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Short Communication

Polystyrene nanoparticles induce anisotropic effects in subcellular fraction of the digestive system of freshwater mussels

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ABSTRACT

The release of nanoplastics (NP) from the weathering and degradation of plastics represents one of the major concerns for the environment given their pervasiveness in cells. A methodology for the detection of anisotropic changes induced by polystyrene NP is proposed using fluorescence polarization. The commercially available probe fluorescein octadecyl ester (FOE) has the property to interact in hydrophobic environments (phospholipids) which could be measured by fluorescence polarization spectroscopy. Although increasing the concentrations of 50 nm polystyrene NP in buffer alone did not change polarization, the addition of the subcellular fraction increased polarisation of the dye in a concentration-dependent manner. The assay was performed in mussels exposed to primary-treated effluents and revealed increased anisotropy of the subcellular fraction of the digestive gland suggesting NP-like effects. Although lipid contents were significantly correlated with fluorescence polarization ($r = 0.65$; $p < 0.001$), the increase in polarization was not entirely explained by changes in lipid droplets. In conclusion, polystyrene NP induces anisotropic effects at the subcellular fraction of the digestive gland as determined with the FOE probe. Mussels exposed to primary-treated effluents displayed NP-like anisotropic effects suggesting that these effluents contain NPs.

KEYWORDS: polystyrene nanoplastics, mussels, fluorescence polarization, liquid crystals, municipal effluents.

INTRODUCTION

It is estimated that plastic pollution in our oceans are in the order 100 million tons representing one of the major threats to marine and freshwater ecosystems [1]. Although plastics are considered relatively inert materials they will degrade in the environment as micro- and nanoplastics (NPs). Microplastics are operationally defined as particles or fibers in the 5-0.001 mm range which are often detected in the environment and in biota [2]. Recent evidence suggests that microplastics are readily captured by wildlife including invertebrates such as mussel and Hydra digestive systems [3, 4]. Recent evidence also suggests that microplastics will continue to degrade into even smaller particles reaching the nano-size range [5]. NPs are particles or fibres with at least one dimension in between 1-1000 nm size range [6]. The toxicity of NPs will differ from microplastics given their ability to permeate not only the tissues but within the cell's environment. Hence, the increasing presence of plastics in the environment could become a Pandora's box problem over time as weathering and degradation processes will liberate tremendous amounts of NPs in the environment. For example, one polystyrene plastic coffee cup cover underwent

weathering/degradation for 52 days and liberated over 125 million nanoparticles with mean size of 224 nm [7]. With over million tons of plastics in oceans, the amount of NPs that could be generated in time is staggering to say the least.

Methods for the detection of the effects of NPs in biological tissues are therefore urgently needed to evaluate the environmental risk. It is hypothesized that the increasing concentration of NP in cells will increase hydrophobic interactions in the cytoplasm and membranes, which could lead to altered internal organization of lipids or other non-polar compounds. For example, polystyrene is one of the major polymers used in the industry (coffee cups, food containers, building insulation), which consists of a polymer of styrene molecule composed of benzene and propylene chain which are hydrophobic molecules. The hydrophobic nature of NPs should favor the formation of a corona composed of lipids and proteins thereby decreasing the entropy of the cytosolic media, thus producing an organized structure associated to liquid crystals [8]. Liquid crystals are liquids composed of organized or oriented structures that forms intermediate phase between solid (crystalline) and liquid (isotropic) phases. The nematic phase of liquid crystals, which are composed of parallel structures that resemble phospholipid bilayer organization in membranes, is formed at biologically compatible temperatures. The detection of nematic liquid crystals could be achieved by fluorescence anisotropy (polarization) with probes that align with these structures leading to polarized fluorescence in space as opposed to all directions when in dissolved state in solution. The purpose of this study was therefore to examine the anisotropic effects of polystyrene NPs using the fluorescent dye fluorescein octadecyl ester (FOE) in the presence of subcellular extracts of digestive glands from mussels. The methodology was tested in mussels exposed to a physico-chemically treated municipal effluent in the attempt to determine if these effects could be detected in municipal effluents which are suspected sources of NPs in the environment.

MATERIALS AND METHODS

Dimethylsulfoxide (DMSO), fluorescein octadecyl ester (FOE) and the non-ionic detergent Tween-20 were purchased at Sigma chemical company (ON, Canada). Uncoated polystyrene plastic nanobeads of 50 nm diameter were purchased at Polyscience (USA). The tissue extracts were prepared from the digestive gland of freshwater mussels (*Elliptio complanata*) as follows. Adult mussels (89 ± 10 mm shell length) were placed on ice and dissected for the digestive gland and homogenized in 100 mM NaCl containing 25 mM Hepes-NaOH, pH 7.4, 1 mM EDTA and 1 mM dithiothreitol. The homogenate was passed through a metallic mesh (0.1 mm) and centrifuged at 15 000 x g for 20 min at 2 °C. The resulting supernatant (subcellular fraction) was collected from the pellet and upper lipid layer and stored at -85 °C until analysis. NP suspensions did not precipitate at this centrifugation speed and freezing at -85 °C did not produce any precipitation after thawing and centrifugation. The subcellular fraction from 3 individuals was pooled for fluorescence studies. Total protein concentrations were determined by the Brillant Blue Coomassie methodology using bovine serum albumin for calibration [9].

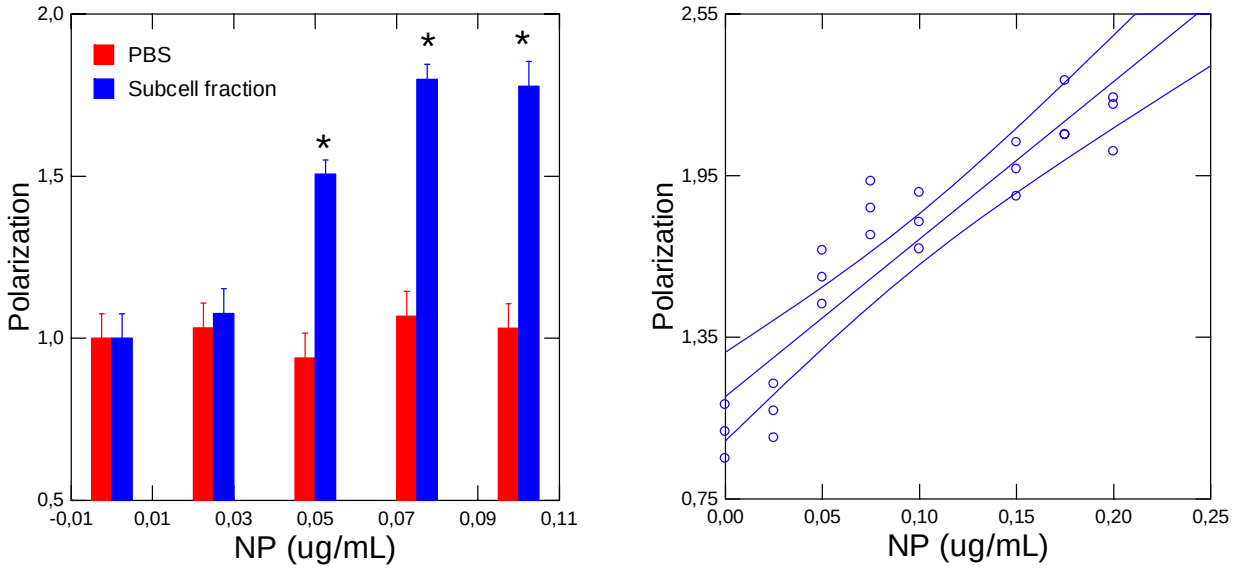
The FOE probe was dissolved in dimethysulfoxide at a concentration of 1 mM and stored in the dark. The FOE probe was diluted at 10 µM in phosphate buffered saline (140 mM NaCl, 5 mM KH_2PO_4, 1 mM $NaHCO_3$, 10 mM Hepes-NaoH at pH 7.4) just before the assays. For fluorescence analysis, the polystyrene NPs (50 nm) and subcellular fraction were analyzed as follows. A sample volume of 50 µL of increasing concentrations of NP (0.025 to 0.2 µg/mL) in phosphate buffered saline or in the presence of a fixed amount of the subcellular fraction (0.1 mg/mL total protein) was mixed with 200 µL of 10 µM FOE probe for 10 min in dark 96-well microplates. Fluorescence polarization measurements were determined at 485 nm excitation and 525 nm emission using microplate fluorimeter (Synergy-4, Biotek, USA). To determine the influence of detergent (Tween-20) on the polarization of the emission signal, the probe (200 µL) was mixed with increasing concentrations of Tween-20 (50 µl sample volume) between 0.1-0.4 mg/mL. The fluorescence polarization data were imported to an Excel Spreadsheet and analysed with SYSTAT software (version 13.2; USA). The theoretical limit of detection was defined as the concentration that produces a fluorescence signal corresponding to 2 times the standard deviation of the FOE blank.

Elliptio complanata mussels were collected from a pristine lake outside the City of Montreal (QC, Canada) and brought back live to the laboratory in ice-boxes at 4 °C. The mussels were placed in a 300 L tank at 15 °C, under constant aeration with water renewal (2 L/hour) and fed three times weekly with commercial preparations of algal suspensions. The aquarium water was dechlorinated tap water of the City of Montréal (Québec, Canada). The mussels were allowed to stand in these pristine conditions for at least one month before the caging experiments. The mussels (N = 20) were caged in cylinder nets (60 cm length x 20 cm width) and placed at 8 km downstream (DOWNS) of a major municipal effluent plume from the City of Montreal (1.5 million inhabitants) for 3 months during the summer (July-end of September) at a water depth of 1-1.5 m. Mussels were also placed at 1 km upstream (UPS) the effluent discharge site as a control site; mussels maintained in the laboratory were also used as reference (REF). At the end of the exposure period, the mussels were collected, brought back to the laboratory and placed in aquarium water overnight to allow elimination of the gut content. The mussels were then processed for fractionation as described above. The levels of lipids in the subcellular fraction were determined using the commercial fluorescent reagent AdipoRed (Lonza; Walkersville, MD, USA). A volume of 5 µL of AdipoRed reagent was added to 100 µL of digestive gland subcellular fraction in a black 96-well microplate. After 10 min under constant agitation, fluorescence was taken at 485 nm excitation and 535 nm emission (Synergy 4, Biotek microplate reader, USA). The data were expressed as relative fluorescence units/mg proteins in the subcellular fraction. The data were obtained from N = 12 individuals for statistical analysis. The data were checked for normality and homogeneity of variance using the Shapiro-Wilks and Levene's tests, respectively. Analysis of variance and Fisher Least Square test were used to highlight significant changes between sites/treatments. Significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

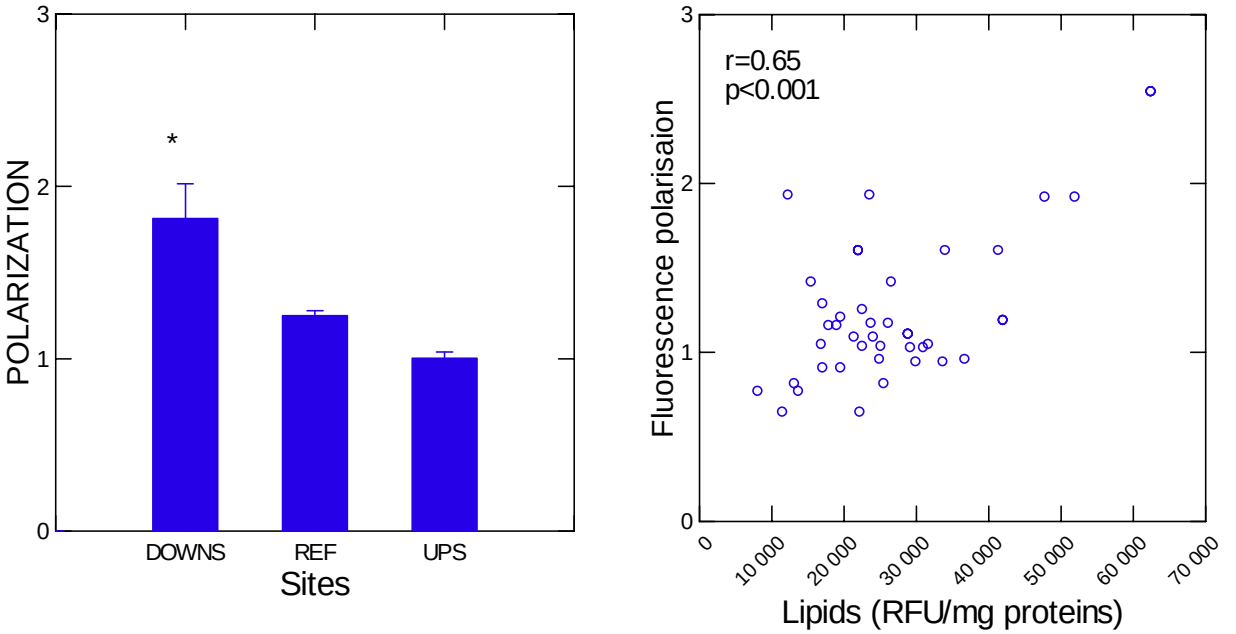
The probe FOE is a lipophilic molecule owing to the 18 carbon aliphatic chain that provides an elongated structure consistent with its affinity with lipophilic liquid crystals. The addition of polystyrene NP to the FOE probe did not induce polarization of fluorescence in phosphate buffered saline (Figure 1A). The addition of Tween-20 at a concentration of 0.4 mg/mL increased polarization only by 7.5% in the mixture. However, in the presence of the subcellular fraction, the addition of NPs readily increased polarization in a concentration-dependent manner (Figure 1A and 1B). In addition, fluorescence polarization was significantly correlated with NP concentration in the subcellular fraction ($r = 0.92$, $p > 0.001$). The detection limit of this fluorescence polarization assay for NPs was estimated at 38 ng/mL. This suggests that components of subcellular fraction (proteins, phospholipids) interact at the surface of the NP forming an organized structure which polarizes FOE. The formation of these organized structures induces anisotropic effects consistent with the properties of liquid crystals [8]. Because the FOE dye is known to interact with lipid structures such as liposomes, the liquid crystal detected in mussel tissue extracts could be of lipid nature. Hence, the introduction of polystyrene NPs in the subcellular fraction produces changes in the lipid organisation (liquid crystal) and polarizes the fluorescence. Crystal suspensions could also damage lysosomes by disrupting the membrane lipid organization [10, 11]. Lysosomes are known to contain many digestive enzymes (proteases, nucleases) and produce reactive oxygen species (nitric oxide synthase, superoxide dismutase etc. for killing foreign particles/microorganisms) making them sensitive targets leading to cell death when membranes are damaged.

Freshwater mussels exposed to a physico-chemically treated municipal effluent in the Saint-Lawrence River were analyzed for changes in anisotropy in the subcellular fraction of the digestive gland (Figure 2A). Municipal effluents are suspected sources of NPs as evidenced by the presence of many plastic-derived compounds such as phthalates and bisphenol A [12, 13]. The analysis revealed that the fluorescence polarization of FOE in the subcellular extracts from mussels exposed to the municipal effluent significantly increased (ANOVA and Fisher Least Square difference at $p < 0.05$) compared to the mussels placed upstream the



(A) **(B)**

Figure 1. Anisotropic effects of NPs in the subcellular fraction of mussel digestive gland.
The subcellular fraction was mixed with increasing amounts of 50 nm polystyrene NP in either saline or in the presence of subcellular fraction (A). The star symbol * indicates significant difference from controls at $p < 0.05$. Linear relationship of NP concentrations in the subcellular fraction, $r = 0.92$, $p < 0.001$, is shown (B).



(A) **(B)**

Figure 2. Anisotropic effects of municipal effluents on freshwater mussels.
Mussels were placed in cages 8 km downstream the municipal effluent dispersion plume for three months. The reference mussels were those maintained in the laboratory. Fluorescence polarization in the subcellular fraction (A) and relationships with lipids (B) are shown. The star symbol * indicates significant difference from controls at $p < 0.05$.

effluent discharge site and the reference mussels kept on the laboratory. The intensity of polarization corresponded to an amount equivalent to 0.1 µg/mL of polystyrene NPs. The levels of lipids were also determined in the subcellular fraction and revealed a significant induction in lipid content in mussels caged downstream the municipal effluent plume. Lipids were in fact significantly correlated with the polarization index ($r = 0.65$, $p < 0.001$, Figure 2B) which highlights the lipophilic nature of the anisotropic response. In the attempt to determine whether increased lipid contents mainly explained the observed polarization, an analysis of covariance was performed with the polarization index with lipid contents as the covariable. The analysis revealed that both lipids and site location were significant but the site location remained significant at $p < 0.05$ suggesting that not only lipid content contributed to polarization but other factors as well. Although we could not determine whether the NP in the effluent-exposed mussels induced FOE polarization, the data suggests that a NP-like effect at least was observed in these mussels. This also suggests that lipid mobilization is involved which could represent a defence mechanism against apolar nanoparticles or other compounds. In a previous study, the microviscosity of the subcellular fraction of the digestive gland of mussels (exposed to the same effluent) was increased using a fluorescent molecular rotor probe (results not shown). The molecular rotor probe was suitable to detect polystyrene NPs in these biological matrices [14]. The interaction of NP with biological membranes was elevated in oyster juveniles exposed to polystryrene NPs [15]. The 50 nm amine-coated polystyrene NP was more toxic than the carboxylated or uncoated NP which suggests that the surface coating properties is an important factor for interaction with membranes. The toxicity of carboxylated and aminated polystyrene NPs towards oyster gametes also differed [16] where the carboxylated NPs increased the production of reactive oxygen species but not the amine-coated NPs. Municipal effluents were also shown to increase adipose deposition in mice drinking municipal effluent-contaminated water [17], which corroborates with our finding in lipid contents in mussels caged in the municipal effluent plume. However, whether plastic nanoparticles contributed to this obesogen effects needs to be confirmed by more investigations.

CONCLUSION

In conclusion, the addition of polystyrene NPs in the subcellular fraction significantly increased FOE polarization suggesting interaction of lipids with NPs consistent with the formation of lipophilic liquid crystals in cells. Mussels exposed to municipal effluents, which are suspect sources of NPs, also showed an increase in anisotropic changes in the subcellular fraction as observed with polystyrene NPs. However, the presence of NPs in effluent-exposed mussels will need further confirmation.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict interest with regard to this publication.

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