ISJ 14: 352-362, 2017 ISSN 1824-307X

RESEARCH REPORT

Toxicological properties of a major release of untreated wastewaters into the St. Lawrence River to quagga mussels *Dreissena bugensis*

F Gagné, C André, M Pilote, P Turcotte, C Gagnon

Aquatic Contaminants Research Division, Environment and Climate Change Canada, 105 McGill, Montreal, QC, Canada

Accepted September 19, 2017

Abstract

Before maintenance work could be carried out on the sewage system in the city of Montreal (Quebec, Canada), 5 billion litres of untreated wastewater had to be released directly into the St. Lawrence River over a five-day period in November 2015. The purpose of this study was to examine the toxicity of untreated wastewaters on quagga mussels. Water samples were collected at various points from the most densely populated downtown area and downstream points: 0 km, 5 km, 12 km, 16 km and 19 km. A river water sample was collected at the opposite shore as a reference site, and aquarium (dechlorinated tap) water was used for controls. Mussels were exposed for four days at 15 ^{o}C to these wastewaters, then examined for biotransformation (CYP1A1 and glutathione S-transferase activities), energy expenses (mitochondria activity and triglycerides), estrogenicity (alkali-labile phosphates) and damage (lipid peroxidation and DNA strand breaks). The data revealed that exposure to the released wastewaters produced changes to all the above biomarkers. CYP1A1 activity, DNA damage and triglyceride levels were the most responsive biomarkers for optimal site classification as determined by discriminant function analysis. CYP1A1 activity, lipid peroxidation and alkali-labile phosphate levels were significantly correlated with distance from downtown, suggesting that population density influenced more directly those effects. In conclusion, exposure to untreated wastewaters could lead to adverse toxic effects in quagga mussels. Mussels could be at risk from the release of untreated wastewaters events given that high-intensity precipitation could also result in the release of untreated wastewaters in these times of climate change.

Key Words: quagga mussels; untreated wastewater; cytochrome P4501A activity; oxidative damage; DNA damage; energy expenses

Introduction

In November 2015, the City of Montreal (Quebec, Canada) had to release billions of litres of untreated wastewaters from its sewage system's southeast interceptor in order to empty it before upgrading the interceptor and a snow collection chute. This resulted in the release of 5 billion litres of untreated wastewater into the St. Lawrence River over a five-day period. Although no evidence of acute toxicity was observed (*e.g.*, from high ammonia or low dissolved oxygen content during the spills), concerns were raised about possible long-term chronic effects on resident sessile organisms such as local mussel populations. Urban wastewaters are sources of many contaminants, ranging from metals, polyaromatic hydrocarbons and pesticides to pharmaceuticals, personal care products and nanomaterials (Holeton *et al*., 2011). These wastewaters contain many endocrine-disrupting compounds, especially those from the estrogen signaling pathways, such as 17α-ethynylestradiol, estradiol-17β, nonylphenol (surfactants) and bisphenol A (Chen *et al*., 2006). These substances are known to induce egg yolk proteins (vitellogenins) in fish and bivalves, leading to feminization of the organisms (Aravindaksham *et al*., 2003; Blaise *et al*., 2003; Tetreault *et al*., 2011). Recent evidence also revealed that toxins in municipal effluents lead to oxidative stress/inflammation, serotonergic and genotoxic effects in fish and bivalves (Gagné *et al*., 2006, 2010, 2011). The release of municipal effluent and untreated wastewater during rain overflow events could pose a risk to local mussel populations.

Corresponding author:
Gagné François
Aquatic Contaminants Research Division
Environment and Climate Change Canada
105 McGill, Montreal, QC, Canada
E-mail: francois.gagne@canada.ca

Moreover, under climate change conditions, more intense precipitation could lead to increased rainfall events (Min *et al*., 2011) that could overwhelm the capacity of existing wastewater treatment plants to handle these inputs of rainfall-driven wastewater. That would lead to increased releases of untreated wastewater at sites where local mussels thrive, and the impacts are unknown. In this respect, the November 2015 major release of untreated wastewater for the purpose of upgrading sewage infrastructure presented a unique opportunity to examine the toxicological properties of released untreated wastewaters in local dresseinid mussels in the St. Lawrence River.

Bivalves are at particularly high risk to aquatic contaminants, given that they are sessile organisms and filter feeders. They are thus exposed to both dissolved and particulate-bound contaminants. Dresseinid mussels are small and formidable aquatic invaders found in many freshwater bodies in Europe and North America. Quagga mussels (*Dresseina burgensis*) are similar to zebra mussels in appearance, but quagga mussel shells are paler toward the hinge and somewhat larger (15 - 25 cm wide). The quagga mussel usually feeds on phytoplankton, algae and other suspended materials in the water column. It is dioecious and a prolific breeder. Fertilization occurs in the water column. A fully mature adult could produce 0.5 million to 1 million eggs per year, depending on food availability and ambient temperature. The veligers and adults adhere to surfaces with byssal threads. Dresseinids were previously used to determine the toxic effects of municipal effluents and urban pollution. Increased expression of metallothioneins and cytochrome P4501A activity, markers of exposure to heavy metals and polyaromatic hydrocarbons respectively, was used to identify the impacts of urban pollution (de Lafontaine *et al*., 2000). Changes in lipid peroxidation (LPO) as a marker of oxidative damage were also monitored in local mussel populations. Dresseinid mussels have previously been used to measure estrogen and a related product, nonylphenol, in municipal effluents (Quinn *et al*., 2004, 2006). Exposure to a tertiary-treated municipal effluent for 112 days resulted in increased levels of egg yolk proteins, as indirectly determined by alkali-labile phosphates (ALP) and gel electrophoresis. Exposure to nonylphenol to 5 µg/L and 500 µg/L for 112 days also resulted in increased ALP and cholesterol levels in mussels. DNA damage in whole tissues of zebra mussels was also monitored in dresseinids using the alkaline precipitation assay (de Lafontaine *et al*., 2000; Ács *et al*., 2016).

The purpose of this study was therefore to examine the toxicity of released untreated wastewater to quagga mussels in the St. Lawrence River (Quebec, Canada). The untreated wastewaters were collected during the major release in November 2015. Quagga mussels were exposed to the undiluted wastewaters for four days and analyzed for toxic effects through monitoring of changes in energy metabolism, xenobiotic biotransformation, oxidative stress and DNA damage.

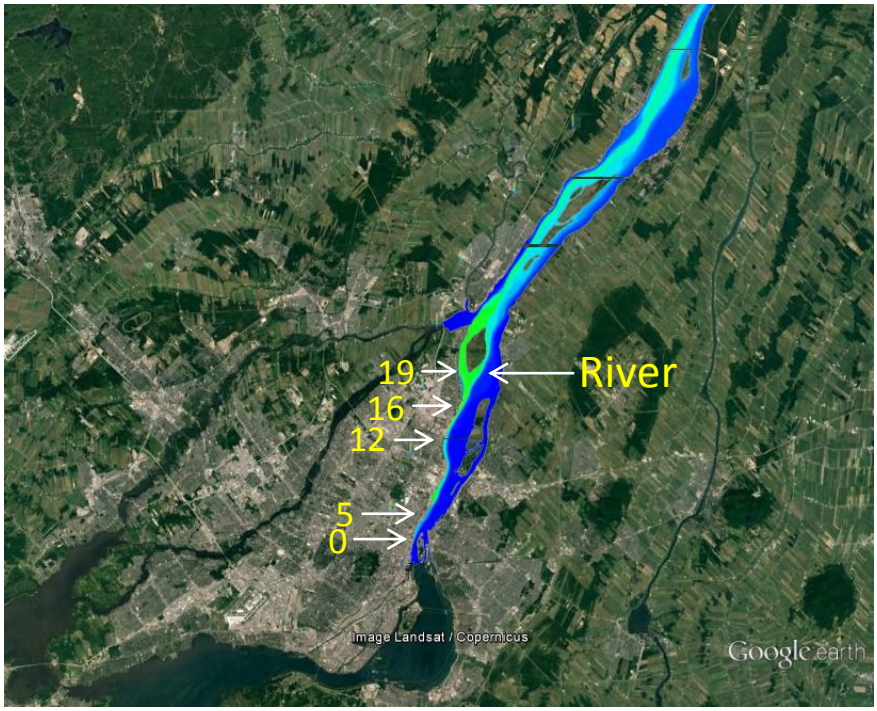

Fig. 1 Map of study sites. The surface waters were collected at five discharge sites located at increasing distances from downtown (0, 5, 12, 16, 19 km). A simulated dispersion plume is shown on the map. The river water samples were collected in the St. Lawrence River near the south shore, which was outside the plume formed by the released untreated water along the north shore of the river. The St. Lawrence River's direction of flow is from the lower left to the upper right of the map. Source: Google Maps.

Materials and Methods

Study sites and mussel exposure

On the third day of the five-day release of untreated waters, surface water samples (40 L) were collected at the mouths of five sewage overflows. The sewage overflows selected were located at increasing distances from Montreal's downtown, starting at 0 km (the most densely populated area) and gradually moving northeast 5 km, 12 km, 16 km and 19 km from downtown (Fig. 1). River water from outside the dispersion plume of the released untreated wastewater was also collected along the south shore. Surface water (five 4-L samples) at each sampling location was collected using an immersion pump lined with Teflon tubing and was stored in 4-L plastic containers that had been washed with 1 % HCl. Quagga mussels were collected along on the south shore of the St. Lawrence River (10 km upstream from the city of Montreal) - a location which was not in direct contact with sources of contamination. The mussels were collected by hand in clumps (held together by byssal threads) and transferred back to the laboratory in coolers (kept at 4 ^{o}C in the dark). At the lab, they were placed in a 20-L aquarium at 15 ^{o}C with an 8/12-h light and dark cycle and fed daily with commercial algal feed. They were kept in those conditions for at least 30 days. The mussels were then delicately separated from each other with a scalpel, and mussels of similar size (0.7 to 1.3 g/shell length) were selected for analysis. The mussel gonads were in the late or early gametogenesis stages (sex was indeterminate). Twelve mussels were placed in 4-L containers filled with each of the surface water samples described above (0 km, 5 km, 12 km, 16 km and 19 km distance, plus the reference river sample taken at 19.5 km on the other side of the St. Lawrence River near the south shore). The control was aquarium water, *i.e*., UV/charcoal-filtered tap water. The mussels were kept for 96 h at 15 ^{o}C under constant aeration, and water samples were changed after 48 h.

Water chemistry

Surface water characteristics (pH, conductivity, total suspended solids, salinity, redox potential and dissolved oxygen) were measured using standard methodology (Hach Laboratories, USA), and total fecal coliform plate counts (APHA, 2012) were performed. Elemental analysis was performed in the dissolved fraction (0.45 µm filtration) by ICP-mass spectrometry (XSERIES 2, ThermoScientific, USA). The following elements were measured: Cd, Co, Cr, Cu, Fe, Li, Na, Ni and Zn. The data were expressed in µg/L.

Toxicity assessment

At the end of the exposure period, mussels were measured (shell length) and weighed. The soft tissues were dissected out on ice, weighed and immediately placed in the four volumes of homogenization buffer (10 mM Hepes-NaOH, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol and 0.1 µg/mL apoprotinin). The whole body tissues were homogenized with a Teflon pestle tissue grinder (five passes at 4 ^{o}C), and a sub-sample of the homogenate was kept aside for lipid peroxidation (LPO), DNA damage, total triglycerides (lipids) and total protein assessments. The other portion was centrifuged first at 1,500*g* for 10 min, then the supernatant was centrifuged at 10,000*g* for 30 min at 2 ^{o}C. The pellet contained the crude mitochondria fraction for mitochondrial electron transport (MET) activity and total protein assessments and was resuspended in 0.25 mL of 100 mM NaCl, 1 mM KH_2PO_4, 1 mM EDTA and 10 mM Hepes-NaOH, pH 7.4, before storing at -85 ^{o}C.

Table 1 Physico-chemical characteristics of released untreated waters

Sites	Distance from downtown (km)	pH	Conductivity ($\mu S*cm^{-1}$)	Total suspended solids (mg/L)	Salinity (mg/L)	REDOX (mvolt)	O_2 (mg/L)	Fecal coliforms (counts/100 mL)
1	0	7.3	505	0.26	0.32	119	8.2	750,000
2	5	8.1	280	0.13	0.18	118	11.1	10,000
3	12	8.1	331	0.16	0.21	137	10.6	10,000
4	16	8.2	308	0.15	0.20	140	10.8	69,000
5	19	7.9	290	0.14	0.18	237	12.6	8,400
Municipal effluent	22	6.8	800	2-4	NA	NA	4-5	300,000 to 500,000
Reference	19.5	8	286	0.14	0.18	211	12.7	5,800

NA: not available.

Table 2 Spearman correlation of water quality properties

	Distance	pH	Cond.	TSS	Salinity	Redox	O_2	Fecal coliforms
Distance	1	0.49	-0.61	-0.58	-0.63	**0.84**	**0.81**	**-0.81**
pH		1	**-0.90**	**-0.91**	**-0.88**	0.13	0.64	**-0.93**
Cond.			1	**0.99**	**0.99**	-0.46	**-0.89**	**0.98**
TSS				1	0.99	-0.42	**-0.87**	**0.98**
Salinity					1	-0.50	**-0.91**	**0.98**
Redox						1	**0.80**	-0.42
O_2							1	**-0.85**

Cond.: conductivity; Redox: redox potential; TSS: total suspended solids.

The 10,000*g* supernatant (S10) was similarly stored for 7-ethoxyresorufin O-deethylase (EROD), glutathione S-transferase (GST) and akali-labile phosphates (ALP) and total proteins. Total proteins were determined using the Bradford (1976) method, and serum bovine albumin was used for calibration. Cellular energy allocation was determined by monitoring changes in MET activity and total triglyceride levels. MET activity was determined using a previously described procedure (André and Gagné, 2017) based on the reduction of marker dye (Frings and Packard, 1975). Briefly, the isolated mitochondria (100 µg/mL total proteins) were mixed in 150 µL of 0.1 M Tris-HCl (pH 8.5) containing 5 % polyvinylpyrrolidone, 0.1 mM $MgSO_4$ and 0.1 % Triton X-100 for 5 - 10 min on ice. Then, 1 mM NADH and 0.2 mM NAPDH were added and the reaction was initiated with 1 mM p-iodonitrotetrazolium. The reaction was allowed to proceed at 20 ^{o}C and absorbance at 520 nm was measured at 5-min intervals for 30 min. The data was expressed as change in absorbance/min/mg proteins. Total triglyceride contents were determined in soft tissue homogenate using an AdipoRed fluorescent assay (Lonza; Walkersville, MD, USA): 5 µL of Adipored reagent was added to 100 µL of homogenate in a 96-well black microplate. The plate was shaken gently to ensure uniform mixing and incubated in the dark for 10 min. The fluorescence was then measured at 485 nm excitation and 535 nm emission in a microplate reader (Synergy 4 Microplate Reader, BioTek, VT, USA). The data were expressed as µg triglycerides/mg total proteins. Xenobiotic biotransformation activity was characterized by the activities of EROD (cytochrome P4501A) and GST. EROD activity was determined in the S10 fraction in the presence of 10 µM 7-ethoxyresorufin, 100 mM NADPH in 50 mM Tris-HCl, pH 7.4, containing 0.1 mM $MgCl_2$. The reaction was allowed to proceed for 40 min at 30 ^{o}C, and fluorescence readings for the formation of product 7-hydroxyresorufin at 540 nm for excitation and 590 nm for emission were taken using a microplate reader (Synergy 4 Microplate Reader, BioTek, VT, USA). Standard solutions of 7-hydroxyresorufin (0.5 - 5 µM) were used for instrument calibration. The data were expressed as fluorescence units formed/min/mg proteins. GST activity was determined in the S10 fraction using the spectrophotometric method (Boryslawskyj *et al*., 1988). Briefly, the S10 fraction (50 µL) was mixed with 150 µL of 0.1 mM of 1-chloro-2,4-dinitrobenzene and 0.1 mM of reduced glutathione. Absorbance readings at 340 nm were taken every 5 min for 30 min in clear microplates (Synergy 4, microplate reader). Data were expressed as the decrease in absorbance/min/mg proteins. The levels of alkali-phosphates were determined in the S10 fraction as a marker of estrogenic properties in dresseinid mussels (Quinn *et al*., 2004), with slight modifications. The S10 fraction was mixed with acetone at 35 % for 5 min on ice and centrifuged at 10,000*g* for 5 min. The pellet was washed in 50 % acetone, centrifuged and resuspended in 1 M NaOH. The mixture was incubated at 60 ^{o}C for 30 min. The levels of inorganic phosphates were then determined using the phosphomolybdate methodology. The NaOH sample was centrifuged at 15,000*g* for 5 min if turbidity was found. The data were expressed as mg phosphate/g soft tissues. Biomarkers of tissue damage were determined by monitoring changes in lipid peroxidation (LPO) and DNA breaks (DB). LPO was determined in tissue homogenates using the thiobarbituric acid reactants (TBARS) methodology as described elsewhere (Gagné, 2014). The levels of TBARS were determined by fluorescence at 520 nm for excitation and 590 nm for emission (Bioscan, USA). Standard solutions of tetramethoxypropane were used for calibration. The data were expressed as µg TBARS/mg proteins. The levels of DNA strand breaks were determined using the alkaline precipitation assay (Benchalgo *et al*., 2014): 25 µL of the homogenate was mixed with 175 µL of 2 % SDS containing 40 mM NaOH, 10 mM EDTA and 10 mM Tris base. After mixing by inversion, 200 µL of 0.12 M KCl was added and placed at 60 ^{o}C in a water bath for 10 min and cooled on ice for 30 min. The mixture was centrifuged at 8,000*g* for 5 min and the supernatant was collected for DNA analysis.

DNA levels in the supernatant were determined using SYBR Green dye in the presence of 0.4 M NaCl, 4 mM sodium cholate and 0.1 M Tris-acetate, pH 8.5, to limit interference with traces of SDS in the supernatant (Bester *et al*., 1994). Fluorescence readings were taken at 485 nm excitation and 520 nm emission, and standard solutions of salmon sperm DNA were used for calibration. The data were expressed as µg DNA/mg proteins in the homogenate.

Data analysis

Mussels were exposed to the untreated waters in triplicate and N = 10 mussels were analyzed for biomarker analyses. Data normality and homogeneity of variance were verified using Shapiro-Wilk and Bartlett tests respectively. The data were log-transformed if proved not normal or presented heterogeneity of variance. The data were then subjected to analysis of variance, and critical difference between controls was determined using the least squares difference test. Correlation analysis was performed using the Pearson-moment procedure. The biomarker data were analyzed using discriminant and factorial analysis to determine differences in the toxicity profile of untreated waters collected at increasing distance from downtown.

Results and Discussion

From among the numerous (150) release points for rainwater and wastewater overflows, we selected locations at increasing distance from downtown Montreal, where the population density is highest. The river water pH was slightly more acidic at the closest (0 km) site (pH 7.1) than at the sites farther downstream (pH 7.9 - 8.2) (Table 1). This value was similar to that of municipal effluent that had undergone primary physical-chemical treatment. Water conductivity was also higher at the site closest to downtown (505 μScm^{-1}) than at the sites farther downstream and in the river water (331 - 280 μScm^{-1}); this value represented 63 % of the conductivity of the treated municipal effluent (which is in the order of 800 μScm^{-1}). The same pattern was found with suspended solids (0.26 mg/L at 0 km, compared to 0.13 - 0.16 mg/L at the downstream sites), salinity (0.32 mg/L at 0 km compared to 0.18 - 0.21 at the downstream sites), dissolved oxygen (8.2 mg/L at 0 km compared to 10.6 - 12.7 mg/L at the downstream sites). The redox potential was 119 mvolt at 0 km and gradually increased, reaching 237 mvolt at 19 km from downtown, which suggests the presence of electron scavengers in the downtown area. The distance from downtown was significantly correlated with redox potential ($r = 0.84$; $p < 0.01$), dissolved oxygen ($r = 0.81$; $p < 0.01$) and total fecal coliforms ($r = -0.81$; $p < 0.01$) (Table 2). The total levels of some metals were also determined in the dissolved fraction of collected surface waters (Table 3). In general, the levels of Na, Cd, Cr, Fe, Co, Ni, Cu and Zn were higher at the site closest to downtown (0 km) than at the downstream sites. Distance from downtown was negatively significantly correlated with levels of Cr ($r = -0.93$, $p < 0.01$), Cu ($r = -0.94$, $p < 0.01$), Zn ($r = -0.83$, $p < 0.05$) and marginally correlated with levels of Ni ($r = -0.75$, $p = 0.08$) and Fe ($r = -0.78$, $p = 0.06$). Correlation analysis with general physical and chemical properties revealed that pH was negatively correlated with Ni ($r = -0.93$; $p < 0.01$), Co ($r = -0.85$; $p < 0.05$), Cu ($r = -0.94$; $p < 0.01$) and Zn ($r = -0.94$; $p < 0.01$) levels. Conductivity was positively correlated with all metals ($r > 0.9$; $p < 0.01$) and sodium ($r = 0.97$; $p < 0.001$). Total suspended solids were also positively correlated with all the metals tested ($r > 0.88$; $p < 0.01$). The redox potential was positively correlated with distance from downtown and not correlated with any of the metals ($r < 0.63$; $p > 0.1$).

Quagga mussels were exposed for 96 h during the five-day release of billions of liters of untreated wastewaters. No changes in the mussel weight-to-shell-length ratio were observed, nor were there any trends based on distance from downtown (results not shown). Changes in the energy status of mussels exposed to the released untreated waters were examined by monitoring MET activity and total triglycerides in mussels (Fig. 2). MET activity was significantly reduced at 0 km, 5 km and 12 km, and in the river samples compared to the control (aquarium water) (Fig. 2A). The lower MET activity in the reference site compared to control aquarium laboratory suggests the presence of agents or contaminants that exists at this urban area site. The maximum decrease, 70 %, was found at the 5 km and

Table 3 Selected dissolved elements of released untreated waters

Sites	Na[1]	Cd	Cr	Fe	Co	Ni	Cu	Zn
1	41.5	0.031	0.22	34	0.068	0.82	8.5	10.8
2	14.4	0.006	0,14	21	0.023	0.57	0.97	1.6
3	13.7	0.005	0.11	17	0.021	0.57	1.1	0.87
4	13	0.007	0.12	22	0.026	0.55	0.83	0.98
5	14	0.006	0.10	17	0.012	0.54	0.82	0.90
Reference	13.2	0.005	0.10	15	0.03	0.55	0.76	0.78

[1]Expressed as the mean from N = 3 determinations in ug/L; coefficient of variations between 5 % and 15 % of the mean.

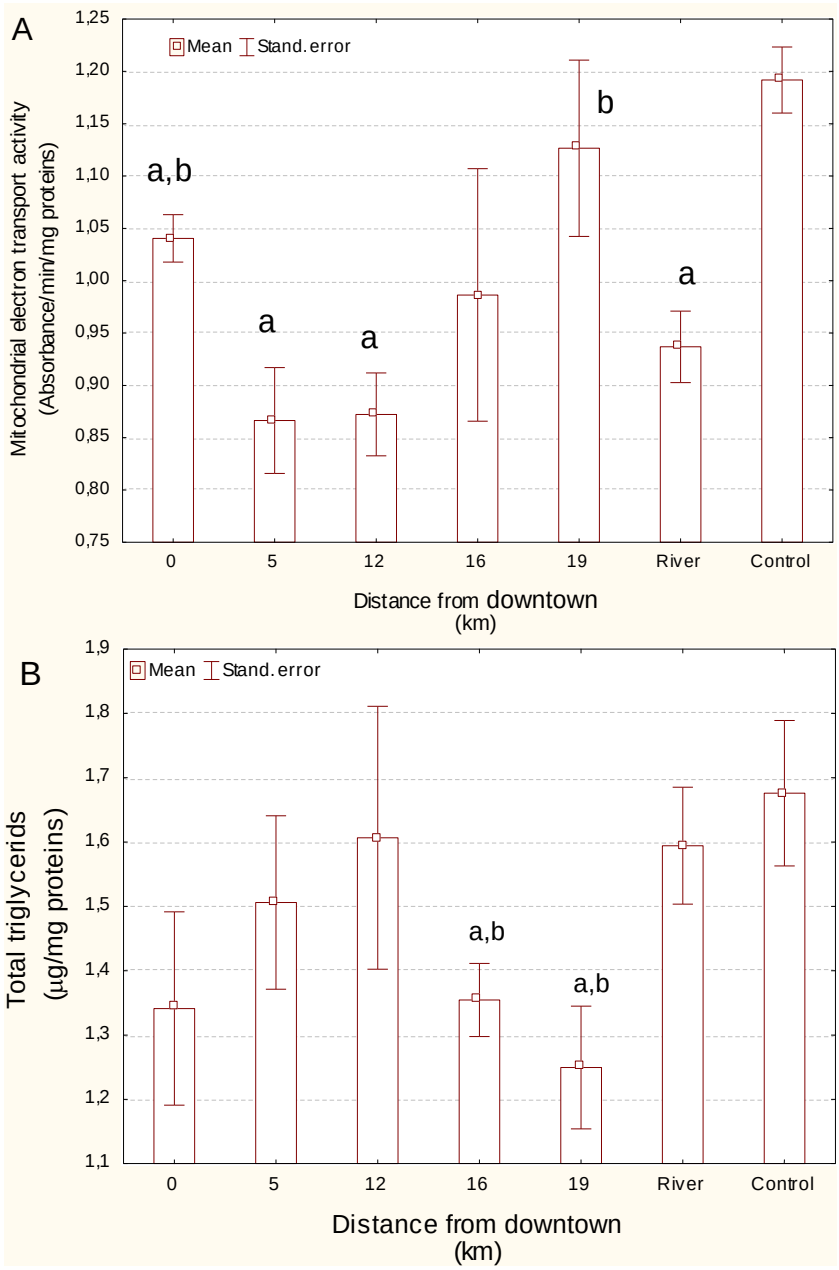

Fig. 2 Energy status of mussels exposed to the untreated wastewaters. Mussels were exposed to the water samples for 96 h. Mitochondrial electron transport activity (A) and total triglycerides (B) were determined in mussel soft tissues. The letter *a* indicates significance when compared to control water; the letter *b* indicates significance when compared to river water at $p < 0.05$.

12 km sites. However, when compared to the river water outside the area of untreated wastewater release, MET activity was significantly increased at 0 km and 19 km. This suggests that an initial suppression in MET activity is observed with the water samples compared to control aquarium water but this is mitigated when compared to river water. Energy reserves were significantly lower at 16 km and 19 km from downtown compared to both control and river water (Fig. 2B). The maximum decrease was 70 % of the control values. Taken together, the increase in MET activity at 0 km and 19 km was associated with lower lipid values when compared to river water, although triglyceride levels for the 0 km site were not significantly different from river water, due to higher variability. However, these changes appeared to be site-specific, since no significant trends based on distance were observed. In a previous study (Gagné *et al*., 2007), MET activity and gonad lipids were higher in mussels exposed to treated wastewater from Montreal. Those effects were reversed following ozonation with 10 - 20 mg/L ozone.

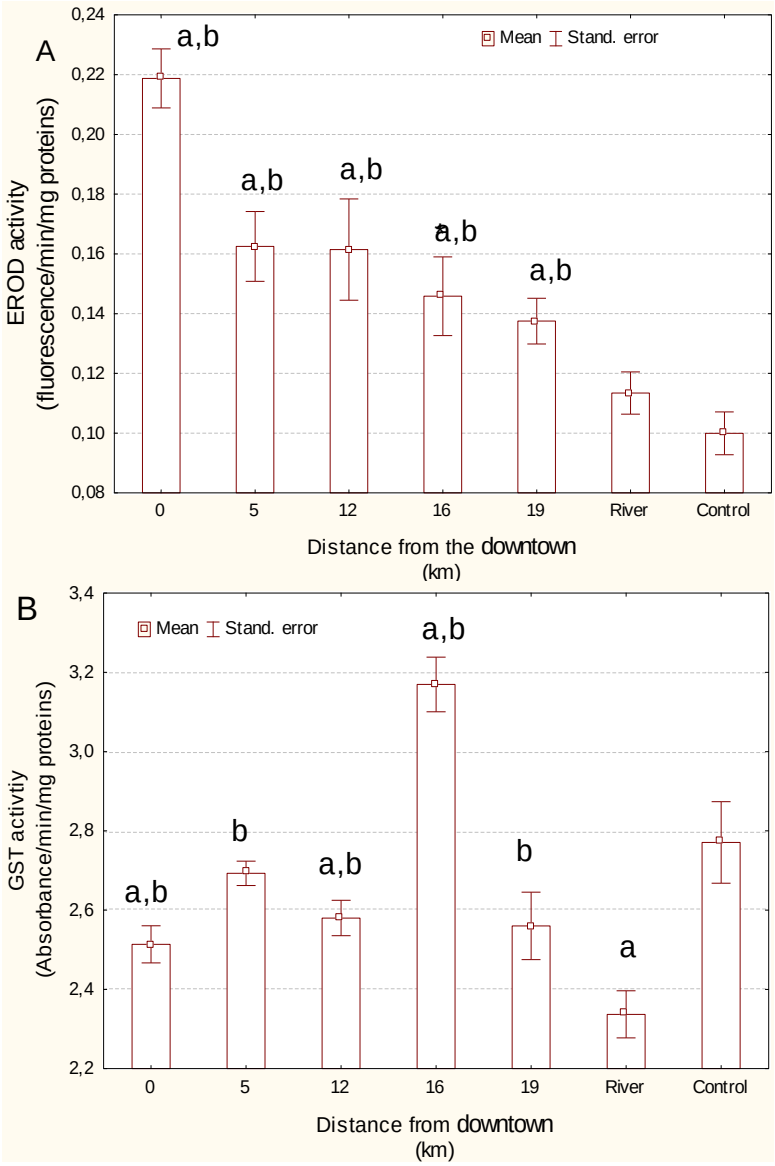

Fig. 3 Biotransformation activity of mussels exposed to the untreated wastewaters. Mussels were exposed to the water samples for 96 h. Phase I and II biotransformation activities were determined by EROD (A) and GST (B) activities respectively. The letter *a* indicates significance when compared to control water; the letter *b* indicates significance when compared to river water at $p < 0.05$.

Biotransformation activity was determined in mussels by measuring changes in EROD and GST activities. EROD activity was significantly increased at all sites compared to both river and control waters (Fig. 3A). The maximal response was found at 0 km, where it was 2.2 times greater than the control/river water samples. EROD activity was significantly correlated with distance from downtown ($r = -0.95$; $p < 0.001$) indicating increased polyaromatic hydrocarbons offshore from at the most densely populated area of the city. GST activity was significantly increased at all sites when compared to the river water, reaching 1.4 times greater at 16 km. The river water sample showed lower GST activity than the laboratory control water, indicating a suppression effect of “natural” waters, as observed for MET activity. It is possible that lower metabolic activity (MET) contributed also to reduce GST activity given its role in the scavenging of reactive oxygen species during respiration. There was no significant correlation with distance from downtown. Primary cultures of rainbow trout hepatocytes were exposed to 12 wastewaters before and after six different treatment processes and had elevated CYP1A1 mRNAs (responsible for EROD activity) nearly 70 % of the time (Gagné *et al.*, 2013). GST gene expression was induced by 70% of the various treated effluents and was significantly related to the expression profiles obtained with the untreated effluent, which suggests that the CYP1A1-inducing properties of the effluent were not produced by the treatment process per se.

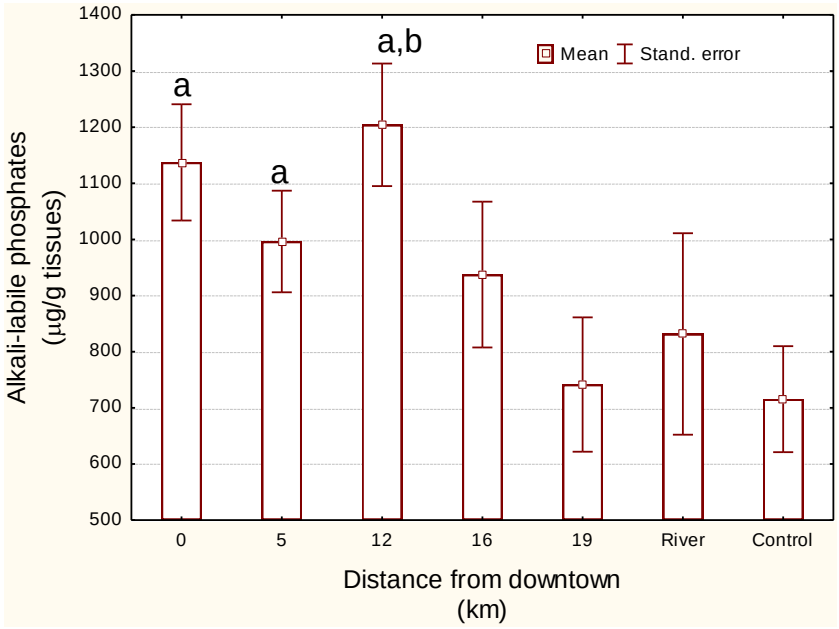

Fig. 4 Changes in alkali-labile phosphates in quagga mussels exposed to the untreated wastewaters. Mussels were exposed to the water samples for 96 h. The levels of vitellogenin-like proteins were determined by the alkali-phosphate method. The letter *a* indicates significance when compared to control water; the letter *b* indicates significance when compared to river water at $p < 0.05$.

The levels in ALP were also examined in mussels exposed to the released wastewaters (Fig. 4). ALP levels were significantly higher at 0 km, 5 km and 12 km than in the controls. There was no significant difference between river water and control water samples, but variability was higher for the river samples. In the river water, only the 12-km site had significantly higher ALP levels. Interestingly, ALP levels were significantly correlated with distance from downtown (r = -079; $p < 0.05$). The ALP levels in dresseinids were associated with egg yolk proteins and were shown to be significantly induced in tertiary-treated municipal effluents (Quinn *et al*., 2004). This suggests that releasing of untreated wastewater from a highly populated area produces estrogenic effects in this species of mussel. Nonyphenol, a common estrogenic compound found in municipal wastewaters, was shown to increase ALP levels in zebra mussels (Quinn *et al*., 2006).

The levels of oxidative damage (LPO) and damage to the genetic material (DD) in mussels were examined. LPO levels were significantly higher at 0 km than in river and control waters (Figure 5A), reaching 1.5 times the control values. There was a significant correlation between distance from downtown and LPO (r = -0.72; $p < 0.05$). This is consistent with previous studies on the oxidative stress/inflammatory effects of municipal effluents on mussels and fish (Gagné *et al*., 2007; Jasinska *et al*., 2015). Caged fathead minnows exposed for four weeks downstream from the City of Edmonton's municipal effluent dispersion plume had elevated levels of oxidized glutathione and higher antioxidant enzyme and glutathione S-transferase activity. Mussels exposed to physico-chemically treated effluent had elevated levels of LPO and DD in gills. For DD, a more complex pattern was observed. The levels of DD in the river water were significantly lower than in the aquarium control water, which suggests a suppressive effect of river water on DNA turnover and repair activity. When compared with river water, DD was significantly induced at 0 km, 12 km and 19 km from downtown while DD was significantly reduced at 16 km from downtown. Caged mussels exposed for one month to waters downstream from Montreal's municipal discharge had elevated DD as determined by the Comet assay (Lacaze *et al*., 2011). Genotoxicity was apparent up to 20 km distance and its intensity was similar to that found in the municipal effluent (< 200 m). This suggests that even with physico-chemical treatment, genotoxic compounds are still released and persist at 20 km downstream from the plume.

Reduced levels of DNA strand breaks were also observed previously in mussels exposed to primary-treated effluent (the same treatment for these wastewaters) (Gagné *et al*., 2011). A biphasic response was observed, with an initial decrease in DNA strand breaks at a low effluent concentration (3 % v/v) followed by a decrease in DNA breaks at higher concentrations (> 10 % v/v). The initial decrease in DNA strand breaks could be associated with the decrease in DNA repair activity, such as reduced precursors for purine synthesis by inhibition of dehydrofolate reductase inhibitors, leading to reduced DNA strand breaks. Decreased DNA repair activity would therefore result in increased DNA damage and increased alkali-labile

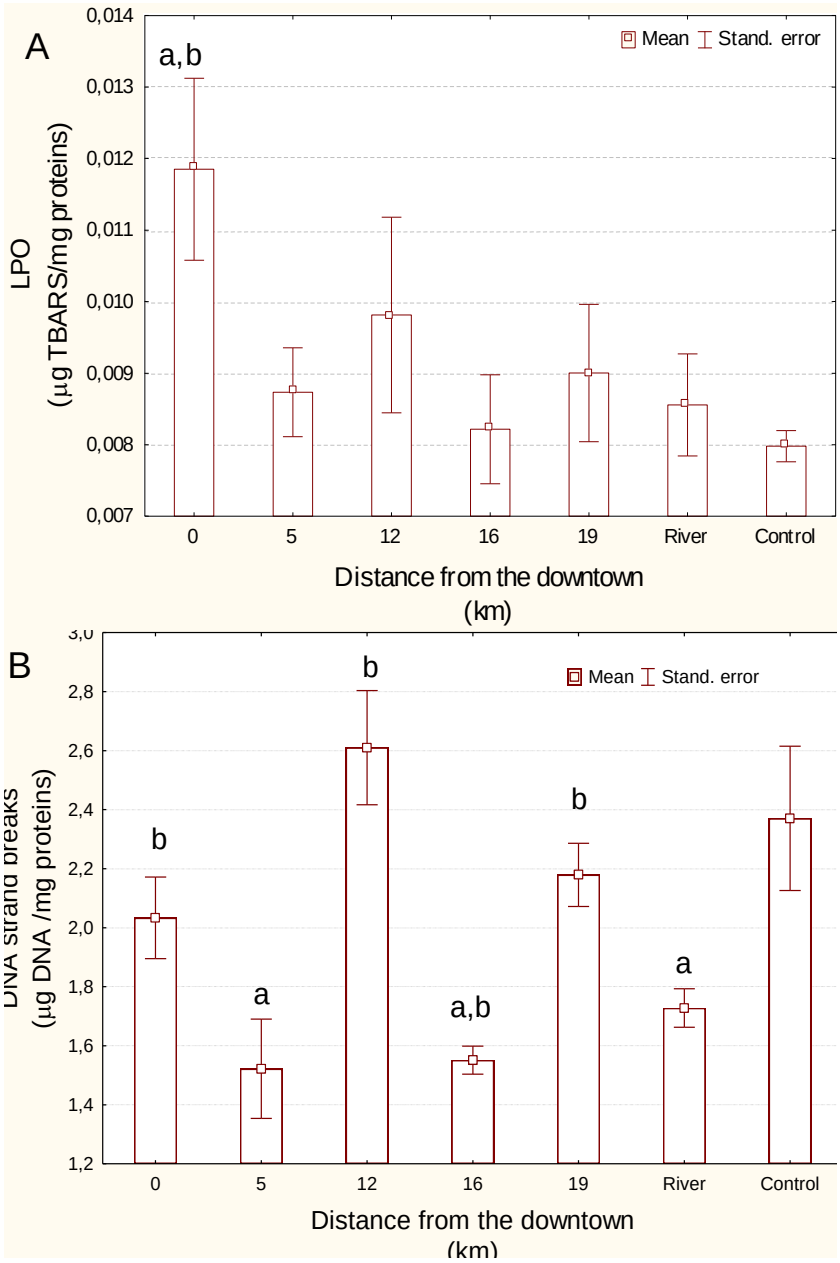

Fig. 5 Oxidative damage and genotoxicity in mussels exposed to the untreated wastewaters. Mussels were exposed to the water samples for 96 h. The levels of LPO (A) and DNA strand breaks (B) were determined in whole tissues of quagga mussels. The letter *a* indicates significance when compared to control water; the letter *b* indicates significance when compared to river water at $p < 0.05$.

sites in DNA at higher effluent concentrations. A previous study (Gagné *et al*., 2007) showed strong inhibition of dihydrofolate reductase activity in *Elliptio complanata* mussels exposed to the same primary-treated municipal effluent and suggested that the dehydrofolate reductase-inhibitors such as the bacteriostatic agent trimethoprim and other similar compounds (methotrexate and pyrimethamine) present in municipal effluents accounted, at least in part, for the observed inhibition. The reported concentration of methotrexate is in the order of 60 ng/L in untreated wastewater from Montreal (Garcia-Ac *et al*., 2009). However, methotrexate was present at concentrations between 20 ng/L and 4,000 ng/L in hospital wastewater effluents (Isidori *et al*., 2016). The concentration of methotrexate required for inhibition in vitro is in the order of 0.5 µg/L to 1 µg/L. This suggests that mussels are able to accumulate this compound or that other inhibitors are also present in municipal effluents and act cumulatively in mussels. Recent evidence suggests that genotoxic compounds are impervious to wastewater treatment even after anoxic-oxic process (Zhang *et*

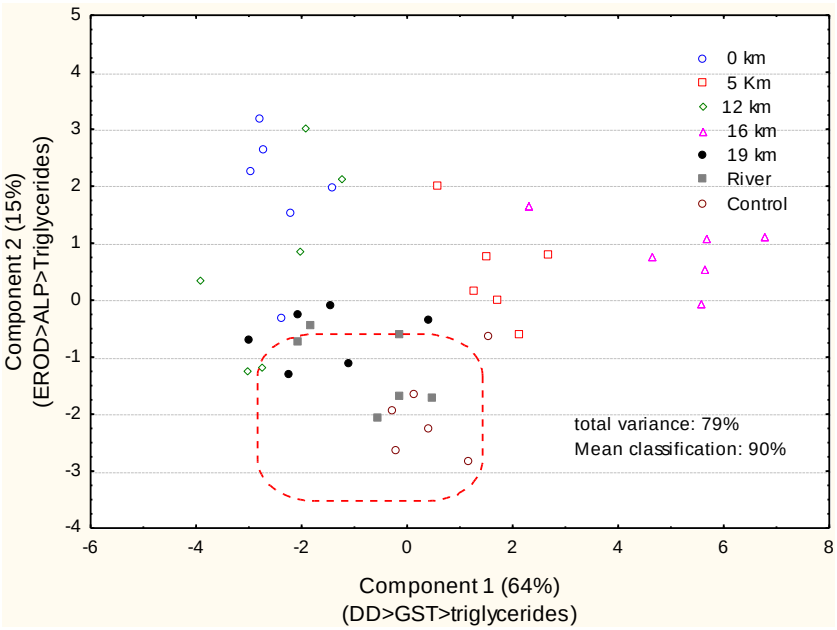

Fig. 6 Discriminant function analysis of biomarker data. Mussels were exposed to the water samples for 96 h. Biomarkers were analyzed using discriminant function and factorial analyses to determine site similarities and the most important biomarkers.

al., 2013). Even though the acute toxicity of municipal wastewater was effectively removed by anoxic/oxic treatment, the DNA-damaging properties (micronucleus and COMET assays) were not affected in zebrafish. Thus, treated and untreated wastewaters represent continuous sources of genotoxic compounds in the environment. Moreover, under climate change conditions where increased precipitation events are likely (Min *et al*., 2011), more frequent rainwater overflows can be expected, given the limited capacity of some wastewater treatment plants to handle more intense rainfall events.

Discriminant function analysis was performed to identify differences between the samples and determine the most responsive biomarkers in achieving site discrimination (Fig. 6). The analysis revealed that 79 % of total variance was explained with a mean classification efficiency of 90 %. Water samples from river and aquarium water were somewhat similar and formed a distinct group from the untreated water samples. The farthest site (19 km) was closely related to the river water sites. Water samples at 0 km, 5 km, 12 km and 16 km formed clusters distinct from the farthest and the river water sites. These were mainly explained by DD, GST and triglyceride levels. The site closest to downtown (0 km) differed from the other untreated waters and from river water in EROD activity, ALP levels and triglycerides (on the y axis). Interestingly, the untreated water samples generally differed from each other from 0 km to 16 km. This suggests some heterogeneity in the water samples which is dependent on distance from downtown. This is consistent with the data showing correlation of distance with EROD, LPO and ALP endpoints.

In conclusion, exposure of quagga mussels to released untreated waters for four days led to toxic effects. The strongest response was increased EROD activity, for which the intensity in activity was proportionally related to the distance from the 0 km point (downtown, the most densely populated part of the city). DD showed a biphasic response with either increased or decreased DNA strand breaks at the release sites when compared with river water outside the area where untreated wastewater was released. Other changes included potential estrogenic effects (ALP), altered energy metabolism and oxidative damage. Exposure to untreated wastewater for relatively brief periods (four days) could negatively impact local mussel populations.

Acknowledgements

This study was supported by the St. Lawrence Action Plan of Environment and Climate Change Canada. Joana Kowalczyk's technical assistance with biomarker analyses was much appreciated.

References

Ács A, Vehovszky Á, Győri J, Farkas A. Seasonal and size-related variation of subcellular biomarkers in quagga mussels (*Dreissena bugensis*) inhabiting sites affected by moderate contamination with complex mixtures of pollutants. Environ. Monit. Assess.188: 426-439, 2016.

American Public Health Association, American Water Works Association, and Water Environment Federation 2012. In Standard Methods for the Examination of Water and Wastewater, 22nd edition, ISBN: 978-0875530130.

André C, Gagné F. Urban pollution increases sensitivity to temperature changes in *Elliptio complanata* mussels exposed to municipal wastewaters. Environ. Pollut. Climate Change 1: 2-9, 2017.

Aravindaksham J, Paquet V, Gregory M, Dufresne J, Fournier M, Marcogliese DJ, *et al*. Consequences of Xenoestrogen Exposure on Male Reproductive Function in Spottail Shiners (*Notropis hudsonius*). Toxicol. Sci. 78: 156-165, 2004.

Benchalgo N, Gagné F, Fournier M. Biomarkers study in rainbow trout exposed to industrially contaminated groundwater. J. Xenobiotics 4: 1-7, 2014.

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

Boryslawskyj M, Garrood AC, Pearson JT. Elevation of glutathione-*S*-transferaseactivity as a stress response to organochlorine compounds, in the freshwater mussel, *Sphaerium corneum*. Mar. Environ. Res. 24: 101-104, 1998.

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254, 1976.

Chen M, Ohman K, Metcalfe C, Ikonomou MG, Amatya PL, Wilson J. Pharmaceuticals and endocrine disruptors in wastewater treatment effluents and in the water supply system of Calgary, Alberta, Canada. Water Qual. Res. J. Canada 41: 351-364, 2006.

de Lafontaine Y, Gagné F, Blaise C, Costan G, Gagnon P, Chan HM. Biomarkers in zebra mussels (*Dreissena polymorpha*) for the assessment and monitoring of water quality of the St Lawrence River (Canada). Aquat. Toxicol. 50: 51-71, 2000.

Gagné F, André C, Cejka P, Gagnon C, Blaise C. Toxicological effects of primary-treated urban wastewaters, before and after ozone treatment, on freshwater mussels (Elliptio complanata). Comp. Biochem. Physiol. 145C: 542-552, 2007.

Gagné F, André C, Bouchard B, Fournier M, Gagnon C. Neurotoxicological effects of aeration lagoons effluents for the treatment of domestic and hospital wastewaters on *Elliptio complanata*. In Ecotoxicology around the Globe. New York: Nova Science Publishers, pp 263-282, 2010.

Gagné F, André C, Cejka P, Hausler R, Fournier M. Alterations in DNA metabolism in *Elliptio complanata* mussels after exposure to municipal effluents. Comp. Biochem. Physiol. 154C: 100-107, 2011.

Gagné F. Oxidative stress. In Biochemical Ecotoxicology: Principles and Methods, Chapter 6, USA: Elsevier Inc., pp 103-116, 2014.

Garcia-Ac A, Segura PA, Gagnon C, Sauvé S. Determination of bezafibrate, methotrexate, cyclophosphamide, orlistat and enalapril in waste and surface waters using on-line solid-phase extraction liquid chromatography coupled to polarity-switching electrospray tandem mass spectrometry. J. Environ. Monit. 11: 830-838, 2009.

Isidori M, Lavorgna M, Russo C, Kundi M, Zegura B, Novak M, *et al.* Chemical and toxicological characterisation of anticancer drugs in hospital and municipal wastewaters from Slovenia and Spain. Environ. Poll. 219: 275-287, 2016.

Jasinska EJ, Goss GG., Gillis PL, Van Der Kraak GJ, Marsumoto J, Machado AAdS., *et al*. Assessment of biomarkers for contaminants of emerging concern on aquatic organisms downstream of a municipal wastewater discharge. STOTEN 530-531: 140-153, 2015.

King F, Packard TT. Respiration and the activity of the respiratory electron transport system in marine zooplankton. Limnol. Oceanogr. 20: 849-854, 1975.

Lacaze E, Devaux A, Bony S, Bruneau A, André C, Pelletier M, *et al.* Genotoxic impact of a municipal effluent dispersion plume in the freshwater mussel *Elliptio complanata*: an in situ study. J. Xenobiotics 3: 14-16, 2013.

Min S-K, Zhang X, Zwiers FW, Hegerl GC. Human contribution to more-intense precipitation extremes. Nature 470: 378-381, 2011.

Quinn B, Gagné F, Costello M, McKenzie C, Wilson J, Mothersill C. The endocrine disrupting effect of municipal effluent on the zebra mussel (*Dreissena polymorpha*). Aquat. Toxicol. 66: 279-292, 2004.

Quinn B, Gagné F, Blaise C, Costello MJ, Wilson JG, Mothersill C. Evaluation of the lethal and sub-lethal toxicity and potential endocrine disrupting effect of nonylphenol on the zebra mussel (*Dreissena polymorpha*). Comp. Biochem. Physiol. 142: 118-127, 2006.

Tetreault GR, Bennett CJ, Shires K, Knight B, Servos MR, McMaster ME. Intersex and reproductive impairment of wild fish exposed to multiple municipal wastewater discharges. Aquat. Toxicol. 104: 278-290, 2011.