Biological Responses in Brook Trout (*Salvelinus fontinalis*) and Rainbow Trout (*Oncorhynchus mykiss*) Caged Downstream from Municipal Wastewater Treatment Plants in the Credit River Watershed, ON

A Report to the Ontario Wildlife Foundation

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Introduction

Many different contaminants are discharged into aquatic ecosystems in the effluents from wastewater treatment plants (WWTPs). Previous studies have shown that these contaminants adversely affect fish living downstream of wastewater discharges, including inducing gonadal intersex, endocrine disruption and oxidative stress (Sumpter and Jobling, 1995; Tetreault et al., 2011; Diamond et al. 2016). The upper reaches of the Credit River watershed are important habitat for cold water fish species, including brook trout (*Salvelinus fontinalis*). Brook trout populations are declining in the tributaries of Lake Ontario (Stanfield et al., 2006). Exposure to contaminants discharged from WWTPs may be contributing to the disappearance of this salmonid species in southern Ontario. Research to evaluate whether WWTP discharges are affecting brook trout will help to develop effective programs for salmonid conservation.

Our previous research has shown that prescription drugs, including various antidepressants are present in rivers downstream of WWTPs and these pharmaceuticals can accumulate in fish tissues (Metcalfe et al., 2010; Chu and Metcalfe, 2007). Our recent work has also shown that illicit drugs, including cocaine, amphetamines and opioid drugs are also present in surface waters impacted by discharges from WWTPs (Rodayan et al., 2016). Studies conducted with mammalian models have shown that antidepressants and illicit drugs can alter the levels of neurotransmitter substances in the brain, including the monoamine neurotransmitters, serotonin and dopamine (Berger et al., 2009; Beaulieu and Gainetdinov, 2011). In fish, the levels of serotonin and dopamine are important neuroendocrine signals for stimulating reproduction (Prasad et al., 2015; Dufour et al., 2010; Mennigen et al., 2010). Thus, it is possible that exposure of fish to antidepressants and illicit drugs in surface waters impacted by wastewater discharges can affect the ability of fish to reproduce.

In this study, conducted through the support from the Ontario Wildlife Foundation, we caged juvenile brook trout and young of year (YOY) rainbow trout (*Oncorhynchus mykiss*) upstream and downstream from the wastewater discharges of two WWTPs that serve small communities in the Credit River watershed. Fish exposed in the caging studies for two weeks were sacrificed and tissues analyzed for biomarkers of exposure to contaminants, including a biomarker for exposure to estrogenic chemicals (i.e. vitellogenin), a biomarker of exposure to chemicals that cause oxidative stress (i.e. TBARS) and levels of dopamine and serotonin in brain tissue. These studies will indicate whether trout exposed to contaminants discharged in municipal wastewater into the Credit River show biological responses that could affect their ability to survive and reproduce. This work was conducted at Trent University, Peterborough, ON by an MSc student, Mr. Stephen McGovarin working under the supervision of Prof. Chris Metcalfe.

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Study sites

The Credit River watershed, located northwest of Lake Ontario is the location that was chosen to study the biological effects of contaminants discharged from WWTPs. Brook trout populations in southern Ontario are mostly restricted to the upper reaches of river systems, such as the Credit River. Within the Credit River watershed, the WWTPs that serve the small communities of Acton and Orangeville, respectively discharge into upper tributaries of the river that are historical habitat for brook trout. The WWTP in Acton has a rated treatment capacity of 4,545 m^3/day and UV disinfection, and the WWTP for the municipality of Orangeville has a rated treatment capacity of 19,850 m^3/day and disinfects with chlorine, followed by dechlorination prior to discharge.

Fish caging and water quality monitoring

All experiments with fish were approved by the Animal Care Committee of Trent University. Working with the Ministry of Natural Resources and Forestry (MNRF), 65 YOY rainbow trout were collected using electrofishing equipment at a site downstream of Black Creek located within the city of Georgetown. In addition, a total of 40 juvenile brook trout were provided by the MNRF's local brook trout stocking program.

In November, 2016, the juvenile brook trout and YOY rainbow trout were caged in 4 L minnow traps for a 2 week time period in locations 40 meters upstream and 100, 200, and 600 meters downstream from the WWTPs serving the municipalities of Orangeville and Acton. The fish cages each contained 4-5 juvenile brook trout and six YOY rainbow trout. After two weeks of deployment, the fish cages were retrieved and all fish were sacrificed. The brain, gill and liver tissues from brook trout were flash frozen and taken back to Trent University for storage in a -80^{o}C freezer. The rainbow trout were sacrificed, and then immediately fixed in buffered formalin.

During the cage deployment period, water samples were collected, and stream flow conditions and water quality parameters were monitored (i.e. pH, Dissolved Oxygen, temperature). The water samples were frozen for later analysis of selected contaminants.

Biomarkers

1) Lipid Peroxidation (TBARS)

Oxidative stress occurs when reactive oxygen species (ROS), that are naturally produced in cells as a result of respiration, are not completely inactivated (Di Giulio et al., 1980). Under normal conditions, ROS are inactivated by antioxidant enzymes in the cell (Winston and Di Giulio, 1991).

However, under conditions of oxidative stress, such as when organisms are exposed to contaminants, antioxidant enzymes are unable to cope with the production of ROS and this leads to oxidative damage to lipid molecules, causing lipid peroxidation (Hageman et al., 1992). Oxidation of fatty acids that are components of cell membranes and organelles could lead to cell death (Oost et al., 2003). A test to determine levels of thiobarbituric acid reactive substances (TBARS) in tissues is a widely-utilized biomarker of lipid damage in the tissues of organisms (Stegeman et al., 1992; Hai et al., 1995). TBARS were analyzed in gill tissue from brook trout using a colourimetric assay with a UV-VIS microplate reader, as describe previously by Diamond et al. (2016).

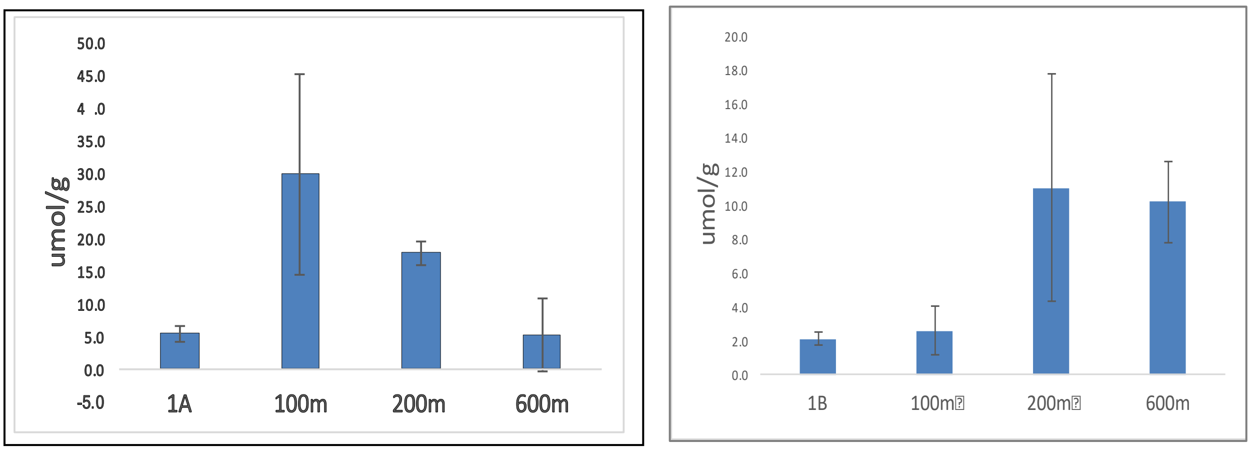

Figure 1. Mean (±SE) levels of TBARs (µmol/g) in gill tissue from brook trout caged upstream and downstream of the WWTPs serving the communities of Orangeville (Left) and Acton (Right).

As shown in Figure 1 (left), brook trout caged downstream from Orangeville's WWTP showed evidence of lipid damage in gill tissues, as indicated by significantly elevated TBARS in fish caged at the two sites closest to the discharge (i.e. 100 and 200 m downstream). In brook trout caged downstream of Acton's WWTP (Figure 1, right) there was also evidence of lipid damage in the gills of trout deployed downstream of the discharge, although this response was not observed in fish caged at the site closest to the WWTP (i.e. 100 m). It is not immediately clear why there was no elevation of TBARs in these fish, while trout caged further downstream showed evidence of damage from oxidative stress.

2) Vitellogenin

Vitellogenin (VTG) is a protein that is used to synthesize yolk in developing eggs (i.e. oocytes) in the ovary of female fish (Sumpter and Jobling, 1995). The synthesis of VTG in the liver is triggered by the elevated levels of the natural estrogen, 17ß-estradial (E2) that occurs when female fish are preparing to spawn (Tetreault et al., 2011). In male fish, the VTG protein is absent due to the naturally low levels of E2. However, a variety of estrogenic substances released from WWTPs can induce synthesis of VTG in male fish (Bahomonde et al., 2014; Tanna et al., 2013; Tetreault et al., 2011). Monitoring levels of VTG in juvenile fish or in mature male fish is widely used as a biomarker of exposure to estrogenic compounds in the aquatic environment. These estrogenic substances can include chemical contaminants (e.g. bisphenol A), natural hormones excreted into wastewater, or pharmaceuticals, such as the active ingredient in the birth control pill (i.e. ethinylestradiol). VTG was analyzed in liver tissue from brook trout using a commercial enzyme linked immunoassay kit from TECO Medical, as measured using a fluorescence plate reader.

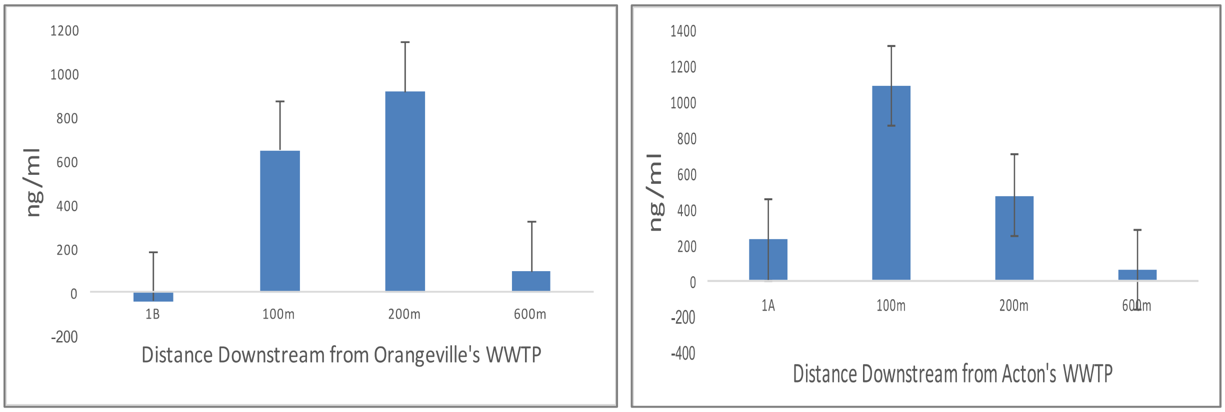

Figure 2. Mean (±SE) levels of VTG (ng/g) in liver tissue from brook trout caged upstream and downstream of the WWTPs serving the communities of Orangeville (left) and Acton (right).

The results from the VTG analysis showed significantly elevated levels of VTG protein in the livers of brook trout deployed downstream of both WWTPs (Figure 2). These data indicate that the fish were exposed to estrogenic substances released from the WWTP's discharges. Prolonged exposure to estrogenic substances could result in the "feminization" of exposed fish and reduced reproduction (Bahomonde et al., 2014; Tanna et al., 2013).

3) Monoamines

Dopamine and serotonin are neurotransmitters that control the behaviour of vertebrates. Antidepressants and illicit drugs directly influence the serotonergic and dopaminergic systems in the mammalian brain (Esposito, 2006; Biesesi et al., 2016). Exposure to antidepressants and illicit drugs discharged from WWTPs may also alter the levels of serotonin and dopamine in fish. Since levels of serotonin and dopamine regulate reproduction in teleosts (Prasad et al., 2015; Dufour et al., 2010; Mennigen et al., 2010), modulation of these neurotransmitters may influence the reproductive capacity of exposed fish. We developed a method for analyzing serotonin and dopamine in the brain tissues of fish that involves extraction of brain tissue homogenates in 0.1 formic acid solvent, centrifugation of the extracts, and analysis of the extracts with the Thermo Q-Exactive Orbitrap mass spectrometer available at Trent University's Water Quality Center. These analyses will be completed in the next few weeks to determine if there is modulation of the levels of serotonin and dopamine in the brain tissues of brook trout downstream of the WWTP discharges.

4) Water Quality Parameters

The water quality data and stream discharge (i.e. flow) rates that were collected in the field at the time of deployment of fish cages show very little differences between sites (Table 1).

Table 1. Water quality parameters and stream discharge recorded from downstream locations below the sites of discharge of the WWTPs in Acton and Orangeville.

Parameters	Acton	Orangeville
Water Temperature (°C)	10	12
pH	7.5	7.9
Dissolved Oxygen (mg/L)	10	9.5
Discharge Downstream from WWTPs (f^3/s)	0.89	1.08

5) Water Samples

Water samples were collected upstream as well as downstream from both Acton and Orangeville's WWTPs. These water samples will be analysed for a variety of pollutants, including illicit drugs (e.g. cocaine, amphetamines), antidepressants (e.g. venlafaxine, fluoxetine, sertraline, citalopram), and compounds that are indicators of wastewater discharges (i.e. caffeine and artificial sweeteners).

6) Brain Imaging

In studies with rats, Gonzalez et al (2011) reported that monoamine concentrations differed among various areas of the brain, and that the ratios of dopamine and serotonin in these regions may control physiological responses. Except for very large fish, it is not practical to prepare extracts from different areas of the teleost brain. However, using the imaging capabilities of a high-resolution mass spectrometer (Buck et al., 2015), we can map out the concentrations of monoamines in various regions of tissue sections prepared from the brains of exposed fish. Although this aspect of the project was not included in the original project proposal, this highly original and innovative study will be conducted over the next few months using the fixed brain tissues from the rainbow trout. The high-resolution mass spectrometer (i.e. FT-ICR/MS) instrumentation for these imaging analyses is available at the Water Quality Centre at Trent University.

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