Current Topics in Toxicology
Vol. 13, 2017

Original Communication

Municipal effluent exposures in fathead minnows during partial life cycle: endocrine disruptive effects and impact on reproduction

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ABSTRACT

Municipal effluents are recognized as major sources of pollutants that could compromise fish health and reproduction. The purpose of this study was to examine and compare the reproductive toxicity of low- and high-risk municipal effluents to fathead minnows (*Pimephales promelas*) exposed for 12 weeks in the laboratory. After the exposure period, reproductive success was determined by following changes in the total number of eggs, egg hatchability/survival and time to hatch. In parallel, the expression of the following was also assessed in adults to gain insights into the pathways involved in toxicity: estrogen receptor alpha (ERα), androgen receptor (Ar1), pregnane X receptor (PXR1), vitellogenin (VTG), CYP3A4 and 17β-hydroxysteroid dehydrogenase (HSD). The results revealed that in the high- and low-risk effluents, egg laying followed a biphasic response, with an initial increase in egg laying followed by a decrease at higher concentrations and stronger amplitudes with the high-risk effluent. Hatching success (i.e., release of viable fish fry) was directly proportional to the decrease in egg production with no hatching of viable fish at 10% and 20% for both effluents. VTG gene expression was significantly increased in females, reaching levels 4 and 3 orders of magnitude greater than in the controls for the high- and low-risk effluent, respectively. VTG gene expression was also found in males but at lower expression levels than for the females. The expression of ERα was significantly correlated with VTG levels, which suggests the presence of estrogenic compounds in municipal effluents. This was further supported by the increased expression of CYP3A4, which is involved in the biotransformation of steroid-like and pharmaceutical compounds. In conclusion, municipal effluents have the capacity to reduce reproduction in fathead minnows and involve estrogenic effects. The high-risk effluent generally displayed stronger effects than the low-risk effluent.

KEYWORDS: wastewater treatment plant effluents, *Pimephales promelas*, endocrine disruption, reprotoxicity, toxicogenomics

1. INTRODUCTION

Municipal wastewaters are of concern due to their continuous release in the environment near urban areas and their potential ecological impacts [1]. Indeed, pollutants such as metals, polyaromatic hydrocarbons,

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plasticizers, hormones, pharmaceuticals, personal care products and illicit drugs are present in these effluents at trace concentrations, ranging from a few ng L^{-1} to several mg L^{-1} [1]. Sewage treatment plants are designed to remove particles and suspended matter, fine particles, and microorganisms, depending on the treatment processes, but not pollutants, especially soluble pollutants and pollutants associated with dissolved organic matter. Although the various treatment processes are not specifically designed to remove trace contaminants, some of them may be better at removing them than others [2, 3]. In general, conventional activated sludge plants are able to remove 80% of the pollutants. Hormones and pharmaceuticals are also removed from water (>70%) by both adsorption to suspended matter and microbial degradation mechanisms [4]. The complexity of municipal effluents has drawn attention to the issue of contaminants of emerging concern (CEC) due to their poorly documented persistence in the environment and the absence of provisions related to them in environmental legislation [5, 6]. It is noteworthy that several CECs like estrogens and estrogen mimics that are present in municipal effluents have endocrine disruptive activity, affect the reproductive health of fishes and can ultimately impact the sustainability of wild fish populations [7]. Recently, 17β-estradiol (E2), 17α-ethinylestradiol (EE2) and diclofenac were included in European legislation as priority hazardous substances and are now subject to regulatory criteria [8, 9]. An increased knowledge of treatment performances for these contaminants and a better understanding of their impacts on aquatic ecosystems would help support the establishment of new regulations targeting municipal effluents. In addition, the extremely variable composition of municipal effluents over time induces constant changes in concentrations of individual contaminants, depending on human use and environmental factors. The complexity of the chemical mixture can lead to unexpected (synergistic, antagonist and additive) effects and thus make it difficult to produce sound risk assessments based on reported levels of CEC. Ultimately, toxicity information incorporating long-term exposure and effects of changing municipal effluent composition is needed, especially in relation to reproductive success and endocrine disruption in addition to the chemical characteristics of the effluent.

Reports on the endocrine disruptive properties of municipal effluents appeared in the literature by the end of the 1990s [10, 11]. Several studies have documented detrimental, developmental, reproductive, and behavioral effects in fish exposed to municipal effluents [1, 12]. In addition, the production of vitellogenin and the feminization of male fish were linked to exposure to estrogens and estrogen mimics at concentrations similar to those found in wastewater effluents [10]. In natural biota, municipal effluents have been found to exert estrogenic effects on common carp and walleye living in waters that receive such discharges [13, 14]. Pharmaceuticals [3], flame retardants such as polybrominated diphenyl ethers (PBDEs) [15], steroid hormones [16] and illicit drugs [17] have been measured in municipal effluents and downstream sediments in the St. Lawrence River (Quebec, Canada). Increased vitellogenin gene expression in males and intersex conditions in local minnow populations (*Notropis hudsonius*) downstream from a municipal effluent dispersion plume were also observed [18]. In this study, we chose a "high" and a "low" risk municipal effluent based on the wastewater treatments used and the volume being discharged into the St. Lawrence River. The "high-risk" effluent consisted of wastewaters from a large city that underwent physico-chemical treatment, while the "low-risk" effluent was treated by a biofiltration process supplemented with UV disinfection during the summer season at a flow rate approximately 10 times less than that of the high-risk effluent. Given that the 3-week survival of fathead minnows (*Pimephales promelas*) is a recommended test in Canada, toxicity was assessed in this species using longer exposure times and the addition of effects' endpoints related to reproduction, endocrine disruption and biotransformation usually observed with municipal effluents [1, 19].

The aim of this study was to determine the effects of municipal effluents subjected to different treatments from two wastewater treatment plants (WWTPs) located along the St. Lawrence River. In a previous study, several endocrine disrupting chemicals, including estrone, progesterone, testosterone, norethindrone, carbamazepine, methylparaben, coprostranol and ethynylestradiol, were detected in these effluents [20, 21]. We examined the reproductive competence of sexually mature adults exposed to these effluents, and endocrine disruptive activity was determined using a suite of gene

expression endpoints associated with reproduction, specifically vitellogenin, estrogen receptor alpha, androgen receptor, 17β-hydroxysteroid dehydrogenase and pregnane X receptor. The pregnane X receptor is involved in the metabolism of steroids. It indicates exposure to hydrophobic aliphatic and aromatic organic compounds. The activation of this receptor leads to the induction of CYP3A, the major drug-metabolism enzyme complex involved in the metabolism and elimination of steroids and pharmaceutical products in mammals, fish and mussels [22, 23]. This will allow us to determine whether these effluents are able to compromise the endocrine systems and identify links between the presence of contaminants and endocrine disrupting activity.

2. MATERIALS AND METHODS

2.1. Experimental set-up and fish exposure

The fish were held in separate aquaria grouped by size and age, using the standardized methodologies for survival and reproductive toxicity assessments [24, 25]. Adult fish were held in 32-L aquaria, juvenile fish more than 7 days old in 16-L aquaria, and embryos and larvae in 500-mL beakers. All aquaria were randomly arranged on shelving units by age category. Control water consisted of reconstituted fresh water with a pH level of 7.5 and hardness of 120 ppm. Major ion concentrations were as follows: Ca^{2+} 70 µM; Cl^- 129 µM; K^+ 12 µM; Mg^{2+} 13 µM; Na^+ 179 µM and SO_4^{2-} 63 µM. Control water was used to dilute the effluents to achieve the various nominal exposure concentrations (1.25%, 2.5%, 5%, 10% and 20% effluent). Fresh wastewater effluent samples were collected weekly in the mid-morning on a weekday from WWTP facilities along the St. Lawrence River (Quebec, Canada) for a period of 5 months. They were sent directly to the laboratory where they were held at 4 °C in polyethylene plastic containers. In the case of adult and juvenile fish, the water was replaced twice weekly by removing >95% of the contents of the aquarium and replacing them with freshly prepared control water or diluted effluent. All the aquaria were continually aerated to maintain maximal oxygen concentrations, and the fish were fed to satiation daily.

Adult fish and embryos were purchased from Aquatic Research Organisms Inc. (Hampton, NH). Adult fish were acclimated for 2 months prior to beginning the 12-week exposures to the high and low-risk effluents (Table 1). Purchased embryos were verified daily and newly hatched larvae were used for larval exposures (7 days) and juvenile exposures (16 weeks). Fecundity of *Primephales pomelas* was checked daily. Eggs produced by fathead minnow (FHM) females exposed to municipal effluents or control water were maintained in the same effluent concentration in which they were laid. Percent egg survival and hatching times were reported daily. Figure 1 shows the experimental set-up and the biological endpoints measured. All procedures were approved by the INRS institution's animal care committee.

2.2. Fish handling and tissue preparation

Adult and juvenile fish were dissected after being humanely euthanized by a blow to the head. The fish were measured (fork length ± 1 mm) and weighed (± 0.1 g) prior to tissue sampling. For each fish, the liver was weighed and divided equally into two samples, one preserved in RNAlater® for toxicogenomics investigation and one for analysis of other non-genomic endpoints. All samples were kept at -80 °C until RNA extraction. Newly hatched and 7-d-old larvae were weighed in batches to determine the average weight for the group and were then euthanized by submerging them in liquid nitrogen. Storage was the same for both adults and juveniles.

2.3. Gene expression endpoints

RNA extraction and reverse transcription

Total RNA was extracted with the RNA Plus Mini Kit (Qiagen) according to the manufacturer's instructions. RNA concentration and purity were estimated using the NanoDrop-1000 spectrophotometer (Thermo Fisher Scientific, ON, Canada). All samples had an A_{260}/A_{280} ratio of 1.9 to 2.1. RNA integrity was verified with the Experion™ Automated Electrophoresis System (Bio-Rad, ON, Canada), using the Experion™ RNA Analysis Kit (Bio-Rad). Reverse transcription was performed with the QuantiTect® Reverse transcription Kit (Qiagen), which ensured the complete removal of genomic DNA, in accordance with the manufacturer's instructions. The cDNA samples generated were stored at -80 °C until quantitative real-time PCR analysis (qPCR).

Table 1. Physico-chemical properties of the municipal effluents.

	High-risk effluent	**Low-risk effluent**
Type of treatment	Physico-chemical process	Biofiltration and UV disinfection process
Flow rate	10	1
Equivalent inhabitants	7	1
	High-risk effluent	**Low-risk effluent**
BOD5	46 mg L^{-1}	19 mg L^{-1}
P_{tot}	0.4 mg L^{-1}	1 mg L^{-1}
Total fecal coliforms	$4.8*10^5$/100 mL	$1.9*10^4$/100 mL
Total nitrogen (NH4+; mg/L)	6.7	12.4
Nitrates	<1 mg/L	<2 mg/L
Suspended matter (mg/L)	18.4	15.8
pH conductivity ($uS*cm^{-1}$)	6.9 700-800	7.4 N.D

N.D: Not determined.
1: To ensure anonymity, only the relative population number and flow rates are provided. For example, the high-risk effluent has 7 and 10 times more population and flow rates than the low-risk effluent.

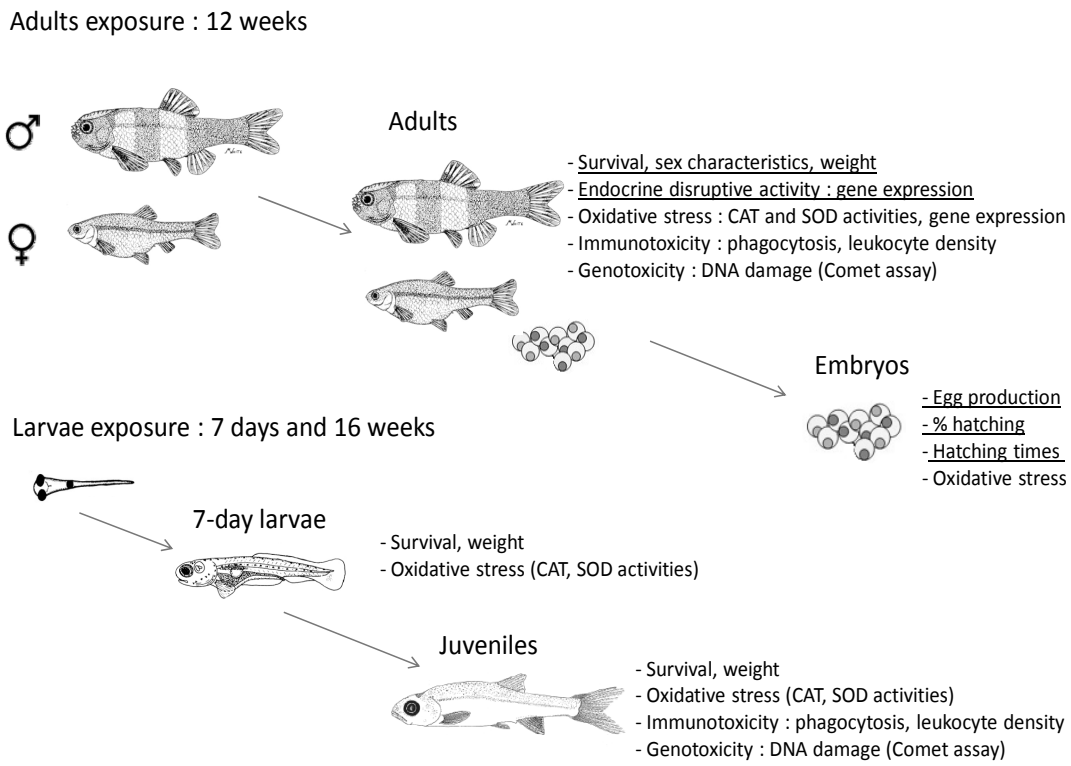

Figure 1. Graphical representation of the experimental set-up with strategic endpoints in adults, juveniles and larvae stages. Biological endpoints measured in the present study are underlined.

Primer design

Gene expression endpoints involved in steroid metabolism associated with reproduction were investigated. The genes selected for this study along with their respective primers are listed in table 2. When no suitable primers were available through the literature, the authors designed them using NCBI's Primer-BLAST (Primer3 with Blast) [26]. The absence of secondary structures was evaluated using NetPrimer (Biosoft, Palo Alto, CA). For each gene, two or more primer pairs were evaluated. Primers were synthesized by IDT (Carlville, IA, USA).

Quantitative real-time PCR

All qPCR analyses were performed using SoFast™ EvaGreen® Supermix (Bio-Rad, Mississauga, ON, Canada) and Mastercycler ep realplex2 (Eppendorf). For each selected primer pair, a calibration curve (starting cDNA concentration: 10 ng, 8 serial dilutions, 5-fold increments) was produced with PCR efficiency values between 95% and 110%, and the limit of detection was determined. Each reaction was run in duplicate and consisted of 5 µL cDNA (equivalent to 5 ng cDNA for the studied samples), 6.5 µL of 2×SsoFast™ EvaGreen® Supermix (Bio-Rad), 300 nM of each primer and DEPC-treated water (Ambion) brought up to a total volume of 13 µL. Cycling parameters were 95 °C for 30 s, then 40 cycles of 95 °C for 15 s and 60 °C for 20 s. Amplification specificity was verified with a melting curve. A no-template control (NTC) was included on each plate.

2.4. Data statistical analysis

The effects of exposure to WWTP effluents on fish reproduction were investigated using a Kruskal-Wallis non-parametric analysis of variance (ANOVA) for each effluent separately. The significance threshold was set at $p = 0.05$. The Mann-Whitney U test was used to test differences between treatments and the control. The statistical analyses were conducted with GraphPad© Prism 5 and Statistica (version 9, Statsoft Inc.). Relationships among biomarker responses were determined by nonparametric Spearman rank correlation (Spearman-moment denoted by ρ). A factorial analysis of the biomarker data (log transformed) was performed to identify the principal components (with factorial weights > 0.7) explaining the total variance.

For gene expression data, acquisition and analysis were performed using the Mastercycler ep realplex software (Eppendorf). The baseline and threshold were set manually. Quantification cycle (Cq) values were then imported into GenEx Enterprise software (MultiD Analyses, AB, Canada) in order to choose the reference genes using geNorm [27]) and Normfinder [28] algorithms implemented in GenEx and to calculate relative expression. The selected reference genes were ribosomal protein l8 (RPL8) and elongation factor 1-alpha (Ef1α). Cq values were corrected when efficiency was not 100%.

3. RESULTS AND DISCUSSION

3.1. Impact on reproduction

The high and low-risk effluents were operationally defined based on the wastewater treatment used, flow rates released in the Saint-Lawrence River and basic physico-chemical properties (Table 1). The high-risk effluent resulted from a primary physico-chemical process of wastewaters from a large city with high flow rates. It contained high conductivity, fecal coliforms and biochecmical oxygen demands compared to the low-risk effluent. The low-risk effluent used a combination of biofiltration and UV disinfection processes of wastewaters of 7 times less inhabitants. The flow rate was also 10 times less than the high-risk effluent and contained circa 25 times less fecal coliforms. The reprotoxic effects of the high- and low-risk municipal effluents were examined in adult FHM. Fish were exposed for 12 weeks and egg production was checked daily. Egg production was increased at low effluent concentrations relative to control fish after exposure to both effluents. A large decrease in the total number of eggs laid was observed for the 2.5% effluent concentration relative to the controls for the high-risk effluent and at a concentration of 10% for the low-risk effluent (Figure 2). Moreover, eggs showed increased rates of larval mortality. No eggs survived at concentrations ≥ 2.5% and ≥ 10% for the high- and low-risk effluents, respectively (Figure 3). Spearman's correlation analysis revealed that egg number was correlated with the survival of newly hatched fry and with hatching time ($\rho = 0.99$, $p < 0.05$ for both). No significant influence on hatching time was found when embryos from the control group were compared with fish exposed to

Table 2. Role of target genes selected in this study.

Target genes	Symbol	Role	PRIMER (5' - 3') (Forward/reverse)
CYP 3A	CYP 3A	Main drug metabolizing enzymes; has 6β-testosterone hydroxylase activity and is under the control of the pregnane X receptor	TGC AGG GAG AAC TGA GAG AGA AG[1] / TCC GTT CGG TCC GAC AAG
Vitellogenin	VTG-1	The egg yolk protein precursor under the control of estrogen receptor signalling	GCT GCT GCT CCA TTT CAA AAG[1] /GTG AGA GTG CAC CTC AAC GC
Estrogen receptor α	Era-1	Estrogen-binding receptor for the detection of estrogen mimics	GGC TGA GAT TTT CGA CAT GCT T[2] /AAA TTC CTC CAG CTT CAG TTT TAG AC
Androgen receptor	AR-1	Receptor for testosterone and corresponding mimics	CGC GAG TGT GGC GAG TT[2] 3TCG CGC TGT CTC CGA AA
Pregnane X receptor	PXR-2	A steroid and xenobiotic sensing receptor for the detection of steroid exposure; involved in the activation of drug biotransformation	CTG GTC ACC GCA CAT CAA AAG[3] 3 ATG AGA GGT TGA CTG TCC GC
Hydroxysteroid dehydrogenase 17β	HSD-2	Gene involved in the reduction of estrone/androstenedione to estradiol-17β/ testosterone, the more active form of the steroid	CCA CCC TTA GAG GCG GAG TAT[3] /CAC AGC CAG TGT TGC TGC TT
Reference genes			PRIMER (5' - 3')
Elongation factor 1-alpha (Efα)	Efa-2	This protein promotes the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes during protein biosynthesis	TGC AGG GAG AAC TGA GAG AGA AG[3] /GGT CGT TCT TGC TGT CTC CA
Ribosomal protein l8	RPL8-2	Involved in the formation of ribosome subunit 18S for protein synthesis	GCT GCT GCT CCA TTT CAA AAG[3] /CGG TAT GGG TCA CGG AAA AC

[1]: [51]
[2]: [52]
[3]: This study

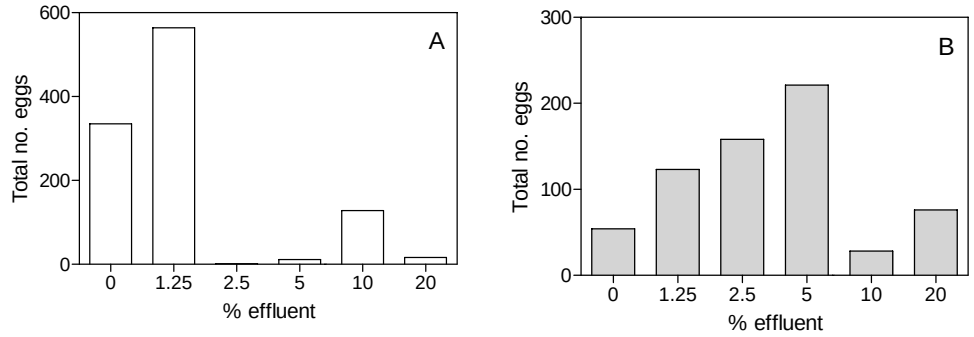

Figure 2. Fecundity of *Primephales promelas* exposed to municipal effluents from city A and city B during 12 weeks. Data expressed as total number of eggs produced during exposure.

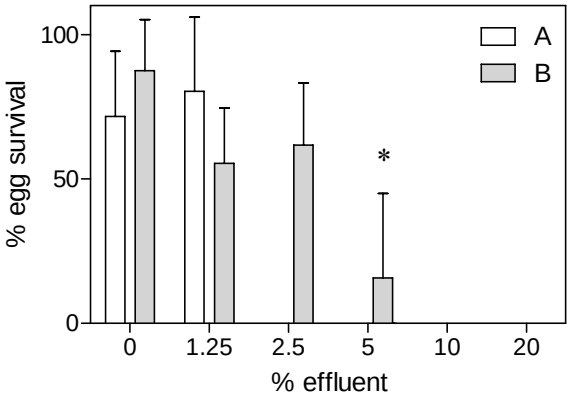

Figure 3. Percent egg survival. Eggs produced by FHM females exposed to municipal effluents were maintained in the same effluent concentration in which they were laid. Values expressed as mean ± standard deviation. The asterisk * indicates significant difference at $\alpha = 0.05$ (Mann Whitney U test).

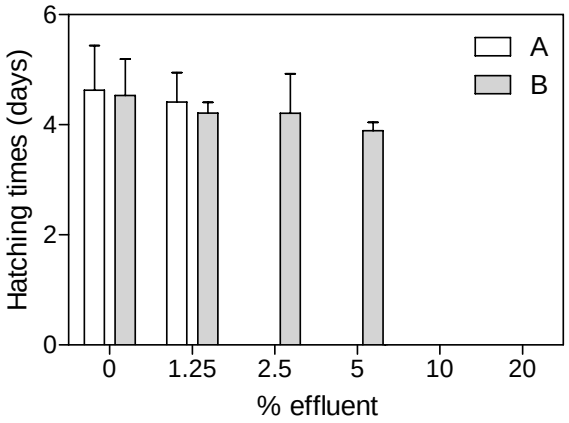

Figure 4. Hatching times of eggs produced by FHM females exposed to municipal effluents and maintained in the same effluent concentration in which they were laid. Values expressed as mean ± standard deviation. Number of exposed eggs for effluent A: 0% effluent n = 291; 1.25% n = 466; for effluent B 0% n = 24; 1.25% n = 60; 2.5% n = 112; 5% n = 220.

the effluent, for all concentrations combined (high-risk effluent: 4.5 ± 0.7 days; low-risk effluent: 4.2 ± 0.6.days) (Figure 4). In FHM exposed to an effluent that underwent physico-chemical treatment, decreased egg production was observed, and significantly increased energy expenditure and vitellogenin protein production was observed in the liver tissue of both males and females [29]. Furthermore, decreased egg production was significantly related to liver oxidative damage as determined by lipid peroxidation, pointing to the toxicity of municipal effluents.

The high-risk effluent was associated with increased embryo mortality at a 2.5% effluent concentration, whereas decreased hatching success was noticed at higher concentrations for the low-risk effluent. Taken together, these results are consistent with a less severe impact on reproduction from the low-risk effluent compared to the high-risk effluent. Reduced fecundity may also have resulted from decreased survival in males exposed to 10% and 20% concentrations of the low-risk effluent, which produced 100% male mortality before the end of the 12 weeks of exposure. It is noteworthy that a biphasic response was observed at low effluent concentrations for egg production. Indeed, an increase in the mean number of eggs occurred at a 1.25% concentration in the high-risk effluent and was followed by a decrease in the number of eggs at concentrations >5%. An identical pattern was

apparent in a previous study in which FHM were exposed to a historically estrogenic WWTP effluent. Cumulative fecundity was significantly increased in fish exposed to 20% effluent, but reduced in those exposed to 100% effluent [30]. The study also showed that the effluent was estrogenic, although estrogen receptor agonists were not the principal causes of reprotoxic effects on egg yield [30]. Indeed, the high-risk effluent resulted from a primary physico-chemical treatment designed to remove solids and suspended material only where pollutants readily remain in the effluent in the St. Lawrence River. Conversely, the low-risk effluent resulted from a more thorough treatment process: biofiltration, capable of removing suspended particles at high efficiency and relatively low volume discharge rates. In fact, this is the hypothesis of the present study and was further examined in the light of sublethal toxicological effects.

Identifying causative factors involved in adverse reproductive outcomes in fish exposed to complex mixtures such as those described in the present study is a challenge. Before examining concentrations of CEC, attention should be paid to abiotic factors like dissolved oxygen, pH and nitrogen concentrations, which have the potential to interfere with reproduction [31, 32]. In our study, basic water quality parameters (pH, NH_4, NO_2/NO_3, suspended matter, phosphates, fecal coliforms and BOD_5) were fairly similar for the different effluents. Thus, they cannot explain the reduced fecundity observed in fish exposed to effluents and can be excluded from the causes of reprotoxic effects. Furthermore, the presence and effects of emerging chemicals likely to have endocrine disrupting activity were assessed in both effluent sources in a previous study [20]. This could provide information on the chemical cues involved in these complex effluents and their effects on reproduction in fish. The study identified 16 compounds in both effluents [20]. Exposure to the high-risk effluents increased AR1 and PXR gene expression (Figure 5A). For the ER, although gene expression levels were higher, no significance was established owing to data variability. In fish exposed to the low-risk effluents, there was no significant difference in gene expression, and the gene expression data for ER also proved highly variable (Figure 5B). Indeed, the high- and low-risk effluents contained numerous compounds known to act as endocrine disrupters and sexual steroid receptor agonists, such as estrone, progesterone, testosterone, bisphenol A (BPA), and other related compounds like cholesterol, coprostanol and diclofenac [33]. Moreover, levels of progesterone, BPA, EE2 and diclofenac were significantly higher in the high-risk effluent than in the low-risk one. Among these four compounds, BPA was found at the highest concentration (593 ± 412 ng/L BPA; 40 ± 22 ng/L diclofenac; 17 ± 9 ng/L progesterone). Interestingly, the high-risk effluent did not always contain higher concentrations of some contaminants than the low-risk effluent. For example, cholesterol and coprostanol were detected at significantly higher concentrations in the low-risk effluent (median concentration = 3168 ng/L and 3684 ng/L, respectively, versus 1194 ng/L and 992 ng/L in the high-risk effluent) [20].

BPA has been proven to elicit endocrine disrupting activity in both *in vitro* and *in vivo* systems. BPA acts on many cellular pathway systems, including the gene expressions regulated *via* ER/AR steroid receptors, and the conversion of testosterone into estrogen by aromatase. It alters the function of PXR-2, which is involved in the production of steroids such as estrogens and the metabolism of steroids and xenobiotic compounds [34]. In fathead minnows, exposure to 10 µg BPA/L altered vitellogenin gene expression and steroid production after 4 days of exposure [35]. Reproduction in FHM was impaired after 21 days of exposure to 344 µg/L BPA [36]. In the present study, the concentration of BPA never exceeded 1.4 µg/L in the high-risk effluent. However, the exposure duration was longer and given the relative non-polarity of BPA (log Kow = 3.32, giving a theoretical bioconcentration factor of 90 [37]) the contribution of this contaminant, in part at least, to reproduction is conceivable. The reported concentrations of EE2 in both effluents would be sufficient to cause reproductive changes in fathead minnows. For example, FHM exposure to EE2 during a whole life cycle engendered detrimental effects at concentrations as low as 0.32 ng/L [38]. At this concentration, the fertilization rate was decreased by 20%, reaching 50% at 0.96 ng/L. No eggs were laid by fish exposed to concentrations >3.5 ng/L EE2 [38]. In another

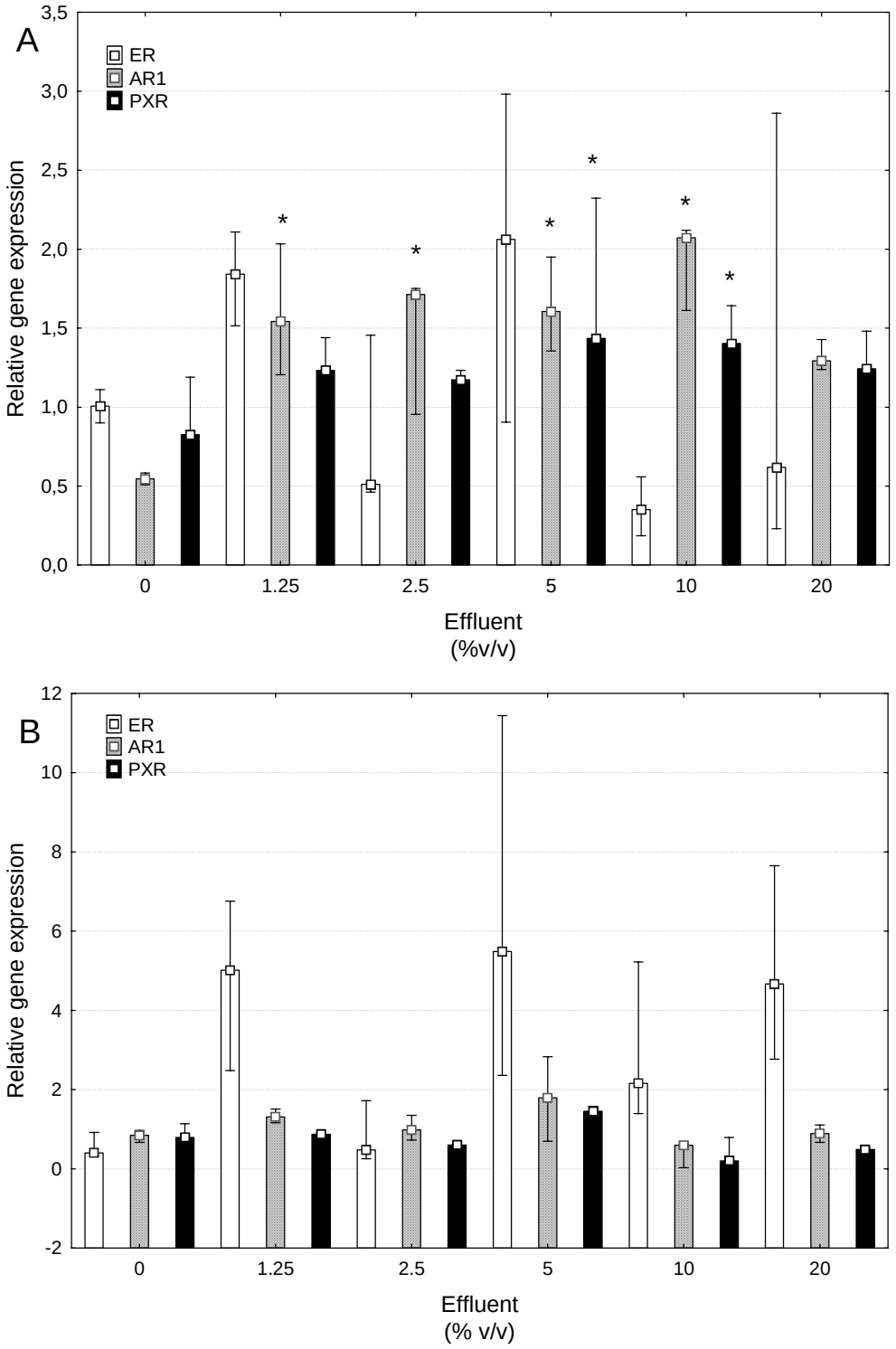

Figure 5. Gene expression of receptors involved in xenobiotic and steroid binding. Specific genes related to steroid hormone synthesis and metabolism were analyzed in the effluent-exposed fish as well as in control fish (0% effluent). The livers were collected and analyzed for expression of the described genes by real-time reverse transcriptase polymerase chain reaction. The data are expressed as the median with 25-75 centiles for the high- (A) and low-risk (B) effluents. The asterisk * indicates significant difference at $\alpha = 0.05$ compared to the control (Mann Whitney U test).

study, a significant increase in the mean number of eggs laid was observed at 0.1 and 1 ng/L EE2, whereas higher EE2 concentrations led to a dose-dependent decrease of fecundity [39]. Lange *et al.* established a no-observable effect concentration of 1 ng/L EE2, while higher concentrations produced negative impacts on growth, development, sexual development and reproductive health [40]. Other estrogenic chemicals could also contribute to the adverse effects on reproduction observed in our study. Indeed, diclofenac, at concentrations of 40 ± 22 ng/L and 32 ± 17 ng/L in the high- and low-risk risk effluents, respectively, was found to inhibit estrogen and androgen receptors in the T47D human cell line [41]. In *Oreochromis niloticus,* exposure to diclofenac at environmentally relevant concentrations (0.1 to 1 µg/L) was involved in estrogenic activity as evidenced by induction of vitellogenin gene expression, sexual differentiation and gametogenesis disorders [42]. Interestingly, the herbicide atrazine and its metabolite desethylatrazine were at higher concentrations in the high-risk effluent and barely detected in low risk effluents [20]. This herbicide is a known endocrine disruptor and negatively impacts FHM reproduction. Cumulative egg production of atrazine-exposed FHM was significantly decreased, by more than 20%, relative to the controls after 20 days of flow-through exposure to 0.5 µg/L atrazine [43]. Moreover, several genes involved in the development of reproductive organs were found to be altered in zebrafish after acute developmental atrazine exposure [44]. In another study, low doses of atrazine were shown to alter expression of steroidogenesis genes in the gonad, estrogen receptor in the liver, and gonadotropins in the brain of exposed Medaka and fathead minnow females, which resulted in a decline in egg production [45]. Illicit drugs along with their main metabolites (cocaine, benzoylecgonine, MDMA, mephedrone, methylephedrine) were detected in both the low and high risk effluents [20]. However, these drugs have been marginally tested for toxicity to aquatic organisms. Toxicity testing of illicit drugs should be expanded to include investigation of their effects on reproduction and their endocrine disrupting potential. Taken together, our results highlight the reprotoxic effects of two types of municipal effluents treated by different processes.

The endocrine disruptive effects of the effluents were tested in adult fathead minnows using a suite of gene expression markers involved in reproduction and steroid biotransformation. Specific genes related to steroid hormone synthesis and metabolism were analyzed by real-time PCR in fish exposed to both effluents: VTG-1, ERα-1, AR-1, HSD-2, PXR-2, and CYP3A (Table 2). AR-1 and PXR-2 gene expression was not significantly altered by the effluent concentration for both types of effluents. Estrogenic activity of the municipal effluents is shown in figure 6, expressed as VTG-1 gene expression for males and females. The high-risk effluent caused a significant increase in VTG-1 expression in females at the lowest concentration used (1.25%). Although VTG-1 expression was increased in males, low replication prevented statistical testing. VTG-1 gene expression was increased 21-fold in males relative to the control group and, on average, 54-fold in females at the 1.25% effluent concentration. The high-risk effluent induced a greater increase in VTG-1 expression than the low-risk effluent. In fact, the low-risk effluent did not cause significant VTG-1 gene induction in males and a moderate 5-fold induction was observed in females at the highest concentration tested (20%). CYP3A gene expression was significantly upregulated in the high-risk effluent only (Figure 7). Although the changes in ERα-1 gene expression were not significantly different owing to large variations, they were significantly correlated with VTG gene expression ($\rho = 0.63$; $p < 0.001$) and HSD ($\rho = -0.59$; $p < 0.001$), and VTG transcripts were significantly influenced by effluent concentrations. Based on VTG, a well-recognized biomarker of exposure to estrogenic contaminants in fish [46, 47], the high-risk effluent appeared to be 10 times more estrogenic than the low-risk effluent. Male FHM exposed to a low dose of EE2 for 35 days (7 weeks) showed a rapid increase in VTG mRNA levels after only 3 days [48]. Municipal wastewaters, ultimately discharged into the St. Lawrence River (Canada), were found to be estrogenic *in vitro*, as determined from the relative increase of VTG mRNA in rainbow trout hepatocytes [23]. Cavallin *et al.* found a significant concentration-dependent response for the male FHM hepatic VTG mRNA levels in fish exposed to a municipal effluent [30]. A recent survey of wastewaters before and after treatments from over 12 cities in Canada revealed that 50% of wastewaters were estrogenic to fish hepatocytes after treatment while 75% of untreated raw

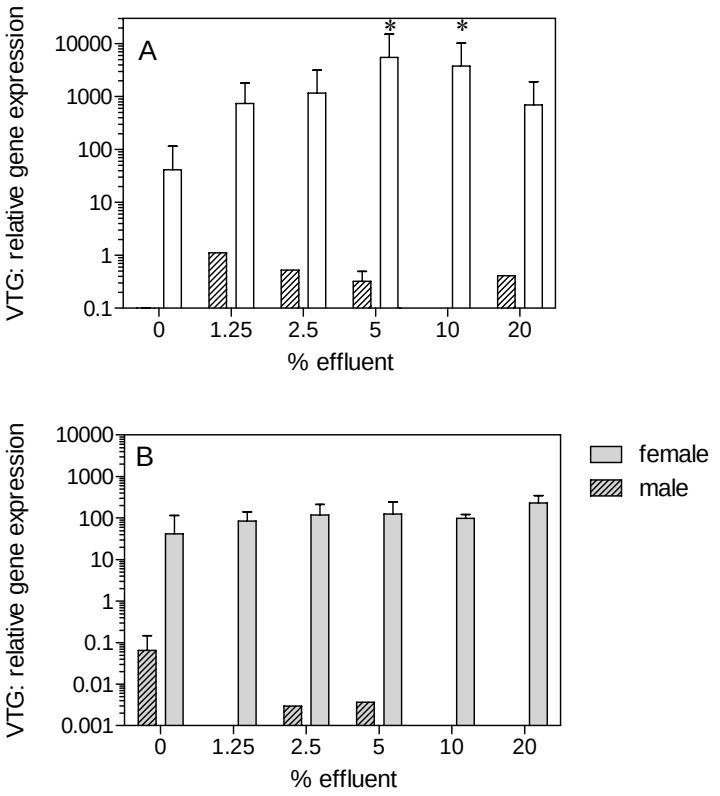

Figure 6. Adult FHM were exposed for 12 weeks to the high-risk (A) and low-risk (B) municipal effluent. The reference genes were elongation factor 1. The asterisk * indicates significant difference at $\alpha = 0.05$ compared to the control (Mann Whitney U test).

wastewaters were estrogenic [49]. The expression of HSD-2 (involved in the conversion of estradiol into estrone, a less active form) and CYP3A (involved in steroid inactivation) genes was measured to examine the xenobiotic and steroid biotransformation activity of the municipal effluents (Figure 6). Consistent with VTG up-regulation, CYP3A transcript abundance was increased after exposure to the high-risk effluent at low concentrations (<10%) ($p < 0.05$ Mann-Whitney U test), whereas no induction was observed for the low-risk effluent. In the low-risk effluent, correlation analysis indicated that HSD-2 gene expression was significantly correlated with CYP3A gene expression ($\rho = 0.7025$, $p < 0.05$). This suggests that HSD-2 and CYP3A4 gene expression are involved in mitigating the amount of estrogenic signalling in the liver of FHM.

The endocrine disrupting activity of the high-risk effluent was further examined through a factorial analysis of reproductive endpoints and gene expression (Table 3). This analysis revealed that 87% of the variance was explained by two factors. The most important factor (48%) implicated VTG-1, AR-1 and CYP3A as genomic effects and egg survival. HSD-2, PXR-2 and the total number of eggs were the major components for the second factor, with factorial weights >0.7, which explained an additional 39% of the variance. In the low-risk effluent, factorial analysis showed that the CYP3A and HSD-2 genes had the highest factorial weights and explained 32% of the variance. The remainder of the variance (30%) was explained by ERα-1 and PXR-2 gene expression, and then by the hatching times (which accounted for 17% of the variance). Based on this analysis, egg survival and hatching time involved CYP3A4, HSD-2 and PXR-2 gene expression. CYP3A gene expression is involved in the metabolism and elimination of steroids and pharmaceutical products, suggesting that this molecular endpoint can be associated with adverse effects at the reproductive level (an adverse outcome pathway). However, linkage

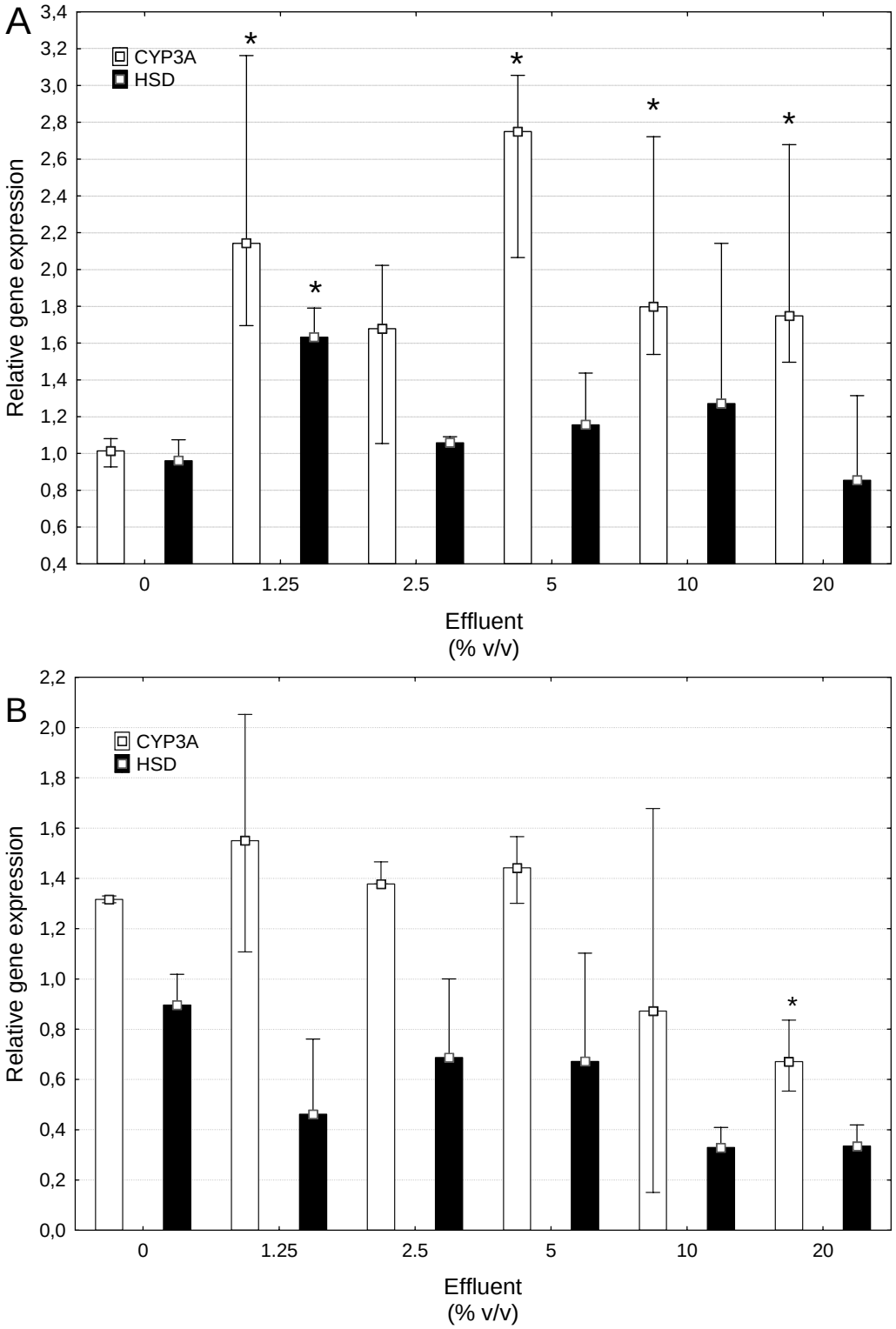

Figure 7. Xenobiotic and steroid biotransformation activity of the municipal effluents A and B. Adult FHM were exposed for 12 weeks to the municipal effluent. The livers were collected and analyzed for gene expression of the described genes by real-time reverse transcriptase polymerase chain reaction. The data represent the media with 25-75 centiles. The asterisk * indicates significant difference at $\alpha = 0.05$ compared to control (Mann Whitney U test).

Table 3. Factorial analysis of biological endpoint results after exposure to high and low-risk effluents. Data in bold indicate factorial weight >0.7, which are considered as principal components.

	High-risk effluent		**Low-risk effluent**		
	Factor 1	**Factor 2**	**Factor 1**	**Factor 2**	**Factor 3**
Cyp 3A	**-0.82**	-0.55	**0.92**	-0.13	0.12
VTG	**-0.99**	0.10	0.67	-0.38	-0.57
AR1	**-0.83**	0.48	0.69	0.56	0.36
Era-1	-0.66	0.57	-0.04	**0.80**	0.38
PXR 2	-0.12	**-0.72**	-0.31	**0.81**	-0.27
HSD	0.27	**-0.89**	**-0.73**	0.32	-0.46
Nb of eggs	0.37	**-0.85**	0.05	0.62	0.10
Egg survival	**-0.82**	-0.55	-0.56	-0.52	0.12
Hatching time	0.52	0.55	-0.41	-0.43	**0.76**
Variance explained (%)	48	39	32	30	17
Cumulative (%)	48	87	32	62	79

between transcriptomic responses and physiological effects must be established with caution. In one study, fecundity changes were not associated with either ER activation or EE2-equivalents in FHM exposed to a WWTP effluent [30]. However, using BPA as model xenoestrogen, Villeneuve *et al*. observed that transcriptomic responses in a short-term study had sensitivity at least comparable to the apical responses in longer-term experiments with FHM and zebrafish [35]. In the present study, both effluents exhibited estrogenic activity based on the results obtained for VTG and ERα gene expression. These results corroborate the findings obtained for the YES-assay in the estrogenicity potential assessment for the same high- and low-risk municipal effluents [20]. Other studies have provided evidence for the hypothesis that effluents resulting from primary treatment of municipal wastewater have been found to retain high levels of natural and synthetic steroidal estrogens [50, 53], whereas more extensive treatment, as in the case of the low-risk effluent may result in greater removal [1].

4. CONCLUSION

This study compared the potency of two wastewater treatments in relation to fish reproduction. Reproductive toxicity was found in the high- and low-risk effluents at corresponding potencies. In addition, VTG, HSD and PXR-1 gene expressions also permitted discrimination between the low- and high-risk effluents in this study and were associated with reproductive endpoints such as egg survival and hatching time.

ACKNOWLEDGMENTS

This research was funded by the Canadian Water Network and the St. Lawrence Action Plan of Environment and Climate Change Canada. We are grateful for the technical assistance provided by Joanna Kowalczyk for fish and liver preparations for gene expression analysis.

CONFLICT OF INTEREST STATEMENT

The authors declare no financial or other conflict of interest.

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