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A multi-level biological approach to evaluate impacts of a major municipal effluent in wild St. Lawrence River yellow perch (*Perca flavescens*)

Magali Houde [a,*], Maeva Giraudo [a], Mélanie Douville [a], Bérénice Bougas [b,c], Patrice Couture [c], Amila O. De Silva [d], Christine Spencer [d], Stéphane Lair [e], Jonathan Verreault [f], Louis Bernatchez [b], Christian Gagnon [a]

[a] Centre Saint-Laurent, Environment Canada, 105 McGill Street, Montreal, QC H2Y 2E7, Canada
[b] Institut de biologie intégrative et des systèmes, Université Laval, 1030, avenue de la Médecine, Québec, QC G1V 0A6, Canada
[c] Institut National de la Recherche Scientifique, Centre Eau Terre Environnement, 490 de la Couronne, Québec, QC G1K 9A9, Canada
[d] Canada Centre for Inland Waters, Environment Canada, 867 Lakeshore Road, P.O. Box 5050, Burlington, ON L7R 4A6, Canada
[e] Centre québécois sur la santé des animaux sauvages, Université de Montréal, C.P. 5000, St-Hyacinthe, QC J2S 7C6, Canada
[f] Centre de recherche en toxicologie de l'environnement (TOXEN), Département des sciences biologiques, Université du Québec à Montréal, C.P. 8888, Succursale Centre-ville, Montreal, QC H3C 3P8, Canada

HIGHLIGHTS

- A multi-level biological approach was used to evaluate impacts of an urban effluent on yellow perch.
- Genes related to immunity, detoxification and retinol metabolisms were impacted.
- Fish exposure to the effluent resulted in increased activity of antioxidant enzymes.
- Lipid metabolism, biosynthesis and aerobic capacities were lower in exposed perch.
- Biological responses correlated to contaminant levels in whole fish homogenates.

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ABSTRACT

The development of integrated ecotoxicological approaches is of great interest in the investigation of global concerns such as impacts of municipal wastewater effluents on aquatic ecosystems. The objective of this study was to investigate the effects of a major wastewater municipal effluent on fish using a multi-level biological approach, from gene transcription and enzyme activities to histological changes. Yellow perch (*Perca flavescens*) were selected based on their wide distribution, their commercial and recreational importance, and the availability of a customized microarray. Yellow perch were sampled upstream of a major municipal wastewater treatment plant (WWTP) and 4 km and 10 km downstream from its point of discharge in the St. Lawrence River (Quebec, Canada). Concentrations of perfluoroalkyl substances (PFASs), polybrominated diphenyl ethers (PBDEs) and metals/trace elements in whole body homogenates were comparable to those from other industrialized regions of the world. Genomic results indicated that the transcription level of 177 genes was significantly different ($p < 0.024$) between exposed and non-exposed fish. Among these genes, 38 were found to be differentially transcribed at both downstream sites. Impacted genes were associated with biological processes and molecular functions such as immunity, detoxification, lipid metabolism/energy homeostasis (e.g., peroxisome proliferation), and retinol metabolism suggesting impact of WWTP on these systems. Moreover, antioxidant enzyme activities were more elevated in perch collected at the 4 km site. Biomarkers of lipid metabolism, biosynthetic activity, and aerobic capacities were significantly lower ($p < 0.05$) in fish residing near the outfall of the effluent. Histological examination of the liver indicated no differences between sites. Correlations between PFAS, PBDE, and metal/trace element tissue concentrations and markers of peroxisomal proliferation, oxidative stress, and retinoid metabolism were found at the gene and cellular levels. Present results suggest that relating transcriptomic analyses to phenotypic responses is important to better understand impacts of environmental contamination on wild fish populations.

* Corresponding author. Tel.: +1 514 496 6774; fax: +1 514 496 7398.
E-mail addresses: magali.houde@ec.gc.ca (M. Houde), maeva.giraudo@ec.gc.ca (M. Giraudo), melanie.douville@ec.gc.ca (M. Douville), berenice.bougas.1@ulaval.ca (B. Bougas), patrice.couture@ete.inrs.ca (P. Couture), amila.desilva@ec.gc.ca (A.O. De Silva), christine.spencer@ec.gc.ca (C. Spencer), stephane.lair@umontreal.ca (S. Lair), verreault.jonathan@uqam.ca (J. Verreault), louis.bernatchez@bio.ulaval.ca (L. Bernatchez), christian.gagnon@ec.gc.ca (C. Gagnon).

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1. Introduction

Municipal wastewater effluents (MWWEs) are complex mixtures of anthropogenic contaminants and microorganisms representing important continuous sources of pollutants to aquatic environments (Chambers et al., 1997). Exposure to MWWEs has been linked to adverse effects in fish including intersex and reproductive impairment (Tetreault et al., 2011, 2012), morphology alterations (Diniz et al., 2005; Tetreault et al., 2012), and impacts on immunological functions (Ménard et al., 2010; Gagné et al., 2013).

The largest primary wastewater treatment plant (WWTP) in North America is located in Montreal (Quebec, Canada). Montreal's MWWE is released in the St. Lawrence River, a major waterway that connects the Great Lakes and tributaries with the Atlantic Ocean. This MWWE has been reported as a source of metal (Gagnon and Saulnier, 2003), pharmaceutical (Lajeunesse et al., 2011) and personal care product (Sabik et al., 2003) contamination for the receiving St. Lawrence River ecosystem. Additionally, perfluoroalkyl substances (PFASs) were reported in northern pike (*Esox lucius*) collected in this urban area of the St. Lawrence River (Houde et al., 2013) as well as polybrominated diphenyl ethers (PBDEs), and other emerging halogenated flame retardants, found at high detection frequency and elevated concentrations in suspended sediment (Pelletier and Rondeau, 2013), predatory fish (Houde et al., 2014), and birds (Champoux et al., 2010; Chen et al., 2012a; Gentes et al., 2012).

In the field, impairment of reproductive function was reported in male spottail shiners collected downstream to Montreal's WWTP (Aravindakshan et al., 2004). Experimental exposure of aquatic organisms to this MWWE has led to alterations of immune function in rainbow trout (Salo et al., 2007; Hébert et al., 2008) and pond snail (Gust et al., 2013) as well as neuroendrocrine alterations and oxidative stress in freshwater mussels (Gagné et al., 2011). Overall, these results indicate that the complex urban mixtures of the Montreal's effluent impact the organisms living in its receiving environment.

The yellow perch (*Perca flavescens*) is indigenous to the St. Lawrence River and a species of great commercial and recreational importance. This species has a wide distribution and its congregation near shore in the spring facilitates its capture. A five-year moratorium on all yellow perch fishing has been established in 2012 following a decline in yellow perch population in Lake St-Pierre, located about 120 km downstream Montreal. Habitat destruction, chemical pollution, competition with the invasive round goby, increase in the predatory double-crest cormorant population, low water levels, and increase in water temperature are all hypotheses that have emerged to explain the decline of yellow perch population in this fluvial lake.

In order to address concern of chemical pollution to aquatic organisms, it is important to evaluate their biological effects on fish. The use of ecotoxicogenomic approaches and microarrays has increased in recent years in non-model aquatic species (Garcia-Reyero et al., 2008b; Vidal-Dorsch et al., 2012). These qualitative genomic analyses can be used to generate hypotheses to test specific mechanisms of toxicity and to direct future investigations. However, the linkage between genomic alterations and biochemical and/or physiological levels is often lacking although necessary in the establishment of the biological relevance of contaminant-induced modifications in gene transcription levels.

Based on this information, and considering the precarious status of yellow perch population in sectors of the St. Lawrence River located downstream of Montreal, an innovative multi-biological level approach (i.e., gene transcription, enzyme activities, and histology) was developed to evaluate the impacts of perch environmental exposure to a major municipal effluent. Relationships between fish biological responses and tissue concentrations of contaminants of concern (i.e., perfluoroalkyl substances, PBDEs, and metals) were also evaluated.

2. Material and methods

2.1. Study site and fish collection

Yellow perch sampling was conducted in June 2012 following reproduction to minimize hormonal variations. Perch, with length ranging from 150 to 210 mm, were collected at three sites in the St. Lawrence River in the vicinity of Montréal, Québec, Canada: Boucherville islands (n = 20), located upstream of Montreal's WWTP, Ilet Vert (n = 20), located 4 km downstream the treatment plant, and the Beauregard island (n = 20) situated at 10 km downstream of the outfall (Fig. 1). Physical barriers (e.g., the waterway) and the sedentary behavior of yellow perch (Aalto and Newsome, 1990) minimized the probabilities of fish to visit more than one site. All fish were captured using a beach seine. Yellow perch were euthanized in a 250 mg/L solution of clove oil, measured (total length) and weighed. The liver was preserved in RNA*later*® and kept at −80 °C until RNA extraction. Additional samples of the liver, muscle, and gonad tissue were preserved at −80 °C or in formaldehyde (10% buffered) for subsequent enzyme analyses, histological examination, and sex determination. Whole fish were put on ice and frozen (−20 °C) upon returning to the laboratory. Whole carcasses were homogenized for chemical analyses. Both opercula were removed from the specimens for age determination by counting the number of growth annuli. The Fulton's condition index (K) was used as an indicator of the general body condition of the fish and calculated as $K = ((\text{weight (g)}) / (\text{total length (cm}^3\text{)})) \times 1000$. Sampling protocols were approved by Environment Canada's Animal Care Committee working under the Canadian Council on Animal Care. Additionally, resources were available to collect and analyze PFSA in surface water collected at the same sites.

2.2. Chemical analyses

Eleven perfluoroalkyl substances (PFASs) were analyzed in perch whole body homogenate (n = 20/site) and thirteen in surface water (n = 3/site) using methods as described by De Silva et al. (2011). All contaminants and their abbreviations are listed in the caption of Fig. 2. PFASs were not detected in blanks and therefore concentrations were not blank corrected. The method detection limits (MDL) were calculated using mean method blank response + 3× standard deviations. All MDLs can be found in the Supplementary information. For compounds which were not detected in blanks, a value of half the LC/MS/MS limit of detection was substituted.

Thirty-eight polybrominated diphenyl ethers (PBDEs) and fifteen non-PBDE flame retardants were analyzed in yellow perch whole body homogenates (n = 6/site) and methods and results were previously published (Houde et al., 2014). Quality control and assurance procedures included method blanks and standard reference material SRM 1947, Lake Michigan fish tissue. Blank correction was performed and concentrations were expressed on a lipid wet basis (ng/g l.w.). Method detection limits (signal to noise ratio = 3) were based on replicate analyses (n = 8) of matrix samples and were of 0.01 ng/g wet weight for all PBDE congeners reported in this article.

Twenty-two metals were analyzed in yellow perch (n = 20/site). Fish homogenates were freeze-dried and grounded with dry ice to homogenize samples. Samples were then acid-digested using microwave heating Teflon bombs or vessels after adding an analytical grade mixture of 8 mL nitric acid, 1 mL hydrochloric acid and 2 mL hydrogen peroxide (Seastar™ Baseline®). The samples were completely digested and diluted to 0.5 N with bi-distilled water. Metals were determined by ICP-MS (Thermal Ash, USA) and standard solutions of these elements were used for calibration. The data were expressed as μg/g dry weight and reproducibility was better than 5%. The recovery for these metals was between 90% and 105%. All MDLs can be found in Table S5.

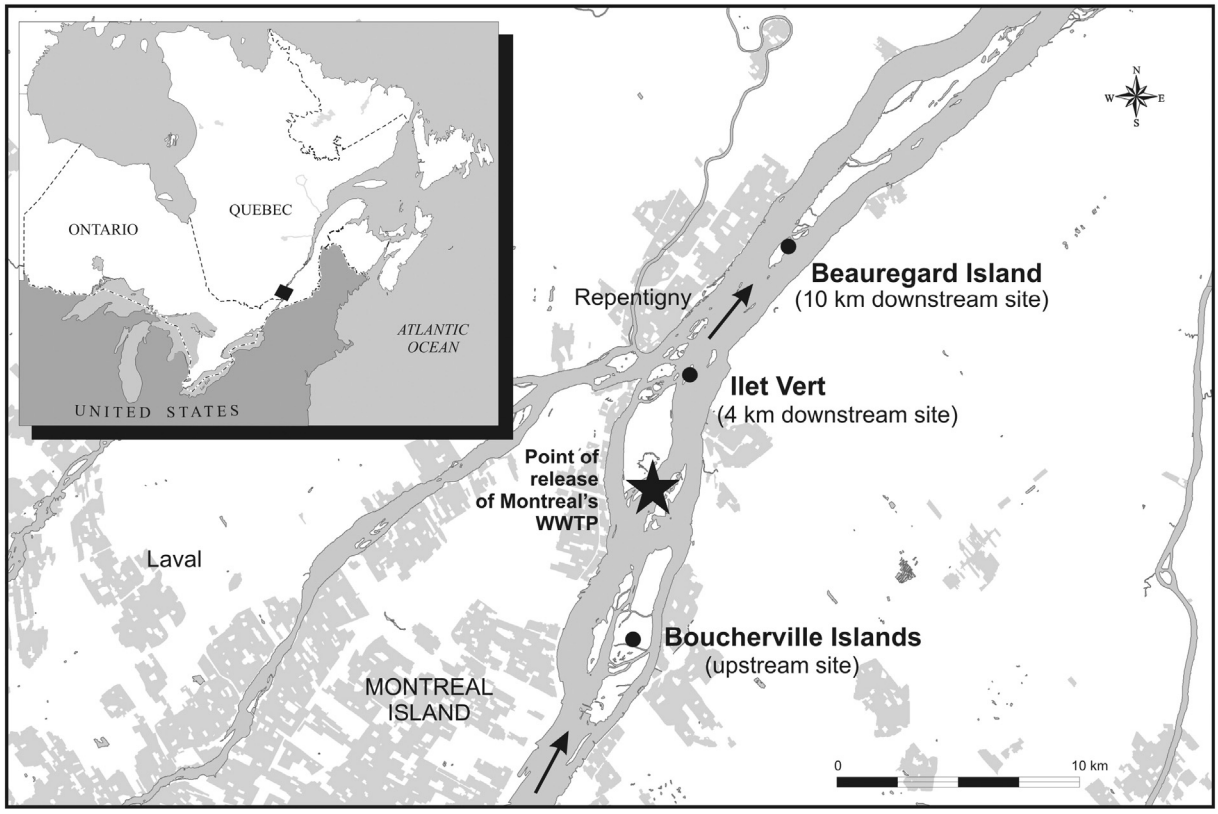

Fig. 1. Sampling sites in the St. Lawrence River, Quebec, Canada (June 2012).

2.3. Microarray

2.3.1. RNA extraction, labeling, and cDNA hybridization

Total RNA from the livers of 24 fish was extracted (n = 8 for each of the three sampling stations) with the PureLink™ Micro-to-Midi Total RNA Purification System Kit and treated using Amplification Grade DNase I (3 unit/μL; Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Total RNA was stored in RNAse free water at −80 °C. Quality and integrity of the total RNA were controlled using an Experion Automated Electrophoresis Station and RNA HighSens Chips (Biorad, Hercules, CA). For each sample, 10 μg total RNA was retro-transcribed and the cDNA samples labeled using Genisphere 3DNA Array 50 Kit, Invitrogen's Superscript II retro-transcriptase and *Cyanine3* and *Cyanine5* fluorescent dyes (Genisphere), following the procedures described at http://genisphere.com/products/3dna-array-detection/support (Genisphere Array 50 Protocol). A loop design included pair-wise direct comparisons among samples of the different sampling stations, for a total of 24 microarrays. Each sample was technically replicated on two bi-colored microarrays and dye-swapped. Information of the 1000-candidate gene yellow perch microarray have been previously published by Bougas et al. (2013) (GEO accession number: GPL17732).

2.3.2. Microarray data acquisition, preparation, and statistical analysis

Microarrays were scanned using a ScanArray scanner (PerkinElmer Life Sciences). Spots were localized and quantified with the QuantArray 3.0 software (PerkinElmer Life Sciences), using the histogram quantification method and using the mean value of intensity for each spot. Data from poor replicate spots were excluded from the data set and local background was subtracted from the signal. All of the 1000 genes were used in the analysis. The data were normalized according to the regional lowess procedure, as implemented in the R/MAANOVA package (Kerr et al., 2000) to remove signal intensity-dependent and region-dependent dye effects on each slide. In order to detect differences in transcription profiles among the conditions, data were analyzed using a mixed model ANOVA (Wolfinger et al., 2001) and the R/MAANOVA package (Kerr et al., 2000, 2002). The presence of significant effects for the different sampling stations (upstream, 4 km, and 10 km) was tested using a mixed ANOVA where the array term was included as random while "dye" and "sampling station" were fixed terms. A permutation-based F-test (Fs, with 1000 sample ID permutations) was then performed and restricted maximum likelihood was used to solve the mixed model equations (Cui et al., 2005). The effect of station was tested with the ANOVA model and used the p-values to determine the significance of differential expression. A False Discovery Rate correction (FDR = 0.1) was applied within the R/MAANOVA package to account for multiple tests. *A posteriori* tests were conducted with 1000 permutations for the three possible comparisons to generate a list of genes with significant differential expression among the different sampling stations (FDR = 0.1).

2.3.3. Functional annotation and pathway analysis

Although the 1000 genes on the array were chosen for their specific function, an additional translated BLAST search (blastx) using NCBI nr database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was conducted on each of the differentially expressed genes in order to retrieve the latest up-to-date annotations. Yellow perch genes were also mapped to their human SwissProt orthologs (e-value $< 1e^{-10}$) for additional functional and pathway annotation using KEGG and GO databases in DAVID (http://david.abcc.ncifcrf.gov/home.jsp) (Dennis et al., 2003). Mapping fish genes to mammalian orthologs enables using the well-annotated databases for mammalian model organisms (Garcia-Reyero et al., 2008a,; Wang et al., 2010), despite limitations of the mapping due to the extra genome duplication events in teleost fish and species-specific differences in gene functions and pathways (Yadetie et al.,

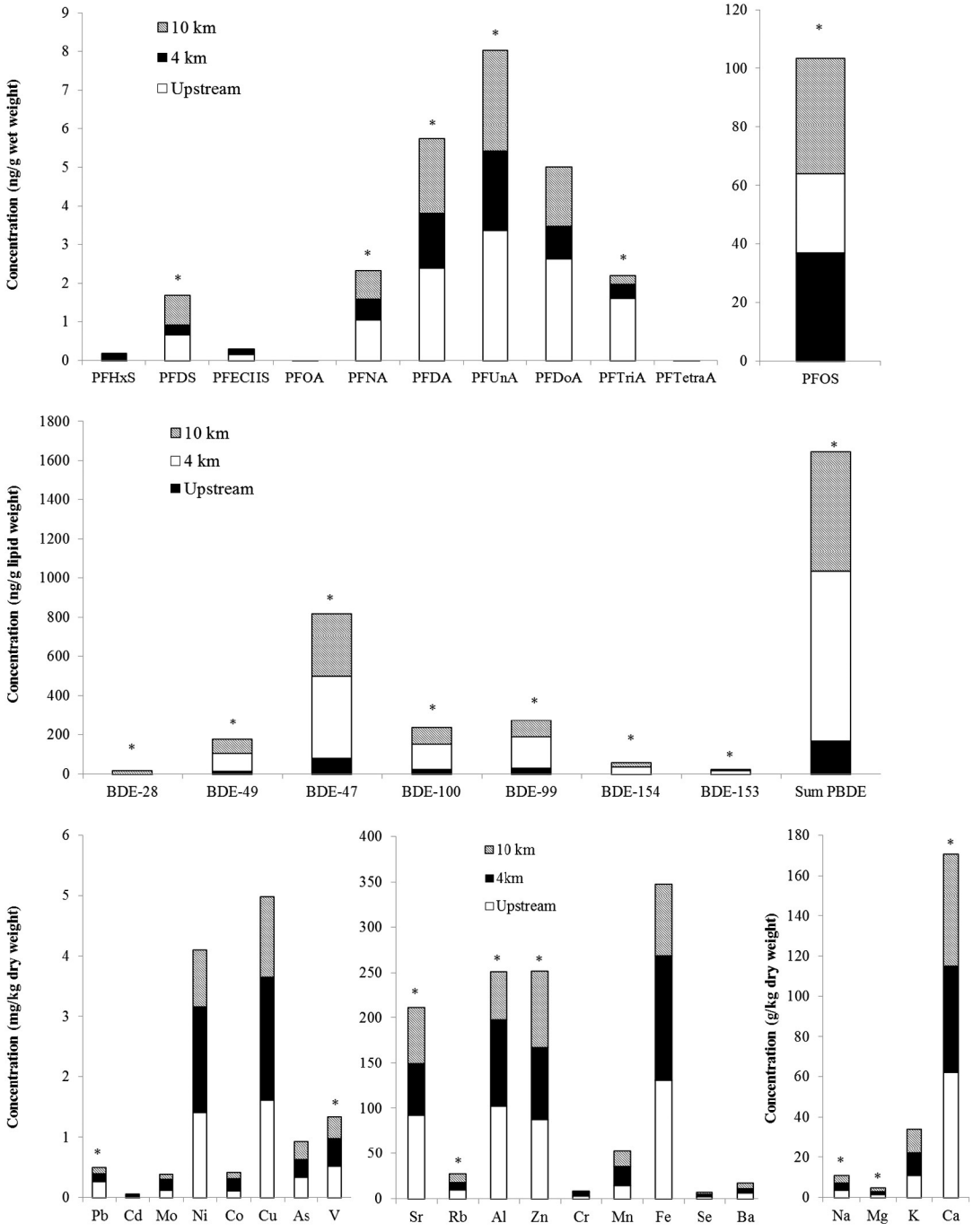

Fig. 2. Concentrations of perfluoroalkyl substances (ng/g w.w.) (top), polybrominated diphenyl ethers (ng/g l.w.) (middle), and metals (mg/kg and g/kg d.w.) (bottom) were detected in whole yellow perch homogenates (n = 20/site) from three St. Lawrence River sites: upstream and 4 km and 10 km downstream a major primary WWTP. * indicates that concentrations were significantly different among sites (Kruskal–Wallis, $p < 0.05$). Information on standard errors can be found in Table S1. Acronyms: PFHxS: perfluorohexane sulfonate; PFOS: perfluorooctane sulfonate; PFDS: perfluorodecane sulfonate; PFECHS: perfluoroethylcyclohexane sulfonate; PFOA: perfluorooctanoate; PFNA: perfluorononanoate; PFDA: perfluorodecanoate; PFUnA: perfluoroundecanoate; PFDoA: perfluorododecanoate; PFTriA: perfluorotridecanoate; PFTetraA: perfluorotetradecanoate; BDE: brominated biphenyl ether; Pb: lead; Cd: cadmium; Mo: molybdenum; Ni: nickel; Co: chromium; cobalt; Cu: copper; As: arsenic; V: vanadium; Sr: strontium; Rb: rubidium; Al: aluminum; Zn: zinc; Cr: chromium; Mn: manganese; Fe: iron; Se: selenium; Ba: barium; Na: sodium; Mg: magnesium; K: potassium; Ca: calcium.

2013). All genes were additionally submitted to a thorough bibliographic search for functional classification.

2.4. Quantitative PCR (qPCR)

2.4.1. RNA extraction and reverse transcription

RNA was extracted from the liver (10–20 mg of tissue) by adding 50 μL of buffer RLT Plus (QIAGEN, Toronto, ON, Canada) and homogenizing on ice (with a sterile RNase/DNase free plastic pestle) with addition of 550 μL of buffer RLT Plus after homogenization. Total volume was put on a QIAshredder spin column (QIAGEN), centrifuged at 16,000 ×*g* for 2 min at room temperature. Total RNA of the lysates was then extracted with the RNeasy® plus mini kit (QIAGEN) following manufacturer's instructions. RNA concentration and purity were estimated with the NanoDrop 1000 (Thermo Fisher Scientific, ON, Canada). All samples had a A_{260}/A_{280} ratio of 1.9–2.1. RNA integrity was verified with the

Table 1
Genes, symbols, and primers used for qPCR analyses in the liver of yellow perch.

Gene name	Symbol		Primers	Efficiency %	Amplicon size (bp)
Target genes					
G-type lysozyme	*LYS*	Forward	GTGGCTCGTCAGAAAGGAC	104	101
		Reverse	TCAAAGCACCCCTTGCCT		
β-Microglobulin	*B2M*	Forward	TCAAGCCAACACCCTCATCT	96	149
		Reverse	CGTGTTTGGTTAGGTGGTAGTG		
Peroxisome proliferator-activated receptor gamma	*PPAR*	Forward	TCGCCAAACTGCTCCAGAAA	95	114
		Reverse	GCAGCAGTGGGTGTAAGCAA		
Acyl-CoA oxidase, palmitoyl	*ACOX*	Forward	GTAGATGCCTTTGACCACCA	108	117
		Reverse	CTCTGTAGCGTTTAGGGGTG		
Glutathione S-transferase theta-1	*GST*	Forward	GAACTCACGGGTCCAAGGT	90	88
		Reverse	GCAGCGTCCATCTTCTCCTT		
Retinol-binding protein II	*RBP*	Forward	GGACAACCGAAAACTCAAGACA	96	82
		Reverse	ACGATTTGCTTTCTCCCCCT		
Vitellogenin C	*Vg*	Forward	GGCACACATTGGATGGGGA	105	96
		Reverse	GCCTTGATTGTTGGCTTGGA		
Catalase	*CAT*	Forward	GTCGTCTCTTCTCCTACCCAG	101	110
		Reverse	ATCACGCTGGTAGTTGCTC		
Extracellular superoxide dismutase	*SOD*	Forward	GCCCCAGGAAGGAAAAGTCA	95	72
		Reverse	CCAATCACAGACACACCCGT		
Cytochrome c oxidase subunit 1	*CCO*	Forward	CCTTGTTCGTATGGGCTGT	109	161
		Reverse	GAATAAGTGTTGGTAAAGGATGGG		
Reference genes					
Actin β	*ACT*	Forward	ATGAGAGGTTCCGTTGCCC	91	92
		Reverse	AATGCTGTTGTAGGTGGTCTC		
Ubiquitin-protein ligase	*UBI*	Forward	CTTCTGTCTGGTGGGGTCAA	106	127
		Reverse	ACAAACCCTTCCTCATCGCA		

Experion™ Automated Electrophoresis System (Bio-Rad, Mississauga, ON, Canada), using the Experion™ RNA StdSens Analysis kit (Bio-Rad).

Reverse transcription was performed with the QuantiTect® Reverse transcription kit (QIAGEN) following manufacturer's instructions for a 40 μL total volume of cDNA. cDNA samples were then stored at −80 °C until real-time quantitative PCR analysis (qPCR). All the primers were designed by the authors using Primer-BLAST from NCBI (Primer3 with Blast, Rozen and Skaletsky, 2000) (Table 1). The presence of secondary structures was evaluated using Netprimer (PREMIER Biosoft, Palo Alto, CA, USA). For each gene, two or more primer pairs were evaluated. Primers were manufactured by IDT (Coralville, IA, USA).

2.4.2. qPCR method

All qPCR analyses were performed using iQ™ SYBR® Green Supermix (Bio-Rad) and CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). For each primer pairs selected (Table 1), a calibration curve (starting cDNA concentration: 10 ng, 8 serial dilutions, 2, 3, 4 or 5-fold) was established in order to obtain PCR efficiency (values between 90 and 110%) and limit of detection. Each reaction was run in duplicate and consisted of 5 μL cDNA (equivalent to 10 ng cDNA), 6.5 μL iQ™ SYBR® Green Supermix (50 mM KCl, 20 nM Tris–HCl, pH 8.4, 0.2 mM of each dNTP, 25 units/mL iTaq DNA polymerase, 3 mM $MgCl_2$, SYBR Green I, and 10 nM fluorescein, final concentrations), primers (300 nM each), and DEPC treated water up to a total volume of 13 μL. Cycling parameters were 95 °C for 2 min, then 40 cycles of 95 °C for 15 s, and 54.8 °C (genes: *GST*, *SOD*), 60 °C (*LYG*, *B2M*, *PPAR*, *RBP*, *Vg*, *CCO*, *ACT*, *UBI*), 61.6 °C (*CAT*), or 64 °C (*ACOX*) depending of primers for 5 s. Amplification specificity was verified with a melting curve. No-template control (NTC) was included on each plate. qPCR analysis of the studied samples was run in duplicate. qPCR conditions for sample analysis were the same as mentioned above, with the exception of cDNA concentration, which was of 5 ng.

Data acquisition and analysis were performed by CFX Manager™ Software (Bio-Rad). Baseline and threshold were set manually. Quantification cycle (Cq) values were then imported into GenEx Enterprise software (MultiD Analyses AB) in order to choose reference genes. Selected reference genes were Actin β (*ACT*) and ubiquitin-protein ligase (*UBI*) (analysis by geNorm and Normfinder). Cq values were corrected when the efficiency was not 100%. The comparative threshold method ($^{\Delta\Delta}$Ct) (Livak and Schmittgen, 2001) was used for relative quantification and results from fish collected at the upstream site were used as reference.

2.5. Biological indicators

A suite of biomarkers involved in protection against oxidative stress (catalase, CAT; superoxide dismutase, SOD) and implicated in energy/lipid metabolism (lactate dehydrogenase, LDH; cytochrome c oxidase, CCO), biosynthetic capacities (nucleoside diphosphate kinase, NDPK), and reproduction (vitellogenin, Vg) were selected *a priori* to be analyzed in the liver and muscle of perch (Section 2.4.1). Liver samples were also used to analyze biological indicators that were identified based on microarray results (Section 2.4.2).

2.5.1. Predetermined biomarkers of exposure

2.5.1.1. Vg. A semi-quantitative ELISA kit from Biosense Laboratories (Bergen, Norway) for fish was used to detect vitellogenin in yellow perch liver samples. Liver homogenates were centrifuged at 15,000 ×*g* for 20 min at 4 °C. Supernatants were diluted 1:50 in coating buffer. One hundred microliters of samples or standards (*Salmo salar* Vg) was added in microplate wells (in duplicate) and incubated at 4 °C overnight. The incubated microplate was washed 3 times with 300 μL washing buffer and then blocked with 200 μL blocking/dilution buffer at room temperature for 45 min. The wells were emptied and incubated 90 min at 37 °C with 100 μL monoclonal primary mouse anti-striped bass vitellogenin antibody ND-3G2 (Biosense Laboratories), diluted 1:100 with the blocking/dilution buffer. The wells were washed 3 times with 300 μL washing buffer and incubated 1 h at 37 °C with the secondary antibody (horseradish peroxidase conjugate) diluted 1:2000 in blocking/dilution buffer. After five successive washes with 300 mL washing buffer, the microplate was developed using 100 μL of OPD-peroxidase substrate for 15 min at room temperature. The color was fixed with 50 μL H_2SO_4 and the absorbance read at 492 nm. The results were expressed as equivalents of μg *S. salar* Vg/mg protein.

2.5.1.2. NDPK, LDH, CCO, CAT, and SOD. Samples for enzyme and protein assays were frozen at −80 °C until analysis. On the day of analysis,

samples were thawed on ice and diluted 25-fold (weight:volume) in buffer (pH 7.5; 20 mM HEPES; 1 mM EDTA; 0.1% Triton® X-100). Samples were homogenized using a Janke & Junkel Ultra-Turrax® T25 (IKA-Labortechnik) by three 10-second pulses at maximal speed (24,000 RPM) with 30-second pauses between pulses. Enzyme activity was measured using 96-well plates with a UV/Vis Varian Cary 50 spectrophotometer at constant (room) temperature (24 °C). Each assay was carried out in triplicates. Assay conditions (see below) were determined after optimization of substrate and homogenate concentrations for each tissue and enzyme. Enzyme activities were expressed as IU/g tissue (IU = international units or μmol of substrate converted to product/min).

Assays for nucleoside diphosphate kinase (NDPK; EC 2.7.4.6), lactate dehydrogenase (LDH; EC 1.1.1.27) and cytochrome c oxidase (CCO; EC 1.9.3.1) were performed as in Gauthier et al. (2008) with the following modifications: for NDPK, thymidine 5′-diphosphate concentration was 5.25 mM for the liver and 3.5 mM for muscle and homogenates were diluted 1:25 for the liver and 1:500 for muscle; for LDH, pyruvate concentration was 5 mM (liver) or 10 mM (muscle) and homogenates were diluted 1:25 for the liver and 1:1000 for muscle; for CCO, homogenates were diluted 1:100 for both tissues. Catalase (CAT; EC 1.11.1.6) and superoxide dismutase (SOD; EC 1.15.1.1) activities were determined following instructions from the kit suppliers (Cayman Chemical Company).

Protein assays were performed on homogenates from enzyme assays frozen at −80 °C until analysis and diluted 1:1000. Analysis of protein concentrations was carried out using the Bio-Rad Protein Assay on 96-well microplates, based on published method (Bradford, 1976), with bovine serum albumin as a standard.

2.5.2. Biomarkers selected from microarray results

Lysozyme (LYS), retinol binding protein (RBP), and peroxisomal acyl-CoA oxidase activity (ACOX) were selected based on impacted gene transcription. Livers were homogenized with a Polytron in 20 mM Hepes–NaOH buffer (pH 7.5), containing 1 mM EDTA and 0.1% Triton X-100 (1 g of tissue:25 mL of buffer), for 30 s. Homogenates were centrifuged at various speeds for the different biomarkers (see details below), and the supernatant was collected and stored at −90 °C. Total protein content was measured on supernatant as described in Section 2.5.1.2.

2.5.2.1. LYS. Lysozyme activity was measured in the liver based on published method (Dautremepuits et al., 2009) with some modifications. Liver homogenates were centrifuged at 3500 ×*g*, for 30 min at 4 °C. Ten microliters of supernatant (in duplicate) was mixed with 200 μL *Micrococcus lysodeikticus* (0.2 g/L in 0.05 M phosphate buffer, pH 6.2). Decrease of absorbance was monitored at 450 nm, every 30 s for 20 min at room temperature. The activity was expressed as Δ absorbance/(min × mg protein).

2.5.2.2. RBP. The fish urinary retinol binding protein ELISA kit from MyBioSource (San Diego, CA, USA) was used to detect retinol binding protein in liver samples from yellow perch. Liver homogenates were centrifuged at a speed of 15,000 ×*g* for 20 min at 4 °C. One hundred microliters of supernatant, or standards, and 100 μL HRP-conjugate were added in microplate wells (in duplicate) and incubated at 37 °C for 60 min. Microplate was washed 3 times with 300 μL wash solution. Fifty microliters Chromagen Solution A and 50 μL Chromagen Solution B were added successively and then incubated 15 min at 37 °C. Fifty microliters Stop solution was added and absorbance read at 450 nm. The results were expressed as μg RBP/(mL × mg proteins).

2.5.2.3. ACOX. ACOX activity was measured in liver tissues as described in Holth et al (2011) with some modifications. Liver homogenates were centrifuged at 500 ×*g* for 15 min at 4 °C. Ten microliters of supernatant was mixed with 190 μL of reaction buffer (in duplicate): 53 μM leuco-dichlorofluorescein (Invitrogen, CA, USA), 12 U/mL horseradish peroxidase (Sigma-Aldrich, USA), 40 mM sodium azide (Sigma-Aldrich, USA) and 0.02% Triton X-100 in 10 mM potassium phosphate buffer, pH 7.4, and incubated 5 min at 25 °C. Twenty microliters of 30 μM palmitoyl-CoA (Sigma-Aldrich, USA) was then added and the increase of absorbance was monitored at 502 nm every 30 s for 20 min. ACOX activity was expressed as IU/mg proteins, calculated using molar extinction coefficient 91,000 M/cm (Köchli and von Wartburg, 1978).

2.6. Stable isotope

Muscle samples were dried at 45 °C to a constant weight and ground into a fine powder using a mortar and pestle. All stable isotope analyses ($\delta^{13}C$ and $\delta^{15}N$) were performed at the University of Waterloo-Environmental Isotope Laboratory on a Thermo Finnigan Mat Delta Plus Mass Spectrometer coupled to a Carlo Erba Elemental Analyzer (NA1500). Results are expressed in parts per thousand (‰) in standard δ notation as $\delta = [(R_{sample} / R_{reference}) - 1] \times 1000$ where $R = {}^{13}C/{}^{12}C$ or ${}^{15}N/{}^{14}N$.

2.7. Histology

Liver and gonad samples were fixed in paraffin, sliced at 4 μm and stained with hematoxylin phloxine saffron. Liver slides were examined semi-quantitatively using photon microscopy following the method proposed by Bernet et al. (1999). The methodology is based on the rating of pathological changes with 'score values' and the pathological importance of each alteration was defined as an 'importance factor'. A 'lesion index' for the liver was calculated using these numeric data. A semi-quantitative evaluation of melano-macrophage centers was also conducted in the liver using the same method. Developmental stages (Blazer, 2002) and gender were determined for gonads.

2.8. Data analysis

Transcriptomic, enzymatic, isotope data, and physiological parameters of fish and contaminant concentrations were compared between sites using ANOVA and Kruskal–Wallis analyses after verification of the homoscedasticity and normality of the data (JMP 10.0, SAS Institute Inc.). Tukey's a posteriori comparisons were used to test the differences between groups. Contaminant concentrations below MDLs were substituted with a randomly generated number between 0 and analyte-specific MDL for statistical analyses. Correlations between contaminant concentrations and fish length, age, and trophic position were evaluated using Spearman correlations. Spearman analyses were also conducted to evaluate relationships between biological responses (transcriptomic data from qPCR) and chemical concentrations. The significant threshold was established at 0.05.

3. Results and discussion

3.1. Chemical analyses in fish

3.1.1. Perfluoroalkyl substances

Concentrations of PFAS were significantly different between sites with highest levels of PFNA, PFDA, PFUnA, and PFTriA found in fish collected upstream the WWTP (Fig. 2, Supplementary information Table S1) and highest tissue concentrations of PFOS and PFDS in fish collected at the most remote downstream site (10 km). Results indicated that PFAS concentration in fish decreases near the point of discharge and tends to re-increase further down the river. Results in fish suggest that the bioavailability of PFAS is impacted by the complex organic mixture in urban effluent resulting in lower bioaccumulation in perch. PFAS concentrations in surface water were all similar among sites (Table S1). Studies have shown that several treatment

techniques were ineffective in removing PFASs from wastewater (Appleman et al., 2014) and that some congeners could be formed during the processes (Guerra et al., 2014). No significant relationships were found between PFAS concentrations in whole fish homogenates and any physiological parameters.

3.1.2. Flame retardants

Ten PBDEs were detected in yellow perch homogenates but none of the non-PBDE flame retardants were found (Houde et al., 2014). All PBDEs were found at significantly higher concentrations in fish downstream the treatment facility (Fig. 2, Table S1). Results suggest that the WWTP is a source of PBDE contamination for this aquatic environment and that these chemicals are readily available for fish. These results corroborate the high concentrations of PBDEs and other alternatives flame retardants detected in suspended matter, fish, and birds analyzed from this area of the river (Gentes et al., 2012; Pelletier and Rondeau, 2013; Houde et al., 2014).

3.1.3. Metals and trace elements

The bioavailability of metals varied among the study sites with significantly higher concentrations of Ag, Al, Ca, Mg, Na, Pb, Rb, Sr, V, and Zn found in perch not exposed to the effluent (Fig. 2, Table S1). Concentrations of Co, Cu, Mn, Se, and Fe were significantly higher in perch from the 4 km downstream site suggesting the effluent as a potential source of contamination for these metals; ferric chloride is also used in the chemical treatment of the Montreal's effluent and therefore an additional source of Fe. Levels of Ag analyzed in caged-mussels exposed to the effluent outfall were lower compared to the upstream reference site and were associated with colloids in water samples collected at the Montreal's WWTP point of discharge (Gagnon et al., 2006). In another cage-study, concentrations of Zn in mussels were also significantly reduced in mussels collected closer to the Montreal's effluent outfall (Gagné et al., 2007). Variations in the partitioning of metals between dissolved and particulate phases, impacting the bioavailability and thus the bioaccumulation of metals, have been reported along this effluent plume (Gagnon and Saulnier, 2003).

3.2. Physical conditions

Results from histological analyses indicated that all fish gonads examined ($n = 51$) were from females which was coincidental and may be related to the choice of capture sites (i.e., shallow vegetated areas) or the length range established beforehand which may have excluded smaller males from captures. The age of 58 perch out of 60 was between 2 and 4 years-old; two individuals were 6 years-old. Based on gonad histology, all females were considered mature in the previtellogenic stage of their annual reproductive cycle except for five fish that were considered as juvenile. Sexual maturity in perch has been reported to generally be around three years-old in males and four years-old in females (Scott and Crossman, 1974) but this may vary with geographical region. Data analyses indicated no clear trend between age of fish and gonad histology; immature fish were in the same age range than other perch considered in the previtellogenin stage of their reproductive cycle (2 to 4 years-old). Given that 96% of fish were within the same 2 to 4 year-old age range and that results of contaminant and biological analyses were also within the same range for all fish, no differentiation was made between age classes during analyses.

Perch weight ($p = 0.12$) and body condition ($p = 0.70$) were similar among sites. However, $\delta^{15}N$ values were significantly different among sites ($p < 0.0001$) with fish captured 4 km downstream the WWTP outfall feeding at lower trophic levels (Table 2). Perch from both downstream sites were also younger and smaller in length compared to the upstream location ($p < 0.0001$). The lower $\delta^{15}N$ values could be related to the younger age of fish and/or to the greater consumption of organic matter found in the effluent compared to the regular diet consisting of invertebrates and small fish.

Table 2
Mean and range of physiological and biometric parameters for yellow perch captured in the St. Lawrence River in June 2012 ($n = 20$/site except for sex determination indicated below).

	Upstream	4 km downstream	10 km downstream
Total length (mm)	168 (150–209)	160 (145–198)	159 (145–199)
Weight (mm)	56.4 (38–112)	49.3 (29–90)	48.5 (31–100)
Girth (mm)	103 (88–132)	96 (85–121)	97 (83–129)
Age (year)	3.2 (2–6)	2 (all 2)	2.4 (2–4)
Female gonads	$n = 14$	$n = 17$	$n = 20$
Fulton's condition index (K)	11.7 (10.5–13.7)	11.9 (9.6–15.4)	11.8 (9.5–14.3)
$\delta^{15}N$ (‰)	13.1 (12.3–13.8)	9.8 (7.9–12.6)	12.5 (11.2–13.7)

3.3. Gene transcription

Microarray analyses indicated that exposure to the urban effluent affected significantly the gene transcription in the liver of yellow perch when compared to fish from the control upstream site. A total of 177 out of the 1000 gene transcripts included in the array were differentially expressed downstream of the effluent: 133 at the 4 km downstream sites and 44 at the 10 km site (Table S2, Fig. S1). Among those genes, 38 were commonly affected in both impacted sites and were mostly involved in the fish immune system (11 genes), lipid metabolism (4 genes), retinol metabolism (3 genes) as well as detoxification processes (2 genes), cellular proliferation, and membrane/cellular transport (2 genes respectively) (Table 3). Results from the quantitative PCR corroborated the data obtained with microarray for most genes, in both direction and magnitude (Table 3). These results suggest that the transcriptional profiles derived from the microarray data are accurate and reproducible.

Most of the biological pathways affected were down-regulated by the effluent (Fig. S2), especially the immune response with 11 genes under-transcribed (Table 3). Several studies report the down-regulation of immune-related genes in fish residing downstream urban WWTPs (Garcia-Reyero et al., 2008a,; Ings et al., 2011) as well as decrease in immune responses at the cellular level (Liney et al., 2005; Müller et al., 2009). Fish rely on their innate immune system as a first line of defense against invading pathogens (Segner et al., 2012). The complement system and lysozyme in particular are major effector mediators of the fish defense against infection (Saurabh and Sahoo, 2008; Zhu et al., 2013). The high number of immune-related genes impacted therefore suggests that MWWE exposure impairs the yellow perch immune system, increasing their susceptibility to pathogens and/or parasites.

Energy homeostasis is important for survival and normal physiology and is mainly mediated by the catabolism of fatty acids by β-oxidation in the mitochondrion and the peroxisome. The Acyl-CoA oxidase, palmitoyl (ACOX) is the first step and rate limiting enzyme in peroxisomal β-oxidation and is used as a biomarker of peroxisomal proliferation (i.e., a cellular perturbation leading to an increase in number and volume of peroxisomes) (Cajaraville et al., 2003). Increased *ACOX* transcription and enzyme activities have been reported in different fish species in response to organic contaminants such as PFAS, sometimes associated with either an increase or a decrease expression of its transcriptional regulators, the peroxisome proliferator-activated receptors (PPAR) (Arukwe and Mortensen, 2011; Holth et al., 2011). An over-transcription of the gene coding for ACOX was found at the downstream sites suggesting that the effluent may result in peroxisome proliferation in the liver of yellow perch (see Section 3.6 for specific correlations between individual compounds and biological responses). Decrease in the transcription of the *PPARϒ* gene, in addition to the Acyl-CoA dehydrogenase (*ACADVL*) gene, which catalyzes the first step of the mitochondrial

Table 3
Annotated differentially transcribed genes (microarray and qPCR) in the liver of yellow perch captured at both downstream sites of the WWTP.

Gene name	Regulation	4 km/upstream		10 km/upstream		Genbank #
		Microarray[a]	qPCR	Microarray[a]	qPCR	
Immunity						
E3 ubiquitin ligase TRIM39	−	0.63		0.69		XM_003200516.2
E3 ubiquitin/ISG15 TRIM25	−	0.92		0.93		XM_005737343.1
E3 ubiquitin ligase MIB2	−	0.96		0.95		XM_004570978.1
C1q-like 1	−	0.75		0.80		EU131872.1
C1q-like 2	−	0.78		0.82		EU131873.1
C1qC-like	−	0.79		0.82		JQ805144.1
G-type lysozyme	−	0.86		0.90		AB355631.1
Beta-2 microglobulin	−	0.77	0.69	0.84	0.81	HQ206491.1
Galectin-9	−	0.87		0.84		XM_003458327.2
Delta-like protein B	−	0.96		0.96		XM_003970746.1
Nuclear factor interleukin (NFIL)-3 regulated protein	−	0.86		0.90		XM_005926735.1
Lipid metabolism						
Peroxisome proliferator-activated receptor (PPAR) gamma	−	0.92	0.88	0.94	0.86	AB574331.1
Acadvl protein	−	0.92		0.90		BC155201.1
Peroxisomal acyl-coenzyme A oxidase 1 (ACOX)	+	1.07	1.63	1.06	1.60	XM_005470198.1
Non-specific lipid-transfer protein	+	1.19		1.17		XM_005742015.1
Detoxification/oxidative stress						
Dimethylaniline monooxygenase	−	0.84		0.87		HQ206476.1
Glutathione-S-transferase (GST)	+	1.28	1.04	1.33	1.34	GU938675.1
Retinol metabolism						
Nuclear receptor NR2F5	−	0.96		0.95		XM_003443141.2
Retinol dehydrogenase 3 (RDH)	+	1.30		1.25		XM_004570405.1
Retinol-binding protein II (RBP)	+	1.28	1.18	1.32	1.28	XM_004562806.1
Membrane/cellular transport						
Kinesin-like protein KIF21a	−	0.82		0.85		HQ206500.1
NA^+/K^+ transporting ATPase	−	0.84		0.89		BT072358.1
Cellular proliferation						
B-cell translocation protein 1	−	0.85		0.87		FJ426150.1
Growth hormone-inducible protein	−	0.88		0.90		DQ470490.1

[a] Significant differential expression values expressed as fold change (FDR < 0.1; p < 0.1).

β-oxidation, was also observed at both downstream sites. These results therefore suggest that the effluent impacts the transcriptional regulation of cellular energy homeostasis. Furthermore, genes related to the pentose phosphate pathway, involved in glucose metabolism (i.e., glucose-6-phosphate dehydrogenase), to the tricarboxylic acid (TCA) cycle (e.g., acyl-CoA synthetase), and to oxidative phosphorylation (i.e., ATP synthase and cytochrome c oxidase) were also over-transcribed in perch from the 4 km downstream site indicating that the energy metabolism may be increasingly impacted in fish residing nearer the effluent point of discharge.

Retinoid levels in organisms have been used as sensitive biomarkers for a wide range of environmental pollutants in wild animal populations (Novak et al., 2008; Chen et al., 2012b). These compounds (which include retinol, retinal, and retinoic acids) relate to the vitamin A activity that is essential for growth, vision, immunity, and reproduction (Defo et al., 2014). An increase of the RBP gene transcription was found in yellow perch exposed to the effluent, along with the over-transcription of the retinol dehydrogenase gene (*RDH*), responsible of metabolizing retinol into retinal in target organs. Retinal is the predominant retinoid form in eggs and oocytes of marine and freshwater fish. High amounts of retinal have been linked to the inhibition of *PPARϒ* in vitellogenogenic zebrafish females (Levi et al., 2012). All fish captured in the present study were females which may explain both increased *RDH* and decreased *PPARϒ* levels observed in the liver. Interestingly, *ACOX*, *RBP*, and *GST* gene transcription (from qPCR analyses) were positively correlated in perch liver.

3.4. Biochemistry

A suite of biomarkers were preselected and analyzed to evaluate the effects of the environmental exposure of perch to the urban effluent on oxidative stress, energy metabolism, and reproduction. Lysozyme (LYS), acyl-CoA oxidase (ACOX) and retinol-binding protein (RBP) were selected based on the microarray data as three potential biomarkers of effects. Lysozyme activity is a convenient biomarker of the environmental impact of contaminants on fish immunity, but the nature of the regulation can be complex (Saurabh and Sahoo, 2008). Effects of the Montreal's MWWE were studied in rainbow trout (Salo et al., 2007). Results indicated that lysozyme-mediated defense decreased in fish experiencing long-term exposure to sewage effluent, similarly to our observation of a decreased gene transcription of g-type lysozyme. On the other hand, lysozyme activity was reported to be higher in the head-kidney of yellow perch, which is the main distribution organ for this molecule, downstream of the effluent (Dautremepuits et al., 2009). Results from the present study indicated no differences between sites for lysozyme activity when results were expressed on a protein basis. Lysozyme activity was measured in the liver of perch, which may explain why no significant differences were found among the sites.

An up-regulation of *RBP* and *RDH* gene transcription at both downstream sites did not result in an increase of RBP content in the liver. RBP binds to retinol and protects it against oxidation and/or degradation (Defo et al., 2014) as RDH catalyzes its transformation into retinal mainly implicated in vision. The increase of *RBP* and *RDH* could therefore reflect an increase of retinol and retinal levels in the liver which were not determined in the present study. Significantly lower ACOX activity in the liver of fish from the 4 km downstream was observed compared to other locations (Fig. 3). The transcription of the ACOX coding gene is known to be induced by PPARs in fish (Yang et al., 2014). Results in perch indicated a significant down-regulation of the *PPARγ* gene liver at downstream sites which could have led to the observed decrease in ACOX enzymatic activities, however, no significant correlations were found between the parameters. Relations between genotypic and phenotypic expression are not linear, indeed several post-transcriptional and post-translational steps exist between the transcription of genes and activities of related proteins. The lower muscle CCO activity observed downstream the outfall, in addition to the decrease in ACOX activity in the liver (4 km site), suggests impacts of the effluent on the energy/lipid metabolism of perch. This conclusion is supported by the

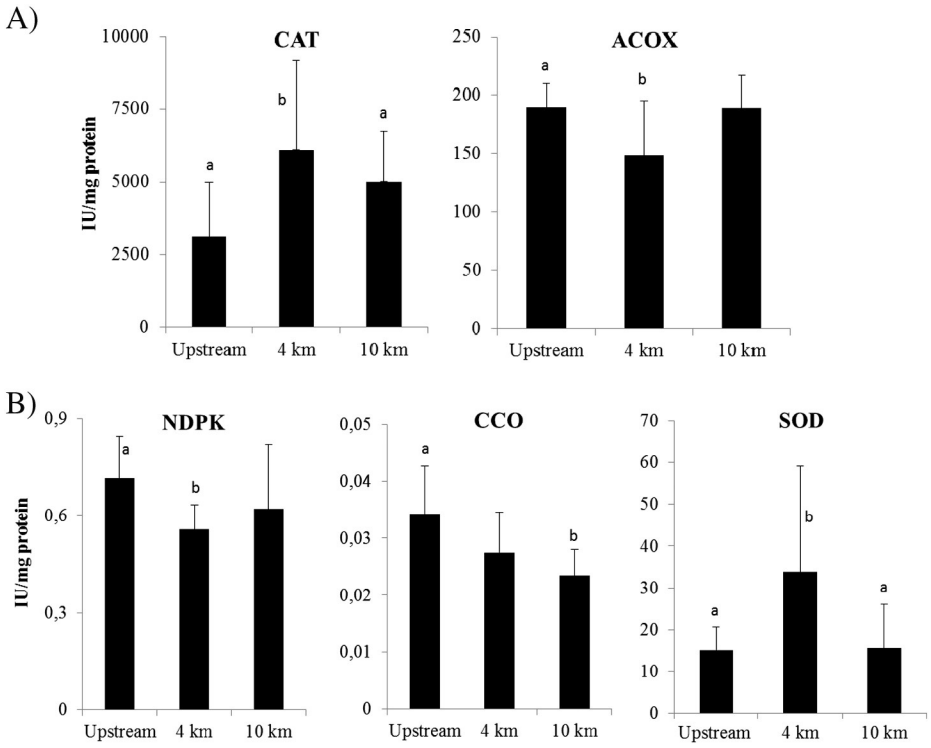

Fig. 3. Enzyme activities ± std error (IU/mg protein; n = 10/site) in A) the liver and B) muscle of yellow perch were significantly different among sampling sites. Different letters indicate significant differences among sites (Tukey's post-hoc tests, $p < 0.05$). CAT: catalase, ACOX: acyl-CoA oxidase, NDPK: nucleoside diphosphate kinase, CCO: cytochrome c oxidase, SOD: superoxide dismutase.

significantly lower activity of muscle NDPK in fish captured at the site 4 km downstream of the effluent (Fig. 3). In yellow perch, NDPK activity is positively correlated with indicators of energy accumulation and growth (Gauthier et al., 2008, 2011) and has been reported to be negatively affected by metal contamination (Audet and Couture, 2003; Gauthier et al., 2011). In addition to an impairment of energy metabolism in effluent-exposed fish, the lower muscle CCO activity in fish downstream of the effluent suggests that muscle aerobic capacities, used for sustained swimming activities like foraging, are negatively affected.

Results also indicated an increase in CAT activity in the liver of perch captured downstream (4 km) the effluent outfall compared to the upstream site (Fig. 3). Catalase is an important enzyme in protecting the cell from oxidative damage by reactive oxygen species and is a known stress response biomarker. An over-transcription of the CAT gene was observed with the microarray for perch from the 4 km site compared to the reference fish (Table S2), corroborating observation on the biochemical level. However, qPCR analyses did not validate this trend. The activity of the antioxidant superoxide dismutase (SOD) in muscle was also found at higher levels in fish captured in waters 4 km downstream the outfall. Additionally, CAT activity in the liver was positively associated with SOD in the liver/muscle (Spearman's correlation, $p < 0.05$). These results indicate that the effluent causes oxidative stress in perch. Lactate dehydrogenase (LDH) activity in perch liver and muscle, indicator of anaerobic ATP production capacities which is often used as an indicator of swimming activity, did not vary among sites nor did liver vitellogenin (Vg), a precursor of egg yolk.

3.5. Histopathology

Histological examination of perch indicated a spectrum of indices: from absence of alterations and mild tissue changes to severe inflammatory reactions and tissue damage in the liver of some fish (Table S3). No statistically significant differences were found for indices of histological changes in fish liver among sites, suggesting that the effluent might not be directly responsible of tissue damage. However, correlations were found in perch between tissue concentrations of BDE-66 and -100 and RES activation (negative relationships) and melano-macrophage centers and Cd (positive relationship). Liver neoplasm, gonadal lesions, and decreased condition factors have previously been reported in St. Lawrence River whitefish, collected in 1996, 200 km downstream of Montreal (Mikaelian et al., 2002). The authors suggested that the histologic changes could be related to the elevated tissue concentrations of organochlorine pesticides and polychlorinated biphenyls. Strong relationships ($p < 0.0001$) were found between the liver lesion, melano-macrophage and RES activation indices, indicating that most histopathological alterations are closely related. The activity of lactate dehydrogenase enzyme (LDH) in perch muscle was also significantly and positively correlated to indices of the liver lesion and plasma alteration and LDH in the liver associated with melano-macrophage centers. LDH is indicative of tissue damage and commonly used as a biomarker in human and animal health assessment to diagnose tissue alterations and diseases. Results from this study therefore corroborate the use of LDH as a useful biomarker of tissue damage in fish (Ramesh et al., 1993).

3.6. Correlations between contaminants and biological parameters

Tissue contaminant concentrations were analyzed in relation to multi-level biological responses in order to identify potential links between specific systems/pathways and compound classes. These analyses were conducted using fish from all sites combined. Such results could be of interest for further controlled studies. All significant correlations can be found in Table S4.

Correlations between PFAS and biological parameters were mostly found on the enzymatic level. Statistical analyses indicated that PFAS tissue concentrations were positively related to ACOX activity (i.e., PFNA, PFDA, PFUnA, PFDoA, and PFOS) and lysozyme (PFDA

and PFDoA) in the liver of perch and SOD (negative relation; PFDoA and PFTriA) and CCO (positive relation; PFNA and PFDA) in fish muscle. At the genomic level, PFDoA also correlated positively with *ACOX* gene transcription. Observations for *ACOX* corroborate information in the literature. PFOS has been reported as a peroxisome-proliferating compound causing increased hepatic ACOX activity in several fish species experimentally exposed in microcosm (Oakes et al., 2005) as also observed for PFOA in the liver of male Japanese medaka (Yang, 2010). Exposure of salmon hepatocytes to PFOS resulted in an increase of *PPAR* gene regulation (Krøvel et al., 2008) and moderate increase in ACOX activity has been observed in zebrafish larvae exposed to PFNA with no direct links to *PPARα* and *PPARγ* gene transcription (Yang et al., 2014). Variations in the *PPAR* gene transcription depended on doses, exposure time, and tissues analyzed in salmon following exposure to PFAS (Krøvel et al., 2008; Arukwe and Mortensen, 2011).

Correlations found in this study suggest that PFAS may also have effects on lipid metabolism that could impact energy metabolism (i.e., CCO in muscle). Disordered glucose metabolism was indeed hypothesized to explain the decrease swimming efficiency of juvenile goldfish exposed to PFOS (Xia et al., 2013). Regarding responses to oxidative stress, acute exposure to PFOS/PFOA resulted in an increase of SOD activity in cultured freshwater tilapia hepatocytes (Liu et al., 2007) as a 7 day exposure to PFOA led to no changes in the liver of Japanese medaka (Yang, 2010). Results of environmentally exposed yellow perch showed negative relationships between SOD and long-chain PFAS which may be caused by long-term exposure to these chemicals and/or interaction with other compounds/pathogens found in the perch environment. It is also important to note that enzyme activities (e.g., ACOX, SOD) have been reported to change depending on tissue analyzed and concentrations used with attenuation of effects with higher doses of PFAS (Oakes et al., 2004; Yang et al., 2014).

Concentrations of individual PBDEs in tissue were positively related to the transcription of *ACOX* (BDE-17, -28, -47, -49, -99, -100, -153, -154), *CAT* (BDE-28, -47), and *RBP* (BDE-17, -28, -49) and negatively to the *B2M* gene (BDE-47, -49, -100, -153, -154). Genomic work with PBDE and fish has focused mainly on the thyroid system known to be affected by these compounds. However, responses at the biochemical level have been reported for enzyme/protein for which correlations with PBDE tissue concentrations were found in perch. For example, a dose-dependent increase in antioxidant CAT activity in the liver of emerald rock cod chronically exposed to a mixture of PBDE was reported (Ghosh et al., 2013) and long-term exposure to brominated flame retardants affected the antioxidant system (including CAT) in freshwater goldfish (Feng et al., 2013). Additionally, an increase in retinol content, with a concomitant increase in transcription of the *RBP* gene and RBP protein content in the liver, was reported in the liver of zebrafish exposed to the commercial mixture DE-71 (Chen et al., 2012b) and acute exposure to PBDE led to disturbance of the retinoid signaling pathway with possible impacts on eye development in zebrafish larvae (Xu et al., 2013).

On higher biological levels, negative relationships were observed between NDPK activity in the liver (BDE-66) and muscle (BDE-126, and -154) indicating possible impacts of PBDE congeners on the protein synthesis of perch. Higher concentrations of BDE-99 and -153 were also associated with lower activation of the reticuloendothelial system (RES activation) in the liver tissue. Few studies have investigated the effects of PBDEs on the integrity of fish tissue. Histological effects of BDE-47 have been evaluated in juvenile turbot and hepatic lesions were characterized by cellular and nuclear hypertrophy, circulatory disturbances and irregular morphology of hepatocytes (Barja-Fernández et al., 2013). Exposure to BDE-209 has led to the liver tissue degeneration in female adult Chinese rare minnow (Li et al., 2014) and weak correlations were found between summed PBDE liver concentrations and macrophage area in largescale sucker from the Columbia River, USA (Torres et al., 2014).

The highest number of correlations for metals was found for the transcription of genes related to lipid metabolism, retinol metabolism, and detoxification (i.e., *ACOX* which negatively related to As, Ca, Cd, Mg, Na, Pb, Sr, V; *RBP* inversely related to Ca, Cd, and Sr; *GST* inversely related to As, Cd, Cu, and V). Results for Cd corroborate the disturbance of genes-related to retinol and energy metabolism in yellow perch environmentally exposed in lakes (Pierron et al., 2011). Vitamin A homeostasis has also been reported to be impacted by chronic exposure to Cd in wild yellow perch exposed to a gradient of metal contamination (Defo et al., 2012).

On the immune enzymatic level, higher tissue concentrations of Cu, Co, and K correlated with lower lysozyme activities. Negative associations have been previously reported in yellow perch for genes involved in immunity after exposure to Cd (Pierron et al., 2011) and suppression of immune-related proteins in gilthead sea bream exposed to Cu (Isania et al., 2011). All relationships between CAT (Ca and Sr) and Vg (Al, Pb, and Fe) were negative. At the tissue level, higher concentrations of Cd were related to the higher melanomacrophage indices. Overall, results from correlation analyses between contaminants and biological parameters may corroborate and support effects previously reported as well as help identify specific pathways of interest for further studies on individual compound groups.

3.7. Conclusion

This study aimed to evaluate impacts of a major WWTP on the health of yellow perch using a multi-level biological approach. Chemical tissue analyses in perch suggest that the bioavailability of PFAS and metals may be impacted by the complex mixture of the effluent. The regulation of genes related to immune function, as well as lipid and retinol metabolism, was impacted by exposure to the effluent in the liver of yellow perch. Phenotypic biomarkers of oxidative stress and lipid/energy metabolism were also different among upstream and downstream sites. Significant correlations were found between PFAS, PBDE, and metal tissue concentrations and multi-level biological responses, which could help guide further experimental studies. A next step to this multi-biological level tool development would be to evaluate yellow perch health at different sites of the St. Lawrence River including Lake St-Pierre where the recruitment of yellow perch is impacted. Additional information, such as parasitology and population structures, should also be added to this continuing project in order to integrate additional stressors and to potentially link molecular changes to higher biological and population levels. The incorporation of field-based studies and the development of multi-biological level approaches are of importance in order to better evaluate impacts of pollution on aquatic organisms.

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Appendix A. Supplementary data

Mean concentrations of all chemicals analyzed in yellow perch homogenates, the regulation and the list of genes differentially expressed in perch liver upstream/downstream the WWTP, histological changes in the liver tissue of yellow perch, correlations found between multi-level biological responses and contaminant tissue concentrations, and method limits of detection can be found in this section. Supplementary data

associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.scitotenv.2014.07.059.

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