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Research Article

Urban Nanoplastic Contamination Profiles And Ecotoxicity In Experimentally Caged Freshwater Mussels Elliptio complanata.

André C., Qchiqach H., GagneF.

Aquatic Contaminants Research Division, Environment and Climate Change Canada, Montréal, Quebec, Canada H2Y 2E7.

Abstract

The release and ecotoxicological impacts of combined sewers overflows and treated municipal effluents in respect to plastic nanoparticles (PNPs) in endemic mussels are not well understood. The purpose of this study was to examine the levels of PNPs and toxicity in feral mussels caged at the 2 rainfall overflow, downstream a largely populated (~2 million inhabitants) city and its municipal effluent dispersion plume sites. The mussels were caged for a period of three months during the summer and analyzed for tissue levels of PNPs, air tolerance, morphological changes, oxidative stress, DNA damage and neuroendocrine effects. The data revealed that mussels from rainfall overflow and downstream the city contained PNPs in tissues, had reduced levels in serotonin, elevated levels of zinc binding proteins (metallothioneins), gonad DNA strand breaks and modulated acetylcholinesterase (AChE) activity,an enzyme reported to interact with various nanoparticles. The levels of PNPs were also related to changes in the progression of the AChE reaction rate from crowding/steric effects. This could lead to an hysteresis behavior of AChE where the enzyme is less sensitive to baseline levels of acetylcholine. Conversely, mussels caged at the municipal effluent dispersion plume had lower PNPs with an increase in the deceleration rates in AChE although mussels accumulated high levels of heterotrophic bacteria. This corroborates previous studies showing that the municipal effluents with low hydraulic residency times can reduce plastic materials in the water column. In conclusion, the occurrence of PNPs in mussels is ubiquitous in an urban area where higher levels are found from rainfall overflow sites and downstream a largely populated city.

Keywords: Municipal effluents, rainfall overflows, mussels. Plastic nanoparticles, toxicity.

INTRODUCTION

Organisms thriving in benthic environments are more at risk to urban pollution since they are sessile and feed on suspended matter, a recognized vector of contamination. Pollutants tend to accumulate at the sediment water interface, where many organisms are found such as annelids (worms), cnidarians (hydra), bivalves (mussels, clams) and microcrustaceans. Hence, mussels are particularly at risk from urban pollution especially wastewaters discharges, combined sewers overflows and rainfall runoffs. Global warming is though to increase rainfall runoffs and combined sewers overflow events thereby exposing locally mussels' habitats (Wang et al., 2023). Sewage wastewater treatment stations have limited capacity to cope with larger intakes of stormwater where excess water is either directly released in the aquatic environment or combined with raw, untreated wastewater before being released in the environment. Although these events are episodic in nature, only occurring for short periods, these events could occur up to hundred of times (100-150 per year) a year depending on the station plant's capacity and geographical region (Statistics Canada: Combined sewer overflow discharge volumes). The realization that plastic materials are observed in almost every ecosystem has raised serious concerns on its potential impact to the environment (Hietbrink et al., 2025). This study revealed that between 1.5 to 32 µg/ L of nanoplastics were found throughout the entire spectrum of water column in the North Atlantic. These were mainly composed of polyethylene terephthalate, polystyrene, and polyvinyl chloride. These values are consisted with those reported in biofilms downstream of large urban (Montreal) in the Saint-Lawrence River (Roubeau Dumont and Gagné, 2024). The evaluation of mussel health status at sites near urban areas near sources of plastic contaminant is warranted to understand the cumulative impacts of urban pollution on an important member of the benthic community, bivalves.

***Corresponding Author:** F Gagné.** François Gagné Researcher at Environment and Climate Change Canada. **Email:** francois.gagne@ec.gc.ca.
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The freshwater mussels *Elliptio complananta* is one of the most prevalent species in the Saint-Lawrence river (Downing and Downing, 1992). This species was selected as a representative species at sites in the vicinity of Montreal city in the Saint-Lawrence River. The longevity of this species is estimated at 15 years and are usually found a water depth of 0.5-3 m (Haag and Rypel, 2011; Gagné et al., 2022). It reproduces sexually with relatively low incidence of intersex (<2%) according to Downing et al (1989). The occurrence of PNPs and other nanomaterials were recognized to produce toxic effects in mussels (Ostapchuk et al., 2025). For example, exposure of *Limnoperna fortunei* mussels to 10 µg/mL dense mesoporous silica nanoparticles for 96h led to increased lipid peroxidation, superoxide dismutase and glutathione S-transferase, markers of oxidative stress and damage in tissues. Exposure of mussels to zinc oxide nanoparticles also revealed changes in neural activity as determined by reduced feeding activity, oxidative stress and modulation of acetylcholinesterase (AChE) activity (Ons et al, 2024). AChE was recently identified as highly sensitive to nanomaterials (Wang et al., 2009) including PNPs (Maria et al., 2024; Gonçalves et al., 2023). Indeed, AChE activity was reduced by either adsorption or inhibition by 8 representative nanoparticles (SiO2, TiO2, Al2O3, Al, Cu, carbon-coated copper), single and multi-walled carbon nanotubes. This makes this enzyme a suitable biomarker of exposure to nanomaterials including PNPs, nano-tin and nano-silver previously reported in municipal effluents (Gagné and André, 2025; Polesel et al., 2018).

The purpose of this study was to examine exposure and cumulative effects of PNPs in mussels caged on freshwater *Elliptio complanata* mussels caged downstream urban sites in the Saint-Lawrence River. Mussels were caged at 2 combined sewers overflows sites, one downstream the City of Montreal before the effluent discharge and one site downstream the municipal effluent dispersion plume. They were exposed as such for 3 months during the summer where frequent storm events occur. It is though that combined sewers overflow sites receive rainfall street run offs containing tire wear particles and chemicals usually associated with such as zinc, sulphur, reductants (1,3-diphenylguanidine) and plastic stabilizers (e.g. dibutylphthalate) (Mayer et al., 2024). Toxicity was examined the oxidative stress, zinc binding proteins (metallothioneins), neural activity (AChE) and general health status to better understand the toxicity of rainfall overflows and from a primary-treated municipal effluent. The objective was to highlight the contribution of PNPs contamination and effects in mussels exposed to combined sewer overflow and treated municipal effluents in an endemic mussel of the St. Lawrence River.

MATERIALS AND METHODS

Mussel collection and handling

Unionid *Elliptio complanata* mussels were collected from a pristine lake (i.e., harbouring no housing or streets) located some 120 km in the Laurentian mountains range north of the city of Montréal (Québec, Canada) in June 2019. Mussels were collected by hand by scuba diving, placed in containers at 4-6°C and transported back to the laboratory the same day. Upon arrival, mussels were submerged in UV-treated and charcoal-filtered tap water in 60 L aquarium with 2-3 cm washed sand base. They were kept at 15 °C for at least 15 days under constant aeration with a feeding regime of 3-4 times/week with a commercial phytoplankton (Phytoplex®) feed. Forty mussels (N=40) were then placed in each of two cylindric nets (1 m long x 0.5 m diameter) (1 cm diameter polyethylene mesh) attached to 5 kg cement block. They were immersed in 1-2 m depth at 2 combined sewers overflow sites (OVF1: 45°38'26.3"N; 73°29'15.6"W; OVF2: 45°36'05.2"N; 73°30'33.6"W), one downstream site located 30 km from the city center of Montreal but upstream the municipal discharge point (DOWNS city: 45°39'28,5"N; 73°28'37,9"W) and one downstream site located 8 km downstream the municipal effluent dispersion plume (Effluent: 45°44'23,9"N; 73°25'43,9"W). This site was previously shown to significantly feminize mussels after one year (Blaise et al., 2003). Hence, the study comprised 4 sites: one downstream of the city center (Downs city) site, one downstream site in the municipal effluent dispersion plume (Effluent) and 2 rainfall overflows (OVF1 and OVF2). The exposure period was 3 months during July-September of 2019 and were checked bi-monthly for any cage loss or mussel mortality. There were no changes in mussel survival between the sites. Following the exposure period, mussels were transported back to the laboratory and allowed to depurate overnight in fresh dechlorinated and UV-treated tap water. A group of 10 mussels per site at the time of cage retrieval were retained for plastic nanoparticles (PNPs) and total heterotrophic bacteria loadings. Another group of mussels (10) were set aside to determine resistance to air emersion. Briefly, mussels were placed in plastic containers at 15°C in 80% air humidity. Mussel weights were measured each day (dehydration rate DHR) and death was measured based on shell opening after handling. The time of death were expressed as days and the DHR at the time of death were determined as follows: 100 x (weight at T0-weight at day of death /weight at T0).

Biomarkers analyses

A sample of 10 mussels per site were randomly selected, weighted, shell length measured and digestive gland /gonad were dissected on ice, weighted and stored at -85°C. For each mussel, the condition factor (CF: mussel weight/shell length),

the digestive- (DGI) and gonad-somatic index (GSI) were determined. organ wet weight Tissues were thawed on ice for 30 min and 4 volumes of ice-cold 100 mM NaCl containing 10 mM Tris-acetate, pH 8, 1 mM EDTA, 1 mM KH2PO4, 1 mM dithiothreitol and 0.1 µg/mL aprotinin were added. Tissues were homogenized using a Teflon pestle tissue grinder (3-4 passes) and 1.5 mL was centrifuged at 12 000 x g for 30 min at 2°C. The supernatant (S12 fraction) was collected for metallothioneines, (MT), glutathione S-transferase (GST), arachidonate cyclooxygenase (aCOX) and acetylcholinesterase (AChE) activity assessments. The samples were stored at -85°C until analysis.

The levels of plastic nanoparticles (PNPs) and total heterotrophic counts were determined in whole soft tissues. For PNPs, the levels were determined using the NaCl/acetonitrile extraction method followed by a nanogold sensor for quantitation (Gagné et al., 2024). Briefly, one volume of the homogenate was mixed with one volume of saturated 5M NaCl followed by the addition of one volume of acetonitrile. After mixing for 5 min, the upper acetonitrile fraction was taken for PNPs assessment using nanoAu (10 nm diameter, Polyscience USA) coated with mercaptoundecanoic acid. The principle of this probe consists in the prevention of nanoAu aggregation (increased 530/620 nm ratio) by binding to PNPs following the addition of 10 mM HCl. Standard solutions of polystyrene (20 nm diameter, Thermofisher USA) nanoparticles were used for calibration. The data were expressed as ug PNPs/g tissues. For total hetorotrophic bacteria counts (HBC), the soft tissues from N=4 separate individuals per site were set to Microbiology laboratory (Eurofins (Longueuil, Québec, Canada). The soft tissues were weighted and homogenized using Teflon pestle tissue grinder to determine the levels of coliform growth at 35°C. The data were expressed as total bacteria counts/g tissues.

The levels of metallothionein (MT), a zinc/copper binding protein, were determined in the digestive gland using the silver saturation assay using non-radioactive silver (Gagné, 2014). Briefly, the S12 fraction was incubated with 2 mg/L of Ag+ at pH 8.5 in 100 mM glycine for 15 min and the excess silver and heat sensitive proteins were removed by two successive additions of hemoglobin followed by heat denaturation at 100°C/centrifugation. The remaining silver in the supernatant was determined by graphite furnace atomic absorption spectrometry with silver nitrate for calibration and rabbit MT-II for validation. A ratio of 17 moles of Ag/mole of MT (molecular weight of 67000 g/mole) was used for validation with rabbit MT. The data were expressed as ng MT equivalents/g digestive gland.

AChE was determined in the gonad tissues (S12 fraction) using acetylthiocholine and Ellman's reagents to detect the formation of thiocholine by spectrometry (Wang et al., 2009). Readings at 412 nm were taken at each 2 min interval for 30min to determine reaction rates and fractal kinetic analysis for crowding effects in enzyme activity (Synergy-4, Bioteck Instrument, USA). The data were expressed as increase absorbance/min/g tissues. The crowding effects on AChE were determined using fractal kinetics to determine the spectral dimension (sD) from the evolution of the reaction rates over time (Kopelman, 1988; Gagné, 2020). The sD depends on the topology of the space or the local distribution of neighbors in a population (crowding), and the diffusion rate of the substrates. More specifically to AChE, sD is related to the loss of reaction rate in time with a slope h (h=1-(sD/2)) and is related to the random walk problem (N(t) ~1/texpsD/2). A decrease in the deceleration rate h results from higher sD where the enzyme is visited less often by its substrates and reaction products indicating an increase complexity in the immediate environment of AChE. In other words, this leads to a situation where AChE is less reactive to changes in basal levels in acetylcholine, hence cloud leads to an hysteresis behavior.

Arachidonate cyclooxygenase (COX) and glutathione S-transferase activities were determined in the S12 fraction by fluorescence (arachidonic acid/2,7-dichlorofluosrescein) and absorbance (GSH/chlorodinitrobenzene)-based assays in 96-well microplates as previously described (Fujimoto et al., 2002; Hoarau et al., 2001). The enzyme activities were expressed as substrate change (relative fluorescence units or absorbance) /min/mg proteins in the gonad or the digestive gland and normalized against the upstream site.

The levels of vitellogenin-like phosphate were also determined in the gonad homogenates following the alkali-labile phosphate (ALP) methodology (Blaise et al., 2003). The S12 fraction were treated in 30 % acetone for 5 min and the pellet obtained by centrifugation for 5 min at 10 000 x g at 4°C. The pellet was washed once with 50 % acetone and the pellet resuspended in 1M NaOH and incubated at 60-70°C for 30 min. Standards of rainbow trout vitellogenin and inorganic phosphate were prepared for quantitation and determined by the phosphomolybdate procedure (Stanton, 1968). Data were expressed as µg of ALP/g gonad.

Lipid peroxidation (LPO) in the digestive gland and gonad tissues was determined according to the thiobarbituric acid methodology (Wills, 1987). The assay was done in 96-well microplate format: 10 µL of homogenates were mixed with 90 µL of water, 50 µL of 10% trichloroacetic acid-1 mMFeSO4 and 100 µL of thiobarbituric acid (0.67%). The mixture was heated for 70–75 °C for 5 min and allowed to cool down at room temperature. Emission was measured at 600 nm following 540 nm excitation in a dark microplate (Synergy-4, Biotek Instruments, USA). Standards of tetramethoxypropane (stabilized form of malonaldehyde-MDA) were used for calibration. The data were expressed as µg of MDA per g gonad or digestive gland (wet weight). The levels of DNA

strand breaks were determined in the homogenates in both tissues by the alkaline DNA precipitation assay as described previously (Debenest et al., 2012). The data were expressed as µg DNA strand breaks/g gonad or digestive gland.

Data analysis

Mussels (N=40 individuals) were placed in each of 2 cages at the 4 sites (OVF1, OVF2, DOWNs city and Effluent). The biomarkers were determined in 10 randomly collected mussels at the end of the exposure period. The data were examined for normality and homogeneity of variance using Shapiro–Wilk and Bartlett tests, respectively. The data were found parametric and analyzed using one-way (sites) ANOVA followed by the Least Square Difference test as the post-hoc test. The star symbol * indicates significance from the DOWNS city center site ($p < 0.05$).

RESULTS

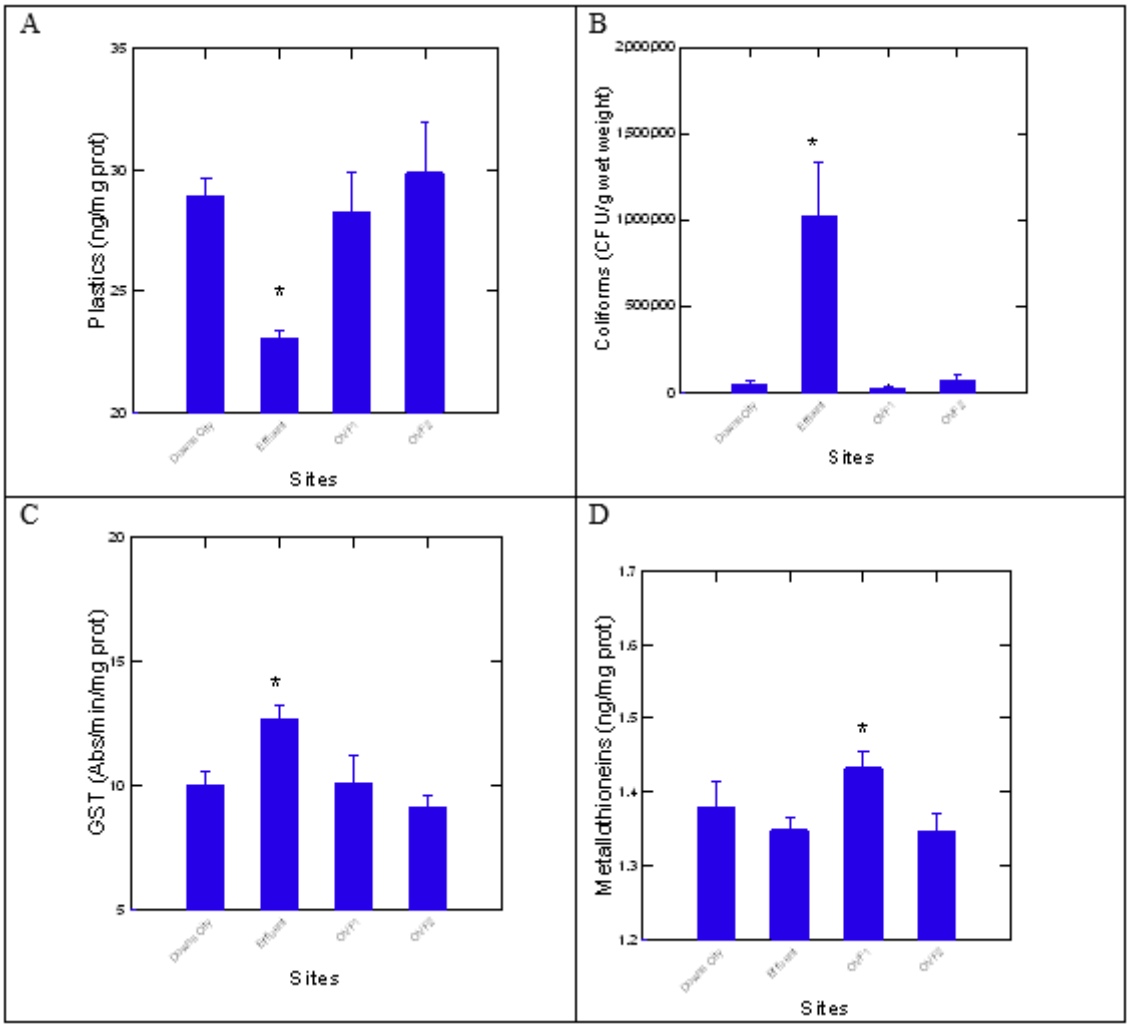
Following 3 months (July-October) exposure of caged mussels at the 4 sites, there was no significant changes in the CF and the SFT (soft tissues weight/total weight) in mussels (**Table 1**). The DgI (digestive gland weight/soft tissue weights) were significantly increased at the municipal effluent dispersion plume site. The air survival time (LT) was significantly increased at the same site; however, the dehydration rate was not altered throughout the sites indicating that lost weight during air emersion was not critical for passing. The CF was significantly correlated with DGi (r=0.78) and LT (r=0.71). The SFT index was significantly correlated with LT (r=-0.54) and DGi (r=-0.66). The DGi was significantly correlated with the LT (r=0.82) suggesting that increased feeding provided some resilience towards air emersion stress.

Table 1. Morphological changes and air survival in caged mussels.

Sites	CF (g mussel /shell length)	SFT (tissues/ total wt)	DgSI (g DG/ g tissues)	TL (days)	DHR %
Downs-City	0.76±0.03	0.85±0.01	0.023±0.002	15.9±5	36±2.6
Overflow 1	0.73±0.03	0.83±0.007	0.023±0.002	12±4.6	32±3
Overflow 2	0.77±0.03	0.85±0.007	0.022±0.002	18±6	34±1
Effluent (8 km downstream)	0.73±0.02	0.81±0.005	0.027±0.002*	30±5*	34±1

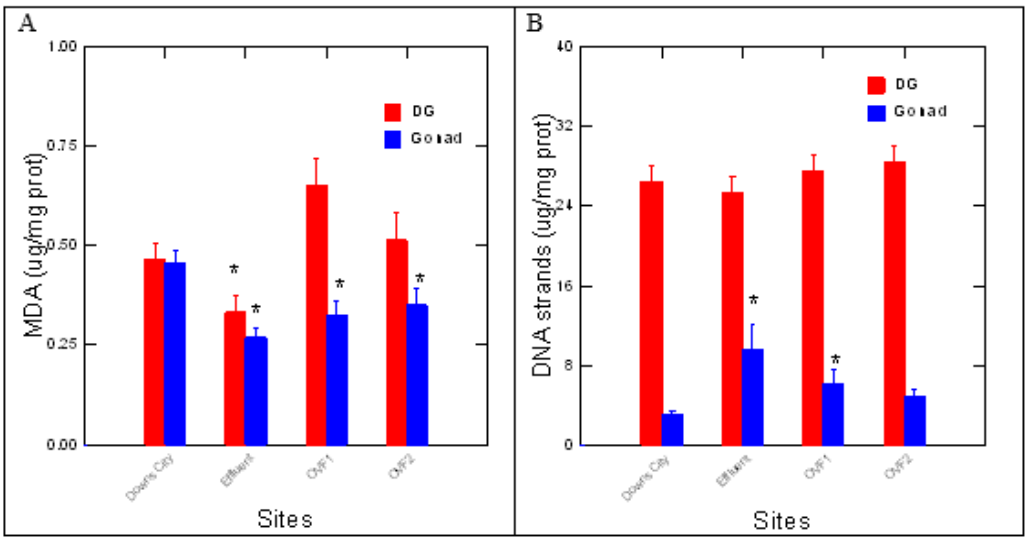
The levels of plastic and heterotrophic bacterial (coliform) counts (HBC) were determined in mussel tissues (**Figure 1A, B**). The levels of PNPs were significantly higher in the downstream city site and the 2 rainfall overflow sites while PNPs levels were the lowest at Effluent site (**Figure 1A**). Conversely, HBC in mussel tissues were significantly lower at these sites compared with the Effluent site which contained up to 1 million HBC per g tissues (**Figure 1B**). The higher HBC corroborates increased DGi in mussels and confirmed exposure to the municipal effluent dispersion plume. Correlation analysis revealed that PSNPs were significantly associated with CF (r=0.62), SFT (r=0.77) and DGi (r=-0.64). In respect to HBC in mussel tissues, they were correlated with SFT (r=-0.54), DGi (r=0.5), LT (r=0.69) and NPs (r=-0.56). Covariance analysis between the LT and HBC in soft tissues revealed that only HBC significantly influenced the LT over the sites where the LT was no longer significantly higher at the Effluent after adjusting the mean squares of LT with HBC. The activity of GST in the digestive gland was measured as an indicator of exposure to miscellaneous organic compounds (pharmaceuticals, PAHs etc) and was significantly increased at the municipal effluent dispersion plume compared to the other sites (**Figure 1C**). Correlation analysis revealed that GST activity was significantly correlated with HBC (r=0.67). The levels of MT were determined to evaluate the exposure to divalent metals such as zinc (a major pollutant released by tire wear dusts and municipal effluents) and copper. MT levels were significantly increased at one of the overflow sites (**Figure 1D**) consistent with tire wear pollution.

Figure 1. Plastic materials, bacterial loadings and biotransformation activity in caged mussels at the urban sites.
The levels of plastic materials (A), total coliforms (B), metallothioneins (C) and GST activity (D) were determined in the digestive gland. The data represents the average with the standard error. The star * symbol indicates difference from the downstream city site (located upstream of the effluent).



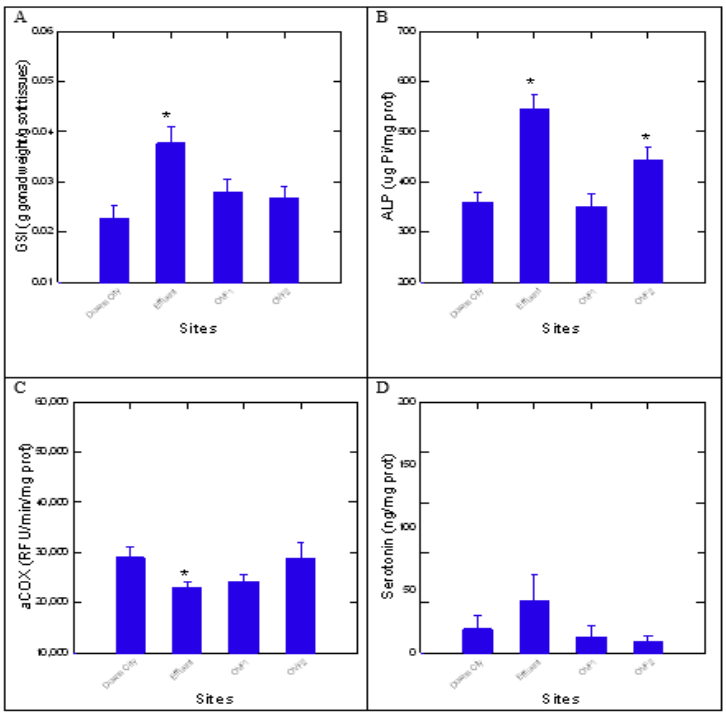
The levels of lipid peroxidation (MDA-malonaldehyde) and DNA strand breaks were determined in freshwater mussel gonads and digestive gland (**Figure 2**). MDA levels were significantly lower at the Effluent (dispersion plume) in the digestive gland compared to the downstream city site (**Figure 2A**). Correlation analysis revealed that lipid peroxidation (MDA) in the digestive gland was significantly associated with MT levels (r=0.9). MDA levels in the gonad were all reduced relative to the downstream city site and were correlated with GSI (r=-0.85), PNPs (r=0.71) and SFTi (r=0.9). DNA strand breaks in the digestive gland were not significantly different (**Figure 2B**). Correlation analysis revealed that DNA breaks in the digestive gland were correlated with DGi (r=-0.71), SFT (r=0.81) and gonad LPO (r=0.75). In respect of DNA, the strand breaks in gonads were significantly higher at the effluent dispersion plume and one overflow site. In gonads, DNA strand breaks were correlated with DGI (r=0.54) with HBC (r=0.91), NPs (r=-0.65) and GST (r=0.91).

Figure 2. Oxidative stress and DNA damage in freshwater mussels.
The levels of lipid peroxidation (MDA; A) and DNA strand breaks (B) were determined in the digestive gland (DG) and gonad in caged mussels. The data represent the mean with the standard error. The star symbol * indicates significant difference from the downstream city site (Down City).



The neuroendocrine effects of reproduction were examined by following changes in GSI, vitellogenin-like proteins, arachidonate cyclooxygenase (aCOX), serotonin levels and AChE in gonad tissues. The GSI was significantly increased at the municipal effluent dispersion plume (Effluent) site compared to the Downs city (**Figure 3A**). Correlation analysis revealed that the GSI was significantly correlated with correlated with HBC (r=0.78), CF/SFTi (r=-0.84/-0.91) and gonad MDA (r=-0.82). The levels of ALP (vitellogenin-like properties) were significantly elevated at the municipal dispersion plume and one OVF sites (**Figure 3B**). ALP levels were correlated with MT (r=0.60). The activity of arachidonate cyclooxygenase (aCOX) was significantly decreased at the municipal dispersion plume site compared to the Downs city center while serotonin levels were higher but not a significance level (**Figure 3C and 3D**). The activity of aCOX was significantly correlated with DGi (r=-0.74), SFTi (r=-0.89), MT (r=0.94), gonad and digestive gland LPO (r=0.92/0.9) and digestive gland DNA (r=0.95). Ser levels were correlated with any of the above biomarkers.

Figure 3. Neuro-endocrine status of mussel caged at polluted sites.
The neuro-endocrine status were determined by following changes in GSI (A), vitellogenin-like proteins (B), arachidonate cyclooxygenase (C) and serotonin levels (D) in the gonad of caged mussels. The data represents the mean with the standard error. The star symbol * indicates significant difference from the downstream city site (Down City).



AChE activity was also determined to determine mussel neural activity and explore the crowding effects of this enzyme given its reported sensitivity to nanomaterials by using fractal kinetics analysis as indicated in the Data analysis section. At first glance, no significant changes in AChE were observed (**Figure 4A**) and was not correlated with any of the above biomarkers. A close examination of reaction rates over time revealed a steeper decrease in the slope h in mussels exposed to the municipal effluent dispersion plume (Effluent) site compared to the overflow sites and the Downs-City sites (**Figure 4B**). The sD of the reaction rate was extracted from the slope h (h =1-[sD/2]) and revealed a significant decrease in the sD at the overflow and Downs City sites compared to the Effluent site. The sD was significantly correlated with NPs (r=-0.62; **figure 4D**), HBC (r=-0.85), ALP (r=-0.68), GST (r=-0.90) and DNAd gonad (r=-0.89). The significant relationship between the sD and PNPs in tissues suggests that PNPs contributed to the crowding effects of AChE in part at least. The decrease in the sD for AChE in mussels caged at the urban sites (Overflows and Downs-City) suggests that this enzyme is visited more by the substrates in time indicating that enzyme activity will decrease less quickly in time, this was confirmed by adjusting AChE activity with the sD where corrected enzyme rate v = v2/sD (Gagné, 2020) as seen in **Figure 4C**. The corrected AChE activity for the sD was significantly higher at the Effluent sites compared to the overflow and Downs City Centre sites suggesting that urban sites near large city and combined sewers overflow sites reduced AChE activity as seen for PNPs and other nanomaterials. AChE corrected for sD was significantly correlated with DGi (r=0.75), HBC (r=0.89), CF (r=-0.78), GSi (r=0.72), GST (r=0.95), gonad DNA damage (r=0.94).

In the attempt to gain a global view of the biomarkers examined in the present study, a principal component analysis was performed (**Figure 5**). The total variance was explained at 60% with the following biomarkers: HBC, PNPs, MT, DHR, ALP(Vtg), gonad and digestive DNAd, gonad and digestive LPO(MDA), GST and COX. The exposure biomarker HBC revealed clear input from the municipal effluent dispersion plume site was directly associated to GSI, DGi and LT while PNPs revealed an input from OVF and the Down city sites and closely related to AChE, sD of AChE (related to reduced deceleration rates of AChE over time).

Figure 4. Ache Activity And Spatial Constraints Assessment In The Digestive Gland Of Freshwater Mussels.
Ache Activity Was Determined In The Digestive Gland Of Mussels (A). Spatial Constraint Analysis Was Performed By Following Changes In Reaction Rates Over Time (B) Giving The Spectral Dimension Sd As Followed: Slope H =1-[Sd/2] (C). Correlation Analysis Between The Sd Of Ache In The Digestive Gland And Pnps In Tissues (D).

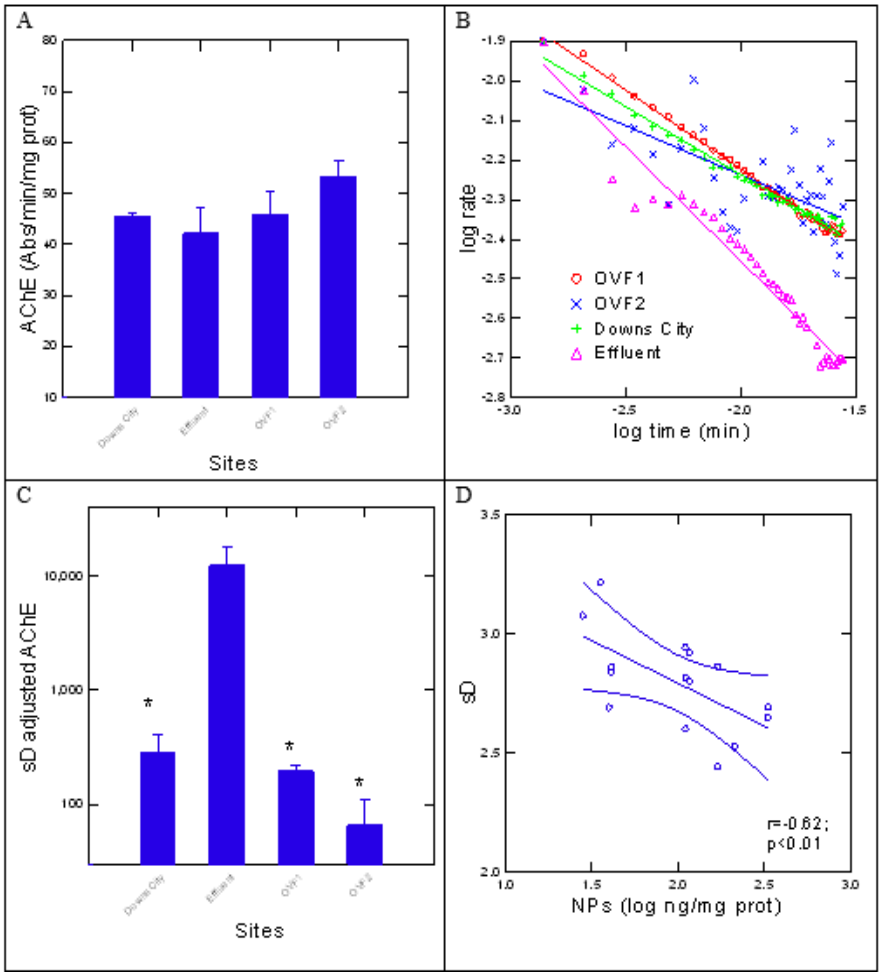
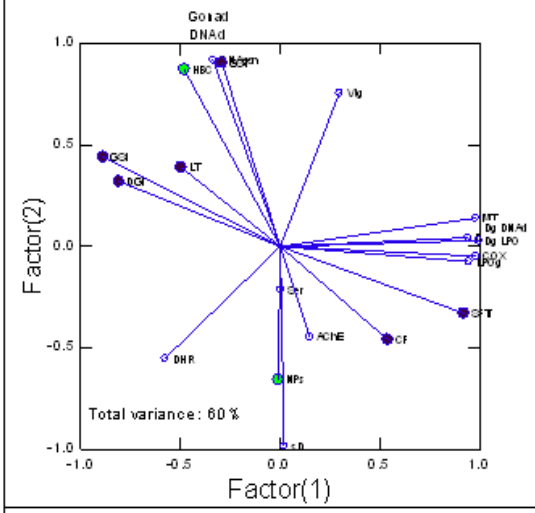


Figure 5. Principal component analysis of biomarker data.
The biomarker data were analyzed using principal component analysis. The total explained variance was 72% with the first 2 factors and the most important biomarkers were given at each factors (x and y axis). The green dots are exposure biomarkers (heterotrophic bacteria counts and NPs in the digestive gland), the black dots correspond to morphometric changes including the LT to air exposure and DHR.



Major biomarkers

Factor coefficients	1	2
MT	**0.13**	0.07
sD(AChE)	-0.04	**-0.18**
PNPs	-0.03	**-0.12**
SFT	**0.10**	-0.02
DHR	-0.1	**-0.13**
ALP	0.07	**0.15**
Gon DNAd	-0.01	**0.16**
DG DNAa	**0.12**	0.05
Gon LPO	**0.12**	0.02
DG LPO	**0.13**	0.05
GST	-0.001	**0.16**
COX	**0.16**	0.03
HBC	-0.03	**0.14**

DISCUSSION

The high levels of HBC in mussel's tissues at the Effluent site confirmed exposure to the municipal effluent plume since the effluent treatment process does not apply any disinfection steps. The DGI and LT were also elevated and suggest that mussels feeding on urban fecal counts did not harm them since they naturally feed on microorganisms in the water column. This further indicates that increased feeding could render the organisms more resistant to air emersion and perhaps the harmful effects of municipal wastewaters contamination. In a previous study, air survival time was associated with mass, length and low oxidative stress as determined by lipid peroxidation (André et al., 2021). This was consistent with lower levels of MDA levels in both the digestive gland and gonad tissues in the present study. In another study, the air survival time in Elliptio sp. exposed to a 20% dilution of a municipal effluent was not significantly different from controls compared to river waters (Gagné et al., 2019).

It was noteworthy that PNPs in tissues were significantly lower at the municipal dispersion plume (Effluent site) compared to combined sewers overflow and the Downs-City sites. This suggests that plastics are mitigated during the wastewater treatment plant where street runoffs contained more plastic materials probably from tire and asphalt erosion and rain-diluted untreated effluents. Indeed, the release of rubber-based particles from street runoffs were the predominant form of plastics released in river water (Haney et al. 2025). Hence, urban rivers are suspected vector of plastic pollution since it is the main sink for increasing precipitation events in these times of global warming. Indeed, macro and microplastics discharges in the river following storm events. The composition of plastic materials also changes during these events where plastics change from fiber/particulate forms to rubber from tire wear dusts. It is also recognized that macro/microplastics are sources of nanoparticles in the aquatic environment (Kharral et al., 2025). PNPs were released from microplastics not only from physical abrasion but also from oxidation chain scission (hydrolysis) reactions. In this context, the removal of large plastic materials by municipal effluent is mandatory to prevent the eventual release of PNPs in the water column during wastewater treatment. During sieving solids and primary treatment (flocs) steps of wastewaters, MP are retained and subject only to mechanical/physical abrasion in low hydraulic residency (hours to days) wastewater treatment plants (Zdarta and Li, 2025). However, MPs could be degraded further (i.e., releasing more PNPs) by chemical means during secondary/tertiary treatments such as advanced oxidation processes, chlorination, and ozonation usually involving increased hydraulic residency times of the wastewaters at the station plant. Hence a paradox could be observed in different wastewaters where municipal effluents with low hydraulic residency times (hours to days) such as physical/chemical (primary) treatments could reduce the levels of both micro and nano plastics, while in small (low population) cities employing passive oxidative processes (aerated lagoons) with higher hydraulic residency times (10-25 days), PNPs contamination could increase. This was corroborated in recent studies in the Saint-Lawrence River showing higher PNPs in small townships

using aeration lagoons and sludges (André and Gagné, 2025). This could explain why tissue PNPs were elevated at the rain overflow and the DOWNs city sites, which receive inputs from smaller cities on the North shore in addition to street runoffs in this area. A more detailed analysis of the plastic types would have been interesting to better understand the paradox of plastic contamination.

The enzyme AChE was inhibited in the presence of plastics and various nanoparticles essentially by adsorption mechanisms (Wang et al. 2009). Among the eight nanoparticles tested (SiO2, TiO2, Al2O3, Al, Cu, carbon-coated Cu, multi-walled carbon nanotubes (MWCNT) and single-walled carbon nanotubes (SWCNT), SWCNT nanotubes had the highest affinity for AChE leading to the highest inhibition (94%). This is particularly of interest since carbon nanotubes share surface hydrophobicity with plastics from both tire wear (carbon black) and some commonly used plastics such as polystyrene (composed of aromatic vinyl chain), which was shown also to decrease AChE activity (Maria et al., 2024). Inhibitions of AChE in gills of the marine mussel were reported following 3 days exposure to 10 µg/L polystyrene nanoplastics (Gonçalves et al., 2023). In the present study, the sD of AChE activity was significantly correlated with the PNPs in tissues (Figure 4D) indicating that higher levels of PNPs were associated to lower sD leading to lower deceleration rates (Figure 4). This suggests that interactions of PNPs with AChE could lead to an hysteresis effect where the enzyme becomes less sensitive to acetylcholine levels. Indeed, correction of this crowding effect revealed significantly lower AChE activity at the urban sites (containing more PNPs) compared to the municipal effluent dispersion plume site. Decreased in uncorrected AChE for the sD was observed in zebrafish larvae exposed to both microplastic and nanoplastics (Chen et al., 2017).

Experiments with 17 α-ethynylestradiol (EE2) and polystyrene nanoplastics revealed that both these compounds activated the estrogen receptor (Lekki-Porębski et al., 2025). The levels of vitellogenin-like phosphates were elevated at the effluent dispersion plume and one of the OVF sites although less PNPs were found in mussels for the former site. This suggests that other estrogens were acting at this site given the absence of correlation between vitellogenin-like phosphates and PNPs or that inhibitors of vitellogenesis were found at the other OVF site. Polyaromatic hydrocarbons found in roads and tire wear dusts, which are inducers of CYP1A, activity antagonize vitellogenesis in fish (Mayer et al., 2024; Navas and Segner, 2000). Another mitigation factor is that rainfall events are episodic with varying amounts of rain, which could modulate exposure to mussels. Typically, about 3 strong rainfall events (>10 mm) per month occurred in July and August (with 1-2 events in September and October) during the duration of the caging experiments. Zinc was detected at ppm levels in tire wear leachates and recognized as a potent inducer of metal detoxication proteins such as MT (Kim et al., 2025). Indeed, zinc was identified as a major component released from tire wastes in the aquatic environment reaching concentrations up to 2.7 mg/L zinc in tire leachates. Vitellogenin was reported to bind zinc and have a role in metal transport in oocytes (Montorzi et al, 1994) where excess zinc and other metals like cadmium could inhibit egg production (vitellogenins). This was corroborated by the significantly higher levels of MTs and vitellogenin-like phosphates at one of the overflow sites in the present study.

In conclusion, freshwater mussels caged at stormwater overflows and municipal effluent discharges revealed various effects. On the one hand, mussels exposed to municipal effluents had elevated levels of bacteria, vitellogenin-like phosphates, GSI, DGI, GST (biotransformation), DNA strand breaks in the digestive gland and survived for longer times in air but contained less inflammation, oxidative stress and PNPs. Conversely, the levels of zinc-binding proteins (MTs), PNPs and gonad DNA strand breaks were significantly elevated at the OVF sites compared to the municipal effluent dispersion plume. AChE activity was significantly lower at the 2 overflows and Downs City sites once the correction against molecular crowding (sD) was applied, indicative of exposure to nanomaterials including PNPs. Indeed, the sD of AChE activity was significantly associated with PNPs in tissues. This study revealed that plastic contamination does not follow contaminant profiles usually associated to municipal wastewaters and appear more strongly associated to rivers downstream cities and stormwater runoffs.

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REFERENCES

1. André C., Bibeault J.F., Gagné F. 2021. Identifying physiological traits of species resilience against environmental stress in freshwater mussels. Ecotoxicology30, 1862-1871.

2. Blaise C, Gagné F, Salazar M, Salazar S, Trottier S, Hansen P-D, 2003. Experimentally-induced feminisation of freshwater mussels after long-term exposure to a municipal effluent. Fresenius Environ. Bull 12, 865-870.

3. Chen C, Gundlach M, Yang S, Jiang J, Velki M, Yin D, Hollert H, 2017. Quantitative investigation of the mechanisms of microplastics and nanoplastics toward zebrafish larvae locomotor activity. Sci Total Environ 2017 Apr 15:584-585:1022-1031.

4. Debenest T, Gagné F, Burgeot T, Blaise C, Pellerin J, 2012. DNA integrity assessment in hemocytes of soft-shell clams (Mya arenaria) in the Saguenay Fjord (Québec, Canada). Environ. Sci. Poll. Res. 20: 621-629.

5. Downing J. A., Downing W. L. (1992). Spatial aggregation, precision, and power in surveys of freshwater mussel populations. Can. J. Fish. Aquat. Sci. 49, 985–991. 10.1139/f92-110.

6. Downing J.A., Amyot J.-P., Perusse M., Rochon Y. 1989. Visceral sex, hermaphroditism, and protandry in a population of the freshwater bivalve Elliptio complanata. J. N. Am. Benthol. Soc. 8, 92-99.

7. Fujimoto, Y., Sakuma, S., Inoue, T., Uno, E. and Fujita, T. 2002. The endocrine disruptor nonylphenol preferentially blocks cyclooxygenase-1. Life Sci., 70:2209–2214.

8. Gagné F. 2014. Metal metabolism and detoxication, Chap V. In: Biochemical Ecotoxicology: Principle and Methods. Elsevier Inc. New York, USA pp. 75-100.

9. Gagné F 2020. Ecotoxicology of altered fractal organization in cells. Am J Biomed Sci Res 8, 498-502.

10. Gagné F, André C, Turgeon S and Menard, N. 2022. An examination of relationships between air-time survival, dehydration rate and morphology in bivalves: implications for climate changes and pollution. Cur Top Toxicol 18, 191.

11. Gagné F, J Auclair J, Turcotte P, Gagnon C, Peyrot C, Wilkinson KJ 2019. The influence of surface waters on the bioavailability and toxicity of zinc oxide nanoparticles in freshwater mussels. Comp Biochem Physiol C Toxicol Pharmacol. 219:1-11.

12. Gagné F., Gauthier, M., André C 2024. Simple detection of polystyrene nanoparticles and effects in freshwater mussels: method development and in situ application to urban pollution. Water Emerg Contam Nanoplast 3, DOI: doi.org/10.20517/wecn.2023.60

13. Gagné, F., André, C. 2025. Evaluation of Surface Water Quality with Biochemical Assays. Adv Earth & Env Sci; 6:1-8.

14. Gonçalves JM, Benedetti M., d'Errico G., Regoli F. , Bebianno MJ., 2023. Polystyrene nanoplastics in the marine mussel Mytilus galloprovincialis. Environ Poll 333, 122104

15. Haney J., Zhu, X., Long, M., B. A. Schwenk, Hoellein, T.J., Wollheim, W.M., Lammers R.B., Zuidema, S., Rochman, C.M. 2025. The influence of flow on the amount, retention and loss of plastic pollution in an urban river. Phil. Trans. R. Soc. A 383, 20230023.

16. Hietbrink SST, Materić, D., Holzinger, R., Groeskamp, S., Niemann, H. 2025. Nanoplastic concentrations across the North Atlantic. Nature 643, 412-420.

17. Hoarau P, Gnassia-Barelli M, Romeo M, Girard JP. Differential induction of glutathione S-transferases in the clam Ruditapes decussatus exposed to organic compounds. Environ Toxicol Chem. 20: 523-529.

18. Kharraf A. L., Rabiller Baudry M.,, Del Real A. P., Sandt, C., Wiesner, M., Nemeth G., Bavay, D., Peraza, K.S., Bossa, N., Davranche, M. 2025. Polyethylene plastic degradation: the dual pathways from macroplastics to nanoplastics, J Hazard Mat doi: https://doi.org/10.1016/j.jhazmat.2025.140200.

19. Kim B, Kim S, Kim R, Heo Y, Jo J, Ahn S, Jeon S, Woo SH, Lee S, Kim JJ, Kim YJ, Shin TH, Park JW, Cho WS. 2025. Comparative toxicity of tire wear particle leachates: Zinc as a key toxicant affecting development and motility in zebrafish larvae. J Hazard Mater. 499: 140296.

20. Kopelman R., 1988. Fractal reaction kinetics. Science 24: 16201626.

21. Lekki-Porębski S.A., Męczyńska-Wielgosz S., Kruszewski M. et al. Silver and polystyrene nanoparticles activate oestrogen signaling via cytoplasmic oestrogen receptor. Sci Rep (2025). https://doi.org/10.1038/ s41598-025-30440-4

22. Maria VL, Santos J, Prodana M, Cardoso DN, Morgado RG, Amorim MJB, Barreto A 2024 Toxicity mechanisms of plastic nanoparticles in three terrestrial species: antioxidant system imbalance and neurotoxicity. Nanotoxicology 18, 299-313.

23. Mayer PM, Moran KD, Miller EI, Brander SM, Harper S, Garcia-Jaramillo M, Carrasco-Navarro V, Ho KT, Burgess RM, Thornton Hampto LM, Granek EF, McCauley M, McIntyre , Edward JK, Kolodzie Pj , Hu X, Williams AJ, Beckingham BA, Jackson ME, Sanders-Smith RD, Fender CL, King GA, Bollman M, Kaushal SS, Cunningham BE, Hutton SJ, Lang J, Goss KV, Siddiqui S, Sutton R, Lin D, Mendez M 2024. Where the rubber meets the road: Emerging environmental impacts of tire wear particles

and their chemical cocktails. Sci Total Environ 2024 Jun 1:927:171153.

24. Montorzi M, Falchuk KH, Vallee BL 1994. Xenopus laevis vitellogenin is a zinc protein. Biochem Biophys Res Commun. 200, 1407–1413. doi: 10.1006/bbrc.1994.1607. [DOI] [PubMed]

25. Navas JM, Segner H. 2000. Antiestrogenicity of β-naphthoflavone and PAHs in cultured rainbow trout hepatocytes: evidence for a role of the arylhydrocarbon receptor. Aquat Toxicol 51, 79-92.

26. Ons B, Abdelhafidh K, Zeineb H, Amine M, Samir T 2024. Biochemical and behavioral effects of zinc oxide nanorods on the freshwater mussel Potomida littoralis Int J Environ Health Res. (2):1180-1190.

27. Ostapchuk GO, Ale A, Andrade VS, Poletta GL, Montalto L, Rojas Molina FM, Desimone MF, Catalano PN. 2025. Impact of morphologically tuned silica nanoparticles on the invasive freshwater bivalve Limnoperna fortunei. Environ Toxicol Pharmacol. 120, 104857. doi: 10.1016/j.etap.2025.104857.

28. Polesel F, Farkas J, Kjos M, Carvalho PA, Flores-Alsina X, Gernaey KV, Hansen SF, Plósz BG, Booth AM 2018. Occurrence, characterisation and fate of (nano) particulate Ti and Ag in two Norwegian wastewater treatment plants. Water Res 141, 19-31.

29. Roubeau Dumont E, Gagné F 2024. Nanoplastic Contamination in Freshwater Biofilms Using Gel Permeation Chromatography and Plasmonic Nanogold Sensor Approaches. Nanomaterials (Basel) 14:1288.

30. Stanton MG. 1968. Colorimetric determination of inorganic phosphate in the presence of biological material and adenosine triphosphate. Anal. Biochem. 22: 27-34.

31. Wang M., Fu X., Zhang D., Chen F., Liu M., Zhou S., et al. (2023). Assessing urban flooding risk in response to climate change and urbanization based on shared socio-economic pathways. Sci. Total Environ. 880, 163470.

32. Wang Z, Zhao J, Li F, Gao D, Xing B, 2009. Adsorption and inhibition of acetylcholinesterase by different nanoparticles. Chemosphere 77, 67-73.

33. Wills ED. 1987. Evaluation of lipid peroxidation in lipids and biological membranes. In: Snell, K., Mullock, B. (Eds.), Biochemical Toxicology: A Practical Approach. Oxford University Press, Oxford, UK, pp. 127–152.

34. Zhao J., Li F., Gao D., Xing B 2009. Adsorption and inhibition of acetylcholinesterase by different nanoparticles. Chemosphere 77, 67-73.

35. Zdarta, A., Li, G. (2025). The Paradox of Microplastic Removal in WWTP: Redistribution of Micropollutants in the Environment. Cur Poll Reports 11, DOI : https://doi.org/10.1007/s40726-025-00370-w