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THE PERSISTENCE OF STEROIDAL ESTROGENS IN THE AQUATIC ENVIRONMENT

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ABSTRACT

The presence and fate of the steroidal estrogens, estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2), in Ottawa and Cornwall wastewater treatment plants (WWTP's), drinking water, and the river water used as the drinking water source, were identified. Estrogens were extracted using accelerated solvent extraction, gel permeation chromatography and solid phase extraction, and identified using gas chromatography-mass spectrometry and internal C-13 standards. E1, E2 and EE2 concentrations ranged from 1.8 to 370, 24.3 to 66.9 and 0.4 to 9.8 ng L^{-1}, respectively, and were affected by weather variables such as temperature and precipitation, and WWTP parameters such as daily flow and carbonaceous biochemical oxygen demand. Photodegradation rate constants under ultraviolet B radiation for E1 were directly proportional to radiation intensity and sample thickness, and inversely proportional to dissolved organic carbon concentration, but EE2 was remarkably persistent. A luciferase reporter gene assay found estrogenicity in both sewage effluent and UVB-exposed samples of estrogens, contributed by the degradation products of steroidal estrogens. Finally, EE2 persistence was also seen in a time-course experiment in which goldfish were exposed to 25 ng L^{-1} EE2. A mass-balance model calculated a bioconcentration factor (BCF) for EE2 in fish blood of 1400, whereas measured data revealed a maximum BCF of only 500.

RÉSUMÉ

La présence et le comportement des estrogènes stéroïdes, estrone (E1), 17β-estradiol (E2) et 17α-ethinylestradiol (EE2), dans les eaux usées et potables ainsi que dans l'eau des rivières utilisée come source d'eau potable, à Ottawa et Cornwall, Ontario, ont été identifiés. Les estrogènes ont été extraits en utilisant l'extraction par solvant à haute pression, la chromatographie sur gel et l'extraction sur phase solide, et ont été séparés et détectés par la chromatographie à phase gazeuse, la spectrométrie de masse et des standards internes de type C-13. E1, E2 et EE2 ont été mesurés à des concentrations de 1.8 à 370, de 24.3 à 66.9 et de 0.4 à 9.8 ng L^{-1}, et ont été affectés par la température et la précipitation ainsi que les paramètres du traitement des eaux useés comme l'écoulement quotidien et la demande biochimique en oxygène. Les constantes de vitesse de photodégradation sous la radiation ultraviolet B pour E1 étaient directement proportionelles à l'intensité de la radiation tandis qu'elles étaient indirectement proportionelles à la concentration de carbone organique dissous. Malgré ceci, EE2 a été remarquablement persistent. Un essai gène indicateur de luciferase a demontré de l'estrogenicité, résultant de produits de dégradation, dans les eaux usées ainsi que dans les échantillons d'estrogènes exposés à la radiation UVB. Finalement, la persistance de EE2 a été observée dans un essai d'exposition de poissons rouges à 25 ng L^{-1} EE2. Un facteur de bioconcentration (BCF) pour EE2 dans le sang de poisson de 1400 a été calculé à partir d'un modèle masse-équilibre, tandis que les données pour les expositions ont révélées un BCF maximum de 500.

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For Toby and M.C.

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LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

AAF	annual average flow (ML)
APEO	alkylphenol polyethoxylate
ASE	accelerated solvent extraction
BAF	bioaccumulation factor
BCF	bioconcentration factor
BFFBB	1,4-bispentafluorobenzoylbenzene
BFTSA	*N, O*-bis(trimethylsilyl)trifluoroacetamide
BMF	biomagnification factor
BPA	bisphenol-A
CAREG	Centre of Advanced Research in Environmental Genetics
cBOD	carbonaceous biochemical oxygen demand (mg L^{-1})
DCM	dichloromethane
DDT	dichloro-diphenyl-trichloroethane
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DOC	dissolved organic carbon (ppm)
DTE	dithioerytrol
E1	estrone
E1-3S	estrone-3-sulfate
E2	17β-estradiol
E3	estriol

EDC	endocrine disrupting compound
EE2	17α-ethinylestradiol
EEq	estrogen equivalent
EI	electron impact
ELISA	enzyme-linked immunosorbent assay
ER	estrogen receptor
ERE	estrogen response element
ESI	electrospray ionization
FE	final effluent
FSH	follicle stimulating hormone
GC	gas chromatography
GPC	gel permeation chromatography
GSI	gonadosomatic index
H	Henry's law constant (Pa m^3 mol^{-1})
HP	high performance
HR	high resolution
HRT	hydraulic retention time (hours)
IDL	instrument detection limit (ng L^{-1})
k_1	uptake rate constant (h^{-1})
k_2	elimination rate constant (h^{-1})
KOH	potassium hydroxide
K_{ow}	octanol/water partition coefficient
LC	liquid chromatography

LH	leutinizing hormone
LOEC	lowest observable effect concentration (ng L^{-1})
LOEL	lowest observable effect limit
M	molecular weight (g mol^{-1})
Masl	meters above sea level (m)
MDL	method detection limit (ng L^{-1})
MRNA	messenger ribonucleic acid
MS	mass spectrometry
MSTFA	*N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide
MTBSTFA	*N*-methyl-*N*-*tert*-butyldimethylsilyltrifluoroacetamide
MW	molecular weight
n	sample size
nav	not available
n/a	not applicable
nd	not detected
NCI	negative chemical ionization
NOEC	no observable effect concentration (ng L^{-1})
NP	nonylphenol
ns	not significant
OP	octylphenol
p	probability of a Type 1 error in t-tests
PFBCl	pentafluorobenzoyl chloride
PFBO	pentafluorobenzoyl chloride

PFPA	pentafluoropropionic acid anhydride
Q	daily plant flow (ML)
RED	reduction equivalent dose
RIA	radioimmunoassay
ROPEC	Robert O. Pickard Environmental Centre
RS	raw sewage
SD	standard deviation
SE	standard error
SIM	selected ion monitoring
SPE	solid phase extraction
STP	sewage treatment plant
T	temperature (°C)
TMSI	trimethylsilylimidazole
TSS	total suspended solids
UV	ultraviolet radiation
VTG	vitellogenin
WWTP	wastewater treatment plant

CHAPTER 1: INTRODUCTION

1.1 GENERAL INTRODUCTION

Estrogenic chemicals are a class of endocrine-disrupting compounds (EDC's) that mimic the function of the principal natural estrogen, 17β-estradiol. 17β-estradiol enters cells freely and then binds to estrogen receptor proteins (ER's) that activate transcription by binding to DNA estrogen response elements (ERE's). The proteins formed from the subsequent translation of mRNA are essential to the functioning of an animal's endocrine system, which controls growth, reproductive development and behaviour (Purves et al. 1997). Elevated estrogen levels increase rates of breast cancer and tumour development, and decrease testicular growth and sterility (Roy and Liehr 1999; Coyle 2004; Jobling et al. 1998; Carreau 2000). Similarly, the presence in the environment of estrogenic chemicals like 17β-estradiol, poses a threat to an animal's growth, development and behaviour (Kang et al. 2002; Palanza et al.1999; Guillette 2000).

The aquatic environment is at particularly high risk for exposure to estrogenic compounds due to the release of domestic sewage effluent, industrial wastewater, and run-off from agricultural lands, which can contain both natural estrogenic compounds and synthetic estrogenic compounds, or xenoestrogens. Natural compounds include the steroidal estrogens estrone (E1), 17β-estradiol (E2), estriol (E3) and testosterone. Xenoestrogens consist of a myriad of chemicals used in pharmaceuticals, industrial processes and agriculture. These include (1) the synthetic steroidal estrogens 17α-ethinylestradiol (EE2), mestranol and estradiol valerate, all used in oral contraceptive

pills; (2) nonylphenol (NP) and octylphenol (OP), the degradation products of alkylphenol polyethoxylate (APEO) surfactants used in nonionic detergents; (4) bisphenol-A (BPA) used in the production of polycarbonate, epoxy and other plastics; (5) the phytoestrogen β-sitosterol produced as a byproduct of the pulp and paper industry; and (6), herbicides and insecticides such as atrazine and DDT (dichloro-diphenyl-trichloroethane) (Metcalfe et al. 2001; Herman and Mills 2003; Bennie et al. 1997; Thiele et al. 1997; Ternes et al. 1999a; MacLachty and van der Kraak 1995.).

In the 1980's, scientists reported elevated vitellogenin in the blood of male rainbow trout (*Oncorhynchus mykiss*) that were exposed to sewage effluent in British streams (Purdom et al. 1994). Vitellogenin (VTG) is an egg yolk protein precursor normally found in female oviparous animals and is a common biomarker for estrogen exposure. Estrogen induces the synthesis of VTG, which is exported from the liver into the blood stream and picked up by the developing oocyte in females (Sherry et al. 1999; Copeland et al. 1986). However, males also possess the VTG gene, and exposure to estrogens will induce VTG synthesis in male fish. Exposure to wastewater effluent increases VTG production in fish, as well as inhibiting testicular growth and producing intersex fish, a disorder where male and female gametocytes are found in a single gonad (Jobling et al. 1998; Tyler and Routledge 1998). An accumulation of VTG can lead to kidney failure and may decrease metabolic energy used for growth and spermatogenesis (Chen 1983; Folmar et al. 2001; Schwaiger et al. 2000). Of all the estrogenic compounds, chemical fractionation and *in vitro* biological screening have identified E1, E2 and EE2 as the three greatest sources of estrogenic activity in domestic sewage effluent (Desbrow et al. 1998; Nakada et al. 2004). Domestic sewage effluent is the

greatest source of steroidal estrogens to the aquatic environment, but runoff from agricultural lands also contributes to their presence. In the last few decades, extensive research has been done on steroidal estrogens, to understand their behaviour in the aquatic environment, and to predict what risks they pose to wildlife populations.

1.2 PHYSICOCHEMICAL PROPERTIES OF STEROIDAL ESTROGENS

The primary female sex hormones are steroidal estrogens. There are three types of naturally occurring estrogens: estrone (E1), 17β-estradiol (E2), and estriol (E3). E1 is the least prevalent of the three hormones, and its derivative, estrone sulfate, mainly acts as a pool to produce the more active E2, which is the main estrogen in humans. E3 is only produced in significant amounts during pregnancy, as it is made by the placenta. Among their many functions, estrogens promote formation of female secondary sex characteristics and are involved in the thickening of the endometrium and other aspects of regulating the menstrual cycle (Purves et al. 1997). They reduce blood concentrations of FSH (follicle stimulating hormone) and LH (leutinizing hormone); oral contraceptives, which contain a combination of estrogen and progestin, use this feedback mechanism to prevent ovulation (Purves et al. 1997). They also play an important role in the development of the mammalian brain and bone formation, and in males, estrogens are important to the maturation of sperm (Cao et al. 2003; Belcher 2008).

Natural steroidal estrogens are synthesized in the gonads using cholesterol as a precursor, whereas EE2 is synthesized in the lab using 17β-estradiol as the initial reagent (Lai et al. 2000). E1, E2 and EE2 are all organic compounds with a tetracyclic structure.

However, small chemical variations in their functional groups produce enormous functional differences (Purves et al. 1997). All contain an aromatic A-ring with a hydroxyl group at the C3 position and a methyl group at the C13 position, but the D-ring structure differs, with a carbonyl, hydroxyl and alkyne group at the C17 position for E1, E2 and EE2 respectively (Figure 1; National Library of Medical Information Specialized Information Services 2006; Wang et al. 2008).

E1, E2, and EE2 are hydrophobic due to their steroid ring structure. Their high octanol/water partition coefficients (K_{ow}) values reveal a strong tendency to bind to organic surfaces (Lai et al. 2000; Lai et al. 2002c). Their low vapour pressures prevent volatilization, unlike for other estrogenic chemicals (Dachs et al. 1999). Table 1 lists some of the physicochemical properties of these three estrogens which may be used to predict their behaviour in the aquatic environment.

1.3 THREAT TO AQUATIC ORGANISMS

The lessons learnt from the study of endocrine-disrupting compounds need to be applied to the study of steroidal estrogens in addressing their threats to aquatic organisms (Sumpter and Johnson 2005). For example, groups of organisms may show dissimilar responses to chemicals, and continual exposure to low concentrations, variable concentrations and mixtures may cause significant effects. These points are of extreme relevance to the exposure of aquatic organisms to steroidal estrogens as the chemicals enter the aquatic environment in a complex mixture (domestic sewage effluent or runoff).

Many laboratory studies clearly illustrate the effects of steroidal estrogens on

aquatic organisms; also important is the field evidence to show that these compounds adversely impact wild populations at low concentrations. To determine the long-term effects of E1, E2 and EE2 on populations, researchers need to determine if intersex organisms can produce gametes and whether these gametes are viable, and they need to consider the effect of sterile organisms on other members of their communities (Sumpter 1998).

Many studies use fish as a model to relate alterations in development, growth and reproduction because they are representative of other species, and there is extensive knowledge of their endocrinology (Blázquez et al. 1998; Tyler et al. 1996). Therefore, the majority of studies addressing the effects of steroidal estrogens on aquatic organisms focus on fish.

EE2 is the more potent steroid estrogen in fish, followed by E2 and E1. The differences in potencies are caused by differences in their estrogen receptor affinities (de Voogt and van Hattum 2003). The lowest observable effect concentration (LOEC) for E1, E2 and EE2 by vitellogenin induction in rainbow trout (*Oncorhynchus mykiss*) is 44, 9 and 0.1 ng L^{-1} respectively (Thorpe et al. 2003; Routledge et al. 1998; Purdom et al. 1994). The responses and relative sensitivities of different animal species to steroidal estrogens may vary, both within and between taxa (Van den Belt et al. 2003). For example, LOEC's are lower for juvenile rainbow trout than adults, at 3.3 ng L^{-1} for E1 (Thorpe et al. 2003). LOEC's for E2 by vitellogenin induction are also lower in rainbow trout than for other species like roach (*Rutilus rutilus*); salmonids like rainbow trout have naturally higher VTG plasma concentrations compared to cyprinids like roach, which may be a consequence of larger egg sizes in salmonids (Routledge et al. 1998).

Invertebrates and amphibians are also affected by steroidal estrogens at similar concentrations (Lafront and Mathieu 2007; Mitsuit 2007). Embryo production in the snail *Potamopyrgus antipodarum* is diminished at E2 concentrations ranging between 4 and 56 ng L^{-1}, and at EE2 concentrations as little as 2 ng L^{-1} (Jobling et al. 2003). Roepke et al. (2005) looked at the effect of E2 and EE2 on the sea urchins *Strongylocentrotus purpuratus* and *Lytechinus anamesus* and found significant development inhibition, where embryos and larvae either did not reach the same stage as controls, or developed abnormally with misshapen spicules, incomplete guts, missing arms or flattened bodies, at low ng mL^{-1} concentrations of E1, E2 and EE2 .

Population-level effects of steroidal estrogen exposure have been observed in a number of studies, in a number of different taxa. In a seven-year study in Northwestern Ontario, a lake population of fathead minnow (*Pimephales promelas*) collapsed after being exposed to weekly additions of 5-6 ng L^{-1} EE2 (Kidd et al. 2007). Male fish showed arrested testicular development and female fish showed delayed ovarian development. Due to reproductive failure and loss of young-of-the-year, the minnow population was near extirpation after three years. A multi-generation exposure of the Chinese rare minnow to EE2 (*Gobiocypris rarus*) shows the reproduction of the F1 generation is completely inhibited at an even lower level, 0.2 ng/L (Zha et al. 2008). Whole-lake addition experiments of 5 ng L^{-1} EE2 disrupted hatching and gonad development of green frog tadpoles (*Rana clamitans*) producing a skewed sex ratio of green frog tadpoles within a year (Park and Kidd 2005).

Another study to address the population-level impacts of steroid estrogens investigated the long-term exposure to EE2 in zebrafish (*Danio rerio*) (Nash et al. 2004).

Like in other studies the authors found exposure to EE2 resulted in disturbed sexual differentiation and sterility; however, they observed regular male-pattern reproductive behaviour during spawning in sterile fish. The result of sterile males competing with healthy males resulted in a disruption of fertilization. This implies that estrogenic compounds may not only reduce reproductive ability in exposed individuals, but in entire populations associated with these individuals.

In the environment, aquatic organisms more commonly encounter several steroidal estrogens in combination, as well as variable concentrations, as the chemicals primarily originate from sewage effluent, which is a complex variable mixture of trace contaminants (Jukovsky et al. 2008; Vermeirssen et al. 2005). Even at no observable effect concentrations (NOEC's), chemicals may induce an effect when in a mixture. Thorpe et al. (2003) showed that a mixture of E2 and EE2 was more potent to juvenile rainbow trout than either of the individual chemicals, indicating that effects caused by steroidal estrogens are additive. Because levels of hormones and VTG in fish fluctuate throughout the year due to the natural reproductive cycle, exposed fish may only receive detrimental concentrations of steroid estrogens during certain periods of the year (Copeland 1986; Blázquez et al.1998). Concentrations of estrogenic compounds will also vary depending on precipitation events, creating water runoff from agricultural fields and dilution of sewage effluent. However, Panter et al. (2000) showed that intermittent exposure of fish to E2 resulted in increased VTG concentrations approximately equal to concentrations in continually exposed fish, concluding that estrogenic effects may last well beyond the time of exposure.

Clearly, assessments that do not take into account the age and taxa of an exposed

organism, or the length and complexity of exposure to estrogen-containing effluent and surface waters, may underestimate the risk of steroidal estrogens to organisms in the environment. Currently, many assessments do not take into account the trophic level of the aquatic organism, and because steroidal estrogens have a high K_{ow}, they may bioaccumulate and biomagnify, increasing the severity of exposure for higher trophic levels.

1.4 RESEARCH OBJECTIVES

My objectives were to investigate factors affecting concentrations of steroidal estrogens in aquatic environments and organisms. First, methods were developed to measure estrone, 17β-estradiol and 17α-ethinylestradiol in water, blood and tissue (Chapter 2). Then sampling was undertaken in sewage treatment plants, rivers and drinking water in Ottawa and Cornwall, Ontario to determine E1, E2 and EE2 concentrations in ambient waters. Population demographic data, city environmental data, sewage treatment plant hydrology and water chemistry data were collected to predict factors that affect these levels in water (Chapters 3 and 4). Finally, fish were exposed to low concentrations of the steroidal estrogens to investigate their bioconcentration dynamics (Chapter 5).

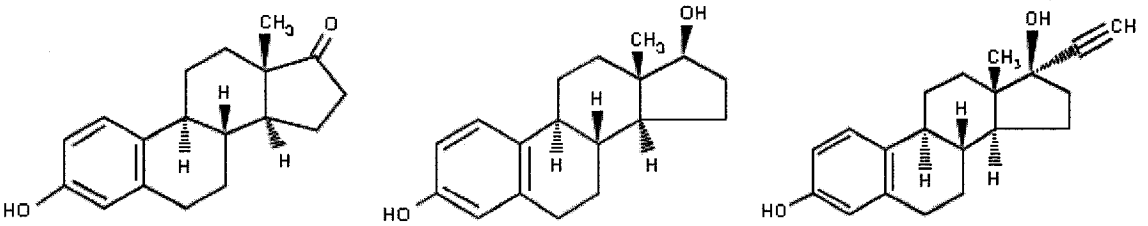
My predictions were as follows: (1) the concentrations of steroidal estrogens found in Ottawa and Cornwall would decrease from raw sewage to final effluent to receiving waters, and to drinking water, more so in Ottawa which has secondary sewage treatment than in Cornwall with only primary treatment; (2) temperature, water flow,

hydraulic retention time, dissolved organic concentration and UVB radiation would affect the persistence of these compounds; and (3), ethinylestradiol, the most hydrophobic of the steroidal estrogens, would bioconcentrate in goldfish.

Table 1.1 **Physicochemical properties of estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2); molecular weight (MW), density, water solubility, Log octanol/water partition coefficient (K_{ow}) and vapour pressure are listed**

Property	E1	E2	EE2
MW (g)	270.37	272.3	296.4
Density (g mL^{-1}	1.16	1.17	1.21
Water solubility (mg L^{-1})	13	13	4.8
Log K_{ow}	3.43	3.94	4.15
Vapour pressure (mm Hg @ 25 °C)	1.54×10^{-8}	9.82×10^{-9}	3.74×10^{-9}

Data based on Wang et al. (2008), Lai et al. (2002c) and ChemZoo (2008)



estrone (E1) 17β-estradiol (E2) 17α-ethinylestradiol (EE2)

Figure 1.1 Molecular structure of steroidal estrogens (National Library of Medical Information Specialized Information Services 2006)

CHAPTER 2: EXTRACTION AND ANALYSIS OF STEROIDAL ESTROGENS

2.1 ABSTRACT

A method was developed to quantify estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) in water samples down to 1.8, 24.3 and 0.4 ng L^{-1} respectively. It was shown that due to the complexity of the matrices involved, many existing methods have serious drawbacks. After performing an isotope dilution, estogens were extracted with solid phase extraction (SPE), and derivatized with pentafluorobenzoyl chloride (PFBCl). Analysis took place with a gas chromatograph/mass spectrometer (GC/MS) in negative chemical ionization mode (NCI). In addition, the extraction of estrogens from fish blood and tissue was carried out, first using accelerated solvent extraction (ASE) and gel permeation chromatography (GPC) to clean up the samples prior to the derivitization reaction. Although the following method is relatively easy to carry out, and allows for easy detection of all three estrogens, it was demonstrated that sensitivity needs to be further improved to allow detection of estrogens at the lowest observable effect limit (LOEL).

2.2 INTRODUCTION

The development of an extraction, separation and detection method for a chemical of interest is the initial step necessary for the risk analysis of any potentially contaminated habitat. For the steroidal estrogens estrone (E1), 17β-estradiol (E2) and

17α-ethinylestradiol (EE2), an additional challenge lies in the development of a method sensitive enough to detect concentrations at the lowest effect limits (LOEL's), which are lower than for other organic environmental contaminants (Lee and Peart 1998).

Published analytical methods for determining estrogen concentrations in environmental samples include high-performance liquid chromatography and gas chromatography, both coupled with mass spectrometry (HPLC/MS and GC/MS), enzyme-linked immunosorbent assays (ELISA's) and radioimmunoassay (RIA's), each with their own advantages and disadvantages (Table 2.1; reviewed by Wang et al. 2008 and Giese et al. 2003). For example, in water samples, the LC/MS and GC/MS methods are more sensitive and selective than the radioimmunoassay techniques, but are also more time consuming. Likewise, *in vivo* and *in vitro* assays are excellent large-scale screening tools, but they may over or under estimate concentrations in wastewater due to cross-reactions by estrogen receptor (ER) agonists or antagonists (Murk et al. 2002).

Generally, chromatographic methods are better used to determine the specific concentrations of a chemical like E1, E2 and EE2 and are the main quantification methods used here. Bioassays are better used to determine the total estrogenicity of a sample, usually as estrogen equivalents (EEq's), which express estrogenic activity of the sample in concentration units of E2 (Figure 2.1), or by screening for a particular mRNA or protein known to be inducible by an estrogenic EDC, but do not provide quantification of specific compounds (Gomes et al. 2003) (Appendix I).

In the preparation of samples for chromatographic quantification, an isotope dilution can correct for losses incurred at every step of the extraction procedure, from binding to the glassware, loss due to degradation, or human error. There is a basic

assumption that the isotope added is distributed in the same way as the native compound. If a pool exists that is not equilibrating, it will not be detected. Isotope dilution is done by adding an internal standard of carbon-13(^{13}C)-labelled E1, E2 and EE2 to the samples prior to the extraction (de Hoffmann and Stroobant 2002). Alternatively, deuterated estrogens have also been in the analysis of environmental samples. Chen et al. (2007), Hernando et al. (2004) and Williams et al. (2003) used this technique to measure steroidal estrogens in final sewage effluent, Stanford et al. (1999) used it for septic and groundwater, and Adlercreutz et al. (2004) applied it to urine samples (Table 2.1).

To determine the total dissolved and sediment-associated concentrations of estrogens, the isotope standard is added to a sample prior to any filtration method; if only one or the other concentration is desired, the standard is added to a second sample after the filtration step, and a subtraction is done. This is followed by an extraction step, carried out using a solid phase extraction (SPE) or liquid-liquid extraction with organic solvents such as acetonitrile, methanol or ethanol, acetone, hexane or dichloromethane (Table 2.1; Wang et al. 2008; Lai et al. 2000; Ternes et al. 2002). Determining the concentration of conjugated estrogens requires an additional step of enzymatic hydrolysis; adding β-glucuronidase overnight to a SPE eluant enables analysis of the glucuronides, also by subtraction (Belfoid et al.1999). SPE is an ideal technique for extracting estrogens from water because minimal clean-up of samples is required before analysis.

Separation by LC/MS is straightforward, but the limit of detection is 200 ng L^{-1} (Croley et al. 2000). GC/MS provides a lower limit of detection, but the method is more tedious due to a derivitization step that must be carried out due to the polar hydroxyl

groups in E1, E2 and EE2 (Hernando et al. 2004). Commonly used derivatives are pentafluoropropionic acid anhydride (PFPA) that converts the hydroxyl groups to perfluoropropionate groups, or pentafluorobenzoyl chloride (PFBCl) that converts the steroid estrogens to a pentafluorobenzolyate ester which will pass through the GC column (Table 2.1; Thiele et al. 1997; Lee and Peart 1998; Kuch and Ballschmiter 2001).

Lee and Peart (1998) report a limit of detection of 5 ng L^{-1} for E1, E2 and EE2 in sewage effluent using GC/MS in negative chemical ionization (NCI) mode, but this has now been improved to 0.15 ng L^{-1} for E1 and E2 and 0.1 ng L^{-1} for EE2 in both sewage effluent and river (Table 2.1; Kuch and Ballschmiter 2001). GC/MS in electron impact (EI) mode also provides a low limit of detection: 0.4-1.0, 0.6-1.0 and 0.6-1.0 ng L^{-1} in sewage effluent for E1, E2 and EE2 respectively (Table; 2.1; Williams et al. 2003).

In this thesis an isotope dilution of environmental water samples was done with a ^{13}C-labelled standard and Lee and Peart's (1998) SPE method was used to extract the estrogens. Then, a derivitization method described by Kuch and Ballschimter (2001) was used to convert the steroidal estrogens into non-polar pentafluorobenzoyl esters. Finally, separation and detection of the esters was achieved with GC/MS in NCI mode with methods adapted from Xiao et al. (2001). As well, a novel method for the extraction of steroidal estrogens from tissue and blood was attempted using accelerated solvent extraction (ASE) and gel permeation chromatography (GPC).

2.3 SAMPLE PREPARATION

2.3.1 Glassware and storage of samples

All glassware was washed with dichloromethane (DCM; ACS grade, Fisher Scientific) and stored in a 200 °C oven overnight. Calibrated equipment and plastic siphons not placed in the oven were instead washed with DCM (HPLC, GC Pesticide Grade, Burdrick & Jackson) for immediate use. While not being extracted, water, blood and tissue samples were stored in a 4°C fridge and in the dark.

2.3.2 Water

2.3.2.1 Isotope dilution and solid phase extraction

An isotope dilution was done immediately after water sampling by adding 80 µL of a 100 µg mL^{-1} internal standard of ^{13}C-labelled E1, E2 and EE2 in acetonitile (E1: 3, 4 - ^{13}C, 90% purity; E2: 3, 4 - ^{13}C, 99% purity; EE2: 20, 21 - ^{13}C, 99% purity; Cambridge Isotope Laboratories, Andover, MA) (Figure 2.2). Samples were filtered using glass fibre filters (Whatman GF/F 47 mm) and celite (Aldrich Grade 545), when needed, to prevent the filter from plugging. Samples were then vacuum-loaded onto 1 gram ENVI-18 tube solid phase extraction (SPE) tubes pre-conditioned with 5 mL acetone, 5 mL methanol and 10 mL HPLC water (HPLC, GC Pesticide Grade, Burdrick & Jackson) using Visiprep Large Volume Sampler 1/8" Teflon tubes and an ENVI-Disk Holder manifold for 6 x 1L samples. The SPE tubes were eluted with 5 mL acetone and 5 mL methanol and extracts were evaporated to dryness under a stream of nitrogen gas using a mini evaporator.

2.3.2.2 Derivitization and clean-up

Residues were re-dissolved in 2 mL HPLC water and a derivitization method was done by adding 10 μL 10% pentafluorobenzoyl chloride (2, 3, 4, 5, 6 - PFBCl) (Sigma, Oakville, ON) (Figure 2.3.) in toluene (Omnisolve, 99.98% purity, EMD) and 50 μL 2 M Potassium Hydroxide (KOH) buffer in water (Aldrich). The organic phase was then extracted twice with 2.5 mL of hexane (HPLC, GC Pesticide Grade, Burdrick & Jackson) and reduced to 0.5 mL with the mini-evaporator. Subsequently, it was passed through a glass pipette column plugged with glass wool (pre-sonicated in DCM) and filled with anhydrous sodium sulphate (ACS Grade, EMD granular) to remove impurities and any traces of water. The sample was dried with the mini-evaporator and resuspended in 200 μL isooctane (2, 2, 4 - trimethylpentane) (Optima Grade, 99% purity, Fisher Scientific).

2.3.3 Blood

As for the water, an isotope dilution with the internal standard of ^{13}C-labelled estrogens was done immediately after sampling. Blood samples were extracted twice with 1:1 hexane:dichlormethane (HPLC, GC Pesticide Grade, Burdrick & Jackson) and dried with the mini-evaporator to obtain a sample volume of 3 mL. A GPC Autoprep 1002A gel permeation chromatograph (GPC) (Analytical Biochemistry Laboratories) was used for sample clean-up. The 51 cm x 2 cm column was packed with 70 g of 200-400 mesh S-X3 Biobeads from Bio-Rad (Hercules, CA), the flow rate and pressure were set at 5 mL min^{-1} and 10 psi, respectively, and an injection volume of 5 mL was used. The chromatographic cycle was set for 28 minutes to waste, 30 minutes to collection vials, and 5 minutes to waste. The elutions in the collection vials were evaporated to dryness

using a turbovap and mini-evaporator. Residues were re-dissolved in HPLC water and the extraction was continued as for the water samples described in section 2.3.2.2, starting with the derivitization step.

2.3.4 Whole tissue

Tissue samples were homogenized at Environment Canada's National Wildlife Research Centre, Ottawa, Ontario, following the standard operating procedure SOP-TP-PROC-07D, and using a Sorvall Omni-mixer or the Robot Coupe (McNeil 2005). Fractions of homogenized tissue were weighed, mixed with hydromatrix, and packed into accelerated solvent extraction (ASE) 33-mL vials. At this point the ^{13}C-labelled E1, E2 and EE2 standard was added in an isotope dilution. A Dionex 200 accelerated solvent extractor was used to extract the estrogens from the tissue by heating for 5 minutes at 70°C, extracting with DCM at 2000 psi and a static hold for 8 minutes. As for the blood samples, extracts were evaporated down to 3 mL for injection onto the GPC column, and subsequently dried before carrying out the derivitization and clean-up steps described in section 2.3.2.2.

2.4 SEPARATION AND DETECTION BY GC/MS

The pentafluorobenzoyl-derivatives of E1, E2 and EE2 were separated and detected by GC-MS (Croley et al. 2000). Samples of 4 µL were injected in splitless mode onto an Agilent 6890 gas chromatograph with a Zebron ZB-5ms column (25 m x 250.00 µm x 0.25 µm), a 10 cm guard, and a methane (CH_4) reaction gas at 65 cm s^{-1}.

The injector was set at 250°C and the oven temperatures were programmed as follows: 80°C min^{-1} for 1 minute, 24°C min^{-1} until 200°C and held for half a minute, 73°C min^{-1} until 245°C and held for 5 minutes, and then 1°C min^{-1} to 260°C. The transfer line temperature was set at 330°C. A Hewlett Packard 5973 mass spectrometer, with a quad and ion-source temperature set at 100°C and 200°C, respectively, was used for quantification of estrogens in negative chemical ionization (NCI) mode. The following quantification ions were used in selected ion monitoring (SIM) mode: *m/z* 464 (C-12 E1), *m/z* 466 (C-13 E1, C-12 E2), *m/z* 468 (C-13 E2), *m/z* 490 (C-12 EE2) and *m/z* 492 (C-13 EE2). Chromatographic data were collected and analyzed using Agilent MSD Chemstation Data Analysis Software.

Instrument detection limits (IDL's) and method detection limits (MDL's) were calculated using the methods outlined by the United States' EPA (1993). IDL's were obtained from seven injections of one water sample extract (prepared from E1, E2 and EE2 standards of 40, 80 and 5 ng L^{-1}, respectively, in 1 L double distilled water) where the standard deviation for the mean concentration was multiplied by three. MDL's were obtained by the injection of seven extracts from seven water samples (prepared from E1, E2 and EE2 standards of 5, 5 and 1 ng L^{-1}, respectively, in 1 L double distilled water) extracted identically, where the standard deviation for the mean concentration was multiplied by the one-tailed t statistic at a 95% confidence level. In addition, the linear responses of steroidal estrogens were determined from sample extracts of 0, 12.5, 25, 37.5 ng L^{-1} of E1 and E2 standards and from sample extracts of 0, 1, 2, 3 and 4 ng L^{-1} of EE2 standards. Blank samples of double distilled water and blank samples of hydromatrix were spiked with the ^{13}C standards and taken through the extraction process

in Sections 2.3.2 and Section 2.3.3, respectively, alongside each series of sample analysis. It was assumed that MDL's for the blood and tissue extraction methods were comparable to the MDL's for the water extraction method.

2.5 RESULTS AND DISCUSSION

The instrument detection limit (IDL) for steroidal estrogens was 1.2, 14.2 and 0.4 ng L^{-1} for E1, E2 and EE2 respectively. The method detection limit (MDL) for the extraction of steroidal estrogens from water was 1.8 and 0.4 ng L^{-1} for E1 and EE2 respectively. The MDL for the extraction of E2 from water was not available because separate E2 peaks did not appear in the chromatograms due to the co-elution of E1 and E2. Increasing the column length from 15 m to 25 m did not increase the separation, and only led to a longer run time for each sample. Instead, a standard curve plotting the IDL versus MDL for E1 and EE2 was used to estimate the MDL for E2 at 24.3 ng L^{-1} (Appendix II). Examining E1 and E2 as a paired set is another option for analysis if co-elution occurs. This option also has the added benefit of taking the reversible oxidation of E2 to E1 into account (Chen et al. 2007). All three estrogens showed a linear response with concentration (E1: $R^2 = 0.960$, $p = 0.004$; E2: $R^2 = 0.961$, $p = 0.003$; EE2: $R^2 = 0.992$, $p < 0.001$). The concentrations of estrogens found in the blank water, blood and tissue samples were below the MDL's and below the lowest observable effect level for vitellogenin synthesis. Nevertheless, any background concentrations determined from the blanks were subtracted from the concentrations measured in samples.

The MDL's for E1 and EE2 are relatively high compared to other MDL's using GC/MS in NIC with SIM (Table 2.1; Kuch and Ballschimter 2001). However, the use of an isotope dilution provided easy identification of the compounds in the chromatograms, and prevented any loss from degradation of the compounds, or errors in extraction; this method provides comparable MDL's to others using an isotope dilution (Table 2.1; Chen et al. 2007; Hernando et al. 2004; Williams et al. 2003).

The isotope dilution was done immediately after sampling for the water and fish blood, but for the tissue, it was done after the homogenization and subsampling of the fish tissue. Consequently, the concentrations of estrogens measured in tissue may be an underestimate if estrogens are degraded by enzymatic activity in tissue or during the grinding and handling of samples at room temperature. Another limitation to the extraction method is its inability to measure steroidal estrogens associated with particulate matter. Celite was required to filter the insoluble organic matter found in the environmental samples, and consequently, the filters could not be easily extracted for the compounds. Other studies measured particulate-bound estrogens by centrifuging samples prior to filtering, and then extracting pellets with acetone, but the extraction of estrogens is still difficult due to the large capacity of absorption of estrogens by sludge (Cicek et al. 2007). The main drawback to this method is the lengthy extraction and derivitization process; therefore, a team effort is recommended for further analysis.

Sensitive analyses are indeed needed for clinical tests (breast, prostate cancer); however not every estrogen analysis needs to be conducted with high sensitivity. The MDL's and IDL's obtained from this analysis are adequate for use in surveying water from environments susceptible to contamination from steroidal estrogens because they do

not use large sample volumes (1-L for water and 1.5 to 2.7 mL for blood) and because they detect E1, E2 and EE2 at concentrations below the LOEL for the common biomarker vitellogenin.

Table 2.1 **Selection of methods used for the detection of estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) in water and sediment; notes on standards, extraction, clean-up, analysis and method detection limits (MDL's) are listed**

Sample type	Internal Standard	Extraction	Clean-up	Derivative	Analysis	MDL (ng L^{-1} or ng g^{-1})			Study
						E1	E2	EE2	
Effluent	n/a	Solid phase on Empore disk, eluted with acetone, DCM and hexane	Anhydrous sodium sulfate, HPLC	n/a	RIA	0.107	nav	0.053	Snyder et al. 1999
Effluent	2, 4, 16, 16-$^{2}D_4$-E1; 3,4-$^{13}C_2$-E2; 2, 4, 16, 16-$^{2}D_4$-EE2	Solid phase with C18 Speedisks, eluted with methanol and DCM	n/a	n/a	LC/MS/MS-ESI	1.31	7.22	1.80	Chen et al. 2007
Effluent	E2-d_2	Solid phase cartridges, eluted with ethyl acetate	n/a	BFTSA	GC/MS-EI (SIM)	8.5	17.0	4.0	Hernando et al. 2004
Effluent	E2-d_2	Solid phase cartridges, eluted with ethyl acetate	n/a	n/a	GC/MS/MS-EI	7.5	27.5	17.5	Hernando et al. 2004
Effluent	n/a	Solid phase with C18 cartridges, eluted with acetone	Back extraction with ethyl acetate, anhydrous sodium sulfate	PFPA	GC/MS-NCI (SIM)	5	5	5	Lee and Peart 1998
Effluent	E2-17-acetate	Solid phase materials, eluted with acetone	Silica gel	MSTFA/TMSI/DTE	GC/MS/MS-EI	1	1	1	Ternes et al. 1999a
Effluent	E1-d_4; E2-d_4; EE2-d_4	Solid phase with C18 cartridges, eluted with methanol	HPLC	MTBSTFA	GC/MS/MS-EI	0.4	0.6	0.6	Williams et al. 2003

Table 2.1 (continued)

Sample type	Internal Standard	Extraction	Clean-up	Derivative	Analysis	MDL (ng L^{-1} or ng g^{-1})			Study
						E1	E2	EE2	
Effluent	E2-17-acetate	Liquid with DCM	Sodium sulfate, Deactivated florisil	BSTFA	GC/HRMS	7.6	7.1	7.1	Fernandez et al. 2007
Effluent River	BFFBB	Solid phase with glass cartridges eluted with acetone and methanol	Back extraction with hexane	PFBCl	HRGC/MS-NCI (SIM)	0.10	0.15	0.10	Kuch and Ballschimter 2001
Ground-water	n/a	Solid phase with C18 cartridges, eluted with methanol and DCM	n/a	PFBO	GC/MS-NCI	0.2	0.03	0.05	Xiao et al. 2001
Drinking	BFFBB	Solid phase with glass cartridges eluted with acetone and methanol	Back extraction with hexane	PFBCl	HRGC/MS-NCI (SIM)	0.05	0.10	0.05	Kuch and Ballschimter 2001
Sludge Sediment	E2-17-acetate	Liquid with methanol and acetone	GPC and silica gel	n/a	n/a	n/a	n/a	n/a	Ternes et al. 2002
Sludge extracts	E2-17-acetate	Solid phase with glass cartridges, eluted with acetone	HPLC	MSTFA/TMSI/DTE	GC/MS/MS-EI	2	2	4	Ternes et al. 2002
Sediment extracts						0.2	0.2	0.4	

BFFBB = 1,4-bispentafluorobenzoylbenzene; BSTFA = *N, O*-bis(trimethylsilyl)trifluoroacetamide; DCM = dichloromethane; DTE = dithioerytrol; EI = electron impact; ESI = electrospray ionization; GC = gas chromatography; GPC = gel permeation chromatography; HP = high performance; HR = high resolution; LC = liquid chromatography; n/a = not applicable; MS = mass spectrometry; MSTFA = *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide; MTBSTFA = *N*-methyl-*N*-*tert*-butyldimethylsilyltrifluoroacetamide; NCI = negative chemical ionization; PFBCl = pentafluorobenzoyl chloride; PFBO = pentafluorobenzoyl chloride; PFPA = pentafluoropropionic acid anhydride; RIA = radioimmunoassay; SIM = selected ion monitoring; TMSI = trimethylsilylimidazole

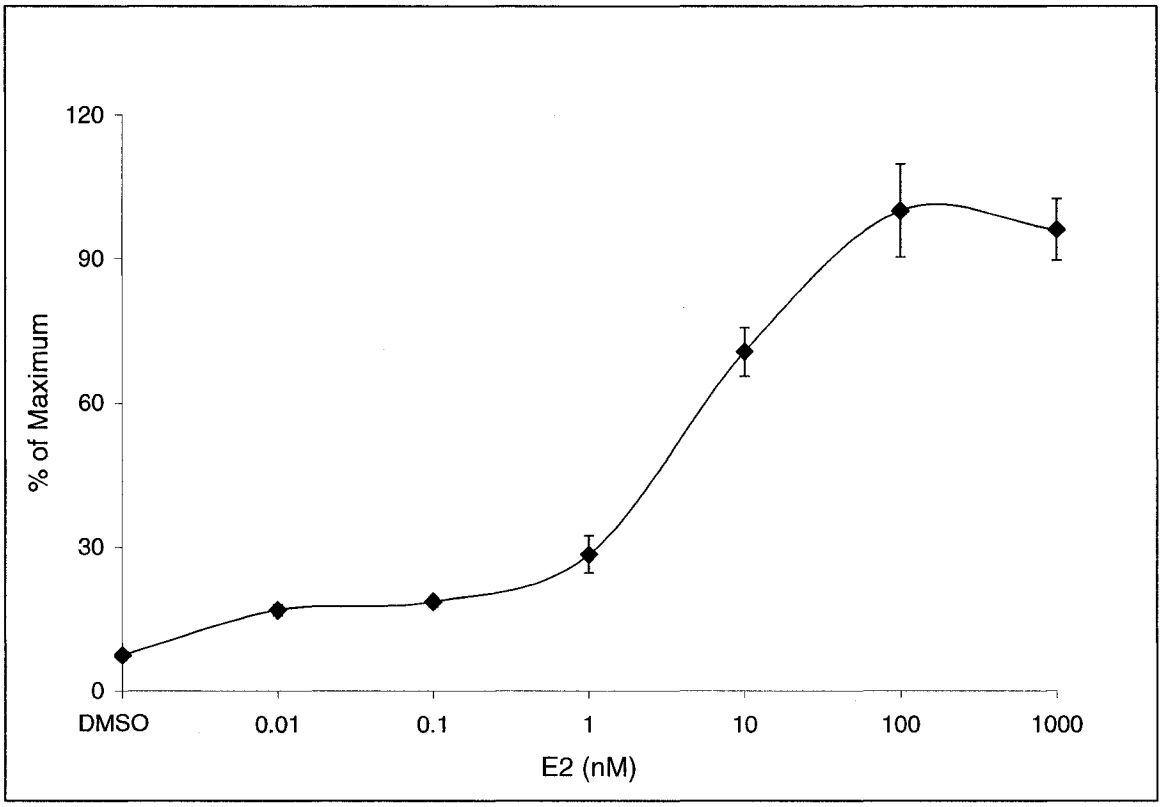


Figure 2.1 **Standard curve for luciferase induction of rainbow trout ER alpha (pSG5) in gonad cell line co-transfected with ERE-TK-Luc (Ackermann et al. 2002; Marlatt 2006)**

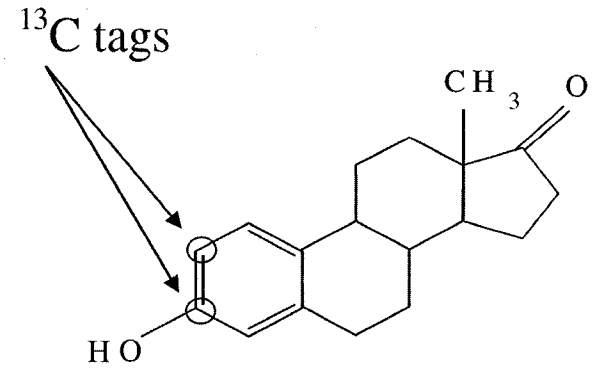


Figure 2.2 **^{13}C-labelled estrone**

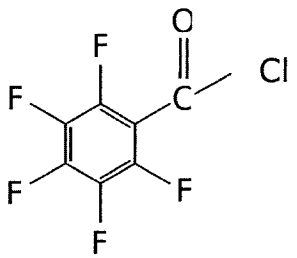


Figure 2.3 **Molecular structure of pentafluorobenzoyl chloride used for derivitization step**

CHAPTER 3: SAMPLING OF WASTEWATER, RIVERS AND DRINKING WATER

3.1 ABSTRACT

Steroidal estrogens were extracted and measured in Ottawa and Cornwall waste, river and drinking water samples using SPE and GC/MS technology. E1, E2 and EE2 were found in raw sewage (RS) at maximum concentrations of 104, 66.9 and 5.7 ng L^{-1} respectively. E1 concentrations in RS were not correlated with concentrations of E2 or precipitation, but did show a significant relationship with environmental temperature and mean daily flow. A multiple regression model incorporating these variables, along with precipitation and carbonaceous biological oxygen demand (cBOD) in RS explained the greatest amount of variation of E1 in RS ($R^2 = 0.967$, $p = 0.015$). The percent difference in cBOD from RS to FE (final effluent) explained the greatest amount of variation in percent difference of E1 from RS to FE ($R^2 = 0.435$, $p = 0.075$). Estrogenicity did not change significantly from RS to FE in Ottawa sewage treatment plant (STP) samples taken on October 11, 2005. In the rivers, the estrogens were found at much lower concentrations and no trends were seen between upstream and downstream sites. In University of Ottawa drinking water, EE2 was detected on all sampling dates. Improvements to steroidal estrogen removal in STP's need to be addressed, as the release of natural estrogens to the aquatic environment cannot be eliminated.

3.2 INTRODUCTION

Steroidal estrogens have been found in wastewater and surface waters all over the world in the ng L^{-1} to μg L^{-1} range. Studies investigating their occurrence generally focus on urban aquatic environments in Europe and North America, but some data are now available for South America and Asia (Table 3.1 and 3.2; Carbella et al. 2004; Labadie and Budzinski 2005; Nakada et al. 2004; Ternes et al. 1999a; Shiwei et al. 2008). Significant progress has been made in quantifying the elimination of steroidal estrogens in wastewater in an effort to both estimate and reduce the risks to aquatic organisms in the environment.

3.2.1 Excretion of steroidal estrogens

Both men and women excrete the natural steroidal estrogens, estrone (E1) and 17β-estradiol (E2), daily. Johnson et al. (2000) estimate that males excrete 3.9 and 1.6 μg of E1 and E2 per day respectively. In women these amounts are higher: 8 and 4.5 μg of E1 and E2 per day is released from a menstruating female (15-59 years), 4 and 2.3 μg of E1 and E2 per day from a menopausal female (over 59 years) and 600 and 259 μg of E1 and E2 per day from a pregnant woman (Table 3.3). Women using the oral contraceptive pill or on hormone replacement therapy are estimated to excrete 26% of their dose of 17α-ethinylestradiol (35 - 27.5 μg EE2) per day (Johnson et al. 2000). Ternes et al. (1999b) estimate similar excretion rates in menstruating women with 3-20 μg of E1 and 0.5-5 μg of E2 daily. A simpler estimate of excretion rates by Christiansen et al. (2002) give values for a whole Danish population at 68.6, 35.7 and 3.2 g daily for E1, E2 and

EE2 respectively (Table 3.3).

E1, E2 and EE2 excretion by an organism initially begins with their conversion to polar compounds that are easier to eliminate by the kidney. Reactions include hydrogenation of the double bonds, hydroxylation, and conjugation of the oxygenated functional groups to generate soluble sulphate and glucuronic acid esters to facilitate their elimination (Wang et al. 1998; Johnson and Sumpter 2001; Honjo et al. 1976). The primary conjugated forms of steroid estrogens released are estrogen glucuronides such as E1-3-glucuronide (E1-3G), E2-3-glucuronide (E2-3G) and E2-17-glucuronide (E2-17G), and estrogen sulfates such as E1-3-sulfate (E1-3S), and E2-3-sulfate (E2-3S) (Figure 3.1; Raftogianis et al. 2000; Fotsis and Adlercreutz 1987; d'Ascenzo et al. 2003).

In wastewater the fecal coliform bacteria (*Escherichia coli*) enzyme, β-glucuronidase, cleaves these esters to release free estrogens (Ternes et al. 1999b). Glucuronated estrogens are readily degraded in wastewater, and free estrogens and sulfated estrogens have been shown to be the dominant estrogen species entering sewage treatment plants, generally in the range of several ng L^{-1} (Table 3.1; D'Ascenzo et al. 2003). Another of the principal transformations by sewage bacteria is the reversible oxidation of E2 to E1, which occurs very quickly under aerobic conditions, so E1 is generally seen at higher concentrations than E2 (Lee and Liu 2002). Concentrations in raw sewage may also reflect the population being served by an STP, such as the high concentrations of E1 (197 ng L^{-1}) found in the raw sewage of a Tokyo plant serving a large population of 709 000 (Nakada et al. 2006). Higher EE2 concentrations measured in one study was explained by the presence of an industrial facility in the community, which produces birth control pills (Cicek et al. 2007). Table 3.1 provides an overview of

the steroidal estrogen concentrations measured in raw sewage around the world.

3.2.2 The sewage treatment plant

Sewage treatment processes are classified as primary, secondary or tertiary. Primary treatment consists of screens and grit removal tanks and sedimentation tanks, which remove settling and floating material, producing a homogeneous mixture that can undergo secondary treatment. The secondary treatment degrades the biological content of wastewater using biological processes, reducing the carbonaceous biological oxygen demand (cBOD) (Figure 3.2; Edexcel Foundation 2003; Joss et al. 2004; Ternes et al. 1999a).

Activated sludge is the most commonly used secondary wastewater treatment process in North America, with a hydraulic retention time (HRT) of 4 to 14 hours, while biological filters, which serve smaller populations and have shorter HRT's of about 0.5 hours, are prevalent in the United Kingdom (Johnson and Sumpter 2001; Lee and Liu 2002). The activated sludge system can use either anaerobic or aerobic digestion to treat solids, and may also include aerated nitrifying tanks, followed by denitrifying, in order to remove ammonia (Andersen et al. 2003; Holbrook et al. 2002). Due to the high K_{ow} of steroidal estrogens, there is partitioning of the compounds to the organic phases in sewage sludge, or sorption to the biosolids (Lee et al. 2003; Bowman et al. 2003). The biosolids are carted away for disposal, and are either incinerated or land-filled after solidification with cement (Isobe et al. 2001).

Tertiary treatment improves the final quality of the effluent by disinfection processes that include phosphate elimination using $Fe(III)Cl_3$, $Fe(II)Cl_2$ or $AlSO_4$,

chlorination by sodium hypochlorite and chlorine, and low and medium-pressure mercury arcs (Ternes et al. 1999a). Effluent may also be filtered through sand before being discharged to the receiving water (Joss et al. 2004).

3.2.3 Elimination of estrogens in sewage treatment plants

Rates of steroidal estrogen biodegradation in STP's vary depending on the compound, and the method of treatment used (Johnson and Sumpter 2001). Biodegradation of E1 and EE2 under aerobic conditions is much slower than for E2 (Ternes et al. 1999b). Ying and Kookana (2003) observe a short lag phase prior to degradation which they attribute to the acclimation by aerobic microorganisms; in river water degradation rates were higher in bacterial assemblages previously exposed to a contaminant than those without previous exposure (Stone and Watkinson 1983). Unlike biodegradation in water, no acclimation stage was seen for aerobic degradation in sediment. Ying and Kookana (2003) hypothesize that is due to a more active and diverse microbial community and richer nutrient levels in the sediment. In anaerobic conditions, degradation rates are much slower in the sediments than in aerobic conditions. The authors indicate that sulfate is the dominant electron acceptor, confirmed by the formation of dark sulfide mineral on the surface of the sediments, and suggest this biodegradation of the estrogens is carried out by sulfate-reducing bacteria. The authors also hypothesize the relative resistance of EE2 to microbial degradation can be attributed to its chemical structure; EE2 has two quaternary carbon atoms directly adjacent to each other.

Generally, activated sludge plants are more effective at removing steroidal

estrogens than biological trickling filters (Johnson et al. (2007). Observed elimination in a Brazilian sewage treatment plant was found to be 83, 99.9 and 78% for the activated sludge system and 67, 92 and 64% for the parallel biological filter for E1, E2 and EE2 respectively (Ternes et al. 1999a). The more efficient removal of the activated sludge system has been attributed to its higher biological activity compared to a trickling filter, as well as the long holding time of sludge (Kirk et al. 2001; Lee and Liu 2002).

In another activated sludge system study, E1 and E2, and EE2 were eliminated with rates that exceeded 98 and 90%, respectively, by the end of the process (Table 3.1; Andersen et al. 2003). In this STP, the denitrifying tanks played an important role in reducing estrogens through oxidation reactions, as before they were installed, only minor reductions of EE2 were observed. It would be expected that removal rates would increase with HRT, allowing more time for the biological reactions to occur, however, no such correlation was seen in a study of southwestern Ontario plants (Lishman et al. 2006).

However, the elimination of steroidal estrogens does not always occur in STP's. In one activated sludge STP, E1 concentrations exceeded those in raw sewage due to the partial oxidation of E2 to E1 (Carbella et al. 2004). In other STP's with trickling filters, E1 and E2 concentrations also increased through the treatment process, hypothesized to be due to the deconjugation of sulfate-glucuronides, along with the transformation of EE2 to E1 and E2 (Schlüsener and Bester 2008). Differences in percent removal may also vary depending on whether particulate-based results are included. When including measurements of estrogens bound to sludge, Cicek et al. (2007) found greater reductions in E1 than E2.

E1 and E2 have been found in British sewage effluent at levels of 1 ng L^{-1} to almost 80 and 50 ng L^{-1}, respectively, and EE2 has been detected at levels of 0.2 to 7.0 ng L^{-1} (Desbrow et al. 1998). Western European sewage effluents contain concentrations of E1, E2 and EE2 as high as 91, 16 and 15 ng L^{-1}, respectively, (Ternes et al. 1999a; Schlüsener and Bester 2008) and Asian effluents contain concentrations of up to 79.7 for E1 and 16.7 ng L^{-1} for E2 (Nakada et al. 2006). A database built from the compilation of pharmaceutical and personal care products in wastewater from 113 research papers lists maximum concentrations of E1, E2 and EE2 of 139, 48.4 and 2.8 ng L^{-1} and 72.8, 5.2 and 1.4 ng L^{-1} in influent and effluent respectively (Miège et al. 2008). Table 3.1 provides further examples of steroidal concentrations found in wastewater around the world.

3.2.4 Steroidal estrogens outside of STP's

In addition to release from domestic wastewater, E1, E2 and EE2 can also enter watersheds through runoff from agricultural land, where sources include animal excrement and fertilizers, although their transport and degradation has not been well investigated (Gagné et al. 2001; Johnson and Sumpter 2001; Khanal et al. 2006). The compounds are immediately diluted in the receiving surface waters, and will undergo the same degradation and removal processes as in the wastewater treatment process. Although all traces of glucuronide conjugates are eliminated through sewage treatment, estrone sulfates are still detected in sewage effluent and may deconjugate in river ecosystems (D'Ascenzo et al. 2003). Jürgens et al. (2002) observed that microorganisms in English river water were capable of transforming E2 to E1, and degrading E1 and EE2, although like in STP's, EE2 was more resistant to biodegradation. Half-lives of these

estrogens in the river water were between 0.2 and 10 days. In contrast, Barel-Cohen et al. (2006) determined that although natural and synthetic estrogen concentrations were reduced by half within 25 km from a sewage effluent source, they could still be detected up to 100 km along the river). The most potent steroidal estrogen, EE2, has even been found in German drinking water.

The incorporation of seasonal variables into models may help explain the variations in the concentrations and removal of contaminants in waste and receiving surface waters. Inflow of steroid estrogens rose in a German treatment plant after a heavy rainfall; it was suggested that sewer sediments were rinsed with rainwater and the hormones were transported to the WWTP (Schlüsener and Bester 2008). The authors also noted that the subsequent elimination rate of E2 was reduced, perhaps because the bacteria responsible for the elimination needed time to adapt to the new situation. Higher concentrations of estrogens were observed in the Jordan River at the end of the dry season which may be due to increased evaporation (Barel-Cohen et al. 2006). Shiwei et al. (2008) reported higher concentrations of E1 in Chinese raw sewage in summer months, attributing this to a more rapid degradation of E2 under higher temperatures. Ternes et al. (1999a) found a much higher elimination of E2 in a Brazilian STP versus a German STP and proposed the higher operational temperature (22°C higher) was increasing microbial activity. Also in the summer, Labadie and Budzinski (2005) found decay rates exceeded that of dilution.

3.2.5 Steroidal estrogens in Canada

In Canada, E1, E2 and EE2 have been detected with similar patterns in concentrations as to those found in Europe. A survey of Ontario sewage treatment plants found all three steroidal estrogens in eight out of the ten sewage effluents (Ternes et al. 1999a). Although the median concentrations of E2 and EE2 were higher than those observed in other surveys, the small number of samples led the authors to suggest further studies to substantiate the evidence of estrogens in Canadian effluent. In Toronto, a study of four wastewater treatment plants observed E1 and E2 in all influent and effluent, with concentrations up to 75 ng L^{-1} E1 and 6.8 ng L^{-1} E2, but did not detect EE2 (Table 3.1; Lee et al. 2004). Steroidal estrogens have also been measured in Burlington, Dundas, Edmonton, Guelph and Brandon effluents (Table 3.1; Lee and Peart 1998; Cicek et al. 2007). Water collected downstream of the Montreal municipal effluent plume was noted to induce vitellogenin synthesis in mussels, which suggests that estrogenic compounds are being released in Montreal effluent (Gagné et al. 2001). In Des Prairies River, to which the Ottawa River drains, and the mouth of the Ottawa River, researchers have observed significantly higher VTG levels in mussels and fish (Aravindakshan et al. 2004). The authors hypothesize xenoestrogens may originate from the Ottawa River.

3.2.6 Steroidal estrogens in Southeastern Ontario

The Ottawa sewage treatment plant, the Robert O. Pickard Environmental Centre, is an activated sludge secondary treatment plant with phosphorus removal and seasonal chlorine disinfection occurring from May 16th to November 15th (Sierra Legal 2004). In 2005, it was reported to serve a population of 786 130, with an annual average flow

(AAF) of 422 ML per day (OCMBP 2006). The City of Cornwall wastewater treatment plant is a primary treatment plant. Despite completing an environmental assessment for the upgrade to secondary treatment in Cornwall, no progress has been made in securing funding for the project (EPA 2008). Published information detailing the processes and parameters in the plant is either limited or is not being made available to the public.

This study will examine concentrations of steroidal estrogens found in wastewater, drinking water, and the rivers receiving the wastewater and providing the source for the drinking water, in areas near Ottawa and Cornwall, Ontario. I predicted that removal of estrogens would be more efficient in Ottawa than in Cornwall due to the more advanced secondary treatment process. In addition, concentrations measured in the receiving waters were predicted to be much lower than in wastewater due to further biodegradation and a dilution effect, and even lower in drinking water due to additional treatment processes.

3.3 METHODS

3.3.1 Selection of sampling sites

The sewage treatment plants (STP's) sampled are located in the City of Ottawa and the City of Cornwall, in southeastern Ontario, Canada. Ottawa's STP, the Robert O. Pickard Environmental Centre (ROPEC), is located in east Ottawa, on 800 Green Creek Drive, south of the receiving Ottawa River. Raw sewage, final effluent, upstream and downstream sampling locations are indicated in Figure 3.3.1 and a schematic of the wastewater treatment process is given in Figure 3.3.2 The location of the raw sewage

composite sampler, downstream of the screening and degritting process, was selected to provide the most representative sample due to its mixture of raw sewage from two collector locations and the location of the final effluent composite sampler was selected to reduce suction lift and eliminate entrained air conditions (City of Ottawa 2005). Cornwall's sewage treatment plant is located in the eastern side of the city at 2800 Montreal Road East. The effluent flows north into the St. Lawrence River, downstream from the river sampling locations (Figure 3.3.3). A schematic of its process is given in Figure 3.3.4. Sampling locations for the Raisin River include headwaters and are indicated in Figure 3.3.5. The drinking water samples were taken from the fourth floor of the Centre of Advanced Research in Environmental Genomics (CAREG), 130 Marie-Curie, at the University of Ottawa (Figure 3.3.6). The latitude, longitude and altitude of all sampling locations are listed in Table 3.4.

3.3.2 Sampling Procedures

3.3.2.1 Safety Concerns

Nitrile gloves were used for sampling, and ethanol was used to wipe down all laboratory surfaces before and after dealing with wastewater samples.

3.3.2.2 Sampling dates

Grab wastewater composite samples were taken between June 2005 and August 2006, on ten occasions at ROPEC, and on two occasions at the Cornwall facility. Both raw sewage and final effluent samples were obtained at the same time in the morning, at approximately 10:30 AM. To account for the hydraulic retention time of ROPEC, on one occasion, May 10, 2006, FE was sampled after the RS by a period of time corresponding

to the hydraulic retention time (HRT), or approximately 17 hours. The rivers were only sampled on two occasions each, and the drinking water, on four occasions. Samples were generally taken in triplicate and, on some occasions, in duplicate (Tables 3.9 and 3.16 to 3.19).

3.3.2.3 Ottawa and Cornwall population demographic data

Estimates of the population being served by ROPEC were obtained from the Association of Municipalities of Ontario and Statistics Canada; 96.8% of the City of Ottawa population is served by ROPEC (Table 3.5; OCMBP 2006; Statistics Canada 2006). The population being served by the Cornwall wastewater treatment plant was assumed to be the total population of the City of Cornwall measured in the Canada 2006 census (Statistics Canada 2006). These data were organized into groups of males and females, with the latter divided into pre-menstruating, menstruating, menopausal and pregnant women. There was a further subdivision of women taking the contraceptive pill, and women on hormone replacement therapy. There was no consideration for males undergoing sex change therapy. Menstruating women were defined as females between the ages of 15 and 59 years, and the proportions of the female population that are pregnant (1/75), use the contraceptive pill (13%) and are on hormone replacement therapy (3%) were defined as reported by Johnson et al. 2000. As well, it was assumed that pre-menstruating females release the same amounts of E1 and E2 as males. This information, along with the steroidal estrogen excretion rates described in Table 3.3, was used to calculate the daily loading of E1, E2 and EE2 in both cities.

3.3.2.4 Ottawa weather data

The daily mean temperature and total precipitation for the City of Ottawa were collected for the five days prior to each Ottawa wastewater sampling date (Environment Canada 2005, 2006). For each sampling event, a mean temperature was calculated and a sum for the total daily precipitation was tabulated (Table 3.6). The five days chosen to calculate the mean temperature and total precipitation for each sampling event was an estimate of the average time it takes for City of Ottawa raw sewage to arrive at the wastewater treatment plant.

3.3.2.5 Hydrology and water chemistry at ROPEC and in Cornwall

The daily wastewater treatment plant flow (Q), hydraulic retention time (HRT), final effluent temperature (FET), and raw sewage and final effluent carbonaceous biochemical oxygen demand (cBOD) for the wastewater sampling dates were obtained from monitoring records provided by the ROPEC technicians and the Laboratory Services of the Environmental Programs and Technical Support (EPTS) (Table 3.7; City of Ottawa 2007). Details on the sampling and analysis of the hydrology and water chemistry data at ROPEC can be found in its Annual Operations Report (City of Ottawa 2005). The average daily flow for 2006, 422 ML, was obtained from the Association of Municipalities of Ontario (OCMBP 2006). This value was used with the daily loading of steroidal estrogens in Ottawa raw sewage (Section 3.3.2.3) and the equations in Appendix III to predict concentrations of E1, E2 and EE2 entering ROPEC.

For Cornwall, the daily flow was calculated from the yearly flow provided in the schematic of the plant (15 966 ML; Figure 3.3.4). Predicted concentrations of E1, E2 and EE2 entering the Cornwall plant were calculated as for ROPEC.

3.3.3 Extraction and analysis

Water samples were extracted and analyzed as outlined in Sections 2.3 and 2.4 of this thesis.

3.3.4 Estrogenicity assay

The luciferase reporter gene assay in Appendix I of this thesis was used to determine the estrogenicity of the raw sewage and final effluent samples obtained from ROPEC on October 11, 2005 (Ackermann et al. 2002; Marlatt 2006).

3.4 RESULTS AND DISCUSSION

3.4.1 Concentrations of steroidal estrogens in wastewater

Estrone is found consistently in all wastewater collected, both in Ottawa and Cornwall. E1 was found at a maximum concentration of 370 ng L^{-1} (SE±26 ng L^{-1}) on August 16, 2006, and at a minimum concentration of 11.2 ng L^{-1} (SE±0.9 ng L^{-1}) in November 30, 2005, both in Ottawa final effluent (Table 3.11.1). Generally, the E1 concentration increases during the treatment process, likely due to both the decoupling of conjugates, and the oxidation of 17β-estradiol and conversion to E1. E2 is found in both sewage treatment plants, at a maximum concentration of 66.9 ng L^{-1} (SE±15.7 ng L^{-1}) in Ottawa raw sewage on September 14, 2005 (Table 3.11.2). E2 was not detected in either STP by the end of the sampling period, but this may be due to problems with the separation of E1 and E2 peaks in the chromatography discussed in Section 2.5 of this thesis. 17α-ethinylestradiol was detected in wastewater on four occasions, with a

maximum concentration of 9.8 ng L^{-1} (SE±8.4 ng L^{-1}) in Cornwall final effluent on June 29, 2005 (Table 3.11.3). Its sporadic occurrences are similar to those reported by Williamson et al. (2003), who hypothesize the compound originates from a point source of sewage effluent due to its occurrence in coordination with boron, a conservative tracer of sewage effluent. The August 2006 Ottawa RS and FE concentrations were left out of the regression analyses due to the unusually high values, over two times greater than for all other values.

The concentrations of steroidal estrogens measured in samples of Ottawa and Cornwall raw sewage and final effluent (Tables 3.11.1, 3.11.2 and 3.11.3) compare well to the values obtained in other Canadian studies (Cicek et al. 2007; Fernandez et al. 2007; Servos et al. 2005) (Table 3.1). The E1 and E2 concentrations measured in raw sewage also correspond well to what would be predicted with a model using the excretion values reported by Johnson et al. (2000) and Christiansen et al. (2002) (Table 3.9 and 3.10). Discrepancies between the predicted concentrations and the measured concentrations may be due to the different sex ratio and age distribution of the Danish and Ottawa populations, along with the difficulties in estimating the number of pregnancies and number of people taking the oral contraceptive pill or undergoing hormonal therapy, and deviations from the average daily flow (Johnson et al. 2000).

The higher daily loading of E1 and E2 in Ottawa wastewater compared to Cornwall is linked to the larger human population being served by the Ottawa sewage treatment plant (Tables 3.9 and 3.10). The removal of E1 and E2 in ROPEC (-340 – 77% and sometimes 100% respectively) is similar to removal in other secondary wastewater treatment plants. The greater percentage difference of 17β-estradiol from raw sewage to

final effluent, and the observed increase of estrone in the Ottawa plant, is likely due to the increased biological activity of the secondary treatment process, compared to the primary treatment process of the Cornwall plant, which allows increased oxidation of E2 to E1.

Estrogens may also not be detected in wastewater because they have sorbed to sewage sludge; EE2 in particular, which has the highest K_{ow}, shows this tendency (Suidan et al. 2005). Difficulties in analyzing sludge-bound estrogens may prevent a complete mass-balance analysis of E1, E2 and EE2 around each treatment unit in a plant. That concentrations in sewage sludge are not well studied is an area of concern, as sludge is frequently applied as fertilizer to agricultural lands. Measurements in the future should also gather composite 24-hour samples. Diurnal cycles of steroidal estrogen inflow have been observed because the excretion of hormones via urine is lower at night while the majority of the population sleeps (Schlüsener and Bester 2008).

3.4.2 Effect of weather and STP parameter on steroidal estrogen concentrations

The City of Ottawa mean temperature and total precipitation calculated for each Ottawa sampling event ranged from a low of –9.3°C to a high of 22.3°C, and from 0 to 38.4 mm respectively (Table 3.7).

ROPEC treatment parameter data for the sampling dates are displayed in Table 3.8. Daily plant flow ranged from 366 to 792 ML, hydraulic retention time from 8.9 to 16.9 hours, FE temperature from 11 to 24°C, RS cBOD from 106 to 220 mg L^{-1}, and FE cBOD from 4 to 15 mg L^{-1}.

A significant positive relationship was found between the concentration of E1 in raw sewage and the mean environmental temperature (Table 3.12, $R^2 = 0.792$, $p = 0.003$; Figure 3.4); this is perhaps due to increased microbial activity at higher temperatures,

allowing for the deconjugation of steroidal esters, and the conversion of E2 into E1 prior to the arrival at the sewage treatment plant. A significant inverse relationship was found between E1 in RS and mean daily flow (Table 3.12, $R^2 = 0.757$, $p = 0.005$; Figure 3.5), which is due to a dilution effect. Adding the total precipitation five days prior to sampling and the cBOD in RS increased the variation of E1 in RS explained by the regression model (Table 3.13; $R^2 = 0.967$; $p = 0.015$); increased precipitation may have created increased additional runoff of steroidal estrogens from agricultural lands. No significant relationships were found between E1 in final effluent (FE) and mean daily flow, FE temperature and FE cBOD (Tables 3.14 and 3.15).

The percent removal in cBOD from raw sewage to final effluent explained some of the variation seen in percent removal of E1 from RS to FE (Table 3.16, $R^2 = 0.435$, $p = 0.075$, Figure 3.6). The inverse relationship is due to the removal of microbial reactions that can break down E1. Advancements in upgrading sewage treatment processes are usually designed to limit biological oxygen demand (BOD), total suspended solids (TSS) and total phosphorus in effluent, and sometimes ammonia nitrogen, *E. coli* and total residual chlorine; the removal of natural hormones is generally not a criterion (Sierra Legal Defence Fund 2006). Here, the use of secondary treatment in improving Ottawa water quality may indirectly decrease the elimination of estrone. However, in general, greater reductions were seen for E2 and EE2 with the Ottawa secondary treatment, than for Cornwall primary treatment.

3.4.3 Estrogenicity of wastewater

The concentrations of estrogens in Ottawa wastewater sampled on October 11, 2005, did not change significantly from raw sewage to final effluent (Tables 3.11.1, 3.11.2, 3.11.3). Similarly, a t-test showed that the percent responses of 100 nM E2 listed in Table 3.17 did not change significantly from raw sewage to final effluent ($p = 0.354$). However, without the analysis of wastewater for a full spectrum of estrogenic chemicals, such as alkylphenols and pesticides like DDT, these results do not show what is contributing to the estrogenicity in the raw sewage and final effluent. Because of lack of change in estrogenicity, the sum of all other estrogenic chemicals present in raw sewage must not have decreased through the treatment process. A more detailed analysis of the chemicals that contribute to estrogenicity in wastewater has been performed by Sun et al. (2008). Here the authors conclude that the low removal of estrogenicity in the Chinese STP is due to the production of unknown estrogenic compounds, as the removal of nonylphenol, octylphenol and bisphenol-A is observed. Due to time constraints, the ER alpha reporter gene assay was only used once throughout the entire sampling period, and should be repeated to uncover trends in estrogenicity through the Ottawa STP.

3.4.4 Concentrations of steroidal estrogens in river and drinking water

No trends in concentration were seen between the upstream and downstream sites in the Ottawa River, the St. Lawrence River, and the Raisin River. Furthermore, there were no differences observed between the different tributaries in the Raisin River. Only E1 and EE2 were found in river samples, with a maximum concentration of 107 ng L^{-1} (SE±35 ng L^{-1}) for E1 in the St. Lawrence River (Tables 3.18 to 3.20). This value

suggests that a source of estrone exists upstream from the Cornwall sewage treatment plant, which may be run-off from fields with farming activity or storm sewer overflow. E1 was detected in samples of drinking water samples obtained from the 4th floor drinking fountain in CAREG on November 5, 2005, and EE2 was detected on all four sampling dates (Table 3.21). The wide range and sporadic occurrence of EE2 is similarly described in Fernandez (2007).

3.5 CONCLUSION

The patterns of occurrence, removal and persistence of estrone, 17β-estradiol and 17α-ethinylestradiol in Ottawa and Cornwall sewage treatment plants, receiving waters, and drinking water, are similar to those found worldwide. Although secondary treatment seems to improve the removal of E2 and EE2, it may indirectly increase the E1 concentrations in final effluent. The sampling results suggest that in addition to sewage treatment plants, there are other sources of estrogens to the Ottawa River and St. Lawrence River which need to be better described (farming activity, storm sewers, coastal currents). Continued monitoring of these steroidal estrogens is necessary in order to improve wastewater treatment guidelines, and ensure the safety of our drinking water.

Table 3.1 **Maximum concentrations of estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) in wastewater and maximum percent difference from raw sewage (RS) to final effluent (FE)**

Location	Type of treatment	RS Concentration ($ng\ L^{-1}$)			FE Concentration ($ng\ L^{-1}$)			% Difference			Study
		E1	E2	EE2	E1	E2	EE2	E1	E2	EE2	
Britain	Various (n = 7)	nav	nav	nav	76.0	48	7	nav	nav	nav	Desbrow et al. 1998
Britain	Secondary (activated sludge, n = 2; trickling filter, n = 1)	nav	nav	nav	12.2	4.3	3.4	nav	nav	nav	Williams et al. 2003
Germany	Various (n = 16)	nav	nav	nav	70	3	15	nav	nav	nav	Ternes et al. 1999a
Wiesbaden, Germany	Secondary, with activated sludge, denitrification and nitrification	76.6	19.5	10.1	<1	<1	<1	98	98	90	Andersen et al. 2003
North Rhine - Westphalia, Germany	Secondary, with activated sludge	130	68	nav	58	19	nav	**92**	**75**	nav	Schlüsener and Bester 2008
	Secondary, with trickling filter	54	10	nav	91	16	nav	**-72**	**-53**	nav	
Frankfurt, Germany	Tertiary, with aerator tank and phosphate elimination	**27**	**15**	nav	nav	nav	nav	68	64	nav	Ternes et al. 1999a
The Netherlands	Secondary, with activated sludge (n = 5)	nav	nav	nav	47	12	7.5	nav	nav	nav	Belfroid et al. 1999
Galacia, NW Spain	Secondary, with activated sludge	2.40	nav	nav	4.40	nd	nav	nav	nav	nav	Carbella et al. 2004
Rome, Italy	Secondary, with activated sludge	**44**	**11**	nav	**17**	**1.6**	nav	**61**	**85**	nav	D'Ascenzo et al. 2003
Wahan, China	Secondary, with activated sludge	62.5	41.3	nav	31.5	8.6	nav	70.1	96.0	nav	Jin et al. 2008
Toyko, Japan	Secondary, with activated sludge	197	25.8	nav	79.7	16.7	nav	90.3	97.9	nav	Nakada et al. 2006
Penha Rio de Janeiro, Brazil	Secondary, with aerator tank and trickling filter	**40**	**21**	nav	nav	nav	nav	83	99.9	78	Ternes et al. 1999a

Table 3.1 (continued)

Location	Type of treatment	RS Concentration ($ng\ L^{-1}$)			FE Concentration ($ng\ L^{-1}$)			% Difference			Study
		E1	E2	EE2	E1	E2	EE2	E1	E2	EE2	
Canada	Primary (n = 1), secondary (n = 10), tertiary (n = 3) and lagoon (n = 4)	**49**	**15.6**	nav	17	1.8	nav	97.8	98.8	nav	Servos et al. 2005
Western Canada	Secondary, with trickling filter	**9**	**5**	**1**	**41**	**5**	**17**	**-370**	**-11**	**-2096**	Fernandez et al. 2007
	Secondary, with activated sludge (n = 2)	33	1	nd	18	nd	nd	70	100	n/a	
	Lagoon	nd	7	nd	56	158	5	**-133**	**-486**	**-699**	
Brandon, Manitoba, Canada	Tertiary, with activated sludge and UV disinfection	72.26	26.45	30.42	4.91	4.43	7.63	93.2	83.3	74.9	Cicek et al. 2007
Ontario, Canada	Various (n = 10)	nav	nav	nav	48	64	42	nav	nav	nav	Ternes et al. 1999a
Southwestern Ontario, Canada	Primary lagoon (n = 3) and secondary with activated sludge (n = 9)	48.7	13.9	nav	38.0	nd	nav	**100**	**100**	nav	Lishman et al. 2006
Toronto, Canada	Secondary, with activated sludge (n = 4)	67	26	nav	75	7.4	nav	nav	nav	nav	Lee et al. 2004

Bold data are mean concentrations and mean percent differences

n = sample size; n/a = not applicable; nav = not available; nd = not detected; UV = ultraviolet radiation

Table 3.2 **Maximum concentrations of estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) in surface waters and drinking water**

Location	Description	Concentration (ng L^{-1})			Study
		E1	E2	EE2	
United Kingdom	River Nene	2.12	nav	4.6	Williams et al. 2003
	River Lea	2.03	nav	4.0	Williams et al. 2003
The Netherlands	Rivers, canals and harbours (n = 11)	3.4	5.5	4.3	Belfroid et al. 1999
France	Jalle d'Eysines River	71	4.4	nav	Labadie and Budzinski 2005
Germany	Rivers and streams (n = 15)	1.6	nd	nd	Ternes et al. 1999a
Israel	Upper Jordan River	nav	6.1 *	6.9	Barel-Cohen et al. 2006
Germany	Drinking water	nav	nav	<0.5	Aherne and Briggs 1989

* E2 value represents (E1 + E2)

n = sample size; nav = not available; nd = not detected

Table 3.3 Daily inputs of estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) by either members of or a total typical Western population

Study	Group	E1 (µg)	E2 (µg)	EE2 (µg)
Johnson et al. 2000	**Individual male**	3.9	1.6	n/a
	Individual pre-menstruating female	3.9	1.6	n/a
	Individual menstruating female	8	4.8	n/a
	Individual menopausal woman	4	2.3	n/a
	Individual pregnant woman	600	259	n/a
	Individual woman on pill	n/a	n/a	9.1
	Individual woman on hormone therapy	n/a	n/a	7.2
Christiansen et al. 2002	**Average individual**	12.8	6.7	0.6

Data for Christiansen et al. (2002) based on population of Denmark in 2001 (Statistics Denmark 2008) and assumes 26% of ingested EE2 is excreted

Table 3.4 Latitude, longitude and altitude of sampling locations

	Sampling Location		Latitude (N)	Longitude (W)	Altitude (masl)
WWTP	**Ottawa**	**Raw sewage**	45° 27' 5.69"	75° 35' 29.29"	54
		Final effluent	45° 27' 50.64"	75° 35' 33.32"	52
	Cornwall	**N/A**	45° 01' 46.21"	74° 40' 42.39"	65
River	**Ottawa**	**Upstream**	45° 27' 55.97"	75° 35' 46.57"	40
		Downstream	45° 28' 45.35"	75° 33' 37.52"	42
	St. Lawrence	**Site 1**	45° 01' 06.57"	74° 41' 45.74"	47
		Site 2	45° 01' 29.82"	74° 40' 56.87"	46
	Raisin	**Site 4**	45° 05' 43"	74° 41' 34.2"	53
		Site 5	45° 08' 6.6"	74° 34' 6.1"	50
		Site 6	45° 08' 00.5"	74° 32' 6.2"	50
		Site 21	45° 07'43"	74° 55' 4.4"	94
Drinking water	**University of Ottawa**	**CAREG, 130 Marie-Curie**	45° 25' 5.42"	75° 40' 5.36"	70

Coordinates and altitudes obtained from Google Earth (2008)

Table 3.5 Estimates of the population demographics for the Ottawa population being served by the Ottawa sewage treatment plant

Age	Total	Male	Female
0 to 4 years	42954	21789	21165
5 to 9 years	44755	22709	22041
10 to 14 years	50471	25802	24674
15 to 19 years	52847	26765	26082
20 to 24 years	56617	28207	28410
25 to 29 years	51686	25105	26581
30 to 34 years	53815	25903	27917
35 to 39 years	57837	28212	29630
40 to 44 years	67590	33197	34393
45 to 49 years	65373	31610	33763
50 to 54 years	57372	27757	29620
55 to 59 years	50858	24800	26063
60 to 64 years	36309	17472	18832
65 to 69 years	27205	12768	14433
70 to 74 years	23086	10667	12419
75 to 79 years	19234	8150	11083
80 to 84 years	15265	5745	9520
85 years and over	12855	3780	9075

Data based on Statistics Canada (2006) and OCMBP (2006)

Table 3.6 Estimates of the population demographics for the Cornwall population being served by the Cornwall sewage treatment plant

Age	Total	Male	Female
0 to 4 years	45965	21795	24170
5 to 9 years	2250	1180	1075
10 to 14 years	2505	1300	1210
15 to 19 years	3065	1540	1525
20 to 24 years	3125	1570	1560
25 to 29 years	2740	1335	1410
30 to 34 years	2325	1100	1230
35 to 39 years	2420	1135	1285
40 to 44 years	2490	1180	1300
45 to 49 years	3520	1650	1875
50 to 54 years	3825	1880	1945
55 to 59 years	3450	1720	1730
60 to 64 years	3235	1565	1670
65 to 69 years	2500	1215	1290
70 to 74 years	2255	1025	1225
75 to 79 years	2020	905	1110
80 to 84 years	1770	720	1045
85 years and over	1325	470	855

Data from Statistics Canada (2006)

Table 3.7 Mean temperature, and total precipitation for the five days prior to sampling dates at the Ottawa sewage treatment plant

Date	Mean temperature (°C)	Total precipitation (mm)
June 9/05	20.5	11
July 20/05	25.2	17.8
Aug. 4/05	22.3	0.8
Sept. 14/05	18.7	0
Oct. 11/05	11.6	28
Nov. 30/05	-1.9	38.4
Jan. 19/06	-9.3	14.6
May 10/06	10.1	7.8
June 6/06	17.8	25
Aug. 16/06	17.8	0

Data from Environment Canada (2005, 2006)

Table 3.8 **Daily plant flow (Q), hydraulic retention time (HRT), final effluent temperature (FE T) and raw sewage (RS) and final effluent (FE) carbonaceous biochemical oxygen demands (cBOD) for sampling dates at the Ottawa sewage treatment plant**

Date	Q (ML)	HRT (hours)	FE T (°C)	RS cBOD (mg L^{-1})	FE cBOD (mg L^{-1})
June 9/05	382	11.9	18	167	5
July 20/05	408	10.9	22	106	4
Aug. 4/05	370	12.3	23	170	4
Sept. 14/05	366	13.7	24	220	6
Oct. 11/05	380	12.9	20	195	4
Nov. 30/05	792	8.9	15	121	11
Jan. 19/06	567	12.5	11	184	15
May 10/06	394	16.9	13	164	9
June 6/06	402	14	18	210	5
Aug. 16/06	378	13.3	23	162	9

Data from City of Ottawa (2007)

Table 3.9 Predicted concentrations of estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) in Ottawa raw sewage

Study	Group	Population	E1 excreted daily (µg)	E2 excreted daily (µg)	EE2 excreted daily (µg)
Johnson et al. 2000	**Males**	380418	1.48×10^6	6.09×10^5	n/a
	Pre-menstruating females	67880	2.65×10^5	1.09×10^5	n/a
	Menstruating Females	257050	2.06×10^6	1.23×10^6	n/a
	Menopausal Women	75362	3.01×10^5	1.73×10^5	n/a
	Pregnant Woman	5409	1.40×10^6	3.25×10^6	n/a
	Woman on Pill	34120	n/a	n/a	3.10×10^5
	Woman on Hormone Therapy	7874	n/a	n/a	5.63×10^4
	Total		5.51×10^6	5.37×10^6	3.67×10^5
	Predicted concentration (ng L^{-1})		**13.1**	**12.7**	**0.9**
Christiansen et al. 2002	**Total**	786130	1.01×10^7	5.25×10^6	4.70×10^5
	Predicted concentration (ng L^{-1})		**23.9**	**12.4**	**1.1**

Data based on Johnson et al. (2000), Christiansen et al. (2002), Statistics Canada (2006) and OCMBP (2006)

n/a = not applicable

Table 3.10 Predicted concentrations of estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) in Cornwall raw sewage

Study	Group	Population	E1 excreted daily (µg)	E2 excreted daily (µg)	EE2 excreted daily (µg)
Johnson et al. 2000	**Males**	21795	8.50×10^4	3.49×10^4	n/a
	Pre-menstruating females	3810	1.49×10^4	6.10×10^3	n/a
	Menstruating Females	13683	1.09×10^5	6.57×10^4	n/a
	Menopausal Women	6345	2.54×10^4	1.46×10^4	n/a
	Pregnant Woman	322	8.35×10^4	1.93×10^5	n/a
	Woman on Pill	1821	n/a	n/a	1.66×10^4
	Woman on Hormone Therapy	420	n/a	n/a	3.00×10^3
	Total		3.18×10^5	3.15×10^5	1.96×10^4
	Predicted concentration (ng L^{-1})		**7.3**	**7.2**	**0.4**
Christiansen et al. 2002	**Total**	45965	5.89×10^5	3.07×10^5	2.75×10^4
	Predicted concentration (ng L^{-1})		**13.5**	**7.0**	**0.6**

Data based on Johnson et al. (2000), Christiansen et al. (2002) and Statistics Canada (2006)

n/a = not applicable

Table 3.11.1 Concentrations of estrone in Ottawa and Cornwall wastewater; mean, standard deviation (SD), sample size (n) and standard error (SE) of raw sewage (RS) and final effluent (FE), and percent difference from raw sewage to final effluent are listed and significant differences between mean RS and FE concentrations are indicated

		RS				FE					
	Date	Mean (ng L^{-1})	SD (ng L^{-1})	n	SE (ng L^{-1})	Mean (ng L^{-1})	SD (ng L^{-1})	n	SE (ng L^{-1})	% Difference	p
Ottawa	**June 9/05**	**47.3**	5.8	2	4.1	**86.8**	10.3	2	7.3	-83.5	ns
	July 20/05	**50.4**	2.6	3	1.5	**20.6**	0.7	3	0.4	59.1	< 0.0001
	Aug. 4/05	**46.7**	2.4	3	1.4	**205**	3	3	2	-340	< 0.0001
	Sept. 14/05	**44.9**	13.2	2	9.3	**158**	4	2	2	-252	0.0071
	Oct. 11/05	**46.0**	5.2	2	3.7	**113**	99	2	70	-148	ns
	Nov. 30/05	**35.0**	2.4	2	1.7	**11.2**	1.6	3	0.9	68.2	0.0009
	Jan. 19/06	**35.2**	1.6	3	0.9	**44.9**	2.2	3	1.3	-27.6	0.0034
	May 10/06	**48.6**	6.1	4	3.0	**11.3**	0.8	3	0.4	76.8	0.0001
	June 6/06	**not sampled**				**15.7**	3.6	3	2.1	n/a	n/a
	Aug. 16/06	**104**	8	3	5	**370**	44	3	26	-257	0.0005
Cornwall	**June 29/05**	**13.1**	2.1	2	1.5	**16.0**	0.2	2	0.2	-22.5	ns
	Aug. 11/05	**29.3**	0.1	2	0.1	**22.9**	0.3	2	0.2	21.9	0.0308

n/a = not applicable; ns = not significant; p = probability value

Table 3.11.2 Concentrations of 17β-estradiol in Ottawa and Cornwall wastewater; mean, standard deviation (SD), sample size (n) and standard error (SE) of raw sewage (RS) and final effluent (FE), and percent difference from raw sewage to final effluent are listed and significant differences between mean RS and FE concentrations are indicated

		RS				FE					
	Date	Mean (ng L^{-1})	SD (ng L^{-1})	n	SE (ng L^{-1})	Mean (ng L^{-1})	SD (ng L^{-1})	n	SE (ng L^{-1})	% Difference	p
Ottawa	**June 9/05**	**24.7**	0.2	2	0.1	**< MDL**	n/a	2	n/a	n/a	n/a
	July 20/05	**< MDL**	n/a	3	n/a	**nd**	0	3	0	100	nc
	Aug. 4/05	**< MDL**	n/a	3	n/a	**nd**	0	3	0	100	nc
	Sept. 14/05	**66.9**	22.2	2	15.7	**< MDL**	n/a	2	n/a	n/a	n/a
	Oct. 11/05	**< MDL**	n/a	2	n/a	**26.7**	3.5	2	2.5	n/a	n/a
	Nov. 30/05	**< MDL**	n/a	2	n/a	**nd**	0	3	0	100	nc
	Jan. 19/06	**nd**	0	3	0	**< MDL**	n/a	3	n/a	n/a	n/a
	May 10/06	**nd**	0	4	n/a	**nd**	0	3	0	n/a	n/a
	June 6/06	**not sampled**				**< MDL**	n/a	3	n/a	n/a	n/a
	Aug. 16/06	**nd**	0	3	0	**nd**	0	3	0	n/a	n/a
Cornwall	**June 29/05**	**< MDL**	n/a	2	n/a	**< MDL**	n/a	2	n/a	n/a	n/a
	Aug. 11/05	**< MDL**	n/a	2	n/a	**< MDL**	n/a	2	n/a	n/a	n/a

MDL = method detection limit; n/a = not applicable; nc = not calculated; nd = not detected; ns = not significant; p = probability value

Table 3.11.3 Concentrations of 17α-ethinylestradiol in Ottawa and Cornwall wastewater; mean, standard deviation (SD), sample size (n) and standard error (SE) of raw sewage (RS) and final effluent (FE), and percent difference from raw sewage to final effluent are listed and significant differences between mean RS and FE concentrations are indicated

		RS				FE					
	Date	Mean (ng L^{-1})	SD (ng L^{-1})	n	SE (ng L^{-1})	Mean (ng L^{-1})	SD (ng L^{-1})	n	SE (ng L^{-1})	% Difference	p
Ottawa	**June 9/05**	**nd**	0	2	0	**nd**	0	2	0	n/a	n/a
	July 20/05	**nd**	0	3	0	**nd**	0	3	0	n/a	n/a
	Aug. 4/05	**nd**	0	3	0	**nd**	0	3	0	n/a	n/a
	Sept. 14/05	**nd**	0	2	0	**0.6**	0.8	2	0.4	n/a	n/a
	Oct. 11/05	**nd**	0	2	0	**nd**	0	2	0	n/a	n/a
	Nov. 30/05	**nd**	0	2	0	**nd**	0	3	0	n/a	n/a
	Jan. 19/06	**1.3**	2.2	3	1.3	**nd**	0	3	0	100	ns
	May 10/06	**nd**	0	4	0	**nd**	0	3	0	n/a	n/a
	June 6/06	**not sampled**				**nd**	0	3	0	n/a	n/a
	Aug. 16/06	**nd**	0	3	0	**nd**	0	3	0	n/a	n/a
Cornwall	**June 29/05**	**5.7**	5.8	2	4.1	**9.8**	11.8	2	8.4	-73.1	ns
	Aug. 11/05	**0.5**	0.1	2	0.1	**1.0**	0.1	2	0.1	-119	ns

n/a = not applicable; nd = not detected; ns = not significant; p = probability value

Table 3.12 **Linear regression analysis results for concentration of estrone (E1) in Ottawa raw sewage (RS) versus mean temperature (T), total precipitation five days prior to sampling, mean daily flow (Q), and raw sewage carbonaceous biochemical oxygen demand (cBOD); R^2, sample size (n) and probability value (p) are listed**

		R^2	n	p
E1 RS (ng L^{-1}) vs.	T (°C)	0.792	8	0.003
	Precipitation (mm)	0.230	8	0.230
	LN[Q (ML)]	0.757	8	0.005
	LN[RS cBOD (mg L^{-1})]	0.001	8	0.947

Table 3.13 **Multiple regression analysis results for concentration of estrone (E1) in Ottawa raw sewage (RS) versus mean temperature (T), total precipitation five days prior to sampling, mean daily flow (Q), and raw sewage carbonaceous biochemical oxygen demand (cBOD); R^2, sample size (n) and probability value (p) are listed**

		R^2	n	p
E1 RS (ng L^{-1}) vs.	T (°C) x Precipitation (mm)	0.796	8	0.019
	T (°C) x LN[Q (ML)	0.870	8	0.006
	T (°C) x LN[RS cBOD (mg L^{-1})]	0.795	8	0.019
	Precipitation (mm) x LN[Q (ML)	0.796	8	0.019
	Precipitation (mm) x LN[RS cBOD (mg L^{-1})]	0.324	8	0.376
	LN[Q (ML) x LN[RS cBOD (mg L^{-1})]	0.963	8	0.001
	T (°C) x Precipitation (mm) x LN[Q (ML)	0.892	8	0.021
	T (°C) x Precipitation (mm) x LN[RS cBOD (mg L^{-1})]	0.796	8	0.073
	T (°C) x x LN[Q (ML) LN[RS cBOD (mg L^{-1})]	0.963	8	0.021
	Precipitation (mm) x LN[Q (ML) x LN[RS cBOD (mg L^{-1})]	0.967	8	0.002
	T (°C) x Precipitation (mm) x LN[Q (ML)] x LN[RS cBOD (mg L^{-1})]	0.967	8	0.015

Table 3.14 **Linear regression results for concentration of estrone (E1) in Ottawa final effluent (FE) versus mean daily flow (Q), final effluent temperature (T), and final effluent carbonaceous biochemical oxygen demand (cBOD); R^2, sample size (n) and probablity value (p) are listed**

		R^2	n	p
E1 FE (ng L^{-1}) vs.	LN[Q (ML)]	0.247	9	0.174
	LN[FE T (°C)]	0.393	9	0.071
	LN[FE cBOD (mg L^{-1})]	0.214	9	0.210

Table 3.15 **Multiple regression analysis results for concentration of estrone (E1) in Ottawa final effluent (FE) versus mean daily flow (Q), final effluent temperature (T), and final effluent carbonaceous biochemical oxygen demand (cBOD); R^2, sample size (n) and probability value (p) are listed**

		R^2	n	p
E1 FE (ng L^{-1}) vs.	LN[Q (ML)] vs. LN[FE T (°C)]	0.419	9	0.196
	LN[Q (ML)] vs. LN[FE cBOD (mg L^{-1})]	0.267	9	0.394
	LN[FE T (°C)] vs. LN[FE cBOD (mg L^{-1})]	0.421	9	0.194
	LN[Q (ML)] vs. LN[FE T (°C)] vs. LN[FE cBOD (mg L^{-1})]	0.756	9	0.257

Table 3.16 **Linear and multiple regression analysis results for percent difference of estrone (E1) during Ottawa sewage treatment process versus hydraulic retention time (HRT) and percent difference carbonaceous biochemical oxygen demand (cBOD); R^2, sample size (n) and probability value (p) are listed**

		R^2	n	p
% Δ E1 vs.	LN[HRT (hours)]	0.032	8	0.672
	% Δ cBOD	0.435	8	0.075
	LN[HRT (hours)] x % Δ cBOD	0.439	8	0.236

Table 3.17 **Responses of extracts of dimethyl sulfoxide (DMSO) control, two raw sewage (RS) and two final effluent (FE) samples taken on October 11, 2005 at ROPEC, Ottawa, ON, in reporter gene assay; mean percent response of 100 nM estradiol, standard deviation (SD), sample size (n) and standard error (SE) are listed**

Sample	Mean response (%)	SD (%)	n	SE (%)
DMSO	**7.4**	7.3	3	4.2
RS – 1	**28.5**	8.7	3	5.0
RS – 2	**33.4**	14.4	3	8.3
FE – 1	**23.4**	7.6	3	4.4
FE - 2	**29.3**	9.1	3	5.3

N.B. ANOVA shows no significant difference between wastewater samples

Table 3.18 **Concentrations of estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) in Ottawa River; mean, standard deviation (SD), sample size (n) and standard error (SE) and difference from upstream to downstream samples are listed**

		Upstream of STP				Downstream of STP				
	Date	Mean (ng L^{-1})	SD (ng L^{-1})	n	SE (ng L^{-1})	Mean (ng L^{-1})	SD (ng L^{-1})	n	SE (ng L^{-1})	Difference (ng L^{-1})
E1	**June 22/05**	**nd**	0	2	0	**< MDL**	n/a	2	n/a	< MDL
	July 20/05	**nd**	0	3	0	**nd**	0	3	0	0
E2	**June 22/05**	**nd**	0	2	0	**nd**	0	2	0	0
	July 20/05	**nd**	0	3	0	**nd**	0	3	0	0
EE2	**June 22/05**	**0.8**	0.4	2	0.3	**2.2**	1.3	2	1.0	-1.4
	July 20/05	**< MDL**	n/a	3	n/a	**< MDL**	n/a	3	n/a	0

N.B. ANOVA shows no significant difference between EE2 samples

MDL = method detection limit; n/a = not applicable; nd = not detected

Table 3.19 **Concentrations of estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) in St. Lawrence River on June 15, 2005; mean, standard deviation (SD), sample size (n) and standard error (SE) are listed**

	Site	Mean (ng L^{-1})	SD (ng L^{-1})	n	SE (ng L^{-1})
E1	**1**	**107**	50	2	35
	2	**nd**	0	2	0
E2	**1**	**nd**	0	2	0
	2	**nd**	0	2	0
EE2	**1**	**0.4**	0.3	2	0.2
	2	**0.7**	< 0.1	2	< 0.1

nd = not detected

Table 3.20 Concentrations of estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) in Raisin River; mean, standard deviation (SD), sample size (n) and standard error (SE) are listed

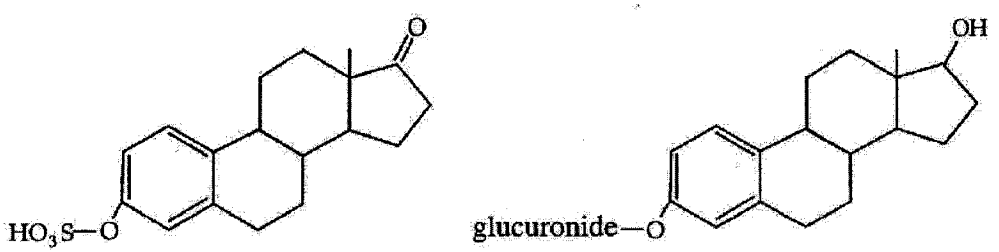
	Site	Date	Mean (ng L^{-1})	SD (ng L^{-1})	n	SE (ng L^{-1})
E1	**4**	**Nov. 30/05**	**nd**	0	2	0
		May 4/06	**< MDL**	n/a	2	n/a
	5	**Nov. 30/05**	**nd**	0	2	0
		May 4/06	**nd**	0	2	0
	6	**May 2/06**	**< MDL**	n/a	2	n/a
	21	**May 2/06**	**3.6**	5.0	2	3.6
E2	**4**	**Nov. 30/05**	**nd**	0	2	0
		May 4/06	**nd**	0	2	0
	5	**Nov. 30/05**	**nd**	0	2	0
		May 4/06	**nd**	0	2	0
	6	**May 2/06**	**nd**	0	2	0
	21	**May 2/06**	**nd**	0	2	0
EE2	**4**	**Nov. 30/05**	**1.7**	0.4	2	0.3
		May 4/06	**nd**	0	2	0
	5	**Nov. 30/05**	**3.0**	2.1	2	1.5
		May 4/06	**nd**	0	2	0
	6	**May 2/06**	**nd**	0	2	0
	21	**May 2/06**	**nd**	0	2	0

MDL = method detection limit; n/a = not applicable; nd = not detected

Table 3.21 **Concentrations of estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) in University of Ottawa drinking water; mean, standard deviation (SD), sample size (n) and standard error (SE) are listed**

	Date	Mean (ng L^{-1})	SD (ng L^{-1})	n	SE (ng L^{-1})
E1	**Sept. 19/05**	**nd**	0	2	0
	Nov. 11/05	**< MDL**	n/a	2	n/a
	April 12/06	**nd**	0	2	0
	May 19/06	**nd**	0	3	0
E2	**Sept. 19/05**	**nd**	0	2	0
	Nov. 11/05	**nd**	0	2	0
	April 12/06	**nd**	0	2	0
	May 19/06	**nd**	0	3	0
EE2	**Sept. 19/05**	**1.3**	0.4	2	0.3
	Nov. 11/05	**< MDL**	n/a	2	n/a
	April 12/06	**0.4**	0.6	2	< 0.1
	May 19/06	**< MDL**	n/a	3	n/a

nd = not detected



E1-3-sulfate (E1-3S) E2-3-glucoronide (E2-3G)

Figure 3.1 Molecular structure of some estrone and 17β-estradiol conjugates (Raftogianis et al. 2000)

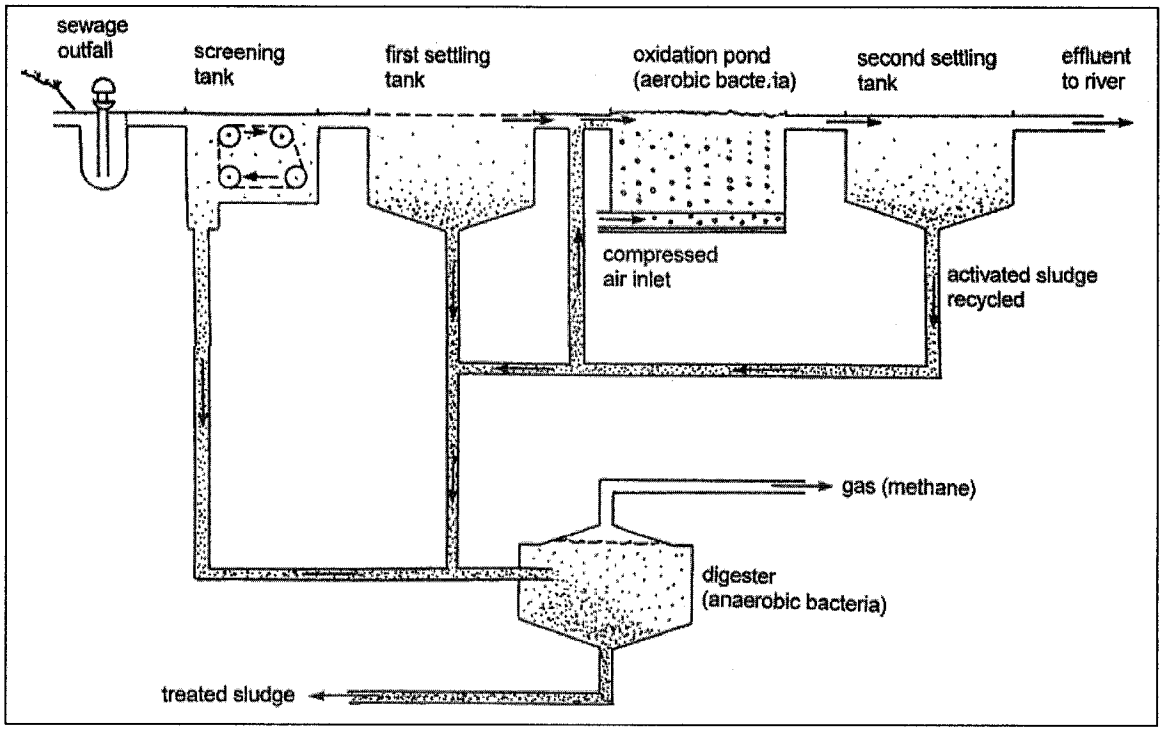


Figure 3.2 **The activated sludge sewage treatment process (Edexcel Foundation 2005)**

Figure 3.3.1 Satellite generated map of the sampling locations at the Ottawa Robert O. Pickard Environmental Centre and in the Ottawa River, Ontario (Google Earth 2008)

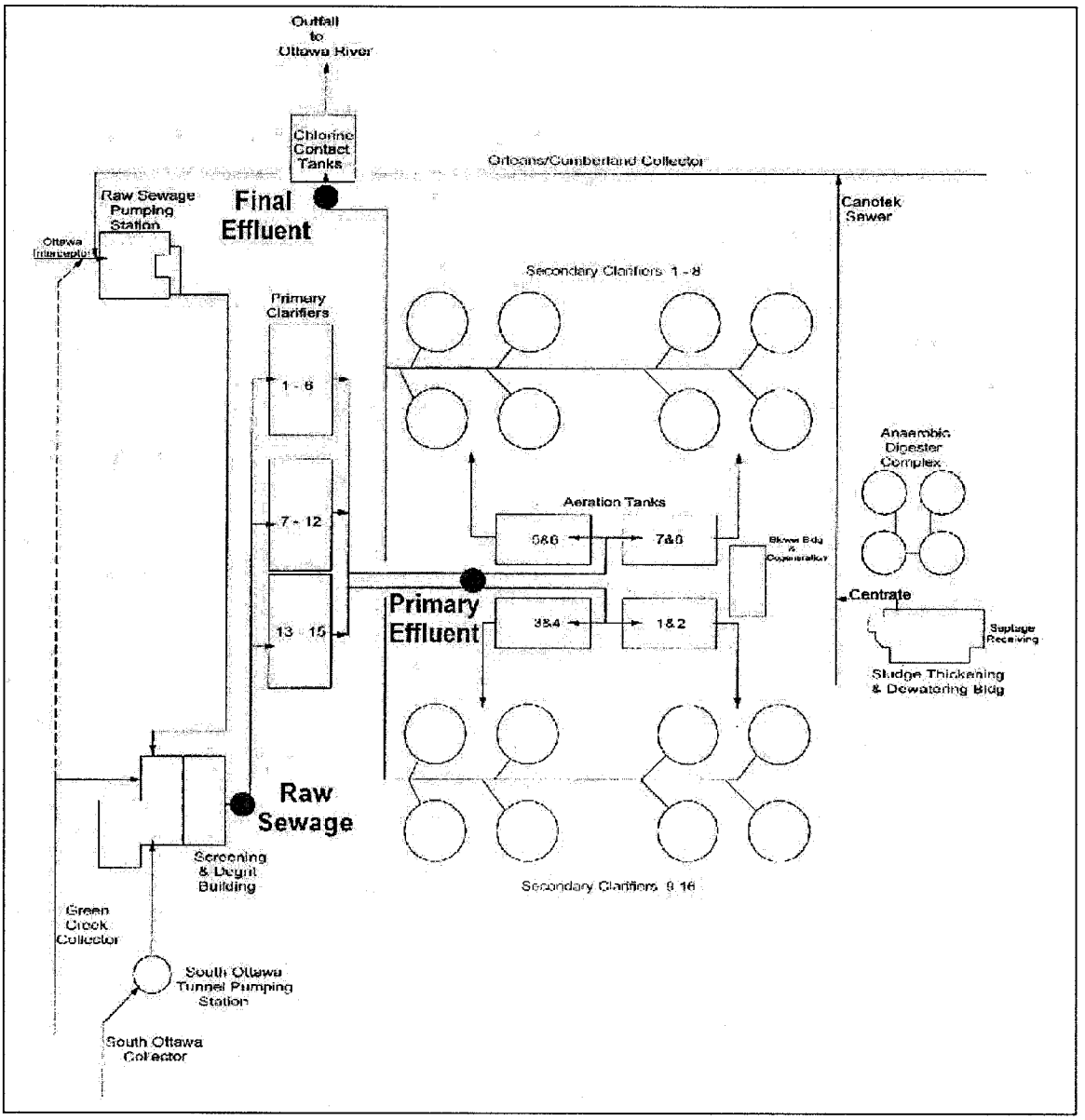


Figure 3.3.2 Schematic of the City of Ottawa Robert O. Pickard Environmental Centre, Ontario with raw sewage and final effluent sampling locations indicated (City of Ottawa 2005)

Figure 3.3.3 Satellite generated map of the sampling locations at the Cornwall sewage treatment plant and in the St. Lawrence River, Ontario (Google Earth 2008)

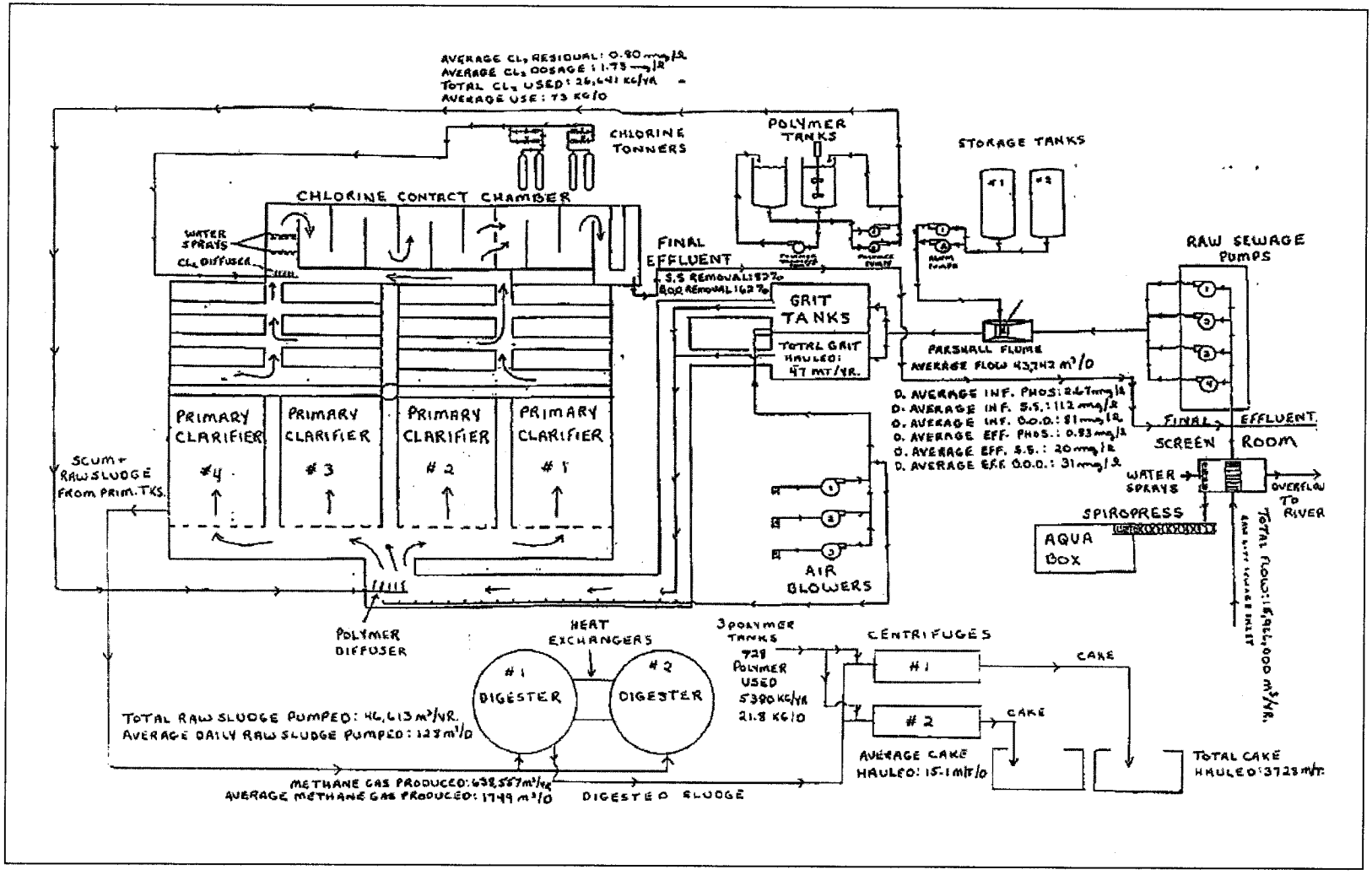


Figure 3.3.4 Schematic of the City of Cornwall sewage treatment plant, Ontario (City of Cornwall 2008)

Figure 3.3.5 Satellite generated map of the sampling locations in the Raisin River, Ontario (Google Earth 2008)

Figure 3.3.6 Satellite generated map of the sampling location at the University of Ottawa, Ottawa, Ontario (Google Earth 2008)

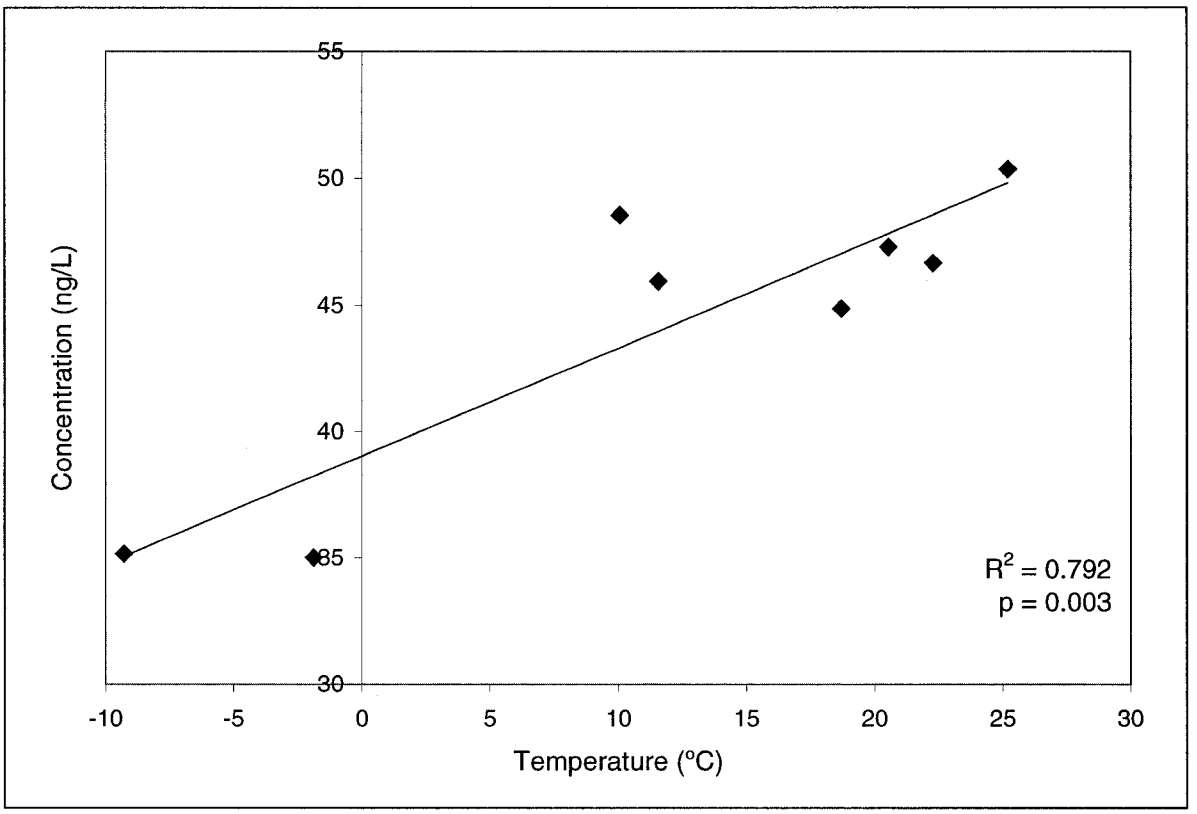


Figure 3.4 **Concentration of estrone in Ottawa raw sewage versus mean environmental temperature five days prior to sampling**

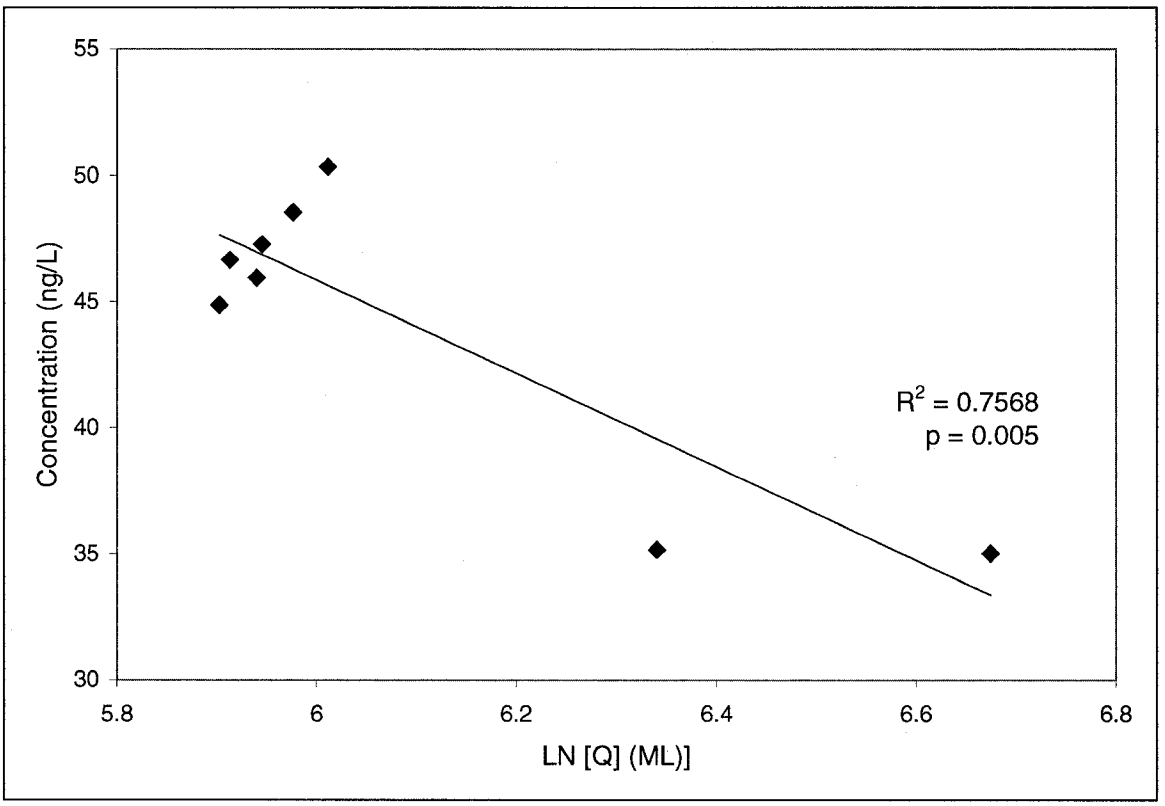


Figure 3.5 **Concentration of estrone in Ottawa raw sewage versus mean daily flow**

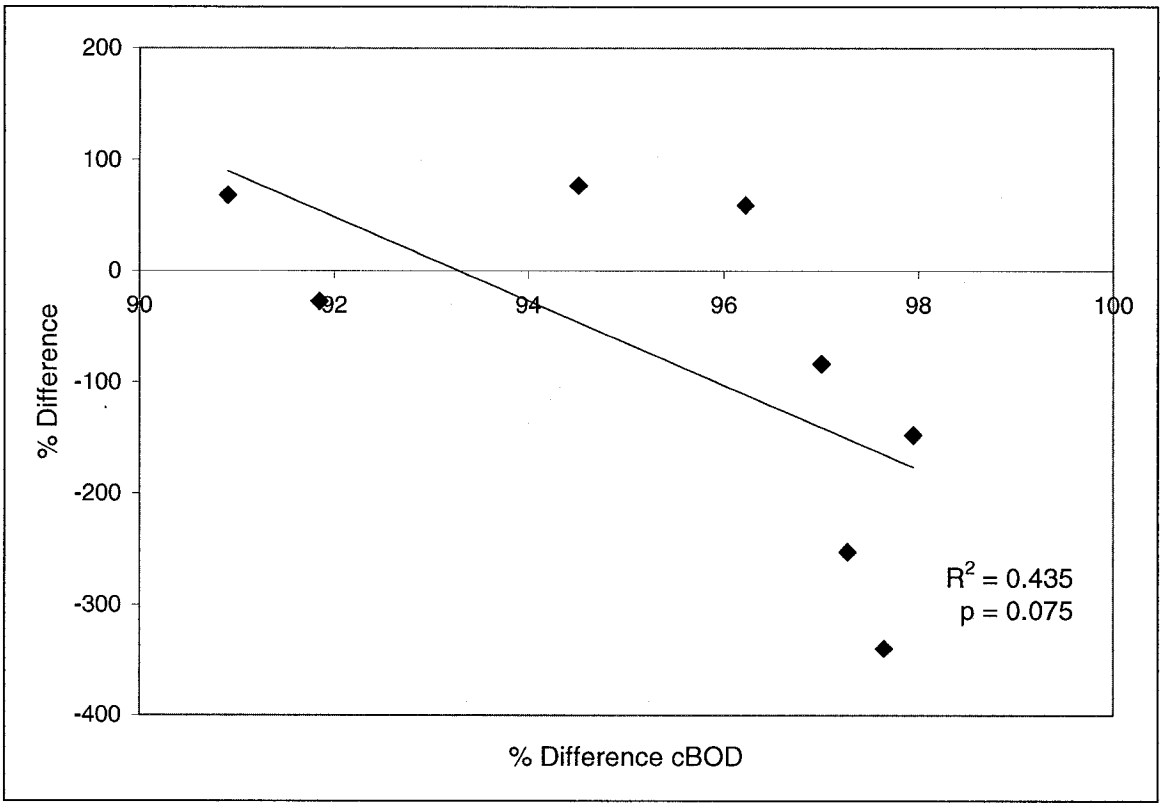


Figure 3.6 **Percent difference of estrone versus percent difference of carbonaceous biological oxygen demand in Ottawa wastewater**

CHAPTER 4: DEGRADATION OF STEROIDAL ESTROGENS UNDER ULTRAVIOLET B RADIATION

4.1 ABSTRACT

The environmental fate and persistence of steroidal estrogens is influenced by their photodegradation in the presence of sunlight. To determine the photodegradation rate of estrone (E1) and 17α-ethinylestradiol (EE2) water samples containing these compounds were exposed to levels of ultraviolet (UV) B radiation, simulating ambient sunlight. E1 degraded with a first-order rate law constant that was directly proportional to UVB radiation intensity (R^2 = 0.999, $p < 0.001$) and inversely proportional to dissolved organic carbon (DOC) concentration (R^2 = 0.812, p = 0.037). DOC acted as a competitive inhibitor to UVB activity. In contrast to E1, EE2 was more persistent under similar UVB treatment. A reporter gene assay showed that the estrogenicity of UVB-exposed estrogens did not decrease relative to non UVB-exposed estrogens, and this may be attributed to the estrogenicity of the E1 degradation by-product, lumiestrone. Even though some modern STP's are incorporating UVB in tertiary treatment, it may not be a viable solution for removing estrogenicity in effluent. These results suggest that environmental degradation rates of steroidal estrogens are predictable from UV intensity reaching surface waters, and DOC concentrations.

4.2 INTRODUCTION

The investigation of steroidal estrogen behaviour under ultraviolet (UV) B radiation (λ = 280-320 nm) is important because of the increasing use of UV radiation in the tertiary sewage treatment plant as a disinfection tool. Furthermore, when estrogens are released to the environment, they are exposed to solar radiation, which includes the UVA wave band of the solar spectrum (λ = 320-400 nm), as well as UVB. Not reaching the earth's surface is the highly destructive UVC band (λ = 240-280 nm) which is removed by the ozone layer; however lamps producing UVC are used in STP treatment. The estrogens estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) are candidates for UV photodegradation because they show maximum absorbance peaks in the UV area of the radiation spectrum (Coleman et al. 2004).

In sewage treatment plants, UV radiation sources are not only a clean alternative to chlorination for removing bacteria and viruses, but also provide additional decontamination. Conventional wastewater disinfection methods use chemical agents such as chlorine, hypochlorites, chloramine, chlorine dioxide, bromine or ozone that destroy genetic material in microorganisms, and sometimes physical methods such as filtration by sand or synthetic membranes (Acher et al. 1997; Ternes et al. 1999a; Das 2001). Regulatory agencies are encouraging the replacement of chemical methods by ecologically friendly disinfection processes because of the toxic by-products found in water treated with chemicals. As well, conventional water treatment systems do not completely remove endocrine-disrupting compounds (EDC's) (Rosenfeldt and Linden 2004). Ultraviolet radiation can degrade contaminants, either via direct photolysis, direct

absorption of light followed by a chemical reaction, or indirect photolysis, where a sensitizer such as dissolved organic matter, nitrate and nitrite, absorbs solar radiation and transfers the energy in a form such as aqueous electrons, superoxide and its products or singlet oxygen (Miyamoto 1996; Lin and Reinhard 2005).

Liu and Liu (2004) investigated the feasibility of the photochemical treatment of estrogens with wavelengths in the UV and visible spectrum. The direct photolysis of E1 and E2 (3-20 mg L^{-1}) occurred under both wavelengths. UV-radiation (intensity = 1500 $\mu W\ cm^{-2}$) was more effective in reducing concentrations than UV-Vis-radiation, and E1 degraded more rapidly than E2. Photodegradation occurred in accordance with a pseudo-first-order law; the higher the initial concentration, the lower the rate of photolysis. Coleman et al. (2004) showed that photodegradation can also reduce the potency of steroidal estrogens, which was measured using a recombinant yeast assay with the reporter gene Lac-Z. A 50% reduction in estrogenicity was observed for E1, E2 and EE2 under UVA radiation (125 W bulb, 3 cm from samples) within 68, 23 and 195 minutes respectively. Cicek et al. (2007) noted that UV doses used in laboratory studies are several-fold higher than commonly encountered in wastewater disinfection applications; in their study of a Brandon, Manitoba STP, the UV process did not have a statistically significant effect on reducing the concentrations of estrogens.

Liu and Liu (2004) also used infra-red (IR) technology to show that photolysis of E1 and E2 causes the breakage of benzene rings and produces carbonyl groups. Because photodegradation rates increased in the presence of a titanium dioxide (TiO_2), which helps generate hydroxyl radicals, the mechanisms for this photooxidation is hypothesized to be initiated at the phenolic hydroxyl groups, which is susceptible to hydroxyl radical

attack (Coleman et al. 2004). In addition, the unconjugated estrogens, E1- and E2-glucuronides, which do not have these phenolic groups, remained after UV irradiation (Mitamura et al. 2004). The catalyst TiO_2 is not found in natural waters, however, this mechanism may help explain why Liu and Liu (2004) report a decrease in photolysis of E1 and E2 at lower pH values; H^+ ions may possibly prevent hydroxyl radicals from attacking the phenolic groups. The higher rates of E1 photodegradation, as compared to EE2, have been suggested to be a result of the E1's carbonyl group, which is excited to its triplet state (Lin and Reinhard 2005).

Factors that affect the reduction equivalent dose (RED), which is the efficacy of a UV disinfection device as measured by a bioassay, are lamp intensity and water transmittance (Sommer et al. 1997). Dissolved and suspended substances within the water column significantly attenuate the penetration of UV radiation, and in water bodies with low turbidity, dissolved organic carbon (DOC) is the major control of UVB attenuation (Brooks et al. 2005). Ahel et al. (1994) looked at the photochemical behaviour of an estrogenic chemical, nonylphenol and its ethoxylates in filtered lake water (DOC = 4 mg L^{-1}) and found degradation was significantly reduced with depth due to light attenuation. In addition, sewage treatment studies have shown that where there are suspended solids in effluent, the UVB dose required to achieve adequate disinfection is significantly higher (Andreadakis et al. 1999). Overall, it has been shown that diminished UV intensity, achieved by diminishing the lamp voltage, has a greater effect on altering the RED than water transmittance, achieved by pumping aqueous sodium thiosulphate solution into the water inflow (Sommer et al. 1997).

One excellent study investigated the photodegradation of estrogens under a number of environmentally relevant conditions: estrogen concentrations typically found in effluent, degradation in river water as opposed to purified water, an intensity of radiation identical to that of midday, midsummer sunlight in California, and different oxygen concentrations (Lin et al. 2005). Half-lives of E1, E2 and EE2 in purified water were 4.7, 41.7 and 28.4 hours, respectively, and in river water disinfected by autoclaving, half-lives decreased. In contrast to Ahel et al.'s alkylphenol degradation study (1994), the presence of DOC in the river water was suggested to increase the degradation rates of estrogens. DOC was hypothesized to act as a photosensitizer, producing excited triplet states and radicals that reacted with the estrogens, causing indirect phototransformation (Canonica et al. 2008). Rates also decreased in the presence of oxygen; although unclear, this decrease was suggested to be a result of enhanced photodecarboxylation. High costs can be involved in UV sewage treatment, so more research needs to be done to determine if UV lamps could significantly increase steroidal estrogen removal compared to biodegradation in a matrix as complex as sewage effluent (Johnson and Sumpter 2001).

Here, photodegradation rates of E1 and EE2 were determined using environmentally relevant UVB intensities in water samples obtained from the Ottawa sewage treatment plant and local rivers and lakes. I predicted that E1 would degrade faster than EE2 and that the presence of DOC in the environmental samples would alter these degradation rates. Two alternatives were anticipated: 1) rates of degradation would decrease in the presence of DOC due to competitive inhibition, or 2) rates would increase since DOC would be acting as a photosensitizer. Furthermore, both increased

UVB intensity and decreased water sample thickness were predicted to increase degradation rates.

4.3 METHODS

4.3.1 UVB absorbance of steroidal estrogens

Estrone, 17β-estradiol and 17α-ethinylestradiol were dissolved in acetonitrile (Burdrick & Jackson) and absorbances were measured with a Cary 300 UV-visible spectrophotometer.

4.3.2 Types of samples used in exposures

5 μL of 500 ng mL^{-1} E1 and EE2 standards (E1-12C, 100% purity; EE2-12C 100% purity; Cambridge, Isotope Laboratories, Andover, MA) were dissolved in 95% ethanol (VWR International) and added to 1-L samples of water to obtain E1 and EE2 concentrations of 500 ng L^{-1}. Five different types of water were used: double distilled water, water collected from the upstream sampling location on the Ottawa River on August 4, 2005 (Figure 3.3.1), water collected from Lake Cromwell, Quebec on October 22, 2005 (Appendix IV), raw sewage collected from the Ottawa Robert O. Pickard Environmental Centre on September 14, 2005 (Figure 3.3.1), and water collected from the Raisin River on November 30, 2005 (Figure 3.3.5). The DOC concentrations were measured using a IO Analytical 1010 total organic carbon analyzer (Graden Instruments, Oakville, ON), and values were calculated using a standard curve constructed from the analysis of five standards (0, 5, 10, 25 and 50 ppm) made from sodium biphthalate. The thicknesses (i.e. depth) of the water samples were also measured prior to UVB exposure.

4.3.3 Set-up of incubator and UVB bulbs

An environmental chamber (Conviron model 125L, Winnipeg, MB), set at a constant temperature of 21°C, was used for the simultaneous exposures of E1 and EE2. The supplementary lighting consisted of two UVB lamps (UBL FS20T12, National Biological Corp. Twinsberg, Ohio) and two soft white lamps (Philips F20 T12). The emission spectra of the UVB lamps was measured using a Luzchem and is illustrated in Figure 4.1. UV and VIS measurements were carried out using a GOLDILUX radiometer/photometer GRP-1 (Model 70237) with an interchangeable UVAR probe GAP-1 (Model 0237), UVBR probe GBP-1 (Model 0238) and a cosine probe GLP-1 (Illuminance/VIS; Model 70235) (ThermoOriel, Stratford, CT). Samples were placed on shelves which were adjusted vertically to attain five different intensities of UVB radiation: 6.7 $\mu W\ cm^{-2}$ (SE $\pm$ 0.3 $\mu W\ cm^{-2}$), 73.3 $\mu W\ cm^{-2}$ (SE $\pm$ 0.5 $\mu W\ cm^{-2}$), 133 $\mu W\ cm^{-2}$ (SE $\pm$ 2 $\mu W\ cm^{-2}$), 500 $\mu W\ cm^{-2}$ and 1000 $\mu W\ cm^{-2}$. The probes were also used to measure midday UVB intensity on a cloudy day near the Rideau Canal by the University of Ottawa; this was approximately 110 $\mu W\ cm^{-2}$.

4.3.4 Extraction and analysis

Water samples were extracted and analyzed as outlined in Sections 2.3 and 2.4 of this thesis.

4.3.5 Estrogenicity assay

The estrogenicity of UVB-exposed (6 hours at 133 $\pm$ 2 $\mu W\ cm^{-2}$) and non-exposed (6 hours at 6.7 $\pm$ 0.3 $\mu W\ cm^{-2}$) samples of E1 standards in double distilled water was

determined using the luciferase reporter gene assay in Appendix I of this thesis (Ackermann et al. 2002; Marlatt 2006).

4.4 RESULTS AND DISCUSSION

4.4.1 Degradation rates of estrone and 17α-ethinylestradiol

Both estrone and 17α-ethinylestradiol were observed to degrade under UVB radiation. The absorbance curves measured for the estrogens were similar to those reported in Lin et al. (2005), with no significant absorbance above λ = 350 nm (Figure 4.2). The absorbance spectrum for E1, E2 and EE2 were virtually identical but E1 showed a slightly higher peak absorbance in the UVB range. It is well known that for a photochemical reaction to occur, energy must be absorbed in the wave band used for the exposure (Figure 4.1). Consequently, degradation would be due principally to wave bands lower than 350 nm. This higher absorbance of E1 is consistent with the observation of a more rapid degradation of E1 compared to EE2 (Figure 4.3; Liu and Liu 2004).

E1 exhibited first-order degradation constants ranging from 0.004 to 0.640 h^{-1} and in 9 of the 16 conditions these were significant at an α of 0.05 (Table 4.1). In contrast, the rate constant was only significant for EE2 in 3 of the 16 cases, ranging from 0.010 to 0.038 h^{-1} (Table 4.1). In Ottawa River water under 133 $\mu W\ cm^{-2}$, which is the intensity of UVB radiation closest to that of midday, midsummer sunlight in Ottawa, the half-life of E1 was calculated to be 5 hours. No significant degradation of EE2 was observed in the exposure period of 6 hours. These half-lives are comparable to those observed in

English rivers for E1 and EE2 which range from 0.2 to 9 days (Jürgens et al. 2002). Overall, a decline in E1 concentration from final effluent to sites downstream from a STP and a persistence of EE2 would be expected from these rate constants; in Chapter 3, E1 is shown to decrease in concentration from Ottawa final effluent to the Ottawa River downstream sampling site, and EE2 is still detected in the river water.

4.4.2 Effect of DOC, UVB intensity and sample thickness on estrone rate constants

The dissolved organic carbon concentrations calculated from the standard curve (Appendix V, $R^2 = 0.999$) ranged from 0.07 ppm for distilled water to 23.9 ppm for the Raisin River water (Table 4.1). The degradation rate constants for E1 show a significant inverse relationship with DOC concentration, and show that DOC is acting as a competitive inhibitor and not as a photosensitizer (Table 4.2, $R^2 = 0.812$, $p < 0.037$; Figure 4.4; UVB intensity = 133 $\mu W\ cm^{-2}$, sample thickness = 23.9 cm). E1 rate constants also show a significant positive relationship with UVB intensity (Table 4.2, $R^2 = 0.941$, $p < 0.030$; Figure 4.5), which was predicted, and with sample thickness (Table 4.2, $R^2 = 0.781$, $p = 0.47$; Figure 4.6), which was not anticipated.

Potential sources of error relate to the changing exposure conditions over time. For example, an increase of water transmittance may occur due to the UVB-degradation of DOC during the exposures; Winch et al. 2002 observed a 20% decrease in DOC in their Raisin River water samples after 10 days of exposure to 138 $\mu W\ cm^{-2}$. Here, DOC concentrations were unchanged in Raisin River water following an 8 hour UVB exposure at 133 $\mu W\ cm^{-2}$, confirming that DOC degradation was insignificant. As well, in samples with a smaller water thickness, significant evaporation of the sample occurred (up to

30%), which may concentrate DOC in the water and decrease transmittance over time. This may explain why a strong positive relationship was seen between the degradation rate constant and the sample thickness. The conditions that are easily kept constant throughout the exposure periods are the UVB intensities, as lamps simply switch on, or off. Perhaps this is why the greatest amount of variability in E1 degradation rate constants is explained with the regression models using UVB as the sole predictor (Table 4.2).

4.4.3 Degradation products of UVB-exposed steroidal estrogens

During separation of the compounds in the E1 and EE2-UVB exposed samples with gas chromatography, a third peak was observed in the chromatograms at approximately 15.8 minutes (Figures 4.7.1 and 4.7.2). Degradation studies of the estrogens separately showed that this peak is produced from UVB degradation of E1 (Figure 4.7.3) and that the ion spectra of the peak is almost identical to that of E1 (Figure 4.8.1 and Figure 4.8.2). These studies also showed that one of the degradation products of EE2 is E1 (Figure 4.7.4); thus the calculated rate constants for E1 may be an underestimate. On the other hand, as the degradation rate of EE2 is so much slower than E1, this error would be very small.

Past methods employing HPLC-NMR and HPLC-MS have proved useful in the characterization of photodegradation products. These techniques have previously been used to identify the photodegradation products of ethinylestradiol, which consist of dimeric compounds and a hyperoxide (Segmuller et al. 2000). In a collaborative study between the University of Ottawa departments of Biology and Chemistry, HPLC-NMR

will be used to isolate and identify the E1 photoproduct, which is characterized as lumiestrone (Trudeau et al. 2008).

Physicochemical properties of any photoproducts need to be investigated to determine their environmental persistence. For example, by-products of UVB exposure could be volatile, resulting in long-range transport in the atmosphere. Previous research has shown that organic compounds may accumulate in pristine higher altitudes due to distillation (Blais et al. 1998). Recently, elevated VTG levels have been reported in fish from alpine lakes in Rocky Mountain National Park, which suggests an estrogenic source is entering the water, yet to be identified (Schwindt et al. 2004).

4.4.4 Estrogenicity of UVB-exposed samples

The estrogenicity assay determined that in addition to the degradation of E1, UVB radiation results in a reduction of estrogenicity after 6 hours. An ANOVA, followed by a Tukey test, revealed a statistically significant difference between the estrogenicity of the non-exposed samples (mean response = 50.3%, SE ±6.6%, mean response 49.2% and SE ±3.6%) and one of the UVB-exposed samples (mean response = 32.7%, SE ±4.5%), or a decline in estrogenicity of approximately 35% (Table 4.3; $p < 0.05$). A t-test also showed a significant difference between the concentrations of E1 at 0 hours (500 ng L^{-1}) and 6 hours (122 ng L^{-1} ±3.1 ng L^{-1}) or a reduction of estrone of approximately 75%.

These results provide evidence that the degradation product observed in the chromatogram in Figure 4.7.2 is estrogenic, contrary to the notion that UV irradiation does not produce any undesirable by-products as in disinfection methods like chlorination (Sommer et al. 1997). No studies showing the effects of UV treated estrogens on aquatic

organisms exist. Some contaminants such as atrazine and polycyclic aromatic hydrocarbons increase in toxicity under UV as they are broken down into photolytic byproducts like hydride ions and oxides (Sharpless et al. 2003; Fernandez and l'Haridan 1994). In addition to the estrogenicity of the by-product, it may also affect aquatic organisms in ways yet not characterized. Results from a microarray analysis indicate hepatic transcriptional responses to lumiestrone that are largely different from that of both steroidal and non-steroidal estrogenic EDCs in *Cyprinidae* species (Trudeau et al. 2008).

4.5 CONCLUSION

Despite the reduction of steroidal estrogen concentrations by UV treatment, UV disinfection has not been seen to have a statistically significant effect on estrogenicity in sewage treatment plants (Cicek et al. 2007). As suggested by Liu and Liu (2004), further research in the area of UV-degradation of estrogens needs to be carried out to address its potential in reducing estrogenic activity, especially with the identification of by-products such as lumiestrone. This study supports both DOC concentration and UVB intensity as strong predictors of steroidal estrogen photodegradation rates. Variability in the temporal and spatial sources of DOC should be investigated (Brooks et al. 2005). In sewage treatment plants, Acher et al. (1997) propose that costs for disinfecting large amounts of effluent are reduced with UV techniques, particularly when filtration is employed before irradiation, and if the water flows in a relatively thin layer with UV fluence lamps immediately mounted above the water surface (Acher et al. 1997). This study supports

these design alterations as methods that will also effectively reduce estrogen concentrations in final effluent.

Table 4.1 **Linear regression analysis results for the concentrations of estrone (E1) and 17α-ethinylestradiol (EE2) versus the length of exposure under different conditions; slope (rate constant), R^2, sample size (n) and probability value (p) are listed**

				E1				EE2			
Type of water	[DOC] (mg L^{-1})	UVB Intensity (μW cm^{-2})	Thickness of sample (cm)	Rate constant (h^{-1})	R^2	n	p	Rate constant (h^{-1})	R^2	n	p
Distilled	0.07	6.68	12.3	**0.010**	0.460	3	0.526	**-0.012**	0.739	3	0.341
		73	12.3	**-0.122**	0.964	11	< 0.001	**-0.004**	0.044	11	0.534
		133	12.3	**-0.208**	0.936	10	< 0.001	**-0.013**	0.401	9	0.067
		500	12.3	**-0.452**	0.909	5	0.012	**-0.038**	0.981	5	0.001
Ottawa River	6.76	6.68	12.3	**0.018**	0.214	3	0.694	**-0.024**	0.864	3	0.240
		73	12.3	**-0.041**	0.737	5	0.063	**0.013**	0.827	5	0.320
		133	12.3	**-0.085**	0.850	7	0.003	**< 0.001**	< 0.001	8	0.983
		500	12.3	**-0.361**	0.991	3	0.062	**-0.030**	0.904	3	0.201
Lake Cromwell	6.85	133	12.3	**-0.087**	0.904	5	0.013	**-0.021**	0.452	5	0.213
Raw sewage	10.78	133	123	**-0.065**	1.000	2	n/a		n/a		
Raisin River	23.90	133	12.3	**-0.004**	0.018	7	0.074	**-0.010**	0.345	8	0.126
		500	12.3	**-0.105**	1.000	3	0.001	**-0.015**	0.975	3	0.102
		500	6.1	**-0.064**	0.927	9	< 0.001	**-0.010**	0.573	7	0.049

Table 4.1 (continued)

Type of water	[DOC] (mg L^{-1})	UVB Intensity (µW cm^{-2})	Thickness of sample (cm)	E1 Rate constant (h^{-1})	E1 R^2	E1 n	E1 p	EE2 Rate constant (h^{-1})	EE2 R^2	EE2 n	EE2 p
Raisin River	23.90	500	3.1	**-0.076**	0.912	5	0.011	**-0.014**	0.881	8	0.001
		500	2.1	**-0.061**	0.540	4	0.265	**-0.380**	0.720	3	0.355
		500	1.5	**-0.046**	0.069	7	0.569	**0.051**	0.062	7	0.590
		1000	12.3	**-0.060**	0.959	5	0.004	**-0.007**	0.687	5	0.083

[DOC] = dissolved organic carbon concentration; n/a = not applicable; UVB = ultraviolet B

Table 4.2 **Linear and multiple regression analysis results for estrone (E1) rate constants versus dissolved organic carbon (DOC) concentration, UVB intensity and thickness of sample; R^2, sample size (n) and probability value (p) are listed**

		Constant variables	Regression equation	R^2	n	p
E1 Rate constant vs.	**[DOC]**	UVB Intensity = 133 μW cm^{-2}	$y = 0.0076x - 0.1629$	0.812	5	0.037
		Thickness = 12.3 cm				
	UVB Intensity	[DOC] = 0.07 ppm	$y = -0.0009x - 0.0406$	0.941	4	0.030
		Thickness = 12.3 cm				
		[DOC] = 6.76 ppm	$y = -0.0008x + 0.0183$	0.999	4	<0.001
		Thickness = 12.3 cm				
	Thickness	[DOC] = 23.9 ppm	$y = -0.0044x - 0.0483$	0.781	5	0.047
		UVB Intensity = 500 μW cm^{-2}				
E1 Rate constant vs.	**[DOC] x UVB Intensity x Thickness**	none	$y = 0.011x_1 - 0.009x_2 + 0.002x_3 - 0.150$	0.580	17	0.009
	[DOC] x UVB Intensity	Thickness = 12.3 cm	$y = 0.012x_1 - 0.000x_2 - 0.126$	0.564	13	0.016
	UVB Intensity x Thickness	[DOC] = 23.9 ppm	$y = -0.000x_1 + 0.004x_2 - 0.154$	0.010	7	0.980

Table 4.3 **Responses of extracts of dimethyl sulfoxide (DMSO) control, two non-UVB-exposed and two UVB-exposed estrone standards in reporter gene assay; mean percent responses of 100 nM estradiol, standard deviation (SD), sample size (n) and standard error (SE) are listed**

Sample	Mean response (%)	SD (%)	n	SE (%)
DMSO	**7.4**	7.3	3	4.2
No UVB – 1	**50.3**	11.2	3	6.5
No UVB – 2	**49.2**	6.2	3	3.6
UVB-exposed – 1	**38.5**	9.7	3	5.6
UVB-exposed – 2	**32.4**	7.6	3	4.4

ANOVA followed by Tukey tests reveal a significant different between both the non-exposed samples and the second UVB-exposed sample

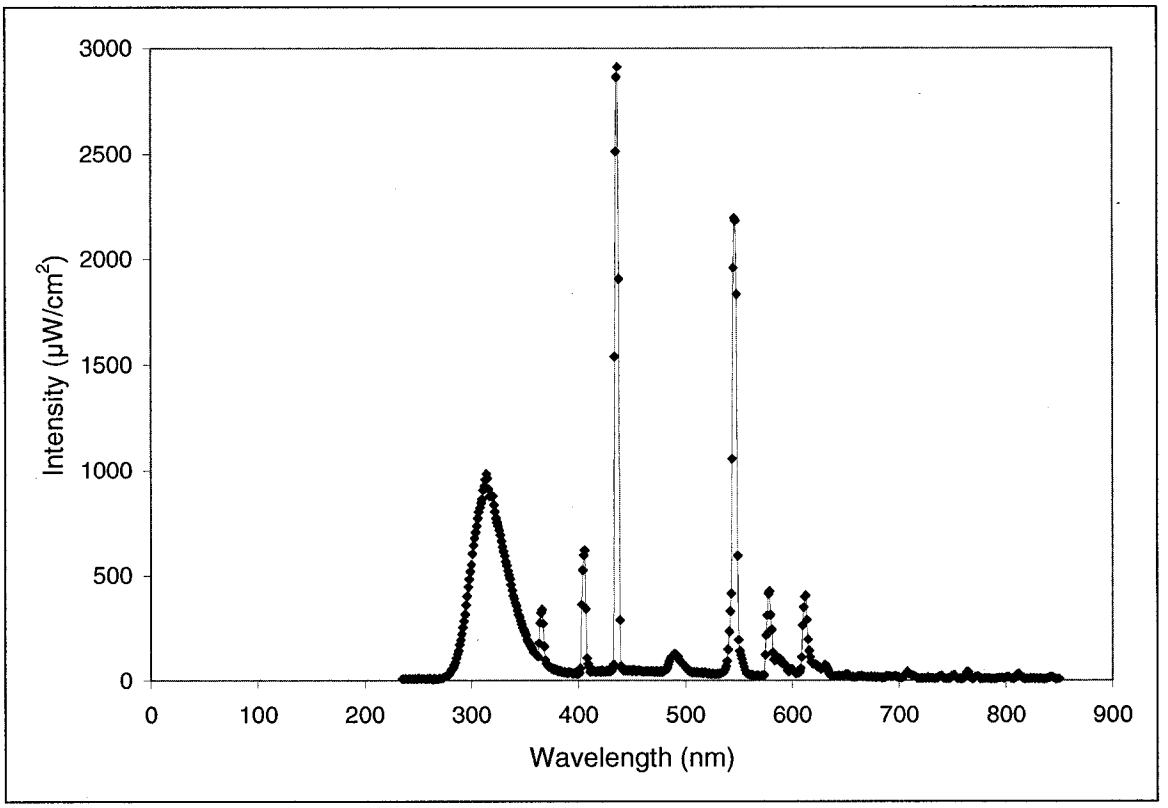


Figure 4.1 **Emission spectra of UVB bulbs used in exposure experiments measured on August 26, 2006 using a LuzChem**

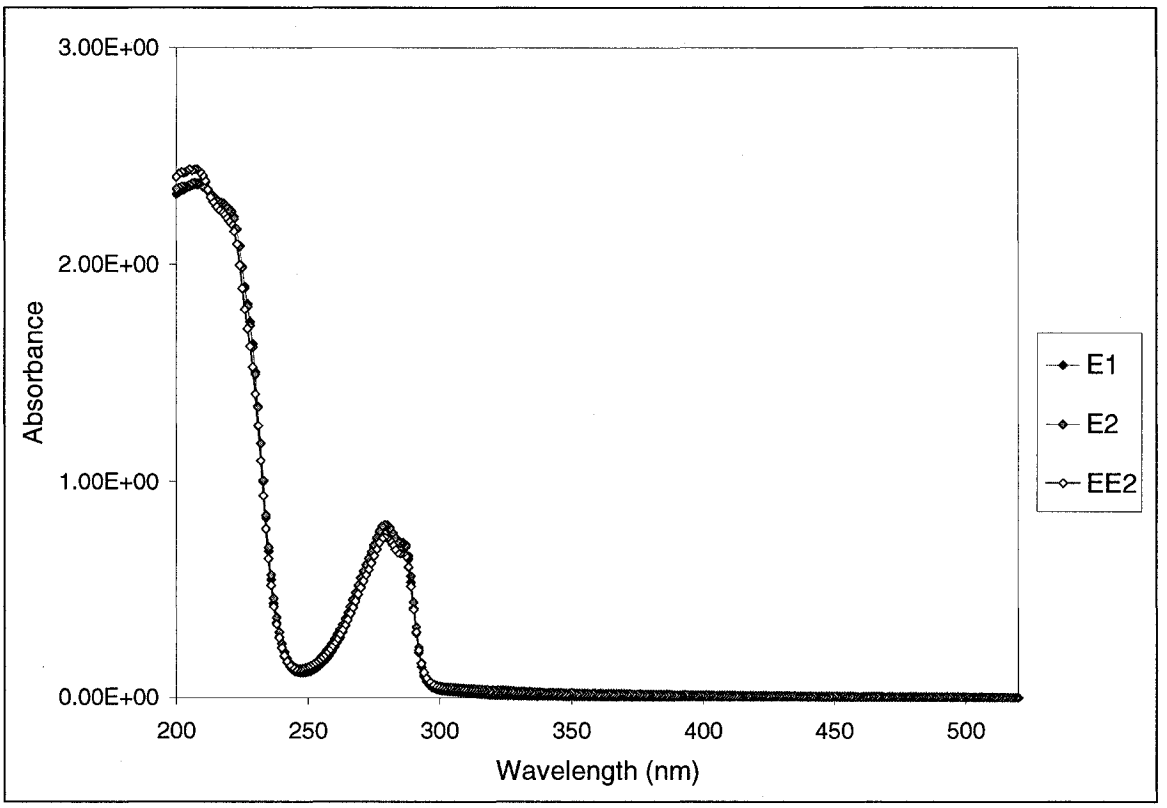


Figure 4.2 **Absorbances of estrone, 17β-estradiol and 17α-ethinylestradiol taken on April 4, 2006 by a Cary 300 UV-visible spectrophotometer**

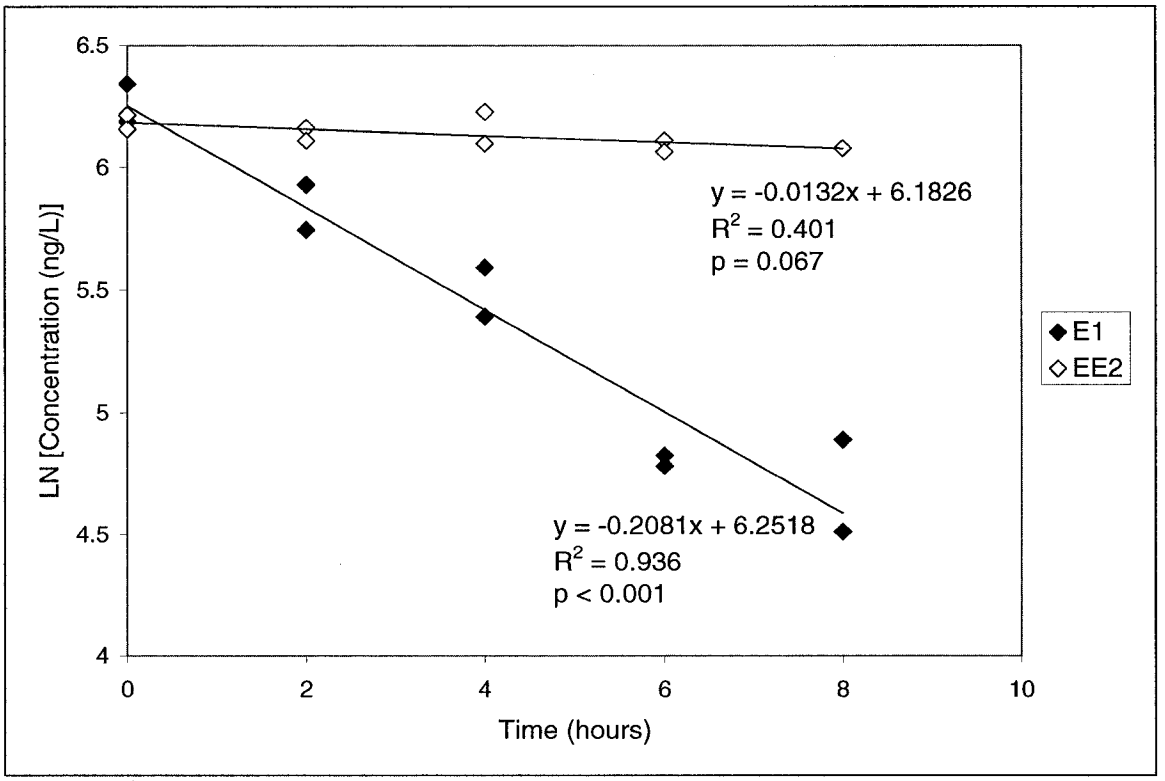


Figure 4.3 **UVB degradation of 500 ng L^{-1} estrone and 17α-ethinylestradiol in distilled water under 133 µW cm^{-2} UVB; data points represent individual samples**

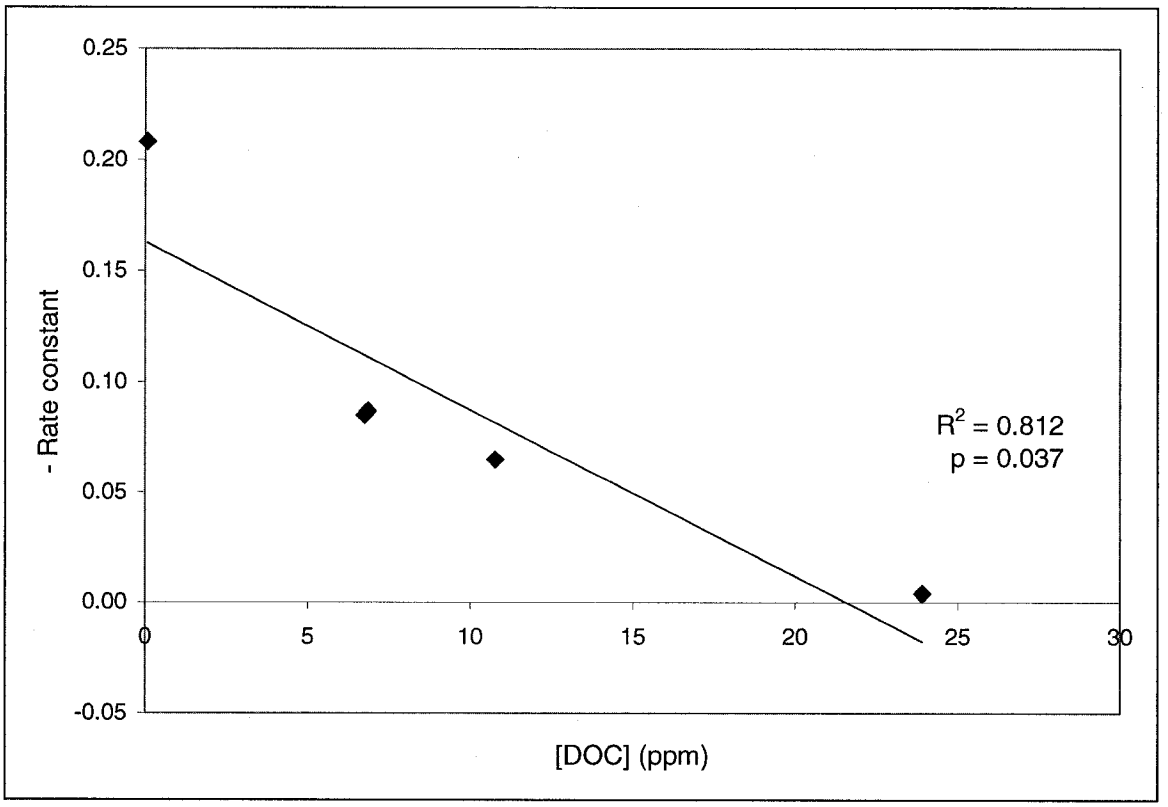


Figure 4.4 **UVB degradation rate constants for estrone versus dissolved organic carbon concentration under 133 μW cm^{-2} UVB; thickness of sample = 12.3 cm**

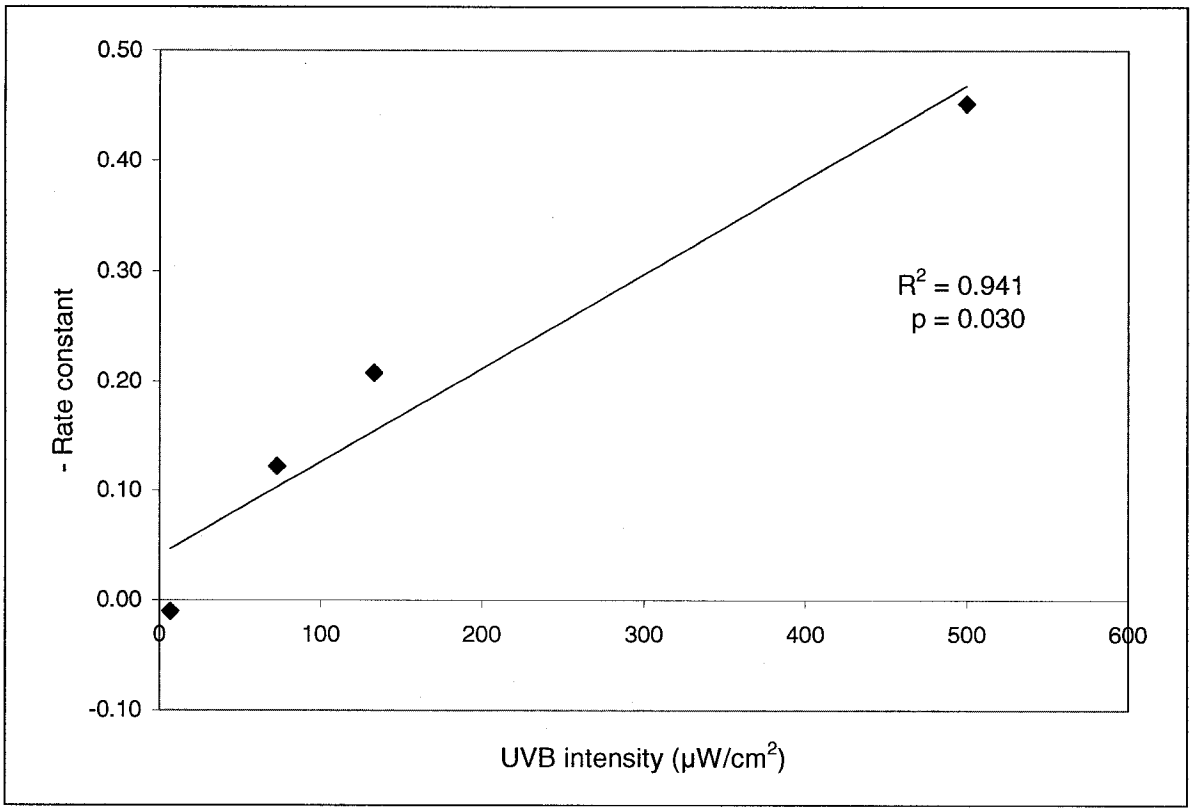


Figure 4.5 **UVB degradation rate constants for estrone in distilled water versus UVB radiation intensity; thickness of sample = 12.3 cm**

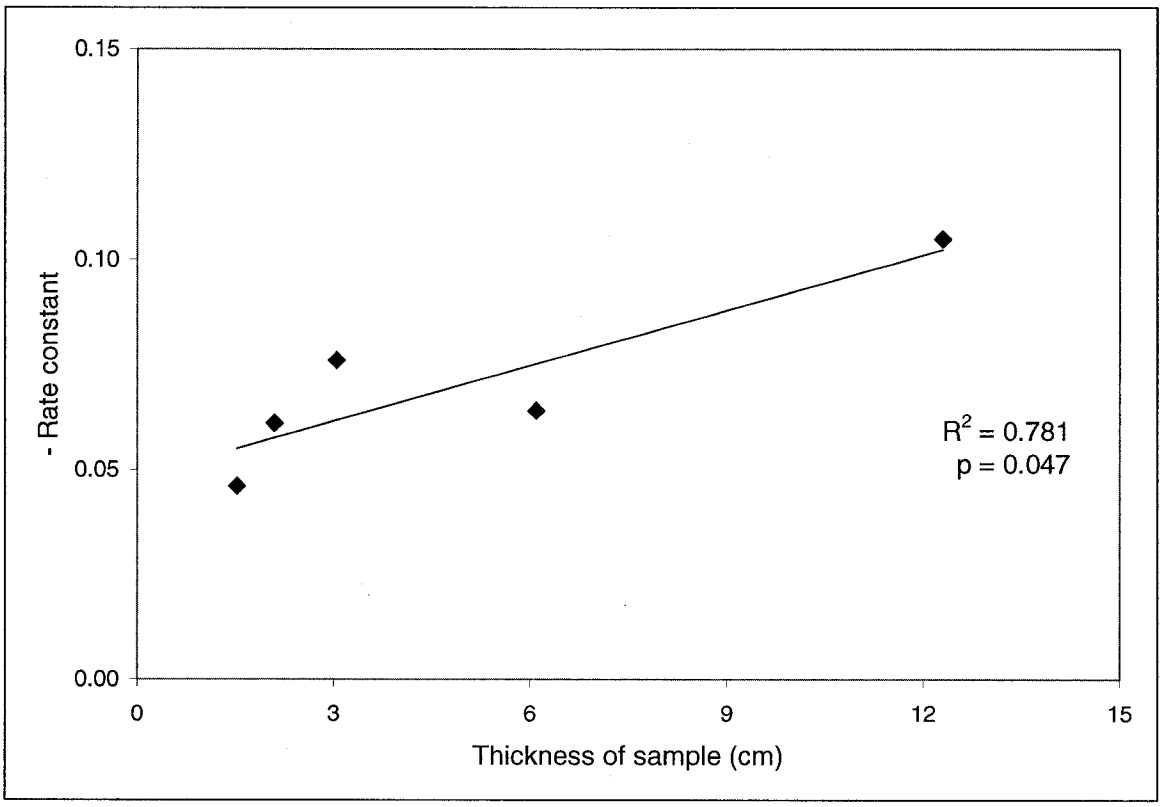


Figure 4.6 **UVB degradation rate constants for estrone in Raisin River water versus thickness of sample under 500 μWcm^{-2} UVB**

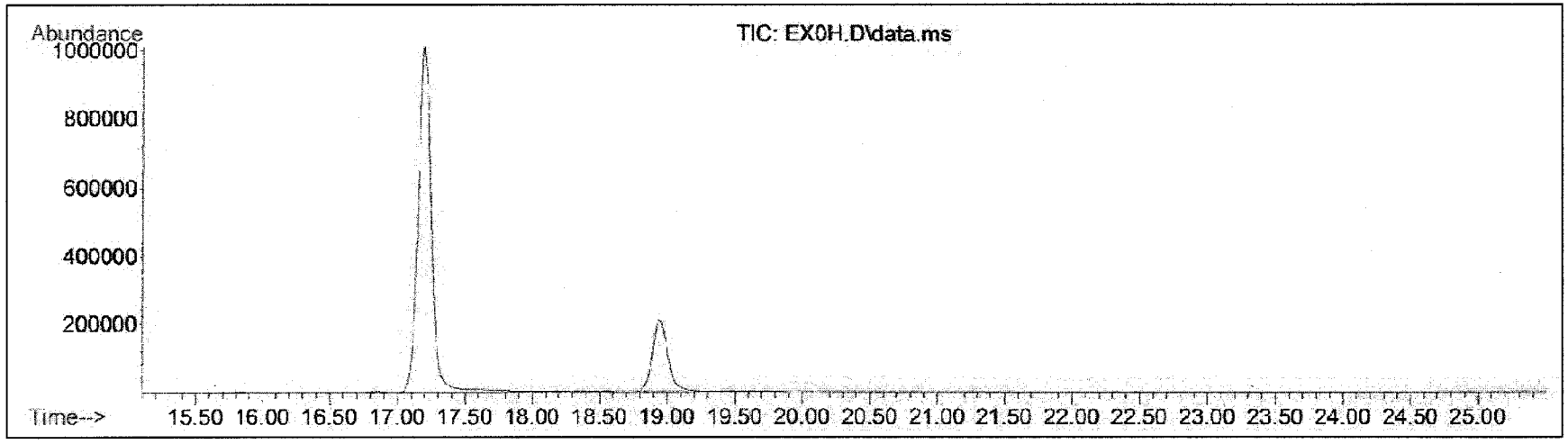


Figure 4.7.1 Chromatogram of estrone and 17α-ethinylestradiol standards in distilled water before UVB exposure; extract ran on GC/MS in SIM mode

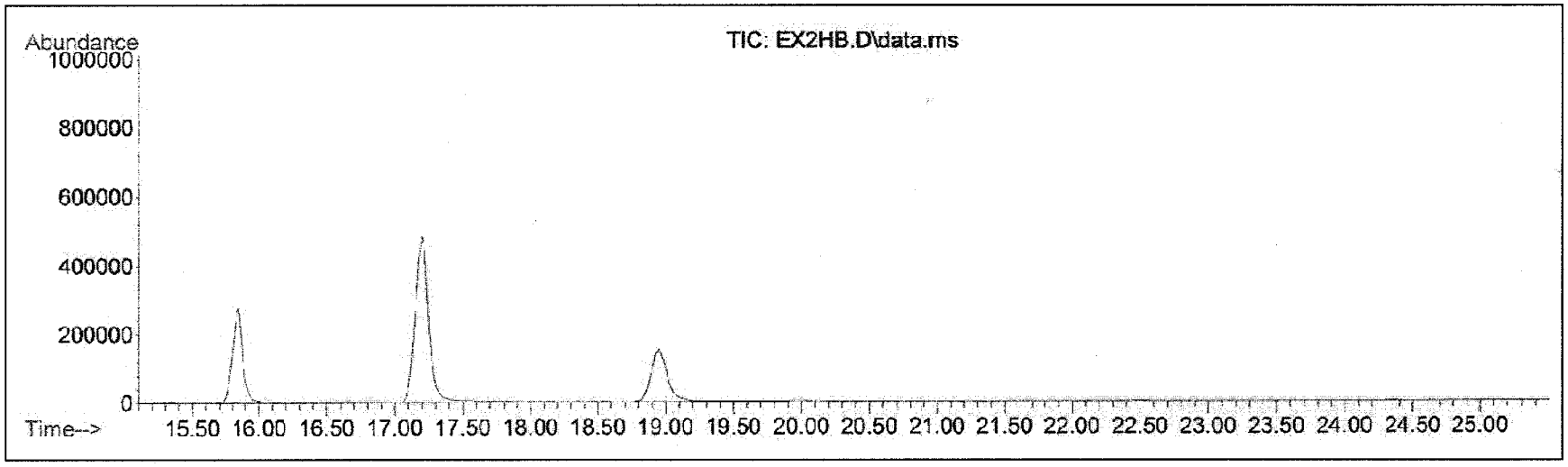


Figure 4.7.2 Chromatogram of estrone and 17α-ethinylestradiol standards in distilled water after simultaneous UVB exposure; extract ran on GC/MS in SIM mode

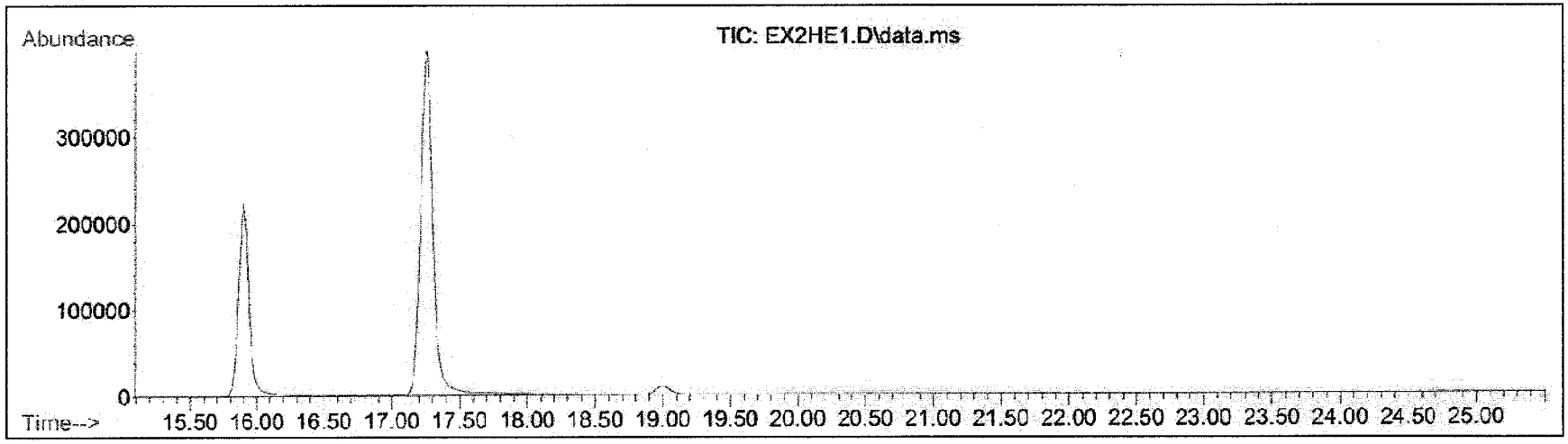


Figure 4.7.3 Chromatogram of estrone standard in distilled water after UVB exposure; extract ran on GC/MS in SIM mode

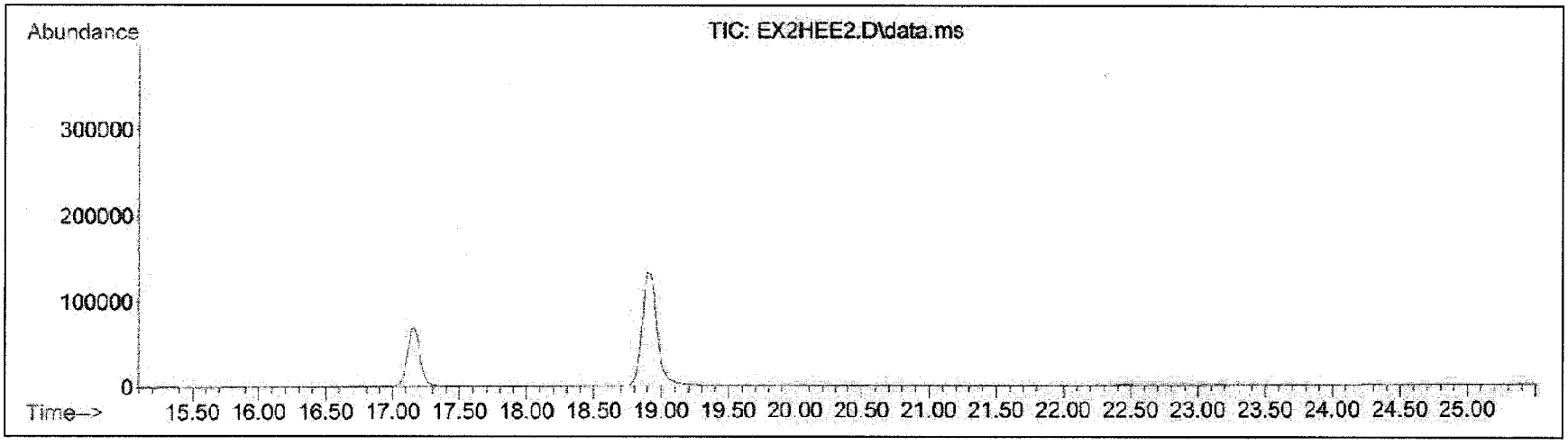


Figure 4.7.4 Chromatogram of 17α-ethinylestradiol standard in distilled water after UVB exposure; extract ran on GC/MS in SIM mode

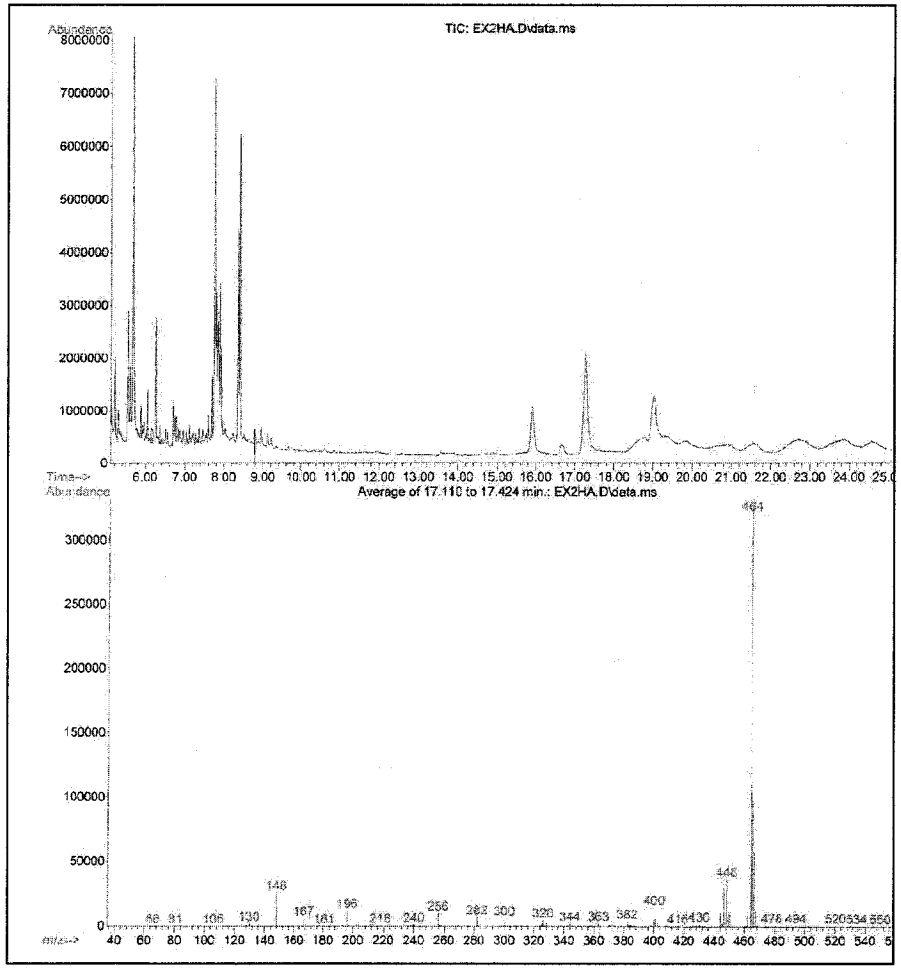


Figure 4.8.1 Chromatogram of UVB-exposed estrone and 17α-ethinylestradiol standards ran on GC/MS in SCAN mode (above) and ion spectra for estrone peak at 17.2 minutes (below)

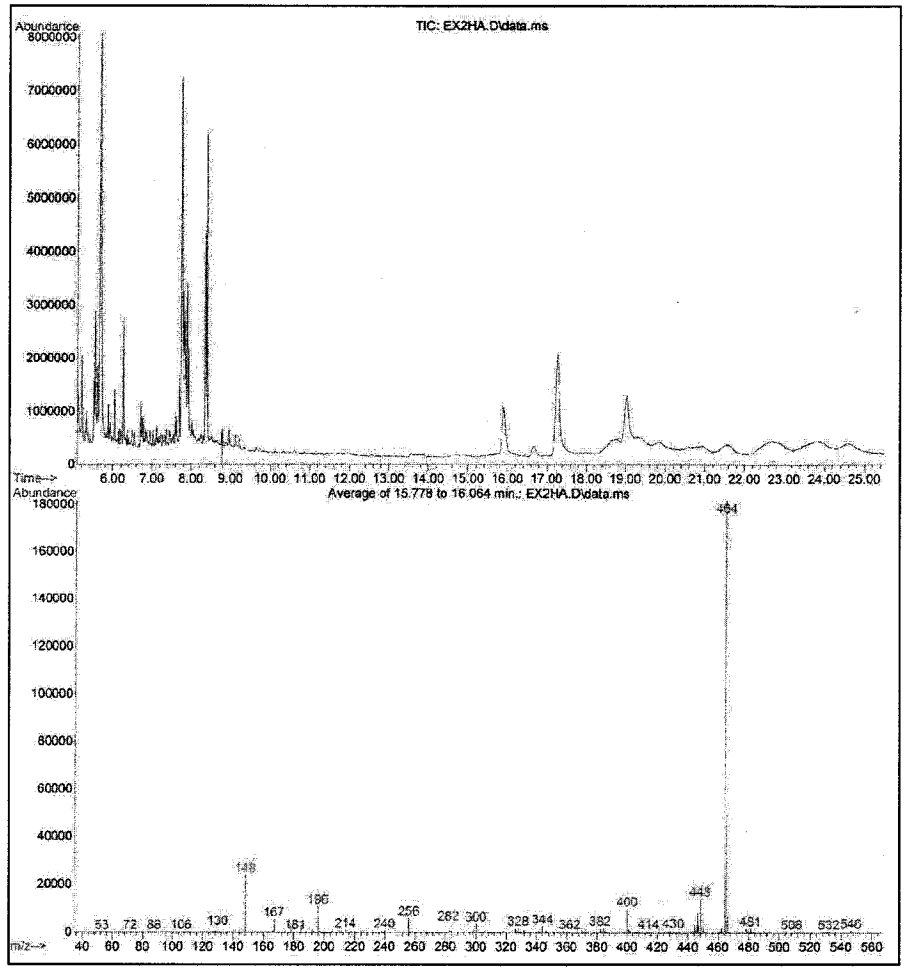


Figure 4.8.2 Chromatogram of UVB-exposed estrone and 17α-ethinylestradiol standards ran on GC/MS in SCAN mode (above) and ion spectra for mystery peak at 15.8 minutes (below)

CHAPTER 5: BIOACCUMULATION OF STEROIDAL ESTROGENS IN GOLDFISH (*CARRASIUS AURATUS*)

5.1 ABSTRACT

The bioconcentration of environmentally relevant concentrations of steroidal estrogens was measured using waterborne exposures of male goldfish (*Carrasius auratus*) at 100 ng L^{-1} of 17α-ethinylestradiol (EE2) and 50% final effluent collected from the Ottawa sewage treatment plant. The fish exposed to 100 ng L^{-1} EE2 were shown to accumulate the steroidal estrogen in both blood and tissue over a period of 72 hours, compared to the ethanol control. A mass-balance model was used to estimate the rate of EE2 uptake by fish, and the rate of EE2 loss from fish to water. This information was then used to calculate a bioconcentration factor (BCF) of 1400. In contrast, the maximum BCF observed in experimental data was 200 at 24 hours, which may be lower because a steady-state was not reached. The fish exposed to 50% effluent, which contained 15.7 ng L^{-1} estrone and 9.5 ng L^{-1} 17β-estradiol, did not accumulate any estrogen in any significant amount, in either blood or whole tissue, over 72 hours. However, the detection of EE2 in the blood was another indication of the bioaccumulation potential of the compound. It was not possible to determine if goldfish metabolize EE2 over time, or transport it for storage in organs such as the liver, due to variation in tissue values.

5.2 INTRODUCTION

It is evident from wastewater sampling studies that aquatic organisms living in systems that receive effluents from wastewater treatment plants are at risk of being exposed to the steroidal estrogens, estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) (Chapter 3). The magnitude of this risk depends not only on the physiological potency and persistence of each chemical, but also on their bioaccumulation potential.

It has been postulated that steroidal estrogens could reach biota through bioconcentration in the food chain due to their hydrophobicities (Murk et al. 2002). The bioconcentration factor (BCF) of a chemical is the ratio of a chemical's steady state concentration in an organism to concentration in water and can be estimated using a chemical's octanol/water partition coefficient (K_{ow}). Using the BCF equations listed in Table 5.1, the steroidal estrogen EE2, with the highest K_{ow}, has the highest potential to bioconcentrate (Lai et al. 2002c; Meylan et al. 1999). A BCF for EE2 in fish of 672 is predicted.

A study done by Lai et al. (2002c) used a model developed by Campfens and Mackay (1997), which has proved to be a more robust tool in predicting the bioaccumulation factors of E1, E2, E3 and EE2. In addition to BCF, they considered the bioaccumulation factor (BAF), which takes the ingestion of a substance into account, and the biomagnification factor (BMF), the increase of a chemical substance in the food chain. In multiple river organisms, including plankton, invertebrates and fish, bioaccumulation of steroidal estrogens did occur, but at a rate orders of magnitude lower

than other endocrine-disrupting compounds (EDC's) such as DDT and chlordane (Devillers 1996). The BCF, BAF and BCM of steroidal estrogens ranged from 1.8 to 332 and were dependent on K_{ow} values, trophic level, metabolic rate, and an organism's association with sediment. BCF increased with the log K_{ow} of each estrogen, particularly in species with higher lipid content. BAF increased with estrogen concentration in sediment for two benthic invertebrate species, suggesting increased exposure risks for bottom-dwelling organisms, and also increased with longer metabolic rates for E2. BMF contributed little to the overall bioaccumulation. Overall, diet was not predicted to be a significant route of estrogen exposure.

The bioaccumulation factors predicted by Lai et al. (2000c) were lower than those predicted by octanol/water partition coefficients, but do support some of the evidence of steroidal estrogen bioaccumulation in the environment. Gibson et al. (2005) found bioconcentration of estrogens in fish exposed to estrogenic wastewater effluent. E1, E2 and EE2 content in rainbow trout (*Oncorhynchus mykiss*) and roach (*Rutilus rutilus*) bile was considerably higher than in clean water. EE2, the estrogen with the highest K_{ow} was also detected in the testes and ovaries of effluent-exposed roach. Larson et al. (1999) reported steroidal estrogen concentrations in the bile of caged juvenile rainbow trout downstream from an STP receiving mainly domestic water, that were $10^4 - 10^6$ times higher than water levels. They also exposed trout to 50000 ng L^{-1} EE2 and found an accumulation of up to 375 ug g^{-1} in bile. Gomes et al. (2004) found a BCF of 228 for E1 in the zooplankton *Daphnia magna*, but a BMF of only 24 via ingestion of the algae *Chlorella vulgaris*. If biomagnification occurs, one would predict top aquatic predators to contain the highest concentrations of steroidal estrogens downstream of a sewage

treatment facility, but in the pike (*Esox lucius*), the top predator in English rivers, no evidence of endocrine disruption was seen (Vine et al. 2005). Fenske et al. (2005) also saw differences in EE2 bioaccumulation in zebrafish exposed to 3 ng L^{-1}, at either a reproductive stage, or from initial fertilization to gonad development.

There are few studies that address the rates at which these compounds accumulate in organisms, and their subsequent elimination pathways. One study of EE2 uptake in the oligochaete *Lambriculus variegates* showed that uptake occurred linearly, and that after 35 days a steady-state was still not achieved. This was hypothesized to be due to the sequestration of contaminants into chloragogen tissue, which acts similarly to the liver in vertebrates (Liebig et al. 2005). When a steady-state is achieved however, the bioconcentration factor can be estimated from the uptake rate constant k_1, and the elimination rate constant k_2, where BCF = k_1/k_2 (Walker 1990).

To assess the dynamics of steroidal estrogen bioconcentration in fish, common goldfish were exposed to both an environmentally relevant concentration of 17α-ethinylestradiol in water and Ottawa sewage effluent. I predicted that of the steroidal estrogens E1, E2 and EE2, EE2 would bioconcentrate to the greatest degree.

5.3 METHODS

5.3.1 Specimens

Common goldfish (*Carassius auratus*) were purchased in June 2006 (Aleong's International Inc, Mississauga, Ontario, Canada), and were lab acclimatized to 18°C in a

holding tank, under a natural photoperiod, and fed with standard floating trout pellets for about three weeks. Male goldfish were chosen for the exposure experiments.

5.3.2 Exposures

5.3.2.1 Time-course exposure to 17α-ethinylestradiol

Four 70-L fiberglass tanks were dosed with 700 μL of an 17α-ethinylestradiol stock (1.11×10^7 ng L^{-1} in ethanol) in tap water, to achieve a concentration of about 100 ng L^{-1}. Groups of 15 goldfish from the holding tank were exposed to the treatment for either 4, 12, 24 or 72 hours.

5.3.2.2 50% final effluent exposure

A 70-L fiberglass tank was half filled with final effluent collected from the Ottawa Robert O. Pickard Environmental Centre (ROPEC) on June 6, 2006 (35 L), and half filled with tap water (35 L). 15 goldfish were transferred from the holding tank to the final effluent tank and left for 24 hours.

5.3.2.3 Control

A control of 15 fish were transferred from the holding tank to a 70-L plastic tank of plain water spiked with 700 μL ethanol and left for 24 hours.

5.3.3 Collection, extraction and analysis

5.3.3.1 Water

After the exposure periods, 1-L samples of water were collected from the four tanks used in the time-course exposures and were extracted and analyzed for EE2, using

the method described in Sections 2.3 and 2.4 of this thesis. Water from the control tank was also analyzed.

5.3.3.2 Fish blood and whole tissue

After the exposure periods, the fish in the time-course tanks, the effluent tank and the control tank were anaesthetized in tricaine methanesulphonate (MS222; 0.1 g L^{-1}) in water and were sacrificed by cervical transaction. Fish and gonadal tissue were weighed to determine the gonadosomatic index (gonad weight/body weight) (GSI). Blood was collected via the caudal vein using syringes and pooled for groups of five fish to give three samples of blood from each tank, which were extracted and analyzed using the methods described in Sections 2.3 and 2.4 of this thesis. Whole tissue samples from the groups of five fish were stored in sealed plastic bags in a -18°C freezer for approximately a month. Following overnight thawing in a 4°C fridge, tissue was homogenized and three sub-samples of fish tissue from each group were extracted, also using the methods from Sections 2.3 and 2.4 of this thesis.

5.3.3.3 Tissue for gene expression profiles

Testis, hypothalamus and telencephalon tissue were immediately removed from two groups of five fish exposed to EE2 for 24 hours, frozen on dry ice, and stored at -80°C until RNA isolation. Gene expression profiles of ER subtypes were conducted by Marlatt et al. 2006 (Appendix I).

5.3.4 Modeling of rate constants

The uptake and elimination rate constants of EE2 were estimated using a model developed by MacKay and Blais (2008). Appendix VI describes the mass-balance equations and assumptions made in the model.

5.4 RESULTS AND DISCUSSION

5.4.1 GSI of goldfish

The GSI for the male goldfish was 2.8 (SE = 0.2%) which indicates the fish were in sexual regression (Marlatt et al. 2008; Ortega-Salas and Reyes-Bustamante 2006).

5.4.2 Time-course exposure to 17α-ethinylestradiol

Concentrations of 17α-ethinylestradiol measured in tank water over time are displayed in Table 5.2. The amount of EE2 stock solution added to the tanks at the beginning of the exposures was designed to achieve an exposure of 100 ng L^{-1}, however measurements taken at t = 4 hours indicated a water concentration of approximately 23 ng L^{-1}, both in the tanks with goldfish and those without. These may be due to the hydrophobic nature of EE2 and its potential to immediately bind to the sides of the tanks, errors in preparing the standard stock solution, or degradation of EE2.

A regression analysis showed a significant decline in EE2 water concentration over time in tanks with goldfish ($R^2 = 0.9998$, $p < 0.0001$) but no trend was seen in tanks without fish ($R^2 = 0.0433$, $p = 0.937$). It appears that an EE2 uptake constant (k_1) from

the water by fish exists. Furthermore, EE2 concentrations measured in fish blood and whole tissue also show that the compound is being taken up by fish from the water.

EE2 was detected in blood samples at every sampling interval (Table 5.3). T-tests revealed that EE2 concentrations in blood were significantly different than the ethanol control at 4, 12 and 24 hours. There is an overall increase of EE2 in blood from 0 to 24 hours, and a subsequent decrease by 72 hours. The decrease of EE2 in blood may be due to its metabolism by the goldfish, or by the transport of EE2 to other tissues for storage. The maximum concentration of EE2 in blood measured was 5177 ng L^{-1} (± 1781 ng L^{-1}) at 24 hours, which is about 500 times the concentration of EE2 in the water at 24 hours; the measured BCF for EE2 in blood is 500.

An ANOVA did not find any significant difference in EE2 concentration between the subsamples taken from the homogenized tissue samples. Therefore, it was appropriate to use the means from three homogenized tissue samples to calculate a mean EE2 concentration in goldfish tissue for each time interval. A maximum concentration of 1.19 ng g^{-1} (SE ± 0.96 ng g^{-1}) was observed at 4 hours (Table 5.4) but a t-test showed that the concentrations were only significantly different from the ethanol control at 12 hours ($p = 0.0164$).

Additional confirmation of EE2 uptake in goldfish tissue was provided by the gene expression profile of the goldfish ER subtypes in multiple tissues. In the liver, a 4.3 and a 7.5 fold increase of the ERα transcript and VTG transcript levels compared to the ethanol control were observed respectively. ER transcript levels were not affected in the testes, hypothalamus and telencephalon (Marlatt et al. 2008).

Standard errors for the whole tissue measurements are high, both within groups of goldfish and between groups of goldfish, due to high sample numbers in which no EE2 was detected. The method may not be detecting low concentrations of EE2 in the tissue samples. Difficulties in extracting accumulated estrogen from small samples of tissue have also been seen in incubation experiments with the green algae *Chlorella vulgaris*, where partitioning of steroidal estrogens occurs but concentrations are below the quantification limit (Lai et al. 2002a). Removing two individual subsample measurements reduced the standard errors for the groups drastically, and revealed that EE2 concentration in tissue is relatively steady between 4 and 24 hours, with a maximum of 0.42 ng g^{-1} (0.10 ng g^{-1}), and showing a slight decrease by 72 hours. Standard errors may also be high if the goldfish exposed were not at similar stages in their reproductive cycle, but the standard error for the GSI measurements was approximately 0.2% (Marlatt et al. 2008; Fenske et al. 2005).

5.4.3 Bioconcentration factor calculated from modeled rate constants

The measured data for EE2 water concentration and EE2 fish blood concentration was extrapolated for time intervals of 0.1 hours using the equations listed in Appendix VI (Figures 5.1 and 5.2). Assuming that when t is very small the only transfer of EE2 occurring is from water to fish, and that a steady-state is achieved at the end of the 72 hour exposure period, the rates of uptake and elimination of EE2 by fish were estimated as 28 and 0.02 h^{-1}, respectively, and the rates of EE2 degradation in water and fish were estimated as 0.015 and 0.001 h^{-1} respectively (Appendix VI). A modeled BCF of 1400 was calculated.

5.4.4 50% final effluent exposures

None of the steroidal estrogens were detected in the 24 hour control water. In the final effluent used for the 50% effluent exposures 15.7 ng L^{-1} (SE ± 2.1 ng L^{-1}) E1 and 9.5 ng L^{-1} (SE ± 2.4 ng L^{-1}) E2 were measured, but EE2 was not detected (Chapter 3, Tables 3.9.1, 3.9.2 and 3.9.3). After 24 hours, no differences were observed between the natural steroidal estrogen concentrations in the blood and tissue of the control group and the effluent-exposed group. However, in one group of effluent-exposed fish, EE2 was detected in the blood at a concentration of 874 ng L^{-1}. Because the 50% final effluent was not extracted and analyzed for estrogens after the 24-hour exposure period with the fish, uptake and elimination rate constant for estrogens cannot be calculated as for the EE2 standard exposure experiment.

5.5 CONCLUSION

These experiments illustrate how the bioconcentration of 17α-ethinylestradiol can occur in the blood of male goldfish, a well-known test organism, but do not provide any details as to the mechanism of EE2 uptake, which could occur via gills or via diet, or the potential for EE2 biomagnification. Like in the oligochaete *Lumbriculus variegates,* it is suspected that goldfish may sequester the chemical in tissue, but its bioaccumulation is not observed to the same degree, perhaps because it is not a benthic species associating with the sediments to which EE2 may partition. It is difficult to confirm whether sequestration might be the mechanism of EE2 elimination from the blood without more sophisticated estrogen detection tools for tissue (Liebig et al. 2005). Other routes of EE2

elimination, such as the conversion of EE2 into other estrogens, should be investigated, as these may be costly for exposed aquatic organisms. As well, future studies need to incorporate lower concentrations of EE2, full life cycles, and both males and females to determine differences in sensitivities to exposure.

Table 5.1 Predicted bioconcentration factors (BCF's) for estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) based on octanol/water partition coefficient (K_{ow})

		BCF		
Organism	**Equation**	**E1**	**E2**	**EE2**
Non-species specific	**Log BCF = 0.79 x log K_{ow} - 0.40**	204	516	756
	Log BCF = 0.86 x log K_{ow} - 0.39	362	996	1510
Fish	**Log BCF = 0.85 x log K_{ow} - 0.70**	164	446	672

Equations based on Lai et al. (2002c) and Meylan et al. (1999)
K_{ow} values listed in Table 1.1

Table 5.2 **Concentration of 17α-ethinylestradiol in water used in time-course exposure experiments; mean, standard deviation (SD), sample size (n) and standard error (SE) are listed**

		Exposure (hours)				
Sample		**0**	**4**	**12**	**24**	**72**
Water – With goldfish	**Mean (ng L^{-1})**	**100**	**22.59**	**17.13**	**10.47**	**1.93**
	SD (ng L^{-1})	n/a	3.64	3.13	1.33	0.99
	n	n/a	3	3	3	3
	SE (ng L^{-1})	n/a	2.10	1.81	0.77	0.57
Water – Without goldfish	**Mean (ng L^{-1})**	**100**	**22.85**	**22.50**	**25.23**	**23.07**
	SD (ng L^{-1})	n/a	1.50	0.40	2.66	1.00
	n	n/a	2	2	2	2
	SE (ng L^{-1})	n/a	1.06	0.28	1.88	0.71

n/a = not applicable

Table 5.3 **Concentration of 17α-ethinylestradiol in goldfish blood from time-course experiment; mean, standard deviation (SD), sample size (n) and standard error (SE) are listed**

		Exposure (hours)				
Sample		**0**	**4**	**12**	**24**	**72**
Goldfish blood	**Mean (ng L^{-1})**	**nd**	**1825**	**4005**	**5177**	**762**
	SD (ng L^{-1})	0	471	2082	3085	670
	n	**3**	3	3	3	3
	SE (ng L^{-1})	0	272	1202	1781	387

nd = not detected

Table 5.4 Concentration of 17α-ethinylestradiol in goldfish whole tissue from time-course experiment; mean, standard deviation (SD), sample size (n) and standard error (SE) are listed

		Exposure (hours)				
Sample		**0**	**4**	**12**	**24**	**72**
Goldfish whole tissue - group	**Mean (ng g^{-1})**	**nd**	**1.19**	**0.42**	**0.30**	**0.68**
	SD (ng g^{-1})	n/a	1.67	0.18	n/a	1.05
	n	1	3	3	1	3
	SE (ng g^{-1})	n/a	0.96	0.10	n/a	0.60
Goldfish whole tissue - A	**Mean (ng g^{-1})**	**nd**	**nd**	**0.21**	**0.30**	**0.16**
	SD (ng g^{-1})	0	0	0.36	0.26	0.28
	n	3	3	3	3	3
	SE (ng g^{-1})	0	0	0.21	0.15	0.16
Goldfish whole tissue - B	**Mean (ng g^{-1})**		**3.10**	**0.50**		**nd**
	SD (ng g^{-1})		5.09	0.50		0
	n		3	3		2
	SE (ng g^{-1})		2.94	0.29		0
Goldfish whole tissue - C	**Mean (ng g^{-1})**		**0.48**	**0.50**		**1.89**
	SD (ng g^{-1})		0.12	0.10		3.27
	n		3	2		3
	SE (ng g^{-1})		0.07	0.07		1.89

Group means based on the means of samples (A, B and C) each obtained from three subsamples; an ANOVA shows no significant difference between subsamples

n/a = not applicable; nd = not detected

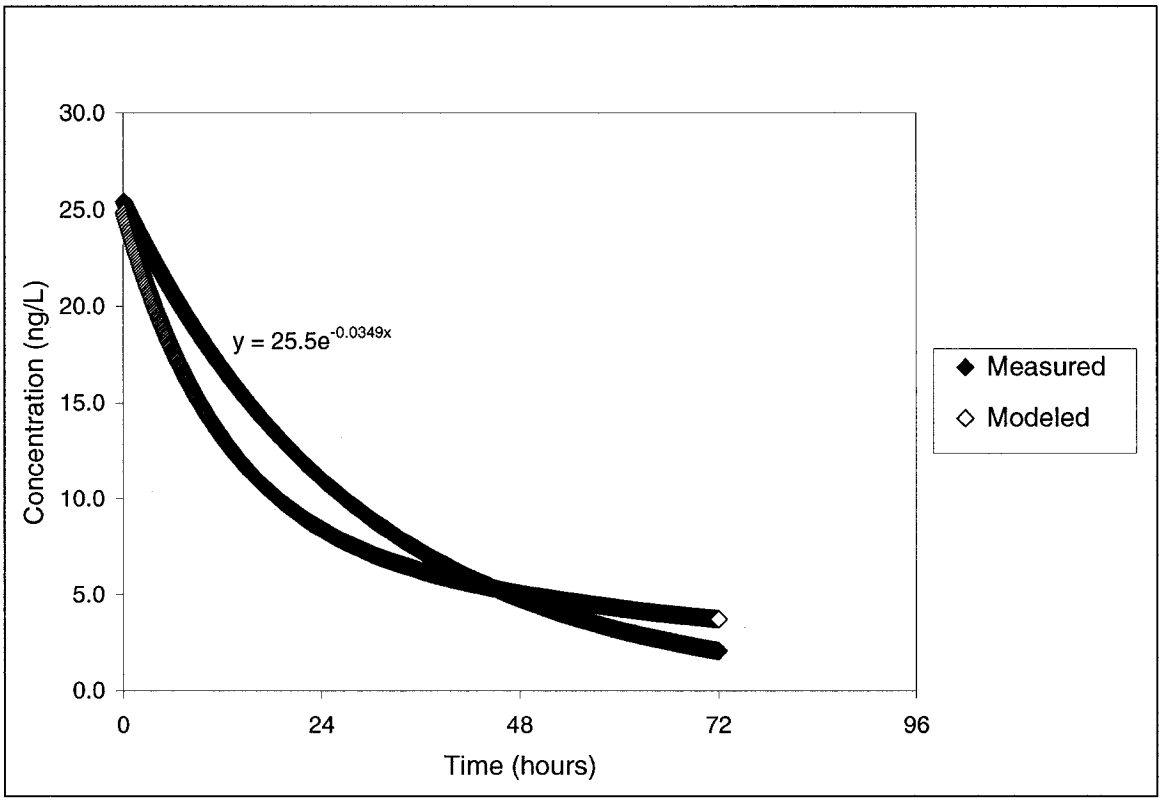


Figure 5.1 **Measured concentration of 17α-ethinylestradiol in tank water (with fish) versus modeled concentration of 17α-ethinylestradiol in tank water from time course exposure experiments**

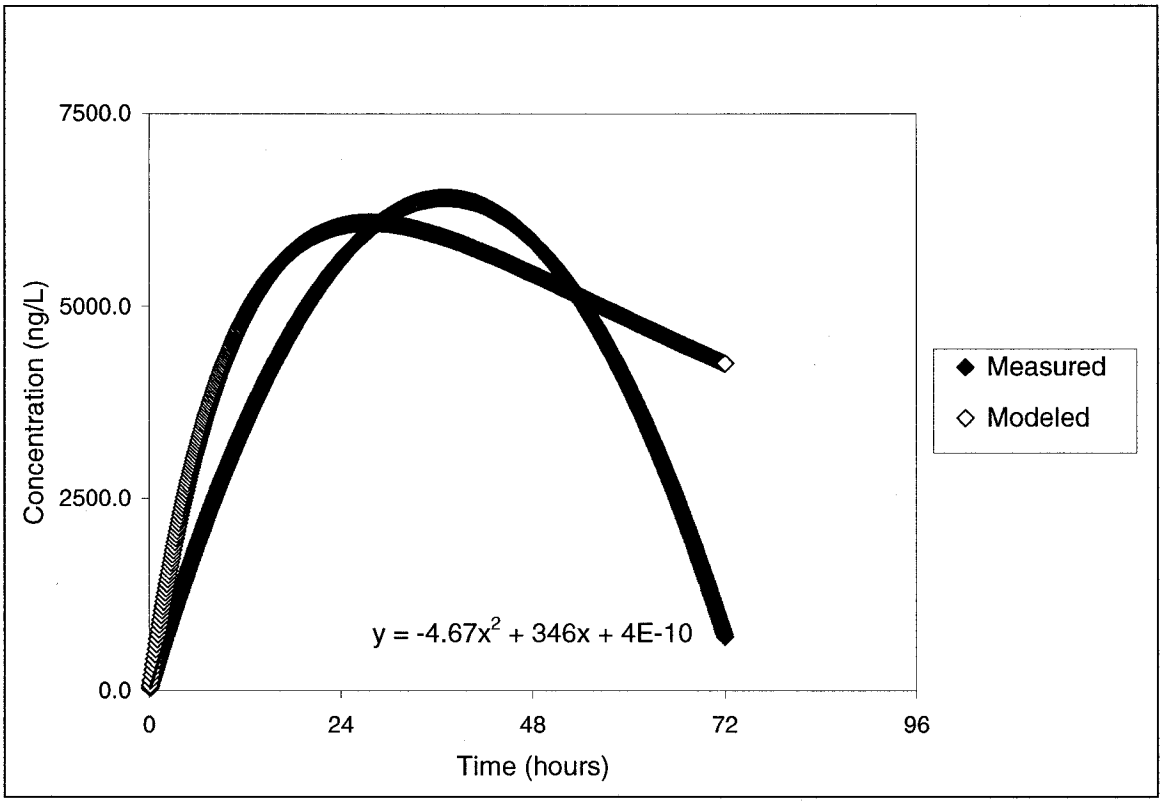


Figure 5.2 **Measured concentration of 17α-ethinylestradiol in goldfish blood versus modeled concentration of 17α-ethinylestradiol in goldfish blood from time course exposure experiments**

CHAPTER 6: FINAL CONCLUSIONS

Estrone, 17β-estradiol and 17α-ethinylestradiol were found in both Ottawa and Cornwall domestic sewage effluent. Furthermore, these compounds were detected in drinking water, and the river water that was used as the source of this drinking water. They are found at biologically relevant concentrations that can disrupt the physiology and reproduction of both fish and aquatic invertebrates, and at similar concentrations and with comparable patterns to those found in other studies around the world.

Trends found in this thesis add to predictive capabilities of steroidal estrogen fate and behaviour in the environment and the location and severity of endocrine disrupting effects (Johnson et al. 2004; Jobling et al. 2006). The data show that the population size being served by a sewage treatment plant, environmental conditions such as temperature and precipitation, and wastewater treatment plant parameters, such as mean daily flow and carboniferous biological oxygen demand, can be used to predict the concentrations of steroidal estrogens entering and leaving a sewage treatment plant. However, the data also show that treatment type (secondary versus primary) does not always help predict the removal of estrogens from wastewater. Once released to the environment, estrone degradation rates will likely be affected by dissolved organic carbon concentrations and UVB intensities. This study also demonstrates that upon entering the environment, the synthetic steroid EE2 will bioconcentrate in fish.

Unlike many synthetic organic contaminants, the release of steroidal estrogens from wastewater and agricultural runoff into the aquatic environment cannot be prevented as E1 and E2 are naturally occurring hormones in human and animal

populations. However, improvements in sewage treatment plant technology are possible. Experimenting with forms of microbial and UV degradation are options that may increase E1 and E2 removal; however the biological activity of the byproducts from the degradation of steroidal estrogens, such as the newly characterized lumiestrone, needs to be further characterized. For E1 and E2, both stormwater overflow, and the application of biosolids to agricultural land as fertilizer, could be other sources of steroidal estrogens to the environment.

Methods for the reduction of E1 and E2 concentrations do not prove helpful for eliminating exposure risks to EE2, due to its relative persistence, high K_{ow} value and high biological potency. Unfortunately, the development of a pharmaceutical such as EE2 takes many years, and long-term environmental risk assessment is one of the last steps of authorization. EE2, a synthetic chemical specifically designed for its high potency and long half-life, is so persistent in the environment that it is detectable at levels above threshold toxicity levels in drinking water for rainbow trout. Its observed tendency to bioaccumulate also means the compound is a threat to the food chain. Mastrup et al. (2005) conclude that the exposure of EE2 from a single portion of fish might be significantly greater than that from the daily portion of drinking water.

For the synthetic compound EE2, short-term measures aimed at changing consumer behaviour and improving safe waste disposal can be applied immediately. The contraceptive ethinylestradiol patch was in fact the first medicinal product assessed by the German Federal Environment Agency (UBA), and based on the published effects on algae, daphnids and fish, appropriate disposal advice was added to the product leaflet to help protect the environment (Adler et al 2008).

It will be necessary to continue monitoring drinking water and other potentially contaminated diet sources for levels of E1, E2 and EE2, keeping in mind the lessons learnt from other EDC's (Sumpter et al 2005), to assess exposure risks of the aquatic environment, as well as humans, to steroidal estrogens.

REFERENCES

Acher, A., Fischer, E., Turnheim, R. and Manor, Y. 1997. Ecologically friendly wastewater disinfection techniques. *Wat. Res.* **31**(6): 1398-1404.

Ackermann, G.E., Brombacher, E. and Fent, K. 2002. Development of a fish reporter gene system for the assessment of estrogenic compounds and sewage treatment plant effluents. *Environ. Toxicol. Chem.* **21**: 1864-1875.

Adler, N.E., Koschorreck, J. and Rochenberg, B. 2008. Environmental impact assessment and control of pharmaceuticals: the role of environmental agencies. *Water Sci. Technol.* **57.1**: 91-97.

Adlercreutz, H., Kiuru, P., Rasku, S., Wähälä, K. and Fotsis, T. 2004. An isotope dilution gas chromatographic-mass spectrometric method for the simultaneous assay of estrogens and phytoestrogens in urine. *J. Steroid Biochem.* **92**: 399-411.

Ahel, M., Scully, F.E., Hoigné, J. and Giger, W. 1994. Photochemical degradation of nonylphenol and nonylphenol polyethoxylates in natural waters. *Chemosphere* **28**(7): 1361-1368.

Aherne, G.W. and Briggs, R. 1989. The relevance of the presence of certain synthetic steroids in the aquatic environment. *J. Pharm. Pharmacol.* **41**:735-736.

Andersen, H., Siegrist, H. and Halling-Sorensen, B. 2003. Fate of estrogens in a municipal sewage treatment plant. *Environ. Sci. Technol.* **37**(18): 4021-4026

Andreadakis, A., Mamais, D., Christoulas, D. and Kabylafa, S. 1999. Ultraviolet disinfection of secondary and tertiary effluent in the Mediterranean region. *Wat. Sci. Tech.* **40**(4-5): 253-260.

Aravindakshan, J., Paquet, V., Gregory, M., Dufresne, J., Fournier, M., Marcogliese, D.J. and Cyr, D.G. 2004. Consequences of xenoestrogen exposure on male reproductive function in spottail shiner (*Notropis hudsonius*). *Toxicol. Sci.* **78**: 156-165.

Barel-Cohen, K., Shore, L.S., Shemesh, M., Wenzel, A., Mueller, J. and Kronfeld-Schor, N. 2006. Monitoring of natural and synthetic hormones in a polluted river. *J. Environ. Manage.* **78**: 16-23.

Belcher, S.M. 2008. Rapid signaling mechanisms of estrogens in the developing cerebellum. *Brain Res. Rev.* **57**(2): 481-492.

Belfroid, A.C., Van der Horst, A., Vethaak, A.D., Schäfer, A.J., Rijs, G.B.J. and

Wegener, J. 1999. Analysis and occurrence of estrogenic hormones and their glucuronides in surface water and waste water in The Netherlands. *Sci. Total Environ.* **225**: 101-108

Bennie, D.T., Sullivan, C.A., Lee, H-B., Peart, T.E. and Maguire, R.J. 1997. Occurrence of alkylphenols and alkylphoneol mono- and diethoxylates in natural waters of the Laurentian Great Lakes basin and the upper St. Lawrence River. *Sci. Tot. Environ.* **193**: 263-275.

Blais, J. M., Schindler, D.M., Muir, Kimpe, L. E., Donald, D.B. and Rosenberg, B. 1998. Accumulation of persistent organochlorine compounds in mountains of western Canada. *Nature* **395**: 585-588.

Blázquez, M., Bosma, P. T., Fraser, E. J., Van Look, K. J. W. & Trudeau, V. L. 1998. Fish as models for the neuroendocrine regulation of reproduction and growth. *Comp. Biochem. Phys. C* **119**: 345-364.

Bowman, J.C., Readman, J.W. and Zhou, J.L. 2003. Sorption of the natural endocrine disruptors, oestrone and 17β-estradiol in the aquatic environment. *Environ. Geochem. Hlth.* **25**: 63-67.

Brooks, P.D., O'Reilly, C.M., Dimaond, S.A., Campbell, D.H., Knapp, R., Bradford, D., Corn, P.S., Hossack, B. and Tennessen, K. 2005. Spatial and temporal variability in the amount and source of dissolved organic carbon: implications for ultraviolet exposure in amphibian habitats. *Ecosystems* **8**: 478-187.

Campfens, J. and Mackay, D. 1997. Fugacity-based model of PCB bioaccumulation in complex aquatic food webs. *Environ. Sci. Technol.* **31**: 577-583.

Canonica, S., Meunier, L. and von Gunter, U. 2008. Phototransformation of selected pharmaceuticals during UV treatment of drinking water. *Wat. Res.* **42**: 121-128.

Cao, L.H., Bu, R.F. Oakley, J.I., Kalla, E. and Blair, H.C. 2003. Estrogen receptor-beta modulates synthesis of bone matrix proteins in human osteoblast-like MG63 cells. *J. Cell Biochem.* **89**(1): 152-164.

Carbella, M., Omil, F., Lema, J.M., Llompart, M., García-Jares, C., Rodríguez, I., Gómez, M. and Ternes, T. 2004. Behaviour of pharmaceuticals, cosmetics and hormones in a sewage treatment plant. *Wat. Res.* **38**: 2918-2926.

Carreau, S. 2000. Estrogens and male reproduction. *Fol. Histoch. Cytochem.* **38**(2):47-52.

ChemZoo. 2008. Chemspider. Web search engine available online at http://www.chemspider.com.

Chen, C.-Y., Wen, T.-Y., Wang, G.-S., Cheng, H.-W., Lin, Y.-H. and Lien, G.-W. 2007. Determining estrogenic steroids in Taipei waters and removal in drinking water treatment using high-flow solid-phase extraction and liquid chromatography/tandem mass spectrometry. *Sci. Tot. Environ.* **378**: 352-365.

Chen, T.T. 1983. Identification and characterization of estrogen responsive gene products in the liver of the rainbow trout. *Can. J. Biochem. Cell Biol.* **61**: 802-810.

Christiansen, L.B., Winther-Nielsen, M. and Helweg, C. 2002. Feminization of fish. Danish Environmental Protection Agency Environmental Project no. 729.

Cicek, N., Londry, K., Oleszkiewicz, J.A., Wong, D. and Lee, Y. 2007. Removal of selected natural and synthetic estrogenic compounds in a Canadian full-scale municipal wastewater treatment plant. *Water Environ. Res.* **79**(7): 795-800.

City of Cornwall, ON. 2008. Personal correspondence from Patrick Carriere, Supervisor, Waste Water Treatment Plant, Department of Infrastructure & Municipal Works.

City of Ottawa, ON. 2007. Personal correspondence from Gary Robidoux, Supervisor Pumping Station/Process Control, Water and Wastewater Services Branch, Public Works and Services Department.

City of Ottawa, ON. 2005. Robert O. Pickard Environmental Centre Annual Operations Report. Public Works and Services Department, Utility Services Branch, Wastewater and Drainage Services Division. 47 p.

Coleman, H.M., Routledge, E.J., Sumpter, J.P., Eggins, B.R. and Byrne, J.A. 2004. Rapid loss of estrogenicity of steroid estrogens by UVA photolysis and photocatalysis over an immobilized titanium dioxide catalyst. *Water Res.* **38**: 3233-3240.

Copeland, P., Sumpter J.P., Walker T.K. and Croft M. 1986. Vitellogenin levels in male and female rainbow trout (*Salmo gairdneri Richardson*) at various stages of the reproductive cycle. Comp. *Biochem. Physiol.* **83B**: 487-493.

Coyle, Y.M. 2004. The Effect of Environment on Breast Cancer Risk. *Breast Cancer Res. Tr.* **84**(3): 273-288.

Croley, T.R., Hughes, R.J., Koenig, B.G., Metcalfe, C.D. and March, R.E. 2000. Mass spectrometry applied to the analysis of estrogens in the environment. *Rapid Commun. Mass Spectrometr.* **14**: 1087-1093.

Dachs, J., Van Ry, D.A. and Eisenreich, S.J. 1999. Occurrence of estrogenic

nonylphenols in the urban and coastal atmosphere of the lower Hudson river estuary. *Environ. Sci. Technol.* **33**(15): 2676-2679.

Das, T.K. 2001. Ultraviolet disinfection application to a wastewater treatment plant. *Clean Prod. Processes* **3**: 69-80.

D'Ascenzo, G., Corcia, A Di, Gentili, A., Mancini, R., Mastropasqua, R., Nazzari, M. & Samperi, R. 2003. Fate of natural estrogen conjugates in municipal sewage transport and treatment facilities. *Sci.Total Environ.* **302**: 199-209.

de Hoffman, E. and Stroobant, V. 2002. Mass Spectrometry: Principles and Applications. John Wiley and Sons. 407 p.

de Voogt, P and van Hattum, B. 2003. Critical factors in exposure modeling of endocrine active substances. *Appl. Chem.* **75**: 1933–1948.

Devillers, J., Bintein, S. and Domine, D. 1996. Comparison of BCF models based of log *P. Chemosphere* **33**: 1047-1106.

Desbrow, C., Routledge, E.J., Brighty, G.C., Sumpter, J.P. and Waldock, M. 1998. Identification of Estrogenic Chemicals in STW effluent. 1. Chemical fractionation and in vitro biological screening. *Environ. Sci. Technol.* **32**(11): 1549-1558.

Edexcel Foundation. 2005. Microorganisms and Disease Booklet. Web publication available online at http://web.ukonline.co.uk/webwise/spinneret/edexcel/index.htm.

Environment Canada. 2006. Canadian Climate Data Online. Website available online at http://www.climate.weatheroffice.ec.gc.ca/climateData/canada_e.html.

EPA. 2008. Lake Ontario Lakewide Management Plan 2008. Web publication available online at http://www.epa.gov/glnpo/lamp/lo_2008/index.html.

EPA. 1993. Guidelines establishing test procedures for the analysis of pollutants. U.S. Code of Federal Regulations, Title 40, Part 136, Appendix B.

Fenske, M., Maack, G., Schäfers, C. and Sagner, H. 2005. An environmentally relevant concentration of estrogen induces arrest of male gonad development in zebrafish, *Danio rerio. Environ. Toxicol. Chem.* **24**(5): 1088-1098.

Fernandez, M.P., Ikonomou, M.G. and Buchanan, I. 2007. An assessment of estrogenic organic contaminants in Canadian waters. *Sci. Total Environ.* **373**: 250-269.

Fernandez, M. and l'Haridan, J. 1994. Influence of lighting conditions on toxicity and genotoxicity of various PAH in the news in vivo. *Mutat. Res.* **298**: 31-41.

Folmar, L.C., Gardner, G.R., Schreibman, M.P., Magliulo-Cepriano L., Mills, L.J., Zaroogian, G., Gutjahr-Gobell, R., Haebler, R., Horowitz, D.B. and Denslow, N. 2001. Vitellogenin-induced pathology in male summer flounder (*Paralichthys dentatus*). *Aquat. Toxicol.* **51**: 431-441.

Fotsis, T. and Adlercreutz, H. 1987. The multicomponent analysis of estrogens in urine by ion-exchange chromatography and GC-MS. 1. Quantitation of estrogens after initial hyrdrolysis of conjugates. *J. Steroid Biochem. Mol. Biol.* **28**(2): 202-213.

Gagné, F., Marcogliese, D.J., Blaise, C. and Gendron, A.D. 2001. Occurrence of compounds estrogenic to freshwater mussels in surface waters in an urban area. *Environ. Toxicol.* **16**: 260-268.

Gibson, R., Smith, M.D., Spary, C.J., Tyler, C.R. and Hill, E.M. 2005. Mixtures of estrogenic contaminants in bile in fish exposed to wastewater treatment works effluents. *Environ. Sci. Technol.* **39**: 2461-2471.

Giese, R.W. 2003. Measurement of endogenous estrogens: analytical challenges and recent advances. *J. Chromatogr. A* **100**: 401-412.

Gomes, R.L., Deacon, H.E., Lai, K.M., Birkett, J.W., Scrimshaw, M.D. and Lester, J.N. 2004. An assessment of the bioaccumulation of estrone in Daphnia magna. *Environ. Toxicol. Chem.* **23**(1): 105-108.

Gomes, R.L, Scrimshaw, M.D. and Lester, J.N. 2003. Determination of endocrine disrupters in sewage treatment and receiving waters. *Trends Anal. Chem.* **22**(10): 697-705.

Google Earth 4.3 (beta) for P.C. 2008. Program available for download online at http://earth.google.com.

Guillette, L.J., Jr. 2000. Contaminant-induced endocrine disruption in wildlife. *Growth Horm. IGF* **Res. 10** (Suppl B): 45-50

Herman, J.S., and Mills, A.L. 2003. Biological and hydrogeological interactions affect the persistence of 17β-estradiol in an agricultural watershed. *Geobiology* **1**: 141-151.

Hernando, M.D., Mezoua, M., Gómez, M.J., Malato, O., Agüera, A. and Fernández-Alba, A.R. 2004. Comparative study of analytical methods involving gas chromatography-mass spectrometry after derivitization and gas chromatography-tandem mass spectrometry for the determination of selected endocrine disrupting compounds in wastewater. *J. Chromatogr. A.* **1047**: 129-135.

Holbrook, R.D., Novak, J.T., Grizzard, T.J., and Love, N.G. 2002. Estrogen receptor

agonist fate during wastewater and biosolids treatment processes: a mass balance analysis. *Environ. Sci. Technol.* **36**(21): 4533-4539.

Honjo, H., Ishihara, M., Osawa, Y., Kirdani, R.Y. and Sandberg A. A. 1976. In vivo and in vitro conjugation and metabolism of estrogens by the baboon kidney. *Endocrinol.* **99**: 1054-1062.

Isobe, T., Nishiyama, H., Nakshima, A., and Takada, H. 2001. Distribution and behavior or Nonylphenol, octylphenol, and nonylphenol monoethoxylate in Tokyo Metropolitan area: their association with aquatic particles and sedimentary distributions. *Environ. Sci. Technol.* **35**(6): 1041-1049.

Jin, S., Yang, F., Liao, T., Hui, Y. and Xu, Y. 2008. Seasonal variations of estrogenic compounds and their estrogenicities in influent and effluent from a municipal sewage treatment plant in China. *Environ. Toxicol. Chem.* **27**(1): 146-153.

Jobling, S., Williams, R., Johnson, A., Taylor, A., Gross-Sorokin, M., Nolan, M., Tyler, C.R., von Aerle, R., Santos, E. and Brighty, G. 2006. Predicted exposure to steroid estrogens in U.K. rivers correlate with widespread sexual disruption in wild fish populations. *Environ. Health Persp.* **114**: 32-39.

Jobling, S., Casey, D., Rodgers-Gray, T., Oehlmann, J., Schulte-Oehlmass, U., Pawlowski, S., Baunbeck, T., Turner, A.P. and Tyler, C.R. 2003. Comparative responses of molluscs and fish to environmental estrogens and an estrogenic effluent. *Aquat. Toxicol.* **65**: 205-220.

Jobling, S., Nolan, M., Tyler, C.R., Brighty, G. and Sumper, J.P. 1998. Widespread sexual disruption in wild fish. *Environ. Sci. Technol.* **32**: 2498-2506.

Johnson, A.C., Williams, R.J., Simpson, P. and Kanda, R. 2007. What difference might sewage treatment performance make to endocrine disruption to rivers? *Environ. Pollut.* **147**: 194-202.

Johnson, A.C. and Williams, R.J. 2004. A model to estimate influent and effluent concentrations of estradiol, estrone, and ethinylestradiol at sewage treatment works. *Environ. Sci. Technol.* **38**: 3649-3658.

Johnson, A.C. and Sumpter, J.P. 2001. Removal of endocrine-disrupting chemicals in activated sludge treatment works. *Environ. Sci. Technol.* **35**(24): 4697-4703.

Johnson, A.C., Belfroid, A. and Di Corcia, A. 2000. Estimating steroid oestrogen inputs into activated stludge treatment works and observations on their removal from the effluent. *Sci. Total Environ.* **256**: 163-173.

Joss, A., Anderson, H., Ternes, T., Richle, P.R. and Siegrist, H. 2004. Removal of

estrogens in municipal wastewater treatment under aerobic and anaerobic conditions: consequences for plant optimization. *Environ. Sci. Technol.* **38**: 3047-3055.

Jukosky, J.A., Watzin, M.C. and Leiter, J.C. 2008. The effects of environmentally relevant mixtures of estrogens on Japanese medaka (*Oryzias latipes*) reproduction. *Aquat. Toxicol.* **86**: 323-331.

Jürgens, M.D., Holthaus, K.I.E., Johnson, A.C., Smith, J.J.L., Hetheridge, M. and Williams, R.J. 2002. The potential for estradiol and ethinylestradiol degradation in English rivers. *Environ. Toxicol. Chem.* **21**(3): 480-488.

Kang, I.J., Yokota, H., Oshima, Y., Tsuruda, Y., Yamaguchi, T., Maeda, M., Imada, N., Tadokoro, H. and Honjo, T. 2002. Effect of 17β-estradiol on the reproduction of Japanese medaka (*Oryzias latipes*). *Chemosphere* **47**: 71-80.

Khanal, S.K., Xie, Bin, Thomspon, M., Sung, S., Ong, S and VanLeeuwen, J. 2006. Fate, transport, and biodegradation of natural estrogens in the environment and engineered systems. *Environ. Sci. Technol.* **40**(21): 6537-6546.

Kidd, K.A., Blanchfield, P.J., Mills, K.H., Palace, V. P., Evans, R.E., Lazorchak, J.M. and Flick, R.W. 2007. Collapse of a fish population after exposure to a synthetic strogen. *Proc. Nat. Acad. Sci.* **104**(21): 8897-8901.

Kirk, L.A., Tyler, C.R., Lye, C.M and Sumpter, J.P. 2001. Changes in estrogenic and androgenic activities at different stages of treatment in wastewater treatment works. *Environ. Toxicol. Chem.* **21**: 972-979.

Kuch, H.M. and Ballschmiter, K. 2001. Determination of endocrine-disrupting phenolic compounds and estrogens in surface and drinking water by HRGC-(NIC)-MS in the pictogram per litre range. *Environ. Sci. Technol.* **35**: 3201-3206.

Labadie, P. and Budzinski, H. 2005. Determination of steroidal hormone profiles along the Jalle d'Eysines River (near Bordeaux, France). *Environ. Sci. Technol.* **39**(14): 5113-5120.

Lafront, R. and Mathieu, M. 2007. Steroids in aquatic invertebrates. *Ecotox.* **16**(1): 109-130.

Lai, K.M., Scrimshaw, M.D. and Lester, J.N. 2002a. Biotransformation and bioconcentration of steroid estrogens by *Chlorella vulgaris*. *Appl. Environ. Microb.* **68**(2): 859-864.

Lai, K.M., Scrimshaw, M.D. and Lester, J.N. 2002b. The effects of natural and synthetic steroid estrogens in relation to their environmental occurrence. *Crit. Rev. Toxicol.* **322**: 113-132.

Lai, K.M., Scrimshaw, M.D. and Lester, J.N. 2002c. Prediction of the bioaccumulation factors and body burden of natural and synthetic estrogens in aquatic organisms in the river systems. *Sci. Tot. Environ.* **289**: 159-168.

Lai, K.M., Johnson, K.L., Scrimshaw, M.D. and Lester, J.N. 2000. Binding of waterborne steroid estrogens to solid phases in river and estuarine systems. *Environ. Sci. Technol.* **34**: 3890-3894.

Larson, D.G.L., Adolfsson-Eric, M., Parkkonen, J., Pettersson, M., Berg, A.H., Olsson, P.E. and Förlin, L. 1999. Ethinyloestradiol – an undesired fish contraceptive? *Aquat. Toxicol.* **45**: 91-97.

Lee, H.-B., Peart, T.E., Chan, J. and Gris, G. 2004. Occurrence of endocrine-disrupting chemicals in sewage and sludge samples in Toronto, Canada. *Water Qu*al. *Res. J. Canada* **39**(1): 57-63.

Lee, H.-B. and Liu, D. 2002. Degradation of 17β-estradiol and its metabolites by sewage bacteria. *Water, Air, Soil Pollut.* **134**: 353-368.

Lee, H.-B. and Peart, T.E. 1998. Determination of 17β-estradiol and its metabolites in sewage effluent by solid-phase extraction and gas chromatography/mass spectrometry. *J. AOAC Int.* **81**: 1209-1216.

Lee, L.S., Strock, T.J., Sarmah, A.K. and Rao, P.S.C. 2003. Sorption and dissipation of testosterone, estrogens and their primary transformation products in soils and sediment. *Environ. Sci. Technol.* **37**: 4098-4105.

Liebig, M., Egeler, P., Oehlmann, J. and Knacker, T. 2005. Bioaccumulation of 14C-17α-ethinylestardiol by the aquatic oligochaete *Lumbriculus variegates* in spiked artificial sediment. *Chemosphere* **59**: 271-280.

Lin, A. Y.-C., and Reinhard, M. 2005. Photodegradation of common environmental pharmaceuticals and estrogens in river water. *Environ. Toxicol. Chem.* **24**(6): 1303-1309.

Lishman, L., S.A. Smyth, K. Sarafin, S. Kleywegt, J. Toito, T. Peart, B. Lee, M. Servos, M. Beland and P. Seto. 2006. Occurrence and reductions of pharmaceuticals and personal care products and estrogens by municipal wastewater treatment plants in Ontario, Canada. *Sci. Total Environ.* **367**(2-3): 544-558.

Liu, B. and Liu, X. 2004. Direct photolysis of estrogens in aqueous solutions. *Sci. Total Environ.* **320**: 269-274.

MacKay, D. and Blais, J. 2008. Canadian Environmental Modeling Centre, Trent University and Ottawa-Carleton Institute of Biology, University of Ottawa.

MacKay, D. and Fraser, A. 2000. Bioaccumulation of persistent organic chemicals: mechanisms and models. *Environ. Poll.* **110**: 375-391.

MacLachty, D.L. and van der Kraak, G.J. 1995. The phytoestrogen β-sitosterol alters the reproductive endocrine status of goldfish. *Toxicol. Appl. Pharm.* **134**: 305-312.

Marlatt, V.L, Lakoff, J., Crump, K., Martyniuk, C.J., Watt, J., Jewell, L., Atkinson, S.K, Blais, J.M., Sherry, J., Moon, T.W. and Trudeau, V.L. 2008. Sex- and tissue-specific effects of waterborne estrogen on estrogen receptor subtypes and E2-mediated gene expression in the reproductive axis of goldfish. Manuscript prepared for submission to *Gen. Comp. Endocr.*

Marlatt, V. L., Martyniuk, C. J., Zhang, D., Xiong, H., Watt, J., Xia, X., Moon, T. and Trudeau, V. L. 2007. Auto-regulation of estrogen receptor subtypes and gene expression profiling of 17b-estradiol action in the neuroendocrine axis of male goldfish. *Mol. Cell. Endocr.* **283**(1-2): 38-48.

Marlatt, V.L. 2006. Ottawa-Carleton Institute of Biology, University of Ottawa.

Mastrup, M., Schäfer, A.I. and Khan, S.J. 2005. Predicting fate of the contraceptive pill in wastewater treatment and discharge. *Water Sci. Technol.* **52**(8): 279-286.

McNeil, R. 2005. Standard Operating Procedure for Tissue Preparation SOP-TP-PROC-07D. Laboratory Services Section, National Wildlife Research Centre, Environment of Canada. 8 p.

Metcalfe, C.D., Metcalfe, T.L., Kiparissis, Y., Koenig, B.G., Khan, C., Hughes, R.J., Croley, T.R., March, R.E. and Potter, T. 2001. Estrogenic potency of chemical detected in sewage treatment plant effluents as determined by in vivo assays with Japanese Medeka (*Oryzias latipes*). *Environ. Toxicol. Chem.* **20**(2): 297-308.

Meylan, W.M., Howard, P.H., Boethling R.S., Aronson, D., Printup, H. and Gouchie, S. 1999. Improved method for estimation bioconcentration/bioaccumulation factor from octanol/water partition coefficient. *Envrion. Toxicol. Chem.* **18**(4): 664-672.

Miège, C., Choubert, J.M., Ribiero, L, Eusèbe, M. and Coquery, M. 2008. Removal efficiency of pharmaceuticals and personal care products with varying wastewater treatment processes and operating condition – conception of a database and first results. *Water. Sci. Technol.* **57**(1): 49-56.

Mitamura, K., Narukawa, H., Mizuguchi, T. and Shimada, K. 2004. Degradation of estrogen conjugates using titanium dioxide as a photocatalyst. *Anal. Sci.* **20**: 3-4.

Mitsuit, N., Tooi, O. and Kawahar, A. 2007. Vitellogenin-inducing activities of natural, synthetic, and environmental estrogens in primary cultured *Xenopus laevis* hepatocytes. *Comp. Biochem. Phys. C* **146**(4): 581-587.

Miyamoto, J. 1996. Environmental and health issues. *Pure & Appl. Chem.* **68**(9): 1737-1748.

Murk, A.J., Legler, J., Van Lipzig, M.M.H., Meerman, J.H.N., Belfroid, A.C., Spenkelink, A., Van Der Burgh, B., Rus, G.B. and Vethaak, D. 2002. Detection of estrogenic potency in wastewater and surface water with three in vitro bioassays. *Environ. Toxicol. Chem.* **21**: 16-23.

Nakada, N., Tanishima, T., Shinohara, H., Kiri, K. and Takada, H. 2006. Pharmaceutical chemicals and endocrine disrupters in municipal wastewater in Tokyo and their removal during activated sludge treatment. *Wat. Res.* **40**: 3297-3303.

Nakada, N., Nyunoya, H., Nakamuru, M., Hara, A., Iguchi, T. and Takadas, H. 2004. Identification of estrogenic compounds in a wastewater effluent. *Environ. Toxicol. Chem.* **23**(12): 2807-2815.

Nash, J.P., Kime, D.E., Van der Ven, L.T.M., Wester, P.W., Brion, F., Maack, G., Stahlschmidt-Allner, P. and Tyler, C.R. 2004. Long-term exposure to environmental concentrations of the pharmaceutical ethinylestradiol causes reproductive failure in fish. *Environ. Health Perspect.* **112**(17): 1725-1733.

National Library of Medical Information Specialized Information Services. 2006. Website available online at http://chem.sis.nlm.nih.gov/chemidplus/.

OCMBP. 2006. Best Practice Summary Report. Water and Wastewater – Alternative Sources of Energy. Case study of Ottawa. Association of Municipalities of Ontario, Toronto. 5 p.

Ortega-Salas, A.A. and Reyes-Bustamante, H. 2006. Initial sexual maturity and fecundity of the goldfish *Carassius auratus* (Perciformes: Cyprynidae) under semi-controlled conditions. *Rev. Biol. Trop.* **54**(4): 1113-6.

Palanza, P., Morellini, F., Parmigiani, S. and vom Saa, F. S. 1999. Prenatal exposure to endocrine disrupting chemicals: effects on behavioral development. *Neurosci. Biobehav. Rev.* **23**: 1011-1027.

Panter, G. H., Thompson, R. S. and Sumpter, J. P. 2000. Intermittent exposure of fish to estradiol. *Environ. Sci. & Tech.* **34**(13): 2756-2760.

Park, B.J. and Kidd, K. 2005. Effects of the synthetic estrogen ethinylestradiol on early stages of mind frogs and green frogs in the wild and in situ. *Environ. Toxicol. Chem.* **24**(8): 2027-2036.

Préfontaine, G. and Bélanger, M. 2003. Bathymétrie du Lac Cromwell. Station de biologie des Laurentides, Université de Montréal.

Purdom, C.E., Hardiman, P.A., Bye, V.J., Eno, N.C., Tyler, C.R. and Sumpter, J.P. 1994. Estrogenic effects of effluents from sewage-treatment works. *Chem. Ecol.* **8**: 275-285.

Purves, W.K., Orians, G.H., Heller, H.C. and Sadava, D. 1997. Life, the Science of Biology, 5th edition. Sinauer Associate, Inc., Sunderland. 1243 p.

Raftogianis, R., Creveling, C., Weinshilbourn, R. and Weisz, J. 2000. Chapter 6: Estrogen metabolism by Conjugation. *J. Natl. Cancer Inst. Monograph.* **27**: 12 p.

Roepke, T.A., Snyder, M.J. and Cherr, G.N. 2005. Estradiol and endocrine disrupting compounds adversely affect development of sea urchin embryos at environmentally relevant concentrations. *Aquat. Toxicol.* **71**(2): 155-173.

Rosenfeldt, E.J. and Linden, K.G. 2004. Degradation of endocrine disrupting chemicals bisphenol A, ethinyl estradiol, and estradiol during UV photlysis and advanced oxidation processes. *Environ. Sci. Technol.* **38**: 5476-5483.

Routledge, E.J., Sheahan, D., Desbrow, C., Brighty, G.C., Waldock, M. and Sumpter, J.P. 1998. Identification of estrogenic chemicals in STW effluent. 2. In vivo responses in trout and roach. *Environ. Sci. Technol.* **32**: 1559-1565.

Roy, D. and Liehr, J.G. 1999. Estrogen, DNA damage and mutations. *Mutat. Res.* **424**:107-115.

Schlüsener, M.P. and Bester, K. 2008. Behaviour of steroid hormones and conjugates during wastewater treatment – a comparison of three sewage treatment plants. *Clean* **36**(1): 25-33.

Schwaiger, J., Spieser, O. H., Bauer, C., Ferling, H., Mallow, U., Kalbfus, W., and Negele, R. D. 2000. Chronic toxicity of nonylphenol and ethinylestradiol: haematological and histopathological effects in juvenile common carp (*Cyprinus carpio*). *Aquat. Toxicol.* **I**: 69-78.

Schwindt, A.R., Schreck, C., Landers, D., Ackerman, L., Usenko, S., Ramsay, J.M., Simonich, S. and Kent, M. 2004. Do Airborne Contaminants Affect Fish in the Lakes of Western U.S. National Parks? Fifth International Symposium on Fish Endocrinology, Castellon, Spain.

Segmuller, B.E., Armstrong, B.L., Dunphy, R. and Oyler, M.R. 2000. Identification of autooxidation and phtodegradation products of ethinylestradiol by on-line HPLC-NMR and HPLC-MS. J. Pharmaceut. Biomed. **23**: 927-937.

Servos, M.R., Bennie, D.T., Burnison, B.K., Jurkovic, A., McInnis, R., Neheli, T., Schnell, A., Seto, P., Smyth, S.A. and Ternes, T.A. 2005. Distribution of

estrogens, 17β-estradiol and estrone, in Canadian municipal wastewater treatment plants. *Sci. Total Environ.* **336**(1-3): 155-170.

Sharpless, C.M., Seibold, D.A. and Linden, K.G. 2003. Nitrate photosensitized degradation of atrazine during UV water treatment. *Aquat. Sci* .**65**(4): 359-366.

Sherry, J., Gamble, A., Fielden, M., Hodson, P., Burnison, B. and Solomon, K. 1999. An ELISA for brown trout (*Salmo trutta*) vitellogenin and its use in bioassays for environmental estrogens. *Sci. Total Environ.* **225**: 13-31.

Shiwei, J., Yang, F., Liao, T., Hui, Y. and Xu, Y. 2008. Seasonal variation of estrogenic compounds and their estrogenicities in influent and effluent from a municipal sewage treatment plant in China. *Environ. Toxicol. Chem.* **27**(1): 146-153.

Sierra Legal Defence Fund. 2006. The Great Lakes Sewage Report Card. Toronto, ON. 57 p.

Snyder, S.A., Keith, T.L., Verbrugge, D.A., Snyder, E.M., Gross, T.S., Kannan, K., and Giesy, J.P. 1999. Analytical methods for detection of selected estrogenic compounds in aqueous mixtures. *Environ. Sci. Technol.* **33**: 2814-2820.

Sommer, R., Cabaj, A., Pribil, W. and Haider, T. 1997. Influence of lamp intensity and water transmittance on the UV disinfection of water. *Wat. Sci. Technol.* **35** (11-12): 113-118.

Stanford, B.D. and Weinberg, H.S. 2007. Isotope dilution for quantification of steroid estrogens and nonylphenols by gas chromatography with tandem mass spectrometry in septic soil and groundwater matrices. *J. Chromatogr. A* **1176**: 26-36.

Statistics Canada. 2006. Community Profile for Ottawa from the 2006 Census of Population. Web-based publication available online at http://www12.statcan.ca/english/census06/data/profiles/community/Index.cfm?Lang=E.

Statistics Denmark. 2008. StatBank Denmark. Web-based database available online at http://www.statistikbanken.dk/statbank5a/default.asp?w=1024.

Stone, C.M., and Watkinson, R.J. 1983. Diphenyl propane: an assessment of ready bioidegradabilty. Sittingbourne Research Center (SBRC), Shell Research Limited. Sittingbourne, U.K. 425 p.

Suidan, M.T., Esperanza, M., McCauley, P., Brenner, R.C. and Venosa, A.D. 2005. Challenges in biodegradation of trace organic contaminants – gasoline oxygenates and sex hormones. *Water. Environ. Res.* **77**(1): 4-11.

Sumpter, J.P. and Johnson, A.C. 2005. Lessons from endocrine disruption and their application to other issues concerning trace organic in the aquatic environment. *Environ. Sci. Technol.* **39**: 4321-4332.

Sumpter, J.P. 1998. Xenoendocrine disrupters – environmental impacts. *Toxicol. Lett.* **102-103**: 337-342.

Sun, Q., Deng, S., Huang, J., Shen, G. and Yu, G. 2008. Contributors to estrogenic activity in wastewater from a large wastewater treatment plant in Beijing, China. *Environ. Toxicol. Pharmocol.* **25**: 20-26.

Ternes, T.A., Anderson, H., Gilbert, D. and Bonerz, M. 2002. Determination of estrogens in sludge and sediments by liquid extraction and GC/MS/MS. *Anal. Chem.* **74**: 3498-3504.

Ternes, T.A., Stumpt, M., Mueller, J., Harbere, K., Wilken, R.-D. and Servos, M. 1999a. Behaviour and occurrence of estrogens in municipal sewage treatment plants – I. Investigations in Germany, Canada and Brazil. *Sci. Total Environ.* **225**: 81-90.

Ternes, T.A., Kreckel, P. and Mueller, J. 1999b. Behaviour and occurrence of estrogens in municipal sewage treatment plants – II. Aerobic batch experiments with activated sludge. *Sci. Total Environ.* **225**: 91-99.

Thiele, B., Gunther, K. and Schwuger, M. J. 1997. Alkylphenol ethoxylates: trace analysis and environmental behavior. *Chem. Rev.* **97**: 3247-3272.

Thorpe, K.L., Cummings, R.I., Hutchinson, T.H., Scholze, M., Brighty, G., Sumpter, J.P. and Tyler, C.R. 2003. Relative potencies and combination effects of steroidal estrogens in fish. *Environ. Sci. and Tech.* **37**(6): 1142-1149.

Trudeau, V.L., Heyne, B., Blais, J.M., Temussi, F., Atkinson, S.K, Pakdel, F., Pepesku, J.T., Marlatt, V., Sciano, T. and Lean, D.R.S. 2008. Lumiestrone is the biologically active photochemically-derived epimer of the human hormone estrone that is being released to the environment without detection. Manuscript prepared for submission to *Science*.

Tyler, C.R. and Routledge, E.J. 1998. Oestrogenic effects in fish in English Rivers with evidence of their causation. *Pure Appl. Chem.* **70**(9): 1795-1804.

Tyler, C.R., van der Eerden, B., Jobling, S., Panter, G. and Sumpter, J.P. 1996. Measurement of vitellogenin, a biomarker for exposure to oestrogenic chemicals, in a wide variety of cyprinid fish. *J. Comp. Physiol. B* **166**: 418-426.

Van den Belt, K., Verheyen, R. and Witters, H. 2003. Comparison of vitellogenin responses in zebrafish and rainbow trout following exposure to environmental estrogens. *Ecotox. Environ. Safe.* **56**: 271-281.

Vermeirssen, E.L.M., Körner, O., Schönenberger, R., Suter, M.J.-F. and Burkhardt-Holm, P. 2005. Characterization of environmental estrogen in river water using a three pronged approach: active and passive water sampling and the analysis of accumulated estrogens in the bile of caged fish. *Environ. Sci. Technol.* **39**: 8191-8198.

Vine, E., Shears, J., van Aerle, R., Tyler, C.R. and Sumpter, J.P. 2005. Endocrine (sexual) disruption is not a prominent feature in the pike (*Esox lucius*), a top predator in English waters. *Environ. Toxicol. Chem.* **24**(6): 1436-1443.

Walker, C.H. 1990. Kinetic models to predict bioaccumulation of pollutants. *Funct. Ecol.* **4**(3): 295-301.

Wang, S., Huang, W., Fang, G., Zhang, Y. and Qiao, H. 2008. Analysis of steroidal estrogen residues in food and environmental samples. *Intern. J. Environ. Anal. Chem.* **1**(15): 1-25.

Williams, R.J., Johnson, A.C., Smith, J.J.L. and Kanda. R. 2003. Steroid estrogens profiles along river stretches arising from sewage treatment works discharges. *Environ. Sci. Technol.* **37**: 1744-1750.

Winch, S., Ridal, J. and Lean, D. 2002. Increased metal bioavailability following alteration of freshwater dissolved organic carbon by ultraviolet B radiation exposure. *Environ. Toxicol.* **17**(3): 267-274.

Xiao, X-Y., McCalley, D.V. and McEvoy, J. 2001. Analysis of estrogen in river water and effluents using solid-phase extraction and gas chromatography-negative chemical ionization mass spectrometry of pentafluorobenzoyl derivatives. *J. Chromatogr. A.* **923**: 195-204.

Ying, G.-G. and Kookana, R.S. 2003. Degradation of five slected endocrine-disrupting chemicals in seawater and marine sediment. Environ. Sci. Technol. **37**: 1256-1260.

Zha, J., Sun, L., Zhou, Y., Spear, P.A., Ma, M and Wang, Z. 2008. Assessment of 17α-ethinylestradiol effects and underlying mechanisms in a continuous multigeneration exposure of the Chinese rare minnow (*Gobiocyprus rarus*). Toxiol. Appl. Pharmacol. **226**: 298-308.

APPENDIX I

Assays for estrogenic activity

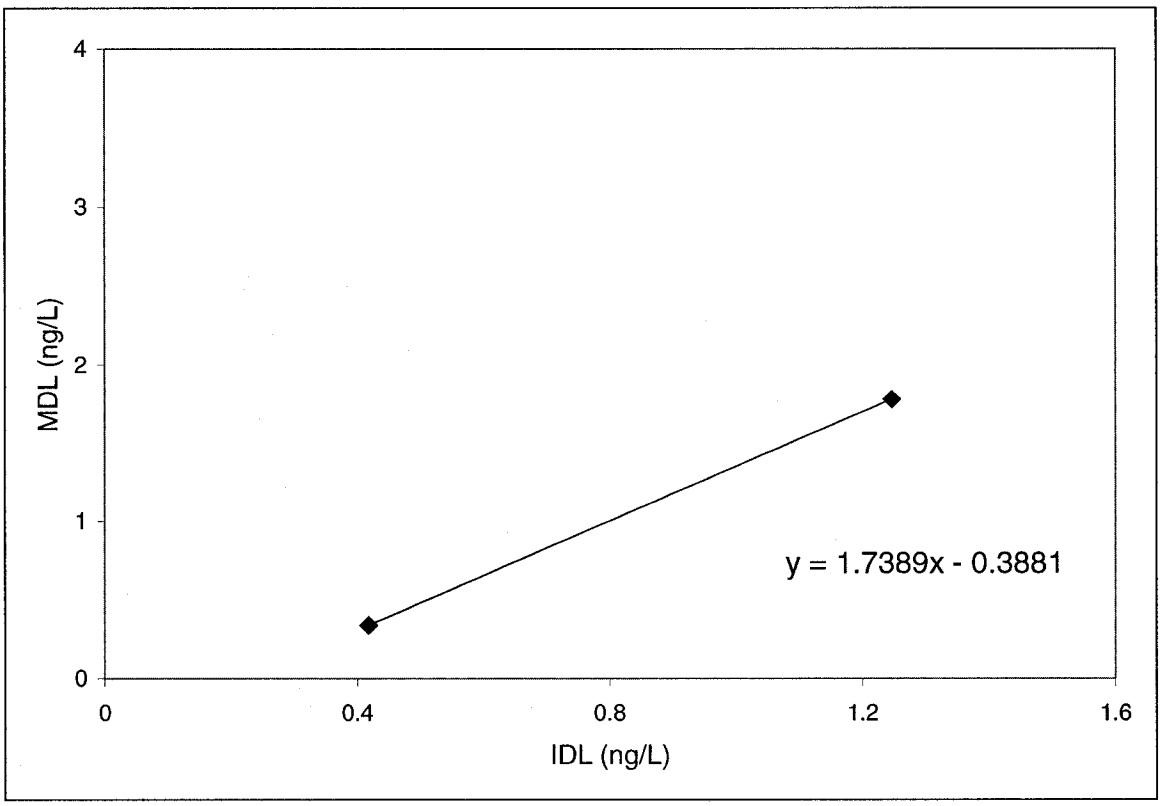
ER alpha reportor gene assay

Water samples were filtered and passed through SPE tubes as described in Secton 2.3.2.1, omitting the isotope dilution step. The dried residues were resuspended in dimethyl sulfoxide (DMSO), a solvent that increases the permeability of cell membranes. The estrogenicity of the eluates was evaluated using a reporter gene assay, which uses a rainbow trout gonad cell line with estrogen receptor (ER) alpha (pSG5) transiently transfected with a plasmid containing an estrogen response element upstream of a reporter gene, luciferase (ERE-TK-Luc) (Ackermann et al. 2002; Figure 2.3). DMSO controls were included in all assays.

Gene expression of ER subtypes

RNA from tissue samples was extracted and prepared for realtime PCR and microarray analysis using methods described by Marlatt et al. (2007).

APPENDIX II



Standard curve used the estimation of a method detection limit for 17β-estradiol

APPENDIX III

Equations used for predicting steroidal estrogens concentrations in raw sewage

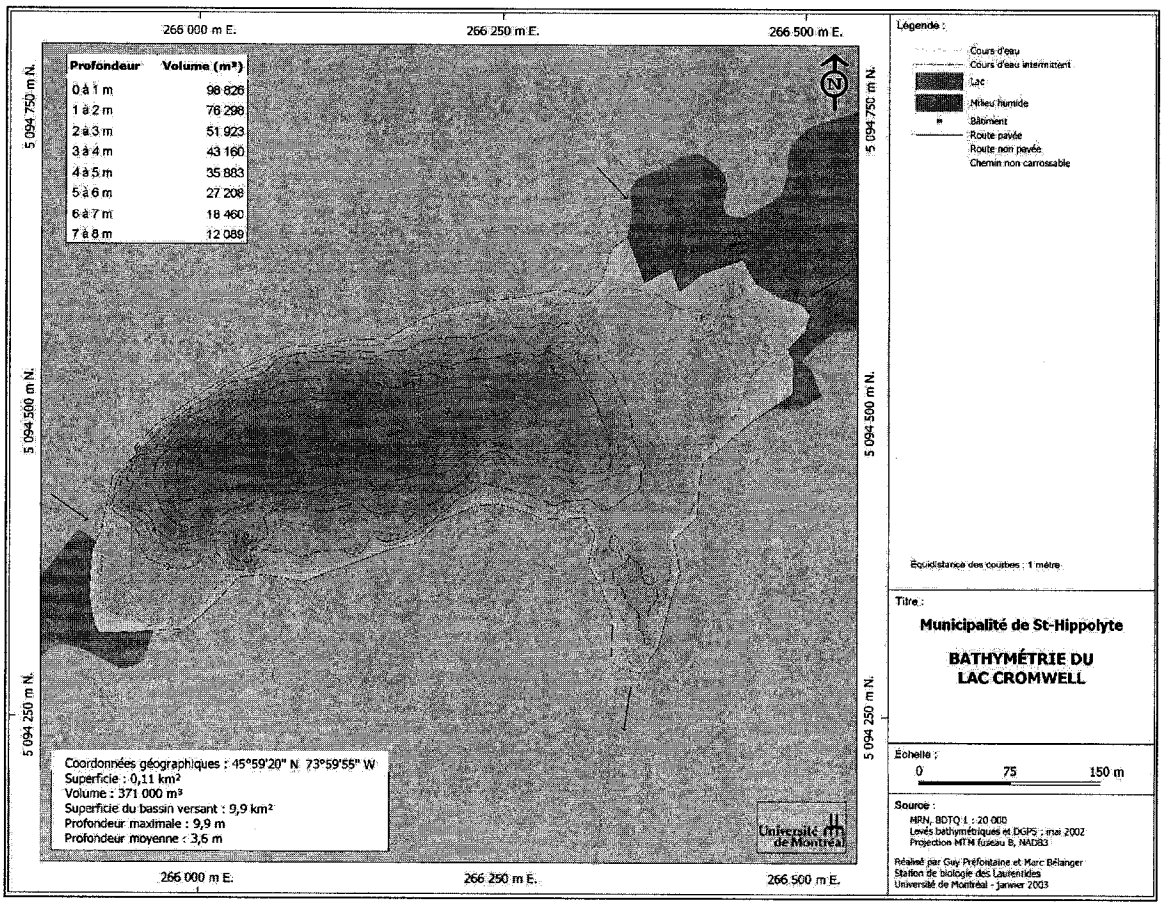
Method using Johnson et al. 2000

$$\text{Estrogen concentration} = \frac{\Sigma\ [\text{daily individual input of estrogen for group x number in population group}]}{\text{daily plant flow}}$$

Method using Christiansen et al. 2002

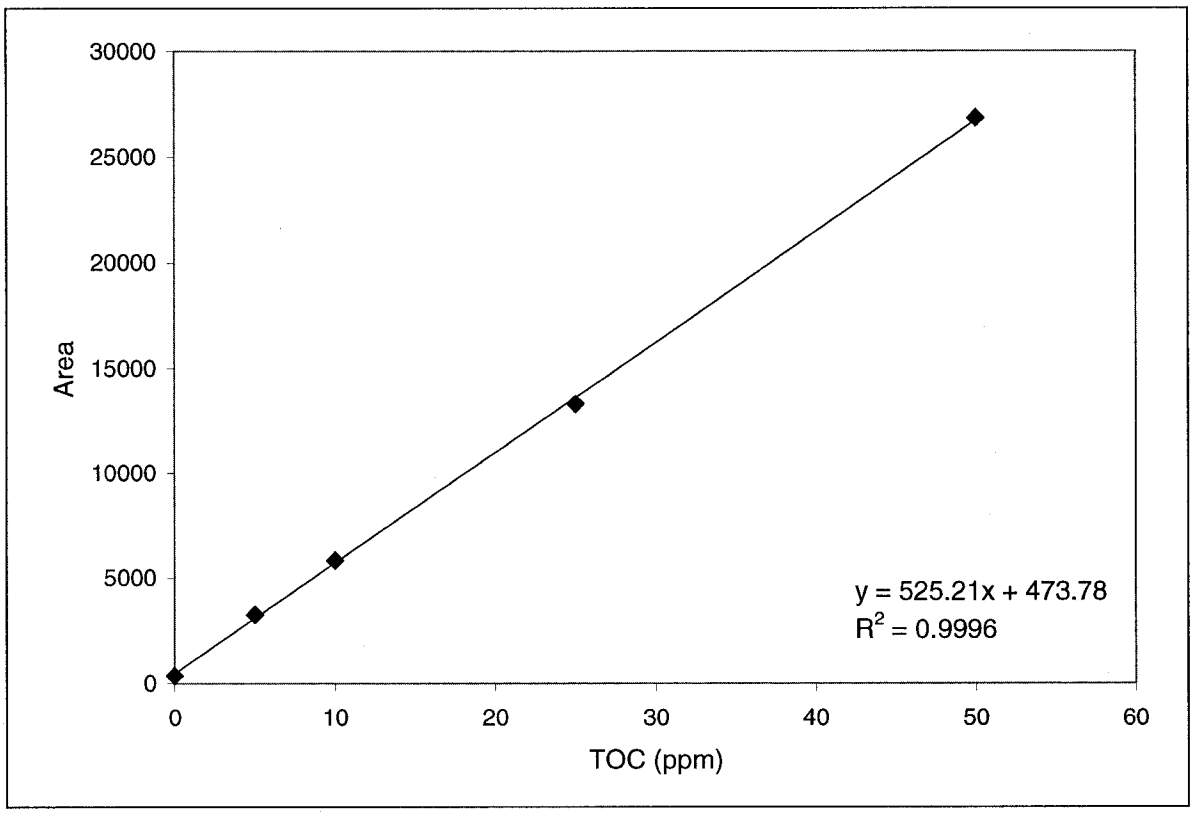
$$\text{Estrogen concentration} = \frac{\text{daily average individual input of estrogen x population}}{\text{daily plant flow}}$$

APPENDIX IV



Map of Cromwell Lake, St-Hippolyte, Quebec where water with a DOC concentration of 6.85 ppm was collected on October 22, 2005 (Préfontaine et Bélanger 2003)

APPENDIX V



Calibration curve used for analysis of dissolved organic carbon concentration in water used for UVB exposure experiments

APPENDIX VI

Equations and values used in mass-balance model calculating bioconcentration factor of 17α-ethinylestradiol in fish blood (Mackay and Blais 2008; MacKay and Fraser 2000)

Measured data

Plot measured data for EE2 concentration in water (C_W) (Table 5.2) versus time (t) and EE2 concentration in fish blood (C_F) (Table 5.3) versus t and take equation of best-fit lines:

$C_W = 25.5e^{-0.0349x}$
$C_F = -4.67x2 + 346x + 4E\text{-}10$

Calculate an approximated experimental C_W and C_F for t = 0 to t = 72 hours at every 0.1 h interval

Modeled data

Mass-balance equations for fish and water

$V_W\, dC_W/dt = V_F\, C_F\, k_2 - V_W\, C_W\, k_{WD} - V_F C_W\, k_1$
$V_F\, dC_F/dt = V_F\, C_W\, k_1 - V_F\, C_F\, k_2 - V_F\, C_F\, k_{FD}$

Where:

k_1 = uptake rate constant (h^{-1})
k_2 = elimination rate constant (h^{-1})
k_{FD} = rate constant for degradation in fish (h^{-1})
k_{WD} = rate constant for degradation in water (h^{-1})

V_W = 70 L
C_{WO} = 25 ng/L
C_{FO} = 0 ng/L

Assumptions and estimates

When t is very small, $k_2 = 0$, $k_{WD} = 0$ and $k_{FD} = 0$

$V_F\, dC_F/dt = -\, V_w\, dC_w/dt$

$V_F = 0.15$ L

Also, when t is very small and $k_2 = 0$, $k_{WD} = 0$ and $k_{FD} = 0$

$V_F \, dC_F/dt = V_F \, C_W \, k_1$
$k_1 = (dC_F/dt)/C_W$
$k_1 = 13 \, h^{-1}$

Now, when t is very large, assuming a steady-state is reached at 72 hours, Cf / Cw is approximately equal to k_1 / k_2.

$k_2 = (Cf / Cw)/ k_1$
$k_2 = 0.04 \, h^{-1}$

Experimental data from tanks without fish show that k_{WD} is very low, which we estimate as $10^{-3} \, h^{-1}$

Assume $k_2 = k_{FD} = 0.04$

Calculating modeled concentrations

For every time interval of t = 0.1 hours, calculate the transfer and losses of EE2

$\Delta Mw = (V_F \, C_F \, k_2 - V_W \, C_W \, k_{WD} - V_F \, C_W \, k_1) \, \Delta t$
$\Delta Mf = (V_F \, C_W \, k_1 - V_F \, C_F \, k_2 - V_F \, C_F \, k_{FD}) \, \Delta t$

$Mw = Mw + \Delta Mw$
$Mf = Mf + \Delta Mf$
$t = t + \Delta t$
$Cw = Mw/Vw$
$Cf = Mf/Vf$
$Mt = Mw + Mf$

Calculate an approximated modeled C_W and C_F for t = 0 to t = 72 hours at every 0.1 h interval

Fitting modeled data to experimental data

The task is to minimize SS_{FISH}.

$\Sigma \, (C_{F \, experimental} - C_{F \, modeled})^2 = SS_{FISH}$

This is done by "tweaking" the k values. For example, by keeping k_1, k_2 and k_{WD} constant SS_{FISH} can be minimized when k_{FD} is 0.015. This is done for each k constant to find the optimized values where:

$k_1 = 28\ h^{-1}$
$k_2 = 0.02\ h^{-1}$
$k_{FD} = 0.001\ h^{-1}$
$k_{WD} = 0.015\ h^{-1}$

Calculating bioconcentration factor

The bioconcentration factor can be expressed as:

$k_1/k_2 = 1400$