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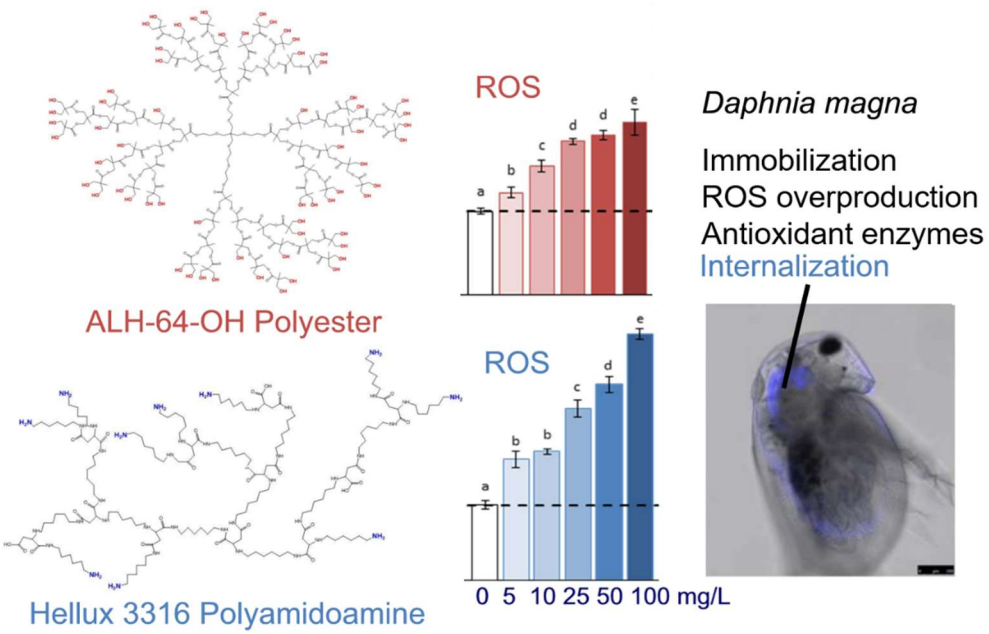

Hyperbranched polymeric nanomaterials impair the freshwater crustacean Daphnia magna

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Abstract

Hyperbranched polymers are nanomaterials belonging to the class of dendritic architectures with increasing applications in many diverse fields. We studied the toxicity of two hyperbranched polymers to the freshwater crustacean *Daphnia magna*. A hyperbranched hydroxyl-terminated polyester and a commercial hyperbranched polyamidoamine, Helux-3316 were tested for the acute immobilization of daphnids, the overproduction of reactive oxygen species and the activity of the antioxidant enzymes catalase and glutathione S-transferase. The effect for *D. magna* immobilization was higher for the hyperbranched polyamidoamine Helux-3316, which was attributed to the presence of primary amino groups on its surface. Following exposure to both hyperbranched polymers, a clear overproduction of reactive oxygen species took place accompanied by concentration-dependent enzymatic antioxidant response. Our results showed that the overproduction of reactive oxygen species activated antioxidant defence mechanisms and was responsible for the immobilization of daphnids exposed to both hyperbranched polymers. We showed evidence of the uptake of fluorescently labelled Helux-3316 that accumulated into the gastrointestinal tract of *D. magna*, and its removal via excretion within fecal pellets. This is the first work reporting the internalization of hyperbranched polymers in aquatic organisms.

Keywords: Hyperbranched polyester; Hyperbranched polyamidoamine; *Daphnia magna* immobilization; Oxidative stress; Enzymatic antioxidant activity; Internalization.

1. Introduction

Hyperbranched polymers belong, together with dendrimers, to the class of dendritic architectures. They are randomly branched molecules growing from a central core but, unlike dendrimers, hyperbranched polymers possess irregular topology and are prepared in one-step, relatively facile synthesis (Zheng et al., 2015). The applications of hyperbranched polymers derive from their characteristics, many of them shared with dendrimers, like high number of reactive groups, low viscosity and high solubility (Gurunathan et al., 2016). The applications of hyperbranched polymers are very wide and cover diverse fields of nanotechnology, biomedical uses, and component of adhesives, composites and coatings (Jeon et al., 2018). Concerning large-volume applications, hyperbranched poly(ethyleneimine) has been used as dispersant in oils spills (Salehi et al., 2017). BASF markets Lupasol®, which is a hyperbranched poly(ethyleneimine) used for crosslinking of epoxy resins (Román et al., 2018). Hyperbranched polyesters from the Boltorn® family are used as flexographic ink component (Żołek-Tryznowska and Izdebska, 2013).

The data available on the toxicity of hyperbranched polymers are very scarce. The toxicity of hyperbranched poly(ethyleneimine) was reported to increase with increasing molecular weight (expressed in mass concentration) with EC_{50} for the 10 kDa polymer of 0.16 mg/L for the 48 h immobilization of *Daphnia magna* (Salehi et al., 2017). The toxicity of hyperbranched polyesters is generally considered very low compared to poly(ethyleneimine) allowing their use for biomedical applications (Reul et al., 2009). As most anthropogenic chemicals, hyperbranched polymers may reach the environment through the discharge of wastewater treatment plants(Gavrilescu et al., 2015). The release of chemicals included in goods during use and improper disposal is another source of pollution and in both cases, the aquatic environment is the receiving main medium (Farré et al., 2008).

In this work, we studied the immobilization of the freshwater crustacean *Daphnia magna* after 48 h exposure periods to two chemically different hyperbranched polymeric nanomaterials. One of them is representative from the family of hyperbranched hydroxyl-terminated polyesters while the other is a commercial hyperbranched polyamidoamine with primary amines as terminal groups. We also studied the overproduction of reactive oxygen species (ROS) and the subsequent antioxidant enzymatic activities in daphnids as well as the internalization of fluorescently-labelled polymers.

2. Materials and methods

2.1. Materials

Potassium dichromate, potassium chloride, dipotassium hydrogen phosphate, potassium dihydrogen phosphate, ethylenediaminotetraacetic acid (EDTA), methanol,

acetone and hydrogen peroxide 30% (w/v) were purchased from Panreac (Spain). Ultrapure water was used in all cases with specific resistance >18 MΩ $cm^{-1}$and total organic carbon < 2 mg/L (Direct-QTM, Millipore). 2′,7′-dichlorofluorescein diacetate (H_2DCFDA), 1-chloro-2,4-dinitrobenzene (CDNB), L-glutathione reduced (GSH) and fluorescamine were acquired from Sigma Aldrich. Pierce™ BCA Protein Assay Kit was obtained from ThermoFisher Scientific. Hyperbranched bis-MPA polyester-64-hydroxyl generation 4 (in what follows, ALH-64-OH) was purchased from Sigma-Aldrich. ALH-64-OH hyperbranched polymer presents multiple surface hydroxyl groups with a theoretical molecular weight of 7323 g/mol (Fig. S1, SM, Supplementary Material). Hyperbranched polyamidoamine (Helux-3316) was supplied from Polymer Factory (Stockholm, Sweden). Helux-3316 has primary amines and carboxylic acids end-groups and its theoretical molecular weight is 5108 g/mol (Fig. S1, SM). The suspensions of nanoparticle polymers were prepared immediately before use as detailed in the experimental section. Dynamic light scattering (DLS) and electrophoretic light scattering were used to measure suspension particle size and ζ-potential respectively. (Zetasizer Nano ZS instrument, Malvern Instruments).

2.2. Acute toxicity tests

The acute immobilization of the cladoceran crustacean *D. magna* (MicroBioTests, Belgium) was tracked following OECD Test Guideline 202 (OECD, 2004). The detailed description of the assay can be found elsewhere (ISO, 1996). *D. magna* was exposed up to 48 h to different exposure concentrations of ALH-64-OH and Helux-3316. Fresh stocks of toxicants were prepared a few minutes before every toxicity assay by dispersing the polymers in Milli-Q water using an ultrasonic bath. The dispersed stock suspensions were diluted in standard freshwater medium, also used as control, to obtain the appropriate testing concentrations. Prior to exposure, pH and dissolved oxygen concentration were adjusted.

For toxicity assays, neonates were obtained by the hatching of ephippia kept 72–80 h at 21 ± 1 °C under continuous illuminations (6000 lux). Neonates were fed with a suspension of the microalga *Spirulina* for 2 h prior to the exposure. Five neonates were transferred per test well of plastic multiwall plates together with 10 mL of aerated hyperbranched suspensions. The plates were incubated at 21 ± 1 °C in darkness for 48 h. All concentrations and blanks were tested in at least three independent tests with four replicates samples per test concentration. The sensitivity of the organisms to potassium dichromate was within the 0.6–2.1 mg/L range for EC_{50} 24 h according to ISO 6341 (ISO, 1996). Dissolved oxygen concentrations and pH were checked at the end of all tests. Dissolved oxygen concentration was always higher than 3 mg/L and no significant changes were recorded between pH before and after exposure.

After 24 h and 48 h exposure, the number of immobilized daphnids was recorded as toxicological endpoint. Neonates unable to swim after gentle agitation of the exposure media for 15 s were considered immobile, even if they could still move their antennae. The relative toxicity of the samples was expressed as the percentage of immobilized organisms compared with the controls. Dose-response curve fitting was performed with the statistical software R “drc” analysis package (RStudio for Windows) (Ritz et al., 2015). The four-parameter log-logistic model (LL.4, drc) was used and 95% confidence intervals were obtained using the function ED.drc 3.0-1 (drc) on fitted LL.4 models (Ritz and Streibig, 2005).

2.3. ROS measurement

ROS production was assessed using neonates exposed for 48 h to different concentrations of ALH-64-OH and Helux-3316. Each concentration was replicated four times with five neonates each. ROS measurement was performed using 2′,7′-dichlorofluorescein diacetate (H_2DCFDA). H_2DCFDA diffuses through cell membrane and is hydrolysed by intracellular esterases to the non-fluorescent 2,7-dichlorofluorescein, which is subsequently oxidized by intracellular ROS to the fluorescent 2,7-dichlorofluorescein (DCF). DCF fluorescence intensity is proportional to the intracellular ROS levels (Gomes et al., 2005). After 24 h and 48 h exposure to the hyperbranched polymers, each surviving daphnid was transferred to a well of a 24-well microplate, and the exposure solution was carefully removed, leaving only the daphnids. 1 mL of 100 μM H_2DCFDA (prepared from 25 mM H_2DCFDA stock solution in methanol diluted with standard freshwater medium) was immediately added and incubated for 4 h in darkness at room temperature. The intracellular generation of DCF was monitored using a fluorimeter (Fluoroskan Ascent™ FL) with excitation and emission wavelengths 485 nm and 538 nm, respectively.

2.4. Antioxidant enzymatic activity

The activities of the antioxidant enzymes glutathione S-transferase (GST) and catalase (CAT) were measured after acute (48 h) exposure to ALH-64-OH and Helux-3316. Three independent experimental replicates with fifty neonates were performed for each exposure concentration. Surviving animals were transferred to Eppendorf tubes and rinsed three times with 100 mM phosphate buffer (pH 7.4), suspended in homogenization buffer (100 mM phosphate buffer, 100 mM KCl, 1 mM EDTA, pH 7.4 containing), and sonicated in ice bath for 30 s using a probe sonicator (Sonics VibraCell, BioBlock). The homogenates were centrifuged for 25 min at 10000 rpm and 4 °C.

GST activity was determined following Habig et al. (1974). Briefly, GST catalyses the conjugation of reduced glutathione (GSH) with the substrate 1-chloro-

2,4-dinitrobenzene (CDNB), forming a dinitrophenyl thioeter (GS-DNB) spectrophotometrically measured at 340 nm. CDNB and GDH concentrations were 1 mM in 100 mM phosphate buffer (pH 7.5). CAT activity was spectrophotometrically measured by recording H_2O_2 decomposition to H_2O and O_2 (Aebi, 1984). 100 µL of protein supernatant were dispersed in 50 mM potassium phosphate buffer (pH 7.0) and 10 mM H_2O_2. The reaction was followed spectrophotometrically at 240 nm for 3 min. Enzymatic activities were calculated as units (U) (µmol of GS-DNB/min for GST and µmol of H_2O_2/min for CAT) and normalized for the protein content (U/mg protein). Protein concentration was measured using a Pierce™ BCA Protein Assay Kit, using bovine serum albumin as a standard.

2.5. Hellux-3316 uptake

The uptake of Helux-3316 was tracked using Helux-3316 labelled with the fluorescent dye fluorescamine. Fluorescamine reacts with primary amines to form stable and highly fluorescent products. Helux-3316-fluorescamine conjugates were prepared using fluorescamine stock solution in acetone and adding it to 1000 mg/mL Helux-3316 stock solution. Labelled Helux-3316 was diluted in standard freshwater medium to obtain the desired exposure concentrations. *D. magna* neonates were exposed to 5 mg/L and 10 mg/L of Helux-3316-fluorescamine conjugates under the same conditions described for 48 h immobilization tests. Control samples without fluorescent Helux-3316-fluorescamine and control samples containing only fluorescamine were also used. Fecal pellets excreted by the organisms exposed to Helux-3316-fluorescamine conjugates were also analysed. After 48 h, daphnids were removed and the exposure medium was collected and filtered through a 0.45 µm nitrocellulose filter. Fecal pellets were identified using an optical microscope. The uptake of fluorescently labelled Helux-3316-fluorescamine and fecal pellets containing Helux-3316-fluorescamine conjugates were visualized by laser scanning confocal microscope (TCS-SP5, Leica Microsystems), at 365 nm excitation and 470 nm emission wavelengths. All comparative images (control and treated samples) were obtained using the same microscope settings.

2.6. Statistical analysis

The statistical analysis consisting of one-way ANOVA and Tukey's HSD tests were performed using R software 3.5.0 and Rcmdr 2.5–1 package (Fox, 2005). A p-value < 0.05 was considered statistically significant.

3. Results and discussion

3.1. Hyperbranched polymers characterization

The physicochemical properties of ALH-64-OH and Helux-3316 hyperbranched polymers in freshwater medium and ultrapure water at pH 7.7 ± 0.1 are shown in Table 1 and Table S1 (SM) respectively. Particle size and ζ-potential were recorded after 24 h and 48 h, corresponding to the exposure times of acute test performed to *D. magna*. DLS size revealed the existence of aggregates in the hundreds of nanometre range, with a tendency towards greater sizes for higher concentrations. The size of the background colloid was 196 ± 18 nm, indicating the heteroaggregation of hyperbranched polymers on freshwater medium particles. In pure water and for Helux-3316, we observed a lower peak at 3.95 ± 0.33 nm representing a small percentage of the intensity, but high in number distribution calculated from Mie's theory. We interpret that this signal corresponds to the individual molecules coexisting with aggregates and most probably indicating the presence of a significant number of individual hyperbranched molecules in solution and in dynamic equilibrium with aggregates. It should be taken into account that DLS is a technique with sensitivity limitations for macromolecules with refractive index not much different to that of water. The

Table 1. Physicochemical properties of ALH-64-OH and Helux-3316 in freshwater medium[a] at different times (ζ-potential measured at pH 7.7 ± 0.1).

	ALH-64-OH					
	1 mg/L		10 mg/L		100 mg/L	
	d_{DLS} (nm)	ζ-potential (mV)	d_{DLS} (nm)	ζ-potential (mV)	d_{DLS} (nm)	ζ-potential (mV)
0 h	228 ± 22	-7.2 ± 0.6	222 ± 19	-6.5 ± 0.6	349 ± 13	-3.5 ± 0.2
24 h	201 ± 12	-6.9 ± 0.5	132 ± 10	-7.2 ± 0.6	375 ± 19	-8.5 ± 0.6
48 h	116 ± 10	-10.2 ± 0.9	130 ± 9	-10.4 ± 0.9	371 ± 25	-11.7 ± 0.3
	Helux-3316					
	1 mg/L		10 mg/L		100 mg/L	
	d_{DLS} (nm)	ζ-potential (mV)	d_{DLS} (nm)	ζ-potential (mV)	d_{DLS} (nm)	ζ-potential (mV)
0 h	205 ± 13	-6.4 ± 0.6	247 ± 13	-4.0 ± 0.4	260 ± 18	+1.5 ± 0.1
24 h	210 ± 9	-9.2 ± 0.8	306 ± 12	-8.0 ± 0.7	309 ± 20	-2.3 ± 0.2
48 h	357 ± 12	-8.1 ± 0.4	387 ± 15	-7.5 ± 0.6	408 ± 16	-3.9 ± 0.3

* Freshwater medium d_{DLS} 196 ± 18 nm; freshwater ζ-potential -6.8 ± 0.4 mV.

colloids formed in water with ALH-64-OH were generally stable along the duration of tests, while a slight size increase was observed for Helux-3316. This was probably a consequence of the negative surface charge of ALH-64-OH, while Helux-3316 displayed positive ζ-potential values in pure water due to the protonation of primary amines (Niu et al., 2003). No significant differences in ζ-potential for both hyperbranched polymer suspensions in freshwater medium were observed, the values being similar to the background colloid.

3.2. Acute toxicity immobilization tests

The results of *D. magna* immobilization are presented in Fig. 1 as percentage of immobilized daphnids with respect to control. For both hyperbranched polymers the immobilization was dose-dependent with higher immobilization for Helux-3316 in comparison to ALH-64-OH for the same tested concentrations and exposure times. The maximum immobilization percentage for *D. magna* exposed to ALH-64-OH was 71.7 ± 5.8 and 88.3 ± 7.6 after 24 h and 48 h, respectively, at the highest tested concentration (500 mg/L) (Fig. 1A). After 24 h, Helux-3316 exposure caused 96.4 ± 3.1% immobilization at 100 mg/L (100% after 48 h, Fig. 1B).

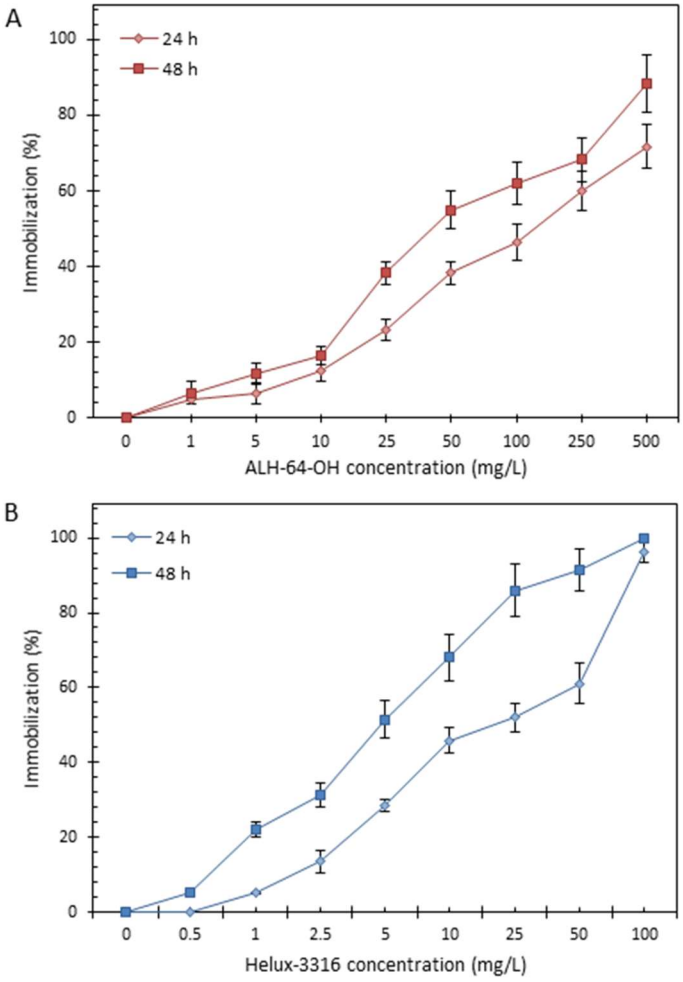

Figure 1. Effect of ALH-64-OH (A) and Helux-3316 (B) on the immobilization of *D. magna* after 24 h and 48 h (Error bars represent standard deviation, n = 12).

The calculated effective concentrations (EC_x) values of ALH-64-OH and Helux 3316 on the immobilization of *D. magna* are summarized in Table 2. The results showed that 24 h EC_{50} and 48 h EC_{50} of ALH-64-OH were 127 ± 6.7 mg/L and 53.7 ± 4.1 mg/L, respectively. The toxicity reported for hyperbranched polyesters is generally low (Reul et al., 2009). However, the EC_{20} values obtained in this work were 17.9 and 9.0 mg/L for 24 and 48 h respectively, which were relatively low and can be used as lowest observed effect concentrations (Warne and van Dam, 2008).

Up to our knowledge, no data have been reported for hydroxyl-terminated hyperbranched polyesters, but comparison can be established to PAMAM G2-OH (3.3 kDa) and G3-OH (6.9 kDa), which were much less toxic than their amino-terminated counterparts (Gonzalo et al., 2015). Helux-3316 was considerably more toxic than ALH-64-OH. 24 h and 48 h EC_{50} values were 16.8 ± 1.3 mg/L (3.29 ± 0.26 μM) and 4.69 ± 0.24 mg/L (0.92 ± 0.05 μM) respectively. EC_{20} values were as low as 1.24 mg/L after 48 h exposure.

These results agree with the presence of primary amines in its chemical structure and consistent with data for similar compounds. Casado et al. reported EC_{50} values of 0.77 mg/L and 0.66 mg/L, for poly(ethyleneimine)-polystyrene 55 nm and 110 nm, respectively (Casado et al., 2013). Naha et al. reported for EC_{50} 1.1 μM (24 h) and 0.7 μM (48 h) for the immobilization of *D. magna* exposed to polyamidoamine (PAMAM) dendrimers of generation G4 (Naha et al., 2009). PAMAM dendrimers show a similar chemical surface to Helux-3316, suggesting that the primary amino groups play the same role in the observed toxicity to *D. magna*. Accordingly, the toxicity of amino functionalized polystyrene and polyethylenimine nanoparticles has been linked to the presence of primary amino moieties (Aravindan et al., 2009; Nasser and Lynch, 2016).

Table 2. EC_X values of hyperbranched ALG-64-OH and hyperbranched Helux-3316 for the immobilization of *D. magna* after 24 and 48 h of exposure.

		ALH-64-OH		
Time points		**EC_{20}**	**EC_{50}**	**EC_{80}**
24	mg/L	17.9 ± 1.5	127 ± 6.7	-
	μM	2.44 ± 0.20	17.4 ± 0.9	-
48	mg/L	9.0 ± 1.2	53.7 ± 4.1	323 ± 45
	μM	1.22 ± 0.16	7.34 ± 0.55	44.0 ± 6.1
		Helux-3316		
		EC_{20}	**EC_{50}**	**EC_{80}**
24	mg/L	3.31 ± 0.40	16.8 ± 1.3	85.4 ± 7.4
	μM	0.65 ± 0.08	3.29 ± 0.26	16.7 ± 1.5
48	mg/L	1.24 ± 0.11	4.69 ± 0.24	17.8 ± 1.4
	μM	0.24 ± 0.02	0.92 ± 0.05	3.49 ± 0.28

The toxicity of amino-functionalized materials has been linked to the interaction of cationic moieties with the negative charge of cell membranes (Nasser and Lynch, 2016). The interaction would cause nanoscale holes, increase membrane permeability and cytotoxicity responses (Hong et al., 2006). *Salehi* et al. indicated that hyperbranched polyethylenimine may interact with the negatively charged antennae, legs, and carapace of *D. magna* (Salehi et al., 2017). The pK_a for the terminal primary amines is ~9, similar to amino-terminated dendrimers. Consistent with this fact, Helux-316 was positively charged at pH 7.7 as shown in Table S1.

3.3. ROS overproduction

The production of ROS was assessed using H_2DCFDA by measuring the intensity of DFC fluorescence after exposure to ALH-64-OH and Helux-3316. Fig. 2 shows the results obtained as percentage with respect to controls. A clear concentration-dependent ROS increase was observed at 24 h and 48 h for the two hyperbranched polymers. Fig. 2A shows significant ROS increase from 10 mg/L of ALH-64-OH after 24 h of exposure and from 5 mg/L after 48 h exposure. As shown in Fig. 2B Helux-3316 also induced a time and concentration dependent increase in ROS generation. The exposure to Helux-3316 caused higher intracellular ROS levels, with significant increase detected at 5 mg/L after 24 h and 2.5 mg/L after 48 h. Higher ROS levels were observed upon exposure to Helux-3316 with respect to ALH-64-OH, with DCF signals reaching levels over 300% that of controls and higher for all tested concentrations.

Increased ROS levels have been reported as part of the toxic response of different nanoparticles to *D. magna* (Klaper et al., 2009). Positively charged nanoparticles were associated to higher ROS levels in the gastrointestinal tract of *D. magna* (Dominguez et al., 2015). ROS generation and oxidative stress are generally associated to the toxic effects of engineered nanoparticles (Xia et al., 2006). Hydroxyl and Amine-terminated dendrimers have been shown to induce oxidative stress and mitochondrial damage causing metabolic impairing and cell apoptosis (Gonzalo et al., 2015). In mammalian cells, PAMAM dendrimers were shown to stimulate ROS overproduction (Mukherjee et al., 2010; Wei et al., 2009). More specifically, ROS overproduction was shown to induce damage to cell structures, nucleic acids, lipids and proteins, causing the immobilization of *D. magna* (Fan et al., 2012). The results were consistent with *D. magna* immobilization shown in Fig. 1 and suggested that oxidative stress was the reason for the observed toxicity. Lower concentrations, 5 mg/L ALH-64-OH and 2.5 mg/L Helux-3316, resulted in limited immobilization of *D. magna* (6.7% and 13% respectively, Fig. 1) coincident with not significant ROS overproduction, which was probably due to activation of antioxidant defences (Ulm et al., 2015).

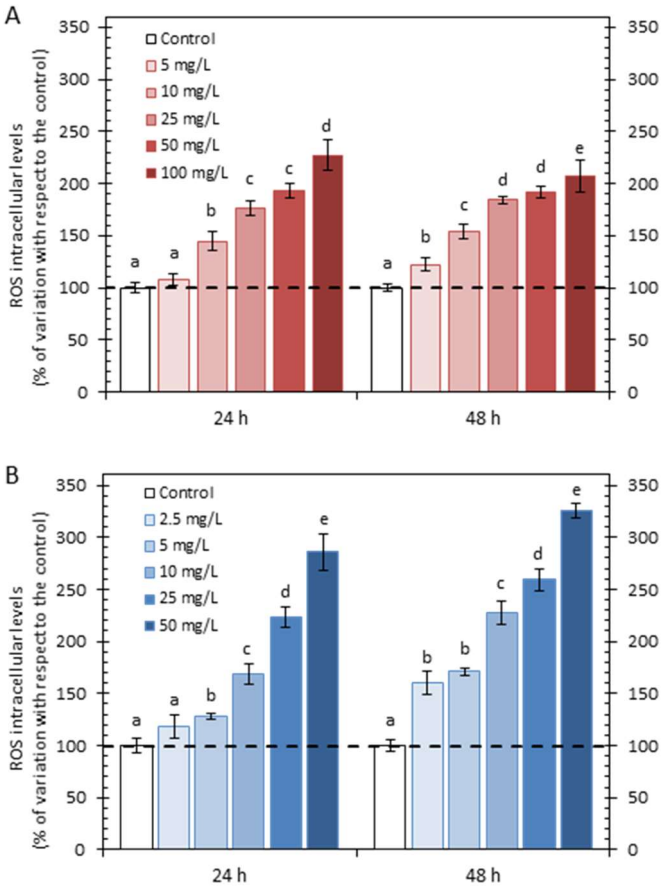

Figure 2. ROS intracellular levels of *D. magna* exposed to ALH-64-OH (A) and Helux-3316 (B). The results are expressed as percent variation of intracellular ROS levels ± SD with respect to controls (100% indicated by the dashed line). Treatments with different letters are significantly different (Turkey's HSD, $p < 0.05$).

3.4. Antioxidant enzyme activities

The activation of defence mechanisms by upregulation of the activity of antioxidant enzymes is an adaptation to prevent ROS damage upon toxicant exposure (Livingstone, 2003). GST and CAT activities were evaluated as antioxidant enzymatic biomarkers for the toxic response to hyperbranched polymers. GST (EC 2.5.1.18) conjugates breakdown products of lipid peroxidation and plays an important role preventing oxidative damage. CAT (EC 1.11.1.6) is a hydrogen peroxide scavenger that prevents ROS formation. GST and CAT have been employed as biomarkers for antioxidant defence and early indicators of acute toxicity in *D. magna* in various studies (Kim et al., 2010; Klaper et al., 2009; Ulm et al., 2015). The increase of GST and CAT activities in *D. magna* and *D. pulex* exposed to nanoparticles was previously reported (Kim et al., 2010; Klaper et al., 2009).

GST and CAT activities were normalized to protein content per sample as protein content in exposed daphnids differed from non-exposed organisms. Protein content decreased in *D. magna* exposed to increasing concentrations of ALH-64-OH and Helux-3316 for 48 h (Fig. S2, SM). The antioxidant enzymatic responses of *D. magna* exposed to ALH-64-OH and Helux-3316

after 48 h are shown in Fig. 3. Concentration-dependent response was observed for GST and CAT activities upon exposure of *D. magna* to ALH-64-OH. GST activity increased significantly at 10 mg/L ALH-64-OH, while significant increase in CAT activity was observed for 5 mg/L ALH-64-OH (Fig. 3A). Concerning Helux-3316, significant increase of GST activity was observed for 5 mg/L. CAT activity increased upon exposure to Helux-3316 up to 5 mg/L to decrease thereafter (Fig. 3B). Such decrease might be a consequence of sufficiently high ROS overproduction able to damage antioxidant defence mechanisms (Regoli and Giuliani, 2014). A similar nanomaterial, the amino-terminated G5-PAMAM dendrimers has been reported to induce GST activity in rainbow trout hepatocytes (Auclair et al., 2016).The higher increase in the activities of antioxidant enzymes induced by Helux-3316 compared to ALH-64-OH was consistent with ROS overproduction and daphnids immobilization results.

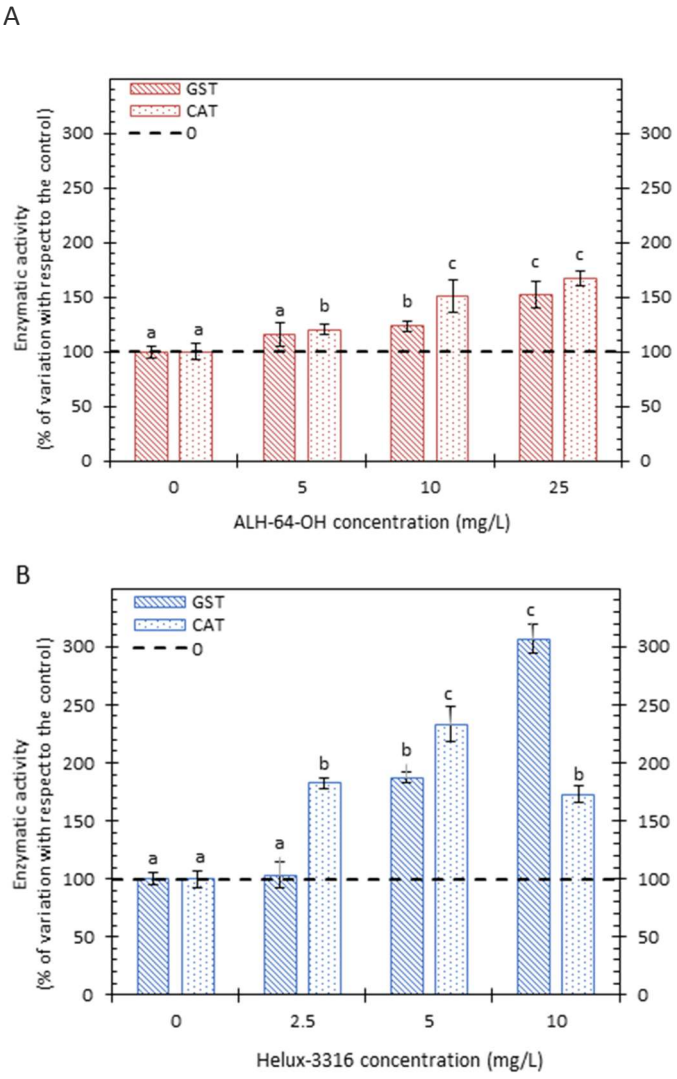

Figure 3. Changes in glutathione-S-transferase (GST) and catalase (CAT) activities in *D. magna* exposed to ALH64-OH (A) and Helux-3316 (B). The results are expressed as percent variation of enzymatic activities (U/mg protein) ± SD with respect to controls (100% indicated by the dashed line). Treatments with different letters are significantly different (Turkey's HSD, $p < 0.05$).

3.5. Helux-3316 uptake

Live daphnids exposed to Helux-3316-fluorescamine conjugates were visualized by confocal microscopy to investigate the potential presence of Helux-3316 hyperbranched material within the animals (Fig. 4). Fig. 4 A, B and C show control organisms displaying a light blue fluorescence on their surface. Organisms exposed only to fluorescamine at concentrations corresponding to the labelling experiment with the polymer exhibited the same light fluorescence and, therefore, were similar to control organisms indicating that the fluorescamine dye did not interact with daphnids (Fig. S3 and S4, SM). The confocal images of *D. magna* show blue fluorescence generally distributed across the surface of daphnids at the beginning of exposure tests with 5 mg/L and 10 mg/L of Helux-3316-fluorescamine conjugates (Fig. 4 D and G). The translucent body of *D. magna* allowed locating the fluorescent Helux-3316 conjugate. Fig. 4 E and H clearly showed the presence of fluorescent Helux-3316-fluorescamine in the gastrointestinal tract of the organisms. The uptake of Helux-3316-fluorescamine in the digestive tract of the exposed daphnids after 24 h indicated that Helux-3316 can be rapidly filtered by filter-feeding aquatic organisms such as *D. magna*. Intense blue fluorescence response from the conjugate was clearly visible within the gastrointestinal tract of *D. magna* after the standard 48-h exposure period (Fig. 4 F and I). After 48 h, the entire gastrointestinal tract contained fluorescent

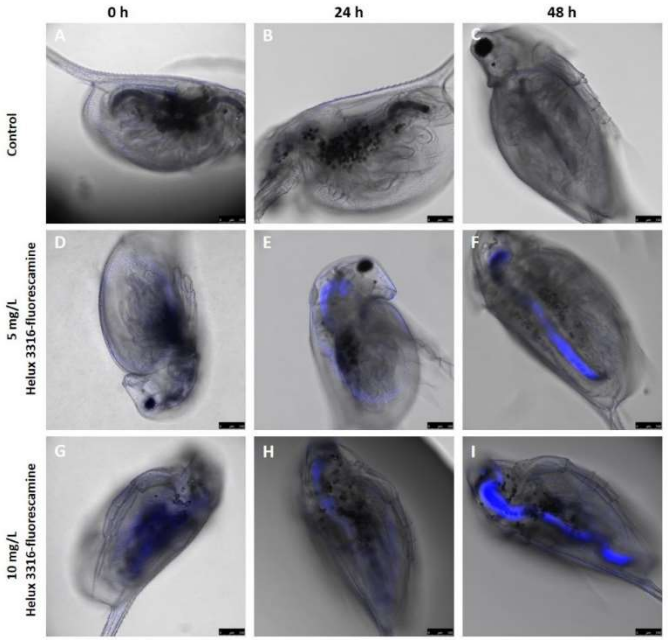

Figure 4. Confocal microscopy images overlaid on bright-field images of *D. magna* neonates (A, B, C) non-exposed, exposed to (D, E, F) 5 mg/L of Helux-3316-fluorescamine and exposed to (G, H, I) 10 mg/L Helux-3316-fluorescamine after 0 h, 24 h and 48 h exposure.

hyperbranched polymers, indicating higher uptake of Helux-3316-fluorescamine after prolonged exposure time. The intense response observed in the gastrointestinal tract of organisms exposed to labelled Helux-3316, was compatible with ingestion through

filtration, which is the most likely route of uptake for *D. magna*, an aquatic organism able to filter particles not exceeding 50–60 μm (Bednarska et al., 2014). Rosenkranz et al. observed accumulation of fluorescent carboxylated polystyrene beads in the gastrointestinal tract of *D. magna*(Rosenkranz et al., 2009). Auffan et al. showed CeO_2 nanoparticles in the gut content of *D. pulex* that were accumulated by ingestion (Auffan et al., 2013). Ingestion of particles is considered the primary route for accumulation in planktonic filter feeders. However, *Brun* et al. reported a route of fluorescent polystyrene nanoparticles accumulation mediated through the brood pouch in *D. magna* (Brun et al., 2017).

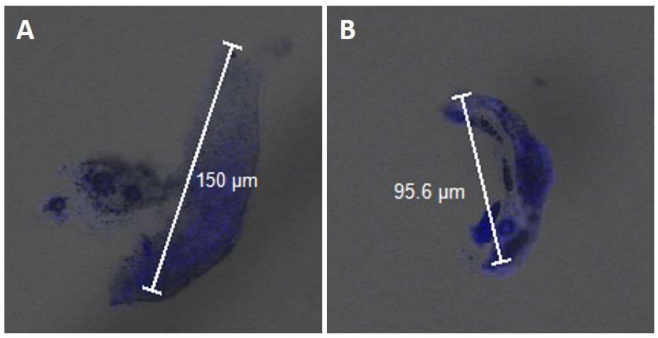

Figure 5. Confocal microscopy images overlaid on bright-field images of *D. magna* fecal pellets collected after 48 h exposure to (A) 5 mg/L of Helux-3316-fluorescamine and to (B) 10 mg/L of Helux-3316-fluorescamine.

Filtration is followed by a depuration period through excretion. Fig. 5 evidenced the presence of fluorescent fecal pellets in the medium of daphnids exposed to Helux-3316-fluorescamine conjugates for 48 h. The fecal pellets were previously identified by optical microscopy after being filtrated from the exposure medium, as shown in Fig. S5 (SM). The use of fluorescent labelling to detect fecal material can be relevant for environmental fate studies. Booth et al. reported the excretion of fluorescent poly-methyl methacrylate plastic nanoparticles in *D. magna* by visualizing fluorescent fecal material (Booth et al., 2016). The physiological process of depuration has been previously described for uptake studies in *D. magna* exposed to carbon nanotubes (Petersen et al., 2009) and ^{14}C-labelled graphene (Guo et al., 2013). Although fecal pellets produced by cladocerans are fragile, the materials regenerated by excretion might remain for a long period of time in the water surface facilitating the interaction with other aquatic organisms. The release of materials from *D. magna* has significant ecological consequences considering the production of fecal pellets as exposure source via food chain in aquatic environment. The observed effects via fluorescently labelled polymers provided useful information for the environmental fate of hyperbranched polymers. Further detailed studies are needed to assess the environmental implications of fecal pellets as carriers of toxicants in freshwater systems.

4. Conclusions

We studied the toxicological effects of two hyperbranched polymeric nanomaterials, the polyester ALH-64-OH and the polyamidoamine Helux-3316 to the freshwater crustacean *D. magna*. The followed the immobilization of daphnids, ROS overproduction and the activity of defence antioxidant enzymes. Our results support the hypothesis that the overproduction of ROS above permissible levels is could result is the cause for the damage in daphnids exposed to both hyperbranched polymers.

EC_{20} for *D. magna* immobilization at 48 h, that could be assimilated to lowest-observed-effect concentration, was 9.0 mg/L for ALH-64-OH and 1.24 mg/L for Helux-3316. The higher toxicity of Helux-3316 could be attributed to the presence of primary amino groups on its positively charged surface. Concentration-dependent antioxidant response was observed for GST and CAT activities with particularly high response (>300% with respect to controls) for CAT in individuals exposed to Hellux-3316.

We showed for the first time evidence of the uptake of fluorescently labelled Helux-3316 into the gastrointestinal tract of *D. magna*, and its removal via excretion within fecal pellets. The information on the toxicity of hyperbranched polymers is limited despite their multiple uses. Our findings indicated that hyperbranched polymers pose a significant ecological risk to aquatic organisms and their presence in water and wastewater should be monitored.

Acknowledgements

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SUPPLEMENTARY MATERIAL

Hyperbranched polymeric nanomaterials impair the freshwater crustacean Daphnia magna

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Contents:

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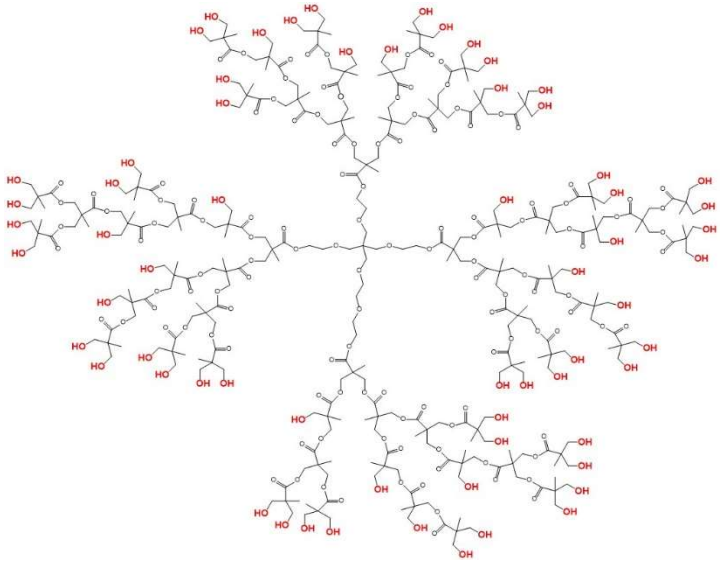
A



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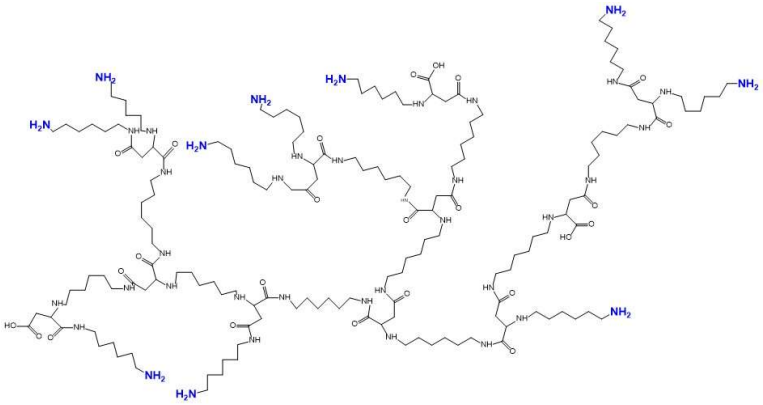

Figure S1. Chemical structure of (A) ALH64-OH and (B) Helux-3316. Hydroxyl surface groups (-OH) are shown in red and primary amine end-groups (-NH_2) in blue.

Table S1. Physicochemical properties of ALH-64-OH and Helux-3316 in ultrapure water at different times. (ζ-potential measured at pH 7.7 ± 0.1).

	ALH-64-OH					
	1 mg/L		10 mg/L		100 mg/L	
	d_{DLS} (nm)	ζ-potential (mV)	d_{DLS} (nm)	ζ-potential (mV)	d_{DLS} (nm)	ζ-potential (mV)
0 h	246 ± 23	-5.3 ± 0.5	274 ± 19	-15.1 ± 0.5	263 ± 22	-25.9 ± 0.5
24 h	272 ± 13	-12.9 ± 1.1	258 ± 13	-30.6 ± 0.4	289 ± 21	-40.5 ± 3.8
48 h	212 ± 8.0	-26.5 ± 2.0	302 ± 27	-30.9 ± 2.5	255 ± 21	-41.7 ± 0.9
	Helux-3316					
	1 mg/L		10 mg/L		100 mg/L	
	d_{DLS} (nm)	ζ-potential (mV)	d_{DLS} (nm)	ζ-potential (mV)	d_{DLS} (nm)	ζ-potential (mV)
0 h	273 ± 21	+8.6 ± 0.4	307 ± 17	+2.6 ± 0.1	314 ± 10	+13 ± 1.1
24 h	296 ± 12	+5.3 ± 0.5	310 ± 20	+2.3 ± 0.2	341 ± 29	+17 ± 1.2
48 h	301 ± 14	+6.5 ± 0.6	372 ± 19	+5.2 ± 0.4	456 ± 36	+16 ± 0.2

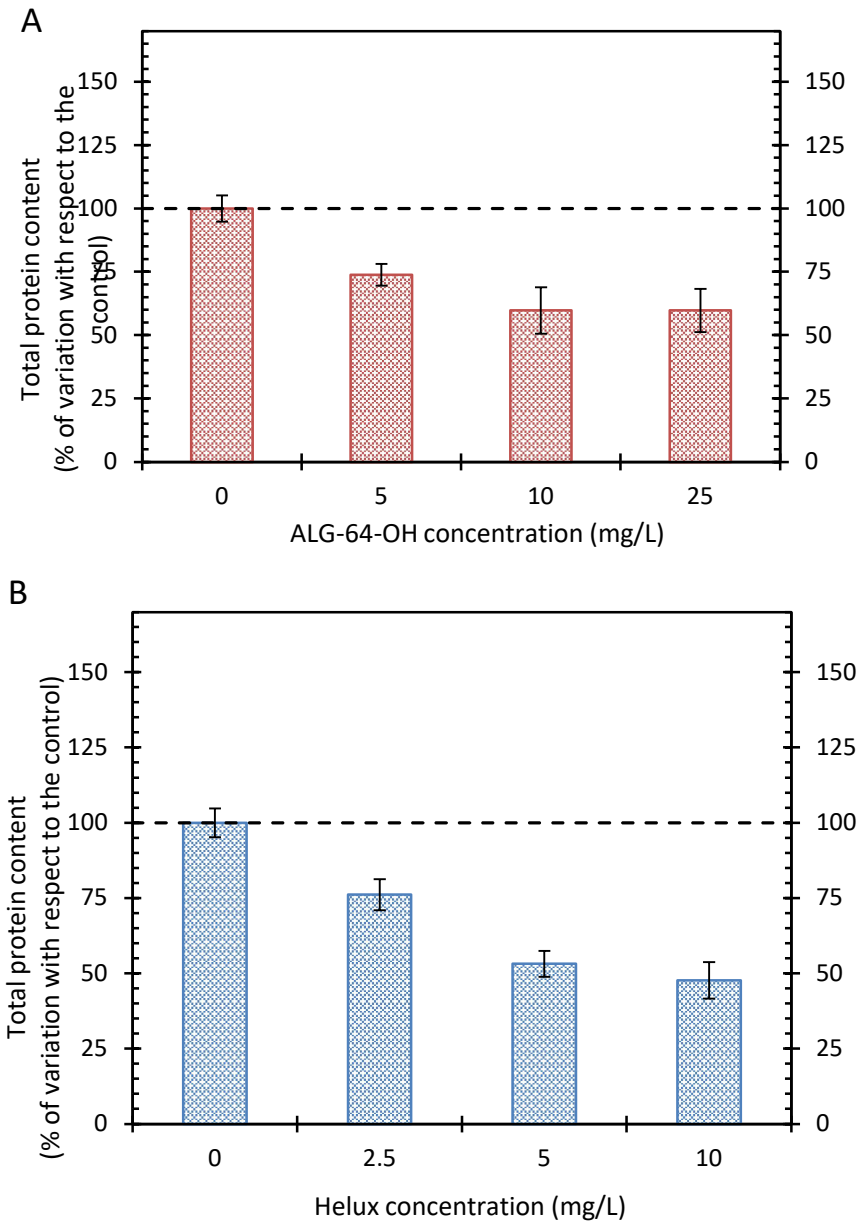

Figure S2. Changes in total protein content in *D. magna* exposed to ALH64-OH (A) and Helux-3316 (B). Results are shown as percentage of variation of protein content ± SD (n=3) with respect to control (the dashed line corresponds to 100%).

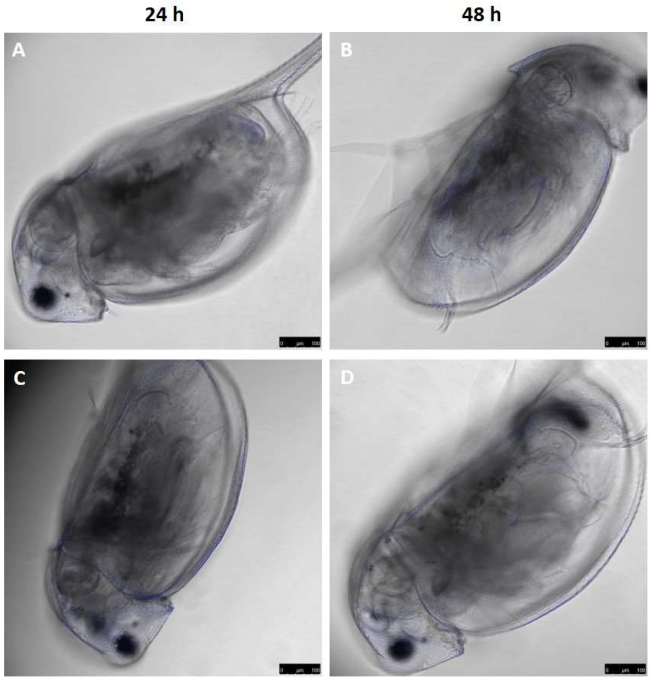

Figure S3. Confocal images of *D. magna* treated only with fluorescamine for 24 and 48 h at the concentrations corresponding to the labelling of 5 mg/L (A-C) and 10 mg/L (B-D) of Helux-3316.

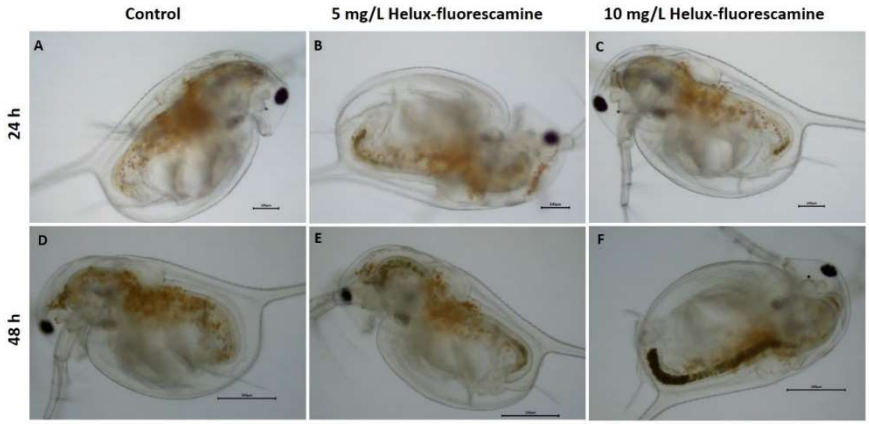

Figure S4. Optical microscopy images of *D. magna* neonates non-exposed (A, D), exposed to 5 mg/L of Helux-3316-fluorescamine (B, E) and exposed to 10 mg/L of Helux-3316-fluorescamine (C, F) after 24 h and 48 h exposure.

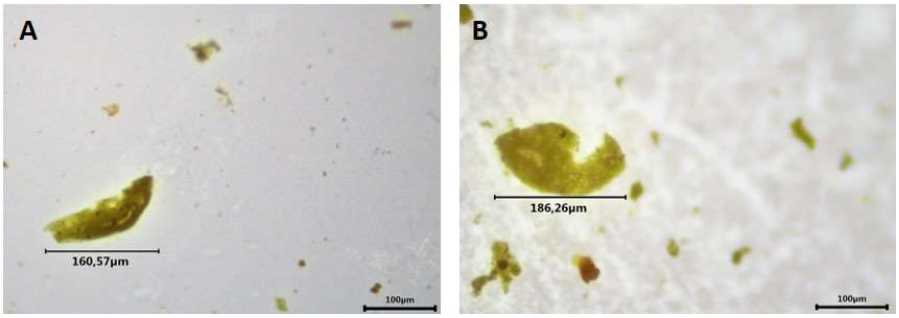

Figure S5. Optical microscopy images of *D. magna* fecal pellets collected after 48 h exposure to (A) 5 mg/L of Helux-fluorescamine and to (B) 10 mg/L of Helux 3316-fluorescamine.