Distribution of antidepressants and their metabolites in brook trout exposed to municipal wastewaters before and after ozone treatment – Evidence of biological effects

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Abstract

This study examined the tissues distribution of selected serotonin reuptake inhibitors (SSRIs) in brook trout exposed for 3 months to continuous flow-through primary-treated effluent before and after ozone treatment. A reliable analytical method was developed for the quantification of trace amounts of antidepressants in small tissue homogenate extracts. Levels of six antidepressants and four of their N-desmethyl metabolites were determined using liquid chromatography–tandem mass spectrometry. Significant amounts of the SSRIs were found in fish tissue—in decreasing order: liver > brain > muscle. Sertraline and its metabolite desmethylsertraline were the predominant substances observed in most tissues (0.04–10.3 ng g⁻¹). However, less SSRIs (0.08–1.17 ng g⁻¹) were bioaccumulated in the ozonated effluent. The early molecular effects of these SSRIs on the Na/K-dependent ATPase pump activity in brain synaptosomes were also investigated in vitro and in fish exposed to the municipal effluents. With respect to their potential biological effects, in vitro exposure to selected SSRIs induced a reduction of the brain Na/K-ATPase activity in synaptosomes in a dose-dependent manner. Results showed that Na/K-ATPase activity was readily inhibited by exposure to municipal effluent before and, to a lesser extent, after ozone treatment. Moreover, the Na/K-ATPase activity was significantly and negatively correlated with brain tissue concentrations of fluoxetine (r = −0.57; p < 0.03), desmethylsertraline (r = −0.84; p < 0.001), and sertraline (r = −0.82; p < 0.001). The present study reveals that SSRIs are readily available in fish, biologically active and corroborates previous findings on the serotonergic properties of municipal effluents to aquatic organisms.

1. Introduction

Over the last decade, pharmaceutical and personal-care products (PPCPs) have been recognized as a major source of pollution for the aquatic environment (Halling-Sørensen et al., 1998; Daughton and Ternes, 1999). Their occurrence in most municipal effluent seems to indicate that conventional wastewater treatments have only a limited ability to remove such substances from sewage. In the 1990s, it was discovered that some active drugs have the capability to interfere with ecosystems in concentrations as low as a few nanograms per liter (Halling-Sørensen et al., 1998). Until now, different pharmaceutically active compounds (PhACs) have been detected in the environment: analgesics, antibiotics, antiepileptics, antidepressants, synthetic and natural hormones (Jones et al., 2006; Lishman et al., 2006). Antidepressant drugs are among the PhACs likely to be found in Canada (IMS Health Canada, 2006), United States (Schultz et al., 2010), and other countries around the world (Calisto and Esteves, 2009). The commonly prescribed antidepressants from the class of selective serotonin reuptake inhibitors (SSRIs) have become the most successful class of marketed antidepressants, with the number of new prescriptions written for these drugs now exceeding that of tricyclic antidepressants (DeVane, 1999).

To date, most analytical methods for determining the presence of antidepressants and their respective metabolites in environmental matrices have been developed for surface waters and wastewater effluents (Lishman et al., 2006; Lajeunesse et al., 2008; Schultz and Furlong, 2008; Metcalfe et al., 2010), sediments (Schultz et al., 2010), and sewage sludge (Radjenović et al., 2009). Due to the complexity of samples, these analyses typically employ liquid chromatography–tandem mass spectrometry (LC–MS/MS).
Since the occurrence of antidepressants in municipal wastewater could lead to physiological and behavioural effects on aquatic organisms (Fong, 1998; Calisto and Esteves, 2009), it is critical that these compounds be measured in organisms using reliable analytical methods. Even so, only a few analytical methods have been reported on antidepressants in aquatic organisms (Brooks et al., 2005; Chu and Metcalfe, 2007; Ramirez et al., 2007; Metcalfe et al., 2010; Schultz et al., 2010). Unfortunately, the required mass of tissues often reached up to 2 g, making difficult the detection of SSRIs and their metabolites with small organisms or limited quantity of target tissues.

As antidepressants are present at relatively low levels in the environment (e.g. ng L−1), risk for acute toxic effects is unlikely, but chronic environmental toxic effects cannot be excluded (Péry et al., 2008; Carlsson et al., 2006). The potential ecotoxicological impacts of neuroactive drugs on aquatic organisms are not well understood at the present time. Moreover, the lack of information about the fate and long-term subtle effects of these compounds and/or their metabolites in aquatic organisms makes accurate risk assessment difficult (Gagné and Blaise, 2004; Stanley et al., 2007; Paterson and Metcalfe, 2008). Hence, the measurement of the Na/K-dependent ATPase pump activity in synapses represents a more specific biomarker of the effects of SSRIs (Kanoh et al., 1998). The serotonin reuptake pump (or transporter) exchanges one K ion per molecule of serotonin. The loss of K ions outside the synapse button is counterbalanced by the Na/K-ATPase pump which maintains Na and K ions respectively outside and inside of the membrane. This process requires energy (e.g. ATP → ADP + inorganic phosphate) that can be easily measured (Kakko et al., 2003). Thus, the presence of SSRIs decreases the serotonin-dependent hydrolysis of ATP in neuron synaptosomes by the competitive inhibition of the transporters. At present, the modulation of Na/K-dependent ATPase activity in nerve synaptosomes by the SSRIs in fish is largely unknown. Despite its possible usefulness, this promising methodology alone cannot bring sufficient information about the exact concentration of drug needed to elicit biological effects in fish. Therefore, a combination of a biomarker of effects and a tissue SSRIs analysis would strengthen the link between the presence of this class of contaminants in target tissues and the resulting early biological effects.

In this study, we report the development of a reliable LC–MS/MS method for the trace detection of antidepressants in tissues of brook trout exposed to treated (ozone 15 mg L−1) and non-disinfected effluent at the Montreal wastewater treatment plant (WWTP). The developed method was applied to determine the concentrations and distribution of antidepressant in liver, brain, and muscle tissues. Based on bioaccumulation results, a new efficient biomarker (Na/K-serotonin-dependent ATPase activity in neurons synaptosomal membranes) is proposed to track the early biological effects of SSRIs.

2. Experimental

2.1. Chemicals and standards

Certified standard (>98% purity grade) fluoxetine (FLU), norfluoxetine (NFLU), paroxetine (PAR), sertraline (SER), and (S)-citalopram (CIT) were provided by Toronto Research Chemical Inc. (North York, Ontario, Canada). Desmethylsertraline (DSER), venlafaxine (VEN), O-desmethylvenlafaxine (DVEN), and propioni-d9 were obtained from Nanjing Jilong PharmaTech (Nanjing, China). Amitriptyline (AMI) and nortriptyline (NTRI), HEPES, sodium chloride, sodium hydroxide, ethylenediaminetetraacetic acid (EDTA), diguithiohethanol, aprotinin, sucrose, magnesium chloride, and the bioluminescence commercial ATP detection kit based on luciferin/luciferase enzymatic system were purchased from Sigma–Aldrich Co. (St. Louis, Missouri, USA), while cis-tramadol13C-d3 was purchased from Cerilliant Corp. (Round Rock, Texas, USA). The high-performance liquid chromatography–grade solvents (methanol and acetonitrile) and ammonium hydroxide were provided by Caledon Laboratories Ltd. (Georgetown, Ontario, Canada). Reagent-grade hydrochloric acid, acetic acid, monobasic potassium phosphate, and ammonium bicarbonate were provided by American Chemicals Ltd. (Montreal, Quebec, Canada). Solid-phase extraction (SPE) cartridges of 6 mL, 500 mg (Strata-SCX™) were purchased from Phenomenex (Torrance, California, USA). Stock solutions of 100 mg L−1 of each substance were prepared in methanol and stored at 4 °C in amber glass bottles. All corrosive and pure standard chemicals were handled carefully under a ventilated fume hood wearing appropriate protection.

2.2. Experimental procedures

2.2.1. Instrumental parameters

A Thermo Finnigan Surveyor liquid chromatograph (LC) quadrupole pump equipped with an autosampler was used in this study. As reported in a previous paper (Lajeunesse et al., 2008), analytes were separated on a C18 column using a binary gradient mode of (a) ammonium bicarbonate (10 mM) pH 7.8, and (b) acetonitrile at a flow rate of 200 μL min−1. Volume of injection was 20 μL for biota extracts. Eluted analytes were monitored by a Thermo Finnigan TSQ Quantum Ultra triple quadrupole tandem mass spectrometer equipped with an electrospray ionization source. The capillary was held at 5.0 kV and the cone was operated at an optimized voltage for each analyte in positive-ion mode (+ESI). Additional instrument parameters and analytical details are available (Lajeunesse et al., 2008). The specificity of all target analytes was insured by the use of two MS/MS transitions in multiple reactions monitoring (MRM) mode. All optimized mass spectrometer parameters are available as Supporting information in Table S1, Appendix A.

2.2.2. Fish collection and preparation

Brook trout (Salvelinus fontinalis), 20 cm long, were obtained from a commercial fish hatchery (Station piscicole Trois-Lacs of Wotton, Quebec, Canada). A total of 50 fish per mesocosm (volumetric capacity of 150 L) were exposed for 3 months at the Montreal WWTP to 10% and 20% v/v of final effluent (mean effluent flow rate of 140 mL min−1). Supplementary hatched trout were exposed to 10% and 20% v/v of effluent previously treated with 15 mg L−1 of ozone. During exposure, around 15% of mortality by microbiological infections was observed in mesocosms containing diluted final effluent. However, no fish mortality was reported in mesocosms containing final effluent treated with ozone. Typically, the Montreal WWTP processes 2.7 million m3 of raw sewage daily, yielding a representative mean effluent discharge of 19.8 m3 s−1. Final effluent is discharged directly into the St. Lawrence River. Montreal’s treatment plant uses a physico-chemical process, which involves a grit removal and a physico-chemical treatment by flocculation and sedimentation to reduce suspended matter and phosphorus content. Dissolved organic carbon (DOC) concentrations and pH values ranged from 90 to 110 mg L−1 and 8.1 to 8.2 respectively (Gagnon et al., 2008). Brook trout were dissected in order to obtain brain, liver, and filet (muscle) tissues. Because of limited quantities, fish homogenates (100 mg) composed of pooled tissue (10 alive specimens for each tested conditions) were diluted in 1.0 mL of an ice-cold 10 mM HEPES–NaOH (pH 7.4) buffer solution containing 150 mM NaCl, 1 mM EDTA, 0.1 mM dithiothreitol, and 1 μg mL−1 aprotinin. All tissue samples were stored at −80 °C until analysis. Total proteins were determined using serum bovine albumin for calibration (Bradford, 1976).
2.2.3. Sample extraction

Fish homogenate samples were combined with 9 mL of a solution composed of methanol/0.1 M acetic acid buffer solution pH 4.0 (1:1 v/v) in a 16 × 150 mm borosilicate glass screw-top conical tube. To each tube were added 100 μL of a surrogate standard solution in methanol (bupropion-d9: 0.25 mg L⁻¹) equivalent to 25 ng. Samples were then shaken vigorously and mixed on a rotary extractor (Caframo REAX 2) for 15 min. After extraction, samples were centrifuged at room temperature and 320g for 5 min. Following the solid-phase extraction (SPE) protocol of Lajeunesse et al. (2008), supernatants were transferred to strong cation exchange cartridges (Strata-SCX, Phenomenex) preconditioned with 4 mL of methanol and at least 8 mL of the same extraction buffer solution. The dried extracts containing 50 μL of cis-Tramadol-d3 in methanol (2.5 mg L⁻¹) as internal standard (ISTD) were reconstituted with 0.5 mL of the mobile phase solution made of ammonium bicarbonate (10 mM) pH 7.8/acetonitrile (1:1 v/v).

2.2.4. Na/K-ATPase activity determinations

Total serotonin content and the activity of Na/K-dependent and Mg-dependent ATPases in synaptosomal membranes were determined in the brain of brook trout based on a previous methodology (Kakko et al., 2003). Brain synaptosomes were prepared from the homogenates as follows: homogenates (a portion was kept for total serotonin) were centrifuged at 3000g for 15 min at 2 °C. The supernatant was overlaid on cold 0.8 M sucrose containing 10 mM HEPES–NaOH and 1 mM EDTA and centrifuged at 10 000g for 20 min at 2 °C. The synaptosomes (at the 0.8 M sucrose fraction) were collected from the mitochondria (pellet) and diluted one third in distilled water. Na/K-ATPase and Mg-ATPase activities were determined in synaptosomes by mixing the synaptosomes with 100 μM ATP with or without 50 μM serotonin in 10 mM HEPES–NaOH buffer at pH 7.4 in the presence of either 140 mM NaCl and 5 mM KH₂PO₄ or 50 mM MgCl₂. The reaction mixture was incubated for up to 30 min at 30 °C, and the levels of ATP were measured using a bioluminescence commercial ATP detection kit based on a luciferin/luciferase enzymatic system. The effects of serotonin and selected SSRIs (CIT, AMI, PAR, and FLU) were also examined in synaptosome from control (unexposed) fish. This process involved pre-treating the membranes to increasing concentrations of PAR and SER for 15 min before taking the ATPase measurements. The data were expressed as ATP hydrolysis min⁻¹ mg proteins⁻¹. Total serotonin levels were also determined using a competitive immunoassay procedure developed in our laboratory. Details about this protocol are available as Supporting information in Appendix B.

3. Results and discussion

3.1. Chemical analysis

3.1.1. LC–MS/MS method validation

Recovery tests were completed on spiked reference brook trout tissues. Additional validation tests were also performed on two different freshwater mussel tissues. The percent recovery of each antidepressant (e.g. 25 ng of antidepressant in 200 mg of tissue) was calculated as follows: the ratio of the spiked sample (area analyte/area ISTD) divided by the ratio of the standard previously prepared by spiking set amounts of analytes in the extracted matrix. Satisfactory results of recovery were obtained for all extracted tissues (data available in Fig. S1, Appendix A).

The developed analytical method enables a sensitive detection of antidepressants in all tissues. Limit of detection (LOD) values were defined as the minimum detectable amount of analyte giving a signal-to-noise ratio (S/N) of 3.1 using the Xcalibur 1.2 software (Thermo Fisher). Mean LOD value of 0.01 ng g⁻¹ (variability: 6.7%) was estimated for fish tissues extracts when 200 mg of wet tissues were spiked with 25 ng of each antidepressant. The higher individual LOD variability (%) was found with brain tissues samples (e.g. SER, up to 19.3%). However, lower variability was observed with liver tissues (e.g. DSer, 0.5%). Following the same procedure, mean LOQ values were determined by a signal-to-noise ratio (S/N) of 10:1. The LOD and LOQ values are given as Supporting material in Table S2, Appendix A. Matrix effects were also investigated on all extracted tissues. To prevent possible losses due to extraction, the ratio of the spiked extracted matrix (25 ng in 200 mg of tissue) minus the ratio of the extracted matrix alone was compared to a 25-ng pure non-extracted standard prepared directly in the mobile phase. For practical purposes, a relative signal response to matrix effects has been reported. Values around 1.0 indicate no appreciable signal suppression or enhancement: results clearly depict limited matrix effects on the overall ion signal of each tissue (Fig. S1, Appendix A). The highest ion suppression occurred within brain tissue samples (~12% versus pure non-extracted standard solution). In order to overcome possible matrix interference within tissues, standard addition curves were used for quantitation. Furthermore, before each extraction, a standard surrogate (bupropion-d9) was added to ensure the quality control of the extraction process. Recoveries of 70–120% are normally obtained for standard surrogate, otherwise the overall sequence is rejected. During quantification process, no adjustments were done for antidepressants against the STD surrogate response. The linearity of antidepressants had been tested across a range of concentrations that was previously reported for pharmaceuticals in fish residues (Ramirez et al., 2007). Hence, the linearity of standard addition calibration curves yielded a correlation coefficient r² > 0.995 for a concentration range of 0.50–50 ng per 200 mg of spiked tissue. The mean ratios of two zero points (e.g. extracts alone without any standard addition) in the calibration curve were used to quantify the analytes in each type of tissue.

3.1.2. Application to fish analysis

Fish that were exposed to ozone- or primary-treated municipal effluents were sampled and investigated for antidepressants. The occurrence of the antidepressant target molecules in liver, brain, and filet (muscle) tissues was confirmed by observing two multiple reactions monitoring (MRM) transitions from mass spectrometer (except FLU, NFLU), and also by their respective average retention times (tₘ) ± 0.20 min. The validated protocol enables the sensitive detection of the eight studied antidepressants using only 100-mg tissue homogenates. The new methodology proved to be a valuable tool in the analytical characterization of antidepressants (e.g. SSRIs, tricyclic) and their metabolites in a variety of biological tissues, especially when the mass of tissues is limited. The relative distribution and concentrations of each detected antidepressant from brook trout exposed to 20% v/v of effluent are represented in Fig. 1.

As described in the previous section, brook trout were exposed for 3 months in a continuous flow-through setup at the Montreal WWTP to 10% and 20% v/v of renewed effluent. The main goal of using a continuous flow-through system was to simulate the dilution factor retrieved in the receiving water of the St. Lawrence River at 0.3 km and 1 km from the effluent outfall. Others studies were also conducted with this system, using 10% and 20% v/v of effluent previously treated with 15 mg L⁻¹ of ozone (Hébert et al., 2008). As shown in Table 1, all studied antidepressants have been detected in final effluent waters used for mesocosm exposition.

The extraction procedure was successfully applied to the analysis of all tissue homogenates (Table 2). An extracted ion chromatogram (EIC) of DSer and SER from LC–MS/MS analysis of brook trout exposed to 20% v/v of effluent is shown in Fig. 2. The highest concentrations of antidepressant were retrieved in liver tissues.
(0.20–10.29 ng g\(^{-1}\)) dissected from fish exposed to the less-diluted primary-treated effluent. Other results revealed that up to 2.08 ng g\(^{-1}\) of DSER was also found in brain tissues from fishes exposed to the same effluent. Therefore, based on those cumulated results after mesocosm exposures, it appears that detected antidepressants could possibly bioaccumulate in aquatic organisms at distances of 0.3 and 1 km from the effluent outfall in the St. Lawrence River. The reported occurrence of antidepressants in the receiving water of the St. Lawrence River (Lajeunesse et al., 2008) strengthened this hypothesis. Whereas our results proved the occurrence of most selected antidepressants in each of the trout tissues, such active drugs may be distributed in other fish species living in an effluent-dominated stream.

Since bioavailability studies are scarce, only limited comparisons could be made on the occurrence of antidepressants in fish tissue extracts. Indeed, it is somehow difficult to make a direct comparison between two or three different studies because mediums of exposure (e.g. mesocosms, lakes, and rivers), ambient concentrations of antidepressant, exposure time, and fish species might be different. Nevertheless, the concentration of DSER in liver tissue was found to be consistent with the concentration previously reported elsewhere (Brooks et al., 2005). Hence, the mean concentration of DSER in liver (10.3 ± 0.8 ng g\(^{-1}\)) was comparable to the observed concentration by Brooks et al. (2005) for three different species of fish in Texas, USA (12.9 ± 10.5 ng g\(^{-1}\)). However, they reported higher concentrations of FLU (1.3 ± 0.7 ng g\(^{-1}\)), N azi fluoxetine (10.3 ± 5.7 ng g\(^{-1}\)) and SER (3.59 ± 1.67 ng g\(^{-1}\)) when compared to our results (FLU, 0.20 ± 0.06 ng g\(^{-1}\); N azi fluoxetine, 0.63 ± 0.17 ng g\(^{-1}\); SER, 0.29 ± 0.05 ng g\(^{-1}\)). In muscles, similar concentrations of FLU, DSER

![Fig. 1. Mean concentrations of antidepressant in 100 mg of tissue extracts from brook trout exposed in mesocosms to 20% v/v of effluent at the Montreal WWTP.](image)
and SER were detected (0.12–0.42 ng g\(^{-1}\)). Recently, Schultz et al. (2010) have reported similar concentration of DSER (1.8–3.0 ng g\(^{-1}\)) in brain homogenates from native White Suckers species exposed downstream to outfalls discharging of two effluent-impacted streams located in Colorado and Iowa, USA. Another study completed in Ontario (Canada) by Metcalfe et al. (2010) reports on the positive detection of five antidepressants cumulated in whole Fathead Minnows species caged at locations downstream of WWTPs. Interestingly, during our bioaccumulation study the N-desmethyl metabolites were mostly measured at higher concentrations in individual liver tissues than their parent molecules. Brooks et al. (2005) and Schultz et al. (2010) observed the same trends for some antidepressants in different fish species. Nevertheless, results have shown much lower concentrations of antidepressants in fish exposed to disinfectant effluent by ozone treatment (15 mg L\(^{-1}\)). A pilot study conducted at the Montreal WWTP in 2005 and 2006 demonstrated the effectiveness of ozone disinfection on several classes of pharmaceuticals (Gagnon et al., 2008). In fact, a removal rate of 70% was achieved for six of the eight pharmaceuticals detected in the effluent when using an optimal ozone concentration of 15–20 mg L\(^{-1}\). Therefore, the remarkable efficiency observed with ozone treatment would certainly decrease the bioavailability of antidepressants to fish exposed to wastewater effluents.

According to our calculated tissue concentrations, the estimated bioconcentration factors (BCFs) for 20% v/v effluent mesocosm (pH: 8.1–8.2) ranged from 2 to 12 250 (Table 1). The highest BCFs were retrieved for liver tissue extracts: DSER (12 250), NFLU (1750), PAR (350), and FLU (345). However, the distribution and bioaccumulation are less important in hydrophobic tissue (e.g. filet) under experimented pH condition (ionized species seem to be much more important than ionized species). Our calculated BCF values for NFLU and FLU were found comparable with those reported by Nakamura et al. (2008) after an exposition of Japanese medaka (Oryzias latipes) at pH 8 during 30 d (NFLU: 3300, FLU: 580). Nevertheless, despite its usefulness, the BCF remained somewhat difficult to calculate. As pointed out by Metcalfe et al. (2010), the origins of metabolites detected in fish tissues could be assigned to direct uptake or by in vivo metabolism. Indeed, a metabolism study conducted by Smith et al. (2010) on rainbow trout liver microsomes showed that hepatic FLU metabolism in fish was much less than that seen in mammals and the major mammalian metabolite, NFLU, was possibly not the primary metabolite of FLU in fish. They also suggested that the low level of metabolism could likely be the reason that fish bioaccumulate FLU in vivo. These assumptions are consistent with our results and could possibly explain why the metabolite NFLU was detected neither in brain nor in filet tissues. However, the physico-chemical properties of the antidepressants and the water pH seem also to play an important role in the molecules distribution (Nakamura et al., 2008; Metcalfe et al., 2010). Therefore, more data and additional information related to the metabolism and the bioaccumulation would be desirable to clarify the possible environmental pathways of antidepressants along their biologically metabolites under given pH conditions.

### 3.2. Na/K-ATPase activity

In addition to using LC-MS/MS for the quantification of bioaccumulated antidepressants, this paper investigated biomarkers of effects sensitive to this class of pharmaceuticals. The potential toxicological effects of the SSRIs in municipal effluents on brook trout were also examined. First, a methodology for evaluating the activity of serotonin reuptake was developed and optimized by following the rate of Na/K-dependent ATPase activity in synaptosomal membrane vesicles. As clearly depicted in Fig. 3A, the addition of 5-hydroxytryptamine or serotonin (10–100 \(\mu\)M) in synaptosomal membrane vesicles induced an increase in ATP hydrolysis, i.e. serotonin stimulation of the ATPase activity to maintain the electromotive gradient in synaptosomal membranes.

Pre-incubation with selected SSRIs (CIT, AMI, PAR, and FLU) was also able to reduce the activity of the ATP-dependent Na/K-ATPase. To verify the validity of the chosen biomarker, Na/K-dependent ATPase, four SSRIs antidepressants were added in synaptosomal membrane vesicles to block serotonin transport. As expected, Na/K-ATPase activity was decreased by the addition of 100 \(\mu\)M of SSRIs to the membrane preparations (see Fig. 3B). The reuptake of serotonin at the membrane interface occurs with the efflux of K\(^+\), which in turn stimulates ATPase to maintain the electroconductive gradient. According to experimental results, the SSRIs reduced Na/K-ATPase activity where PAR and FLU were the most potent ones. In addition, Mg-dependent ATPase activity was also affected by these drugs, but to a lesser extent. Among the four antidepressants tested, PAR was found to be the most potent SSRI for brook trout, followed by FLU and AMI.

Total serotonin and Na/K-ATPase activity was analyzed in brain synaptosomes from trout exposed to primary-treated effluent before and after ozone treatment. Total serotonin content remain constant in fish tissues (ANOVA \(p = 0.24\), results not shown). However, the Na/K-dependent ATPase activity was readily inhibited by the municipal effluent before and after ozone treatment (Fig. 4). Ozone treatment seemed to mitigate this effect, but not completely; Na/K-dependent ATPase activity was still inhibited at
20% v/v effluent dilution. This effect is consistent to the presence of SSRIs in urban effluent. This was further supported by the significant correlation between SSRIs in tissues and Na/K-ATPase in synaptosomes. However, this relationship cannot rule out the possibility that other compounds in effluent might have contributed to the observed responses. Considering the importance of eventually defining a hazard assessment for SSRIs to fish, the reported methodology for measuring toxicological effects caused by antidepressants may represent a promising means of determining whether these changes are linked with drug content in studied tissues.

The Na/K-dependent ATPase activity was negatively correlated with brain tissue concentrations of FLU ($r = -0.57; p < 0.03$), DSER ($r = -0.84; p < 0.001$), and SER ($r = -0.82; p < 0.001$). These results suggest that the presence of SSRIs in the brain, in part at least, was associated to lower Na/K-ATPase activity in synaptosomal membranes, which is in agreement with the mode of action of these drugs and the observed responses during the in vitro experiments (Fig. 3). In keeping with biomarkers used for pharmaceuticals in the environment (Gagné and Blaise, 2004), physiological biomarkers that respond to the mode of action of drugs and address the

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**Fig. 3.** Modulation in vitro of Na/K-dependent and Mg-dependent ATPase activity in the brain tissues by selected SSRIs following (A) added serotonin, and (B) pre-treatment with antidepressants.
toxic consequences are needed in environmental studies. Based on the present study, the Na/K-ATPase activity biomarker was shown to respond to these bioaccumulated SSRIs and other compounds targeting electrolyte balance in membranes. This highlights the need to strengthen the relationships between the presence of serotonergic-like compounds and serotonergic effects in complex environmental mixtures. The data presented for the effluent-exposed fish highlighted the evidence that municipal effluents contained serotonin mimicking compounds (Gagné and Blaise, 2003). Indeed, solid phase C-8 extracts of surface water downstream the same municipal effluent of the present study were capable of activating human serotonin receptors and incubation of female ripe gonads of Elliptio complanata to the filtrated municipal effluent, significantly stimulated egg release (Gagné et al., 2004).

4. Conclusion
A sensitive and rugged LC–MS/MS method has been developed for the simultaneous analysis of antidepressants along with their respective N-desmethyl metabolites in limited fish tissues extracts samples. The results reported supports the observation that antidepressant may accumulate in fish living in an effluent-dominated stream. In the present study, most drug residues partitioned in liver and brain tissues depicting the possibility of distribution in specific tissues. The higher concentrations of antidepressant were observed in liver tissues when the brook trout were exposed to 20% v/v of effluent. Results have shown much lower concentrations of antidepressants in fish exposed to disinfected effluent by ozone treatment (15 mg L⁻¹).

However, these interesting observations cannot rule out other contaminants given the complex nature of municipal effluents. The combination of tissue loadings and early biological effects provide useful information on potential ecotoxicological impacts of discharged drugs in urban effluents to aquatic life.

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Appendix A. Supplementary material

References


