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RESEARCH REPORT

Long-term and comparative impacts of combined sewers and municipal effluents to freshwater mussels

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Abstract

Excess rainfall events could lead to overflows and combined sewer overflows, which could threaten local mussel populations. This study sought to compare the long- term effects of combined sewer overflows and treated municipal effluents in caged *Elliptio complanata* mussels. Mussels were caged at 2 overflow sites, one downstream site of a major municipal effluent dispersion plume and a reference upstream site for 3 months during the summer. At the end of the exposure period, mussels were collected, analyzed for municipal contaminants (including pharmaceuticals), and effects biomarkers based on endocrine disruption (vitellogenin expression), xenobiotic detoxification (glutathione S-transferase and metallothioneins), oxidative stress/inflammation (cyclooxygenase and lipid peroxidation) and DNA damage. The data revealed that surface waters contained less pharmaceutical products than the downstream site but atrazine and its metabolite were at higher levels in overflow sites. Mussels contained elevated amounts of total heterotrophic bacteria, caffeine, acebutolol and venlafaxine at the downstream site relative to the upstream site where caffeine was higher at one of the overflow site. The levels of vitellogenin gene expression were significantly increased in both sexes of mussels caged at the downstream site only. Multivariate analysis revealed that the biomarker responses were completely separated between upstream, overflow and downstream sites. The site discrimination was based on vitellogenin, metallothioneins, DNA damage in gonad and digestive gland, gonad lipids/proteins reserves, lipid peroxidation, gonado-somatic index and condition factor. Adverse outcome pathways analysis using the power law approach revealed that most changes in the biomarkers identified by discriminant function analysis were significantly scaled to gonad energy reserves, tissue/ mussel size and loss of weight following air emersion stress. In conclusion, the toxic effects of mussels caged at overflow sites generally displayed lower responses than mussels caged downstream a treated municipal effluent suggesting that these overflows pose a lower risk than the continuous exposure to treated municipal effluent.

Key Words: *Elliptio complanata*; combined sewers; municipal effluent; vitellogenin; oxidative stress

Introduction

Freshwater mussels constitute an important group of the invertebrate community and are ubiquitous in many lakes and rivers. Because of their sessile lifestyle and high water filtering capacity for feeding, these organisms are susceptible to local sources of pollution such as municipal effluents and agriculture runoffs, which can be exacerbated by heavy rainfall events. This situation is likely to worsen by global warming which could bring low water levels thereby increasing the concentration of contaminants and drought punctuated by periods of intense precipitation (Min *et al*., 2011). Important rainfall events albeit occurring for short periods (hours to days) could overwhelm the wastewater treatment plants capacity to handle excess rain and sometimes combines with untreated wastewater before discharging in the nearby water bodies. From a wastewater risk management perspective, the comparative and cumulative effects of combined sewers effluent following rain events and the continuous release of treated municipal effluents should be better understood.

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Table 1 Physico-chemical characteristics and occurrence of pharmaceutical products in surface waters

Parameter	Upstream	OVF1	OVF2	Downstream
Rainfall events (mm)[1] Open time (min)[2]	249 --	249 719	249 860	249 --
pH	8.38	8.4	8.45	8.39
Conductivity	291 ± 10	292 ± 25	283 ± 20	296 ± 20
TSM (mg/L)	9 ± 1	13 ± 1	3 ± 0.5	85 ± 10*
DOC (mg/L)	2.7 ± 0.5	2.8 ± 0.5	2.9 ± 0.5	3.4 ± 0.5
Ammonia (mg/L)	0.005 ± 0.001	0.01 ± 0.001	0.004 ± 0.001	0.1 ± 0.01*
Heterotrophic bacteria - mussels (counts/mussels)	30000 ± 1400	22000 ± 1500	27200 ± 1500	80800 ± 2000*
Caffeine –water (ng/L) - mussels (ng/g)	0.074 ± 0.067 4.2 ± 2	0.039 ± 0.004* 3.5 ± 1	0.034 ± 0.014* 3.7 ± 1.6	0.864 ± 0.006* 4 ± 1.5
Acebutolol-water (ng/L) - mussels (ng/g)	0.26 ± 0.005 0.01 ± 0.007	0.43 ± 0.01 0.01 ± 0.007	0.28 ± 0.04 0.005 ± 0.003	2.7 ± 0.13* 0.06 ± 0.03*
Venlafaxine-water (ng/L) -mussels (ng/g)	1 ± 0.05 1 ± 0.6	1.8 ± 0.03 1.1 ± 0.3	1.2 ± 0.03 0.9 ± 0.4	7.7 ± 0.3* 2 ± 0.9
Desvenlafaxine-water (ng/L) -mussels (ng/g)	4.2 ± 0.25 6.5 ± 4	7.5 ± 2 4.7 ± 3	5 ± 2 4.8 ± 3	16+2* 4.1 ± 2
Ibuprofen-water (ng/L) -mussels (ng/g)	6 ± 0.9 <LOD	7 ± 0.026 <LOD	6.7 ± 0.95 <LOD	81 ± 2* <LOD
Estrone-water (ng/L)	<LOD <LOD	0.45 ± 0.04* <LOD	<LOD <LOD	0.56 ± 0.05* <LOD
Atrazine-water (ng/L)	58+0.9 <LOD	82 ± 2* <LOD	56 ± 0.2 <LOD	59 ± 0.8 <LOD
Desethylatrazine-water (ng/L)	64 ± 1 <LOD	89 ± 1.3* <LOD	60 ± 0.9 <LOD	48 ± 5 <LOD

[1] Sum of rainfall received in this area from July to September 2016 (mm).
[2] Valve open time of rainfall overflows (min).
The star symbol * indicates significance from the upstream site ($p < 0.05$)

The freshwater mussel *Elliptio complanata* (*E. complanata*) is commonly found in lakes and rivers of the Eastern part of North America (Downing and Downing, 1992). It is one of the most abundant species of freshwater mussels in the Saint-Lawrence River system and was chosen as a representative bivalve of the invertebrate community to study the cumulative impacts of water quality and quantity of urban and agricultural areas. This species could live more than 10 years and are sexually dimorphic with distinct male and female gonads with low occurrence of intersex (van der Shalie, 1970). The synthesis of vitellogenin, the egg yolk proteins for the developing embryo, occurs in follicular tissues in gonads tissues (Pipe, 1987) and recent studies have shown that long-term exposure to estrogens and municipal effluents could lead to vitellogenesis and feminization (Gagné *et al.*, 2011).

Indeed, wild *E. complanata* mussels collected downstream the municipal effluent plume had 85 % of females compared to upstream sites where males and females gonad expressed vitellogenin-like proteins in male individuals. Exposure of caged *E. complanata* mussels for one year to a municipal effluent dispersion plume increased the proportion of females from 40 % to 70 % and vitellogenin-like proteins were increased in both sexes. Long-term exposure to estrogens also induced vitellogenin in both males and females oysters (Andrew *et al.,* 2010). These effects are reversible when the estrogenic stimulus is stopped and from the capacity of mussels to change sex over its lifetime.

Cities are known to discharge a plethora of contaminants through discharges of treated wastewaters and combined sewer overflows following heavy rainfall events (Holeton *et al*., 2011; Haji-Mohamad *et al*., 2014). These overflows also contain large amounts of contaminants of various industrial and consumer products origins such as caffeine, acetaminophen, DEET, atenolol, progesterone and the persistent drug carbamazepine (Haji-Mohamad *et al.,* 2014). Because these rainfall overflows occur only during short periods of time following intense precipitations, it is difficult to compare the long-term ecotoxicological impacts of these overflows with the continuous release of treated municipal effluents. However, in the context of global warming, the increase of warmer temperatures (low water levels) punctuated with strong precipitations will led to a situation where mussels have to endure long periods of drought (low water levels with sites exposed directly to air) with increased output of rainfall overflows. The long-term consequence of these stressors are largely ununderstood at the present time. Urban pollution are well known to produce many other ecotoxic effects to fish and invertebrates besides endocrine disruption (Lacaze *et al.,* 2013; Marcogliese *et al*., 2014; Petrie *et al.,* 2015). Municipal effluents are known to cause oxidative stress, inflammation, DNA damage/decreased DNA repair, reduced immunocompetence and neurotoxicity to invertebrates (Gust *et al*., 2013; Lacaze *et al*., 2013; Gagné *et al.*, 2015). These effects arise from exposure to treated effluents suggesting that many contaminants still passes through the wastewater treatment system. This could contribute to the observed general declines in mussels populations nearby urban and agriculture areas (Lydeard *et al.*, 2004; Nobles and Zhang, 2015).

The purpose of this study was therefore to compare the long-term effects of combined sewers overflows and municipal effluents to freshwater *E. complanata* mussels. Mussels were caged at sites downstream combined sewer overflows and the corresponding treated effluent for 3 months during the summer where water fluxes and quality are quickly changing. Toxicity was examined at different scales i.e., the biochemical levels, energy reserves, tissue weights, mussel size and general health status in the attempt to understand not only the mode of action of these urban contaminants but to better understand adverse outcomes in individuals. An attempt was made to highlight toxic profiles associated to combined sewer overflows and treated municipal effluents in resident mussels. This information is of importance to decision making whether increasing wastewater overflows retention capacity or optimizing wastewater treatments should be prioritized together or separated.

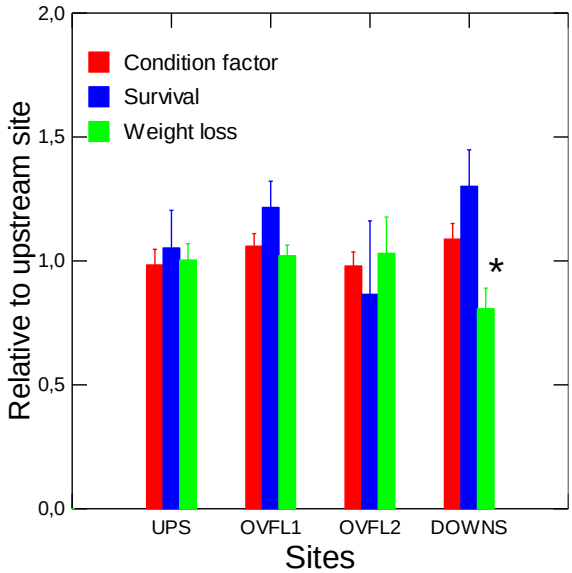


Fig. 1 Morphological parameters and air survival of caged mussels. The data for condition factor (mussel weight/shell length), air survival (days) and weight loss at time of death in air (%) were normalized to the upstream site. The data represent the mean with standard error. The star symbol * indicates significance at $p > 0.05$

Table 2 Correlation analysis of biomarker data

	CF	Air	WL	DNA Dg	LPO dg	COX	GST	MT	GSI	Prot	DNAg	LPOg	Lipids	Sugars	ALP	βact	VTG
CF	1																
Air	-0.17	1															
WL	0.18	-0.35	1														
DNAdg	0.18	0.13	**0.64**	1													
LPOdg	0.21	0.29	0.34	**0.65**	1												
COX	0.27	0.48	-0.45	-0.17	0.28	1											
GST	-0.17	-0.32	0.08	0.07	-0.15	-0.19	1										
MT	0.29	-0.11	0.37	0.47	0.08	0.02	0.06	1									
GSI	0.23	0.17	-0.37	-0.39	0.01	0.38	-0.06	0.13	1								
Prot	0.01	0.30	**-0.64**	-0.47	0.00	0.28	-0.43	**-0.57**	0.22	1							
DNAg	0.27	0.15	-0.02	0.32	0.26	0.15	-0.34	0.74	0.23	-0.11	1						
LPOg	-0.07	0.14	-0.51	-0.47	-0.22	0.46	-0.38	-0.24	0.14	**0.65**	-0.17	1					
Lipids	-0.39	0.27	**-0.54**	-0.35	-0.37	-0.03	0.37	-0.41	0.26	0.41	-0.47	0.37	1				
Sugars	-0.22	0.28	-0.26	0.14	-0.05	-0.25	0.38	-0.26	0.07	0.29	-0.28	0.04	**0.84**	**1**			
ALP	0.15	0.03	-0.45	-0.18	-0.09	0.37	0.12	-0.39	-0.17	0.28	-0.23	0.10	0.08	0.03	1		
Bact	-0.26	0.24	**-0.70**	**-0.54**	-0.27	0.32	-0.03	**-0.58**	-0.09	**0.78**	-0.38	**0.70**	**0.57**	0.31	0.42	1	
VTG	-0.53	0.37	**-0.56**	-0.43	-0.41	0.07	0.22	-0.46	0.20	0.43	-0.52	0.50	**0.95**	**0.68**	0.1	**0.63**	1
GSTmRNA	-0.03	-0.09	-0.11	-0.01	-0.32	-0.39	0.04	-0.22	**-0.59**	0.32	-0.10	-0.04	0.11	0.27	0.37	0.44	0.06

Significant correlations are highlighted in bold.
Abreviations: condition factor: mussel weight/shell length (CF), Air survival (Air), Weight loss at time of death during the air survival test (WL), DNA breaks in digestive gland (DNADg), digestive gland LPO (LPOdg), arachidonate cyclooxygenase (COX), GST activity (GST), metallothioneins (MT), gonado-somatic index (GSI), gonad proteins (Prot), gonad DNA breaks or LPO (DNAg, LPOg), alkali-labile phosphates (ALP), total heterotrophic bacteria (Bact), vitellogenin gene expression (VTG), GST gene expression (GSTmRNA)

Materials and Methods

Mussels collection and handling

Elliptio complanata mussels were collected in a pristine lake i.e., under no direct contact of anthropogenic activity in the Laurentians (Québec, Canada) in the first week of June 2016. Mussels were transported in a humidified container (wet paper towels with lake water) at 4 °C. At the laboratory, mussels were placed in aquariums containing UV-treated and charcoal-filtered tap water at 15 °C for at least 15 days. They were fed three times a week in the morning with a commercial feed of phytoplankton (Phytoplex®) and laboratory-cultured *Pseudokirchneriella subcapitata* algae (50-100 billion algae in 60 L aquarium). Mussels (40 individuals per cage) were then placed in cylindric (1 m long x 0.5 m diameter) polyethylene nets (1 cm diameter mesh) attached to a 1 kg cement block. They were immersed at least in 1 m depth at 2 rainfall overflow sites: overflow site 1 (OVF1; 45°38'26.3"N; 73°29'15.6"W) and overflow site 2 (OVF2; 45°36'05.2"N; 73°30'33.6"W). One upstream (1.5 km) site of a major municipal effluent dispersion plume (45°39'28,5"N; 73°28'37,9"W), considered the reference site, and one downstream site located 8 km downstream the municipal effluent plume (45°44'23,9"N; 73°25'43,9"W). This site was previously shown to induce vitellogenin-like proteins in gonads and mussel feminization after one year exposure (Blaise *et al.*, 2003). Hence, the study comprised 4 sites: one upstream site, one downstream site in the municipal effluent dispersion plume and 2 rainfall overflows. The mussels remained at these sites for 3 months during July-September of 2016. The cages were inspected every two weeks to ensure no important mussel mortality or cage disturbances. No significant mortality was observed between the sites. At the end of the exposure period, mussels were transported back to the laboratory and allowed to depurate overnight to rinse the mussel's shells and tissues and empty the gut contents. A group of 10 mussels per site and surface waters (4L) at the time of cage retrieval were retained for chemical analysis of surface waters and mussel tissues using high performance liquid chromatography mass spectrometry (Boisvert *et al.*, 2012; Darwano *et al.*, 2014). The following compounds were determined in surface waters and mussels: methotrexate, carbamazepine, estriol, estradiol, estrone, ethinylestradiol, norethindrone, levonorgestrel, testosterone, progesterone, atrenogest, caddeine, acebutolol, (des)venlafaxine, atrazine (and its metabolite desehtylatrazine), sulfamethoxazole, diclofenac, clarithromycine, ibuprofen and carbamazepine.

Another group of mussels (10) were set aside to determine the stress on stress test (air surivival) as general indicator of mussel health. The mussels were placed in individual plastic cups and held at 20 °C in air saturated at 80 % humidity in an incubator. Mussels weights were measured each day and death was measured based on shell opening after handling. The time of death were expressed as days and the percentage of weight loss were determined as follows: 100 x (weight beginning-weight at death /weight beginning). Basic surface water characteristics were measured at the 4 sites after one month of cage deployment: pH, conductivity, total suspended solids, dissolved organic carbon and ammonia levels (AWWA, 2017).

LC-MS/MS analysis

A method involving on-line solid-phase extraction (on-line SPE) coupled to ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS Thermo TSQ

Quantiva) was used to analyse the targeted contaminants. The on-line enrichment was performed on a Thermo Hypersil GOLD aQ SPE column, while compound separation was performed on a Thermo Hypersil GOLD analytical column. Water samples were passed through glass fiber membrane filters for particle removal. An exact volume of 5 mL was transferred to an amber glass vial and isotope-labeled internal standards were added. A sample volume of 2 mL was injected to the on-line SPE – UHPLC/MS/MS workflow; negative mode and positive mode analytes were analysed within the same run using a heated electrospray ionization source operated in polarity-switching mode. Further details can be found in the related analytical method developments (Montiel-Leon *et al*., 2018; Goeury *et al*. 2019). The method has been validated for both wastewater and surface water matrices and the different criteria (recoveries, accuracy, precision and linearity) were compliant with US EPA guidelines (US EPA, 2007). Each site was sampled in duplicate and an aliquot from each bottle was also analysed in duplicate; the values reported thus account for sampling and analytical variation. LOD's for targeted analytes in the water samples are in the order to low ng/L (or sub ng/L).

For solid samples (mussel tissues), an solvent extraction method using ultrasonication was used, followed by a QuEChERS clean-up protocol adapted from previous work (Martinez Bueno *et al*. 2013). Freeze-dried samples were ground until homogenized. Water and acetonitrile were added along with various QuEChERS salts (Na_2SO_4, NaCl, $Na_3Cit{:}2H_2O$ and $Na_2HCit{:}3H_2O$) and the tube was vortex-mixed. After extraction, a centrifugation proceeded and a fraction of the supernatant was transferred to a second tube containing clean up reagents (Na_2SO_4, PSA, C18 and formic acid). 1 mL of supernatant was collected and evaporated to dryness, reconstituted in injection solvent, and analyzed by UHPLC-MS/MS using a similar method as that for water samples, but using a lower injection volume and without on-line SPE. Whole-method recoveries were between 60 and 120%. LOD's in mussel samples were in the order of pg/g to ng/g.

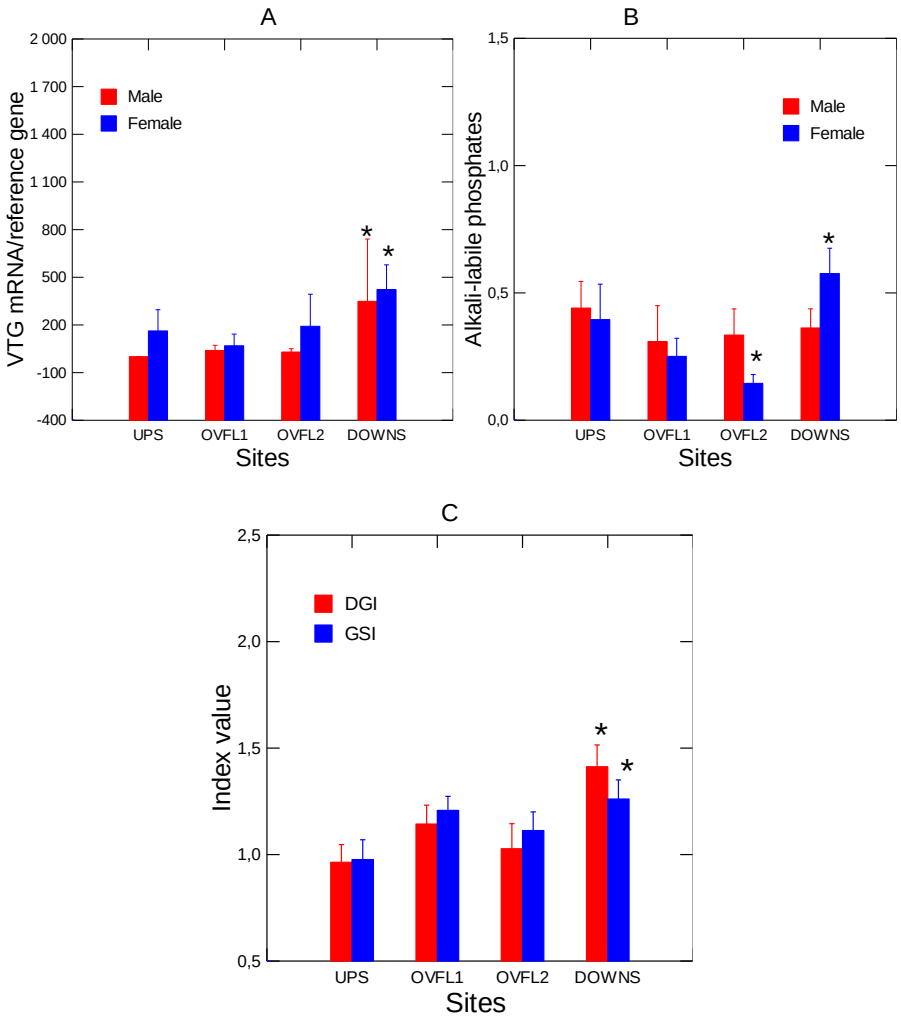


Fig. 2 Gametogenesis in caged mussels exposed to rainfall overflows and municipal effluent. The levels of VTG mRNA (A), alkali-labile phosphates (B) and the digestive gland index (DGI)/ gonado-somatic index GSI (C) were determined in mussels. The star symbol * indicates significance from the upstream site

Biomarkers analyses

A sample of 12 mussels per site were randomly selected, weighted, measured and the digestive gland and gonad were dissected on ice. Mussels and tissue weights were determined and the shell length recorded. For each mussel, the digestive gland and gonad were dissected out and the gonad tissues were separated in 2 portions: one preserved for RNA extraction (RNALater) for vitellogenin gene expression analysis and the other portion for biomarker analyses.

The tissues were thawed on ice for 30 min and 4 volumes of the buffer containing 140 mM NaCl containing 10 mM Tris-acetate, pH 8, 1 mM EDTA, 1 mM KH_2PO_4, 0,1 mM dithiothreitol and 1 µg/mL apoprotinin were added. Tissue homogenates were prepared using a Teflon pestle tissue grinder (5 passses) and a portion was centrifuged at 12000 x g for 30 min at 2 °C. The supernatant (S12 fraction) was collected for MT, GST and COX activity assessments. The samples were stored at -85 °C until analysis.

Energy reserves in the gonad homogenates were determined by determining total proteins, sugars and lipids. Total proteins were determined according to the procedure of Bradford (1976) using serum bovine albumin for quantitation. The homogenate was diluted 1/20 in 10 mM NaOH and incubated for 1 hour at room temperature to allow protein denaturation. Then, 5 µL of the dilution was tested for proteins in 200 µL in clear microplates. The data was expressed as mg proteins/g gonad or digestive gland and normalized against the upstream site which is the reference site in the present field study. Total sugars were determined in the gonad according to the anthrone reaction (Jermyn, 1975). The reaction was adapted to 96-well microplates for rapidity and reduction of reagents (waste). Glucose standards were used for calibration and the data were expressed as µg sugars or glucose equivalents/gonad weight and normalized against the upstream site. Total lipids were also determined in the gonad according to the phosphovanilate reaction (Frings *et al*., 1972) which was also adapted to 96-well microplates. Standard solutions of Triton X-100 were used for calibration. The data were expressed as µg of lipid equivalent/ gonad weight and normalized with the upstream (reference) site. The levels of vitellogenin-like proteins were also determined according to the alkali-labile phosphate in acetone-fractionated proteins with slight modifications (Gagné, 2014a). The high molecular weight proteins were precipitated in 30 % acetone and the protein pellet were washed once in 5 0% acetone (and recentrifuged at 10000 x g for 5 min) before the NaOH addition step. 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 alkali-labile phosphate/mg protein in gonad and normalized against the upstream site in females and males.

Lipid peroxidation (LPO) in gill and gonad tissues was determined according to the thiobarbituric acid methodology (Wills, 1987). The assay was also adapted to 96-well microplate where 10 µL of homogenates were mixed with 90 µL of water, 50 µL of 10 % trichloroacetic acid containing 1 mMFeSO4, and 100 µL of thiobarbituric acid (0.7 %). The mixture was heated for 70–75 °C for 5 min and allowed to cool down at room temperature. Fluorescence was measured with a microplate reader at 540 nm excitation and 600 nm emission (Synergy-4, Biotek Instruments, USA). Standards of tetramethoxypropane (stabilized form of malonaldehyde) were used for calibration. The data were expressed as µg of thiobarbituric acid reactants per mg of total proteins and normalized against the upstream (reference) site. The levels of

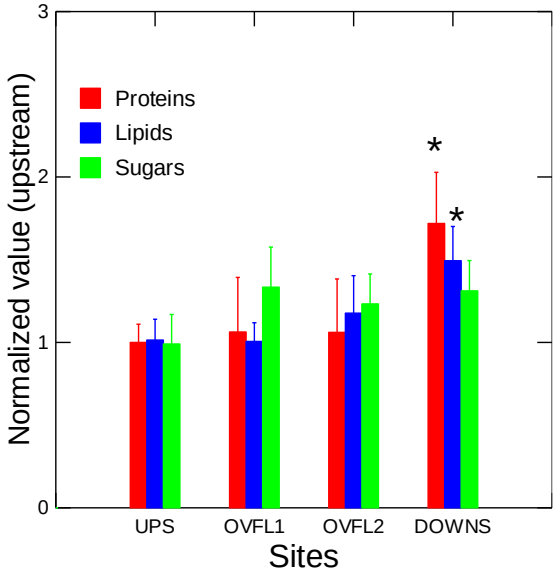


Fig. 3 Energy reserves in mussel gonad tissues. The levels of proteins, lipids and carbohydrates were determined in gonads. The data represent the mean with standard error. The star symbol * indicates significance from the upstream site

DNA strand breaks were determined in the homogenates by the alkaline DNA precipitation assay as described previously (Debenest *et al*., 2012). The data were expressed as µg DNA strand breaks/mg proteins in gonad or digestive gland and normalized against the upstream site.

The levels of metallothioneins (MT) were determined in the digestive gland using the silver saturation assay using non-radioactive silver (Gagné, 2014b). 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 additions of hemoglobin followed by heat denaturation. The remaining silver was determined by graphite furnace atomic absorption spectrometry with standards of rabbit MT and silver nitrate for calibration. A ratio of 17 moles of Ag/mole of MT standard was used for validation. The data were expressed as µg MT equivalents/mg proteins and normalized against the upstream site. COX and GST activities were also determined in the S12 fraction by fluorescence and absorbance-based assays in 96-well microplates as already described (Gagné *et al*., 2007). The enzyme activities were expressed as substrate change/min/mg proteins in the gonad or the digestive gland and normalized against the upstream site.

GST and Vitellogenin gene expression were determined in E. complanata using the recently developed transcriptomics data in Gene Bank (project ID PRJNA575711). The primers used were as follows, for GST: forward primer 5'-TACCCAGGTCTTTCGGTTTCC-3', reverse primer 5'-CCTTCACCGCCTCAGTTACA-3' and for Vitellogenin: forward primer 5'-GTGTCCTGGGGCTTTATGCT-3' and reverse primer 5'-GCGTTTCATCATTGGGGTGG-3'.

Total RNA was extracted in the gonad after sexing the mussels using the commercial RNeasy plus mini kit (Quiagen, Canada). RNA concentration (A260) and purity (A260/A280) were estimated by UV scanning between 220-320 nm using the NanoDrop 1000 instrument (Thermo Fisher Scientific, ON, Canada) and RNA integrity was checked by electrophoresis (Experion™ Automated Electrophoresis System, Bio-Rad, ON, Canada). Synthesis of cDNA was produced also with a commercial kit (QuantiTect® Reverse transcription kit, Qiagen, Canada). The cDNA samples served as the template DNA for amplification using quantitative real-time polymerase chain reaction (qPCR). The qPCR analyses were performed using SsoFast™ EvaGreen® Supermix and CFX96 real-time PCR detection system (Bio-Rad, Mississauga, On, Canada). Calibration was achieved using serial dilutions of cDNA (10 ng) with amplification efficiency values between 96 % and 102 %. The amplification reactions were carried out in the following conditions: 5 ng cDNA, 6.5 µL of 2×SoFast EvaGreen Supermix (Bio-Rad), 300 nM of each primer and DEPC treated water (Ambion) up to a total volume of 13 µL. Cycling temperatures were 95 °C for 30 sec, then 39 cycles of 95 °C for 5 sec and 60 °C for 10 sec. Amplification specificity was verified using melting curve analysis. Three reference genes were tested: ribosomal 18S, 60S ribosomal protein L8 and hypoxanthine-guanine phosphoribosyltransferase and the one showing the least variance between samples was selected for normalization. The Cq values (the cycle with a significant increase in fluorescence) and the Cq for the reference gene was used for comparison between sites. The gene expression data was also normalized to the upstream site responses for inter-site comparisons.

Data analysis

The mussels were randomly collected using a shell length range between 5-8 cm to minimize outliers to ensure an homogenous distribution of mussel size across the sites. The data were analyzed for normality and homogeneity of variance using the Shapiro-Wilks and Bartlett tests. In the case that the data were homogenously distributed and normal, the data was subjected to an analysis of variance (ANOVA) followed by the Least Square Difference (LSD) test to highlight significant changes relative to the upstream (reference) site in this caging experiment in the Saint-Lawrence River. In the case that the data were not homogenous or deviated from normality, the data was analyzed using rank ANOVA followed by the Conower-Inman non-parametric test for comparisons relative to the upstream site. Correlations between the data were determined by the Pearson-moment test. Principal component and discriminant function analyses were also performed to determine the most important biomarkers that could identify specifically rainfall overflow and downstream sites. The propagation of effects at different scales (molecular responses, energy reserves, organ and body sizes) was studied using the power law paradigm (West *et al*., 2002) to find potential adverse outcome pathways. The power law is defined by $y(x) = cx^a$ or $\log y(x) = a\log x + \log c$ where a is the scaling exponent and c a constant, y the biomarker responses at a lower scale and x the corresponding biomarker at a higher scale. The significance of the scaling exponent a was determined by linear regression on the log transformed data where only significant slopes were considered. For example, the expression of LPO in gonad (y) is examined in terms of lipids levels in the gonad or gonad size (x) using the above power relationship. Significance was set at $p<0.05$ and all tests were performed using the SYSTAT software package (version 13, USA).

Results

The surface waters physico-chemical characteristics and occurrence of some pharmaceuticals were determined in surface waters and mussels at the end of exposure time (Table 1). During the 3 months exposure period, the sites received 290 mm of rainfall and the release valves at the overflow sites were opened for 719 and 860 min which represented 0.7 % of total time. The total suspended solids (TSS) and ammonia were circa 10 times higher at the downstream site compared to the upstream and rainfall overflows sites. The water pH, conductivity and dissolved organic carbon (DOC) were not significantly different. Mussels accumulated significant amounts of total heterotrophic bacteria (includes coliforms) at the downstream site with 80800 counts/mussel compared to 30000 counts/mussels at the upstream

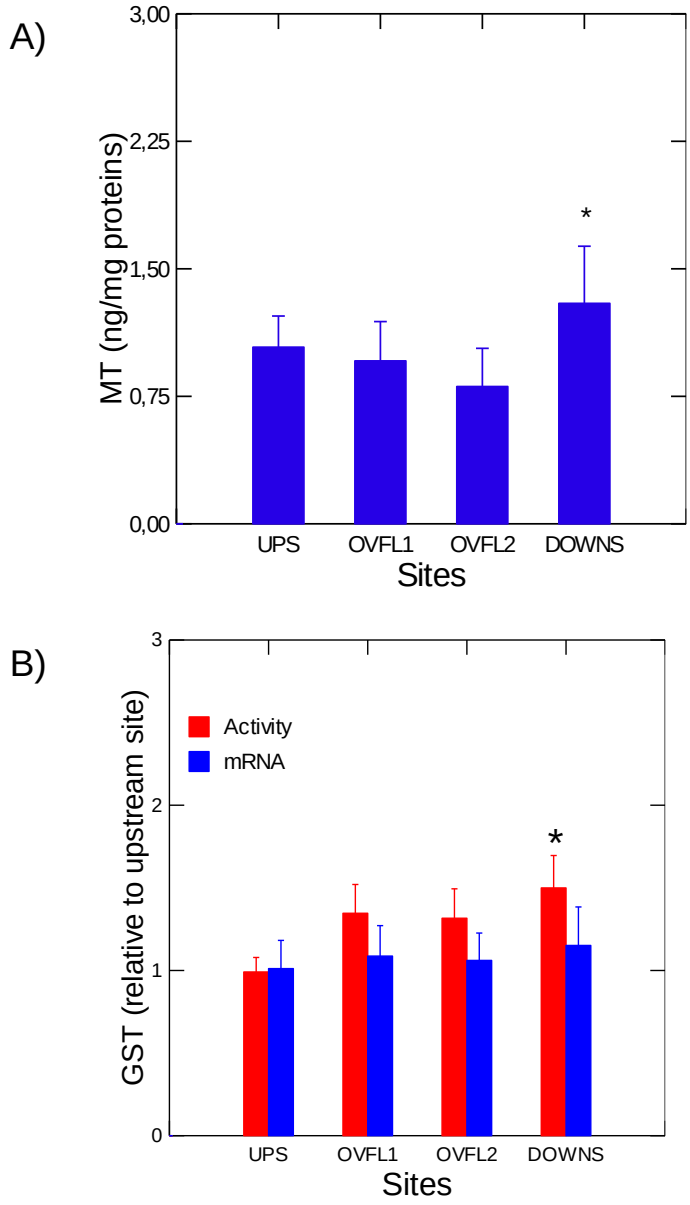


Fig. 4 Metal sequestration and xenobiotic biotransformation activity of mussels exposed to municipal effluent plume and rainfall overflows sites. Xenobiotic biotransformation where determined by following MT levels (A) and GST at the enzyme and mRNA levels (B) in the digestive gland. The star symbol* indicates significance at $p<0.05$

site. Water caffeine levels at the downstream site (0.9 pg/L) were significantly higher than the upstream (0.07 pg/L) and rainfall overflows sites (0.035 pg/L), respectively. However, no significant changes in caffeine contents in mussels were observed. Acebutolol levels in surface waters were circa 10 times higher at the downstream site (0.9 pg/L) compared to the upstream and rainfall overflows sites. Mussels accumulated more acebutolol at the downstream (0.06 ng/g) compared to the upstream (0.01 ng/g) site. Venlafaxine levels were also elevated at the downstream sites reaching 8 pg/L compared to the other sites. The mussels did not significantly accumulate more of the drug at the downstream site compared to the other sites. The levels in ibuprofen and estrone were also significantly increased in surface waters at the downstream sites compared to the upstream site. However, their levels in mussel tissues remained below the method reporting limit.

The condition factor and resistance to air emersion were not significantly affected in cages mussels (Figure 1). However, the weight loss was significantly lower in mussel caged at the downstream site which suggests that mussels were less able to loss weight during the air challenge. Correlation analysis (Table 2) revealed that weight loss was significantly correlated with bacterial

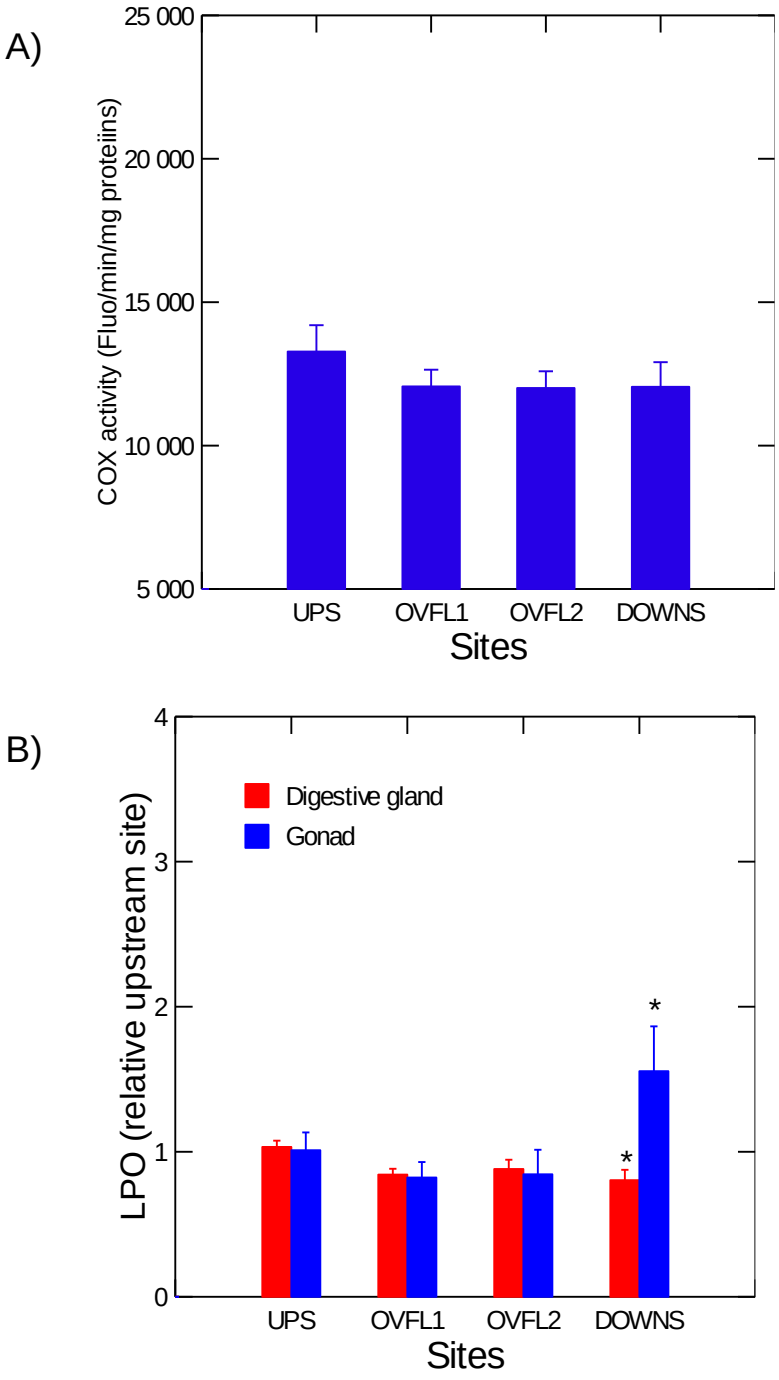


Fig. 5 Oxidative stress and damage in mussels exposed to municipal effluent plume and rainfall overflows sites. COX activity (A) and LPO levels (B) were determined in caged mussels. The data represent the mean with standard deviation. The star symbol * indicates significance from the upstream site

loadings (r = -0.70). Gonad activity in caged mussels was studied by following changes in VTG gene expression, ALP (vitellogenin-like proteitn) and GSI. Mussels caged at the downstream site of a municipal effluent had elevated levels of VTG gene expression in both males and females (Figure 2A). The rainfall overflows had no effects. The levels of ALP revealed that only females had elevated levels at the downstream site and females at OVFL2 had decreased levels compared to females at the upstream site (Figure 2B). Mussels caged at the downstream site had significantly higher levels of GSI compared to the upstream site (Figure 2C). Correlation analysis revealed that VTG gene expression was correlated with condition factor (r = -0.51), weight loss during air survival (r = -0.56)

and bacterial loadings (r = 0.63) indicating that mussels weight/shell length and bacterial loadings tended to be higher at the downstream site were less able to lose weight during air challenge. The DGI was significantly correlated with the CF (r = 0.73). Energy reserves in gonad tissues were also determined in caged mussels (Figure 3). Total proteins and lipids were significantly elevated in the gonad of mussels caged at the downstream site. No change was observed in mussels caged at the rainfall overflow sites. Correlation analysis revealed that gonad proteins were correlated with bacteria levels (r = 0.9), weight loss (r = -0.85), which was correlated with total gonad lipids (r = -0.54). Gonad lipids were significantly correlated with total gonad sugars (r = 0.84), bacteria levels (r = 0.57) and VTG gene expression (r = 0.9). Gonad sugars were correlated with VTG gene expression (r = 0.68).

Xenobiotic biotransformation was determined by following changes in MT levels and GST enzyme activity and mRNA levels. MT levels in the digestive gland were significantly increased at the downstream site compared to the upstream site but not in mussels exposed to the rainfall overflow sites (Figure 4A). Correlation analysis revealed that MT levels were significantly correlated with gonad proteins (r = -0.57) and bacteria levels (r = -0.58). The activity of GST in the digestive gland was also significantly increased at the downstream site only compared to upstream site (Figure 4B). GST mRNA levels were not affected in mussels. Correlation analysis revealed that MT levels were correlated with total proteins in the gonad (r = -0.57) and bacterial loadings (r = -0.58). GST enzyme activity was correlated with total proteins in the digestive gland (r = 0.68).

Oxidative stress was determined by following changes in COX activity and LPO in tissues of caged mussels. COX activity was not significantly influenced by the sites in respect to the upstream site (Figure 5A). The LPO levels were significantly increased and decreased at the downstream site in the gonad and digestive gland respectively (Figure 5B). No changes were observed in mussels caged at the rainfall overflow sites. Correlation analysis revealed that gonad LPO was significantly correlated with bacteria levels (r = 0.70) and gonad protein levels (r = 0.65). DNA strand breaks were also determined in tissues. DNA strand breaks were reduced in the digestive gland in mussels caged at one overflow site and reduced in the gonad of mussels caged at the downstream site (Figure 6). A decreased in DNA strands indicates that DNA repair activity and turnover are reduced. Correlation analysis revealed that DNA strand breaks in the digestive gland were significantly correlated with LPO in the digestive gland (r = 0.65), gonad proteins (r = -0.68), bacteria levels (r = -0.54) and weight loss during air emersion (r = 0.64). DNA breaks in gonad were significantly correlated with gonad proteins (r = -0.7), MT in the digestive gland (r = 0.74) and VTG gene expression (r = -0.52).

In the attempt to gain a global understanding on the various responses in mussels caged at a downstream and rainfall overflow sites, a multivariate and effects scaling analyses were performed to determine the major biomarkers than

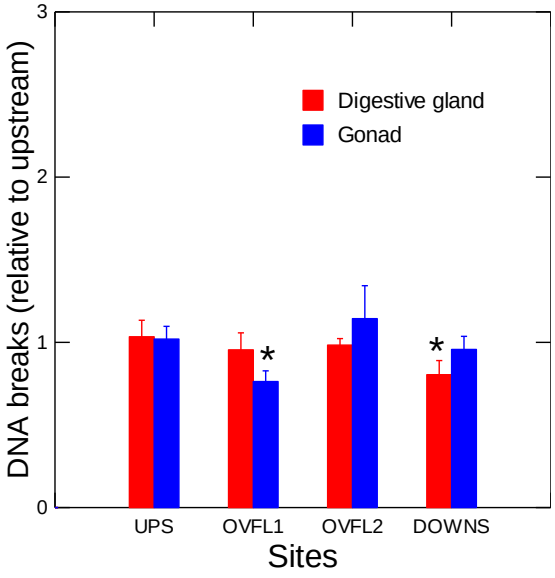


Fig. 6 DNA damage in mussels exposed to rainfall overflows and a municipal effluent plume. DNA strand breaks were determined in the digestive gland and gonad tissues in mussels. The data represent the mean with standard deviation. The star symbol * indicates significance from the upstream site

can discriminate between downstream and rainfall overflow sites and adverse outcome pathways, respectively. Principal components revealed that 60% of the variance was explained by 3 factors (Figure 7 A). The most important biomarkers (biomarkers with the highest factorial weights) associated to these 3 factors were gonad lipids, DNA strands in digestive gland and gonads, VTG gene expression, MT, gonad LPO, gonad proteins, GSI and CF. Discriminant function analysis was used to determine if the downstream, rainfall overflows and upstream sites have distinct toxicity profiles and which effects were involved for site discrimination (Figure 7B). The analysis revealed that the downstream, overflows and upstream sites were completely distinct (100 % classification efficiency) from each other. The biomarkers involved for site discrimination were GST activity, air survival time, LPO and DNA damage in the digestive gland, protein gonads and GSI. In the attempt to determine whether effects observed at the molecular level are manifest at higher levels of complexity (adverse outcome pathways), the biomarker data were analyzed using the power law paradigm as described in Methods (Table 3). The analysis revealed that some biochemical responses were significantly scaled to effects at higher levels of biological organization. MT, gonad LPO, GST activity, APA in both tissues, VTG gene expression and ALP levels were significantly scaled to energy reserves in the gonad (proteins, sugars and lipids).

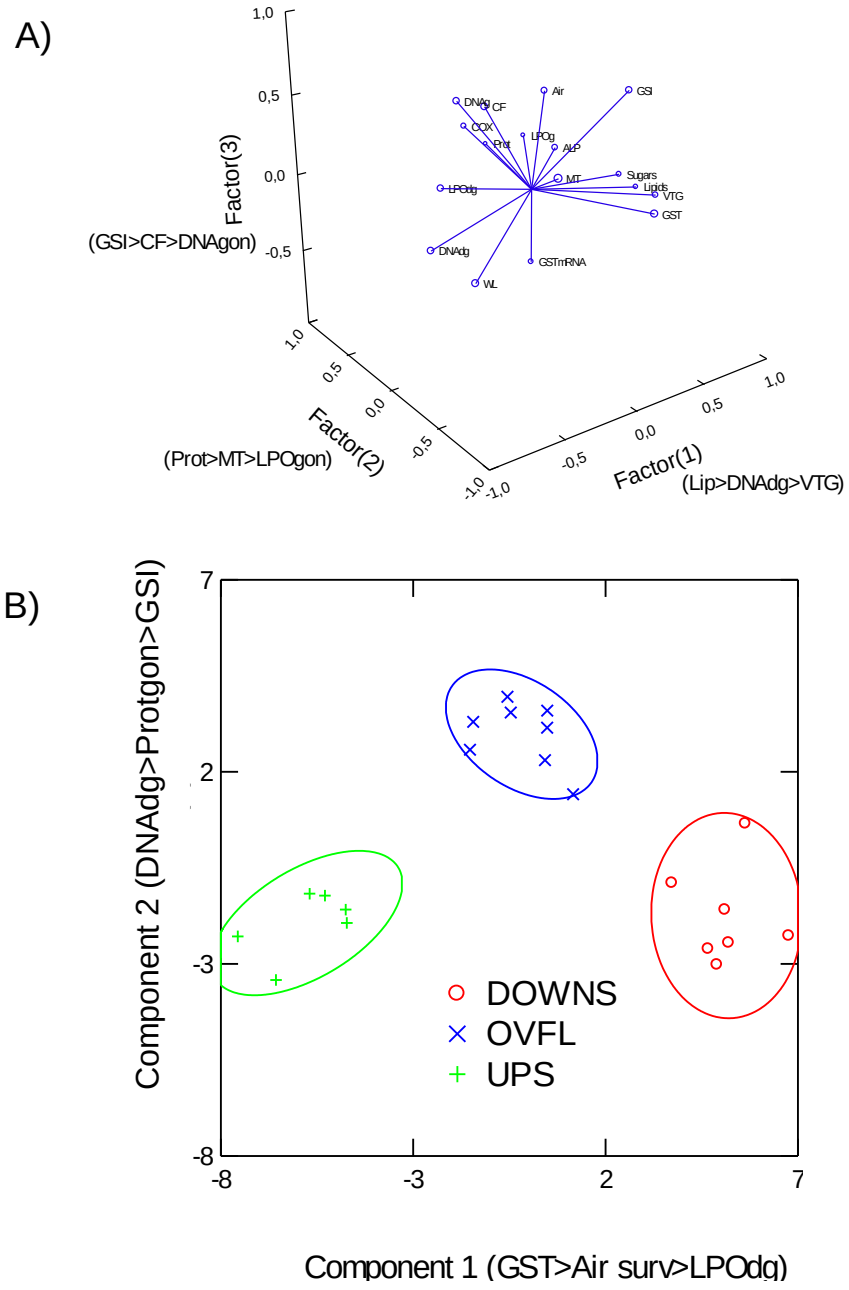
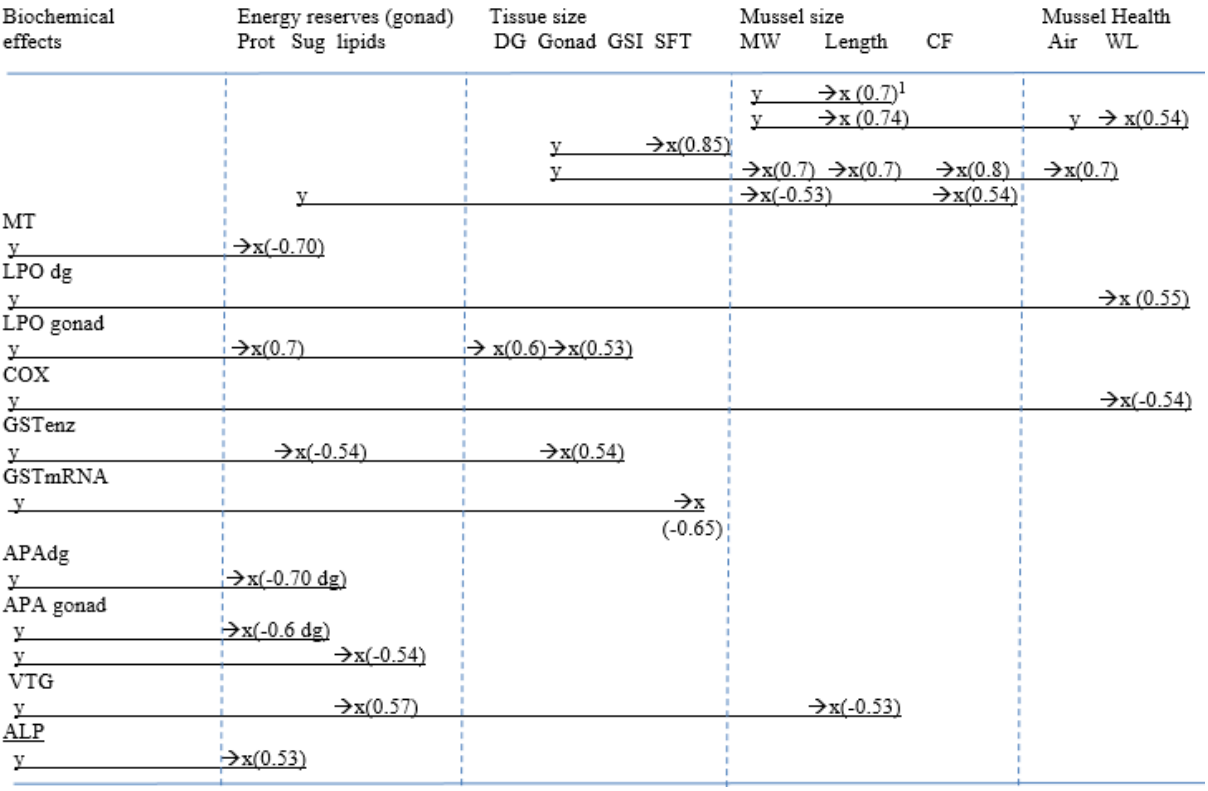


Fig. 7 Multivariate analysis of biomarker data. Biomarker responses were analyzed using factorial (A) and discriminant function (B) analyses. Digestive gland LPO (LPOdg), air survival (Air surv), Proteins in gonads (Protgon)

A subgroup of these biomarkers was also scaled at higher levels: digestive gland LPO (to weight loss), gonad LPO (to digestive gland and gonad size), GST activity (to gonad size), GST mRNA (to soft tissues mass) and VTG gene expression (to shell length). COX activity was also associated to weight loss during air stress. The scaling analysis also revealed that changes occurring at the energy reserves levels were significantly scaled to changes at higher biological level as well. Indeed, gonad sugars levels were associated to mussel size and condition factor. Gonad size was significantly scaled to mussel weight, shell length and condition factor and to soft tissues weight/mussel weight ratio. Based on this analysis, an adverse outcome pathway is proposed as follows. The effects observed at the biochemical levels resonate at energy stores especially at total proteins and lipids levels, which in turn is associated to gonad weight level and it is the changes in gonad size that is associated to mussel size, condition factor and general health indicators (air survival and weight loss).

Table 3 Scaling of effects analysis for adverse outcome pathways identification



1. The annotation y → x (h scaling exponent) is modeled using the power law scaling of effects: $y = kx^h$ where y is an effect at a lower level of complexity and x the effect observed at higher level of complexity (usually body mass, organ size or health index). The scaling exponent h is calculated the log transformation where log y = h log x + log k (a constant). Only significant slopes were used

Discussion

The effective protection of water bodies in urban areas requires knowledge about the relative contamination loads and toxic effects of municipal wastewaters discharges and rainfall overflows. For example, a predictive model was developed to predict changes in chemical oxygen demand and total suspended solids based on the water depth and maximum intensity of rainfall (Brzesinska *et al.*, 2018). In the present study, the surface waters at the overflow sites contained significantly higher levels of caffeine, estrone, atrazine and its metabolite desethylatrazine relative to upstream waters, which suggests that these sites were partially contaminated by wastewaters. These levels were somewhat lower than the surface waters at the downstream site the municipal effluent plume (8 km) with the exception of atrazine and its metabolite, which were highest at the overflow sites. The levels of pesticides represented an important component of hazardous substances from urban wet weather discharges (Gosset *et al.*, 2017). It appears that stormwater is an important input of pesticides and pharmaceutical products in aquatic ecosystems downstream urban area. This suggests that the combined sewer overflows are not simply a diluted version of municipal effluent by stormwater. Based on the effects measured in this study, only decreased DNA strand breaks were significantly influenced in one of the overflow sites compared to the upstream site which suggests that long-term exposure to an episodic release of combined sewer during the summer months do not produce toxic effects as observed in mussels downstream the municipal plume. This was shown by discriminant function analysis which showed that the responses pattern of the overflow sites where closer to the upstream site compared to the downstream site (Figure 7B). It will be interesting to study this further under different rainfall volume scenario over time. However, other effects were also involved to discriminate the overflow sites from the upstream and downstream sites: DNA strand breaks in digestive gland, gonad proteins and GSI. Decreased DNA strand breaks (repair activity) was also observed in quagga mussels exposed to combined sewer discharges during a major release of untreated wastewaters (Gagné *et al.*, 2017). Chronic toxicity testing of combined sewer overflows following rainfall events revealed some effects towards fish *Pimephales promelas* and daphnids

Cerodaphnia dubia (Gooré *et al*., 2015). Exposure to the combined sewer overflows for 6 and 7 days resulted in decreased survival and reproduction in daphnids at 31% v/v of the effluent and growth and survival in fish at 41% v/v effluent volume. Although a continuous exposure a 6-7 days exposure to the combined sewer overflows is unlikely especially in dynamic river systems i.e., intense rainfalls rarely occurs continuously for long periods of time and the released effluents will be diluted by the river's currents which is also increased during rain. This approach could be of value from the perspective that toxic combined sewer overflows should not be released directly in the receiving water to begin with based on the precautionary principle. Combined sewer overflows are environmental source of hormones and other micropollutants (Phillips *et al*., 2012). The study revealed that concentrations of estrogens (estradiol-17β), androgens (testosterone) and other contaminants such as caffeine and coprostanol could reach 10 times the concentrations found in effluent after treatment. However, this discharge is restricted in time compared to the continuous release of effluents but contribute to the global contamination picture of the urban space. In the present study, the overflows valves were open for only 860 min over the 3-month exposure period representing about 0.6 % of the total time. Estrone was detected at similar concentrations in surface waters at 0.45 and 0.56 ng/L in one of the overflow site and downstream site but vitellogenin gene expression and ALP levels (a measure of estrogenicity) were significantly increased at the downstream site only. At the overflow site, mussels were exposed to estrone only 0.6% of the times while at the downstream site, exposure to the effluents was continuous (all the times) albeit the levels could have change during the exposure period. This was similar with heterotrophic bacteria in mussel's tissues, which was 3.3 times higher in tissues in mussels caged at the downstream site than in tissues of mussels from the upstream site albeit both combined sewers overflow and this primary treated municipal effluents contains bacteria (Gibson *et al*., 2017). It was noteworthy that total bacteria count in mussels were strongly correlated with proteins in gonads and VTG gene expression (Table 2) suggesting that other factors than estrogenicity was at play in caged mussels. VTG is usually expressed following the activation of VTG receptors by estrogens but recent studies revealed that VTG could be involved in the immune response against foreign bacteria (Bouchard *et al*., 2009; Li *et al*., 2019). VTG was shown to possess bacteriostatic properties, which could assist the phagocytes to remove bacteria in the hemolymph and tissues.

Adverse outcomes analysis based on power law scaling revealed that biochemical changes was involved at changes at higher scales (levels of biological organization) from energy reserves in gonads up to mussel condition, size and health status. This is consistent to the observation that local mussel populations are scarce downstream municipal effluent in this study based on field observations. This was also shown in previous study in another river system in Canada (Gillis *et al*., 2017). Mussels surveys in the Grand River (Ontario, Canada) revealed that seven species of mussels were found at sites upstream of municipal effluent discharges while no live mussels were found for 7 km downstream a municipal effluent discharges. This suggests that the continuous release of municipal effluents could produce harmful effects to mussels, which threaten maintenance of local populations. This is consistent with a previous study with *E. complanata* mussels where mussels collected downstream to cities in the Mille-Isles River (Québec, Canada) were feminized at 85 % with males showing increased vitellogenin-like proteins (Gagné *et al*., 2011). Clams and mussels were caged upstream and downstream a tertiary treated municipal effluent for 72 days in a small stream to find changes in survival, growth and condition (Nobles and Zhang, 2015). Survival and growth to the non-native Asian clam and growth and condition of three ridge mussel species were significantly lower in caged animals at downstream site of the wastewater treatment plant compared to mussels caged upstream. They also found an absence of native mussels at downstream site further supporting that municipal effluents have negative impacts to mussel abundance and diversity. It was found that *Corbicula fluminea* clams accumulate rapidly pharmaceuticals at downstream sites of wastewater treatment plants (Burk *et al*., 2019). Indeed, a 42 days *in situ* exposure with caged clams, the following compounds were detected in tissues: acetaminophen, caffeine, carbamazepine, dilitiazem, diphenhydrame, fluoxetine, norfluoxetine, sertraline, desmethylsertraline and methylphenidate. Sertraline had the highest values at 341 ng/g tissues. This drug is a selective serotonin reuptake inhibitor which could stimulate spawning in mussels (Ram *et al*., 1993). In addition to serotonin, exposure of gonad tissues to a filtered (0.2 um membrane) municipal effluent from the same city in the present study also stimulated spawning in *E. complanata* mussels showing the serotonergic effects of this municipal effluent (Gagné *et al*., 2004). This effect was measured at a threshold concentration of 3 % corresponding to a distance of 4-6 km downstream the plume which is close to the 8 km downstream site in this study. Moreover, exposure for 45 days of mussels exposed to a static-renewal test to the same municipal effluent in this study lead to increased serotonin and dopamine in mussel gonad tissues with an increase in monoamine oxidase activity, an enzyme involved in the elimination of serotonin and dopamine. The increase levels in serotonergic drugs such as sertraline, venlafaxine and perhaps other (fluoxetine, paroxetine) is consistent with the reported serotonergic properties of the primary-treated municipal effluent. The increase levels in MT and GST in the digestive gland suggests that perhaps other compounds than pharmaceuticals (metals and polyaromatic hydrocarbons) were at play. The increased levels in MT and GST activity were also observed in *E. complanata* exposed to the same municipal effluent for 7 days in the laboratory (Gagné *et al*., 2015). MT and GST could also be involved in the removal of reactive oxygen species leading to oxidative stress

and damage (LPO) (Giannetto *et al*., 2017). An analysis of covariance was performed with MT or GST levels with site location and LPO as the grouping variable and covariate respectively and revealed that MT was mostly explained by oxidative stress not by sites. LPO levels had no significant effects on GST activity in the digestive gland. This suggests that MT was also involved in oxidative stress in mussels exposed to a municipal effluent as suggested previously (Gagné *et al*., 2007) and GST was more associated to xenobiotic conjugation than oxidative stress.

In conclusion, the study revealed that mussels caged 8 km downstream site of municipal effluent plume led to increased accumulation of heterotrophic bacteria, acetobutolol and somewhat with venlafaxine in tissues and produced a number of effects at the biochemical level such as endocrine disruption (vitellogenin), oxidative stress (LPO and MT), xenobiotic biotransformation (GST) and DNA damage. These effects were also scaled at higher levels of biological organization such as gonad energy reserves, tissue and mussel size and health status as determined by resistance to air and weight loss before death corroborating the scarcity of local mussel populations downstream municipal effluents. Based on these responses, the toxicity of downstream site produced generally stronger effects than the overflows sites in mussels exposed for 3 months, which received 249 mm of rain but also showed some difference from the upstream and downstream sites based on DNA damage in the digestive gland, gonad protein and GSI. Further studies with different rainfall scenario will be needed to confirm these observations.

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