Chapter

Bioavailability and Effects of Polystyrene Nanoparticles in *Hydra circumcincta*

Joëlle Auclair, Brian Quinn and François Gagné

Abstract

The release of nanoplastics (NPs) from the weathering and degradation of plastics in the environment is an important concern to aquatic ecosystems. The purpose of this study was to examine the bioavailability and toxicity of 50 and 100 nm fluorescently labeled polystyrene nanoplastics (NP) to the invertebrate *Hydra attenuata*. The hydrae were exposed to increasing concentrations of 50 and 100 nm NPs (1.25-80 mg/L) for 96 h at 20°C. A subgroup of hydra was depurated in media to determine the persistence of effects. The results revealed that the animals accumulated detectable amounts fluorescent NP and produced morphological changes at a threshold concentration between 5 and 10 mg/L. The hydrae were able to eliminate 76 and 78% of the 50 and 100 nm NPs, respectively. A characteristic tentacle detachment from the body was observed. Biochemical markers were also determined in exposed organisms and increased glutathione S-transferase (GST) activity, oxidative damage and neutral lipids levels that persisted after the 24 h. In conclusion, NPs are bioavailable to *Hydra*, produce morphological changes and increase oxidative stress and neutral lipids. The formation of neutral lipids could be the result of reduced food assimilation or a means for the elimination of NPs.

Keywords: nanoplastics, polystyrene, hydra, oxidative stress, neutral lipids

1. Introduction

The occurrence of plastics in the environment represents a major contamination problem, particularly for the aquatic environment. Indeed, it is estimated that over 8 million tons of plastic are released in oceans per year including micro- and nanoplastic (NP) fragments [1, 2]. Microplastic and NP are operationally defined as particles in the size range of 5 mm to 1 μm and 100–1 nm respectively, but plastics between 0.1 and 1 μm are considered by some authors to be NP [2, 3]. Given their small size, NPs can permeate cells and interact with biological macromolecules, possibly leading to unexpected long-term effects. This is further emphasized by the fact that at present NPs are difficult to measure in organisms due to a lack of bioanalytical methods. Recently, a method based on a fluorescent molecular rotor probe was proposed for NP bioanalysis in the attempt to address this bottleneck [4]. Among plastic polymers found in the marine environment, polystyrene, composed of vinylarene repeats, is the most abundant type accounting for up to 8% of total plastic production worldwide, with an annual production of more than 23 million tons per year [5, 6]. Plastics are subject to both abiotic and biotic weathering leading

to degradation into the micro- and nanoscale, dramatically increasing the number of plastic-based nanomaterials in the environment.

As mentioned above, the small size of NPs allows them to be internalized by cells which could lead to severe cell damage such as apoptosis, protein denaturation/fibrillation and the formation of hydrophobic liquid crystals [7–9]. A number of laboratory studies have investigated the aquatic toxicity of NPs, primarily focusing on marine life, i.e. phytoplankton, zooplankton (crustacean, sea urchins) and bivalves (filter-feeders) [10–13]. NPs were shown to reduce algal growth, the development of sea urchin embryos and shrimp survival and produce immune-related effects in bivalves. These studies show that NPs could be harmful to marine organisms, but to date less information exists for freshwater invertebrates. For this reason, it is of value to include freshwater organisms when assessing the risks of nanomaterials given the potential for their behaviour to be influenced by surface water properties such as salinity, pH and organic matter [14, 15]. For example, in high-salinity environments, e.g. saltwater, low Zeta potential (surface charges), nanoparticles would tend to form aggregates, whereas aggregation would be less likely in low salt conditions [16].

The cnidarian *Hydra circumcincta Schulze*, *1914*, is ubiquitously found in freshwater ecosystems and is often used as sensitive test species for toxicity assessments [17]. Hydra is considered an immortal organism which can regenerate for decades and is composed of a tube-like body (foot and digestive tube) with a head surrounded by six or more antennae to catch food [18]. The severity of toxic effects can be easily observed through characteristic morphological changes [19] (Figure 1S, supplementary data). Moreover, the impacts of chemicals or other stressors can be conveniently followed at multiple levels of biological organization such as behaviour (feeding activity), change in antennae and foot morphology, growth, regeneration, asexual reproduction and various biochemical markers (gene expression, proteomics, oxidative stress, genotoxicity, etc.) [20]. Hydrae are easy to maintain in culture and show unique regeneration activity. Recently, hydrae were used to investigate the effects of microplastics [21]. The study revealed that hydrae were able to ingest small microplastic fragments from facewash soaps that compromised feeding activity and displayed toxicity by morphological changes (antenna regression). The purpose of the current study was therefore to determine the bioavailability and toxicity of fluorescently labeled polystyrene NPs to hydra. Hydrae were exposed to 50 and 100 nm polystyrene NP and analysed for toxicity using morphological changes and biomarkers of energy metabolism (neutral lipids) and oxidative stress to relate the presence of NPs in hydra with toxicity responses.

2. Materials and methods

2.1 Preparation and exposure of NPs to hydra

Fluorescently labeled polystyrene nanoplastics were purchased from PolyScience (USA). Hydrae were maintained in 20-cm diameter crystallization bowls at 20°C in the following medium: 1 mM $CaCl_2$ and 0.5 mM TES (N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid), pH 7.0, as previously described [17]. They were fed daily with *Artemia salina* brine shrimps. Exposure tests were conducted in 12-well microplates with three animals placed in each well (n = 9 organisms per treatment) in 4 mL of media. The NP suspension was pre-diluted at 0.5 g/L in Milli-Q water, and the final dilutions were prepared in hydra media. Controls consisted of the hydra media only. The exposure concentrations were 0, 1.25, 2.5, 5, 10, 20, 40 and 80 mg/L corresponding to 0.04, 0.079,

0.159, 0.318, 0.636, 1.27 and 2.54 × 10^{11} nanoparticles/L for 100 nm NP and 0.32, 0.64, 1.29, 2.57, 5.15, 1.03 and 2.06 × 10^{12} nanoparticles/L for 50 nm, respectively. Hence, on a mass basis, 50 nm NP contains eight times more particles than the 100 nm NP.

After a 96 h exposure at 20°C, changes in hydra morphology were determined based on the Wilby scale [18] from controls (normal-sized animals with long and slender tentacles) to sublethal effects (clubbed or reduced tentacles) and lethal effects (tulip or disintegrated stages). Representative examples for each morphological changes are represented in the supplementary data section (**Figure 1**). These morphological features were scored under a stereomicroscope at 6× magnification where the number of hydra with morphological changes is the endpoint used to calculate the 50% effect concentration (EC50) or 50% lethal concentration (LC50). A $ZnSO_4$ (LC50 of 1 mg/L; 0.7–1.3 mg/L 95% confidence interval) test was used to ensure reproducibility of culture conditions. At the end of the morphological assessment, the NP containing media was removed from the wells. The hydrae were washed in 1 mL of media, detached from the culture plate, suspended in groups of three organisms in 250 μL of a homogenization buffer and stored at −85°C for NP uptake and biomarker analyses. The homogenization buffer consisted of 50 mM NaCl containing 10 mM Hepes-NaOH, pH 7.4, 0.1 mM dithiothreitol and 0.1 μg/mL apoprotinin (protease inhibitor). A subset of hydrae at low concentrations (1.25, 2.5 and 5 mg/L) were allowed to depurate in clean media for 24 h before freezing to determine the elimination potential of the NPs and the toxicity potential.

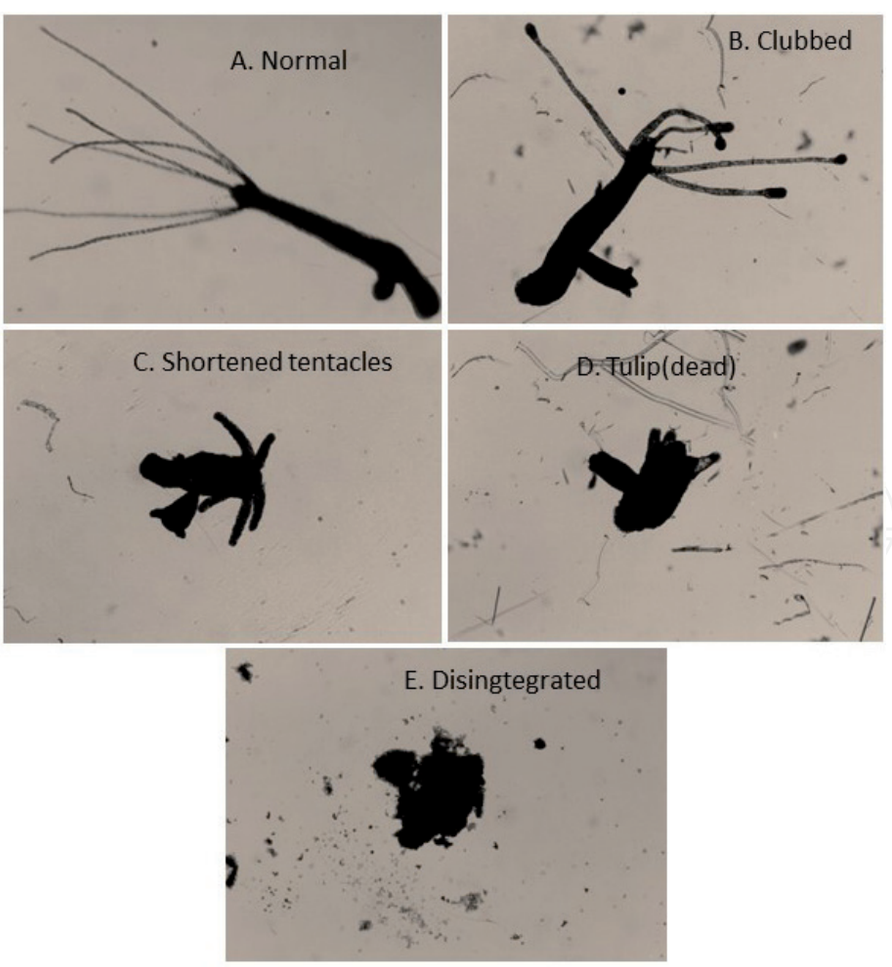


Figure 1.
Morphological stages Hydra attenuate after exposure to REEs for 96h (magnification x40): healthy polyp (A); clubbed tentacles (B); shortened tentacles (C); tulip stages (D) and disntegrated stages (E).

2.2 NP uptake and sublethal effect assessments

The organisms were thawed on ice for 15 min in the homogenization buffer and homogenized using a Teflon pestle handheld tissue homogenizer. Total protein content was determined using the Coomassie Brilliant Blue method in clear 96-well microplates [22]. Serum bovine albumin was used for calibration. The uptake of fluorescently labeled NP was determined in the homogenates using fluorescence spectrometry. A 100 μL volume of the homogenate was placed in 96-well dark microplates, and fluorescence levels were determined at 485 nm excitation and 520 nm emission (Synergy 4, BioTek Instruments, USA). Fluorescence concentration was determined using the standard addition methodology with freshly prepared fluorescence standards. The data were expressed as μm fluorescence/mg proteins. The activity of glutathione S-transferase (GST) was determined by a miniaturized spectrophotometric methodology in 384-well microplates (50 μL assay volume) [23]. The homogenate was mixed with 0.1 mM of reduced glutathione and 2,4-dichloronitrobenzene (DCNB) in 50 mM NaCl containing 10 mM Hepes-NaOH, pH 7.4, and incubated at 30°C for 30 min. The increase in absorbance at 340 nm was determined using a microplate reader every 5 min. The data were expressed as the increase in absorbance at 340 nm/min/mg of proteins. Lipid peroxidation (LPO) was determined in hydra homogenates using a miniaturized version of the thiobarbituric acid method [24]. A volume of 10 μL of the homogenate was mixed with 20 μL of 10% trichloroacetic acid containing 1 mM $FeSO_4$ and 20 μL of 0.7% thiobarbituric acid in 384-well dark microplates. The mixture was heated at 75°C for 10 min and cooled at room temperature. Fluorescence readings were taken at 540 nm excitation and 600 nm emission using standard solutions of tetramethoxypropane (stabilized form of malonaldehyde) for calibration in the blank media (homogenization buffer only). Results were expressed as μg thiobarbituric acid reactants (TBARS)/mg total protein in the homogenate. Finally, neutral lipids (triglycerides) in homogenates were determined using an AdipoRed fluorescent reagent (Lonza; Walkersville, MD, USA). A volume of 5 μL of AdipoRed reagent was added to 10 μL homogenate and 35 uL Milli-Q water in a black 384-well microplate. After 10 min under constant agitation, fluorescence was measured at 485 nm excitation and 535 nm emission (Synergy 4, BioTek microplate reader, USA). Data were expressed as relative fluorescence units (RFU)/mg proteins in the homogenate.

2.3 Data analysis

The study design examines the bioavailability and toxicity of 50 and 100 nm NPs in hydra exposed to seven NP concentrations. The 96-h toxicity (50% lethal concentration or LC50) was determined using the Spearman-Karber method [25] and the 96-h sublethal effect concentration (50% effect concentration or EC50) using the log-logit method. Data normality and homogeneity of variance were verified using the Shapiro–Wilk and Bartlett tests, respectively. The data were analyzed using analysis of variance and critical differences between the controls, and exposure concentrations were determined by least square difference (LSD) test. The trends between the data were also analysed using Pearson-moment correlation and hierarchical tree tests. All tests were performed using the SYSTAT software (version 13). Significance was set at $\alpha < 0.05$.

3. Result and discussion

The stock solutions of the fluorescently labeled polystyrene NPs contained 6.7×10^{14} and 8.3×10^{13} particles/mL for the 50 and 100 nm diameter NPs, respectively.

Hence, on a mass basis, there were eight times more 50 nm NPs than 100 nm NPs. The levels of fluorescently labeled NP were quantified in hydra exposed to increasing concentrations of NPs after 96 h and following a 24 h depuration for the low-exposure concentrations (1.25–5 mg/L) only (**Figure 2**). The hydra contained NPs in a concentration-dependent manner for concentrations >2.5 mg/L and >5 mg/L for 50 and 100 nm diameter NPs, respectively. Based on the ratio of the fluorescence label on each NP sizes, the hydra accumulated in the order of 6.448×10^7 NP per hydra exposed to 80 mg/L 50 nm NP and 2.605×10^8 NP per hydra exposed to 80 mg/L 100 nm NP. This suggests that the 100 nm NP were more bioavailable to hydra than the 50 nm NP. It is expected from the low salt content in the incubation media that the hydrodynamic behaviour of the NP would not overly change and minimal aggregation would occur [26]. The toxicity was determined in hydra based on morphological changes (**Table 1**). Although the lethal toxicity was not detected at concentrations below 80 mg/L based on the formation of tulip and disintegrated stages, the occurrence of the last morphological changes before reaching irreversible/lethal stage (short tube with receded tentacles) occurred at EC50 values of 16.2 and 8.7 mg/L for the 50 and 100 nm NPs, respectively. Based on the confidence intervals, the 100 nm NP was more toxic than the 50 nm NPs. A peculiar morphological response was observed during the depuration step in hydra exposed to 1.25–5 mg/L NPs for 24 h in clean media. Many hydrae lost some of their antenna during this period, and they appeared more fragile during removal for tissue processing as evidenced by fragmentation of the tentacles and body. Interestingly, the levels of NPs were still significantly higher albeit at lower concentrations than controls at 5 mg/L NPs for both sizes. It was estimated that it remained 3.34×10^3 particles per hydra for the 100 nm NPs and 2.6×10^5 particles per hydra for the 50 nm NPs. This represents an elimination potential of 79 and 76% for the 100 and 50 nm NPs, respectively. Hence, although the 100 nm NP were more bioavailable, the 50 nm NP were more slowly eliminated in hydra indicating a size-dependent effect. In a recent study, the 48 h lethal concentration (LC50) of a 75 nm diameter polystyrene NP was 77 mg/L in *Daphnia pulex* [27] which is in the same order with the observed toxicity at 8.7 and 16.2 mg/L for 100 and 50 nm NPs, respectively. This study also found that stress defense genes for superoxide dismutase and GST activity followed a biphasic response, i.e. were first induced and then inhibited in daphnids. The biphasic responses in GST activity and LPO levels in hydra exposed to the 50 nm NPs occurred at concentrations approaching manifestation of morphological changes in hydra.

Oxidative damage and xenobiotic conjugation activity were determined in hydra exposed to the NPs by following changes in GST activity and LPO, respectively (**Figure 3**). In hydra exposed to 50 nm NP, GST activity followed a biphasic response where a significant (LSD test, $p < 0.05$) decrease in activity was observed at low concentrations (2.5 mg/L) followed by an increase at higher concentrations compared to 2.5 mg/L. A decrease in GST activity was observed after the 24 h depuration period. In respect to LPO, an inverse trend was observed with a significant increase in LPO was found at low concentration of 50 nm NPs (5 mg/L) with a significant decrease at 10 mg/L. In the attempt to determine whether the observed changes in GST activity was related to LPO levels, i.e. involved in reactive oxygen species scavenging, an analysis of covariance was performed with LPO as the covariate and the exposure concentration as the main factor. The analysis revealed that GST activity was significantly related with exposure concentration and LPO had no significant effect on its activity. However, during the depuration period, both GST and LPO activity were increased which suggests a delayed stress response. Based on analysis of covariance, LPO did not influence GST activity during the depuration period as well which suggests that GST activity was more involved in

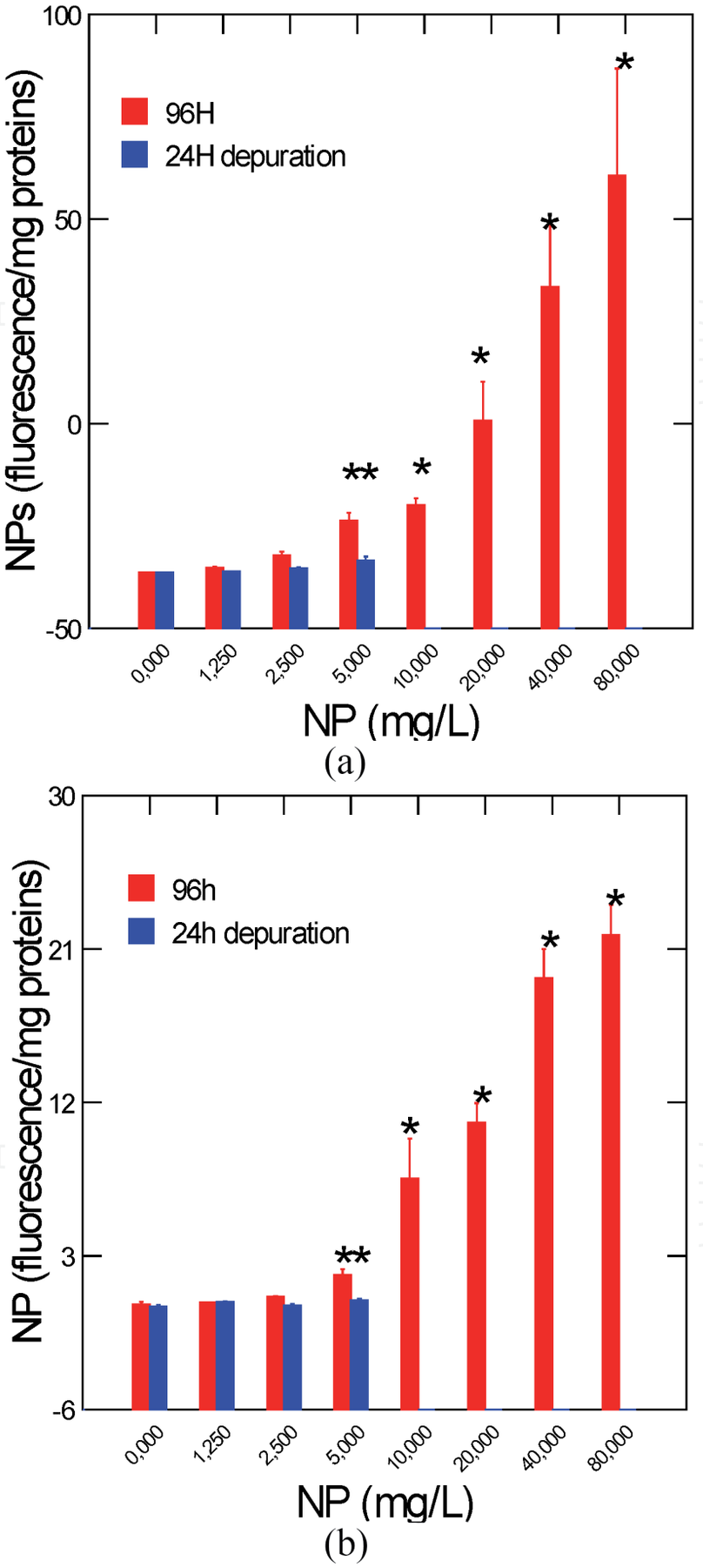


Figure 2.
Bioavailability of NP in Hydra attenuata *exposed to increasing concentrations of 50 nm (A) and 100 nm (B) polystyrene nanoplastics for 96 h at 20°C. A subsample of organisms were placed in clean media for 24 h (depuration). *indicates significance from controls (0) at $p < 0.05$. ** = $p < 0.01$.*

NP (nm)	LC50 in mg/L (95% confidence)	EC50 in mg/L (95% confidence)	Antennae loss in mg/L (amputation) during depuration
50	>80	8.7 (7–10)	1.25–5
100	>80	16.2 (11–26)	1.25–5

Time required for the manifestation of sublethal and lethal effects.

Table 1.
Toxicity data of 50 and 100 nm polystyrene NPs.

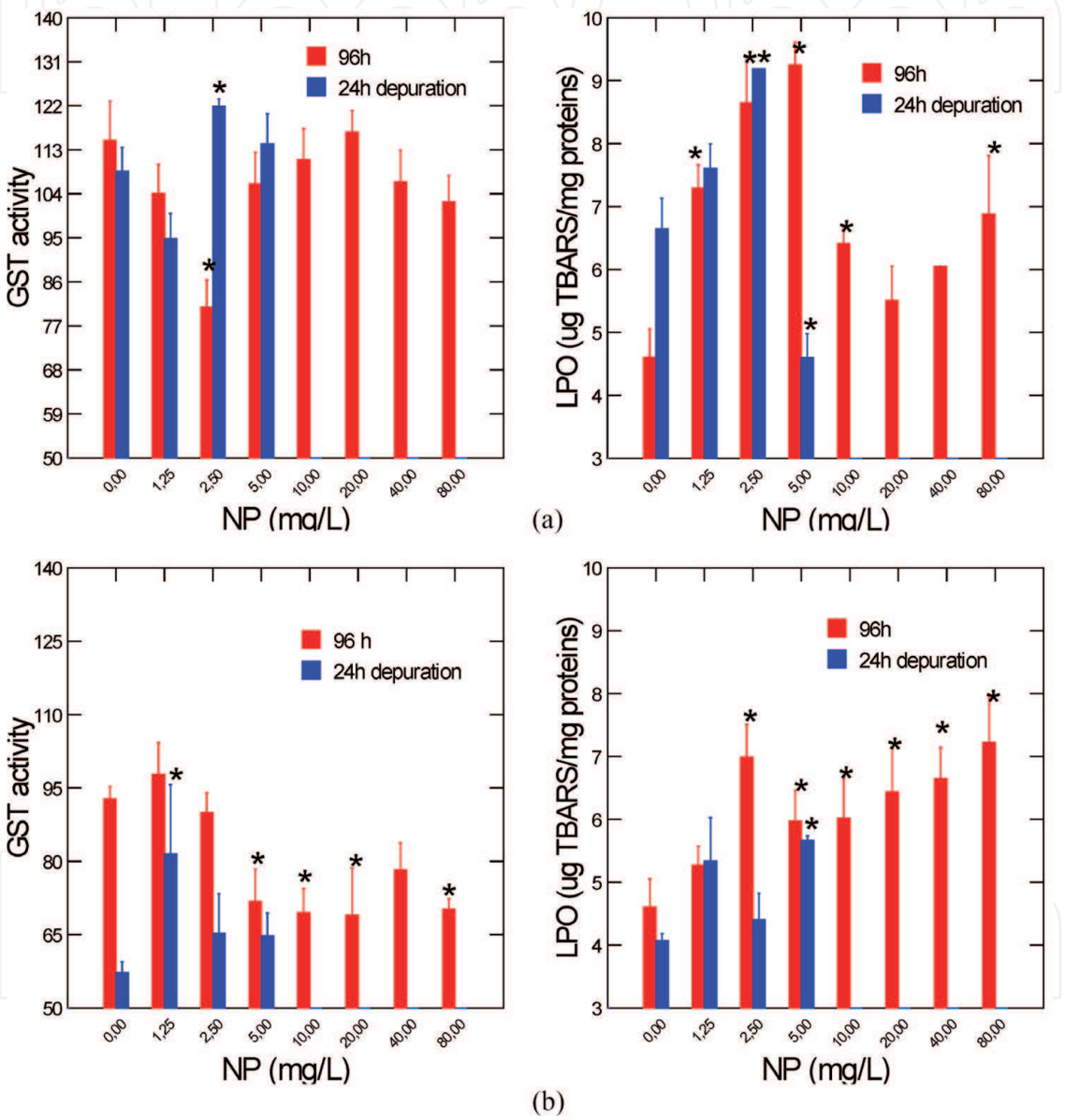
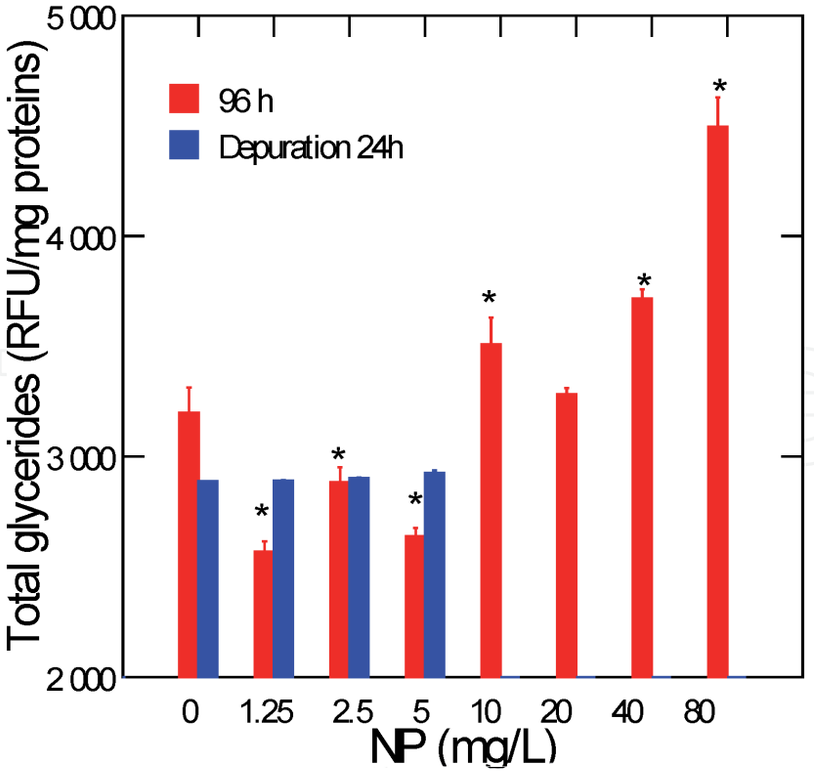


Figure 3.
Oxidative damage in Hydra attenuata *exposed to polystyrene NP. Hydrae were exposed to increasing concentration of 50 nm (A) and 100 nm (B) polystyrene NP for 96 h t 20°C. A subsample of hydrae were placed in clean media for 24 h (depuration). *indicates significance from the controls at p < 0.05.*

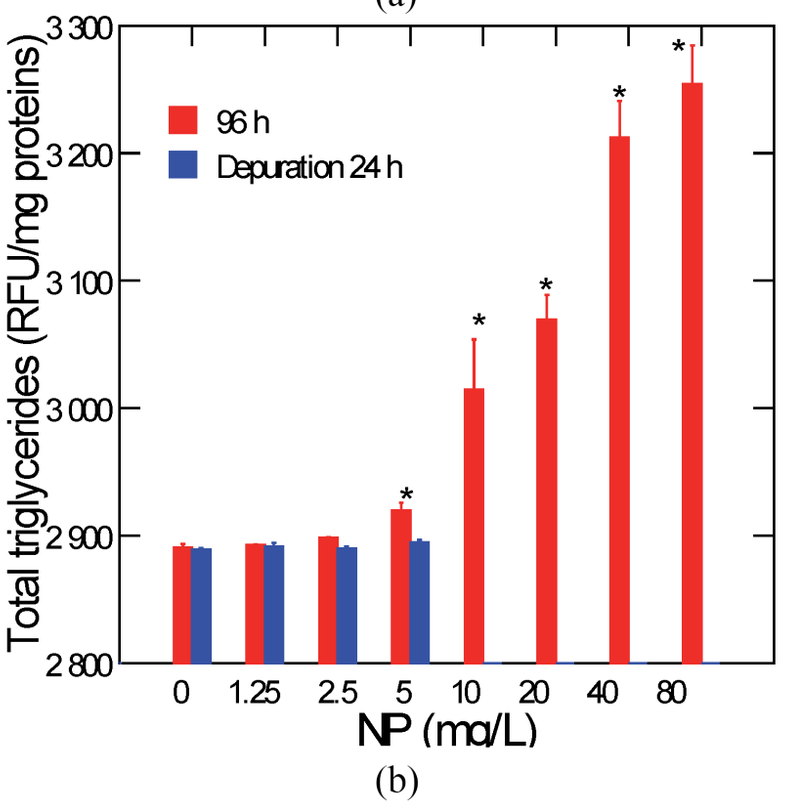
xenobiotic conjugation activity than in oxidative stress. In the case of the 100 nm NPs, overall GST activity was significantly decreased (ANOVA $p < 0.05$) with the concentrations used. A significant reduction in GST activity was observed at all concentrations >5 mg/L after 96 h exposure. LPO levels were significantly higher (ANOVA $p > 0.05$) than controls at concentrations >2.5 mg/L following the 96 h

exposure. Since LPO results generally followed the same trend as the GST results, we performed an analysis of covariance of GST activity with LPO levels as the covariate to determine the contribution of oxidative stress on GST activity. The analysis revealed that both the exposure concentration and LPO levels significantly influence GST activity but the activity was more strongly associated with exposure concentration than LPO levels. This suggests that GST activity was not solely involved in handling oxidative stress but in conjugation activity as well. During the 24 h depuration period, both GST activity and LPO levels were decreased following exposure to 100 nm NPs suggesting a post-exposure stress reaction for the elimination of NPs. Exposure of hydra to NPs revealed that oxidative stress was readily increased by both diameter sizes. Moreover, LPO levels were related with neutral lipids in hydra suggesting that uncharged polystyrene NPs could lead to oxidative lipid damage in hydra. Oxidative stress was also found in the gut of *Daphnia magna* exposed to 100 nm NP [25]. Increased in LPO was also observed in *Artemia salina* shrimps exposed to 50 mg/L of polystyrene nanospheres [28]. Moreover, a reduction in GSH levels was observed which is consistent with the observed decreased in GST activity (depleted GSH?) in hydra exposed to the 100 nm; albeit a transient decrease in the activity was found with the 50 nm at low concentrations.

The levels of neutral lipids were also determined in hydra exposed to NPs (**Figure 4**). For the 50 nm NPs, the neutral lipids levels significantly decreased at low concentrations (1.25–5 mg/L) followed by an increase at higher concentrations where morphological changes appear (with the exception of 20 mg/L). Neutral lipids were highly correlated ($r = 0.81$, $p < 0.001$) with NP levels in hydra. After the 24 h depuration period, lipid levels remained significantly elevated at 5 mg/L NP. For the 100 nm NP, the lipid levels significantly increased at concentrations ≥5 mg/L following 96 h exposure. Neutral lipids were strongly correlated ($r = 0.99$, $p < 0.001$) with NPs, GST activity ($r = 0.48$, $p < 0.05$), and LPO ($r = 0.49$, $p = 0.01$) in hydra. After the 24 h depuration period, the neutral lipid levels were still significantly elevated at 5 mg/L. There was no significant correlation between neutral lipid levels with either GST activity or LPO levels with the 50 and 100 nm NPs. In the attempt, to gain some overview in the responses of hydra exposed to NPs, a cluster tree analysis was performed (**Figure 5**). The analysis revealed two response patterns. The first response pattern was that NP levels in hydrae were closely associated with neutral lipids for NP of both diameter sizes. The second pattern was that GST activity was closely related with LPO with the 100 nm NPs only. The involvement of neutral lipids with NP exposure and accumulation suggests altered metabolisms leading to increased neutral lipid mobilization. In the nematode *C. elegans* exposed to polystyrene NPs, there was increased gene expression of FOXO transcription factor suggesting that the insulin signaling pathway was affected [29]. Moreover, downstream genes in oxidative stress (superoxide dismutase and metallothionein) were involved which supports the hypothesis that polystyrene NP induces oxidative stress in invertebrates. It was found that a mutation of the FOXO gene increased the toxicity of NP which suggests that metabolic energy was required for protection against nanotoxicity of plastics. The increase in neutral lipid levels in hydra exposed to the NPs could be involved in detoxification/protection mechanisms to remove hydrophobic NP from cells. It was suggested that altered lipid metabolism could be obtained from the long-term exposure to both microplastics and NPs [30]. It is possible that the physical obstruction of the digestive track by NPs could lead to decreased food assimilation which could lead to increased mobilization of lipid energy reserves. Another explanation to increased neutral lipids in hydra is that it could represent a means of elimination of neutral hydrophobic nanoparticles composed on polystyrene (vinylarene polymer). Indeed, lipids could form a corona on theses NPs which could favor elimination by fusion to autophagosome and lysosome



(a)



(b)

Figure 4.
Neutral lipid content in Hydra attenuata *exposed to NPs. The levels of neutral lipids were determined in hydra exposed to 50 nm (A) and 100 nm (B) polystyrene NP for 96 h t 20°C. *indicates significance ($p < 0.05$) relative to controls.*

organelles. This is in agreement with a previous study showing that the addition of lipids and proteins to 50 nm NP suspension in the presence of a membrane-selective probe leads to anisotropic changes [9]. Moreover, fluorescent polarization induced by NPs was significantly correlated with lipid contents ($r = 0.65$, $p < 0.001$) of the subcellular fraction of digestive glands of freshwater mussels. In conclusion,

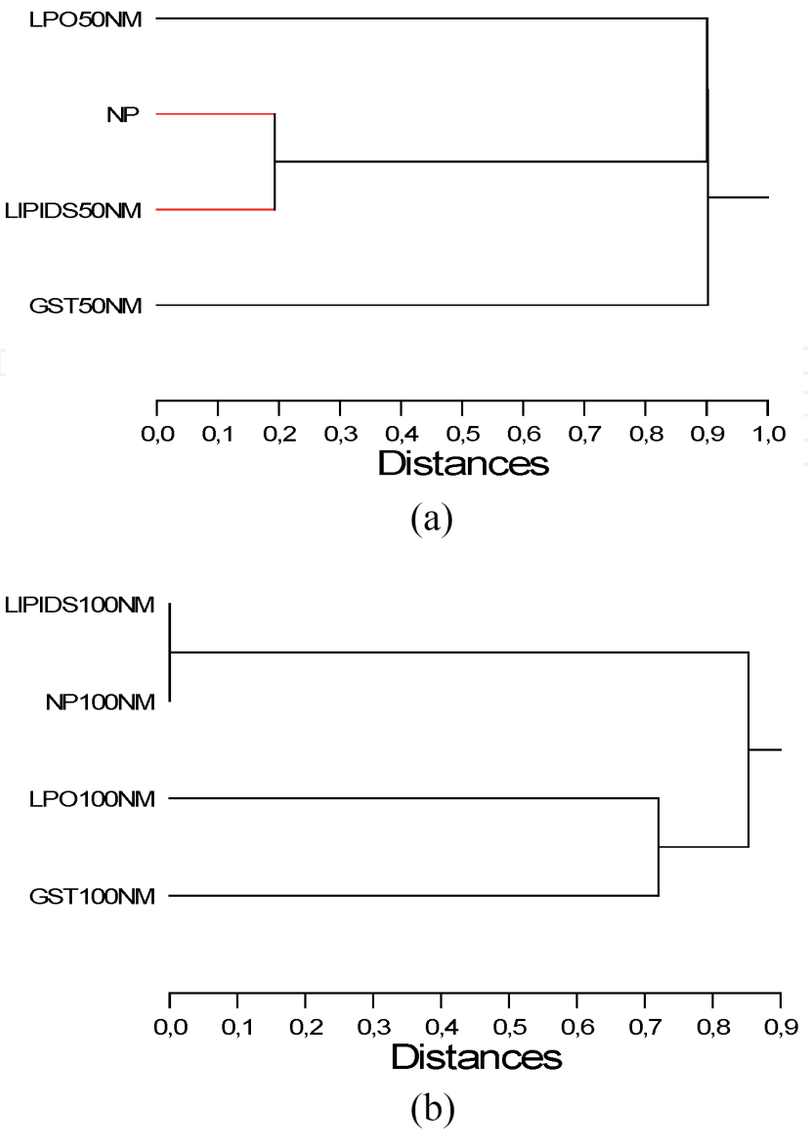


Figure 5.
Cluster analyses of biomarker data in Hydra attenuata *exposed to NPs. The biomarker responses were analysed using cluster analysis with the Euclidian methodology for distances (horizontal axis). The analysis for 50 nm (A) and 100 nm (B) NPs is shown.*

exposure of NPs to hydra led to a significant increase in NP accumulation which was still detected after 24 h depuration period in clean media. Hydrae exposed to NPs were more fragile and lost some antennae during depuration. The exposure also led to increased levels in neutral lipids, oxidative stress and xenobiotic conjugating activity which was also detected after 24 h depuration. NPs are bioavailable to hydra and produce morphological changes and sublethal effects such as oxidative stress.

Acknowledgements

The authors thank Pascale Gagnon from the Quebec Bioanalytical Laboratory of Environment and Climate Change Canada for performing the hydra exposure tests.

Author details

Joëlle Auclair[1], Brian Quinn[2] and François Gagné[1]*

1 Aquatic Contaminants Research Division, Environment and Climate Change Canada, Québec, Canada

2 School of Health and Life Sciences, University of the West of Scotland, Scotland, United Kingdom

*Address all correspondence to: francois.gagne@canada.ca

References

[1] Jambeck JR, Geyer R, Wilcox C, Siegler TR, Perryman M, Andrady A, et al. Marine pollution. Plastic waste inputs from land into the ocean. Science. 2015;**347**:768-771

[2] Mattsson K, Hansson LA, Cedervall T. Nano-plastics in the aquatic environment. Environmental Science: Processes and Impacts. 2015;**10**:1712-1721

[3] Gigault J, Halle A, Beaudrimont M, Pascal P-Y, Gauffre F, Phi T-L, et al. Current opinion: What is a nanoplastic? Environmental Pollution. 2018;**235**:1030-1034

[4] Gagné F. Detection of polystyrene nanoplastics in biological tissues with a fluorescent molecular rotor probe. Journal of Xenobiotics. 2019;**9**:8147-8149

[5] Verschoor A, ban Herwijnen R, Postuma C, Klesse K, Werner S. Assessment document of land-based inputs of microplastics in the marine environment. In: Environmental Impact of Human Activities Series. United Kingdom: OSPAR; 2017. https://www.ospar.org/documents?v=38018

[6] Lithner D, Larsson A, Dave G. Environmental and health hazard ranking and assessment of plastic polymers based on chemical composition. Science of the Total Environment. 2011;**409**:3309-3324

[7] Wang F, Bexiga MG, Anguissola S, Boya P, Simpson JC, Salvati A, et al. Time resolved study of cell death mechanisms induced by amine-modified polystyrene nanoparticles. Nanoscale. 2013;**5**:10868-10876

[8] Colvin VL, Kulinowski KM. Nanoparticles as catalysts for protein fibrillation. PNAS. 2009;**104**:8679-8680

[9] Gagné F, Auclair J, André C. Polystyrene nanoparticles induce anisotropic effects in subcellular fraction of the digestive system of freshwater mussels. Current Topics in Toxicology. 2019;**15**:43-49

[10] Sjollema SB, Redondo-Hasselerharm P, Leslie HA, Kraak MHS, Vethaak AD. Do plastic particles affect microalgal photosynthesis and growth? Aquatic Toxicology. 2016;**170**:259-261

[11] Canesi L, Ciacci C, Bergami E, Monopoli MP, Dawson KA, Papa S, et al. Evidence for immunomodulation and apoptotic processes induced by cationic polystyrene nanoparticles in the hemocytes of the marine bivalve Mytilus. Marine Environmental Research. 2015;**111**:34-40

[12] Cole M, Galloway TS. Ingestion of nanoplastics and microplastics by pacific oyster larvae. Environmental Science and Technology. 2015;**49**:14625-14632

[13] Cole M, Lindeque P, Fileman E, Halsband C, Galloway TS. The impact of polystyrene microplastics on feeding, function and fecundity in the marine copepod Calanus helgolandicus. Environmental Science and Technology. 2015;**49**:1130-1137

[14] Nasser F, Lynch I. Secreted protein eco-corona mediates uptake and impacts of polystyrene nanoparticles on Daphnia magna. Journal of Proteomics. 2016;**137**:45-51

[15] Canesi L, Ciacci C, Fabbri R, Balbi T, Salis A, Damonte G, et al. Interactions of cationic polystyrene nanoparticles with marine bivalve hemocytes in a physiological environment: Role of soluble hemolymph proteins. Environmental Research. 2016;**150**:73-81

[16] Parsai T, Kumar A. Understanding effect of solution chemistry on heteroaggregation of zinc oxide and copper oxide nanoparticles. Chemosphere. 2019;**235**:457-469

[17] Blaise C, Kusui T. Acute toxicity assessment of industrial effluents with a microplate-based *Hydra attenuata* assay. Environmental Toxicology and Water Quality. 1997;**12**:53-60

[18] Dańko MJ, Kozłowski J, Schaible R. Unraveling the non-senescence phenomenon in Hydra. Journal of Theoretical Biology. 2015;**382**:137-149

[19] Wilby OK. The Hydra regeneration assay. In: Proceedings of the Workshop Organised by Association Francaise de Teratologie. France; 1989. pp. 108-124

[20] Quinn B, Gagné F, Blaise C. *Hydra*, a model system for environmental studies. International Journal of Developmental Biology. 2012;**56**:613-625

[21] Murphy F, Quinn B. The effects of microplastic on freshwater *Hydra attenuata* feeding, morphology and reproduction. Environmental Pollution. 2018;**234**:487-494

[22] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry. 1976;**72**:248-254

[23] Boryslawskyj M, Garrood AC, Pearson JT. Elevation of glutathione-s-transferase activity as a stress response to organochlorine compounds, in the freshwater mussel, *Sphaerium corneum*. Marine Environmental Research. 1988;**24**:101-104

[24] Gagné F. Oxidative stress (Chapter 6). In: Biochemical Ecotoxicology: Principle and Methods. New York: Elsevier Inc.; 2014. pp. 103-115

[25] Finney DJ. Statistical Methods in Biological Assay. 2nd ed. London, England: Griffin; 1964

[26] Zhang F, Wang Z, Wang S, Fang H, Wang D. Aquatic behavior and toxicity of polystyrene nanoplastic particles with different functional groups: Complex roles of pH, dissolved organic carbon and divalent cations. Chemosphere. 2019;**228**:195-203

[27] Liu Z, Yu P, Cai M, Wu D, Zhang M, Huang Y, et al. Polystyrene nanoplastic exposure induces immobilization, reproduction, and stress defense in the freshwater cladoceran Daphnia pulex. Chemosphere. 2019;**215**:74-81

[28] Mishra P, Vinayagam S, Duraisamy K, Patil SR, Godbole J, Mohan A, et al. Distinctive impact of polystyrene nano-spherules as an emergent pollutant toward the environment. Environmental Science and Pollution Research International. 2019;**26**:1537-1547

[29] Shao H, Han Z, Krasteva N, Wang D. Identification of signaling cascade in the insulin signaling pathway in response to nanopolystyrene particles. Nanotoxicology. 2019;**13**:174-188

[30] Jovanović B. Ingestion of microplastics by fish and its potential consequences from a physical perspective. Integrated Environmental Assessment and Management. 2017;**13**:510-515