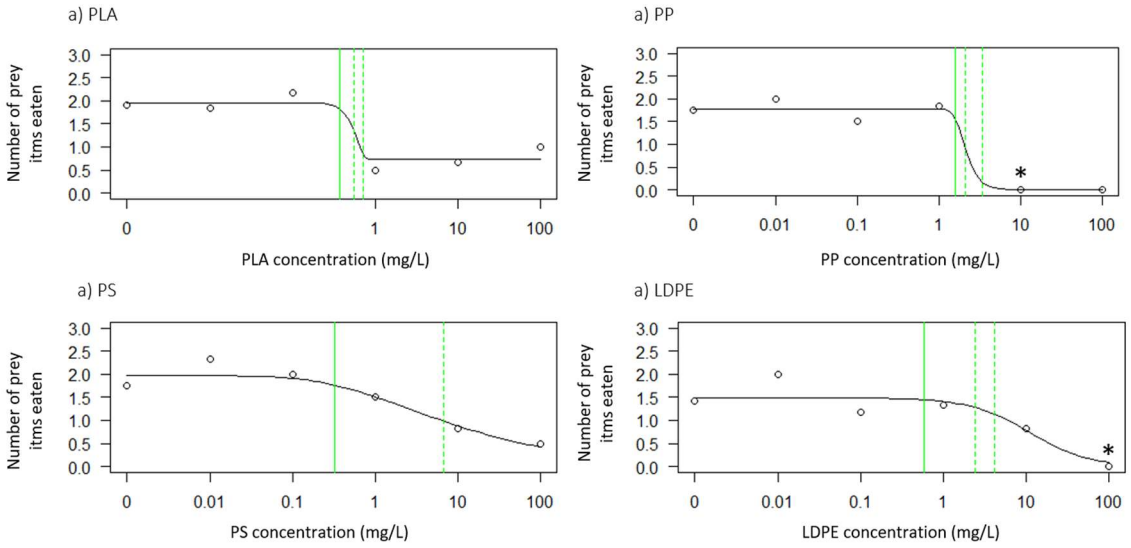

Figure S4: Data from post-exposure feeding assay with the freshwater cnidarian *H. viridissima* after a 96 h exposure period to four different types of polymers (PLA, polylactic acid; PP, polypropylene; PS, polystyrene; and LDPE, low-density polyethylene). Open dots correspond to the average number of prey items eaten during the duration of the assays (30 minutes) and the continuous horizontal line represents the model fit curve. Vertical green lines correspond (from the left to the right) to the feeding EC_{10}, EC_{50}, and EC_{90}, respectively. No data is presented for PP at 100 mg/L as all organisms died. No EC_{90} was possible to compute for PS (out of range). *Indicates statistically different from the control (CTR) after Dunn's ($p < 0.05$).

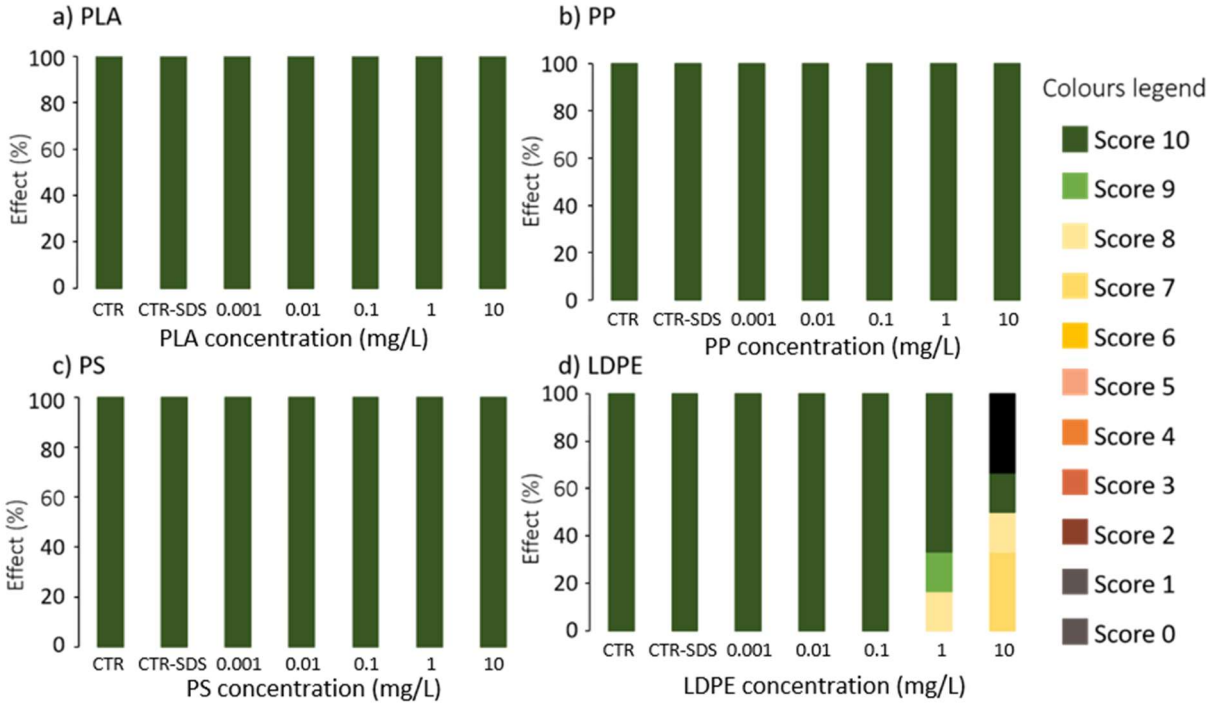

Figure S5: Regenerative morphological evaluation of hydras according to Wilby, (1988), after 96 h exposure to NPLs of different polymers: polylactic acid (PLA), polypropylene (PP), polystyrene (PS) and low-density polyethylene (LDPE) NPLs, presenting the proportion at each scoring. A control with organisms exposed to 0.0005 % w/w of SDS (denoted as CTR-SDS) and a negative control (organisms without NPLs, denoted as CTR) were tested.

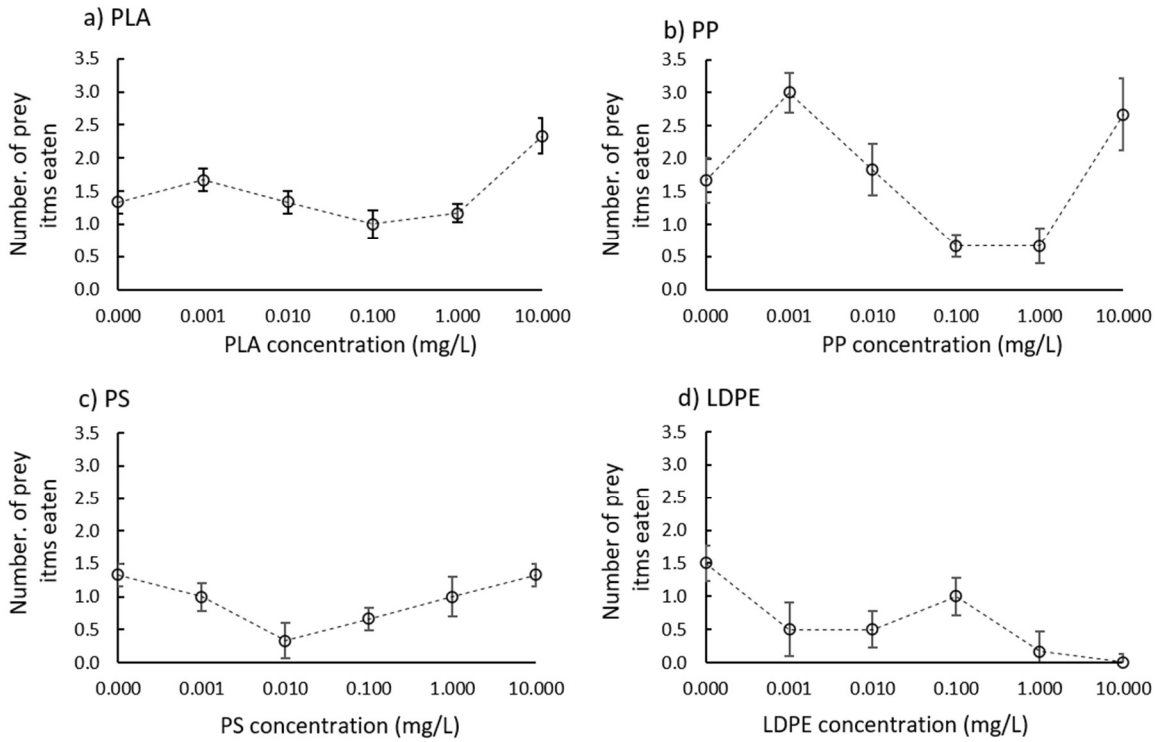

Figure S6: Feeding (in 30 minutes) of *H. viridissima* in the regeneration test, after a 96 h exposure period to NPLs of four different polymers (PLA, polylactic acid; PP, polypropylene; PS, polystyrene; and LDPE, low-density polyethylene). Data are represented as average number of prey items eaten in 30 minutes ± SE.

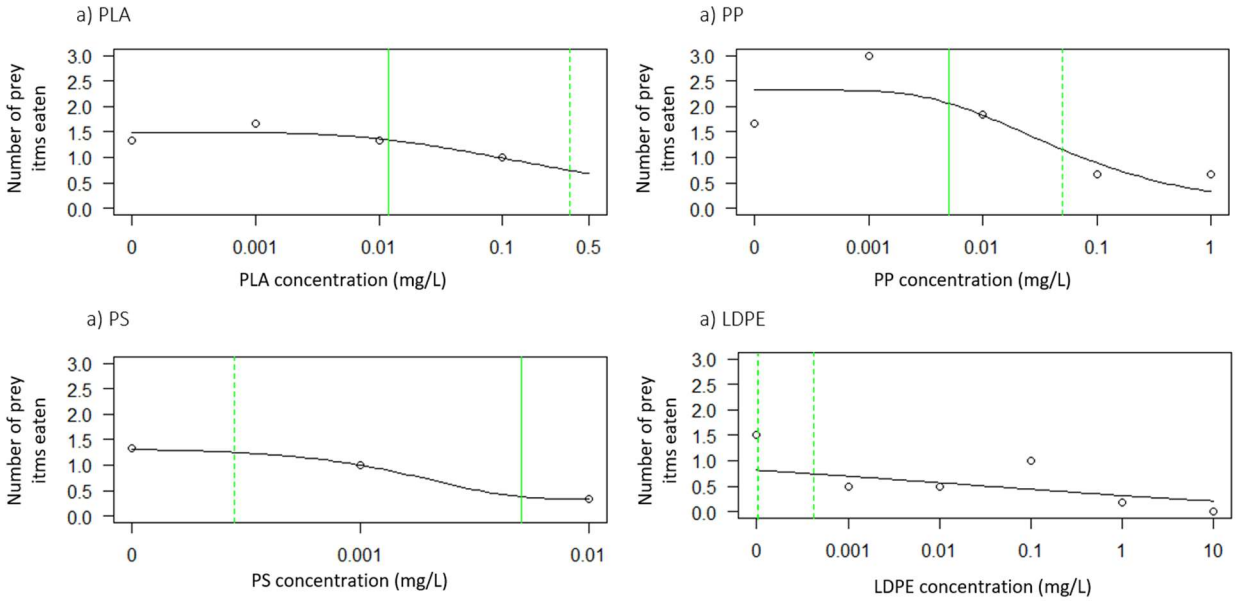

Figure S7: Data from the post-regeneration feeding assay with the freshwater cnidarian *Hydra viridissima* after a 96h exposure period to four different polymers (PLA, polylactic acid; PP, polypropylene; PS, polystyrene; and LDPE, low-density polyethylene). Open dots correspond to the average number of prey items eaten in the 30 minutes period of the test and the continuous horizontal line represents the model fit curve to the data set (since there were a shift in the trend of prey eaten with increasing concentration, only data that negative correlate with the polymer concentration was used for the fitting, raw data are shown in Figure SI5. Vertical green lines correspond (from the left to the right) to the EC_{10} and EC_{50}, respectively, on feeding. No reliable EC_{90} was possible to obtain owed to the trend shift observed in the response when increasing the concentration. *Indicates statistically different from CTR after Dunn's ($p < 0.05$).

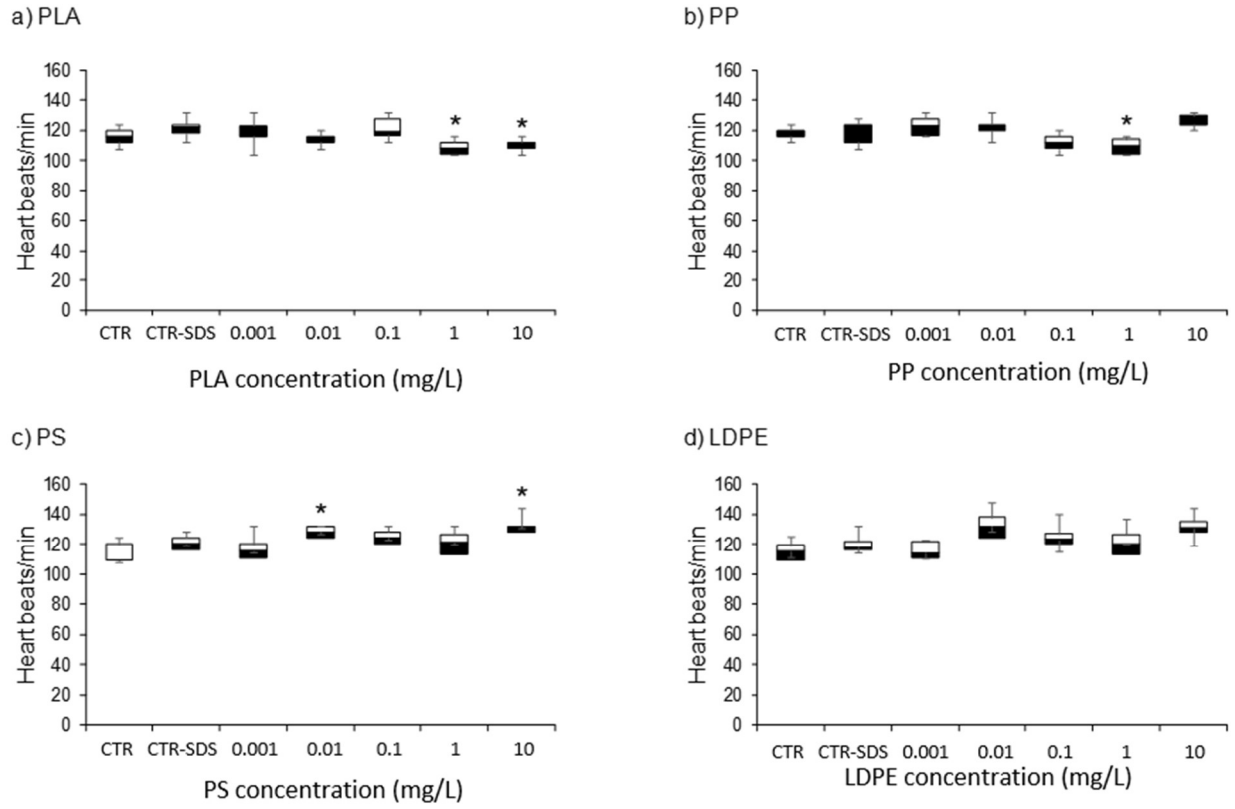

Figure S8: Heartbeat rate of zebrafish embryos after 48 h of exposure to four different types of NPLs. Control without and with 0.0005 % w/w of SDS (CTR and CTR-SDS, respectively). Statistically significant differences were not found between both. * Indicates statistical differences between CTR and the treatments after Dunn's ($p < 0.05$). PLA - polylactic acid; PP - polypropylene; PS - polystyrene; and LDPE - low-density polyethylene.

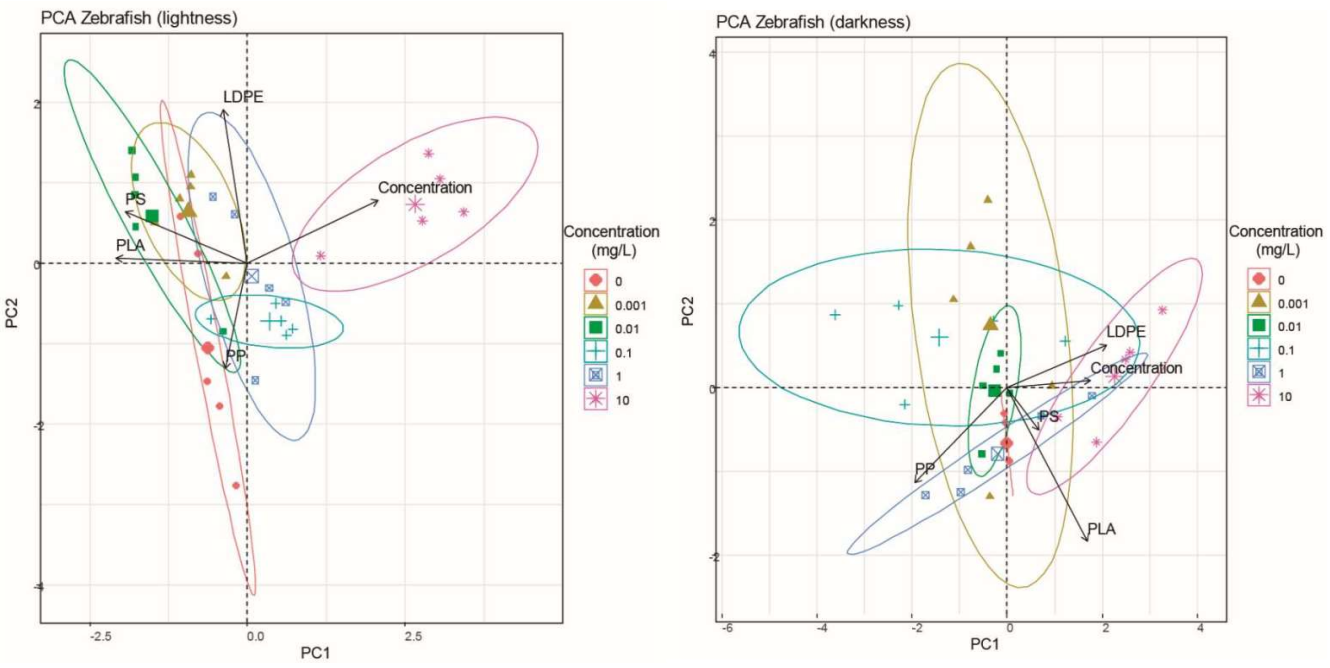

Figure S9: Principal component analysis (PCA) of the locomotor activity response of *D. rerio* exposed to 0.001-10 mg/L range of four types of nanoplastics: Polylactic acid (PLA), polypropylene (PP), Polystyrene (PS) and Low-Density Polyethylene (LDPE), under lightness (left) and darkness (right) conditions.

Table S1: Poly Dispersity Index (PDI) of the nanoplastic suspensions. Results are presented as mean ± standard deviation. PLA - polylactic acid; PP - polypropylene; PS - polystyrene; and LDPE - low-density polyethylene).

	PDI in ultra-pure water				
	0 h	**24 h**	**48 h**	**72 h**	**96 h**
PLA	0.6 ± 0.1	0.6 ± 0.1	0.5 ± 0	0.5 ± 0	0.5 ± 0.1
PP	0.7 ± 0.1	0.5 ± 0.1	0.6 ± 0	0.5 ± 0.1	0.5 ± 0
PS	0.8 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0	0.4 ± 0
LDPE	0.8 ± 0.2	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
	PDI in *Hydra viridisima* culture medium				
	0 h	**24 h**	**48 h**	**72 h**	**96 h**
PLA	0.8 ± 0.2	0.6 ± 0	0.7 ± 0.2	0.7 ± 0.1	0.6 ± 0.1
PP	0.6 ± 0.1	0.4 ± 0	0.5 ± 0.1	0.4 ± 0.1	0.6 ± 0.2
PS	0.7 ± 0.2	0.5 ± 0.1	0.6 ± 0.1	0.3 ± 0.1	0.4 ± 0.1
LDPE	0.8 ± 0.1	0.5 ± 0	0.6 ± 0.1	0.3 ± 0.1	1 ± 0
	PDI in *Danio rerio* culture medium				
	0 h	**24 h**	**48 h**	**72 h**	**96 h**
PLA	0.9 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.4 ± 0.2
PP	0.9 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.5 ± 0.1	0.6 ± 0.1
PS	0.8 ± 0.1	0.5 ± 0	0.5 ± 0	0.6 ± 0	0.6 ± 0.1
LDPE	0.4 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1

Table S2: Behavioral endpoints results of *D. rerio* during light period. Control without and with 0.0005 % w/w of SDS are denoted as CTR and CTR-SDS, respectively. Sadistically significant differences were not found between both. The CTR (control organisms not exposed to the NPLs) was used for the statistical analysis. *Indicates statistical differences after Dunnett's test ($p < 0.05$). PLA - polylactic acid; PP - polypropylene; PS - polystyrene; and LDPE - low-density polyethylene).

		LIGHT						
	Behavioral endpoint	**CTR**	**CTR-SDS**	**0.001**	**0.01**	**0.1**	**1**	**10**
PLA	Activity counts (n)	32.5 ± 1	30.7 ± 2.9	37.4 ± 1.4 *	31.7 ± 2.1	15.5 ± 1.1 *	28.2 ± 1.9	16.5 ± 1.2 *
	Swimming time (s)	13.8 ± 0.6	16.5 ± 1.5	17.8 ± 1	16.2 ± 1.1	8.9 ± 0.6 *	11.9 ± 0.8 *	7.2 ± 0.5 *
	Swimming distance (mm)	49.2 ± 3.3	53.6 ± 6.1	61.5 ± 5.4	59.3 ± 4.6	28.8 ± 2.7 *	32.4 ± 2.2 *	20.3 ± 1.5 *
	Swimming speed (mm/s)	3.6 ± 0.13	3.24 ± 0.33	3.36 ± 0.13	3.29 ± 0.09	3.47 ± 0.1	2.7 ± 0.08 *	2.99 ± 0.1
	Turning frequency (turns/s)	58.6 ± 2.7	58.9 ± 3.4	70.3 ± 3 *	66.8 ± 3 *	32.7 ± 1.8 *	26.1 ± 1.4 *	15.4 ± 0.9 *
PP	Activity counts (n)	32.6 ± 1	41.2 ± 1.4	34.5 ± 1.9	32.7 ± 0.8 *	33.4 ± 1.2 *	19.9 ± 1.3 *	28.8 ± 0.5 *
	Swimming time (s)	18.9 ± 0.4	23.4 ± 0.5	14.2 ± 0.7 *	16.4 ± 0.3 *	16.5 ± 0.4 *	11.2 ± 0.5 *	15.2 ± 0.4 *
	Swimming distance (mm)	74.1 ± 1.6	87.1 ± 2.2	41.1 ± 2.3 *	56.2 ± 0.7 *	52.6 ± 2.3 *	45.9 ± 2.3 *	54.7 ± 0.9 *
	Swimming speed (mm/s)	3.75 ± 0.09	3.51 ± 0.08	2.92 ± 0.09	3.32 ± 0.09	3.14 ± 0.1	4.07 ± 0.15 *	3.63 ± 0.08
	Turning frequency (turns/s)	89.3 ± 1.5	114.9 ± 1.7	45 ± 1.8 *	64.6 ± 1.2 *	62.1 ± 2.2 *	64.1 ± 1.7 *	63 ± 1.4 *
PS	Activity counts (n)	35.9 ± 1.5	22 ± 1.6	29.9 ± 1.8	40.5 ± 1.2	23.4 ± 1.1	31.5 ± 1.1	12.3 ± 1.7
	Swimming time (s)	18.6 ± 0.7	11.3 ± 0.8	15.6 ± 0.9 *	22.3 ± 0.7 *	13.1 ± 0.5	16.9 ± 0.6 *	6.6 ± 0.9 *
	Swimming distance (mm)	76.2 ± 3.7	46.6 ± 3.9	55.4 ± 3.6	85.9 ± 3.1 *	49.1 ± 2.6	62 ± 1.8 *	26.9 ± 2.5 *
	Swimming speed (mm/s)	4.15 ± 0.12	4.08 ± 0.28	3.53 ± 0.13	3.86 ± 0.06	4.19 ± 0.12	3.69 ± 0.07	4.23 ± 0.25
	Turning frequency (turns/s)	91.3 ± 3.1	56 ± 2.9	66.7 ± 2.2 *	105.6 ± 2.7 *	66.4 ± 2.1 *	77.3 ± 1.5 *	31.9 ± 1.2 *
LD	Activity counts (n)	34.7 ± 0.9	30.1 ± 1.6	38.2 ± 2.3 *	33.8 ± 0.9	25.6 ± 0.7	29.1 ± 2.1	29.6 ± 0.8

	Swimming time (s)	20.5 ± 0.4	17.7 ± 0.7	20.5 ± 1	20 ± 0.7	15.3 ± 0.4 *	16.2 ± 0.9	19.9 ± 0.7
	Swimming distance (mm)	83.2 ± 2.1	79 ± 2.5	67.1 ± 3.2	79.7 ± 3.3	60.8 ± 1 *	61.8 ± 2.5 *	83.2 ± 3.7
	Swimming speed (mm/s)	3.98 ± 0.08	4.26 ± 0.1	3.31 ± 0.07 *	3.98 ± 0.06	3.95 ± 0.08	3.86 ± 0.09	4.21 ± 0.08
	Turning frequency (turns/s)	100 ± 2	111.3 ± 2.1	63.5 ± 2 *	98.5 ± 2.7	82.5 ± 2.1 *	84.3 ± 1.7 *	104.4 ± 2.4

Table S3: Behavioral endpoints results *D. rerio* during dark period. Control without and with 0.0005 % w/w of SDS are denoted as CTR and CTR-SDS, respectively. Sadistically significant differences were not found between both. The CTR control (organisms not exposed to the NPLs) was used for the statistical analysis. *Indicates statistical differences after Dunnett's test ($p < 0.05$). PLA - polylactic acid; PP - polypropylene; PS - polystyrene; and LDPE - low-density polyethylene).

	DARK							
	Behavioral endpoint	**CTR**	**CTR-SDS**	**0.001**	**0.01**	**0.1**	**1**	**10**
PLA	Activity counts (n)	34 ± 3.9	37.9 ± 3.9	35.8 ± 3.5	33.6 ± 5.4	37.1 ± 4.5	40.9 ± 2.8	40.9 ± 4.2
	Swimming time (s)	20.8 ± 2.1	23 ± 2.5	14.9 ± 2.1 *	19.1 ± 3.5	20.5 ± 2.4	23.7 ± 2.3	23.9 ± 2.8
	Swimming distance (mm)	90.6 ± 8.9	98.9 ± 11.1	70.3 ± 10.6 *	83.6 ± 15	72.5 ± 9.8 *	97.2 ± 12.3	103.2 ± 13.7
	Swimming speed (mm/s)	43.2 ± 0.7	43.2 ± 0.9	47.3 ± 1.5	43.7 ± 1.2	36 ± 1.3 *	41.1 ± 2.3	43.1 ± 1.9
	Turning frequency (turns/s)	123 ± 5.7	133.9 ± 6.2	100.6 ± 6.5 *	116.3 ± 7.8	86.3 ± 4.8 *	134 ± 7.5	141.6 ± 8
PP	Activity counts (n)	38.4 ± 2.6	40.6 ± 1	40.8 ± 2.6	42 ± 2.2	39.6 ± 1.3	34.9 ± 1.8	40.8 ± 3.6
	Swimming time (s)	26.3 ± 2.1	25.2 ± 1.2	23.4 ± 1.9	24.8 ± 1.5	25.3 ± 1.8	23.5 ± 1.1	22.8 ± 2.1
	Swimming distance (mm)	119.6 ± 10.8	105.9 ± 8.6	98.7 ± 10.9	107 ± 7.3	116.7 ± 12.7	114.7 ± 7.4	97.7 ± 10.6
	Swimming speed (mm/s)	4.55 ± 0.19	4.14 ± 0.22	4.23 ± 0.21	4.31 ± 0.09	4.61 ± 0.24	4.91 ± 0.1 *	4.27 ± 0.12
	Turning frequency (turns/s)	165.8 ± 6.6	148.5 ± 5.9	140.2 ± 7	138.6 ± 5	175.2 ± 8.8 *	164 ± 5.2	119.7 ± 5.4 *
PS	Activity counts (n)	39.5 ± 2.7	35.1 ± 2.6	44.1 ± 1.7 *	44.9 ± 1.8 *	29.9 ± 1.4	41.4 ± 1.7	35.7 ± 3.8
	Swimming time (s)	24.7 ± 1.6	21.2 ± 1.6	22 ± 1.3	28 ± 1.6 *	14.4 ± 0.7 *	24.2 ± 1.2	19.7 ± 2.1
	Swimming distance (mm)	106.2 ± 7.9	90.2 ± 7.9	91.2 ± 7.7	118.1 ± 6.5	52.2 ± 3.4	95.6 ± 5.5	79.5 ± 7.9
	Swimming speed (mm/s)	4.3 ± 0.07	4.31 ± 0.08	4.15 ± 0.12	4.22 ± 0.06	3.7 ± 0.12	3.98 ± 0.07	4.03 ± 0.15
	Turning frequency (turns/s)	127.2 ± 4.7	112.3 ± 4.6	125 ± 5.2	149.8 ± 3.6 *	62.8 ± 1.9 *	117.9 ± 3.4	99.1 ± 4.3
LDPE	Activity counts (n)	44.2 ± 2.6	36.1 ± 3.7	45.3 ± 1.6 *	39.7 ± 2.4	48.2 ± 3.6 *	40 ± 1.4	39.9 ± 1.8
	Swimming time (s)	24.9 ± 1.4	23.9 ± 2.2	26.1 ± 1.3	24.3 ± 1.4	26.2 ± 1.6	24 ± 1.2	28 ± 1.5
	Swimming distance (mm)	104.1 ± 7.1	107.6 ± 9.4	103.1 ± 7.9	103.1 ± 6.5	98.4 ± 7.3	96.7 ± 5.9	130.9 ± 9.1
	Swimming speed (mm/s)	4.15 ± 0.09	4.52 ± 0.08	3.96 ± 0.14	4.25 ± 0.07	3.75 ± 0.08	4.07 ± 0.06	4.66 ± 0.13
	Turning frequency (turns/s)	123.7 ± 5.1	149 ± 5.4	123.7 ± 4.6 *	133.1 ± 4	115.5 ± 4.4 *	131.4 ± 3.6	180.6 ± 6.1 *

Table S4: Correlation coefficients between the assessed endpoints (AC: activity counts, TST: swimming time, TSD: swimming distance, TSS: swimming speed, TF: turning frequency) and the exposure concentrations by polymer type (PLA - polylactic acid; PP - polypropylene; PS - polystyrene; and LDPE - low-density polyethylene).

	PLA		**PP**		**PS**		**LDPE**	
	Light	**Dark**	**Light**	**Dark**	**Light**	**Dark**	**Light**	**Dark**
AC	0.23	0.91	-0.99	-0.97	-0.11	0.22	-0.16	-0.40
TST	-0.18	0.98	-1.00	-0.95	-0.18	0.15	-0.41	-0.53
TSD	-0.48	0.85	-0.96	0.40	-0.25	0.10	-0.53	-0.76
TSS	-0.95	0.10	0.96	0.90	-0.70	0.02	-0.98	0.07
TF	-0.69	0.73	0.25	0.30	-0.33	0.05	-0.48	0.35