

BIOACCUMULATION OF 17 α -ETHINYLESTRADIOL IN FISH

By:

AHMED AL-ANSARI

Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies University Ottawa

In partial fulfillment of the requirements for the Ph.D. degree Chemical and Environmental
Toxicology Program Ottawa-Carleton Institute of Biology

Thèse soumise à la
Faculté des études supérieures et postdoctorales Université d'Ottawa

En vue de la obtention du doctorate Programme en toxicologie chimique et environnementale
L'institut de biologie d' Ottawa-Carleton

© Ahmed Al-Ansari, Ottawa, Canada, 2012

EXAMINERS

This thesis has been examined and approved by:

Dr. Jules M. Blais
Supervisor

Dr. Vance L. Trudeau
Supervisor

Dr. Aaron T. Fisk
External Member (University of Windsor)

Dr. John T. Arnason
Examining Member (University of Ottawa)

Dr. Frances R. Pick
Examining Member (University of Ottawa)

Dr. Banu Örmeci
Examining Member (Carleton University)

Abstract

The active synthetic constituent of oral contraceptives 17 α - ethinylestradiol (EE2) and its natural steroidal estrogen analogues are being released into the aquatic environment mainly via wastewater treatment plants (WWTPs). Although steroidal estrogens have been frequently reported in very low concentrations in the environment, they have been placed at the top of the list of endocrine disrupting chemicals (EDCs) for their high estrogenic activity in non-target aquatic species like fish and frogs. Almost 30 years worth of intensive research has moved the problem of endocrine disruption in the aquatic environment from being a hypothesis to a well-known cause-and-effect story. However, the fate and effects of many pharmaceuticals, including EE2, remain poorly understood. Thus, the main objective of this thesis was to investigate EE2 bioaccumulation in fish by field and laboratory studies.

An optimized sample preparation and analytical method protocol was achieved to detect EE2 in its biologically active form in whole fish tissue at trace levels (ng/g). Shorthead redhorse suckers (ShRHS) (*Moxostoma macrolepidotum*) collected in proximity to a WWTP in the St. Clair River have significantly accumulated EE2 with an average of 1.5 ng/g. EE2 bioaccumulation was limited to fish exhibiting intersex and with induced vitellogenin (VTG). EE2 was absent in fish from a reference site. Positive correlations between EE2 and lipid content as well as $\delta^{15}\text{N}$ supported the hypothesis of EE2 bioaccumulation in wild ShRHSs. A wider survey was carried out to explore patterns of EE2 bioaccumulation in the pelagic and benthic food-web of the St. Clair

River, Ontario. In the WWTP effluents and nearby surface waters, EE2 levels were extremely low and below our method detection limits. All of the seven sampled species from different trophic levels and the sediment samples collected from the impacted sites and the reference sites did not have any measurable EE2 concentrations. A laboratory controlled study where male goldfish (*Carassius auratus*) were exposed to EE2 via water versus food demonstrated that fish can rapidly accumulate EE2 by both routes of exposure. The uptake constant for water was 45 h^{-1} and the bioconcentration factor for EE2 in fish was 377. The fast uptake rate of EE2 via water coincided with a much slower elimination rate constant of 0.0786 h^{-1} . The assimilation efficiency of 0.106 for EE2 by goldfish was determined by EE2 dietary exposure, which was used to predict EE2 accumulation under different exposure scenarios. The work presented here was the first demonstration of EE2 bioaccumulation in wild fish and the first to model EE2 bioaccumulation from water and dietary exposure in laboratory-exposed fish.

Résumé

Le composé synthétique actif des contraceptifs oraux, le 17 α -éthynylestradiol (EE2), ainsi que ses analogues stéroïdiens, sont relâchés dans les milieux aquatiques principalement par les stations d'épuration des eaux usées (STEP). Ces œstrogènes stéroïdiens ont fréquemment été observés à de faibles concentrations dans l'environnement, cependant, ils sont en tête de liste des perturbateurs endocriniens (PE) à cause de leur activité hormonale élevée chez plusieurs espèces de poisson et d'amphibiens. Trente années de recherche intensive sur le problème des perturbateurs endocriniens dans les milieux aquatiques en ont changé sa perception, allant d'une simple hypothèse à une cause à effet reconnue, en ce qui concerne leurs effets néfastes. Toutefois, le destin et les effets de produits pharmaceutiques, incluant le EE2, demeurent mal comprise. Ainsi, le principal objectif de cette thèse est la compréhension du phénomène de bioaccumulation de l'EE2 dans le poisson, par le biais d'études en laboratoire et sur le terrain.

Les protocoles décrivant la préparation des échantillons et la méthode analytique ont été optimisés pour détecter l'EE2 dans sa forme biologiquement active, à des niveaux trace (ng/g), dans les tissus de poissons entiers. Les poissons de type chevaliers rouges (CR) (*Moxostoma macrolepidotum*) échantillonnés près d'une STEP de la rivière St. Clair, Ontario ont accumulé l'EE2 de façon significative, la moyenne étant de 1.5 ng/g. La bioaccumulation de EE2 était limitée aux poissons intersexués et avec induction de vitellogénine (VTG). Les poissons provenant du site de référence n'étaient pas contaminés par l'EE2. Des corrélations positives entre la quantité en EE2 et la teneur en

lipide ainsi qu'en $\delta^{15}\text{N}$ ont supporté l'hypothèse de bioaccumulation d'EE2 dans le CR sauvage. Une étude plus poussée a été effectuée pour explorer les modèles de bioaccumulation dans les chaînes alimentaires pélagique et benthique de la rivière St. Clair. Dans les effluents de la STEP, ainsi que dans les eaux de surface à proximité, les quantités de EE2 étaient très faibles, sous la limite de détection de la méthode. Les sept espèces échantillonnées, provenant de niveaux trophiques différents, ainsi que les sédiments provenant de sites contaminés ou de référence, ne possédaient pas des concentrations détectables d'EE2. Une étude effectuée en milieu contrôlé (en laboratoire) sur des mâles de *Carassius auratus* (poisson rouge) exposés à l'EE2, soit par l'eau ou par la nourriture, a montré une accumulation rapide de l'EE2 par les deux voies d'exposition. La constante d'absorption pour l'eau est de 45 h^{-1} et le facteur de bioconcentration (FBC) de l'EE2 dans le poisson était de 377. Le taux d'assimilation rapide de l'EE2 par l'eau coïncidait avec un taux d'élimination plus lent de 0.0786 h^{-1} . L'efficacité de l'assimilation de l'EE2 par les poissons rouges a été déterminée par une expérience d'exposition à la nourriture, qui a été utile pour prédire les modèles d'absorption, d'élimination et de bioaccumulation, avec une approche de modélisation dans laquelle l'absorption peut provenir de sources multiples. Le travail présenté dans cette thèse démontre la bioaccumulation de EE2 dans le poisson sauvage à proximité des sorties des effluents et dans les poissons exposés en laboratoire.

Acknowledgments

It has been a long journey since I first left my beloved hometown Jeddah, the “Bride of the Red Sea” as everybody calls in Saudi Arabia to begin my eight years higher education adventure toward my Ph.D. As an international student and after all of this wonderful time, it will be extremely difficult to leave my Canadian beloved hometown, Ottawa. Thank you wonderful Canadian people and universities you really made me feel home here in Ottawa. I am thankful to you Canadians for your hospitality and kindness.

I am very grateful to my supervisors, Drs. Jules Blais and Vance Trudeau who guided me to discover their interesting areas of research where I gained a lot of knowledge and useful experience that will surely benefit me in my future academic career. I greatly appreciate the opportunity that they provided me to be a member of their fascinating work teams. I also highly appreciate and value the laboratory training and help provided by Linda Kimpe and Dr. Ammar Saleem who benefited me by their tremendous expertise and knowledge. A special thank to my lunch and coffee companion Dr. Asim Mohammad for performing the NMR analysis.

Thank you to the field collaborators from Environment Canada Dr. Jim Sherry and Mark McMaster for providing the wild-fish samples and some field data for the St. Clair River study and also thanks to Jim for joining us in the 2010 sampling trip. I appreciate Dr. Gilbert Cabana from Université du Québec à Trois-Rivières for giving me the chance to be with him in one of his field trips. Special thanks to my thesis advisory committee members Drs. John Arnason and Thomas Moon from the University of Ottawa and Dr. Steven Cook from Carleton University for their helpful comments,

criticism, and suggestions. I also would like to thank all lab mates in Blais and Trudeau labs and specially Dr. Jamie Doyle, Rachelle Gendron, Dr. Laia Navarro-martin and David Eickmeyer for their field and laboratory assistance. Also, I thank Claudine Lefebvre for the French translation of my thesis abstract.

My sincere thanks and gratitudes to the Environmental Science Department at King Abdulaziz University, Jeddah, Saudi Arabia for offering me such a prestigious scholarship. A special thank is also due to the Royal Embassy of Saudi Arabia and specifically to the Cultural Bureau staff in Canada who are doing all they can to help Saudi students thrive in their academic studies. Canadian funding agencies NSERC Canada, Environment Canada, and the Great Lakes Action Plan are highly acknowledged and appreciated for their support.

My extended family members back home also deserve to be appreciated for all kinds of support, advice, and prayer. Special recognition is due to my uncle Dr. Faisal Al-Haddad who encouraged and assisted me to pursue my graduate studies. I will always remember my father in law who was waiting this achievement, but unfortunately he passed away. My deepest emotions will not be sufficient to express my gratitude and love to my wife Amrah, my son Mohammed, and my daughter Danah who inspired me and stood beside me throughout my scholarship and particularly at the time of discouragement. They were always there whenever I needed them. Finally, a heart-felt thank you is due to my parents who always prayed to see a successful person. Proudly now, I can say your prayers were accepted by the Almighty God and here I am as you always love to see me.

Table of Contents

Abstract	Page III
Résumé	V
Acknowledgments	VII
Table of Contents	IX
List of Figures	XII
List of Tables	XVII
List of Abbreviations	XVIII

Chapter 1

Introduction	1
1.1. General overview	1
1.2. Bioaccumulation	2
1.3. Environmental sources of steroidal estrogens	3
1.4. Occurrence and fate	4
1.4.1. Effluents and surface water	4
1.4.2. Sediments	5
1.4.3. Stability and degradation	6
1.5. Physiochemical properties	7
1.6. Exposure and effects	8
1.6.1. A historical perspective	8
1.6.2. Intersex frequencies	9
1.6.3. Vitellogenin induction	10
1.7. Rationale for research	12
1.8. Thesis main objectives	13

Chapter 2

Use of an Accelerated Solvent Extractor (ASE) in the Sample Preparation for Steroidal Estrogens Analysis from Whole Fish Tissue: A Comparative Study of Two Different Applications	21
2.1. Abstract	21
2.2. Introduction	23
2.3. Experimental	25
2.3.1. Standards and reagents	25
2.3.2. Fish	25
2.3.3. Exposure experiment	26
2.3.4. Accelerated solvent extraction	26
2.3.5. Sample preparation	27
2.4. Results and discussion	31
2.4.1. In cell-clean up (protocol #1)	31
2.4.2. Exhaustive extraction (protocol #2)	33
2.4.3. Validation of the sample preparation method (protocol # 2)	34
2.5. Conclusions	35

2.6. Acknowledgments	36
----------------------	----

Chapter 3

Bioaccumulation of the Pharmaceutical Ethinylestradiol in Shorthead Redhorse Sucker (<i>Moxostoma macrolepidotum</i>) from the St. Clair River, Canada	43
3.1. Abstract	43
3.2. Introduction	45
3.3. Materials and methods	48
3.3.1. Study site	48
3.3.2. Chemicals	48
3.3.3. Sample collection and preparation	49
3.3.4. HPLC-APCI-MSD analysis	50
3.3.5. Lipid determination and $\delta^{15}\text{N}$ analysis	51
3.3.6. Vitellogenin analysis	52
3.3.7. Statistical analysis	52
3.4. Results and discussion	53
3.4.1. Gonadosomatic Index (GSI) and Hepatosomatic index (HSI), lipid content and $\delta^{15}\text{N}$	53
3.4.2. Total vitellogenin in ShRHS	54
3.4.3. Detection and Bioaccumulation of EE2 in wild ShRHS	55
3.4.4. The correlation of EE2 concentrations with GSI, HSI, content and $\delta^{15}\text{N}$	57
3.5. Conclusions	58
3.6. Acknowledgements	59

Chapter 4

Does the Pharmaceutical 17α-Ethinylestradiol Bioaccumulate in a River Food-web? An Investigation of Seven Fish Species in the St. Clair River, Canada	67
4.1. Abstract	67
4.2. Introduction	69
4.3. Materials and methods	71
4.3.1. Study site	71
4.3.2. Chemicals	71
4.3.3. Sample collection	72
4.3.4. Sample preparation	73
4.3.5. Statistical analysis	76
4.4. Results and discussion	77
4.4.1. Trophic levels and stable isotopes	77
4.4.2. Effect of sewage effluents on stable isotopes	78
4.4.3. Total mercury	79
4.4.4. Ethinylestradiol	81
4.5. Conclusions	83
4.6. Acknowledgements	83

Chapter 5	
Dynamics of uptake and elimination of 17α-ethinylestradiol in male goldfish	98
(<i>Carassius auratus</i>)	98
5.1. Abstract	98
5.2. Introduction	100
5.3. Materials and methods	102
5.3.1. Experimental animals	102
5.3.2. Exposure and experimental set-up	103
5.3.3. Lipid determination	105
5.3.4. Water sampling and extraction	105
5.3.5. Tissue extraction	106
5.3.6. Chemical analysis	106
5.3.7. Toxicokinetic model	107
5.3.8. Statistical analysis	109
5.4. Results and discussion	110
5.4.1. EE2 levels in water	110
5.4.2. Uptake and accumulation	111
5.4.3. Depuration	113
5.4.4. BCF and BAF	115
5.4.5. Modeling EE2 uptake and elimination	116
5.5. Conclusions	118
5.6. Acknowledgements	118
Chapter 6	133
General Conclusions and Discussion	133
References	139
Appendix	163
Appendix A	163
Appendix B	165
Appendix C	167
Appendix D	169
Appendix E	171

List of Figures

	Page
Figure 1.1: A schematic showing the journey of environmental estrogens from their sources to the drinking water. EE2: ethinylestradiol, OC: oral contraceptives. Figure adapted from Wise et al. (2011).....	16
Figure 1.2: Structure of steroidal estrogens and their precursor cholesterol.....	17
Figure 2.1: A schematic for the two sample preparation protocols employing in the cell clean-up (protocol # 1) and the exhaustive extraction (protocol # 2) using accelerated solvent extractor.....	37
Figure 2.2: A chromatogram showing lipid and cholesterol removal from whole fish extracts as performed in protocol # 2 A) a preparative-HPLC spectrum of 1 mL sample injection, B) an NMR spectrum of the collected fraction showing cholesterol as a co-eluted matrix. The dashed line indicates the time based fraction collection window.....	38
Figure 2.3: Extracted ion chromatogram (EIC) showing the response of A) EE2 standard injected at 1 ng on column and B) E1 standard injected at 0.2 ng on column. Panels C and D show a representative 2 g goldfish extracts showing the internal EE2 ¹³ C spiked standard spiked at 50 ng and the quantified EE2 in the sample respectively.....	39
Figure 3.1: The sampling sites where the ShRHSs were sampled in 2002. Stag Island near Corunna wastewater treatment plant was considered as an exposed site and Port Lambton was considered a downstream reference site.....	60
Figure 3.2: VTG levels expressed as percent total protein of ShRHS samples from St. Clair River. The black bars indicate Port Lambton (reference site) and the white bars indicate Stag Island (exposed site). No VTG was detected from any male from the reference site.....	61
Figure 3.3: Linear regressions showing correlation of a) EE2 concentrations expressed as ng/g whole tissue with lipid content (%) ($R^2 = 0.512$, $p < 0.01$, $n = 10$) b) EE2 lipid normalized concentrations expressed as ng/g lipid with the trophic level $\delta^{15}\text{N}$ (‰) values ($R^2 = 0.436$, $p < 0.05$, $n = 10$).....	62
Figure 3.4: A representative ShRHS sample chromatogram showing detected EE2 in 20g of extracted whole fish homogenate as in peak (1) the total ion chromatogram (TIC). Peak (2) indicates the extracted ion chromatograms (EIC) of EE2 ¹³ C spiked standard (m/z 297) at the concentration of 50 ng per sample and peak (3) indicates the signal of the quantified EE2 (m/z 295) in the wild fish extracts.....	63

Figure 4.1: A) an aerial view of the St. Clair River (64 km) flowing from Lake Huron south to Lake St. Clair. B) A closer zoom showing Stag Island, Corunna STP, and the exposure site sampling locations SIE: Stag Island East and SIW: Stag Island West. C) Shows the reference site Chenal Ecarte (CE) sampling locations CEN: Chenal Ecarte North and CES: Chenal Ecarte South (Google Earth, 2011).....86

Figure 4.2: $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ isotope values (‰) showing food-web structure of several fish species from the St. Clair 2010 (n = 79). Filled symbols represent samples collected from Stag Island (exposed) and Port Lambton (reference) whereas open symbols represent samples collected only from one site. RG, round goby; YP, yellow perch; ES, emerald shiner; CC, common carp; ShRHS, shorthead redhorse sucker; SS, spottail shiner; LP, logperch.....87

Figure 4.3: Mean $\delta^{13}\text{C}$ (‰) $\pm$ SD (upper panel) and mean $\delta^{15}\text{N}$ (‰) $\pm$ SD (lower panel) plotted against different species. Groups that do not share the same letters are statistically different (one-way ANOVA). RG: round goby (n = 33), ShRHS: shorthead redhorse sucker (n = 3), YP: yellow perch (n = 24), ES: emerald shiner (n = 24).....88

Figure 4.4: Spatial variations in $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ isotope values (‰) within species showing the difference between the exposed site (Stag Island) and reference site (Port Lambton).....89

Figure 4.5: Mean $\delta^{13}\text{C}$ (‰) $\pm$ SD (upper panel) and mean $\delta^{15}\text{N}$ (‰) $\pm$ SD (lower panel) showing the difference within each species from Stag Island (exposed site) compared to Port Lambton (reference site) (one-way ANOVA). Double asterisk (**) indicate $p < 0.01$, (***) $p < 0.001$. RG: round goby (Stag Island, n = 25; Port Lambton, n = 8), YP: yellow perch (n = 16 and 8), ES: emerald shiner (n = 7 and 9).....90

Figure 4.6: A food-web correlation between $\delta^{15}\text{N}$ (‰) values and THg levels (ng/g) dry weight in several fish species from the St. Clair 2010. Filled symbols represent samples collected from Stag Island (exposed) and Port Lambton (reference) whereas open symbols represent samples collected only from one site. RG, round goby; YP, yellow perch; ES, emerald shiner; ShRHS, shorthead redhorse sucker; SS, spottail shiner; LP, logperch.....91

Figure 4.7: Mean $\delta^{15}\text{N}$ (‰) $\pm$ SD (upper panel) and mean THg (ng/g dw) $\pm$ SD (lower panel) plotted against different species. Groups that do not share the same letters are statistically different (one-way ANOVA). RG: round goby (n = 33), ShRHS: shorthead redhorse sucker (n = 3), YP: yellow perch (n = 24), ES: emerald shiner (n = 24).....92

Figure 4.8: $\delta^{15}\text{N}$ (‰) values and THg levels (ng/g) dry weight relationships showing the difference between the exposed site (Stag Island) and reference site (Port Lambton) within three different species. RG, round goby ($R^2 = 0.08$, $p = 0.12$, $n = 33$). YP, yellow perch ($R^2 = 0.002$, $p = 0.85$, $n = 24$). ES, emerald shiner ($R^2 = 0.63$, $p < 0.001$, $n = 16$).....93

Figure 4.9: Mean THg (ng/g dw) $\pm$ SD (upper panel) the difference within each species from Stag Island (exposed site) compared to Port Lambton (reference site) as indicated by asterisk. Double asterisk (**) mean $p < 0.01$, (***) mean $p < 0.001$ (one-way ANOVA). Significant differences between species within a site are indicated by different letters. RG: round goby (Stag Island, $n = 25$; Port Lambton, $n = 8$), YP: yellow perch ($n = 16$ and 8), ES: emerald shiner ($n = 7$ and 9).....94

Figure 4.10: The relationship between EE2 and THg in all fish samples collected from the St. Clair River in 2010 and the ShRHS samples in dark circles collected in 2002.....95

Figure 5.1: A schematic for the water exposure flow-through experimental set-up.....119

Figure 5.2: Panel (A) shows the measured water concentrations in ng/L within the experimental set-up during the exposure gray circles are mean ($n = 6$) concentrations of EE2 in the mixing tank (without fish) before and after the exposure and the dark circles show the mean ($n = 3$) of EE2 levels in the exposure tank (with fish) at the end of each time interval. Panels B (ng-EE2/g tissue) and panel C (ng-EE2/g lipid) are the uptake curves of EE2 in goldfish. Each fish data point shows a mean concentration of three samples $\pm$ standard deviation.120

Figure 5.3: The uptake and elimination curve of EE2 after the feeding experiment. The filled gray and black circles show duplicate samples taken from the tank at each time interval and the open circles show the duplicate average concentration of EE2 in the whole fish tissues.....122

Figure 5.4: Panel (A) shows the measured water concentrations in ng/L within the experimental set-up during the exposure black circles are mean ($n = 6$) concentrations of EE2 in the mixing tank (without fish) before and after the exposure. The gray diamonds show the mean ($n = 3$) of EE2 levels in the exposure tank (with fish) while the black triangles show EE2 levels in the depuration tank at the end of each time interval. Panels B (ng-EE2/g tissue) and panel C (ng-EE2/g lipid) are the depuration curves of EE2 in goldfish. Each fish data point shows a mean concentration of three samples $\pm$ standard deviation.....123

- Figure 5.5:** The depuration profile of EE2 in goldfish. Each data point represents the natural logarithm of the average EE2 whole tissue concentration (ng/g). The slope of the regression line represents the elimination rate constant (k_2) of EE2 in goldfish.....125
- Figure 5.6:** The measured EE2 concentrations (black squares) in goldfish whole tissue homogenates $\pm$ the standard deviation ($n=3$) of the means values compared to a hypothetical extrapolation of the modeled concentrations at 0.5 h time intervals (the dashed line). The data was generated after a flow-through exposure to EE2 at approximately 134.9 ng/L for 72 h. Model inputs: uptake rate constant (k_1) = 45 h^{-1} , elimination rate constant (k_2)= 0.0786 h^{-1} increasing with 0.0015 h^{-1} increments every 0.5 h.....126
- Figure 5.7:** The average measured EE2 concentrations (gray circles) in goldfish whole tissue homogenates from duplicate samples at each time point compared to a hypothetical extrapolation of the modeled concentrations at 0.5 h time intervals (the dashed line). The data was generated after a dietary exposure to EE2 at approximately 231.84 ng/g of food for 72 h. Model inputs: assimilation efficiency (α)= 106, ration (R) =0.031, elimination rate constant (k_2) = 0.0786 h^{-1} increasing with 0.0035 h^{-1} increments every 0.5 h.....127
- Figure 5.8:** The optimization of EE2 assimilation efficiency (α) (0.106) which was achieved by minimizing its sum of square values after the feeding exposure experiment.....128
- Figure 6.1:** The maximum predicted EE2 tissue concentrations in fish assuming exposure via environmentally relevant levels of EE2. WF-High (5 ng/L) and WF-Low (1 ng/L) indicate exposure to EE2 via water plus variable 1, 10, 100, 1000, and 10000 ng/kg via food. Water: indicates exposure to EE2 via water only (1, 2, 3, 4, and 5 ng/L) and Food indicates exposure to EE2 via food only (1-10000 ng/kg). The dashed line shows our MDLs (0.67 ng/g).....138
- Figure B1:** Lipid recovery of a known lipid content ShRHS sample (0.218 g/g) extracted by dichloromethane (DCM) with cells filled with hydromatrix (HM). Hexane flush with a cell filled with HM and filled with HM plus (4 g) diatomaceous earth (DE). The lipid retention capacity of the following packing: alumina (11.2 g) versus silica (SiO_2) (5.0 g) with alumina (1.6 g).....165
- Figure B2:** Optimization of the number of ASE cycles for total lipid removal from the sample during the hexane flush (step 1) extraction.....166
- Figure C1:** EE2 uptake curve obtained from (2 g) whole tissue goldfish extracts after a flow-through exposure to a nominal of 273 ng/L. Duplicate samples were removed from the 70-L exposure tank. The bar figure shows levels of EE2 in 1 L water samples showing a decline in EE2 concentration overtime. This experiment

was redesigned as illustrated in Chapter 5 to stabilize EE2 water levels during the whole time course and to determine whether EE2 decline was caused by metabolism. Sample preparation was carried out according to methods described in Chapter 5.....	167
Figure C2: EE2 uptake curve obtained from (2 g) whole tissue goldfish extracts after a flow-through exposure to a nominal of 273 ng/L plus 50 ng/g food. Duplicate samples were removed from the 70-L exposure tank. The bar figure shows levels of EE2 in 1 L water samples showing a decline in EE2 concentration overtime. This experiment was redesigned as illustrated in Chapter 5 to stabilize EE2 water levels during the whole time course and to determine whether EE2 decline was caused by metabolism. Sample preparation was carried out according to methods described in Chapter 5.....	168

List of Tables

	Page
Table 1.1: Some known and suspected endocrine disrupting chemicals.....	18
Table 1.2: Physiochemical properties of steroidal estrogens.....	19
Table 1.3: List of some studies that examined the feminization of wild fish sampled from U. K. Rivers that were impacted by municipal sewage effluents.....	20
Table 2.1: Accelerated solvent extraction conditions used during method development for protocol #1 and protocol # 2.....	40
Table 2.2: In cell clean-up method (protocol #1) overall performance assessed by spike-recovery of triplicate samples after the two step extraction. Packing material shown below is the sample cleaning adsorbent layer used after the lipid removal in step 1.....	41
Table 2.3: Exhaustive extraction method (protocol # 2) overall performance assessed by spike recovery of triplicate samples.....	42
Table 3.1: Sample weights and total length.....	64
Table 3.2: GSI, HSI, lipid content (%) and $\delta^{15}\text{N}$ (‰) of ShRHS samples from the St. Clair River compared by two way ANOVA followed by Tukey's test.....	65
Table 3.3: Estimated concentrations of EE2 ng/g wet weight and ng/g lipid weight in 10 of 20 ShRHS samples from Stag Island.....	66
Table 4.1: Field data including sample size, fork length, and wet weight for all fish collected from St. Clair River in 2010 in mid July 2010.....	96
Table 4.2: EE2 levels in the environmental samples collected from the St. Clair River in summer 2010.....	97
Table 5.1: Fish related weights and EE2 concentrations (ng/g tissue and ng/g lipid). Data is expressed as mean $\pm$ standard deviation of three samples in whole Goldfish sampled at each time interval	129
Table 5.2: Summary of toxicokinetic studies performed in whole fish tissues for a number of environmental pollutants.....	130

List of Abbreviations

ASE	Accelerated solvent extractor
AOC	Area of concern
ANOVA	Analysis of variance
APCI	Atmospheric chemical ionization
α	Assimilation efficiency
BCF	Bioconcentration factor
BAF	Bioaccumulation factor
BMF	Biomagnification factor
$\delta^{13}\text{C}$	Stable isotopic composition of carbon
CC	Common carp
CEN	Chenal Ecatre North
CES	Chenal Ecatre South
DCM	Dichloromethane
DE	Diatomaceous earth
EDCs	Endocrine disrupting chemicals
EE2	17 α -ethinylestradiol
E1	Estrone
E2	17 β -estradiol
E3	Estriol
EDSTAC	Endocrine Disruptors Screening and Testing Advisory Committee
ELISA	Enzyme-linked immunosorbent assay
EPA	Environmental protection agency
ER	Estrogen receptor
ES	Emerald shiner
GC/MS	Gas chromatography/mass spectrometry
GPC	Gel permeation chromatography
GSI	Gonadosomatic index
HPLC	High performance liquid chromatography
HSI	Hepatosomatic index
K_{ow}	Octanol water partitioning coefficient
LC/MS	Liquid chromatography mass spectrometry
LP	Logperch
$\delta^{15}\text{N}$	Stable isotopic composition of nitrogen
NCI	Negative chemical ionization
NMR	Nuclear magnetic resonance
MeHg	Methyl mercury
PFBCl	Pentafluorobenzoyl chloride
R	Ration
RG	Round goby
RoC	Reports on carcinogens
RSD	Relative standard deviation

SDS	Sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE)
ShRHS	Shorthead redhorse sucker
SIE	Stag Island East
SIW	Stag Island West
SIM	Selected ion monitoring
STP	Sewage treatment plant
SPE	Solid phase extraction
SS	Spottail shiner
THg	Total mercury
YES	Yeast estrogen screening bioassay
YP	Yellow perch
VTG	Vitellogenin
WWTP	Wastewater treatment plant

Chapter 1

Introduction

1.1 General overview

In 1998, the Endocrine Disruptors Screening and Testing Advisory Committee (EDSTAC) of the U. S. EPA assigned the following definition to an emergent group of environmental pollutants: *“The EDSTAC describes an endocrine disruptor as an exogenous chemical substance or mixture that alters the structure or function(s) of the endocrine system and causes adverse effects at the level of the organism, its progeny, populations, or subpopulations of organisms, based on scientific principles, data, weight-of-evidence, and the precautionary principle.”* (U.S. EPA, 1998). This definition produced a massive number of environmental chemicals to be listed as endocrine disrupting chemicals (EDCs). Table 1.1 shows a partial list of some suspected or confirmed EDCs.

Steroidal estrogens, including 17 β -estradiol (E2), estrone (E1), estriol (E3) and the pharmaceutical 17 α -ethinylestradiol (EE2), (Figure 1.2) are considered amongst the most significant sub-classes of EDCs as defined by the EDSTAC. These chemicals have been characterized as the major contributors to the estrogenic activity and potency associated with the wastewater treatment plant (WWTP) effluents (Fernandez et al., 2007; Desbrow et al., 1998). Once released in the aquatic environment, environmentally available steroidal estrogens are potentially capable of modulating or mimicking the action of endogenous steroidal estrogens in the exposed biota and thus disrupting the natural function of endocrine systems and may cause adverse health effects in aquatic

species (Campbell et al., 2006; Jobling and Tyler 2003). The rest of this chapter will provide some insights and literature review in the current knowledge on the environmental sources of steroidal estrogens, their occurrence and fate in various environmental media, and their major effects in exposed aquatic organisms.

In this thesis, I hypothesize that the biologically active form of EE2 bioaccumulates to measurable concentrations in fish caught near municipal effluents containing EE2. However, such bioaccumulation of EE2 will not lead to its biomagnification in freshwater food-webs.

This chapter will review current knowledge on EE2 in the environment and develop the rationale of this thesis to test the hypothesis of EE2 bioaccumulation in fish.

1.2 Bioaccumulation

Bioaccumulation of a certain chemical in an aquatic organism can be defined as the net available amount of the chemical following a period of uptake, elimination, and metabolism. Bioaccumulation is usually associated with environmental exposure where biota is exposed through multiple pathways such as ambient water, food, sediment, etc. The magnitude of a chemical to bioaccumulate is usually measured by its bioaccumulation factor (BAF) which can be calculated by dividing the chemical residues in the body at steady state and at constant exposure from food and water divided by its steady concentration in water (Gobas and Marrison, 2000). Bioconcentration is similar to bioaccumulation, however the only source of exposure considered in this case is water. The bioconcentration factor (BCF) of a chemical is also calculated similarly to the BAF from a laboratory controlled experiment. The tendency of a chemical to biomagnify is

determined by the increase in the concentration of a chemical in organisms with each stepwise increase in trophic position for that food-web. In this case, biomagnification factor (BMF) is calculated by dividing the concentration of the chemical in the body over its concentration in its diet, which may consist of a composite of organisms, including insects, invertebrates, crayfish, or other fish (Gobas and Marrison, 2000).

1.3 Environmental sources of steroidal estrogens

Natural and synthetic sex hormones can be introduced to the aquatic environment via various routes as illustrated in Figure 1.1. For example, several point and non-point sources have been identified through which natural and synthetic steroidal estrogens can find their way to aquatic environments (Wise et al., 2011). In cities, raw sewage discharges (Saravanabhavan et al., 2009) and sewage treatment plant (STP) effluents (Wierman 2007; Lishman et al. 2006; Jobling et al. 2002a) are considered the primary point sources that contaminate the receiving water bodies with various steroidal estrogens and other EDCs as well. Livestock also contribute significantly to the environmental input of natural sex hormones such as E2, E1, E3, and testosterone from animal waste contaminating surface and groundwater (Soto et al., 2004; Arnon et al., 2008; Bartelt-Hunt et al., 2011). Naturally occurring estrogens are also released in the aquatic environment by spawning fish and thus have been detected in surface water during and after fish reproduction seasons. Such levels are comparable to those usually originating from WWTP effluents (Kolodziej et al., 2004). Leaching from municipal landfills may contain a suite of various EDCs such as bisphenol-A, nonylphenol and octylphenol and

they could also contain E2 and EE2 (Behnisch et al., 2001). Urban runoffs are also a source of a cocktail of EDCs, including E1 and E3 (Xu et al., 2011).

1.4 Occurrence and fate

1.4.1 Effluents and surface water

With a particular focus on WWTP effluents as the major source, and considering that WWTPs can successfully achieve a steroidal estrogen removal efficiency of 60 – 90 % (Christiansen et al., 2002), these chemicals are nevertheless detected in effluents at biologically active concentrations (Desbrow et al., 1998). Estrogenically active WWTP effluents have been reported worldwide in countries such as the United Kingdom, Norway, Sweden, Denmark, Germany, France, the United States (Falconer et al., 2006) and Canada (Lishman et al. 2006; Servos et al., 2005). Levels of steroidal estrogens, whether in sewage effluents or in receiving waters, can be highly variable and affected by many factors. Upstream human population density could be linked to endocrine disruption in aquatic biota (van Aerle et al., 2001; Jobling et al., 2006; Desforges et al., 2010). Also, the type of waste treatment applied (e.g. primary treatment vs. secondary treatment) by the WWTP is a significant factor since it is realistic to predict that the more advanced the STPs operations, the higher their removal efficiency would be (Khanal et al., 2006). The flow rate and dilution factor of receiving waters would also affect the final concentrations of steroidal estrogens in a given water body (Jobling et al., 2006; Desforges et al., 2010). Seasonality and weather conditions were also important factors to take into account in order to explain the variability of steroidal estrogens in the environment (Jin et al., 2008).

In 1999 – 2000, the U.S. Geological Survey launched a nationwide investigation to measure 95 organic wastewater contaminants in 139 U.S. streams in 30 states. Among the investigated contaminants, steroidal estrogens scored the highest frequency of detection (~ 90 %). The frequency of detection of EE2 was 15.7 % with maximum level of 0.831 µg/L (831 ng/L) and median of 0.073 µg/L. 10 %, 0.093 µg/L, and 0.009 µg/L were the frequency of detection, maximum and median levels for E2 whereas 7.1 %, 0.112 µg/L, 0.027 µg/L were reported for E1 (Kolpin et al., 2002). Such concentrations of steroidal estrogens in the environment were considered very high, however the authors attributed such high levels to particulate matter because the samples were analyzed without filtration (Kolpin et al., 2002). The most frequently reported levels of steroidal estrogens in the environment were mostly low ng/L levels. Among 30 different EDCs surveyed in four different WWTPs, Fernandez and colleagues (2007) reported steroidal estrogens in effluents reaching ~5 ng/L for EE2, the most frequently detected estrogen, whereas E1, E2, and E3 were all below 20 ng/L. An earlier survey of 18 WWTPs across Canada showed lower average concentrations of 1.8 and 17 ng/L of E2 and E1 (Servos et al. 2005). Within a similar range, E2 and E1 were detected at mean concentrations of 4 and 12 ng/L respectively at five of the eight surveyed WWTPs effluents in the U.S. (Kolodziej et al. 2003).

1.4.2 Sediments

Once it became evident that steroidal estrogens were introduced appreciably to the aquatic environment (Figure 1.1), efforts were made to determine their accumulation in sediment and other media. A study by Peck et al. (2004) showed that sediment was the

major sink of steroidal estrogens in two U.K. rivers. E1 and E2 were found to be responsible for the estrogenic activity of the sediment extracts upstream and downstream of two STPs. Their yeast estrogen-receptor screening bioassay showed that the estrogenic activity of E2 and E1 can reach 24 and 12 times higher in the river sediment samples than the STP effluents (Peck et al., 2004). Marine sediments collected close to a primary STP outfall releasing into the ocean contained up to 1170, 2480 and 500 ng/kg of E1, E2, and EE2 respectively (Braga et al., 2005). Similarly E2 and EE2 sediment concentrations reached 860 and 280 ng/kg respectively in Halifax Harbor (Robinson et al., 2009). Such high concentrations of steroidal estrogens deposited to marine sediment were thought to be associated with sewage particulate matter (Braga et al., 2005; Robinson et al., 2009). Thus, benthic organisms might be at a higher risk of exposure to steroidal estrogens than pelagic organisms (Holthaus et al., 2002)

1.4.3 Stability and degradation

Investigations on the stability of steroidal estrogens within WWTP facilities, receiving waters and sediments have been conducted to understand the behavior and fate of these chemicals once introduced to the aquatic environments. In river water, microbial activity could result in a rapid biodegradation of E2 to E1 with an estimated half-life of 1.7 days for E2 and 1.8 days for E1, whereas EE2 was more persistent with a half-life of 17 days (Jürgens et al., 2002). In sea water, a similar half-life of 1 day was reported for E2, whereas EE2 had a half-life of 5 days (Robinson and Hellou, 2009). In groundwater, while E2 had a half-life of 2 days, EE2 persisted much longer with a half-life of 81 days under aerobic conditions (Ying et al., 2003). Sorption experiments with sediment showed

that steroidal estrogens, especially EE2, were resistant to biodegradation in both aerobic and anaerobic conditions. In aerobic marine sediments, E2 and EE2 half-lives of 4.4 days and > 20 days were observed. In contrast, incubating the sediment samples under anaerobic conditions increased E2 half-life to 70 days and EE2 was not degraded during the experimental time course (Ying and Kookana, 2003). Moreover, EE2 showed similar persistence in lake sediment under different anaerobic microbiological treatments up to three years (Czajka and Londry, 2006).

1.5 Physiochemical properties

Steroid hormones have a characteristic structure of three six membered and five membered rings (Figure 1.2). Cholesterol is known to be the precursor of steroid hormones produced during steroidogenesis. All steroidal estrogens (E2, E1, E3 and EE2) are structurally related and share the same phenol group (A-ring) as well as B and C rings. The structural variations usually occur in functional groups within two carbon atoms C-16 and C-17. Estradiol has a hydroxyl group at its C-17 which must be converted to a ketone group to produce E1. An addition of another hydroxyl group to C-16 of E2 produces E3. In contrast, a semi synthetic addition of an ethinyl group at C-17 of E2 produces EE2 which makes it a very stable compound (Neef, 1999; Nash et al., 2004; Örn et al., 2006).

Steroidal estrogens are the primary female sex hormones that are critical for the growth, differentiation, and function of many tissues in humans and vertebrates. The most potent natural estrogen is E2 and E1 is the second in potency and least in abundance among natural estrogens. Both are released mostly by the ovaries of sexually mature

females under the influence of the hypothalamus, pituitary, and gonad axis. Estriol is produced in large quantities by the placenta during pregnancy (Wood and Strauss 2002; Metallinou et. al., 2007). The active ingredient of oral contraceptives, EE2 is used to prevent pregnancy by inhibiting ovulation. The potency of EE2 is about 30 times higher than E2 (Nash et al. 2004; Örn et al. 2006; Van den Belt et al. 2004). Table 1.2 lists some selected physiochemical properties of steroidal estrogens. Natural steroidal estrogens, E2, E1, and E3 are all odorless white to creamy-white crystalline powders. Ethinylestradiol is an odorless powder which is usually a yellowish-white crystal. Whereas the water solubility of steroidal estrogens is extremely low, they are soluble in organic solvents such as methanol, ethanol, acetone and acetonitrile (RoC, 2011). The vapor pressure of steroidal estrogens is extremely low and almost negligible making them unlikely to volatilize. The high lipophilicity of steroidal estrogens is reflected by their octanol/water partitioning coefficient (K_{ow}). Differences in functional groups affect the lipophilicity of estrogens, with EE2 being the highest and E2, E1, and E3 following in descending order (RoC, 2011).

1.6 Exposure and effects

1.6.1 A historical perspective

Professor John P. Sumpter, at Brunel University in the U.K, one of the pioneers in the field of endocrine disruption, established endocrine disruption as a new field in 1978 when first documenting endocrine related abnormalities in wild fish. Such observations were believed to be caused by substances released from WWTPs. Three years later, a broader survey was conducted to confirm the preliminary data. However, it took until the

early 90s for the effect of STP effluent on wild fish to appear in scientific publications (Sumpter and Johson, 2008). Feminization or intersex, defined as the simultaneous presence of both testicular and ovarian tissues in the same gonad (Kavanagh et al., 2004), was abundant in various fish species in British rivers and was strongly believed to be a prevalent phenomenon (Jobling et al., 2002a; Gross-Sorokin et al., 2006). All U.K. rivers were to some extent polluted by EDCs (Purdom et al. 1994; Kuster et al., 2004).

1.6.2 Intersex frequencies

Since the onset of endocrine disruption as an emerging area of research in the field of ecotoxicology, laboratory and field studies conducted on various aquatic species have provided strong evidence that chronic exposure to environmental estrogens can adversely affect their reproduction and morphology (Örn et al. 2006; Balch et al., 2004; Lye et al., 1997). Such adverse effects could lead to feminization of male fish populations which has been demonstrated as a distinct biological endpoint of exposure to the environmental estrogens.

Table 1.3 summarizes results on intersex frequencies in fish from the U.K. Environmentally occurring steroidal estrogens as low as 1 ng/L can cause feminization and definite sex reversal from male to female fish can occur at a range of 5 – 10 ng/L (Croley et al., 2000). In England, Jobling et al. (1998) found that 100 % of adult male roach (*Rutilus rutilus*) sampled from rivers Nene and Aire downstream of WWTPs were intersex. In another study, histological analysis showed that male roach (*Rutilus rutilus*) from the Nene, Arun, Aire and Calder rivers in the U.K. had 100 % , 96 % , 93 % , and 92 % intersex frequency, respectively (Jobling et al., 2002b). In a third study, they sampled

142 juvenile roach (*Rutilus rutilus*) from seven different affected sites and found that the percentage of sexually developing fish with female-like reproductive ducts and ovarian cavities ranged between 85 – 95% in five sites and the remaining 5 – 15% were developing as normal males (Beresford et al., 2004). It was not possible to observe sperm ducts within the ovarian cavities, however there was evidence that all intersex roach had ovarian cavities (Beresford et al., 2004). Flounders (*Platichthys flesus*) were also investigated for intersex in various studies. Allen et al. (1999a) and (1999b) reported that only (20 %) of the adult male fish population sampled from the river Mersey in the U.K. had oocytes in their testes whereas a lower percentage was found in river Mersey (9 %) and river Tyne (7 %). Simpson et al. (2000) found that all 410 flounder that they sampled from two sites contaminated by WWTP effluents over a period of two years had normal gonads. Also in the U.K., 12, 13, and 14 % of gudgeon (*Gobio gobio*) populations collected from three different contaminated sites at river Aire were intersex (van Aerle et al., 2001). In Hamilton Harbor (Ontario, Canada), white perch (*Morone americana*) showed intersex frequencies of up to 32 % of the sampled fish (Kavanagh et al., 2004). Moreover, 32 % of spottail shiner (*Notropis hudsonius*) sampled from the St. Lawrence River downstream of a wastewater treatment plant were intersex (Aravindakshan et al., 2003).

1.6.3 Vitellogenin induction

Vitellogenin (VTG) is an egg yolk protein produced by fish liver in response to their endogenous estrogen, E2. Like many other estrogen-responsive genes, VTG is regulated by the estrogen receptors (ERs) (Marlatt et al., 2008; Skillman et al., 2006). In

bony fish, three nuclear ER subtypes have been identified and characterized ER α , ER β 1, and ER β 2 or ER γ . In the liver, ER α was shown to be the most abundant ER subtype (Marlatt et al., 2008; Socorro et al., 2000). Environmental estrogens such as EE2 can induce both ER α and VTG mRNA in hepatocytes (Skillman et al., 2006). Consequently, VTG is commonly used as a biomarker in male and juvenile fish species in response to xenoestrogen (Örn et al., 2006; Kavanagh et al., 2004; Jobling et al., 2002a; Jobling et al., 2002b; Croley et al., 2000; Sumpter and Jobling, 1995). Interestingly, male and juvenile fish of both sexes do not normally synthesize considerable amounts of VTG (Kavanagh et al., 2004; Allen et al., 1999a). However male fish have the gene encoding for VTG (LeGuellec et al., 1988) but it appears to be silent unless they are exposed to exogenous estrogen. The plasma VTG concentrations in male fish exposed to environmental estrogens may reach the same concentrations as in females (Korte et al., 2000). Low levels of environmental estrogens detected in the WWTPs effluents can induce VTG synthesis and subsequently could lead to feminization of male fish (Croley et al., 2000; Metcalfe et al., 2001; Peck et al., 2004; Sumpter and Jobling 1995). Although, depuration can significantly reduce the concentration of plasma VTG, decline to baseline concentration may require several months (Hemmer et al., 2002; Rodgers-Gray et al., 2001). Exposure experiments conducted on species like plaice (*Pleuronectes platessa*), flounder (*Platichthys flesus*) and sand goby (*Pomatoschistus minutus*) exposed to 20 ng/L of EE2 for 21 days followed by 31 days of depuration illustrated increased levels of plasma VTG preceded by a slight increase in hepatic VTG mRNA. The mean half life of the VTG mRNA was 3.4 days whereas that of the protein ranged from (15 – 34.6) days (Craft et al, 2004). Determination of hepatic VTG mRNA and plasma levels in male

flounder caught in five British estuaries showed variable results in mRNA induction and/or plasma VTG (Craft et al., 2004). Sand gobies exposed to 6 ng/L of EE2 and 0.3 % dilution of sewage effluent for 7 months induced VTG mRNA (Robinson et al., 2003). Desforges et al. (2010) recently showed that VTG production in male teleost fish populations from 43 rivers across the globe correlated with the upstream human population size.

1.7 Rationale for research

Despite all we know about endocrine disruption in aquatic environments, there are still “*a number of difficult, but extremely important, questions*” to answer such as “*Do any of the chemicals (referring to EDCs) bioaccumulate in biota? If so, which ones, and to what degree?*” and “*Do the chemicals enter biota (across the gills of fish, for example) at similar, or different rates?*” (Sumpter and Johson, 2008). The occurrence and the biological consequences of exposure to low levels of environmentally occurring steroidal estrogens on aquatic species have received a great deal of attention. However, evidence showing that these environmental estrogens can actually bioconcentrate or bioaccumulate in fish is very limited (Lai et al., 2002). Only a few laboratory and field studies have demonstrated that environmental estrogens bioconcentrate or bioaccumulate in the bile of fish prior to excretion and hence, the detection of high concentrations in this specific medium is expected (Gibson et al., 2005; Larsson et al., 1999). Gibson et al. (2005) exposed rainbow trout (*Oncorhynchus mykiss*) and roach (*Rutilus rutilus*) to 100 % WWTPs effluents (exposure group), tap water, and river water (control groups) for 10 days. After an enzymatic hydrolysis of the extracts, high concentrations of environmental

estrogenic chemicals, including E1, E2, EE2, NP, and NPnEOs, were detected in the bile and the gonads of exposed animals, whereas only low levels of E1 and E2 were found in the control groups. High performance liquid chromatography (HPLC) fractionation coupled with the yeast estrogen screening (YES) bioassay approach was used to quantify estrogenic potency, whereas gas chromatography/mass spectrometry (GC/MS) was used for the identification of the YES active fractions. Similarly, the bile of rainbow trout (*Oncorhynchus mykiss*) caged for two weeks downstream of WWTP effluents contained $10^4 - 10^6$ times higher concentration of estrogens than that of the effluent which were 4.5, 5.8, and 1.1 ng/L for EE2, E1, and E2 respectively (Larsson et al., 1999). Whole body concentrations of EE2 measured by radioimmunoassay (RIA) in fathead minnow (*Pimephales promelas*) exposed to nominal concentrations of EE2 (64, 16, 1.0, 0.2 ng/L) via water reached 31 ng/g after 153 days post-hatching with an estimated bioconcentration factor (BCF) of 660 (Länge et al., 2001). Lai et al. (2002) used a mass balance food-web model developed by Campfens and Mackay (1997) to predict the bioaccumulation factors of natural and synthetic estrogens in river systems. Among all steroidal estrogens, EE2 had the maximum bioaccumulation factor (BAF) of 332 in fish feeding low on the trophic level (Lai et al., 2002).

1.8 Thesis objectives

The objectives of my study were to determine the bioaccumulation of the bioactive form EE2 in fish. I also studied the uptake-elimination kinetics of EE2 in laboratory controlled experiments to parameterize EE2 bioaccumulation by fish. In order to accomplish these objectives my thesis was divided into four main projects.

- **Objective #1** was intended to develop analytical tools for sample preparation and chemical analysis to allow detection of trace levels (low ng/g) of EE2 in whole fish homogenates with high sensitivity and selectivity. Such method development is lacking in the scientific literature and was critical to answer my main thesis questions (Chapter 2).
- **Objective #2** was to test the hypothesis of EE2 bioaccumulation in feral bottom feeding fish. Whole fish homogenates from shorthead redhorse sucker (ShRHS) (*Moxostoma macrolepidotum*) sampled from the St. Clair River were analyzed for EE2 concentrations in an exposed site and a reference site. I also determined whether bioaccumulation was correlated with total lipid content and trophic position (Chapter 3).
- **Objective #3** was a wider survey to investigate the potential of EE2 bioaccumulation in the St. Clair River food-web. Round goby (*Neogobius melanostomus*), yellow perch (*Perca flavescens*), emerald shiners (*Notropis atherinoides*), common carp (*Cyprinus carpio*), shorthead redhorse sucker (*Moxostoma macrolepidotum*), Spottail shiner (*Notropis hudsonius*) and Logperch (*Percina caprodes*) were all analyzed for EE2. The samples were also analyzed for the stable nitrogen ($\delta^{15}\text{N}$) and carbon ($\delta^{13}\text{C}$) ratios to identify trophic position ($\delta^{15}\text{N}$ increases 3 – 5 ‰ on average per trophic level, and $\delta^{13}\text{C}$ provides information on carbon sources (Ikemoto et al., 2008; Bertrand et al., 2011). Total mercury was measured as a reference biomagnifying substance to compare with EE2 accumulation in this food-web. I also analyzed EE2 in water and sediment (Chapter 4).

- **Objective #4** was a laboratory controlled experiment where male goldfish (*Carassius auratus*) were exposed to EE2 via water by a flow-through system and via diet in an attempt to parameterize the uptake and elimination kinetics of EE2 in fish (Chapter 5).

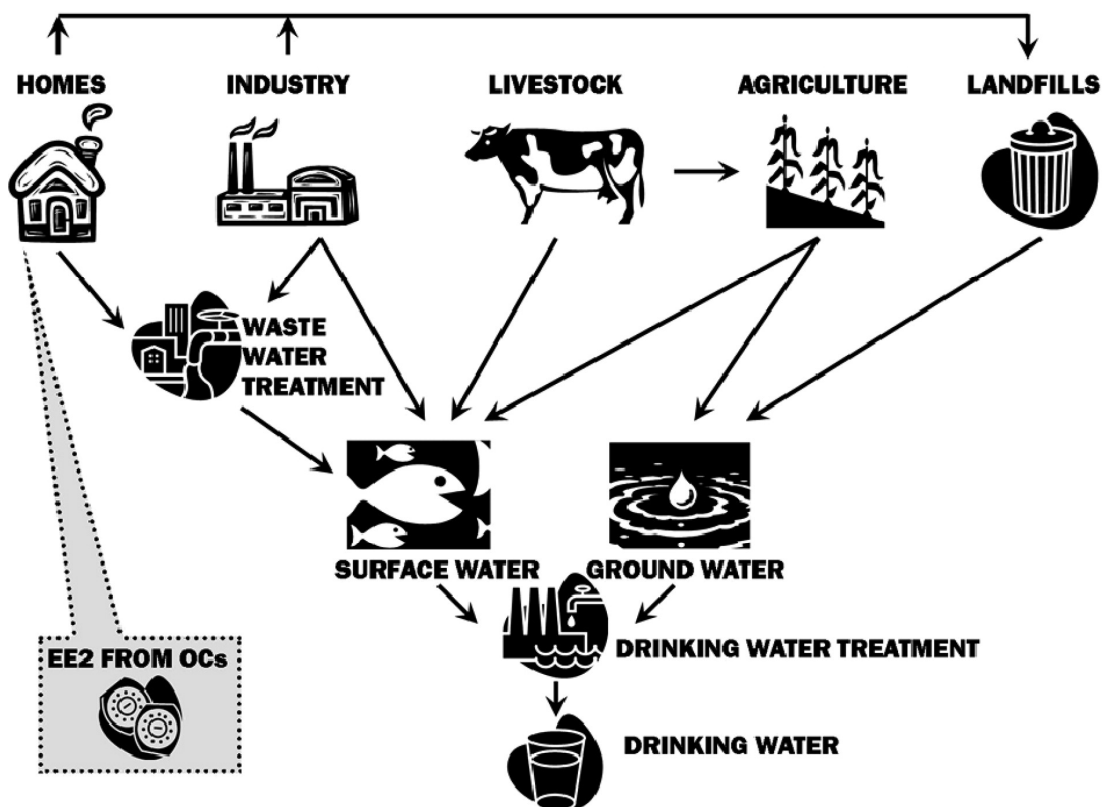


Figure1.1: A schematic showing the journey of environmental estrogens from their sources to the drinking water. EE2: ethinylestradiol, OC: oral contraceptives. Figure adapted from Wise et al. (2011).

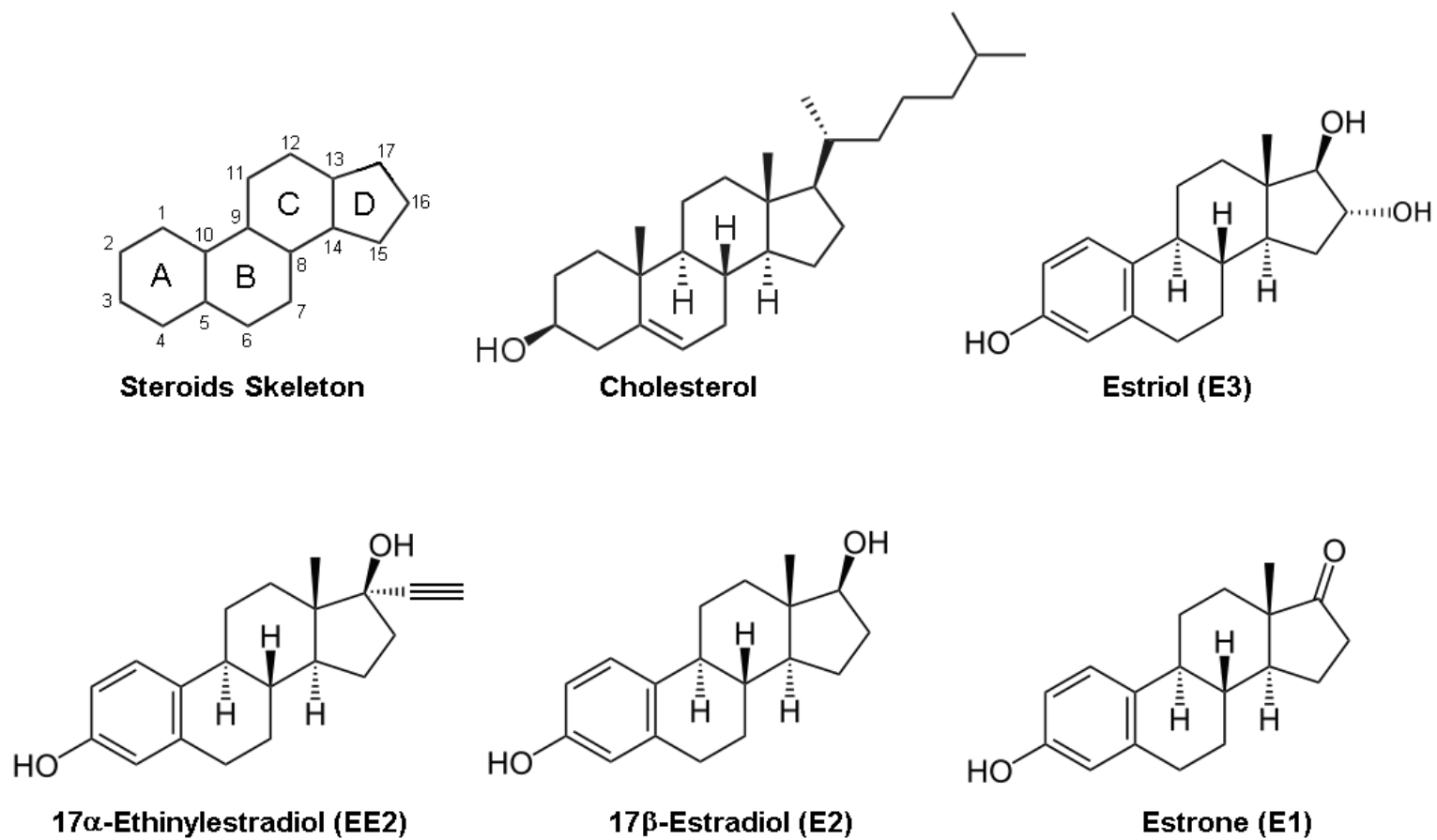


Figure 1.2: Structure of steroidal estrogens and their precursor cholesterol.

Table 1.1

Some known and suspected endocrine disrupting chemicals.

Compounds	Occurrence
Natural and synthetic hormones	Natural hormones, augmented by hormonal drugs such those used as oral contraceptives, are excreted by humans and animals and occur in sewage
Phytoestrogens	Natural constituents of many foodstuffs including beans, sprouts, cabbage, spinach, soybean, grains and hops. The major classes are lignans and isoflavones (e.g. daidzein and genistein)
Mycotoxins	Produced by fungi which can contaminate crops. Some, such as zearalenone, are oestrogenic
Organochlorine pesticides	DDT, lindane and beta-HCH are common, persistent environmental pollutants
Polychlorinated biphenyls (PCBs)	Widespread, persistent environmental pollutants
Alkylphenol polyethoxylates (APEs)	Non-ionic surfactants used in detergents, paints, herbicides, pesticides and plastics. Breakdown products, such as nonylphenol and octylphenol, are found in sewage and industrial effluents
Dioxins	Products of combustion of many materials
Phthalate esters	Widely used as plasticisers for PVC. Common environmental pollutants
Bisphenol A	A component of polycarbonate plastics and epoxy resins used to line food cans

Adapted from Phillips and Harrison, 1999.

Table 1.2

Physiochemical properties of steroidal estrogens

Property	E2	E1	E3	EE2
Formula	$C_{18}H_{24}O_2$	$C_{18}H_{22}O_2$	$C_{18}H_{24}O_3$	$C_{20}H_{24}O_2$
Molecular weight (g/mol)	272.3	270.37	288.39	296.4
Solubility (mg/L)	13	13	13	4.8
Vapor pressure (Pa)	3×10^{-8}	3×10^{-8}	9×10^{-13}	6×10^{-9}
Log K_{ow}	3.94	3.43	2.81	4.15

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

Table 1.3

List of some studies that examined the feminization of wild fish sampled from U. K. Rivers that were impacted by municipal sewage effluents

River	Species	n	Intersex frequency (%)
Aire	roach (<i>Rutilus rutilus</i>)	NS	100 ^a
		95	100 ^b
	roach (<i>Rutilus rutilus</i>) J	20	95 ^c
Nene	roach (<i>Rutilus rutilus</i>)	NS	100 ^a
		111	100 ^b
Dearn	roach (<i>Rutilus rutilus</i>) J	19	95 ^c
Tees		20	95 ^c
Rother		21	86 ^c
Don		20	85 ^c
Mersey	flounder (<i>Platichthys flesus</i>)	NS	20 ^d
		65	9 ^e
Tyne		65	7 ^e
Aire I	gudgeon (<i>Gobio gobio</i>)	105	14 ^f
Aire II		15	13 ^f
Aire III		83	12 ^f
Lea		83	6 ^f

References: Jobling et al. 1998 (a), Jobling et al. 2002 (b), Beresford et al. 2004 (c), Allen et al. 1999a (d), Allen et al. 1999b (e), vAn Aerle et al. 2001 (f). J: Juvenile, NS: not specified shown as 60 – 100 samples. Aire I – III were different sites impacted by different WWTPs on the same river.

Chapter 2

Use of an Accelerated Solvent Extractor (ASE) in the Sample Preparation for Steroidal Estrogens Analysis from Whole Fish Tissue: A Comparative Study of Two Different Applications

A shortened version of this chapter published as: Ahmed M. Al-Ansari, Ammar Saleem, Linda E. Kimpe, Vance L. Trudeau, Jules M. Blais, 2011. The Development of an Optimized Sample Preparation for Trace Level Detection of 17 α -Ethinylestradiol and Estrone in Whole Fish Tissue. Journal of Chromatography B 879, 3649-3652.

2.1 Abstract

Analysis of whole fish tissue extracts containing estrogens can be challenging in the presence of co-extracted lipids and proteins that may result in poor recovery and detection limits. The purpose of this study was to develop an optimized method for the extraction and determination of 17 α -ethinylestradiol (EE2) and estrone (E1) in whole fish tissues at ng/g levels. The optimized procedure for sample preparation included extraction of tissue by an accelerated solvent extractor (ASE-200), followed by lipid removal using a gel permeation chromatography (GPC) approach, and then a further cleanup step to remove any trace of lipids by acetonitrile precipitation followed by a hexane wash step. Another approach of sample preparation based on in cell clean-up was attempted but poor recovery not exceeding 19 % was determined for both analytes.

Analysis was performed by gas chromatography/mass spectrometry (GC/MS) in negative chemical ionization mode (NCI) after samples were derivatized with pentafluorobenzoyl chloride (PFBCl). The method was developed using high lipid content wild fish spiked with tested analytes. The whole procedure recoveries ranged from 74.5 – 93.7 % with relative standard deviation (RSD) of 2.3 – 6.2 % for EE2 and 64.8 – 91.6 % with RSD of 9.46 – 0.18 % for E1. The method detection limits were 0.67 ng/g for EE2 and 0.68 ng/g for E1 dry weight (dw). The method was applied to determine EE2 levels in male goldfish (*Carrasius auratus*) after a 72 h dietary exposure. EE2 was detected in all samples averaging 1.7 ng/g ($\pm$ 0.29 standard deviation, n = 5). This was the first optimized protocol for estrogen extraction from whole fish tissue. Due to the high sensitivity and remarkable recovery, the developed method will improve our knowledge of the environmental uptake of estrogens in fish populations.

Keywords: 17 α -ethinylestradiol; whole tissue extraction; sample preparation; goldfish

2.2 Introduction

Natural and synthetic steroidal estrogens excreted by humans were among the most potent endocrine disruptors (Fernandez et al., 2007). Municipal wastewater treatment plant (WWTP) effluents have been recognized as the primary source by which these estrogens were introduced to the aquatic environment after primary or secondary treatment (Pereira et al., 2011).

In the last two decades, notable progress has been made in the development of sophisticated techniques to extract, clean-up, and analyze estrogens from various environmental matrices such as surface water, ground water, sewage effluents, sewage sludge, soil, and sediment (Gabet-Giraud et al., 2010; Comerton et al., 2009; Beck et al., 2008; Briciu et al., 2009). However, very few studies have focused on the analysis of these molecules in invertebrates (Cailleaud et al., 2011; Dussault et al., 2009; Liebig et al., 2005) or fish (Al-Ansari et al., 2010; Gibson et al., 2005; Länge et al., 2001). To date, optimized sample preparation protocols for the extraction of steroidal estrogens from fish tissues with high recoveries were not available.

Fish tissue can be a very complex matrix to deal with because of its richness in lipids and proteins. Finding an efficient extraction method that successfully maximizes the extraction of the target analytes and minimizes matrix effects is a challenge (Gabet-Giraud et al., 2010). In a study by Gibson et al. (2005), sonication in methanol followed by solid phase extraction (SPE) purification and enzymatic hydrolysis were the main steps used to detect three steroidal estrogens (estrone E1, estradiol E2, and ethinylestradiol EE2) in roach (*Rutilus rutilus*) gonads exposed to sewage effluents for 10 days. The chemical analysis that was performed by GC/MS in this study aimed to

confirm the identity of the active fractions detected by estrogen receptor transcription screening assay rather than to quantify the detected estrogens. Hence, no recovery and sensitivity information was documented. Radioimmunoassay was the technique by which Länge et al. (2001) measured EE2 concentrations in whole body fathead minnow (*Pimephales promelas*) after a laboratory controlled exposure. However, the main criticism for bioassays for steroidal estrogen analysis is low accuracy and limited sensitivity (Giese, 2003). In our laboratory, ASE-200, lipid removal by gel permeation GPC, and Florisil clean-up prior to the analysis by LC/MS were the main sample preparation steps previously employed to measure EE2 for the first time in wild fish sampled near a WWTP (Al-Ansari et al., 2010; Chapter 3). Although successful analysis was achieved, the major drawback for that method was low recoveries which ranged from 25 – 30 %.

Here we develop an optimized sample preparation procedure for the detection and quantification of the steroidal estrogens EE2 and E1 in whole fish homogenates. Accelerated solvent extractor was the preferred extraction technique due to its robustness, full automation, and lower solvent and time consumption (Runnqvist et al., 2010; Beck et al., 2008). We investigated two applications of ASE (Figure 2.1). Protocol# 1, was an in cell clean-up approach which utilized ASE as an extraction and clean-up system. Protocol# 2, was an exhaustive extraction approach where the samples went through harsh pressure and temperature conditions to maximize the extraction efficiency and external clean-up steps were applied afterward (Runnqvist et al., 2010). Whole method recovery was the main criterion for method validation. This study was the first to provide

an optimized sample preparation protocol with high recovery rates and sensitivity for EE2 and E1 using ASE and GC/MS.

2.3 Experimental

2.3.1 Standards and reagents

^{12}C and ^{13}C standards for E1 (99 % purity) and EE2 (99 % purity) were all purchased from Cambridge Isotope Lab., Inc. (Andover, MA, USA). Stock solutions were prepared at 500 ng/mL in nonane (Sigma-Aldrich, St. Louis, MO, USA.) and kept in amber vials in the fridge. All solvents for sample preparation and analysis were of high purity. Dichloromethane (DCM), methanol (MeOH), acetone and hexane, were Optima[®] grade (Fisher Scientific, Brockville, ON, Canada) while acetonitrile and water were Chromsolv grade (Fluka, Mississauga, ON, Canada). Pentafluorobenzoyl chloride (PFBCl), nonane, diatomaceous earth (DE), silica, acidic alumina, anhydrous Na_2SO_4 , and potassium hydroxide (KOH) were all from Sigma-Aldrich (St. Louis, MO, USA). Hydromatrix was from Varian Inc. (Mississauga, ON, Canada).

2.3.2 Fish

Shorthead redhorse sucker (*Moxostoma macrolepidotum*) (ShRHSs) samples (n = 3) used in the spike recovery experiments were collected in June 2009 by trawl net near the City of Montreal sewage treatment plant outfall. The fish were a subsample from a larger study. Goldfish (*Carassius auratus*) were purchased from Aleong's International Inc. (Mississauga, Ontario, Canada). Before the exposure, fish were acclimatized to $18 \pm$

1°C in 70-L holding tanks, under a natural photoperiod, and fed with standard floating trout pellets for about three weeks. Mature male goldfish were chosen for the exposure experiment which was conducted under a protocol approved by the University of Ottawa Animal Care Protocol Review Committee.

2.3.3 Exposure experiment

Male goldfish ($n = 5$) weighing $23.89 \text{ g} \pm (4.21)$ were exposed to EE2 through food. The hormone (EE2) was dissolved in 99% ethanol and mixed with fish food to achieve 100 ng/g. Ethanol was completely evaporated before the exposure. Fish were fed 1 % body weight three meals a day for three days. Anesthetized fish with MS-222 (Sigma Aldrich, St. Louis, MO, USA) were sacrificed by trans-spinal sectioning, put in 50 mL falcon tubes, and then immediately placed vertically on ice for about 10 min to allow blood to coagulate. The head was cut and left in the tube and then gastrointestinal tracks of all samples were carefully removed (see Appendix D). Samples were frozen at -20°C and subsequently freeze-dried using Supremoudylo freeze dryer Thermo-Fisher Scientific (Brockville, ON, Canada).

2.3.4 Accelerated solvent extraction

All extractions were performed with ASE-200 (Dionex Corporation, Sunnyvale, CA, USA). Stainless-steel extraction vessels of two sizes 33 mL and 11 mL were used. ASE conditions used for the method development were listed in Table 2.1. All extractions for the method development were carried out using 1 – 2 g freeze-dried high lipid content ($> 21 \%$, dw) ShRHS homogenates.

2.3.5 Sample preparation

2.3.5.1 In cell clean-up approach (protocol#1)

2.3.5.1.1 Extraction and lipid removal

The initial conditions used in this study such as sorbents selection and amount, solvent composition and the number of ASE cycles were all adopted from Hansen et al. (2010). To assess the quality of this method performance and suitability, 1 g samples were spiked with both analytes at either 10 or 250 ng/g in triplicate. A dual extraction approach was employed in which the first step was intended to eliminate the non-polar matrix, mainly lipids, from the samples while retaining the estrogen on a DE layer. Hexane was used as a lipid removal solvent. The second step was intended to elute and clean up the retained analytes. In the 1st step, 33 mL cells were packed top to bottom as follows: a mixture of sample and DE (1:2), 4 g DE, cellulose filter, 3 g hydromatrix, and cellulose cellulous filter. In 2nd step, extraction cells were opened from the bottom to discard the hydromatrix layer and replace it by different combinations of silica, graphite, and alumina to serve as a cleaning layer. Samples were reextracted with a mixture of acetone/ MeOH (1:1) to elute the retained analytes. Extracts were evaporated to dryness, reconstituted in 1mL MeOH and further cleaned with solid phase extraction (SPE) cartridges.

2.3.5.1.2 SPE clean-up

Supelclean ENVI-18 SPE cartridges (product # 505706) were mainly used to separate the target compounds from the polar matrix since most of the non-polar matrix was cleaned by hexanes. SPE cartridges were conditioned with 6 mL MeOH twice

followed by sample loading. Serial washes with 8 mL of each solvent HPLC water, 5 % MeOH, and 5 % acetone. Cartridges were dried under vacuum for 30 min before elution with 6 mL of MeOH and then samples were dried under N₂ until ready for derivatization and analysis.

2.3.5.2 Exhaustive extraction (protocol#2)

2.3.5.2.1 Extraction

Two main experiments were performed to assess the effect of the extraction cell size and hydromatrix on analyte recovery. In the 1st experiment, 2 g freeze dried tissue were mixed with 6 g hydromatrix and spiked with 50 ng/g and 125 ng/g of both E1 and EE2 and placed in the 33 mL extraction cell. In the 2nd experiment, 2 g tissue was placed in the 11 mL cells without hydromatrix and spiked with E1 at 1.25, 2.5, 5 ng/g and EE2 at 12.5, 25, 50 ng/g. Hydromatrix and Na₂SO₄ were used to produce the method blanks for quality control. Dichloromethane was the extraction solvent. Extract volume (approximately 20 mL) was reduced by Turbo-Vap[®] II (Zymark Hopkinson, MA, USA) to about 1 mL at 35 °C after filtration with 0.45 µm polytetrafluoroethylene (PTFE) by 3 mL disposable syringes (cat. # 1481727) (Henke Sass Wolf, Germany). Evaporation tubes were rinsed with 2 mL DCM and pooled with the samples that were collected in 6 mL preparative-LC vials and kept in the fridge at 4 °C.

2.3.5.2.2 Gel-permeation chromatography

Samples were subjected to GPC for lipid removal using an U.S. EPA method 3640A modified in our laboratory. Briefly, samples were fractionated by two Envirogel[™] GPC cleanup columns connected in tandem (19 × 150 mm → 19 × 300 mm)

(Waters Corporations, MA, USA). DCM was the mobile phase delivered at a flow rate of 7 mL/min and pressure of 57 ± 1 bars using an Agilent 1200 Series preparative HPLC system comprising a binary pump (flow rate range 5 – 100 mL/min), an autosampler with a 5 mL loop, a diode array detector with a flow cell (path length 10 mm and maximum pressure limit 120 bar) and a fraction collector (40 mL collection tubes). Fractions were collected at timed intervals at 284.4 nm UV detection (Figure 2.2). Three to four injections were done per sample and the injection volume was optimized based on the column loading limits of 10 mg/100 μ L (lipid/solvent).

2.3.5.2.2 Sample clean-up

Proton nuclear magnetic resonance (NMR) analysis was performed using a Bruker Avance 400 NMR spectrometer to elucidate the major matrix factor within the GPC cleaned fractions. The obtained NMR spectra were identified as cholesterol (Figure 2.2) by a comparative approach with previously reported data (Sawan et al., 1979). Thus, all fractions for each sample were pooled in Turbo-Vap[®] tubes, evaporated to dryness, reconstituted in 0.5 mL MeOH, and transferred by Pasteur pipettes to a 16 mL disposable PYREX test tube (cat. # 99445-16) (Corning Incorporated, NY, USA), which permitted removal of any precipitates. Evaporation tubes were washed with 3 mL 1 % formic acid in acetonitrile, combined with samples in disposable test tubes, and then placed in a – 20 °C freezer for at least 1 h or overnight to precipitate cholesterol out of the solution. After this step, samples were centrifuged for 15 min at 4000 rpm at 4 °C and then transferred to clean tubes. Cholesterol appeared as suspended white flakes during the freezing step (see Appendix E). A 2 mL hexane wash step was applied twice to liquid-liquid extract any trace of the non-polar matrix. After a short gentle vortex, phase separation occurred and

the upper hexane layer discarded. Samples were dried under a gentle stream of N₂ and kept at 4 °C until derivatization.

2.3.5.3 Derivatization

Derivatization was performed for the GC analysis as described previously by Kuch and Ballschmiter (2001). Briefly, 2 mL of HPLC water was used to reconstitute the samples. A volume of 50 µL of 2 M KOH and 10 µL of a 10 % solution of PFBCl in toluene was added and then samples were gently vortexed for 2 min. The derivatized estrogen was then extracted by the addition of 2 mL of hexane twice and collected in new 16 mL test tubes (see Appendix E). Samples were then evaporated to dryness and reconstituted in 100 µL of isooctane that was transferred to GC vials for analysis.

2.3.6 GC/MS analysis

The PFBCl-derivative of estrogen was separated and detected by GC/MS according to method modified from Kuch and Ballschmiter (2001). Samples of 4 µL were injected in splitless mode onto an Agilent 6890 gas chromatograph with a Zebron ZB5-MS column (10 cm guard + 29.7 m × 250.00 µm × 0.25 µm), Phenomenex Inc. (Torrence, CA, USA). Methane gas pressure was set at 65 cm sec⁻¹. The injector was set at 280 °C and the oven temperatures were programmed as follows: 80 °C min⁻¹ for 1.24 min, 24°C min⁻¹ until 200 °C and held for 0.5 min, 73 °C min⁻¹ until 245 °C and held for 5 min, 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 EE2 in NCI mode. The

following quantification ions were used in selected ion monitoring (SIM) mode: m/z 490 (^{12}C EE2), m/z 492 (^{13}C EE2), m/z 464 (^{12}C E1), m/z 466 (^{13}C E1). Chromatographic data were collected and analyzed using Agilent MSD Chemstation Data Analysis Software. All quantifications were based on isotopic dilution method described by de Hoffmann and Stroobant (2002).

2.4 Results and discussion

2.4.1 In cell-clean up (protocol #1)

As shown in Table 2.2, when samples were spiked with 10 ng/g of E1 and EE2 and cleaned by layers of silica (3.5 g), graphite (0.25 g), and alumina (6.0 g), EE2 was not recovered at all whereas E1 recovery was $12.4 \% \pm (2.54, \text{standard deviation})$. When silica was eliminated and the cleaning layer contained graphite (0.25 g) and alumina (12.0 g), both EE2 and E1 were still poorly recovered: $1.6\% \pm (0.22)$ and $9.18 \% \pm (0.69)$. The last packing that was tried in this experiment contained only alumina (12.0 g) and the recovery was $3.4 \% \pm (1.23)$ for EE2 and $6.88 \% \pm (0.68)$ for E1. The main conclusions drawn from this pilot experiment were that either the spiking concentration was too low to develop the method with or the spiked estrogen was weakly retained by the DE layer during the removal of the non-polar matrix in the 1st step of extraction. Thus, the spiking concentration was increased from 10 to 250 ng/g in the second experiment where only alumina (12.0 g) was used for clean up. However, higher spiking did not improve the recovery of both analytes since the recovery was $8.75 \% \pm (1.33)$ and $7.80 \% \pm (1.82)$ for EE2 and E1 respectively. Substituting alumina with hydromatrix (3 g) resulted in $7.16 \% \pm (0.90)$ and $7.80 \% \pm (1.82)$ for EE2 and E1. From these

observations we concluded that the optimal conditions used for lipid removal in the 1st extraction step resulted in a considerable loss of the spiked analytes. Initial experiments revealed that a minimum of three ASE cycles were required to extract 99.7 % of the tissue lipid. Besides using alumina as a pigment removal adsorbent, it can also be used as a retainer adsorbent for lipids (technical note, TN-210, Dionex Inc.). Adding a layer of alumina 12 g to 1 g sample mixed with 6 g hydromatrix and extracted with DCM according to the exhaustive extraction protocol (Table 2.1) resulted in a slightly improved recovery of EE2 $19.01 \% \pm (0.34)$ and E1 $11.15 \pm (1.51)$.

Hansen et al. (2010) developed an in cell clean-up method to extract steroidal estrogens from pig manure with > 75 % recovery. The poor recovery adopting Hansen and co-workers approach in our study can be attributed to a number of factors. Matrix difference was likely the major factor affecting the recovery in our case. Fish tissue lipid includes wax esters, sterols, steryl esters, triacylglycerides, diacylglycerides, diacylglyceryl esters, monoacylglycerides, phospholipids, and free fatty acids (Indrasena et al., 2005; Keriko et al., 2010). Pig's manure lipid composition however may be influenced by glycolipids and phospholipids that comprise about 95 % of total lipid in a pig's diet (Mackie et al., 1990). Therefore, manure may require much softer lipid removal conditions than those required for fish tissue. Optimization of the pressure, temperature, number of cycles, extraction solvent and even the amount and nature of adsorbents used for ASE can play a critical role in the recovery of the tested analytes (Runnqvist et al., 2010). Overall, our spike-recovery data suggested that an in cell clean-up approach was not a feasible option to prepare fish tissue for steroidal estrogen analysis.

2.4.2 Exhaustive extraction (protocol #2)

As shown in Table 2.3, EE2 recovery was 27 % $\pm$ (5.8) while E1 recovery was 25.2 % $\pm$ (3.3) and when 33 mL cells were packed with 2 g tissue (ShRHSs, 21.7 % lipid content) mixed with 6 g hydromatrix and spiked with 50 ng/g of each estrogen. Recoveries increased at higher spiking concentrations when steroidal estrogens were spiked at 125 ng/g, EE2 recovery was 43 % $\pm$ (9.2) while E1 recovery was 54.5 % $\pm$ (11.1).

To further improve the overall method recovery of our target compounds, another experiment was conducted in which hydromatrix was eliminated and the smaller 11 mL cells were used instead. This approach significantly improved the recovery of both analytes. The recovery of EE2 was linearly correlated with the spike concentration ($R^2 = 0.975$): 74.53 % $\pm$ (1.72) for 12.5 ng/g, 81.45 % $\pm$ (2.62) for 25 ng/g, and 93.7 % $\pm$ (5.8) for 50 ng/g while E1 recoveries were 64.8 % $\pm$ (6.13) for 1.25 ng/g, 57 % $\pm$ (8.7) for 2.5 ng/g, and 91.64 % $\pm$ (0.17) for 5 ng/g. The RSD values for EE2 below 6.2 % for EE2 and 15.3 % for E1 indicating good reproducibility.

Recovery improvement was achieved after the optimization of some critical steps during sample preparation. Firstly, freeze drying the whole fish homogenates was appropriate in light of the low vapor pressure of steroidal estrogens ($3 \times 10^{-8} - 9 \times 10^{-13}$ Pa) (Lai et al., 2002), and to avoid multiple freeze-thaws during sample preparation, which should reduce enzymatic activity that might affect the stability of these analytes. Freeze-drying was associated with high steroidal estrogen recoveries in sediment samples (Zhang et al., 2011; Peng et al., 2007; Braga et al., 2005). Secondly, the elimination of hydromatrix used as an inert dispersing agent (Runnqvist et al., 2010) to fill the cell

empty space during the extraction process resulted in higher recoveries suggesting that it binds with our analytes. Thirdly, the NMR identification of cholesterol as the major matrix after the GPC fractionation enabled us to successfully remove it by the acetonitrile precipitation step. The significance of this step is even more apparent when the sample is analyzed by liquid chromatography because the polar mobile phase may precipitate cholesterol in the separation column over time and reduce its lifespan. Lastly, the hexane wash was also a critical step to help remove any non-polar matrix residuals that could easily form emulsions. Qualitative full scan analysis showed that after the derivatization step, the samples were fairly clean however, the samples may require additional clean-up if liquid chromatography is chosen for analysis.

2.4.3 Validation of the sample preparation method (protocol # 2)

Prior to the analysis of either the spike-recovery samples or the laboratory EE2 exposed goldfish, the GC/MS linearity and limits of detection were assessed by standards injections. Within the tested range (0.004 – 1.0 ng, on column) for EE2 and (0.0008 – 0.2 ng, on column) for E1 the linearity was remarkable with R^2 values of 0.999 for both analytes. RSD values ranged from 7.5 – 4.3 % for EE2 and 11.7 – 4.3 % for E1 of 4 consecutive injections. The instrument detection limits (LODs, 3:1 signal: noise) were 4 and 0.8 pg (on column) whereas the quantification limits (LOQs, 10:1 signal: noise) were 12 and 3.2 pg (on column) for EE2 and E1 respectively. The method detection limits (MDLs) was 0.67 ng/g for EE2 and 0.68 ng/g for E1 respectively.

The commercial trout pellets used as fish diet that were spiked with EE2 contained 108 ng/g $\pm$ (3.4) which was very close to the target concentration of 100 ng/g.

The results shown in Table 2.3 demonstrated that EE2 can be extracted and quantified not only from spiked wild-fish samples but also from laboratory exposed goldfish tissue. Our optimized sample preparation method described in protocol 2 showed good accuracy, selectivity, and sensitivity. All five exposed goldfish samples accumulated EE2 to measurable levels whereas two unexposed samples did not show any detectable EE2. The maximum determined EE2 concentration was 2.2 ng/g whereas the minimum concentration was 1.53 ng/g and the average was $1.7 \text{ ng/g} \pm (0.29)$ (dry weight) (Figure 2.3).

2.5 Conclusions

A sample preparation method consisting of ASE, GPC fractionation, sample purification, and GC/MS (SIM) analysis was described. The proposed method enabled the determination of estrogen levels in whole fish tissues at very low ng/g dry tissue. To date, this method was the first optimized protocol for the determination of EE2 in whole fish tissue with high recovery and low method detection limits. This method was also expected to work with other types of samples like fish organs or invertebrates exposed to EE2 without considerable modifications. This method will help us to further expand our knowledge of the uptake and bioaccumulation of EE2 as an important endocrine disruptor. The method has been successfully applied to measure EE2 levels in laboratory exposed goldfish where it was detected at an average concentration of $1.7 \text{ ng/g} \pm (0.29)$.

2.6 Acknowledgments

The authors acknowledge Dr. Asim Muhammad for his help in the NMR analysis and also thank Dr. Gilbert Cabana for providing the shorthead redhorse sucker samples. This research was supported by the Natural Sciences and Engineering Research Council of Canada (Strategic Grants to J. Blais and V.L. Trudeau), the scholarship fund (for Al-Ansari A.M.) from King Abdulaziz University, Jeddah, Saudi Arabia, and the Great Lakes Action Plan.

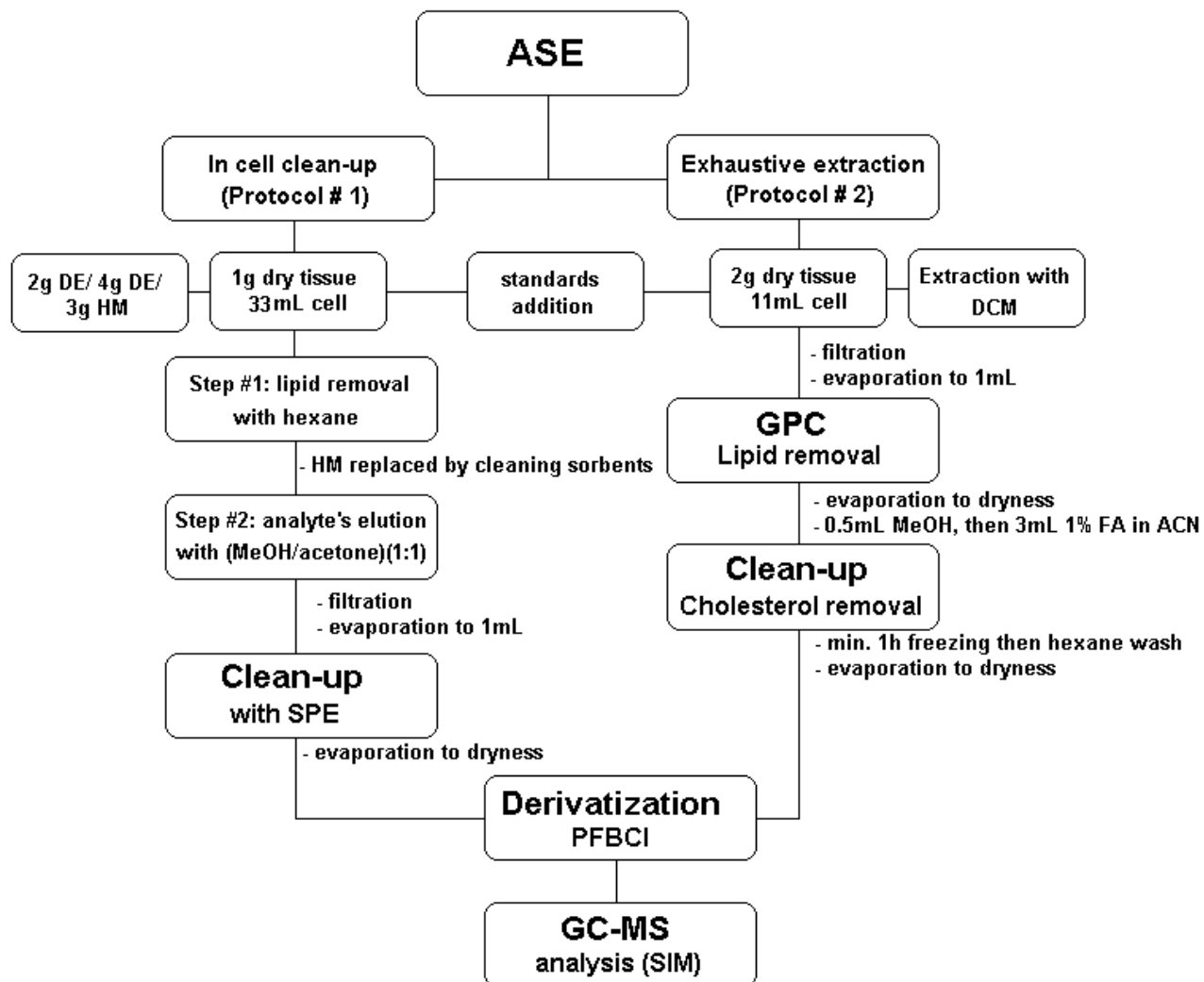


Figure 2.1: A schematic for the two sample preparation protocols employing in the cell clean-up (protocol # 1) and the exhaustive extraction (protocol # 2) using accelerated solvent extractor.

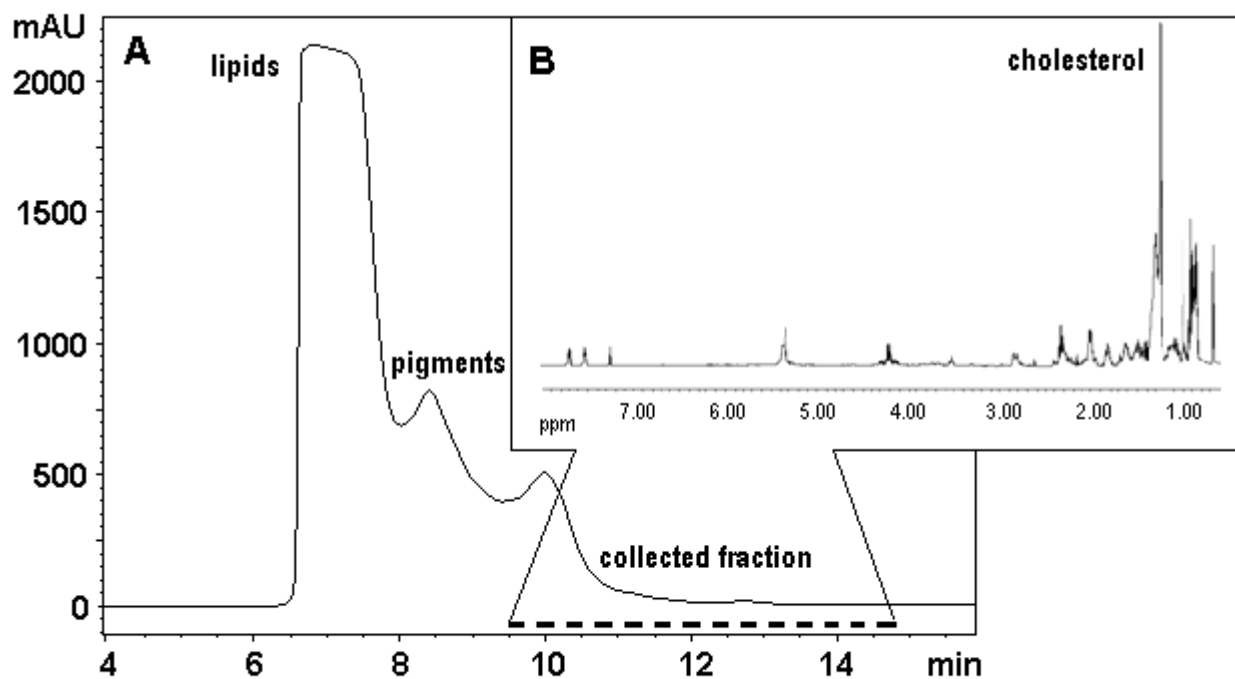


Figure 2.2: A chromatogram showing lipid and cholesterol removal from whole fish extracts as performed in protocol # 2 A) a preparative-HPLC spectrum of 1 mL sample injection monitored at 284.4 nm, B) an NMR spectrum of the collected fraction showing cholesterol as a co-eluted matrix. The dashed line indicates the time based fraction collection window.

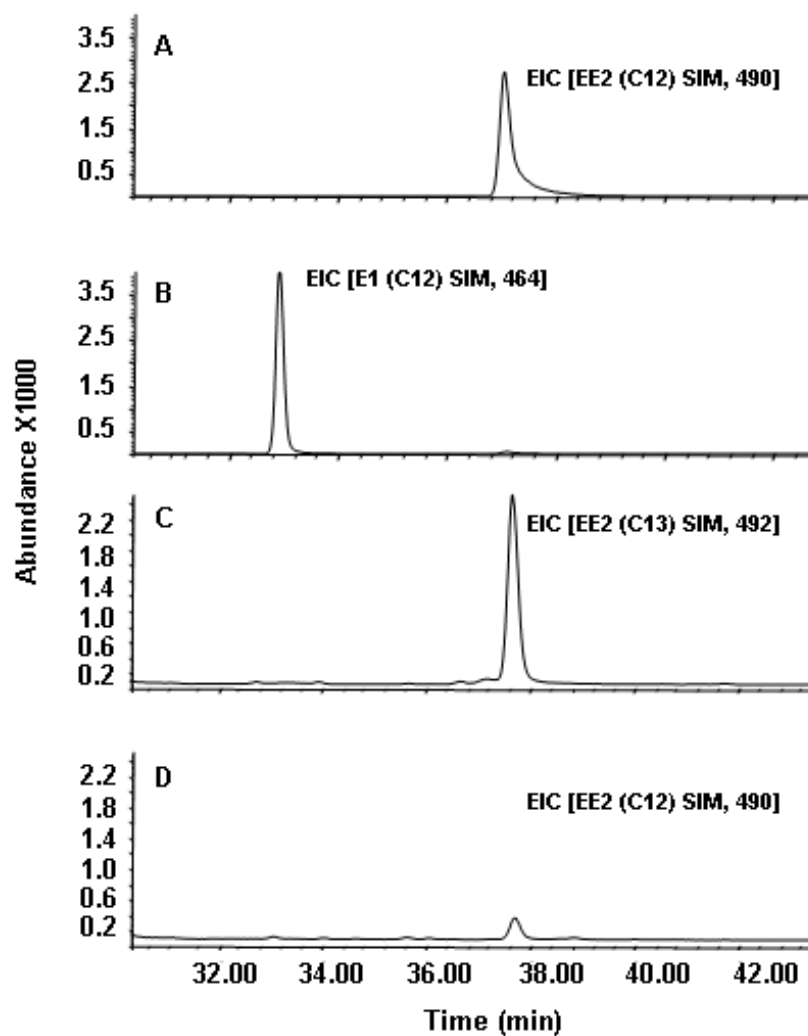


Figure 2.3: Extracted ion chromatogram (EIC) showing the response of A) EE2 standard injected at 1 ng on column and B) E1 standard injected at 0.2 ng on column. Panels C and D show a representative 2 g goldfish extracts showing the internal EE2 ^{13}C spiked standard spiked at 50 ng and the quantified EE2 in the sample respectively

Table 2.1

Accelerated solvent extraction conditions used during method development for protocol #1 and protocol # 2

Extraction conditions	In cell clean-up (protocol # 1)		Exhaustive extraction (protocol # 2)
	lipid removal (step # 1)	elution (step # 2)	
Static (min)	3	3	8
Cycles	3	3	1
Solvent	hexanes	acetone: MeOH (1:1)	DCM

Constant conditions in both protocols are:

- 1- Heat 5 min.
- 2- Temperature 70 (°C)
- 3- Pressure 2000 (psi)
- 4- Purge 120 (sec)

Table 2.2

In cell clean-up method (protocol #1) overall performance assessed by spike-recovery of triplicate samples after the two step extraction. Packing material shown below is the sample cleaning adsorbent layer used after the lipid removal in step 1

Packing material	Amount (g)	Spiking level (ng/g)	Rec. (%)	SD	RSD (%)	Rec. (%)	SD	RSD (%)
			EE2			E1		
1- S, G, A	3.5, 0.25, 6	10	ND	n/a	n/a	12.4	2.54	20.48
2- G, A	0.25, 6	10	1.6	0.22	14.04	9.18	0.69	7.5
3- A	6	10	3.4	1.23	36.15	6.88	0.68	9.85
4- A	6	250	8.75	1.33	15.21	7.65	0.29	3.83
5- H	3	250	7.16	0.90	12.56	7.80	1.82	23.33
6- H, A	6, 12	250	19.01	0.34	1.78	11.15	1.51	13.50

S: Silica, G: graphite, A: alumina, H: hydromatrix, Rec.: Recovery (%), SD: Standard deviation, RSD: relative standard deviation, ND, not detected, n/a: not available.

6: alumina was used to reduce lipid and DCM to extract the sample according to protocol # 2.

1g dry ShRHS samples mixed with 2 g DE were followed by a layer of 4 g DE were consistent in both extraction steps

Table 2.3

Exhaustive extraction method (protocol # 2) overall performance assessed by spike recovery of triplicate samples

Packing material	Amount (g)	ASE cell size (mL)	Spiking level (ng/g)		Rec.	SD	RSD	Rec.	SD	RSD
					(%)		(%)	(%)		(%)
					EE2		E1			
<u>Non-optimized</u>										
T, H	2, 6	33	50		27.74	5.8	21	25.18	3.3	13.27
T, H	2, 6	33	250		43.02	9.2	21.3	54.50	11.1	20.3
			EE2	E1						
<u>Optimized</u>										
T	2	11	12.5	1.25	74.53	1.72	2.31	64.8	6.13	9.46
T	2	11	25	2.5	81.45	2.62	3.21	57.01	8.7	15.26
T	2	11	50	5	93.70	5.8	6.18	91.64	0.17	0.18

Rec.: Recovery (%), SD: Standard deviation, RSD: Relative standard deviation, T: tissue from ShRHS, H: hydromatrix

Chapter 3

Bioaccumulation of the Pharmaceutical Ethinylestradiol in Shorthead Redhorse Sucker (*Moxostoma macrolepidotum*) from the St. Clair River, Canada

Chapter published as: Al-Ansari AM, Saleem A, Kimpe LE, Sherry JP, McMaster ME, Trudeau VL, Blais JM. 2010. Bioaccumulation of the Pharmaceutical Ethinylestradiol in Shorthead Redhorse Sucker (*Moxostoma macrolepidotum*) from the St. Clair River, Canada. Environmental Pollution 158, 2566-2571.

3.1 Abstract

17 α -ethinylestradiol (EE2), a synthetic estrogen prescribed as a contraceptive, was measured in Shorthead Redhorse Suckers (ShRHSs) (*Moxostoma macrolepidotum*) collected near a wastewater treatment plant (WWTP) in the St. Clair River (Ontario, Canada). We detected EE2 in 50 % of the fish samples caught near the WWTP (Stag Island), which averaged 1.6 ± 0.6 ng/g (wet weight) in males and 1.43 ± 0.96 ng/g in females. No EE2 was detected in the samples from the reference site (Port Lambton) which was 26 km further downstream of the Stag Island site. Only males from Stag Island had VTG induction, suggesting the Corunna WWTP effluent as a likely source of environmental estrogen. EE2 concentrations were correlated with total body lipid content ($R^2 = 0.512$, $p < 0.01$, $n = 10$). Lipid normalized EE2 concentrations were correlated with

$\delta^{15}\text{N}$ ($R^2 = 0.436$, $p < 0.05$, $n = 10$), suggesting higher EE2 exposures in carnivores. Our data support the hypothesis of EE2 bioaccumulation in wild fish.

Keywords: bioaccumulation, *Moxostoma macrolepidotum*, St. Clair River, ethinylestradiol

3.2 Introduction

Environmental estrogens are a subclass of a larger family of chemicals known as endocrine disrupting chemicals (EDCs) that are capable of adversely affecting the function of the endocrine system leading to alteration in growth, development, and reproduction in the exposed animals (Mills and Chichester, 2005). These EDCs include the pharmaceutical 17 α -ethinylestradiol (EE2) (the active ingredient in birth control pills) and its natural analogues 17 β -estradiol (E2), estrone (E1), and estriol (E3) (Larsson et al., 1999; Fernandez et al., 2007). Estrogens are metabolized to their water soluble inactive conjugated forms and may be activated into their free forms during sewage treatment due to bacterial modification (Bodzek and Dudziak, 2006).

Estrogenic chemicals released from municipal effluents discharged into the aquatic environments have been shown to cause abnormal biological responses in exposed species (reviewed in Gross-Sorokin et al., 2006; Tyler et al., 1998). For instance, male fish with partial feminization, or intersex, and the induction of vitellogenin (VTG), a precursor egg yolk protein have been recognized as distinct biological endpoints of the exposure to xenoestrogens (Kavanagh et al., 2004; Gross-Sorokin et al., 2006). Furthermore, many EDCs act as agonists or antagonists to the estrogen receptor (Mills and Chichester, 2005). Steroidal estrogens have been detected at low ng/L levels in the effluents of wastewater treatment plants (WWTPs), surface water, and sediment (Fernandez et al., 2007; Falconer et al., 2006; Peng et al., 2006). Yet, such low concentrations are still biologically active and can result in adverse effects. In fish, exposure to 0.2-10 ng/L EE2 induced VTG synthesis in male zebrafish (*Danio rerio*), Japanese Medaka (*Oryzias latipes*) and rare minnow (*Gobiocypris rarus*) (Xu et al.,

2008; Ma et al., 2007; Zha et al., 2007). Abnormal reproductive and developmental effects such as skewed sex ratios and development of mixed secondary sexual characteristics such as feminine papillae and a masculine anal fin in the same fish have also been reported at 0.3-10 ng/L (Xu et al., 2008; Larsen et al., 2008; Parrott and Blunt, 2005). Altered male courtship behavior leading to spawning failure in male zebrafish was observed at 5 ng/L EE2 concentration (Larsen et al., 2008). Likewise, decreased fecundity in fathead minnow (*Pimephales promelas*) was observed at EE2 concentrations ranging between 0.3-5 ng/L. (Parrott and Blunt, 2005; Nash et al., 2004). Feminization can occur as low as 0.05-5 ng/L in lifetime exposed species such as zebrafish and fathead minnow (Larsen et al., 2008; Nash et al., 2004). The lowest observed effect concentration of EE2 was determined by Länge et al. (2001) as 4 ng/L in fathead minnow for lifetime exposure, indicating that reproductive disruption by environmentally relevant concentrations of EE2 is possible (Tyler et al., 1998). Even more convincing is the study by Kidd et al. (2007) who experimentally contaminated a large (34 ha) freshwater lake in the Experimental Lakes Area of Canada with EE2. This led to the feminization of male fathead minnows as indicated by the production of VTG and near extinction of the species from the lake (Kidd et al., 2007).

Although the occurrence and implications of steroidal estrogens in the environment has received a great deal of attention, evidence showing that such chemicals can actually bioconcentrate or bioaccumulate in wild fish is limited. Only in a few cases have laboratory studies shown that environmental estrogens bioconcentrate in the bile of fish prior to excretion and hence, the detection of high concentrations in this specific medium is expected (Gibson et al., 2005; Larsson et al., 1999). Gibson et al. (2005)

exposed rainbow trout (*Oncorhynchus mykiss*) and roach (*Rutilus rutilus*) to 100% WWTPs effluents (exposure group), tap water, and river water (control groups) for 10 days. After enzymatic hydrolysis of the extracts, higher concentrations of environmental estrogens including E1, E2, EE2, nonylphenol, and nonylphenol ethoxylates were detected in the bile and the gonads of the exposed animals whereas only low levels of E1 and E2 were found in the control samples. Similarly, the bile of rainbow trout caged downstream from a WWTP effluent for two weeks contained 10^4 - 10^6 times higher concentration of estrogens than those of the water which were 4.5 ng/L, 5.8 ng/L, and 1.1 ng/L for EE2, E1, and E2 respectively (Larsson et al., 1999). Whole body concentrations of EE2 measured by radioimmunoassay in fathead minnow exposed to nominal concentrations of EE2 (64, 16, 1.0, 0.2 ng/L) via water reached at most 31 ng/g after 153 days post-hatching with estimated bioconcentration factor (BCF) of 660 (Länge et al., 2001).

Here we test the hypothesis that EE2 is accumulating significantly in wild Shorthead Redhorse Sucker (ShRHS) (*Moxostoma macrolepidotum*). First, we investigated whether EE2 is detectable in sexually mature ShRHSs sampled near the effluent of the Corunna WWTP on the St. Clair River, Ontario, and a control site further downstream. Then, we determined VTG levels in ShRHSs from both sites to evaluate the impact of the WWTP effluent. We also examined correlations between EE2 in ShRHS and gonadosomatic index (GSI), hepatosomatic index (HSI), total lipid content, and the stable isotopic composition of nitrogen ($\delta^{15}\text{N}$) which may reveal nitrogen source information and/or carnivory among individuals. This is the first report of EE2 concentrations in wild caught fish.

3.3 Material and methods

3.3.1 Study site

The St. Clair River is a natural border line between the province of Ontario (Canada) and the state of Michigan (USA) serving as an important shipping channel a number of industries and the broader Great Lakes Seaway system (Mayne, 2003). The river is 64 km in length, connecting Lake Huron to Lake St. Clair (Figure 3.1). Its average flow rate is 5153 m³/s (U.S Geological Survey, 2000). St. Clair River is a source of drinking water for a population of 170,000 (Mayne, 2003). Due to high concentrations of contaminants from industrial and municipal effluents released into the river, it was designated as an Area of Concern (AOC) by the International Joint Commission in the 1940s. Remedial action plans have been proposed to rehabilitate St. Clair River AOC by governmental agencies, industries, municipalities and the public in Canada and the U.S (Mayne, 2003).

3.3.2 Chemicals

All solvents for sample preparation, including acetone, hexane, petroleum ether and dichloromethane (DCM) were Optima[®] grade and anhydrous granular sodium sulfate (Fisher Scientific, Brockville, ON, Canada) while acetonitrile and water for LC/MS analysis were LC/MS grade (Fluka, Mississauga, ON, Canada). Hydromatrix for ASE extraction was from Varian Inc., Mississuga, ON, Canada. Florisil (PR 60/100 mesh) was from Supelco, Bellefonte, PA, USA. EE2-¹²C and EE2-¹³C (99% grade) were from

Cambridge Isotope Lab., Inc., Andover, MA, USA. Nonane was from Sigma Aldrich, St. Louis, MO, USA.

3.3.3 Sample collection and preparation

ShRHSs were collected by electrofishing at Stag Island, St. Clair River, in 2002 downstream of the Corunna WWTP (n = 20) and from Port Lambton as a reference site (26 km downstream of the nearest WWTP at Stag Island) (n = 16). Physical characteristics including sex, weight and fork length were determined (Table 3.1). Blood was sampled to determine plasma VTG levels and then fish were kept frozen at -20°C and shipped on dry ice to the University of Ottawa for EE2 analysis. Individual fish samples were sliced and ground entirely using a manual meat grinder. A 20 g tissue sample was homogenized with petroleum ether rinsed hydromatrix, split into two equal portions, and extracted at 70 °C by an accelerated solvent extractor (ASE-200, Dionex Corporation, Sunnyvale, CA, USA) in 33 mL ASE cells for 5 min . A 100 µL of the ¹³C labeled EE2 (500 ng/mL) in nonane was spiked onto each sample as an internal standard in order to calculate EE2 concentration based on the isotopic dilution method as described in (de Hoffmann and Stroobant, 2002). The extraction was carried out with DCM at 138 bars for 8 min. Method blanks (n = 3) containing only hydromatrix spiked with 100 µL of working standard were extracted in parallel to the samples. Extracts were filtered through 0.45 µm and 0.2 µm PTFE filters (Chromatographic Specialist Inc. Brockville, ON.). Extracts (approximately 90 mL per sample) were concentrated to 3 mL by a Turbo Vap[®] II, (Zymark Hopkinson, MA, USA), and fractionated by gel permeation chromatography as described in U.S. EPA method 3640A (U.S. EPA, 1994). The

collected fractions (approximately 35 mL each) were evaporated down to 0.5 mL by TurboVap[®] II and eluted through 1 g florisil packed in Pasteur pipettes and topped with anhydrous Na₂SO₄ (0.25 g). Prior to loading the sample, columns were equilibrated with 5 mL hexane followed by loading the sample (0.5 mL) and eluted with 9 mL hexane into glass centrifuge tubes. Samples were evaporated to dryness, reconstituted in 300 µL 90 % LC/MS grade methanol and transferred to 1.5 mL amber vials for HPLC analysis.

3.3.4 HPLC-APCI-MSD analysis

The analysis of EE2 was performed on an Agilent 1100 series HPLC-DAD-APCI/MS system (Agilent Technologies Inc. CA, USA). The system was equipped with an online degasser, a quaternary pump (maximum pressure limit 400 bars), an autosampler with a 100 mL injection loop, a column thermostat compartment, and on line APCI-MS (version 1964C VL), and Agilent Chemstation version B.03.02). The elution of EE2 was carried out on a Pinnacle DB Biphenyl column (50 mm × 2.1 mm I.D., particle size 1.9 µm (product # 9409252, lot # 070425R, Restek Corporation, Bellefonte, PA, USA).

The optimized conditions of the instrument operated in SIM mode in negative polarity were: fragmentor voltage 80 V, gain 1.0 EMV, nitrogen gas temperature 350 °C, vaporizer temperature 420 °C, drying gas flow 5 L/min, nebulizer pressure 87 bar, capillary voltage 4000 V and corona current 10 µA. EE2 elution was achieved at isocratic mobile phase composition of 55 % acetonitrile and 45 % water at a flow rate of 0.3 mL/min and at column thermostat temperature of 30 °C. The detection of EE2-¹²C was achieved by monitoring the selected ions: 295.3 for EE2-¹²C and 297.1 for spiked EE2-

^{13}C . Extracted ion chromatograms of each sample, injected in triplicate, were compared with the commercially available EE2- ^{12}C and EE2- ^{13}C standards analyzed within the same sequence. Method blanks were injected to determine the background signal. Quantification was performed by isotope dilution method (de Hoffmann and Stroobant, 2002). Seven replicates of the sample showing lowest level of EE2 was used to establish MDL and the standard deviation.

3.3.5 Lipid determination and $\delta^{15}\text{N}$ analysis

Approximately 1 g wet weight fish samples were extracted with DCM by ASE 200 for the determination of total lipid. Samples were heated for 5 min at 50 °C and then flushed with DCM for 1 min at 138 bars modified from Dionex (2004). The fractions were collected in pre-weighed 40 mL glass vials, air dried at room temperature for approximately 72 h and further dried overnight at 60 °C and lipid content was calculated based on the difference of pre and post weight of the glass vials.

Stable isotope ratio $\delta^{15}\text{N}$ analysis was performed to verify the trophic level of ShRHS samples (Peterson and Fry, 1987). Sub-samples of ground whole fish were freeze dried, finely homogenized, and analyzed on a Vario EL III Elemental Analyzer (Elementar, Germany) followed by gas chromatograph separation and on-line analysis by continuous-flow with a DeltaPlus Advantage isotope ratio mass spectrometer coupled with a ConFlo III (ThermoFinnigan, Germany). Data were normalized using internal standards previously calibrated with International standards IAEA-CH-6, IAEA-NBS22, IAEA-N1, IAEA-N2, USGS-40, USGS-41.

3.3.6 Vitellogenin analysis

Vitellogenin analysis was performed in Environment Canada's Laboratories (Burlington, Ontario) to confirm whether ShRHSs were affected by estrogenic EDCs discharged in the vicinity of Stag Island. Briefly, blood samples were collected upon capture from the caudal peduncle using heparinized syringes and centrifuged at 10,000 g for 2 min at 4°C. The plasma was removed, flash frozen in liquid nitrogen, and then stored at -80°C in cryovials. Since there was no ELISA commercially available for the quantitative measurement of VTG in ShRHS, the analysis was performed by gel electrophoretic separation of protein bands (Gallagher, 2007) as previously described and validated (Kavanagh et al., 2004) with the use of silver staining to detect protein bands (Dell' Angelico and Bonifcino, 2000). The VTG values were derived from optical density measurements of VTG bands obtained by sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) in 4 % gels (McDaniel et al., 2008), and are presented as percentage VTG relative to the total protein content of the sample (Medberry and Gallagher, 2002).

3.3.7 Statistical analysis

Analysis of variance (ANOVA) followed by Tukey's test was performed using Origin Lab 7 software (Origin Lab Corporation MA, USA). A two-way ANOVA was used on the mean values of gonadosomatic index (GSI), and hepatosomatic index (HSI) lipid content, and $\delta^{15}\text{N}$ data sets respectively to test the comparison factors sex, sampling site, and sex site interactions. Linear regression analysis was used to test for EE2 correlations with GSI, HSI, lipid content, and $\delta^{15}\text{N}$.

3.4 Results and discussion

3.4.1 Gonadosomatic Index (GSI) and Hepatosomatic index (HSI), lipid content and $\delta^{15}\text{N}$

GSI was higher for females ($7.64 \% \pm 2.49$, $n = 18$) than males ($5.98 \% \pm 0.97$, $n = 18$) (two-way ANOVA $p = 0.02$) but no difference was observed between the fish collected from the reference site and the exposed site (two-way ANOVA $p = 0.38$) (Table 3.2). In addition, no interaction was observed between sex and site ($p = 0.82$). Although seasonal variation in GSI has been shown in some species (Trudeau, 1997), all samples in this study were collected within a two week period in November prior to the spawning season which starts from late April to late May (Scott and Crossman, 1973). Our results show no effect of WWTP effluent on the GSI.

Similarly, HSI was higher for females ($1.45\% \pm 0.30$, $n = 18$) than males ($1.16 \% \pm 0.31$, $n = 17$) (two-way ANOVA $p = 0.01$) but no difference was observed between the reference site and the exposed site ($p = 0.22$) (Table 3.2), and no interaction was found between sex and site ($p = 0.54$). Allen et al. (1999) indicated that in male flounder (*Platichthys flesus*), high HSI can be attributed to VTG synthesis in contaminated sites with estrogenic substances or exposure to other chemicals that may cause liver hypertrophy. Gross-Sorokin et al. (2006) also found higher HSI in females than in males, which they attributed to VTG synthesis in females as a normal reproduction event but not in males.

Total lipid content was not different between males and females (two-way ANOVA, $p = 0.92$), but fish sampled from the exposed site had significantly higher lipid

content ($3.19 \% \pm 2.35$, $n = 20$) than those sampled from the reference site ($1.42 \% \pm 0.95$, $n = 16$) ($p = 0.01$) (Table 3.2). No interaction was observed between sex and site ($p = 0.87$). This clear difference in lipid content may be explained by nutrient enrichment in WWTP effluents (Santos et al., 2008).

Delta¹⁵N values in fish homogenate ranged between 5.19 ‰ and 12.52 ‰, but no differences were observed between males and females ($p = 0.47$). However, we observed lower $\delta^{15}\text{N}$ values ($9.76 \text{ ‰} \pm 0.85$, $n = 20$) in ShRHS samples from the WWTP effluent receiving site than samples from the reference site ($10.63 \text{ ‰} \pm 1.73$, $n = 16$) ($p = 0.06$) (Table 3.2). Typically, invertebrates in river waters affected by agriculture and urban development have elevated baseline $\delta^{15}\text{N}$ (Anderson and Cabana, 2007). An important exception to this pattern is near sewage discharges where suspended organic matter is characterized by very low $\delta^{15}\text{N}$ (deBruyn et al., 2003). The incorporation of organic matter into the food-web downstream from a major WWTP release point is expected to result in low $\delta^{15}\text{N}$ in consumers (invertebrates, fish). Indeed, deBruyn et al. (2003) observed lower $\delta^{15}\text{N}$ values in primary and secondary consumers (invertebrates and fish) from WWTP receiving waters up to 10 km downstream from the effluent site. Likewise, here we found similar differences in $\delta^{15}\text{N}$ between ShRHS samples from the WWTP effluent exposed site versus the reference site.

3.4.2 Total vitellogenin in ShRHS

The estimated plasma VTG levels are expressed as a percentage of total protein. As shown in Figure 3.2, all females from both sites had VTG induction averaging $6.33 \% \pm 1.78$ ($n = 8$) with a concentration range of 4.9 % to 10.5 % in Port Lambton samples

and slightly lower VTG levels averaging $5.43\% \pm 1.20$ ($n = 10$) with a concentration range of 3.9 % to 7.3 % in Stag Island. Eight out of ten males captured from the Stag Island had detectable plasma VTG averaging $1.71\% \pm 1.26$ ($n = 10$) with a concentration range of 1 % to 4.2 %. In contrast, no plasma VTG was detected in any male from Port Lambton ($n = 8$). Even though there are no studies reporting the concentrations of steroidal estrogens in the St. Clair River, the detection of VTG in Stag Island males confirms the presence of estrogenic EDCs in this specific site whereas its absence in Port Lambton males reflects the absence or the presence at substantially lower levels of such chemicals. Many studies have reported ng/L levels of steroidal estrogens in sewage effluents and receiving waters (Falconer et al., 2006). Consequently, male fish caught from contaminated waters with estrogenic substances have elevated plasma VTG levels similar to or even higher than females (Allen et al., 1999; Kavanagh et al., 2004). Here, we observed high variability in VTG concentrations among individuals, which is somewhat expected. White Perch (*Morone americana*) collected near Hamilton Harbour showed similar variability in VTG levels (Kavanagh et al., 2004). However, such variability in male VTG responses to EDCs in laboratory exposure studies is well known (Ackermann et al., 2002), and reflects differences in the physiological status of the animals at the time of exposure.

3.4.3 Detection and bioaccumulation of EE2 in wild ShRHS

The pharmaceutical EE2 was detected in 50 % ($n = 20$) of the ShRHS samples from the exposed site, however, no detectable levels were observed in the samples from the reference site ($n = 16$). The estimated mean concentrations of EE2 were similar

among males ($1.64 \text{ ng/g} \pm 0.63$ wet weight) and females ($1.43 \text{ ng/g} \pm 0.88$ wet weight) (Table 3.3 and Figure 3.3). The method detection limit (MDL) was $0.45 \text{ ng/g} \pm 0.145$. The detection of EE2 in ShRHSs from the exposed site only and its absence in the fish from the control site is in agreement with our VTG data since all males from the reference site were normal with no detected VTG induction (Figure 3.2). This suggests that individuals with detectable EE2 are most likely accumulating this estrogen from the Corunna WWTP effluents that serves the St. Clair Township (Environment Canada), despite some industrial discharges in the vicinity of Stag Island. Interestingly, EE2 accumulation in male ShRHSs ($n = 5$) was limited to those samples with a minimum VTG concentration of 1.9 % or higher whereas EE2 and VTG did not show any similar observation in females. As shown in Table 3.3, the detection of EE2 in 10 of 20 fish collected from Stag Island is most likely due to variable exposure to the estrogenic substances from the effluent outfall, though variability in the uptake and elimination rates, metabolic biotransformation, growth dilution, and fecal egestion (Arnot and Gobas, 2006) are other potential factors that may affect EE2 bioaccumulation in these fishes.

The sources of EE2 accumulating in these ShRHS samples are yet to be clarified however, studies like Peng et al. (2006) and Braga et al. (2005) detected EE2 in sediments at ng/g levels near WWTP effluent receiving sites. It is well known that the members of the Catostomidae family including the ShRHSs have bottom feeding behaviors and their main prey include benthic invertebrates (Scott and Crossman, 1973). Liebig et al. (2005), and Dussault et al. (2009) provided evidence that such endobenthic organisms can be a possible source of EE2 transfer to benthivores and subsequent

secondary predators. Further studies should examine the role of benthic feeding on the delivery of EE2 to the aquatic food chain.

3.4.4 The correlation of EE2 concentrations with GSI, HSI, lipid content and $\delta^{15}\text{N}$

EE2 concentrations were not correlated to GSI ($R^2 = 0.12$) or HSI ($R^2 = 0.08$) in ShRHS samples that had detectable levels of EE2 ($n=10$). Angus et al. (2005) examined sexual development of male western mosquito fish (*Gambusia affinis*) exposed to 0.1-10 $\mu\text{g/g}$ EE2 in their diet and found that all fish exposed to EE2 had lower GSI than control fish. The absence of a correlation between EE2 and somatic indices in our study may be attributed to a number of factors, including differences in dose, and the nature of exposure, possible species differences, or the timing of sampling relative to seasonal gonadal cycles.

A significant positive correlation of EE2 concentration and lipid content was found with an R^2 of 0.512 ($p < 0.05$, $n=10$) (Figure 3.4A), which is the classic relationship expected for a bioaccumulating substance (Kelley et al., 2008; Arnot and Gobas, 2006). EE2 has a relatively high log octanol-water partitioning coefficient ($\log K_{ow}=4.15$) which places it among the hydrophobic chemicals that may bioaccumulate (Lai et al., 2002). The $\log K_{ow}$ is a useful physiochemical property that provides a practical prediction of EE2 partitioning into the lipid fraction or to organic carbon and hence, accumulation in biota or sediment (Liebig et al., 2005).

Another interesting correlation was established between lipid normalized concentrations of EE2 and $\delta^{15}\text{N}$ with R^2 value of 0.436 ($p < 0.05$, $n = 10$) (Figure 3.4B). It is not clear yet whether biomagnification of EE2 may be occurring in aquatic food-webs,

but it suggests that individuals at higher trophic position accumulate more EE2 than those feeding at a lower trophic position. Lai et al. (2002) model predicted that the bioaccumulation of EE2 will be limited to a narrow food-web which is topped by bottom feeders like ShRHS in our case. The model also showed that the BAF of EE2 decreased toward top predators in the food chain (Lai et al., 2002). Such prediction may apply in our case because the mature members of the sucker family are relatively free of predators except the larger northern pike or muskellunge (Scott and Crossman, 1973). Studies examining trophic transfer of persistent organic pollutants in food-webs may cover a wider range of species (Houde et al., 2008; Ikemoto et al., 2008), and the accumulation of EE2 in food-webs should now be expanded to a greater range of species.

3.5 Conclusions

We show that the synthetic hormone and the active ingredient of oral contraceptives, EE2 can be detected in wild fish collected downstream of municipal effluents. Our data also supports what has been modeled by Lai et al. (2002) who proposed the possibility of EE2 bioaccumulation in fish species that feed low in the food-web. Similarly, we provide important data that substantiates similar suggestions by Liebig et al. (2005) and Dussault et al. (2009) that EE2 is a potential candidate for bioaccumulation in higher predators, especially bottom feeding fishes. We suggest that EE2 is a pharmaceutical EDC that bioaccumulates in benthic feeding fish.

3.6 Acknowledgements

This research was supported by the Natural Sciences and Engineering Research Council of Canada (Strategic Grants to J. Blais and V.L. Trudeau), the scholarship fund (for Al-Ansari A.M.) from King Abdulaziz University, Jeddah, Saudi Arabia, and the Great Lakes Action Plan.

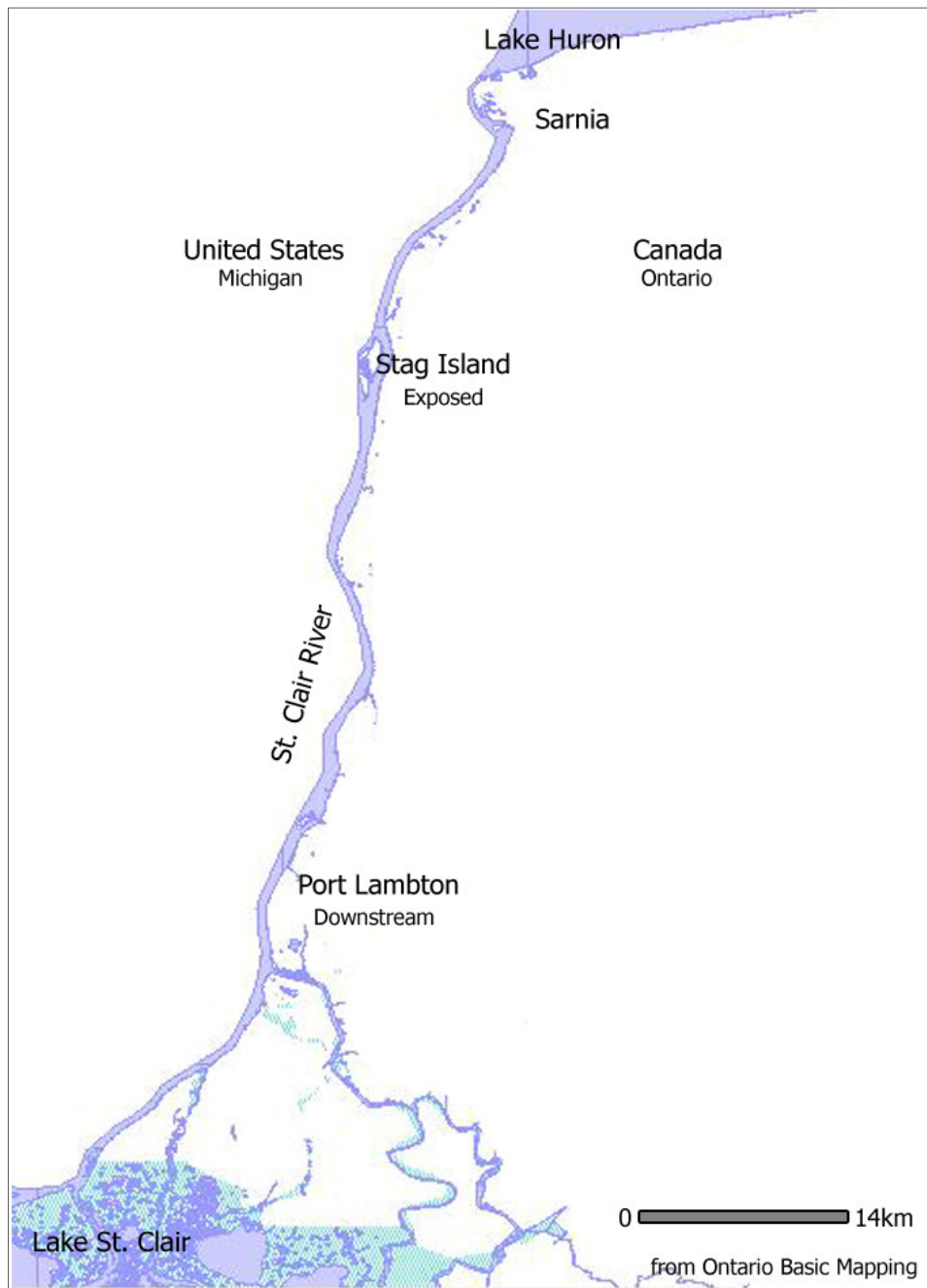


Figure 3.1: The sampling sites where the ShRHSs were sampled in 2002. Stag Island near Corunna wastewater treatment plant was considered as an exposed site and Port Lambton was considered a downstream reference site.

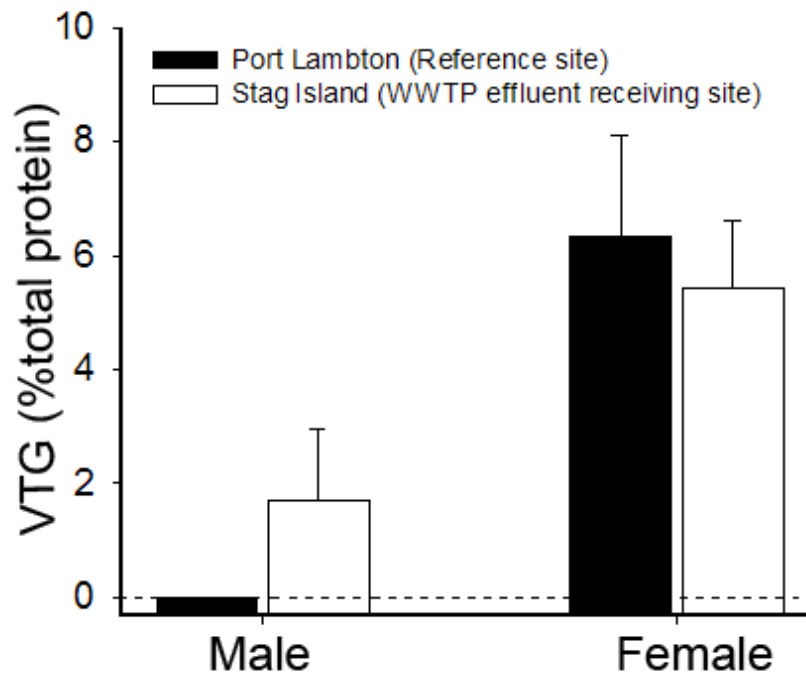


Figure 3.2: VTG levels expressed as percent total protein of ShRHS samples from St. Clair River. The black bars indicate Port Lambton (reference site) and the white bars indicate Stag Island (exposed site). No VTG was detected from any male from the reference site.

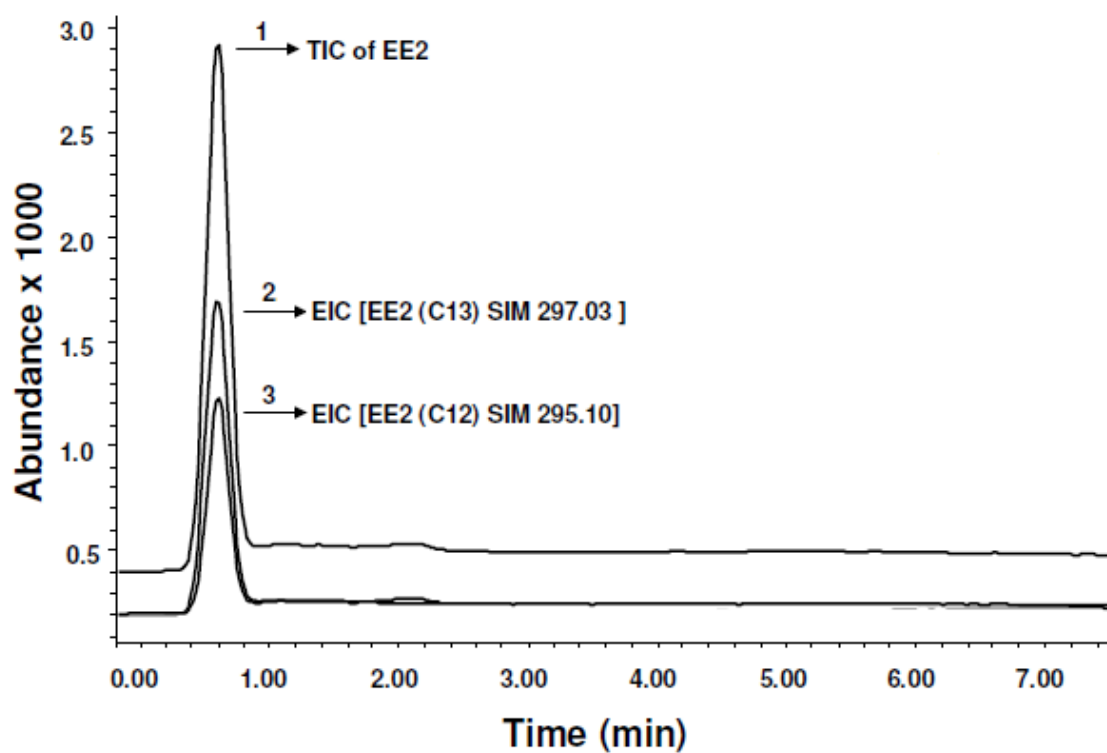


Figure 3.3: A representative ShRHS sample chromatogram showing detected EE2 in 20g of extracted whole fish homogenate as in peak (1) the total ion chromatogram (TIC). Peak (2) indicates the extracted ion chromatograms (EIC) of EE2 ¹³C spiked standard (m/z 297) at the concentration of 50 ng per sample and peak (3) indicates the signal of the quantified EE2 (m/z 295) in the wild fish extracts.

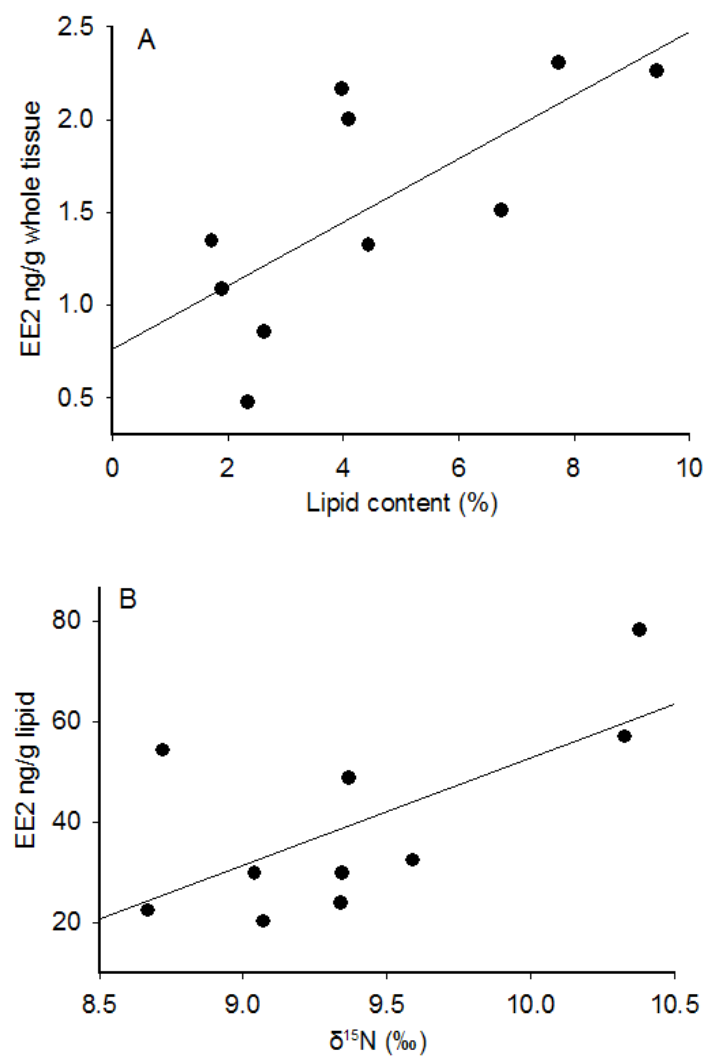


Figure 3.4: Linear regressions showing correlation of a) EE2 concentrations expressed as ng/g whole tissue with lipid content (%) ($R^2 = 0.512$, $p < 0.01$, $n = 10$) b) EE2 lipid normalized concentrations expressed as ng/g lipid with the trophic level $\delta^{15}\text{N}$ (‰) values ($R^2 = 0.436$, $p < 0.05$, $n = 10$).

Table 3.1

Sample weights and total length

Site	Sex	n	Weight (g) $\pm$ (SD)	Fork length (cm) $\pm$ (SD)
Port Lambton Reference	M	8	658.75 (145.79)	36.24 (2.73)
	F	8	866.88 (173.47)	39.15 (2.36)
Stag Island Exposed	M	10	712.13 (171.88)	36.69 (3.36)
	F	10	828.13 (182.87)	37.94 (2.53)

Table 3.2

GSI, HSI, lipid content (%) and $\delta^{15}\text{N}$ (‰) of ShRHS samples from the St. Clair River compared by two way ANOVA followed by Tukey's test.

	Port Lambton Reference site		Stag Island Exposed site		
	Mean ± (SD)		Mean ± (SD)		P value
GSI ^a	6.49	(2.13)	7.07	(2.00)	0.38
HSI ^b	1.38	(0.36)	1.26	(0.30)	0.22
Lipid	1.42	(0.95)	3.19	(2.35)	0.01
δ ¹⁵ N	10.63	(1.73)	9.76	(0.85)	0.06
	Male		Female		
GSI	5.98	(0.97)	7.64	(2.49)	0.02
HSI	1.16	(0.31)	1.45	(0.30)	0.01
Lipid	2.37	(1.99)	2.44	(2.15)	0.92
δ ¹⁵ N	10.31	(0.90)	9.99	(1.72)	0.47

GSI^a gonado somatic index; HSI^b hepato somatic index

GSI, lipid content, and $\delta^{15}\text{N}$: Stag Island (n=20), Port Lambton (n=16), Male (n=18), Female (n=18) HSI: Male (n=17), Female (n=18)

Table 3.3

Estimated concentrations of EE2 ng/g wet weight and ng/g lipid weight in 10 of 20 ShRHS samples from Stag Island.

Sample	Sex	[EE2] ng/g (ww) ^a	[EE2] ng/g (lw) ^b
1	M	2.26	23.95
2	M	2.17	54.32
3	M	1.35	78.15
4	M	1.33	29.83
5	M	1.08	56.96
6	F	2.30	29.78
7	F	2.00	48.75
8	F	1.51	22.39
9	F	0.85	32.40
10	F	0.48	20.27

ww^a wet weight; lw^b lipid weight

No EE2 was detected in the other 10 ShRHS samples from the Stage Island and all samples (n=16) from Port Lambton (reference site). Method detection limit (MDL) is 0.45 ng/g $\pm$ 0.145.

Chapter 4

Does the Pharmaceutical 17 α -Ethinylestradiol Bioaccumulate in a River Food-web?

An Investigation of Seven Fish Species in the St. Clair River, Canada

4.1 Abstract

We previously demonstrated bioaccumulation of 17 α -ethinylestradiol (EE2) in the benthic feeder, shorthead redhorse sucker (*Moxostoma macrolepidotum*) (ShRHSs), a benthic feeder in the St. Clair River (Chapter 3). The present study was designed to extend the existing information about EE2 bioaccumulation with a wider follow-up study. Seven fish species spanning different trophic levels were sampled in summer 2010 from Stag Island (exposed site) and Port Lambton (reference site) on the St. Clair River, Ontario, Canada. Sewage effluents from Corunna STP and surface water were sampled along with grab sediment samples from different locations in the Stag Island and Port Lambton areas. All fish (n = 79), effluent (n = 4), surface water (n = 3), and sediment samples (n = 9) were analyzed for EE2 concentrations. Whole fish tissues were analyzed for stable isotopes of nitrogen ($\delta^{15}\text{N}$) and carbon ($\delta^{13}\text{C}$), total mercury (THg), and total lipid content. Surface water and effluent samples contained < 0.1 ng/L EE2 whereas only shorthead redhorse suckers (ShRHSs) contained EE2. Rapid EE2 uptake was believed to occur at least from the water however, the tissue levels were below the detection limit. The $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values and total Hg revealed an aquatic food-web spanning at least two trophic levels, and clear biomagnification was seen for THg, which correlated

strongly with $\delta^{15}\text{N}$. However, we did not observe any correlations between THg and either $\delta^{15}\text{N}$ or EE2 in this food-web. This study suggested EE2 residues did not extend into pelagic food-webs and likely remained confined to the benthic food-web of the St. Clair River.

Keywords: 17 α -Ethinylestradiol, Stable isotopes, Total mercury, Food-web

4.2 Introduction

Birth control pills, antibiotics, anti-inflammatories, anti-depressants, and lipid regulators are examples of the most common pharmaceuticals that are being released to the aquatic environment (Trudeau et al., 2005; Koplin et al., 2002; Falconer, 2006). These drugs are usually detected in the surface water and/or sewage influents or effluents at $\mu\text{g/L}$ to ng/L concentrations (Trudeau et al., 2005; Koplin et al., 2002; Falconer, 2006). However, the effects of endocrine disruptors and pharmaceuticals on the aquatic organisms have started to emerge only over the last twenty years (Kleywegt et al., 2007). Recent advancements in analytical methodologies have resulted in tremendous improvement of the sensitivity and selectivity of such methods and thus have made it possible to elucidate the identity and quantity of some drugs not only in the aqueous medium, but also in biota (Kleywegt et al., 2007). Environmental uptake mechanisms and bioaccumulation of pharmaceuticals are critical aspects to be considered when studying the environmental fate and potential risk associated with these chemicals (Sumpter and Johnson, 2008). Evidence that pharmaceuticals can bioaccumulate in aquatic biota has started to appear in the literature over the last decade (Mimeault et al., 2005; Paterson and Metcalfe, 2008; Al-Ansari et al., 2010).

Mimeault et al. (2005) studied the human lipid regulator, gemfibrozil (GEM) and found that GEM can bioconcentrate in goldfish (*Carassius auratus*) plasma with a bioconcentration factor (BCF) of 113 after a 14 day waterborne exposure. Another more recent example was a study by Paterson and Metcalfe (2008) to determine the uptake and depuration kinetics of the anti-depressant fluoxetine in Japanese medaka (*Oryzias latipes*). Whole body levels of the drug were 80 times higher than its concentration in

water after seven days of exposure. Although pharmaceuticals are designed to affect and target a specific pathway and be eliminated rapidly (Trudeau et al., 2005), at least 92 of the 275 pharmaceuticals that have been detected in the environment and 58 of the high volume production pharmaceuticals that have never been reported in the environment are predicted to be bioaccumulative in biota based on quantitative structure activity relationships (QSARs) (Howard and Muir, 2011). Fluoxetine and its metabolite norfluoxetine have been detected in wild fish tissues at concentrations ranging from 1 – 10 µg/kg (Brooks et al., 2005; Chu and Metcalf, 2007). Similarly, EE2, the active ingredient in oral contraceptives, was taken up by roach (*Rutilus rutilus*) and detected in its gonads after a ten-day exposure to sewage effluent (Gibson et al., 2005). The bile of rainbow trout (*Oncorhynchus mykiss*) caged downstream from a wastewater treatment plant (WWTP) for two weeks contained 10^4 – 10^6 times higher concentrations of steroidal estrogens including EE2 than that of the effluents (Larsson et al., 1999). A BCF of 660 was determined for EE2 in fathead minnow (*Pimephales promelas*) (Läng et al., 2001) while the model by Lai et al. (2002) predicted that fish feeding at a low trophic level would bioaccumulate EE2 with an estimated bioaccumulation factor (BAF) of 332. Al-Ansari et al. (2010) (Chapter 3) demonstrated that EE2 was a bioaccumulative chemical in wild ShRHSs collected downstream from a WWTP in the St. Clair River.

The main objective of this study was to test the hypothesis of EE2 bioaccumulation in different fish species from the St. Clair River aquatic food-web. Since Al-Ansari et al. (2010) (Chapter 3) showed evidence of EE2 bioaccumulation in a bottom feeder species from the St. Clair River, it was necessary to expand the scope of the study to include both pelagic and benthic species in an effort to determine the range of EE2

accumulation in this food-web. Here I returned to the same site to collect fish samples from different trophic levels near sewage effluents, as well as sampling surface water and sediment. The collection included sites immediately downstream from sewage effluents as well as reference sites further downstream in the St Clair River. In addition to EE2 analysis in fish, sediment and water, we analyzed all fish samples for their total lipid content and their stable isotopic composition ($\delta^{15}\text{N}$ and $\delta^{13}\text{C}$). Total mercury (THg) analysis was also performed to test for any correlations between its well known biomagnification tendency to that of EE2. Our hypothesis was that EE2 whole fish tissue concentrations would correlate positively with $\delta^{15}\text{N}$ and lipid content. Furthermore, THg was used as a model bioaccumulative substance for comparison. To our knowledge, this was the first study to investigate EE2 biomagnification in the St. Clair River aquatic food-web.

4.3 Material and methods

4.3.1 Study site

The description of the study site (St. Clair River) was provided in Chapter 3 of this thesis.

4.3.2 Chemicals

All solvents and dry chemicals used for sample preparation in this study were of high purity. The ^{13}C labeled EE2 (99 % purity) used as an internal standard was purchased from Cambridge Isotope Lab., Inc. (Andover, MA, USA). Solvents including dichloromethane (DCM), methanol (MeOH), acetone and hexane, were Optima[®] grade

(Fisher Scientific, Brockville, ON, Canada) while acetonitrile and water were Chromsolv grade (Fluka, Mississauga, ON, Canada). Pentafluorobenzoyl chloride (PFBCl), nonane, anhydrous Na₂SO₄, and potassium hydroxide (KOH) were all from Sigma-Aldrich (St. Louis, MO, USA). Hydromatrix was from Varian Inc. (Mississauga, ON, Canada).

4.3.3 Sample collection

All fish, water, and sediment samples used in this study were collected from various locations on the St. Clair River (Figure 4.1A). The sampling sites around Corunna WWTP (Stag Island, SI) were the exposed sites, whereas two locations on Chenal Ecarte (CE) (Port Lambton, PL area) were the reference sites. All sample collections were performed in summer 2010 from July 13 – 16.

4.3.3.1 Fish

A total of 88 fish samples spanning seven different species were collected from the exposed and reference sites (Figure 4.1A) using either seine netting, minnow traps and rod and reel. Dog biscuits and locally purchased nightcrawlers were used for bait. Fork length, wet weight, and species of fish, were recorded on site (Table 4.1). Smaller individuals were pooled by species to contain a minimum of 20 g. Samples were then immediately frozen on dry ice. The species that were collected included round goby (RG) (*Neogobius melanostomus*) (n = 38), yellow perch (*Perca flavescens*) (YP) (n = 28), emerald shiners (*Notropis atherinoides*) (ES) (n = 16), ShRHS (n = 3), common carp (*Cyprinus carpio*) (CC) (n = 1), spottail shiner (*Notropis hudsonius*) (SS) (n = 1) and logperch (*Percina caprodes*) (LP) (n = 1).

4.3.3.2 Sewage effluent and surface water

Composite (4 L/day) sewage effluent samples were collected daily from Corunna STP between July 13th until 16th 2010. Total of three surface water samples were collected from sites around Stag Island and Chenal Ecarte. The first site was Stag Island East (SIE) where the STP diffuser pipe released the effluents into the river. The second site was Stag Island West (SIW), which was suspected to receive effluents from other STPs within the region (Figure 4.1B). The third water sample was taken from Chenal Ecarte (CE) at Port Lambton area (Figure 4.1C). All samples were collected in 4 L amber bottles previously washed with DCM. All samples were kept cold until processed in the field within few hours of collection.

4.3.3.3 Sediment samples

Three grab sediment samples were collected by Ekman dredge from each of the following sites: SIW, SIE, and CEN. After recording the wet weight, the samples were immediately frozen on dry ice until transferred to the laboratory for analysis.

4.3.4 Sample preparation

4.3.4.1 Fish

All fish samples were prepared for analysis as previously described (Al-Ansari et. al., 2011). Briefly, approximately 2 g of finely ground freeze-dried tissues were spiked with 50 ng ¹³C labeled EE2, extracted with accelerated solvent extractor (ASE-200) in 11 mL cells using 100 % dichloromethane (DCM) as an extraction solvent. Gel-permeation chromatography (GPC) was used to remove the lipid from the samples which were

further cleaned by an acetone precipitation step before it was further washed by hexanes and dried under a gentle stream of N₂.

4.3.4.2 WWTP effluents and surface water

All WWTP effluent and surface water samples were filtered by 1.2 µm Whatman GF/C microfibre filter and then divided in 1 L subsamples in amber bottles. Each subsample was extracted with a SupercleanTM Envi-18 solid phase extraction (SPE) cartridge (bed wt. 1 g, volume 6 mL, product # 505706; Sigma-Aldrich St. Louis, MO, USA). Using a 12-port manifold apparatus, the SPE cartridges were conditioned with 12 mL methanol (MeOH) followed by 12 mL HPLC water. Fifty µL of ¹³C EE2 (99 %; 500 ng/mL in nonane; Cambridge Isotope Lab., Inc., Andover, MA, USA) was spiked as an internal standard for each of the effluent subsamples whereas the same concentration of the internal standard was spiked in only one SPE cartridge. Samples were extracted at a flow rate of approximately 10 mL/min. After the extraction, the cartridges were kept wet and serial washes with 10 mL of HPLC water, 10 mL 5 % MeOH, and 10 mL 5 % acetone then samples were vacuum dried for 10 min and before applying the last wash step with 10 mL of hexanes and finally cartridges were dried for 30 min. The elution was done three volumes of 3 mL of 100 % MeOH for each effluent subsamples which were then combined and treated as one sample. To increase the chances of EE2 detection in surface water, subsamples of each surface water sample were pooled again after the elution. The elution solvent was evaporated under N₂ to dry the samples and store them at 4°C until ready for chemical analysis.

4.3.4.3 Sediment

Freeze-dried sediment samples (20 g) were mixed thoroughly with sodium sulfate (Na_2SO_4) placed in 11 mL ASE cells, spiked with 100 μL of ^{13}C EE2 (500 ng/mL in nonane) and extracted following the conditions described in Céspedes et al. (2004). The extraction was performed using DCM at 75 °C, 1500 psi; the heating time was 5 min and two cycles of static extraction, each for 5 min. As a final step the cell was purged with gaseous nitrogen. The total volume of the extract was ~20 mL per sample. The extracts were evaporated to approximately 3 mL by a Turbo Vap[®] II (Zymark Hopkinson, MA, USA). Samples were then filtered with by 0.45 μm PTFE and fractionated by GPC as described in Chapter 2 to remove sulfur. GPC fractions were evaporated to 0.5 mL and then loaded on 1 g Superclean[™] Envi-18 solid SPE cartridges to be cleaned-up and eluted after the serial washes and elution steps described in section (4.2.4.2).

4.3.4.4 GC/MS analysis

Prior to chemical analysis, all samples were derivatized and analyzed as described in Al-Ansari et al. (2011) (Chapter 2 of this thesis).

4.3.4.5 Total mercury analysis

Total mercury analysis was done as previously described by Choy et al. (2010). Briefly, 10-50 mg freeze-dried whole fish tissue was analyzed at the University of Ottawa using a Nippon Hg Analyser SP-3D (Nippon Instruments Corporation, Osaka, Japan).

All samples were run in duplicates. At the start of each run, duplicate blank runs preceded the samples, and at the end of each day, duplicate blank runs and a certified

reference material (DORM-3) certified reference material (0.382 ± 0.06 mg/kg; National Research Council of Canada, Ottawa, ON) were run for quality control. The mean recovery of DORM-3 was 97.5% (0.372.4 mg/kg) with a CV of 3 %.

4.3.4.6 Stable isotope analysis

Stable isotope ratio $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ analysis was performed to assess trophic position and carbon source of each species (Peterson and Fry, 1987; Bertrand et al., 2011). Sub-samples of ground whole fish were freeze-dried, finely homogenized, and analyzed on a Vario EL III Elemental Analyzer (Elementar, Germany) followed by gas chromatograph separation and on-line analysis by continuous-flow with a DeltaPlus Advantage isotope ratio mass spectrometer coupled with a ConFlo III (ThermoFinnigan, Germany). Data were normalized using internal standards previously calibrated with International standards IAEA-CH-6, IAEA-NBS22, IAEA-N1, IAEA-N2, USGS-40, USGS-41.

4.3.5 Statistical analysis

One-way analysis of variance (ANOVA) followed by Tukey's test was done to compare stable isotope ratios and THg among all fish species (Origin Lab 7 software, Origin Lab Corporation MA, USA). Site specific comparisons within each species were also performed. Simple linear regression analysis was also used to test for THg correlations with $\delta^{15}\text{N}$.

4.4 Results and discussion

4.4.1 Trophic levels and stable isotopes

The profile of $\delta^{15}\text{N}$ isotope ratios for the analyzed fish showed that all species ($n = 79$) were within the range of 6.53 ‰ to 10.94 ‰ (Figure 4.2). Delta ^{15}N analysis is usually used to verify the trophic position of an organism in food-web studies while $\delta^{13}\text{C}$ analysis is used to track potential carbon sources, whether pelagic or benthic, (Anderson and Cabana, 2007; Burgess and Hobson, 2006; Bertrand et al., 2011). In this study, the ShRHS were found to have the lowest $\delta^{15}\text{N}$ (Figure 4.2). As shown in Figure 4.2, separation of the $\delta^{15}\text{N}$ between the YP and the ES and ShRHS suggested a difference in trophic position of these species.

Mean $\delta^{15}\text{N}$ value comparison between species did not show significant differences between RG ($9.08 \text{ ‰} \pm 1.18$ standard deviation, $n = 33$) and YP ($9.43 \text{ ‰} \pm 0.56$, $n = 24$) (Figure 4.3). Similarly no differences were found between ES ($7.43 \text{ ‰} \pm 0.66$, $n = 16$) and ShRHS ($6.89 \text{ ‰} \pm 0.33$, $n = 3$). However, both RG and YP were statistically higher in $\delta^{15}\text{N}$ values than ES and ShRHS ($p < 0.001$, one-way ANOVA) (Figure 4.3). Although YP and ES were pelagic species, differences in the $\delta^{15}\text{N}$ signature could be due to feeding behaviors. Yellow perches feed primarily on large insects and invertebrates and also feed on other fish while ES feed on zooplankton and algae (Scott and Crossman, 1973). The difference in $\delta^{15}\text{N}$ between the bottom feeders RG and ShRHS may be related to differences in age or size and feeding behaviors (Burgess and Hobson, 2006). In the present study, the sampled ShRHSs were juvenile with $\delta^{15}\text{N}$ of $6.89 \text{ ‰} \pm 0.33$ whereas the mature ShRHSs from the same site reported in Al-Ansari et al. (2010)

(Chapter 3) had higher $\delta^{15}\text{N}$ ($10.15 \text{ ‰} \pm 1.36$, $n = 36$). Thus, mature RG and ShRHS could be feeding at a similar trophic level and have similar $\delta^{15}\text{N}$.

The $\delta^{13}\text{C}$ isotope ratios for the analyzed fish species ($n = 79$) were within the range of -23.39 ‰ to -18.11 ‰ (Figure 4.2). No clear distinction of the carbon source (i.e. pelagic vs. benthic) was seen in $\delta^{13}\text{C}$ between the RG ($-20.13 \text{ ‰} \pm 0.85$, $n = 33$) and the YP ($-20.1 \text{ ‰} \pm 1.32$, $n = 24$). In a study by Bertrand et al. (2011), YP demonstrated a gradient in $\delta^{13}\text{C}$ values which ranged between -38.4 ‰ near shore of Lake St. Pierre (Québec, Canada) to -13.1 ‰ toward the central river channel of the St. Lawrence River (Québec, Canada). Such wide variation in the YP $\delta^{13}\text{C}$ values was attributed to variable feeding strategies due to fish mobility (Bertrand et al., 2011). As shown in Figure 4.3, no statistically significant difference in $\delta^{13}\text{C}$ was found between RG, YP, and ShRHS whereas ES had significantly lower $\delta^{13}\text{C}$ values. Interestingly, all of the pelagic species including ES ($-21.67 \text{ ‰} \pm 1.03$, $n = 16$), SS (-20.03 ‰ , $n = 1$), and LP (-20.99 ‰ , $n = 1$) had a $\delta^{13}\text{C}$ value less than -20 ‰ with the exception of YP. Similarly, all of the benthic species including ShRHS ($-19.68 \text{ ‰} \pm 0.15$, $n = 3$) and CC (-18.59 ‰ , $n = 1$) had $\delta^{13}\text{C}$ value higher than -20 ‰ with the exception of RG.

4.4.2 Effect of sewage effluents on stable isotopes

Our stable isotope data suggested that nutrient-enriched sewage effluents can significantly affect the $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ isotopic signatures at least in one of the investigated species (Figure 4.4; Figure 4.5). However, the $\delta^{15}\text{N}$ data for RG were contrary to what we expected since Stag Island species affected by sewage effluents had higher $\delta^{15}\text{N}$ values ($9.4 \text{ ‰} \pm 1.18$, $n = 25$) than those collected from Port Lambton, the downstream

reference site ($8.06\text{‰} \pm 0.27$, $n = 8$). No difference was seen in $\delta^{15}\text{N}$ of YP between the two sites (Figure 4.4; Figure 4.5). However, the sewage effluent derived nutrients may have slightly affected the $\delta^{13}\text{C}$ ratio in YP. Interestingly, a $\delta^{15}\text{N}$ of ES was significantly higher at Stag Island compared to Port Lambton ($p < 0.001$) (Figure 4.4; Figure 4.5). Furthermore, a significant shift in $\delta^{13}\text{C}$ was observed between the two sites. Stag Island had $\delta^{13}\text{C}$ of ($-22.65\text{‰} \pm 0.67$, $n = 25$) whereas Port Lambton had $\delta^{13}\text{C}$ of ($-20.91\text{‰} \pm 0.43$, $n = 8$) ($p < 0.001$, one-way ANOVA). The low $\delta^{15}\text{N}$ values of ES we have seen in this study were consistent with previous work showing that invertebrates and fish from different trophic levels collected from sewage receiving site had lower $\delta^{15}\text{N}$ signals than from the reference site (deBruyn et al., 2003; Al-Ansari et al., 2010).

4.4.3 Total mercury

The THg concentrations of all fish samples ($n = 79$) analyzed in this study ranged from 2.04 to 121.28 ng/g (dw) (Figure 4.6). No statistically significant difference was observed in THg between RG ($11.89\text{ ng/g} \pm 4.98$, $n = 33$), YP ($12.14\text{ ng/g} \pm 3.61$, $n = 24$) and ES ($10.97\text{ ng/g} \pm 6.81$, $n = 16$) with the exception of ShRHS which had the lowest THg ($3.30\text{ ng/g} \pm 0.89$, $n = 3$) ($p < 0.05$, one-way ANOVA) (Figure 4.7). The clear difference in THg between the benthic species RG and ShRHS may indicate a possible biomagnification since there was a significant difference in trophic position among the two species based on their $\delta^{15}\text{N}$ values. No obvious difference was seen in THg in the pelagic species, YP and ES although their $\delta^{15}\text{N}$ signals were significantly different (Figure 4.7). The high variability in THg concentrations could have masked the biomagnification trend in the YP and the ES. Poor correlation between the $\delta^{15}\text{N}$ values

and the THg was observed in the RG ($R^2 = 0.08$, $p = 0.12$, $n = 33$) and the YP ($R^2 = 0.002$, $p = 0.85$, $n = 24$), suggesting that these individuals were likely feeding at similar trophic positions. In contrast, a significant correlation between $\delta^{15}\text{N}$ and THg was shown in ES ($R^2 = 0.63$, $p < 0.001$, $n = 16$) (Figure 4.8).

Separating the THg data for RG, YP, and ES based on sampling site showed no difference in the THg between RG ($11.84 \text{ ng/g} \pm 5.5$, $n = 25$) and YP ($10.31 \text{ ng/g} \pm 2.03$, $n = 16$). In contrast, ES ($6.03 \text{ ng/g} \pm 2.11$, $n = 7$) was had much lower THg in Stag Island samples than YP and RG ($p < 0.01$, one-way ANOVA) which may indicate THg biomagnification in the pelagic species YP and ES (Figure 4.9). No significant difference was found between RG, YP, and ES collected from Port Lambton.

Although Stag Island received industrial and municipal effluents from multiple sources (Mayne 2003), this site had significantly lower THg in YP ($p < 0.001$) and ES ($p < 0.01$) than the less industrialized Port Lambton site (Figure 4.9). Mercury is one of the environmental pollutants that has natural and anthropogenic sources (Cheng et al., 2011) and thus there could be other unidentified sources in the Port Lambton area that may have contributed to higher THg. In general, THg concentrations in fish tissue were lower or within the range of previously published data reporting THg in different fish species from various locations (Swanson et al., 2011; Cheng et al., 2011; Wyn et al., 2010; Burgess and Hobson, 2006). The low THg may reflect the effort to control and reduce the emissions of environmental contaminants including THg in St. Clair River as an area of concern (Mayne, 2003).

4.4.4 Ethinylestradiol

The effluents of Corunna sewage contained up to 40 pg/L of EE2 during the consecutive four day sampling (Table 4.2). Ethinylestradiol was detected at 10 pg/L in both the first and the second day of sampling and 40 pg/L for the fourth day. However, no EE2 was detected in the third day sample. Two river water samples collected from two sites close to the Stag Island contained 80 pg/L EE2 for SIE and 70 pg/L EE2 for SIW. The low concentrations of EE2 in the river water was most likely due to the dilution factor resulting from the fast flow of the river which averaged 5153 m³/s (Healy et al., 2007). Our data were consistent with the Belfroid et al. (1999) study where EE2 concentrations in surface water from the Netherlands were below their EE2 MDLs (0.3 ng/L) which was identical to our EE2 MDLs. Ethinylestradiol levels ranging from 2.21 – 0.07 ng/L were reported in a previous study in our laboratory (Atkinson, 2008). Improvements to the Corunna WWTP, which were in progress during our sampling, could have resulted in an improved removal efficiency of steroidal estrogens including EE2. Thus, reduced concentrations in final effluents would be anticipated.

Although EE2 was detected in both the river water and the final effluents at extremely low levels, EE2 was not detected in the analyzed sediment samples (n = 9) that were collected from both the exposed Stag Island site and the reference Port Lambton site. Furthermore, EE2 was not detected in any fish sample from any sampling site. Although careful analysis was performed, we did not find a single sample showing any measurable EE2 except the adult ShRHS from the same site in 2002 at approximately 1.53 ng/g wet weight (Al-Ansari et al., 2010). Therefore, THg food-web bioaccumulation comparison to that of EE2 was not applicable (Figure 4.10).

The EE2 mass balance model of Lai et al. (2005) suggested that EE2 could have a BAF reaching 330. Such bioaccumulation was predicted to be restricted to the benthic food-webs. In other words, EE2 food-web bioaccumulation was not predicted by the model which may explain why EE2 was not seen in species like ES and YP. Bertrand et al. (2011) showed that YP were feeding and moving within 2 km along the shoreline of Lake St. Pierre (Québec, Canada) by using $\delta^{13}\text{C}$ values which clearly varied in a spatial manner. In contrast, the variability in $\delta^{13}\text{C}$ values of YP, RG, ES and ShRHS in this study was minor (Figure 4.3) and $\delta^{13}\text{C}$ spatial variation was clear in YP and ES (Figure 4.4) and therefore the fish were expected to be feeding within no more than 2 km of the capture site.

A recent report by Liu et al. (2011) demonstrated that EE2 was bioaccumulative in three wild-fish species collected from a Chinese lake. The study suggested that EE2 whole body levels can reach 11.3 ng/g dw in silvery minnows (*Anabarilius alburnops*). The tissue distribution of EE2 in the crucian carp (*Carassius auratus*) and carp (*Cyprinus carpio*) showed that the highest bioaccumulation in the liver followed by gill and muscle samples (Liu et al., 2011). Despite the fact that EE2 was not shown to biomagnify in this study, Liu et al. (2011) supported our previous finding of EE2 bioaccumulation in free living wild fish (Al-Ansari et al., 2010).

4.5 Conclusions

This study found evidence of EE2 bioaccumulation only in ShRHS, however no food-web transfer of EE2 was evident in the St. Clair River fish. Although our $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ stable isotope data suggested that species selection was suitable to test EE2 food-

web transfer hypothesis, with the exception of the ShRHS from 2002 sampling all of the tested species did not contain any detectable EE2 despite surface water and sewage effluents having very low levels of EE2. Therefore, we were not able to conduct any correlations between EE2 as a potential bioaccumulative chemical with mercury, a well-characterized bioaccumulating substance. This conclusion was in agreement with the predictions of Lai et al. (2002) suggesting that EE2 biomagnification was unlikely to occur, however, bioaccumulation in the benthic food-web was possible.

4.6 Acknowledgements

This research was supported by the Natural Sciences and Engineering Research Council of Canada (Strategic Grants to J. Blais and V.L. Trudeau), Canadian Water Network (V.L. Trudeau), the scholarship fund (for Al-Ansari A.M.) from King Abdulaziz University, Jeddah, Saudi Arabia, and the Great Lakes Action Plan.





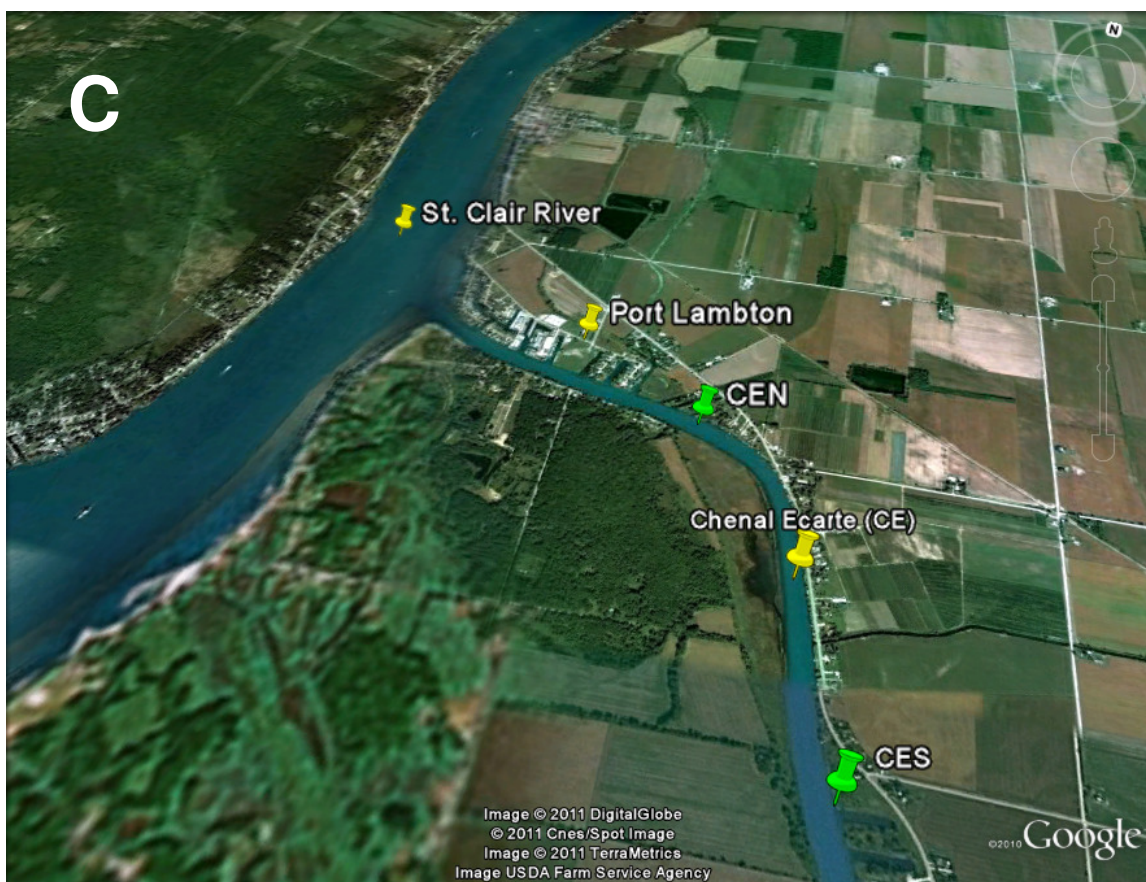


Figure 4.1: A) an aerial view of the St. Clair River (64 km) flowing from Lake Huron south to Lake St. Clair. B) A closer zoom showing Stag Island, Corunna STP, and the exposure site sampling locations SIE: Stag Island East and SIW: Stag Island West. C) Shows the reference site Chenal Ecarte (CE) sampling locations CEN: Chenal Ecarte North and CES: Chenal Ecarte South (Google Earth, 2011).

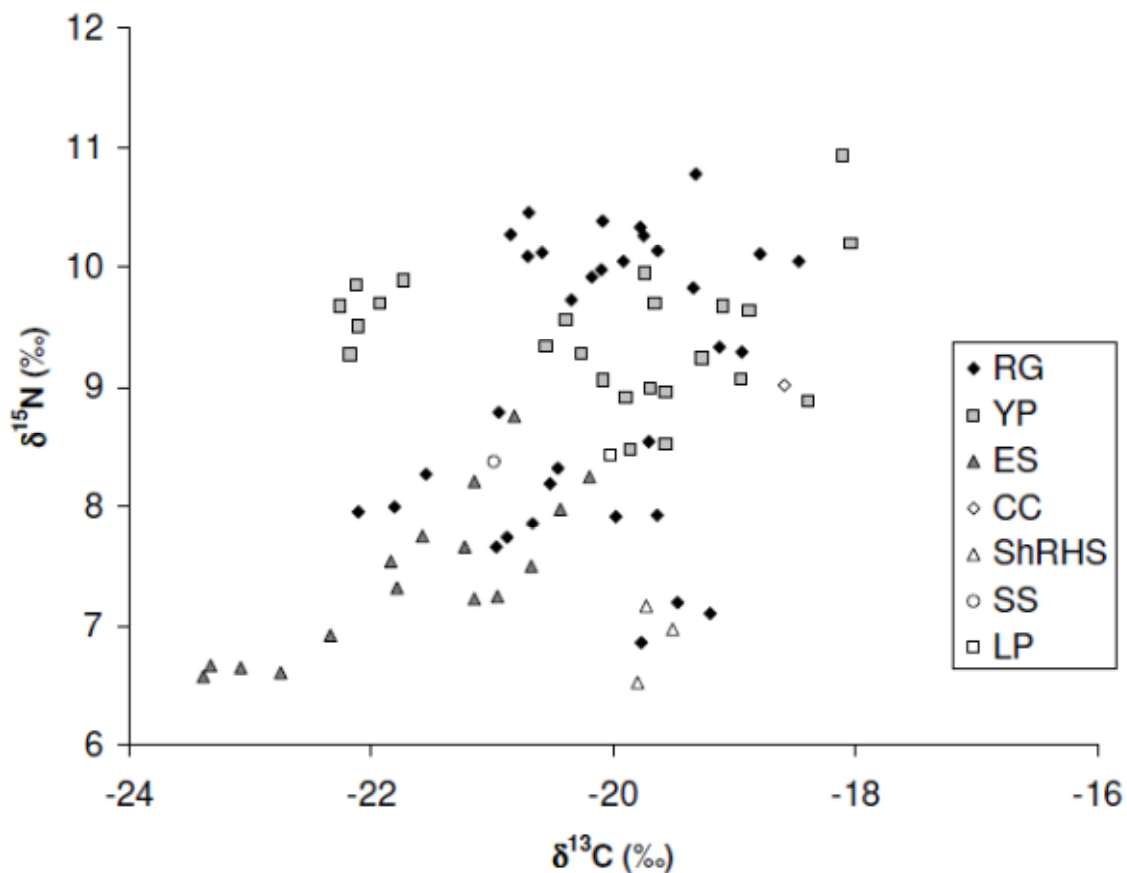


Figure 4.2: $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ isotope values (‰) showing food-web structure of several fish species from the St. Clair 2010 (n = 79). Filled symbols represent samples collected from Stag Island (exposed) and Port Lambton (reference) whereas open symbols represent samples collected only from one site. RG, round goby; YP, yellow perch; ES, emerald shiner; CC, common carp; ShRHS, shorthead redhorse sucker; SS, spottail shiner; LP, logperch.

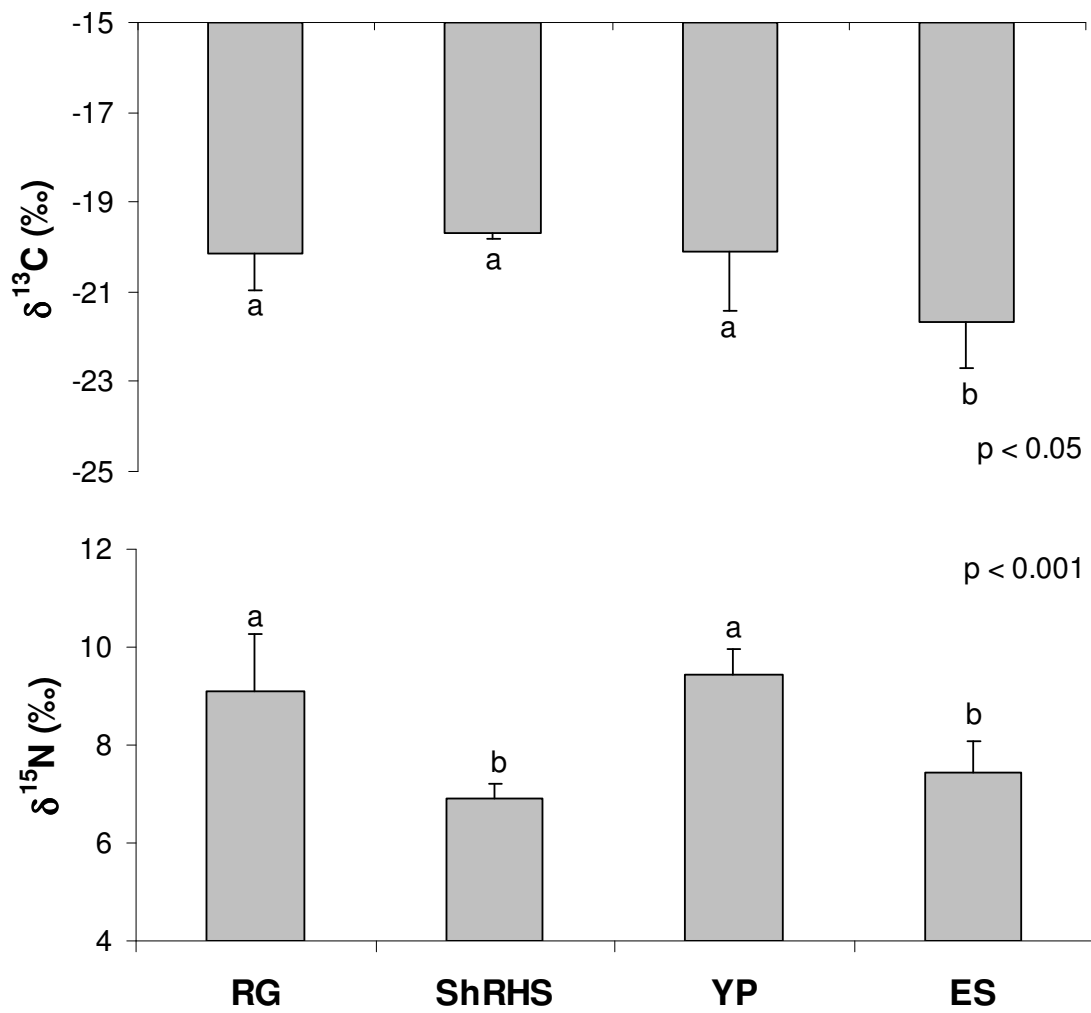


Figure 4.3: Mean $\delta^{13}\text{C}$ (‰) $\pm$ SD (upper panel) and mean $\delta^{15}\text{N}$ (‰) $\pm$ SD (lower panel) plotted against different species. Groups that do not share the same letters are statistically different (one-way ANOVA). RG: round goby (n = 33), ShRHS: shorthead redhorse sucker (n = 3), YP: yellow perch (n = 24), ES: emerald shiner (n = 24).

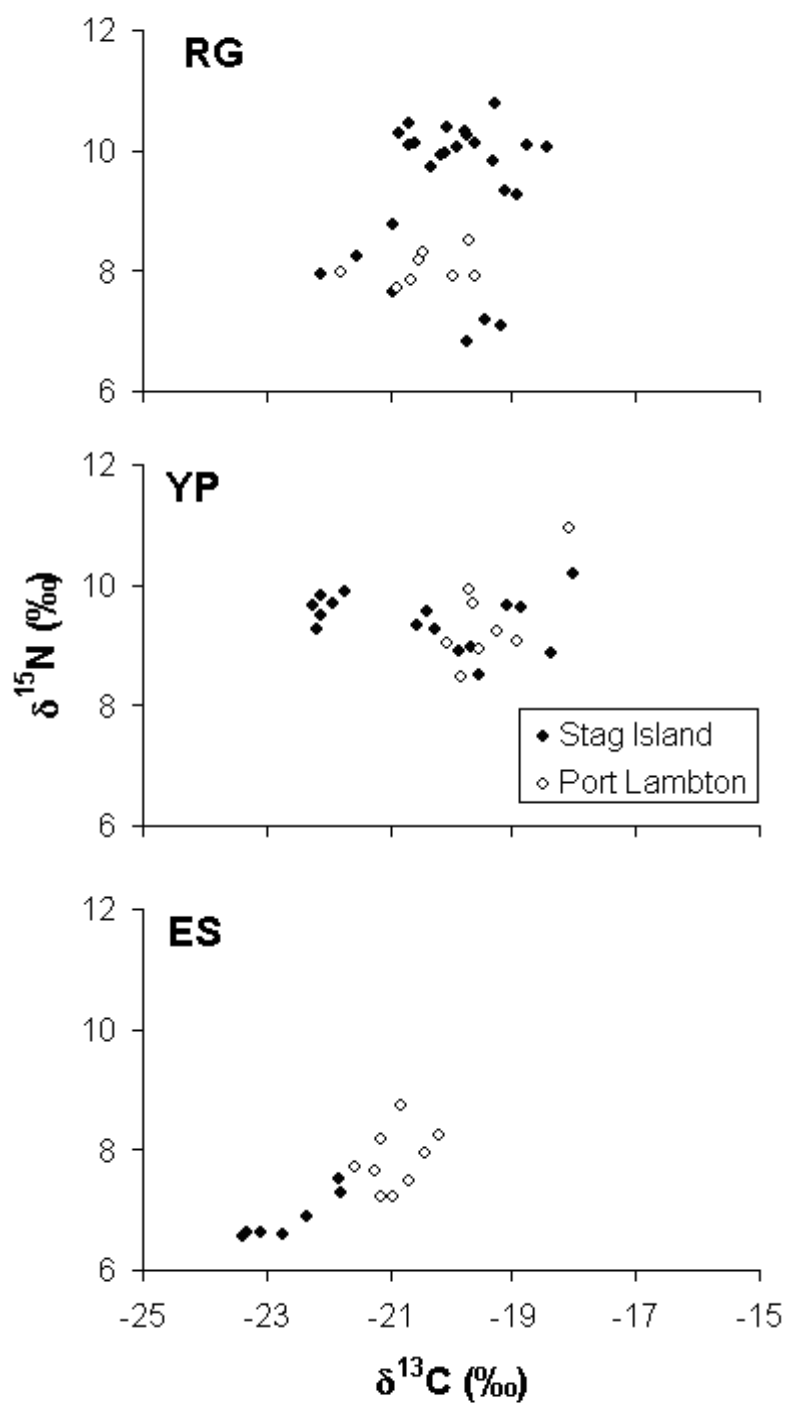


Figure 4.4: Spatial variations in $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ isotope values (‰) within species showing the difference between the exposed site (Stag Island) and reference site (Port Lambton).

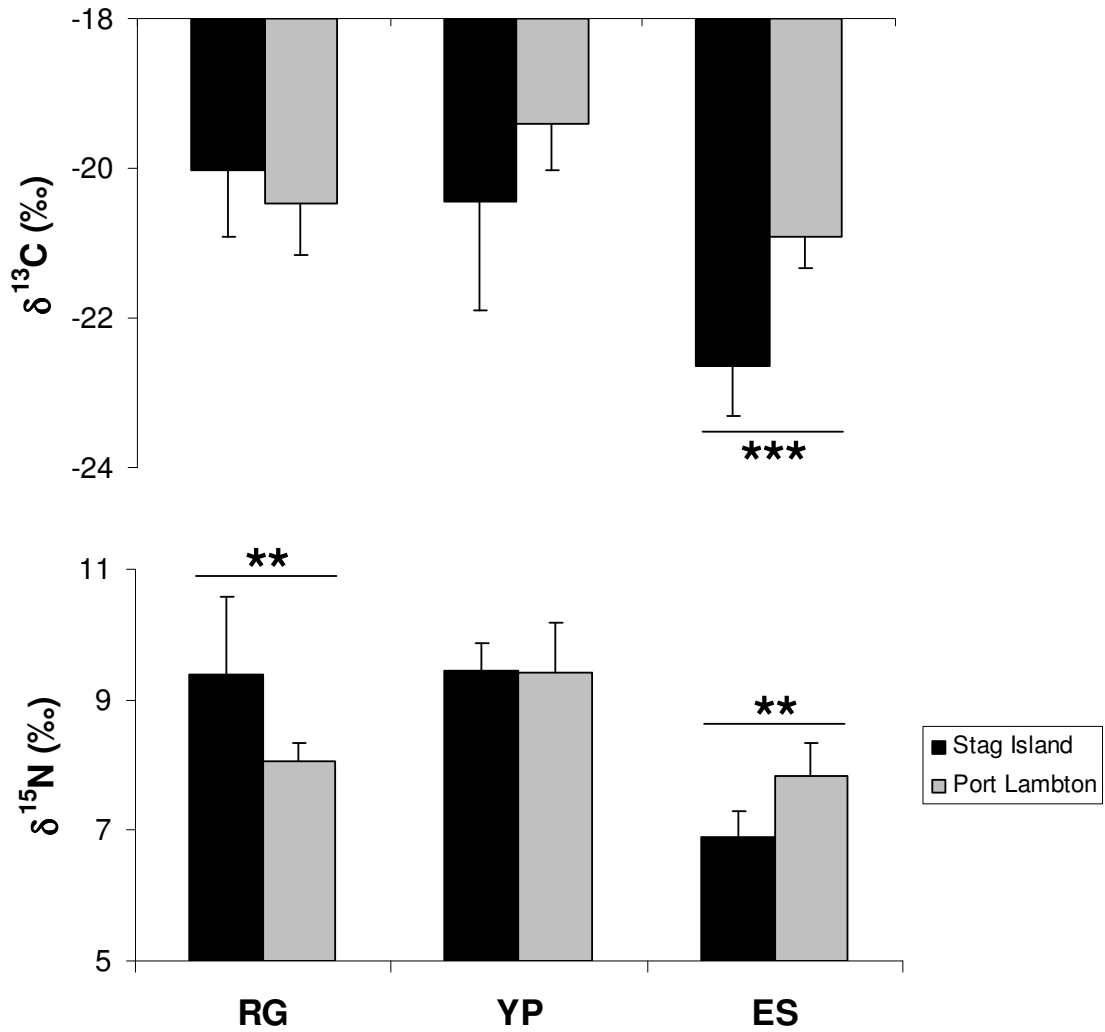


Figure 4.5: Mean $\delta^{13}\text{C}$ (‰) $\pm$ SD (upper panel) and mean $\delta^{15}\text{N}$ (‰) $\pm$ SD (lower panel) showing the difference within each species from Stag Island (exposed site) compared to Port Lambton (reference site) (one-way ANOVA). Double asterisk (**) indicate $p < 0.01$, (***) $p < 0.001$. RG: round goby (Stag Island, $n = 25$; Port Lambton, $n = 8$), YP: yellow perch ($n = 16$ and 8), ES: emerald shiner ($n = 7$ and 9).

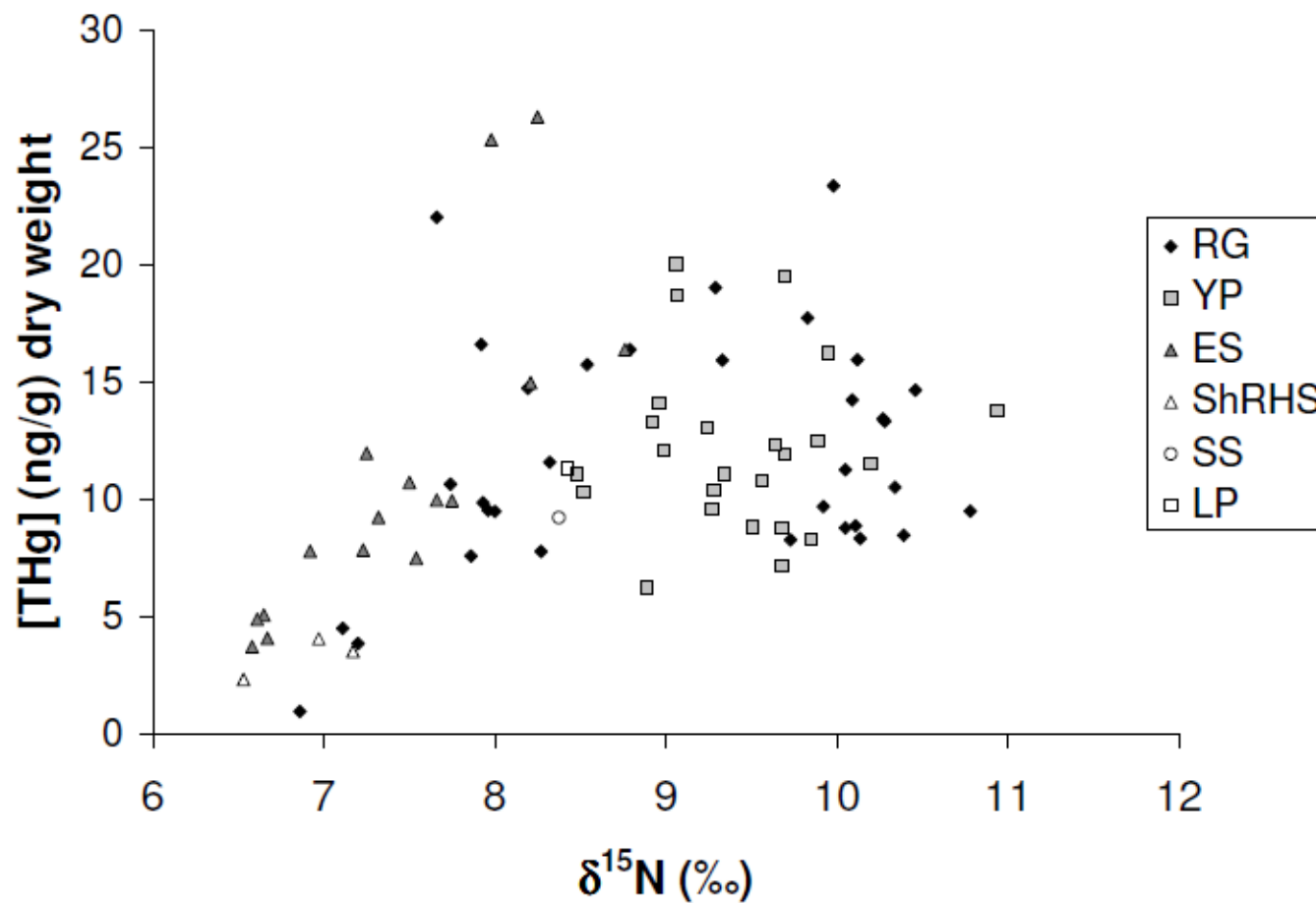


Figure 4.6: A food-web correlation between $\delta^{15}\text{N}$ (‰) values and THg levels (ng/g) dry weight in several fish species from the St. Clair 2010. Filled symbols represent samples collected from Stag Island (exposed) and Port Lambton (reference) whereas open symbols represent samples collected only from one site. RG, round goby; YP, yellow perch; ES, emerald shiner; ShRHS, shorthead redhorse sucker; SS, spottail shiner; LP, logperch.

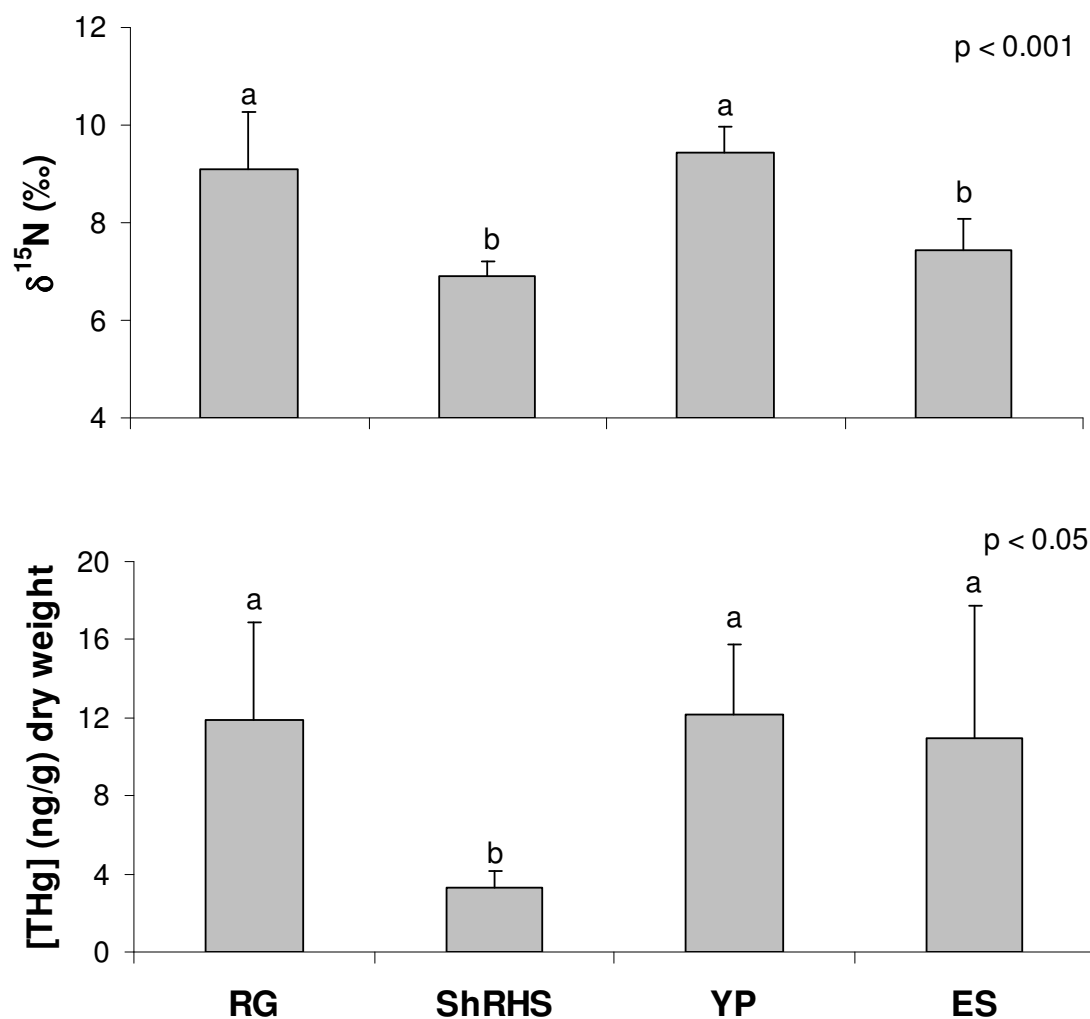


Figure 4.7: Mean $\delta^{15}\text{N}$ (‰) $\pm$ SD (upper panel) and mean THg (ng/g dw) $\pm$ SD (lower panel) plotted against different species. Groups that do not share the same letters are statistically different (one-way ANOVA). RG: round goby (n = 33), ShRHS: shorthead redhorse sucker (n = 3), YP: yellow perch (n = 24), ES: emerald shiner (n = 24).

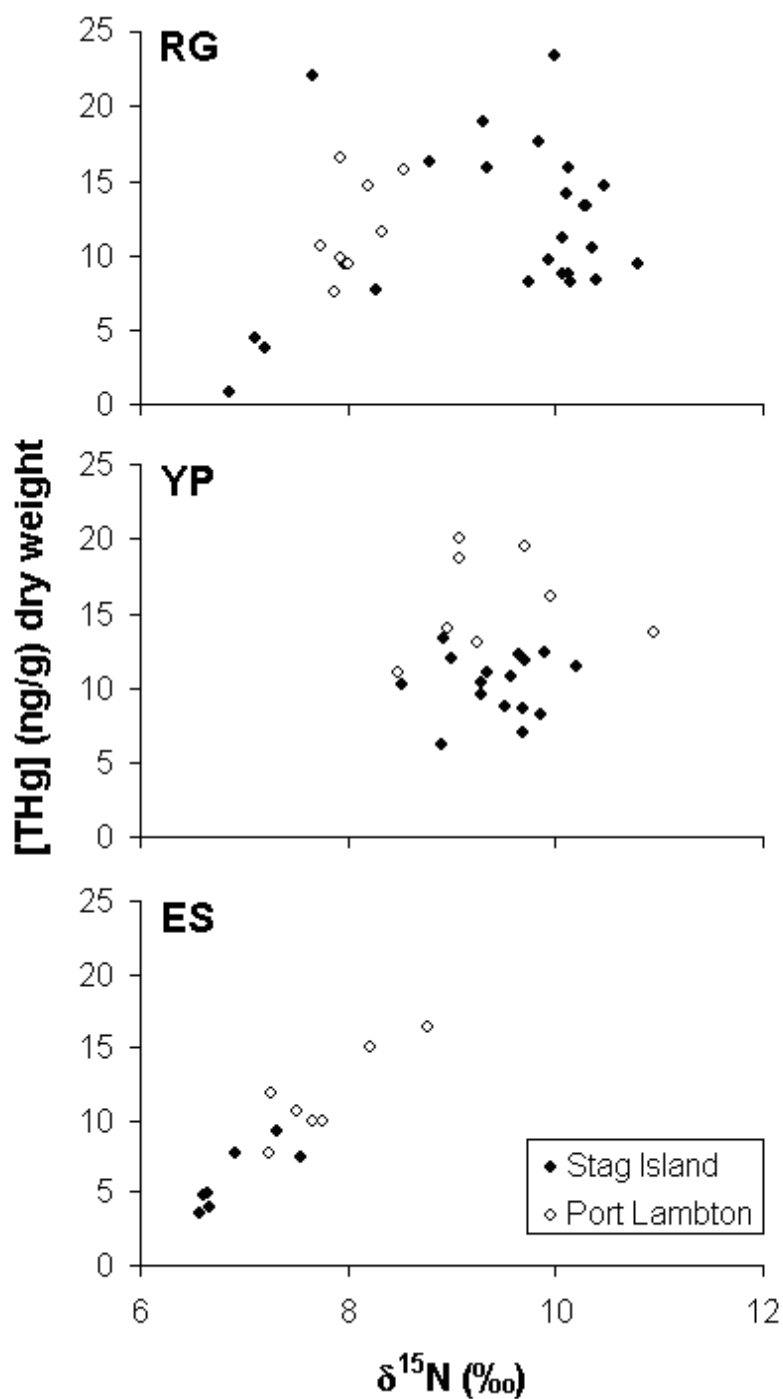


Figure 4.8: $\delta^{15}\text{N}$ (‰) values and THg levels (ng/g) dry weight relationships showing the difference between the exposed site (Stag Island) and reference site (Port Lambton) within three different species. RG, round goby ($R^2 = 0.08$, $p = 0.12$, $n = 33$). YP, yellow perch ($R^2 = 0.002$, $p = 0.85$, $n = 24$). ES, emerald shiner ($R^2 = 0.63$, $p < 0.001$, $n = 16$).

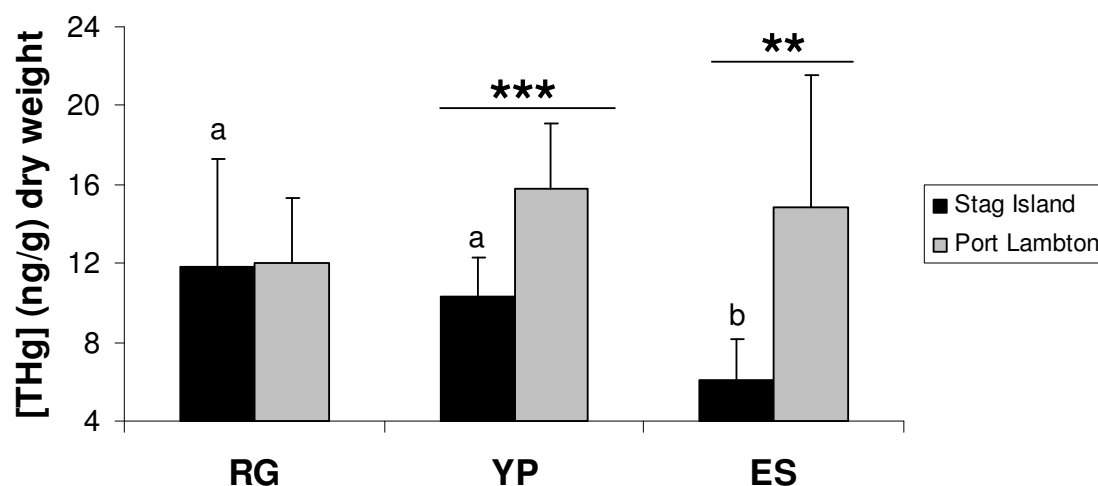


Figure 4.9: Mean THg (ng/g dw) $\pm$ SD (upper panel) the difference within each species from Stag Island (exposed site) compared to Port Lambton (reference site) as indicated by asterisk. Double asterisk (**) mean $p < 0.01$, (***) mean $p < 0.001$ (one-way ANOVA). Significant differences between species within a site are indicated by different letters. RG: round goby (Stag Island, $n = 25$; Port Lambton, $n = 8$), YP: yellow perch ($n = 16$ and 8), ES: emerald shiner ($n = 7$ and 9).

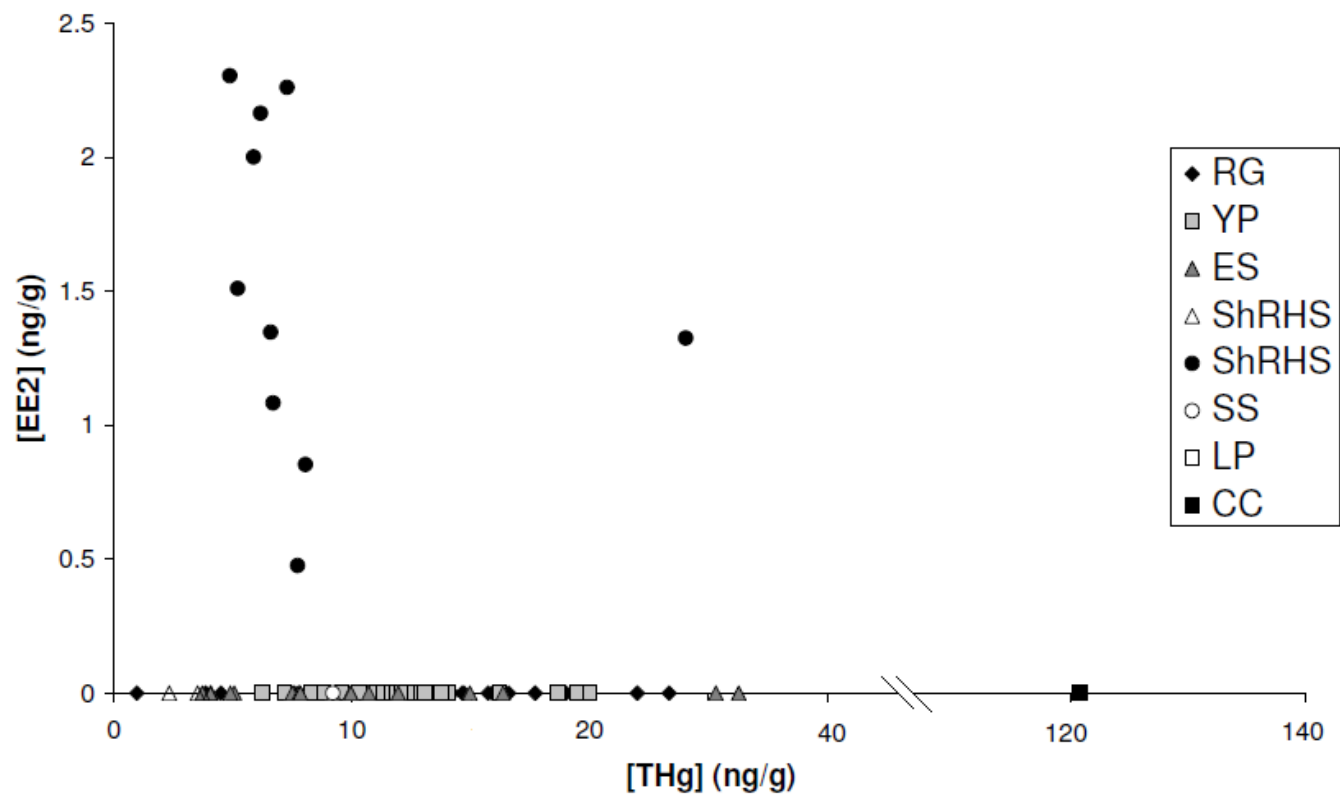


Figure 4.10: The relationship between EE2 and THg in all fish samples collected from the St. Clair River in 2010 and the ShRHS samples in dark circles collected in 2002.

Table 4.1

Field data including sample size, fork length, and wet weight for all fish collected from St. Clair River in mid July 2010

Species	Site	n	Fork length (mean $\pm$ SD, cm)		Wet weight (mean $\pm$ SD, g)	
RG	SIE	27	11.13	(1.72)	22.32	(7.92)
	SIW	3	n/a	(n/a)	22.0	(2.6)
	CEN	4	9.6	(n/a)	19.0	(5.42)
	CES	4	10.28	(2.63)	25.1	(5.05)
YP	SIE	4	12.8	(0.93)	22.75	(5.25)
	SIW	15	13.19	(3.68)	42.83	(21.89)
	CEN	7	11.61	(1.85)	19.67	(5.06)
	CES	2	10.75	(3.46)	18.75	(14.5)
ES*	SIW	7	n/a	(n/a)	24.0	(0.0)
	CES	9	n/a	(n/a)	23.67	(1.87)
CC	SIW	1	46.0	(n/a)	1294	(n/a)
ShRHS	SIW	3	16.0	(1.0)	11.17	(0.46)
SS	SIW	1	10.7	(n/a)	18.0	(n/a)
LP	SIW	1	22.0	(n/a)	n/a	(n/a)

Abbreviations: n/a, not available; RG, round goby; YP, yellow perch; ES, emerald shiner; CC, common carp; ShRHS, shorthead redhorse sucker; SS, spottail shiner; LP, logperch; SD, standard deviation; SIE, Stag Island East; SIW, Stag Island West; CEN, Chenal Ecatre North; CES, Chenal Ecatre South. A single asterisk (*) indicates pooled samples to reach minimum of 20 g wet weight.

Table 4.2

EE2 levels in the environmental samples collected from the St. Clair River in summer 2010.

Sample	Site	n	Extracted amount L or g	[EE2] (SD) pg/L or ng/g
<u>WWTP effluents</u>				
	SIE	4	4	15 (17.32)
<u>Surface water</u>				
	SIE	1	4	80
	SIW	1	4	70
	CEN	1	4	ND
<u>Sediment</u>				
	SIE	3	20	ND
	SIW	3	20	ND
	CEN	3	20	ND
<u>Fish</u>				
RG	SIE	27	2	ND
	SIW	3	2	ND
	CEN	4	2	ND
	CES	4	2	ND
YP	SIE	4	2	ND
	SIW	15	2	ND
	CEN	7	2	ND
	CES	2	2	ND
ES	SIW	7	2	ND
	CES	9	2	ND
CC	SIW	1	2	ND
ShRHS	SIW	3	2	ND
ShRHS*	SIE	20	20	1.53 (0.63)
ShRHS*	CES	16	20	ND
SS	SIW	1	2	ND
LP	SIW	1	2	ND

Abbreviations: ND, not detected; RG, round goby; YP, yellow perch; ES, emerald shiner; CC, common carp; ShRHS, shorthead redhorse sucker; SS, spottail shiner; LP, logperch; SD, standard deviation; SIE, Stag Island East; SIW, Stag Island West; CEN, Chenal Ecatre North; CES, Chenal Ecatre South. ShRHS with asterisk (*) were from 2002 field work (Al-Ansari et al., 2010).

Chapter 5

Dynamics of uptake and elimination of 17 α -ethinylestradiol in male goldfish

(*Carassius auratus*)

Chapter to be submitted as: Ahmed M. Al-Ansari, Jamie R. Doyle, Vance L. Trudeau,

Jules M. Blais

5.1 Abstract

We used male goldfish (*Carassius auratus*) as a model species to determine the uptake, elimination, and bioaccumulation of 17 α -ethinylestradiol (EE2). Goldfish were exposed to EE2 via two different routes: water (150 ng/L, nominal) for 72 h and food (250 ng/g, nominal) for 120 h. Whole tissue EE2 levels revealed a rapid uptake of EE2 in goldfish, and reached 8.43 ng/g ($\pm$ 1.22, standard deviation, dry weight) after the first hour of exposure with an uptake rate constant (k_1) of 45 h⁻¹. The elimination rate constant (k_2) was 0.0786 h⁻¹. In the feeding experiment, EE2 concentrations increased rapidly, reaching an average of 1.4 ng/g after 3 h following the first dietary exposure with estimated assimilation efficiency (α) of 0.106. An average of (2.66 ng/g $\pm$ 0.87, n=8) was determined in the 24–72 h samples, whereas EE2 concentrations in fish were below our method detection limits (0.67 ng/g) for all samples after 72 h. The calculated EE2 bioconcentration factor (BCF) for the water exposure was 377. A good fit was observed between the experimental and modeled data. The modeling was critical to predict the

extent by which EE2 bioaccumulation would occur at variable environmental concentrations. Our data suggested that EE2 can be rapidly taken up by fish via gill absorption and gastrointestinal absorption. To our knowledge, this was the first report to describe EE2 uptake and elimination dynamics, as well as providing important data to model EE2 kinetics following both water and dietary exposures in goldfish.

Keywords: 17 α -ethinylestradiol, uptake and elimination, goldfish (*Carassius auratus*)

5.2 Introduction

The synthetic steroidal estrogen, 17 α -ethinylestradiol (EE2) is used as an oral contraceptive to inhibit ovulation (Pérez-Campos, 2010), and in hormone replacement therapy to treat postmenopausal disorders (Notelovitz 2006). EE2 is ranked 14th among the top 200 prescribed pharmaceuticals (Prescription drug info., 2011). Total worldwide annual production and consumption of EE2 was estimated to be around 907.18 kg (Vosges et al., 2008).

DiLiberti and colleagues (2011) observed a rapid absorption of EE2 (30 μ g) given orally to human subjects. EE2 concentrations in plasma peaked after 2 h and declined to minimal concentration by 24 h with a half-life of approximately 18 h. Variability in EE2 half-lives among individuals may vary between 6-27 h (Pérez-Campos, 2010). In rabbits, EE2 reached its maximum concentration in the plasma only after 9.5 min and declined to the minimal concentration by 250 min with a half-life of approximately 40 min after an oral dose of 1 mg/kg EE2 via food (Fernandez et al., 1998). Schultz and colleagues (2001) demonstrated a positive correlation between plasma EE2 concentration and its half-life after an intra-arterial administration in male rainbow trout. The reported half-lives were 132, 30, and 20 h after dosing trout with 10, 1, and 0.1 mg/kg respectively. The literature lacks studies addressing EE2 uptake and elimination kinetics in fish after environmentally relevant exposures such as exposure via water and/or food.

Toxins and chemicals including steroidal estrogens can enter fish through different routes such as gills and/or skin via ambient water and also gastrointestinal absorption via food ingestion (Lien and McKim, 1993). Fish populations living downstream from sewage treatment plants may be chronically exposed to low nanogram

per liter/gram concentrations of EE2 and natural steroidal estrogens such as estradiol (E2) and estrone (E1) (Flores-Valverd et al., 2010) via water (Pereira et al., 2011; Khanal et al., 2006), algae (Della Greca et al., 2008), microorganisms (Cailleaud et al., 2011; Dussault et al., 2010), and sediment (Peng et al., 2006). Such environmental exposures may result in appreciable EE2 bioaccumulation in wild-fish as observed for shorthead redhorse suckers (*Moxostoma macrolepidotum*) sampled near a sewage treatment plant on the St. Clair River, Canada (Al-Ansari et al., 2010; Chapter 3), and in three different species (Crucian carp; *Carassius auratus*, common carp; *Cyprinus carpio*, and silvery minnows; *Anabarilius alburnops*) from Dianchi Lake in China (Liu et al., 2011a). Previous reports (Gibson et al., 2005; Larsson et al., 1999) on the concentrations of steroidal conjugates in the bile indicated that estrogens were taken up by fish via WWTP effluents and water respectively. Estrone, E2, and EE2 were found to bioaccumulate in rainbow trout (*Oncorhynchus mykiss*) and roach (*Rutilus rutilus*) bile after exposure to sewage effluents for 10 days (Gibson et al., 2005). Similarly, caged juvenile rainbow trout (*Oncorhynchus mykiss*) downstream of a STP were found to bioaccumulate steroidal estrogens in their bile 10^4 - 10^6 times higher than the water levels (Larsson et al., 1999). Skillman et al. (2006) briefly investigated EE2 uptake in male rainbow trout exposed to 125 ng/L for 61 days and found rapid uptake of EE2 from water and equilibrium within 16 h in the plasma. However, no toxicokinetic analysis was associated with their study.

The rapid uptake of EE2 by fish is likely linked to its high octanol water partitioning coefficient ($\log K_{ow}$). Compared to its natural analogues, EE2 has the highest $\log K_{ow}$ (4.15) and the lowest solubility in water (4.8 mg/L). Such important

physiochemical properties place EE2 among those compounds suspected to bioconcentrate rapidly in biota and in fish in particular (Lai et al., 2002).

In order to evaluate EE2 bioaccumulation in fish, and whether that bioaccumulation may lead to its biomagnification in the food-web, it is critical to understand its basic kinetics, not only from aqueous exposure but also from dietary exposure because no such data are yet available. Therefore, the chief goal of this study was to establish some key kinetic parameters such as uptake (k_1) and elimination (k_2) rate constants and the assimilation efficiency for EE2, and also determine its BCF in a model species like goldfish to help us better understand the potential environmental fate of EE2 as one of the most critical and potent endocrine disruptors in the aquatic environment.

5.3 Material and methods

5.3.1 Experimental animals

Goldfish (*Carassius auratus*) were obtained from a commercial fish supplier (Aleong's International, Mississauga, ON). Upon arrival, fish were acclimated at $18 \pm 1^\circ\text{C}$ in 70-L holding tanks, under a natural simulated photoperiod typical of Ottawa Canada in May, and fed with standard floating trout pellets for three weeks prior to experimentation. Total of 63 mature male goldfish were used in all experiments. The fish averaged 24.8 g wet weight $\pm$ (4.5) ($n = 18$) for the food uptake experiment. In the water exposures, the fish averaged 24.8 g wet weight $\pm$ (5.9) ($n = 24$) for the uptake phase and 17.6 g wet weight $\pm$ (2.19) ($n = 21$) for the depuration phase. All experiments were conducted under

a protocol approved by the University of Ottawa Animal Care Protocol Review Committee.

5.3.2 Exposure and experimental set-up

5.3.2.1 EE2 uptake experiments

Two experiments were conducted separately, uptake via water only (150 ng/L of EE2) and uptake via food only (250 ng/g). The feeding exposure was performed in May 2010 while the water exposure was done in May 2011.

The flow-through exposure system was operated by Masterflex[®] pumps (model # 7524-50; Cole Parmer, USA) and set-up as illustrated in Figure 5.1. A stock of EE2 (35.35 µg/L) was prepared in MilliQ and 4.6 mL/L of 95 % ethanol. The final volume of ethanol in the whole exposure system never exceeded 0.00001 %. The hormone stock was kept mixing gently with a stirring bar and delivered constantly at 0.001 L/min to a mixing tank which also received clean water from an adjunct tank at 0.209 L/min. The high flow rate of the clean water and the gentle air flow helped EE2 to mix well in the mixing tank to reach the target concentration of 150 ng/L. The mixing tank was allowed to reach minimum volume of 50 L before its water containing the test compound was pumped into a third tank (15 L volume, 0.210 L/min) designated for the exposure using a. The exposure system was equilibrated for a minimum of three days prior to the beginning of the exposure. Consequently, fish were exposed for 0, 1, 3, 6, 12, 24, 48, and 72 h. For each time interval triplicate fish were exposed. The exposure was designed this way to insure a constant concentration of EE2 in the presence of fish.

For the feeding experiment, EE2 was dissolved in 99 % ethanol and mixed with fish food to achieve 250 ng/g fish food. Ethanol was evaporated completely in a dessicator at ambient room temperature prior to the dietary exposure. Fish were exposed to EE2 by feeding them 3 meals per day (09:00, 13:00 and 17:00) achieving a total of 3 % body weight per day. The target amount of EE2 was a total of 150 ng per fish per day. Prior to the beginning of the exposure, fish were offered the same amount of food without EE2 daily for 7 days. Subsequently, fish were exposed to EE2 via diet, and duplicate fish samples were withdrawn from the tank at 0, 3, 6, 12, 24, 48, 72, 96, and 120 h. Time zero fish were sacrificed prior to the first EE2 exposure. The water was pumped into the dietary exposure tank at a flow rate of 0.0729 L/min to replenish the tank with fresh clean water every 16 h.

5.3.2.2 EE2 depuration experiment

The depuration experiment was performed in December 2011 with the same water exposure set-up described above (Figure 5.1) with an additional tank designed similar to the exposure tank and containing clean water to allow the fish depurate EE2. Prior to the depuration time course, all fish were exposed to EE2 via water for 24 h with the same uptake exposure approach and then depuration lasted 0, 3, 6, 12, 24, 48, and 72 h.

At the end of each time interval in all EE2 uptake and depuration experiments, fish were anaesthetized and sacrificed, and the gastrointestinal tract of each was removed as previously described in Al-Ansari et al. (2011). The general health of the fish was observed to be good during the entire study.

5.3.3 Lipid determination

Total lipid content was determined gravimetrically samples as described in Al-Ansari et al. (2010).

5.3.4 Water sampling and extraction

The EE2 concentration was monitored in both the mixing tank (without fish) and the exposure tank (with fish) during the uptake and depuration experiments. Triplicate 0.25 L water samples were collected in 250 mL amber glass bottles from the mixing tank prior to each time point. Another set of triplicate water samples were taken from the mixing, the exposure, and the depuration tanks after each time point. During the dietary exposure, total of three (1 L) water samples were collected at 24, 72, and 120 h from the exposure tank to determine whether EE2 leaked from the food into water. Consequently, samples were filtered on 1.2 μ m Whatman GF/C microfibre filters and extracted with SupercleanTM Envi-18 solid phase extraction (SPE) cartridges (bed wt. 1 g, volume 6 mL, product # 505706; Sigma-Aldrich St. Louis, MO, USA). Using a 12-port manifold apparatus, the SPE cartridges were conditioned with 12 mL methanol (MeOH) followed by 12 mL HPLC water. 50 μ L (500 ng/mL in nonane) of ¹³C EE2 (99 %) (Cambridge Isotope Lab., Inc., Andover, MA, USA) was spiked as an internal standard. Samples were extracted at a flow rate of approximately 10 mL/min. After extraction, the cartridges were kept wet and serial washes with 10 mL of HPLC water, 10 mL 5 % MeOH, and 10 mL 5 % acetone were applied before they were vacuum dried for 30 min. The elution was done by 100 % MeOH (3 $\times$ 3mL washes), and samples were then dried under N₂ until ready for chemical analysis. Average percent recovery was 99 % $\pm$ (1.37). Concentration of

EE2 in water was determined from 1 L aliquots of water taken from the exposure tank after 24, 72, and 120 h. Samples were prepared as described above.

5.3.5 Tissue extraction

The sample preparation for EE2 extraction and analysis from whole fish tissues followed an optimized protocol described in detail elsewhere (Al-Ansari et al., 2011; Chapter 2). Briefly, 2 g dry tissue was extracted with DCM by an accelerated solvent extractor (ASE-200, Dionex Corporation, Sunnyvale, CA, USA) in 11 mL extraction cells. Extracts were concentrated and fractionated by gel-permeation chromatography to remove excess lipids. Samples were further cleaned by removing cholesterol by an acetonitrile precipitation step before they were derivatized with pentafluorobenzoyl chloride and then analyzed by GC/MS.

5.3.6 Chemical analysis

The pentafluorobenzoyl-derivative of EE2 from water, fish tissue, and fish food samples was separated and detected by GC/MS. The analysis was performed on an Agilent 6890 gas chromatograph with a Zebron ZB5-MS column (29.7 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 280 °C and the oven temperatures were programmed as follows: 80 °C min^{-1} for 1.24 min, 24°C min^{-1} until 200 °C and held for half a minute, 73 °C min^{-1} until 245 °C and held for 5 min, 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 quadrupole and ion-source temperature set at 100 °C and 200 °C, respectively, was used for quantification of EE2 in

negative chemical ionization (NCI) mode. The following quantification ions were used in selected ion monitoring (SIM) mode: m/z 490 (^{12}C EE2), m/z 492 (^{13}C EE2). Chromatographic data were collected and analyzed using Agilent MSD Chemstation Data Analysis Software. All quantifications were based on an isotope dilution method as previously described by de Hoffmann and Stroobant (2002).

5.3.7 Toxicokinetic model

The first order elimination rate constant, k_2 (h^{-1}) of EE2 was determined from the slope of $\ln(\text{concentration})$ -time relationship determined by an independent depuration experiment:

$$\ln C_F(t) = \ln C_F(0) - k_2 \cdot t \quad (1)$$

Where $C_F(0)$ and $C_F(t)$ were the concentrations of EE2 (ng/kg , dw) in the whole fish body at time zero depuration and at a given time (Gobas and Morrison, 2000).

The elimination half life, $t_{1/2}$ (h) was calculated from the following equation:

$$t_{1/2} = 0.693 / k_2 \quad (2)$$

The uptake rate constant, k_1 (h^{-1}) was calculated by fitting the experimental data to the following equation:

$$k_1 = k_2 \cdot (C_F / C_{WD}) \cdot (1 - \exp^{-k_2 t})^{-1} \quad (3)$$

Where C_{WD} was the freely water dissolved concentration of EE2 (ng/L) in the exposure tank. The C_{WD} was assumed constant during the exposure period.

The feeding exposure allowed the determination of the assimilation efficiencies (α) required to incorporate diet as another source of EE2 uptake in fish. The minimum sum

of the squares (SS_{Fish}) (equation 4) was used to estimate α by as shown in Figure 5.6. (Atkinson, 2008).

$$SS_{\text{Fish}} = \sum (C_{\text{F measured}} - C_{\text{F modeled}})^2 \quad (4)$$

The modeled C_{F} was determined from equation (6). The following equations 5, 6, 7 were used to model and reproduce EE2 uptake from water and dietary exposure experiments.

$$C_{\text{F}} = C_{\text{WD}} \cdot k_1/k_2 \cdot (1 - \exp^{-k_2 t}) \quad (5)$$

$$C_{\text{F}} = \alpha R C_{\text{D}} / k_2 \cdot (1 - \exp^{-k_2 t}) \quad (6)$$

$$C_{\text{F}} = (C_{\text{WD}} \cdot k_1 + \alpha R C_{\text{D}}) / k_2 \cdot (1 - \exp^{-k_2 t}) \quad (7)$$

Equation (5) modeled the uptake from water only, equation (6 and 7) modeled EE2 uptake from the food exposure. Equation (6) assuming $C_{\text{WD}} = 0$ while equation (7) considered $C_{\text{WD}} = 4.88 \text{ ng/L}$ to include the amount of EE2 that leaked from the food into the tank water (Newman and Unger, 2003). The experimental rate of food intake, ration (R) was determined as the (0.031 g food/h) and C_{D} (ng/kg) was the measured concentration of EE2 in the fish diet.

The bioconcentration factor (BCF) and the dietary bioaccumulation factor (BAF) were calculated from the following equations:

$$\text{BCF} = C_{\text{F}} / C_{\text{WD}} \quad (8)$$

$$\text{BAF} = C_{\text{F}} / C_{\text{D}} \quad (9)$$

The fitting of the experimental data to modeled data was performed with Stella software. The best fit was achieved when k_2 was allowed to increase with increments of 0.0015 h^{-1} over time for the water exposure and 0.0035 h^{-1} for the dietary exposure.

5.3.8 Statistical analysis

One-way analysis of variance ANOVA (Origin Lab 7 software, Origin Lab Corporation MA, USA) was used to compare the EE2 uptake by after 24h in the uptake phase experiment in relation to the depuration phase. The sample wet weight and the total lipid content data were also compared. Polynomial regression analysis was performed on the uptake curve of EE2 resulted from the water exposure. Multiple linear regression analysis was performed on the depuration data to determine the elimination rate constant of EE2.

5.4 Results and discussion

5.4.1 EE2 levels in water

Measurements of EE2 in the water during the exposure experiments were close to the targeted nominal concentration of 150 ng/L (Figure 5.2A and 5.4A). The concentration of EE2 in the mixing tank water (C_W) averaged 145.1 ng/L $\pm$ (15.75, standard deviation). The overall variation in EE2 water concentrations expressed as coefficient of variation (CV) of was 10.85 % in the mixing tank. The C_{WD} of EE2 in the exposure tank averaged 134.9 ng/L $\pm$ (12.8) and CV was 9.5%. During the depuration experiments (Figure 5.4A), EE2 C_W averaged 156.3 ng/L $\pm$ (2.26) and CV was 1.45% and the C_{WD} averaged 145.5 ng/L $\pm$ (4.0) and CV was 2.75%. The depuration tank water had no detectable levels of EE2. In all experiments, the CV values of EE2 water concentration never exceeded 10.85%. Our EE2 water stability data were comparable or even better than Skillman et al. (2001) study who exposed rainbow trout to a nominal concentration of 125 ng/L in a flow-through system and reported a measured concentration ranging 117 ng/L to 150 ng/L and CV of 17.22%. In similar uptake-depuration experiments where the water concentration must be maintained constant during the time course, up to 20% CV in the test compound concentration was considered acceptable (Arnot and Gobas 2006). Our The experimental set-up (Figure 5.1) for the water exposures allowed us to simultaneously monitor both EE2 C_W and C_{WD} in all tanks at each time interval and therefore determine the effect of the presence of fish on EE2 concentrations in the water. In two independent experiments (Figure 5.2A and 5.4A), the C_{WD} of EE2 was clearly lower than C_W by 7.03% and 6.91% in the uptake and the

depuration exposures respectively. Such loss in water dissolved EE2 between the mixing tank (without fish) and the exposure tank (with fish) was most likely due to the particulate organic matter (POM) consisting of fecal matter and the fish food (Gobas and Morrison, 2000). Therefore, the EE2 C_{WD} was expected to show a decreasing relationship with an increasing POM during static or slow flow-through exposure conditions. During the feeding experiment (Figure 5.3), EE2 was detected in three water samples (1 L) at 4.88 ng/L (1.99). Leaching of EE2 from the fish food was one potential source of EE2 in water since none of the water blank samples contained any measurable EE2. Excretion and deconjugation of EE2 by fish was another potential source of EE2 to the water.

5.4.2 Uptake and accumulation

The concentrations of EE2 in fish increased over the exposure duration in water (Figure 5.2) and diet (Figure 5.3). In the water exposure, EE2 accumulated rapidly in fish within 1 h reaching 8.43 ng/g (1.22) dry weight and 46.44 ng/g (27.37) lipid weight (Table 5.1). In the feeding experiment, EE2 in fish averaged 1.35 ng/g dry weight after the first feeding (Figure 5.3).

The uptake curve (Figure 5.2B), showed a third order polynomial regression ($R^2 = 0.989$, $p = 0.0004$, $n = 8$). A rapid uptake of EE2 coincided with a slow elimination due to low body residues of EE2. However, whole body EE2 increased and reached a maximum concentration by 24h of exposure. The elimination rate continued to increase as shown by declining EE2 concentrations after 24 h of exposure, although the fish were still exposed to a stable and constant concentration of EE2.

Within the time course of the water exposure, no clear equilibrium was observed and 24 h was sufficient to reach the maximum EE2 concentration of $50.95 \text{ ng/g} \pm (2.68)$ dry weight and $238.93 \text{ ng/g} \pm (85.51)$ lipid weight. The uptake curve generated from lipid normalized data (Figure 5.2C) followed the same third order polynomial regression ($R^2 = 0.954$, $p = 0.004$, $n = 8$) that we observed for the whole tissue (Figure 5.2B) properly due to the similarity in the lipid content of the exposed species during the exposure period (Table 5.1). In a previous exposure experiment with slightly different experimental conditions we observed similar uptake and elimination patterns of EE2 (Appendix C, Figure 1 and 2). However, we noticed a clear drop in aqueous EE2 during the exposure that made it difficult to distinguish whether the elimination of EE2 from the fish was driven by endogenous metabolic activities or due to the decline in water levels.

With respect to the dosage and route of exposure, our data were consistent with the EE2 pharmacokinetics studies performed on human and mammalian subjects in which rapid uptake was followed by slower elimination (DiLiberti et al., 2011; Pérez-Campos, 2010; Fernandez et al., 1998). In the feeding experiment, the measured EE2 concentration in three food samples was $231.84 \text{ ng/g} \pm (41.82)$ which was close to the nominal concentration (250 ng/g). Although the variation was small between the duplicate samples after 3 h of exposure, we observed much higher variability in the fish sampled after 6 h and 12 h (Figure 5.3) which could be from variable dietary exposure. Goldfish sampled after 24, 36, 48, and 72 h showed less variability in EE2 concentration, and averaged $2.66 \text{ ng/g} \pm (0.87)$ ($n=8$). However, an apparent decline in EE2 concentrations was also observed in the fish sampled after 96 h and 120 h, with values below our MDLs (0.67 ng/g). Approximately 93.7 % of EE2 was eliminated during the

feeding exposure (24 – 72 h) such that the EE2 measured during this time represents only the residual 6 % of EE2. The persistence of EE2 residues during this time period may be due to a physiological process known as enterohepatic recirculation (Schultz et al., 2001). Although EE2 was heavily biotransformed, only a small fraction of EE2 conjugates in the bile was eliminated while the rest was deconjugated and reabsorbed into the blood stream (Schultz et al., 2001). None of the samples collected prior to EE2 exposure (at time zero, n = 5) had any measurable EE2 in both the water and the food exposures.

5.4.3 Depuration

As illustrated by Figure 5.4, goldfish demonstrated the ability to eliminate EE2. Only after 3h of depuration, whole fish tissue burden of the parent EE2 dropped from 9.61 ng/g $\pm$ (0.46) dry weight and 94.53 ng/g $\pm$ (24.68) lipid weight to 5.78 ng/g $\pm$ (1.67) dry weight and 70.62 ng/g $\pm$ (41.02) lipid weight (Table 5.1). The elimination of EE2 continued in a progressive manner until 48 and 72h when the residues were below detection limit. A first order elimination rate constant, k_2 was determined as 0.0786h⁻¹ (Figure 5.5) and the $t_{1/2}$ was 8.8 h⁻¹. During the depuration experiment, EE2 tissue concentration was significantly lower at zero time (24 h exposure) (9.61 ng/g $\pm$ 0.46, n=3) (Figure 5.4B) than what we measured during the uptake experiment after 24 h (50.95 ng/g $\pm$ 2.68, n=3) (Figure 5.2B) (one-way ANOVA, p=0.00001). Such significant difference in EE2 uptake by goldfish in two independent experiments might be explained by seasonality effect, lipid content data, and fish wet weight. The uptake experiment was performed in May 2011 while the depuration experiment was performed in late December 2011. The photoperiod difference between the two experiments was

approximately 6 h. Variation in environmental factors such as salinity and temperature was shown to affect EE2 uptake by in male killifish (*Fundulud heteroclitus*) (Blewett, 2011). The depuration fish samples were also lower in total wet weight ($17.53 \text{ g} \pm 0.93$, $n = 3$) than the uptake experiment samples which weighted ($20.01 \text{ g} \pm 1.01$, $n = 3$) (one-way ANOVA, $p < 0.05$). Consequently, the lipid content was also lower during the depuration experiment ($0.11 \text{ g/g} \pm 0.02$, $n = 3$) compared to ($0.23 \text{ g/g} \pm 0.07$, $n = 3$) during the uptake experiment (one-way ANOVA, $p < 0.05$).

Generally, the toxicokinetic parameters of EE2 were compared in the light of some physiochemical properties with different environmental pollutants (Table 5.2). Chemicals including Fluoxetine (Paterson and Metcalf (2008), Tebuconazole (Andreu-Sanchez et al., 2012), Trichlorophenol (Call et al., 1980), Anthracene (Spacie et al., 1983), and Tributyltin were all tested on different fish species to determine k_1 , k_2 , $t_{1/2}$, and the BCF using experimental conditions similar to ours. The $\log K_{ow}$ is a key physiochemical property that determines the lipophilicity of a certain chemical and the degree of bioconcentration (Gobas and Marrison, 2000). Although the $\log K_{ow}$ values of the above listed chemical were close to that of EE2, the dynamics of these chemicals in the exposed fish were not similar. The elimination half lives ranged from (1/hour) to (1/day) which demonstrated that the aquatic organisms ability to biotransform environmental toxins was highly variable and dependant on many factors such as exposure duration, toxicity, water quality, salinity, and temperature (Gobas and Marrison, 2000; Arnot and Gobas 2006; Blewett, 2011).

5.4.4 BCF and BAF

Our water exposure results were comparable to Skillman and coworkers (2006) who continuously exposed rainbow trout (*Oncorhynchus mykiss*) to 125 ng/L of EE2 in a flow-through system for about 60 days and measured EE2 in plasma at specific time intervals. Rapid uptake of EE2 was noticed and it was detected within 15 minutes of the initial exposure. EE2 in plasma appeared to reach equilibrium by 16 h in the Skillman et al. (2006) study, although a decline in EE2 was evident up to 100 h before concentrations rose again and varied between 60 – 90 ng/mL. Similar declines in EE2 were apparent in our study on goldfish. However, no rise in EE2 whole tissue concentrations was found, contrary to Skillman et al. (2006), which might be due to differences in exposure duration, analyzed tissue, and fish species. Assuming similar equilibrium after 16 h exposure, EE2 BCFs of 377, 325, and 211 can be reported at 24 h, 48 h, and 72 h respectively. These values were lower than Skillman et al. (2006), where EE2 in plasma was found to be 480 – 720 times higher than water concentrations. Another study by Lång et al. (2000) who estimated a whole body EE2 BCF of 660 after exposing fathead minnows (*Pimephales promelas*) to 64 ng/L of EE2 in water for 153 days, and also a BCF of 610 with 16 ng/L exposure for 245 days. Although our BCF values were lower than Skillman et al. (2006) and Lång et al. (2000), they were much closer to the predicted BAF of 330 of EE2 modeled by Lai et al. (2001).

The rough estimation of EE2 BAF was 0.011 based on ratio between the average measured tissue EE2 divided by the food concentration although EE2 was detectable in all exposed samples at ng/g levels (Figure 5.3). This could mean that dietary exposure alone may not lead to a significant bioaccumulation of EE2 in aquatic species and

therefore biomagnification would not apply. Some hydrophobic chemicals with log K_{ow} values ranging from 3.98 – 7.55 tend to be taken up by gills more effectively than diet in juvenile rainbow trout as demonstrated by Qiao et al. (2000). Moreover, Dussault et al. (2009) reported a similar observation after exposing the benthic invertebrates *Chironomus tentans* and *Hyalella azteca* to EE2 to range of 0.02 – 3.1 mg/L via water only in a flow-through system and also exposed the two species via sediment to 10 µg/g dry weight and the exposure in both cases was 21 days. The highest BAF was 215 for the *Chironomus tentans* exposed to 0.56 mg/L EE2 and a BAF of 142 was calculated for at an exposure concentration of 0.02 mg/L. However, the biota sediment accumulation factors (BSAF) were below 1 for both species exposed to 1.5 µg/g. A similar study reported a much higher BSAF of 254 in the oligochaete *Lumbriculus variegatus* after exposure to a nominal concentration of 0.5 µg/g for a 35 days exposure (Liebig et al., 2005). The high BSAF reported by Liebig et al. (2005) may be explained by two reasons 1) not allowing the worms to purge their gut content before analysis and 2) the failure of the radioimmunoassay to distinguish the free bioactive from of EE2 from the conjugated form.

5.4.3 Modeling EE2 uptake and elimination

The first order uptake models (5, 6, 7) shown above with the kinetic parameters obtained from the uptake and depuration experiments were able to reproduce the EE2 uptake curves in both the water and food exposures (Figure 5.6 and Figure 5.7). The model predicted that maximal EE2 tissue concentrations would reach 49.73 ng/g after 21.5 h of the exposure whereas a slightly higher maximum concentration was measured

at 50.94 ng/g after 24h (Figure 5.6). A nice fit between the modeled and the measured data was obtained for all data points. Studies investigating EE2 uptake and depuration dynamics in fish were limited. Skillman et al. (2006) was the only available study showing EE2 uptake curve in rainbow trout plasma after a water exposure (125 ng/L) however, k_1 and k_2 data were not reported by this study. The only fish study reporting some kinetic parameters such as the $t_{1/2}$ values of EE2 in rainbow trout plasma was accomplished by Schultz et al. (2001) who studied VTG and EE2 dynamics after injecting rainbow trout with increasing doses of the synthetic estrogen. The elimination rate constants (k_2) values in Schultz et al. (2001) study (0.0052, 0.0225, and 0.0345 h⁻¹) were lower than what we observed in goldfish (0.0786 h⁻¹). This may be due to differences in dosage since Schultz et al. (2001) noticed an inverse correlation between EE2 dose and the ability of the fish to eliminate it. The route of exposure may also result in some difference in EE2 dynamics between EE2 injected fish in comparison to water exposed fish. However, EE2 elimination in fish might be slower than exposed mammals (DiLiberti et al., 2011; Pérez-Campos, 2010; Fernandez et al., 1998).

The average EE2 in fish after dietary exposure showed also a reasonable agreement with the modeled data (Figure 5.7). Despite our observation that some EE2 (4.88 ng/L) was leached from the food into the water such leakage of EE2 was negligible as an additional source of EE2. We determined assimilation efficiency (α) for EE2 by calculating the minimum sum of the squares (SS_{fish}) for a range of α values (Figure 5.8).

Applying the modeling approach described here could be very useful to predict EE2 total body burden in environmentally exposed fish

5.5 Conclusions

The data presented here showed that goldfish were able to accumulate the synthetic estrogen EE2 from different exposure routes, and were also able to eliminate it. Our exposure experiments supported the suggestions that aqueous uptake may dominate over dietary uptake for some chemicals like EE2 in fish and invertebrates (Qiao et al., 2000; Dussult et al., 2009) and furthermore, modeling data supporting this observation were provided in Chapter 6. This was the first study to investigate EE2 uptake and depuration kinetics from water and food exposures.

5.6 Acknowledgments

This research was supported by the Natural Sciences and Engineering Research Council of Canada (Strategic Grants to J. Blais and V.L. Trudeau), Canadian Water Network (V.L. Trudeau), the scholarship fund (for Al-Ansari A.M.) from King Abdulaziz University, Jeddah, Saudi Arabia, and the Great Lakes Action Plan.

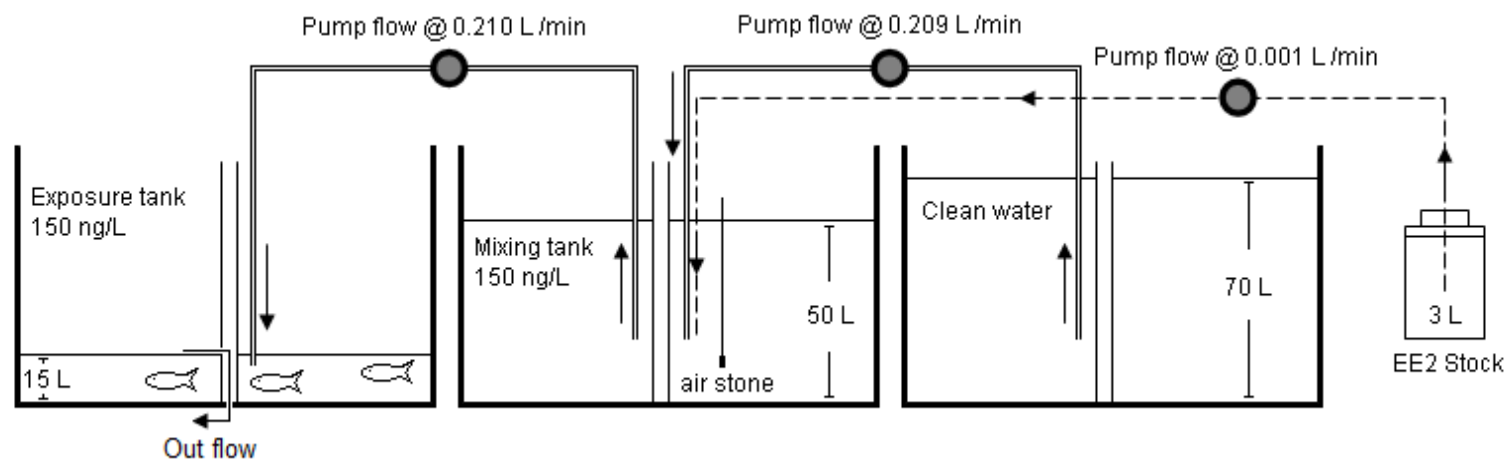


Figure 5.1: A schematic for the water exposure flow-through experimental set-up.

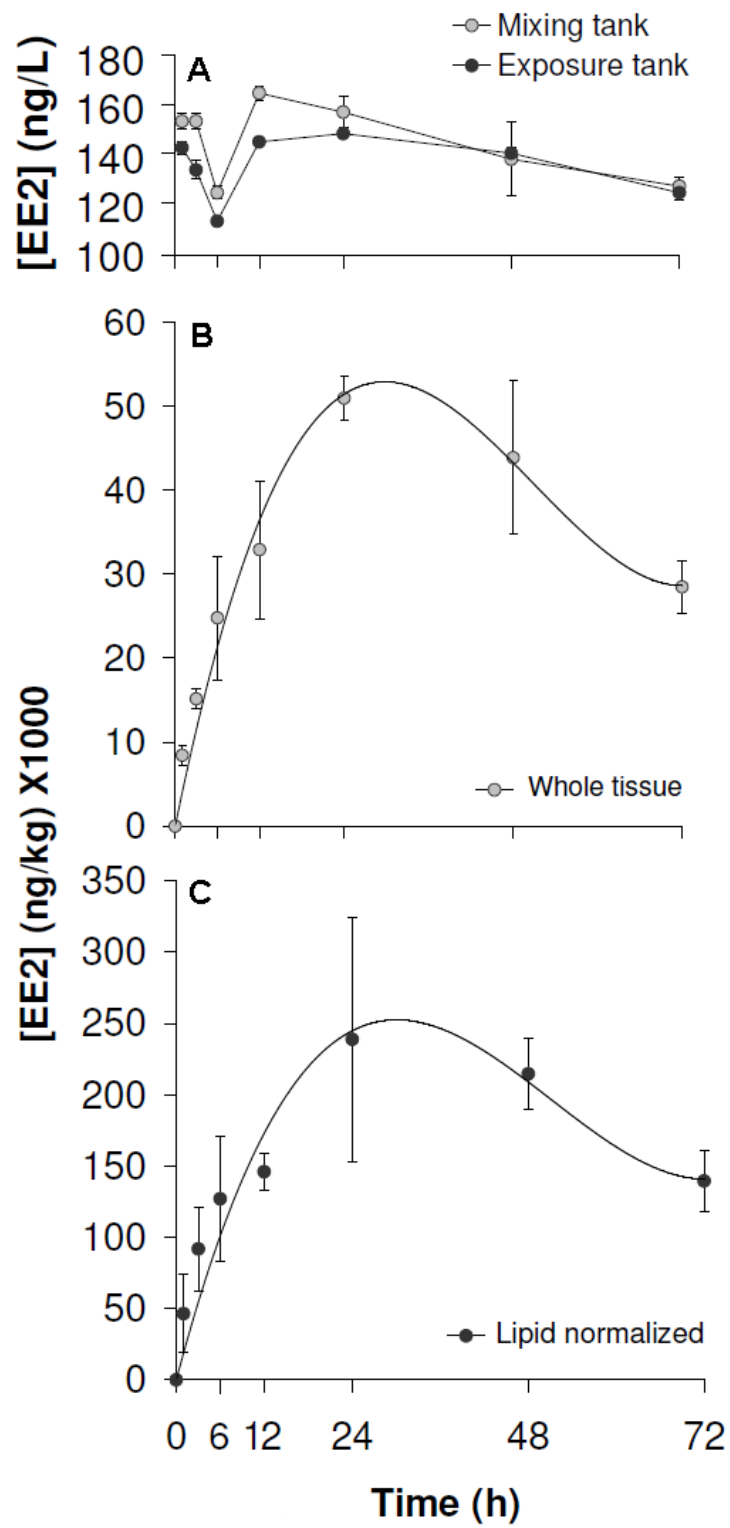


Figure 5.2: Panel (A) shows the measured water concentrations in ng/L within the experimental set-up during the exposure gray circles are mean ($n = 6$) concentrations of EE2 in the mixing tank (without fish) before and after the exposure and the dark circles show the mean ($n = 3$) of EE2 levels in the exposure tank (with fish) at the end of each time interval. Panels B (ng-EE2/g tissue) and panel C (ng-EE2/g lipid) are the uptake curves of EE2 in goldfish. Each fish data point shows a mean concentration of three samples $\pm$ standard deviation.

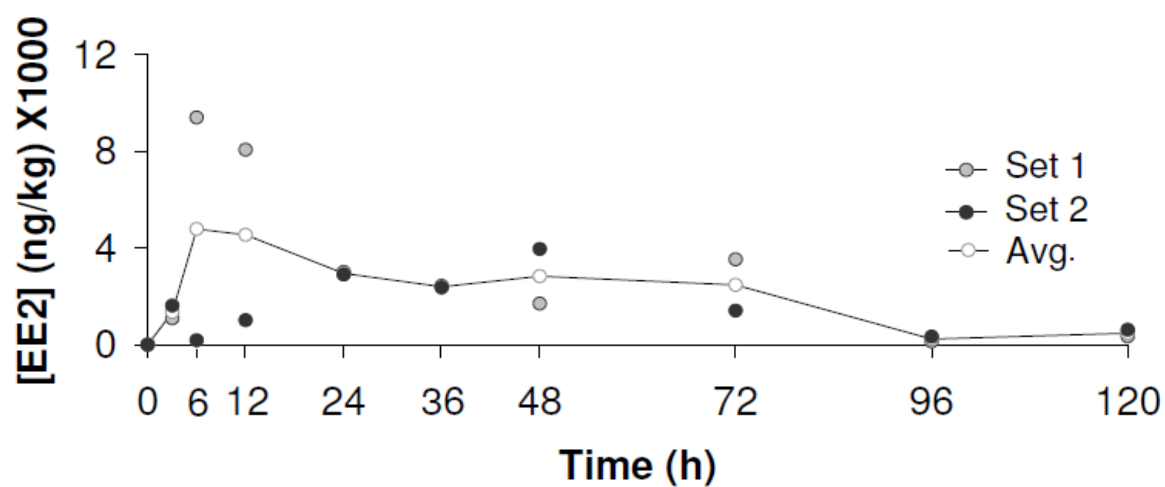


Figure 5.3: The uptake and elimination curve of EE2 after the feeding experiment. The filled gray and black circles show duplicate samples taken from the tank at each time interval and the open circles show the duplicate average concentration of EE2 in the whole fish tissues.

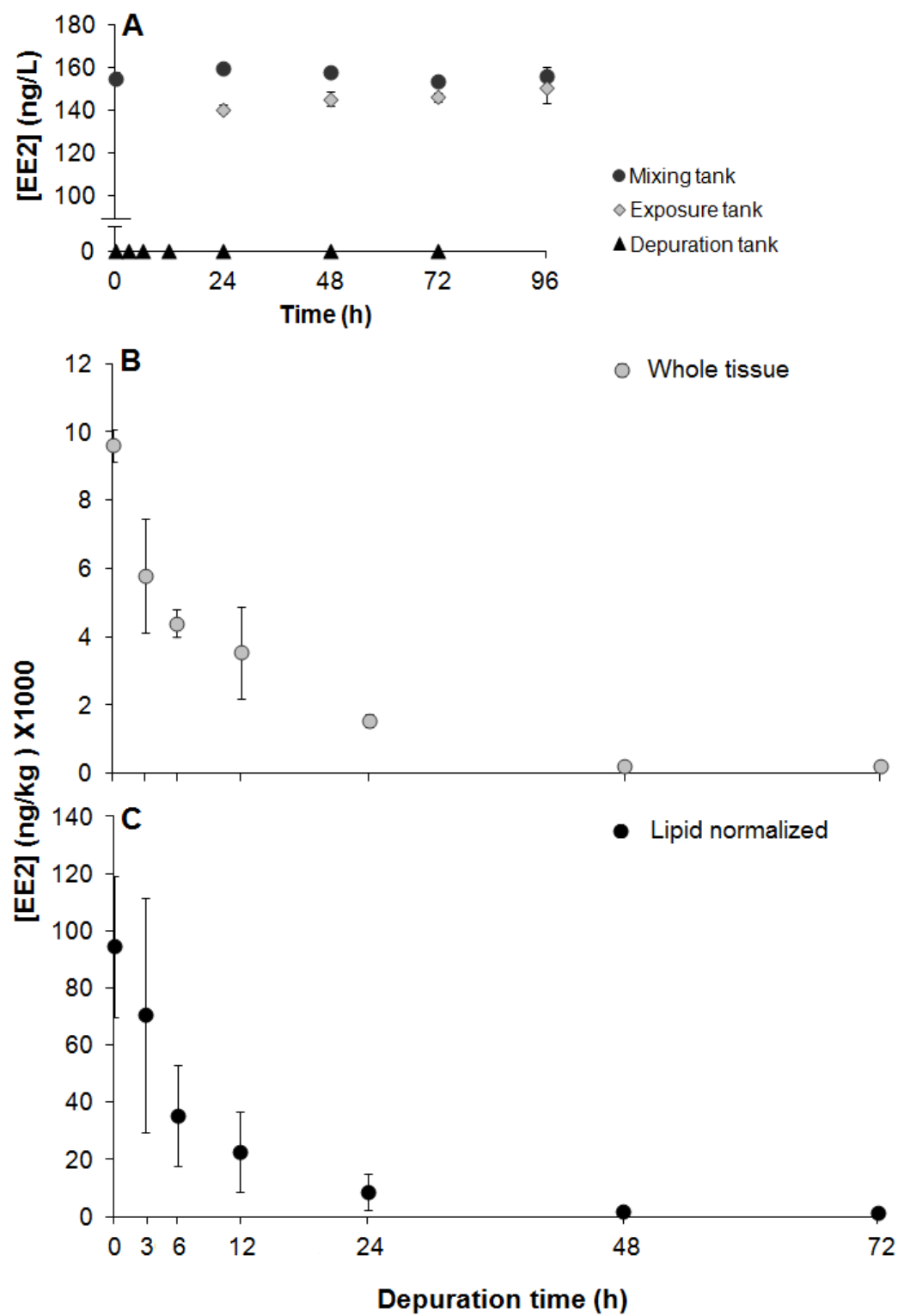


Figure 5.4: Panel (A) shows the measured water concentrations in ng/L within the experimental set-up during the exposure black circles are mean ($n = 6$) concentrations of EE2 in the mixing tank (without fish) before and after the exposure. The gray diamonds show the mean ($n = 3$) of EE2 levels in the exposure tank (with fish) while the black triangles show EE2 levels in the depuration tank at the end of each time interval. Panels B (ng-EE2/g tissue) and panel C (ng-EE2/g lipid) are the depuration curves of EE2 in goldfish. Each fish data point shows a mean concentration of three samples $\pm$ standard deviation.

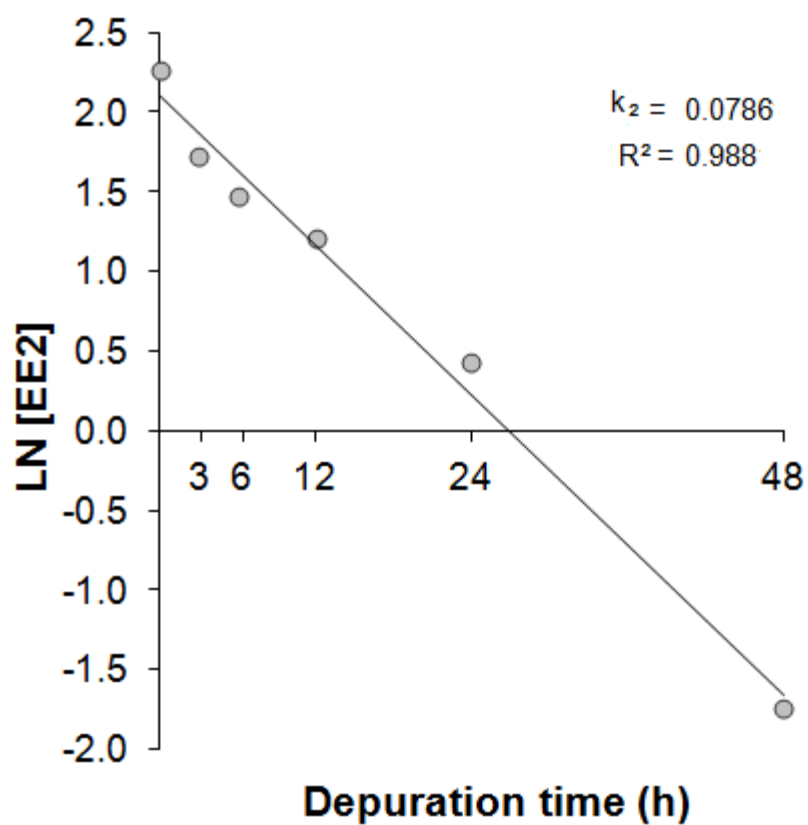


Figure 5.5: The depuration profile of EE2 in goldfish. Each data point represents the natural logarithm of the average EE2 whole tissue concentration (ng/g). The slope of the regression line represents the elimination rate constant (k_2) of EE2 in goldfish.

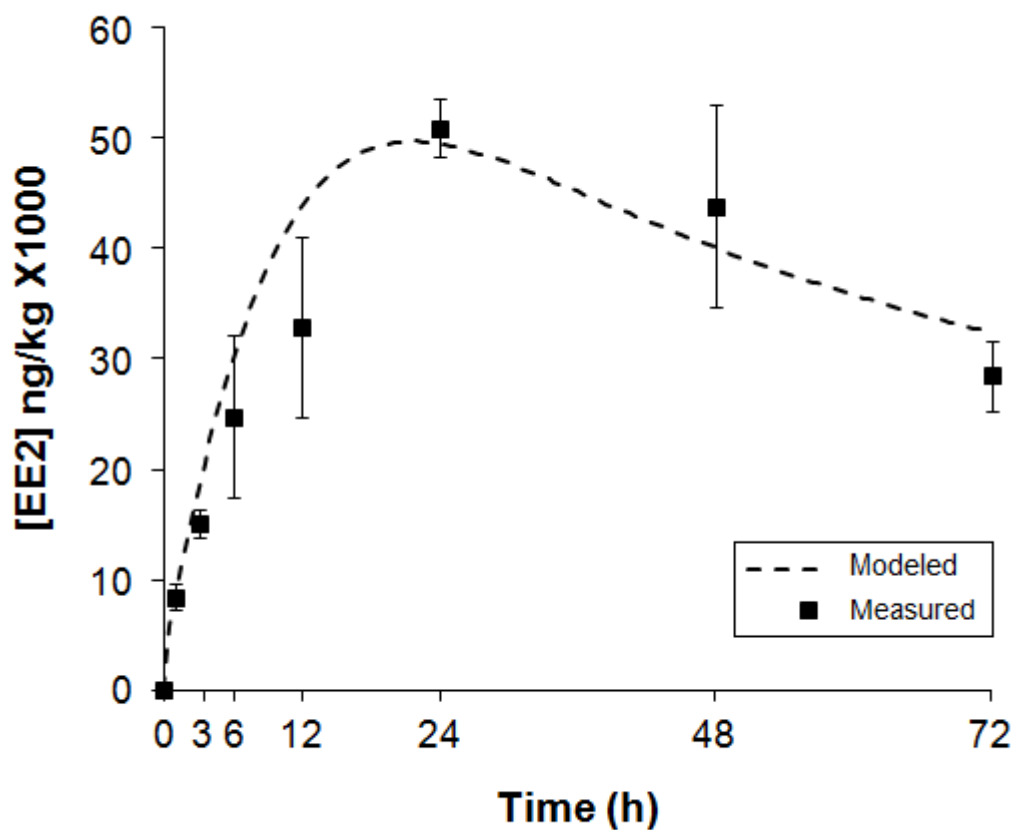


Figure 5.6: The measured EE2 concentrations (black squares) in goldfish whole tissue homogenates $\pm$ the standard deviation ($n=3$) of the means values compared to a hypothetical extrapolation of the modeled concentrations at 0.5 h time intervals (the dashed line). The data was generated after a flow-through exposure to EE2 at approximately 134.9 ng/L for 72 h. Model inputs: uptake rate constant (k_1)= 45h^{-1} , elimination rate constant (k_2)= 0.0786 h^{-1} increasing with 0.0015 h^{-1} increments every 0.5 h.

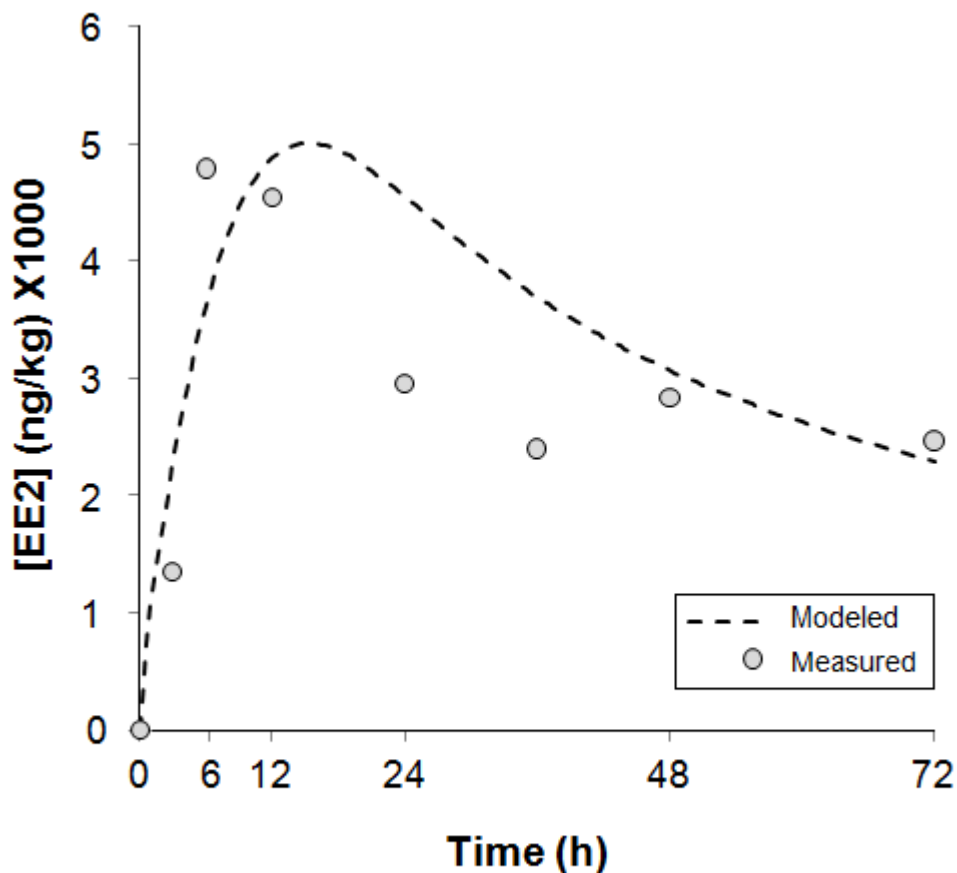


Figure 5.7: The average measured EE2 concentrations (gray circles) in goldfish whole tissue homogenates from duplicate samples at each time point compared to a hypothetical extrapolation of the modeled concentrations at 0.5 h time intervals (the dashed line). The data was generated after a dietary exposure to EE2 at approximately 231.84 ng/g of food for 72 h. Model inputs: assimilation efficiency (α)= 106, ration (R) =0.031, elimination rate constant (k_2) = 0.0786 h⁻¹ increasing with 0.0035 h⁻¹ increments every 0.5 h.

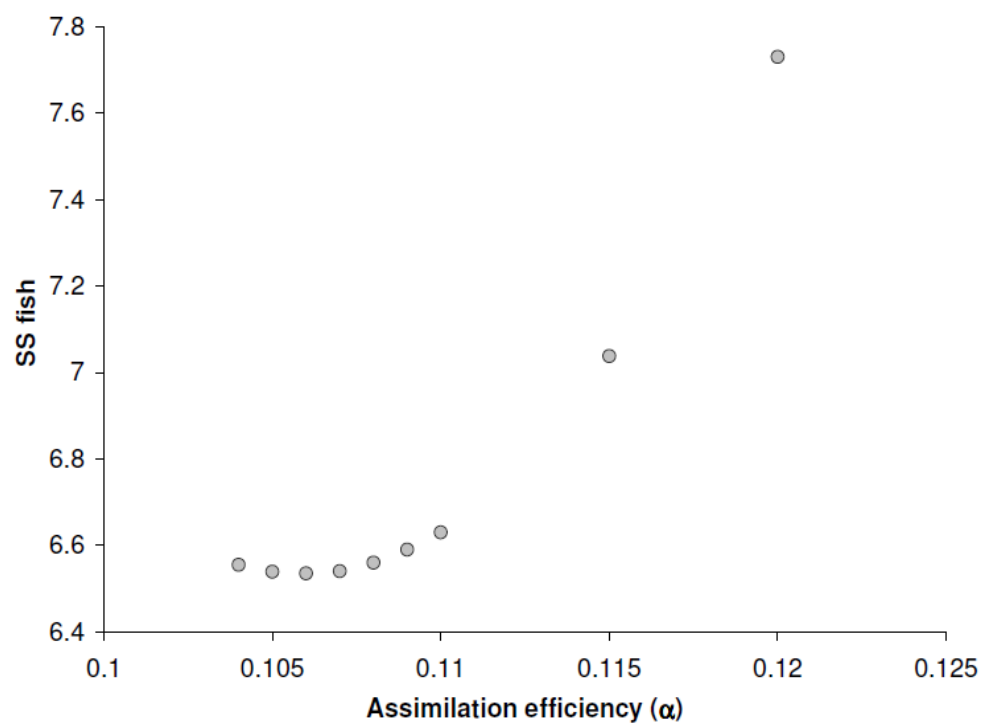


Figure 5.8: The optimization of EE2 assimilation efficiency (α) (0.106) which was achieved by minimizing its sum of square values after the feeding exposure experiment.

Table 5.1

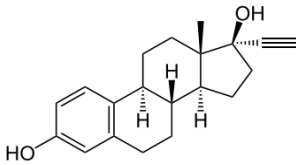
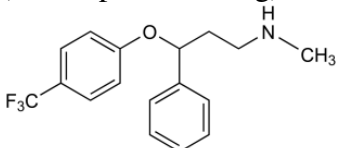
Fish related weights and EE2 concentrations (ng/g tissue and ng/g lipid). Data is expressed as mean ($\pm$ standard deviation) of three samples in whole Goldfish sampled at each time interval

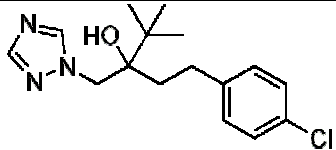
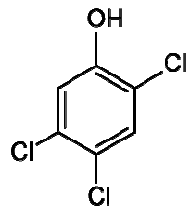
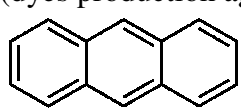
Time (h)	Weight (g)				EE2 (ng/g)	
	Tissue (wet)	GI*	Tissue (dry)	Lipid**	Tissue (dry)	Lipid
<u>Uptake phase</u>						
0	29.1 (8.49)	2.62 (0.94)	6.6 (1.91)	0.22 (0.05)	ND	ND
1	22.8 (6.44)	1.18 (0.42)	5.8 (1.93)	0.21 (0.08)	8.43 (1.22)	46.44 (27.37)
3	25.4 (6.38)	1.11 (0.39)	6.8 (1.48)	0.18 (0.07)	15.14 (1.23)	91.87 (29.66)
6	28.8 (7.08)	1.95 (0.50)	6.8 (1.73)	0.20 (0.02)	24.80 (7.37)	127.04 (43.67)
12	23.2 (5.68)	1.86 (0.26)	5.5 (1.35)	0.23 (0.07)	32.92 (8.20)	145.91 (12.69)
24	20.0 (1.10)	1.66 (0.20)	4.6 (0.56)	0.23 (0.07)	50.95 (2.68)	238.93 (85.51)
48	24.4 (3.45)	1.24 (0.03)	5.8 (1.11)	0.21 (0.06)	43.87 (9.16)	214.67 (25.08)
72	24.6 (7.75)	1.42 (0.68)	5.8 (1.65)	0.21 (0.01)	28.48 (3.14)	139.53 (21.32)
<u>Depuration phase</u>						
0	17.5 (0.9)	0.7 (0.1)	3.6 (0.2)	0.08 (0.02)	9.62 (0.46)	118.17 (30.86)
3	19.9 (6.3)	1.1 (0.2)	4.5 (2.0)	0.10 (0.10)	5.80 (1.67)	88.27 (51.28)
6	16.9 (3.6)	1.1 (0.1)	3.8 (1.2)	0.12 (0.70)	4.40 (0.40)	44.18 (22.17)
12	18.1 (3.9)	1.0 (0.1)	4.4 (1.4)	0.14 (0.03)	3.50 (1.34)	28.21 (17.66)
24	20.2 (3.6)	0.8 (0.3)	5.2 (1.2)	0.21 (0.16)	1.50 (0.20)	10.78 (7.82)
48	13.6 (1.1)	1.0 (0.2)	2.8 (0.3)	0.08 (0.02)	0.20 (0.12)	2.36 (0.95)
72	17.1 (1.6)	1.0 (0.1)	4.0 (0.5)	0.12 (0.03)	0.20 (0.17)	1.78 (1.69)

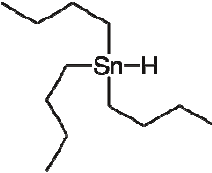
* GI, Gastrointestinal track; **Lipid, expressed as dry g/g tissue

Table 5.2

Summary of toxicokinetic studies performed in whole fish tissues for a number of environmental pollutants

Chemical ⁽¹⁾	Fish Species ⁽²⁾	Aqueous exposure ⁽²⁾	Toxicokinetic parameters ⁽²⁾	References
17α-ethynilestradiol (oral contraceptives)  MW: 296.4 g/mol VP: 6×10^{-9} Pa WS: 4.8 mg/L Log K_{ow}: 4.15	Goldfish (<i>Carassius auratus</i>)	150 ng/L (nominal) flow-through 72h uptake 72h depuration	k_1 : 45 h ⁻¹ k_2 : 0.0786 h ⁻¹ $t_{1/2}$: 8.8 h C_{max} : 50.95 μ g/kg dw BCF : 377	⁽¹⁾ Lai et al 2003 ⁽²⁾ Current study
Fluoxetine (anti-depressant drug)  MW: 309.33 g/mol WS: 50 g/L Log K_{ow}: 1.25-4.30	Japanese medaka (<i>Oryzias latipes</i>)	640 ng/L (nominal) static 7days uptake 21days depuration	k_1 : 5.9 d ⁻¹ k_2 : 0.074 d ⁻¹ $t_{1/2}$: 9.4 d C_{max} : 49.4 μ g/kg ww BCF : 74	⁽¹⁾ Brook et al (2003) ⁽²⁾ Paterson and Metcalf (2008)
Tebuconazole (fungicide)	Zebrafish (<i>Danio rerio</i>)	0.2 mg/L (nominal) static 24days uptake only	k_1 : 745 h ⁻¹ k_2 : 205 h ⁻¹ $t_{1/2}$: 24 d	Andreu-Sánchez et al (2012)

 MW: 307.8 g/mol VP: 1.7×10^{-6} Pa WS: 36 mg/L Log K_{ow}: 3.7			C _{max} : 7500 µg/kg ww BCF : 38.8	
2,4,5, Trichlorophenol (germicide)  MW: 197.45 g/mol VP: 1.25 Pa WS: 0.434 mg/L Log K_{ow}: 3.69	Fathead minnows (<i>Pimephales promelas</i>)	5 and 50 µg/L (nominal) flow-through 28d uptake 28h depuration	at 5 µg/L k ₁ : 200 h ⁻¹ k ₂ : 0.08 h ⁻¹ t _{1/2} : 12 h BCF : 503 at 50 µg/L k ₁ : 3400 h ⁻¹ k ₂ : 0.1 h ⁻¹ t _{1/2} : 12 h BCF : 662	Call et al (1980)
Anthracene (dyes production agent)  MW: 178.2 g/mol VP: 0.001 Pa WS: 4.5×10^{-2} mg/L Log K_{ow}: 4.54	Bluegill Sunfish (<i>Lepomis macrochirus</i>)	0.7 µg/L (nominal) static 4h uptake 48h depuration	k ₁ : 36 h ⁻¹ k ₂ : 0.04 h ⁻¹ t _{1/2} : 17 d C _{max} : 94 µg/kg ww BCF : 675	⁽¹⁾ MacKay (2001) ⁽²⁾ Specie et al (1983)

Tributyltin (antifouling agent)  MW: 595.62 g/mol VP: 100 Pa WS: 4 mg/L Log K_{ow}: 4.14	Rainbow trout (<i>Salmo gairdneri</i>)	0.36 µg/L (nominal) static 64d uptake 32d depuration	k₁: 5.11 h⁻¹ k₂: 0.011 h⁻¹ BCF : 406	⁽¹⁾EXTOXNET (1996) ⁽²⁾Martin et al (1989)
---	---	---	---	--

Chapter 6

General Conclusions and Discussion

The overall objective of this thesis was to determine the bioaccumulation of the synthetic steroidal estrogen and active ingredient in oral contraceptives 17 α -ethinylestradiol (EE2) in its bioactive form in fish through field and laboratory studies. EE2 was selected for its environmental uptake in fish based on various reasons including:

- 1- Environmental persistence and high potency as an endocrine disruptor
- 2- Frequent detection in surface water and sewage effluents
- 3- It is an anthropogenic substance that does not occur naturally in the environment
- 4- Its physiochemical properties suggest high potential for rapid uptake

The studies presented in this thesis were designed to test the hypothesis that the biologically active form of EE2 used by humans to prevent pregnancy can bioaccumulate in laboratory exposed fish as well as wild caught fish near sewage effluents. Further, our results suggested that environmental bioaccumulation of EE2 in the St. Clair River might be restricted primarily to bottom (benthic) food-webs.

To fulfill the thesis general objectives, developing a sample preparation and analysis protocol for sensitive and selective detection and determination of EE2 in fish tissue was a critical step (Al-Ansari et al., 2011; Chapter 2) because optimized methods were not available. The ability of environmentally available EE2 to be taken up by fish from sewage effluents were previously reported (Larsson et al., 1999; Gibson et al., 2005) and an estimate of its bioconcentration factor (BCF) reaching 660 was also proposed (Länge

et al., 2001). Furthermore, EE2 mass balance modeling in freshwater food-web suggested that species feeding at a low trophic level could reach a bioaccumulation factor (BAF) of 332 (Lai et al., 2002). This growing body of literature supporting EE2 accumulation in the aquatic environment prompted us to examine these processes in more detail in this thesis.

Measurements of EE2 in shorthead redhorse sucker (ShRHS) caught downstream of a sewage treatment plant provided the first evidence that EE2 can be detected in whole tissue of free living wild fish at appreciable concentrations (1.53 ng/g wet weight), the first study of its kind (Al-Ansari et al., 2010; Liu et al., 2011a). The main source of EE2 detected in the ShRHS samples was unknown. I speculated that dietary sources may have resulted in EE2 bioaccumulation in the ShRHS. Doyle et al. (2011) showed that sediment constituted up to 46% of the diet in ShRHS, based on their stomach contents. Furthermore, it has been shown that EE2 was found in freshwater and marine sediments (Peck et al., 2004; Braga et al., 2005; Robinson et al., 2009).

A follow up study was initiated to investigate the potential for EE2 bioaccumulation in seven fish species ranging in dietary habits, from planktivores to benthic feeders and spanning different trophic levels. The fish were sampled along with sediment, sewage effluents and surface water samples in the St. Clair River (Chapter 4) within the same sampling sites as the ShRHS considered in (Al-Ansari et al., 2010; Chapter 3). Only sewage effluents and surface water samples close to a point source had detectable EE2. However, none of the additional fish species considered here contained any detectable EE2. Even the investigated bottom feeder species, the round goby, did not have any detectable EE2. This is may not be surprising because the sediments sampled in this

investigation also did not contain detectable EE2. Since St. Clair River is still considered an area of concern (Mayne et al., 2003), clean-up efforts and STPs operation upgrades may have resulted in a noticeable difference in the level of steroidal estrogens in the sewage effluents, surface water, sediment and biota in the eight years between collection of the ShRHS and our wider survey in 2010. It should be emphasized that other studies reporting steroidal estrogens in the St. Clair River environment were not available and it was not known whether such levels vary seasonally. EE2 was not found in any of the investigated seven species and thus EE2 biomagnification was supported.

To better understand uptake and elimination kinetics of EE2 in fish, a controlled laboratory exposure experiment was performed (Chapter 5). A short exposure of goldfish to EE2 via water, food, and water and food together was undertaken. EE2 uptake via water was most apparent with a BCF reaching 377, while the dietary BAF remained always < 1 during a 120 h exposure. The exposure experiment showed that the EE2 uptake rate constant (k_1) was higher than its elimination rate constant (k_2), indicating that EE2 accumulation was expected.

Using uptake and elimination rate constants calculated in Chapter 5, three different exposure scenarios were hypothesized and illustrated in Figure 6.1. The model predicted the amount of EE2 accumulation over a 72 h time course at environmentally relevant EE2 concentrations. Exposure via water only, and water and food would be the most significant pathways by which EE2 can reach nano gram per gram tissue levels with a BCF/BAF reaching 312. Modeled uptake from food only did not result in substantial EE2 accumulation (Figure 6.1). Our model predictions were supported by Liu et al. (2011a) who provided additional evidence of EE2 bioaccumulation in wild caught fish. Crucian

carp (*Carassius auratus*), common carp (*Cyprinus carpio*), and silvery minnows (*Anabarilius alburnops*) from Dianchi Lake in China all contained measurable EE2 (Liu et al. 2011a). The highest EE2 concentration (11.3 ng/g dw) was reported in silvery minnows. EE2 in the crucian carp and carp had the highest EE2 bioaccumulation in liver, followed by gills and muscles. The study also reported an EE2 concentration of 4.4 ng/L in lake water (Liu et al., 2011a), which was much higher than our measurements of EE2 in the St. Clair River.

The studies presented in this thesis increase our understanding about the bioaccumulation of EE2 in fish as one of the most important endocrine disruptors (Fernandez et al., 2007; Desbrow et al., 1998; Kidd et al., 2007). This thesis contributed to the field of endocrine disruption and the fate of endocrine disruptors in the environment by demonstrating the first evidence of EE2 bioaccumulation in wild fish (Al-Ansari et al., 2010). Furthermore, a sensitive analytical technique to study the bioaccumulation of steroidal estrogens in fish was shown (Al-Ansari et al., 2011). A better understanding of the dynamics of EE2 in fish after exposure via environmentally relevant routes and important information for modeling EE2 uptake, elimination, and bioaccumulation were also achieved. The recent studies by Liu et al. (2011a; 2011b) supported our findings and demonstrated that our work may attract more research in this area.

Future studies should examine EE2 uptake and elimination dynamics in fish exposed to EE2 in clean water compared to water containing different dilutions of sewage effluents. Such experiment should reveal whether a complex mixture of chemicals found in natural waters receiving sewage effluents would enhance or inhibit EE2 uptake and

elimination in the environment. Furthermore, risk assessment studies will also be important to determine the potential health effects associated with the consumption of EE2 contaminated fish, especially in children.

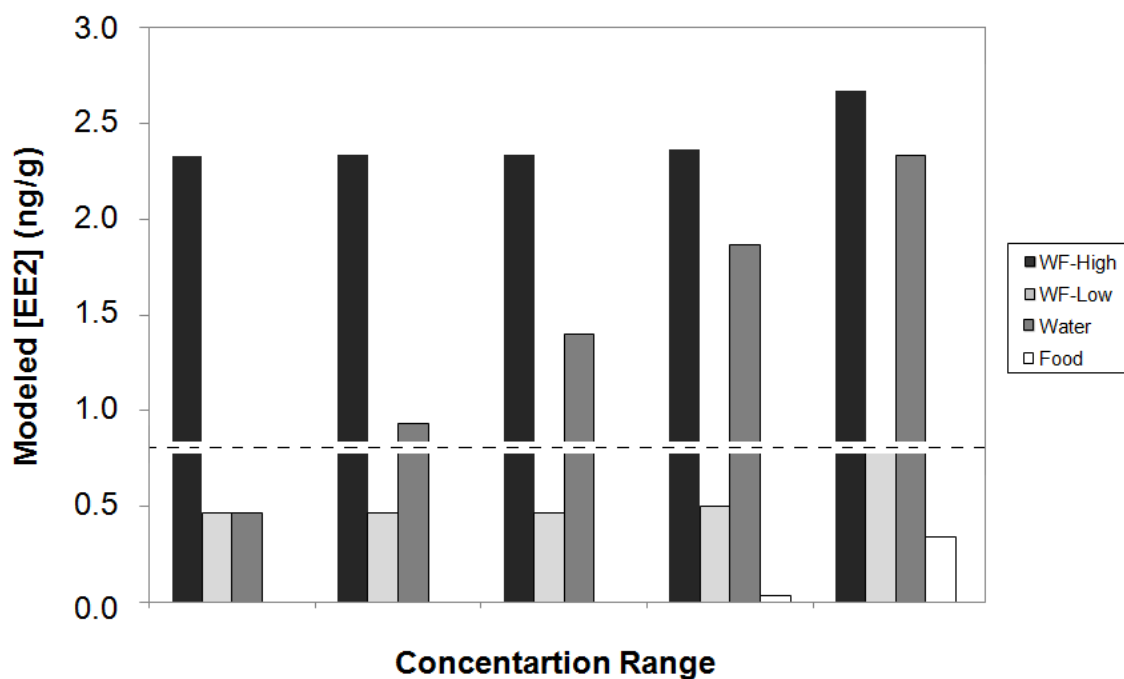


Figure 6.1: The maximum predicted EE2 tissue concentrations in fish assuming exposure via environmentally relevant levels of EE2. WF-High (5 ng/L) and WF-Low (1 ng/L) indicate exposure to EE2 via water plus variable 1, 10, 100, 1000, and 10000 ng/kg via food. Water: indicates exposure to EE2 via water only (1, 2, 3, 4, and 5 ng/L) and Food indicates exposure to EE2 via food only (1-10000 ng/kg). The dashed line shows our MDLs (0.67 ng/g).

References

- Ackermann, G.E., Schwaiger J., Negele, R.D., Fent, K., 2002. Effects of long-term nonylphenol exposure on gonadal development and biomarkers of estrogenicity in juvenile rainbow trout (*Oncorhynchus mykiss*). *Aquatic Toxicology* 60, 203-221.
- Al-Ansari, A. M., Saleem, A., Kimpe, L.E., Sherry, J.P., McMaster, M.E., Trudeau, V.L., Blais, J.M., 2010. Bioaccumulation of the pharmaceutical 17 α -ethinylestradiol in shorthead redhorse suckers (*Moxostoma macrolepidotum*) from the St. Clair River, Canada. *Environmental Pollution* 158, 2566-2571.
- Al-Ansari, A. M., Saleem, A., Kimpe, L.E., Trudeau, V.L., Blais, J.M., 2011. The development of an optimized sample preparation for trace level detection of 17 α -ethinylestradiol and estrone in whole fish tissue. *Chromatography B* 879, 3649-3652.
- Allen, Y., Matthiessen, P., Scott, A.P., Haworth, S., Feist, S., Thain, J.E., 1999a. The extent of oestrogenic contamination in the UK estuarine and marine environments- further surveys of flounder. *Science of the Total Environment* 233, 5-20.
- Allen, Y., Scott, A.P., Matthiessen, P., Haworth, S., Thain, J.E., Feist, S., 1999b. Survey of estrogenic activity in United Kingdom estuarine and coastal waters and its effects on gonadal development of flounder *platichthys flesus*. *Environmental Toxicology and Chemistry* 18, 1791-1800.

- Anderson, C., Cabana, G., 2007. Estimating the trophic position of aquatic consumers in river food-webs using stable nitrogen isotopes. *Journal of North American Benthological Society* 26, 273-285.
- Andreu-Sánchez, O., Paraíba, L.C., Jonsson, C.M., Carrasco, J.M., 2012. Acute toxicity and bioconcentration of fungicide tebuconazole in zebrafish (*Danio rerio*). *Environmental Toxicology* 27, 109-116.
- Angus, R.A., Stanko, J., Jenkins, R.L., Watson, R.D., 2005. Effects of 17alpha-ethynylestradiol on sexual development of male western mosquitofish (*Gambusia affinis*). *Comparative Biochemistry and Physiology, Part C* 140, 330-339.
- Aravindakshan, J., Paquet, V., Gregory, M., Dufresne, J., Fournier, M., Marcogliese, D.J., Cyr, D.G., 2004. Consequences of xenoestrogen exposure on male reproductive function in spottail shiners (*Notropis hudsonius*). *Toxicological Sciences* 78, 156-165.
- Arnon, S., Dahan, O., Elhanany, S., Cohen, K., Pankratov, I., Gross, A., Ronen, Z., Baram, S., Shore, L. S., 2008. Transport of testosterone and estrogen from dairy-farm waste lagoons to groundwater. *Environmental Science and Technology* 42, 5521-5526.
- Arnot, J.A., Gobas, F.A.P.C., 2006. A review of bioconcentration factor (BCF) and bioaccumulation factor (BAF) assessments for organic chemicals in aquatic organisms. *Environmental Reviews* 14, 257-297.
- Atkinson, S.K., 2008. The persistence of steroidal estrogens in the aquatic environment. M. Sc. Thesis, University of Ottawa, Ottawa, Ontario, Canada.

- Balch, C.D., MacKenzie, C.A., Metcalfe, C.D., 2004. Alterations to gonadal development and reproductive success in Japanese medaka (*Oryzias latipes*) exposed to alpha-ethinylestradiol. *Environmental Toxicology and Chemistry* 23, 782-791.
- Bartelt-Hunt, S., Snow D. D., Damon-Powell, T., Miesbach, D., 2011. Occurrence of steroid hormones and antibiotics in shallow groundwater impacted by livestock waste control facilities. *Journal of Contaminant Hydrology* 123, 94-103.
- Beck, J., Totsche, K.U., Kögel-Knabner, I., 2008. A rapid and efficient determination of natural estrogens in soils by pressurised liquid extraction and gas chromatography-mass spectrometry. *Chemosphere* 71, 954-60.
- Behnisch, P.A., Fujii, K., Shiozaki, K., Kawakami, I., Sakai, S., 2001. Estrogenic and dioxin-like potency in each step of a controlled landfill leachate treatment plant in Japan. *Chemosphere* 43, 977-984.
- Belfroid, A.C., van der Horst, A., Vethaak, A.D., Schafer, A.J., Rijs, G.B.J, Wegener, J., Cofino, W.P., 1999. Analysis and occurrence of estrogenic hormones and their glucuronides in surface water and waste water in the Netherlands. *Science of the Total Environment* 225, 101-108.
- Beresford, N., Jobling, S., Williams, R., Sumpter, J.P., 2004. Endocrine disruption in juvenile roach from English rivers: a preliminary study. *Journal of Fish Biology* 64, 580-586.
- Bertrand, M., Cabana, G., Marcogliese, D.J., Magnan P, 2011. Estimating the feeding range of a mobile consumer in a river–flood plain system using $\delta^{13}\text{C}$ gradients and parasites. *Journal of Animal Ecology*, doi: 10.1111/j.1365-2656.2011.01861.x.

- Blewett, T., 2011. Environmental influences on the accumulation of ethynylestradiol in marine teleosts. M.Sc. Thesis, McMaster University, Hamilton, Ontario, Canada.
- Bodzek, M., Dudziak, M., 2006. Elimination of steroidal sex hormones by conventional water treatment processes. *Desalination* 198, 24-32.
- Braga, O., Smythe, G.A., Schäfer, A.I., Feitz, A.J., 2005. Steroid estrogens in ocean sediments. *Chemosphere* 61, 827-833.
- Briciu, R.D., Kot-Wasik, A., Namiesnik, J., 2009. Analytical challenges and recent advances in the determination of estrogens in water environments. *Journal of Chromatographic Science* 47, 127-139.
- Brooks, B.W., Chambliss, C.K., Stanley, J.K., Ramirez, A., Banks, K.E., Johnson, R.D., Lewis, R.J., 2005. Determination of selected anti-depressants in fish from an effluent dominated stream. *Environmental Toxicology and Chemistry* 24, 464-469.
- Brooks, B. W., Foran, C.M., Richards, S.M., Weston, J., Turner, P.K., Stanley, J.K., Solomon, K.R., Slattery, M, La Point, T.W., 2003. Aquatic ecotoxicology of fluoxetine. *Toxicology Letters* 142, 169-183.
- Burgess, N.M., Hobson, K.A., 2006. Bioaccumulation of mercury in yellow perch (*Perca flavescens*) and common loons (*Gavia immer*) in relation to lake chemistry in Atlantic Canada. *Hydrobiologia* 567, 275-282
- Cailleaud, K., Budzinski, H., Lardy, S., Augagneur, S., Barka, S., Souissi, S., Forget-Leray, J., 2011. Uptake and elimination, and effect of estrogen-like contaminants in estuarine copepods: an experimental study. *Environmental Science and Pollution Research International* 18, 226-36.

- Call, D.J., Brooke, L. T., Lu, P., 1980. Uptake, elimination, and metabolism of three phenols by fathead minnows. *Archives of Environmental Contamination and Toxicology* 9, 699-714.
- Campbell, C.G., Borglin, S.E., Green, F.B., Grayson, A., Wozei, E., Stringfellow, W.T., 2006. Biologically directed environmental monitoring, fate, and transport of estrogenic endocrine disrupting compounds in water: A review. *Chemosphere* 65, 1265- 1280.
- Comerton, A.M., Andrews, R.C., Bagley, D.M., 2009. Practical overview of analytical methods for endocrine-disrupting compounds, pharmaceuticals and personal care products in water and wastewater. *Philosophical Transactions. Series A, Mathematical, Physical, and Engineering Sciences* 367, 3923-3939.
- Céspedes, R., Petrovic, M., Raldúa, D., Saura, U., Piña, B., Lacorte, S., Viana, P., Barceló, D., 2004. Integrated procedure for determination of endocrine-disrupting activity in surface waters and sediments by use of the biological technique recombinant yeast assay and chemical analysis by LC-ESI-MS. *Analytical and Bioanalytical Chemistry* 378, 697-708.
- Cheng, Z., Liang, P., Shao, D.D., Wu, S.C., Nie., X.P., Chen, K.C. Li, K.B., Wong, M.H., 2011. Mercury biomagnification in the aquaculture pond ecosystem in the Pearl River Delta. *Archives of Environmental Contamination and Toxicology*, 61, 491-499.
- Choy, E.S., Gauthier, M., Mallory, M.L., Smol, J.P., Douglas, M.S.V., Lean, D., Blais, J.M., 2010. An isotopic investigation of mercury accumulation in terrestrial food-

- webs adjacent to an Arctic seabird colony. *Science of the Total Environment* 408, 1858-1867.
- Christiansen, L.B., Winther-Nielsen, M., Helweg, C., 2002. Feminization of fish: The effect of estrogenic compounds and their fate in sewage treatment plants and nature. Danish Environmental Protection Agency. Environmental Project No. 729.
- Chu, S., Metcalfe, C.R., 2007. Analysis of paroxetine, fluoxetine and norfluoxetine in fish tissues using pressurized liquid extraction, mixed mode solid phase extraction cleanup and liquid chromatography–tandem mass spectrometry. *Journal of Chromatography A* 1163, 112-118.
- Craft, J.A., Brown, M., Dempsey, K., Francey, J., Kirby, M.F., Scott, A.P., Katsiadaki, I., Robinson, C.D., Davies, I.M., Bradac, P., Moffat, C.F., 2004. Kinetics of vitellogenin protein and mRNA induction and depuration in fish following laboratory and environmental exposure to oestrogens. *Marine Environmental Research* 58, 419-423.
- Croley, T.R., Hughes, R.J., Koenig, B.G., Metcalfe, C.D., March, R.E., 2000. Mass spectrometry applied to the analysis of estrogens in the environment. *Rapid Communications in Mass Spectrometry* 14, 1087-1093.
- Czajka, C. P., Kathleen L. Londry, K. L., 2006. Anaerobic biotransformation of estrogens. *Science of the Total Environment* 367, 932 -941.
- deBruyn, A.M.H., Marcogliese, D.J., Rasmussen, J.B., 2003. The role of sewage in a large river food-web. *Canadian Journal of Fisheries and Aquatic Sciences* 60, 1332-1344.

- de Hoffmann, E., Stroobant, V., 2002. Mass spectrometry: principals and applications. Chichester, John Wiley & Sons.
- Desbrow, C., Routledge, E.L., Brighty, G.C., Sumpter, J.P., Waldock, M., 1998. Identification of estrogenic chemicals in sewage treatment water effluent. 1. chemical fractioning and in vitro biological screening. *Environmental Science and Technology* 32, 1549-1558.
- Desforbes, J. P. W., Peachey, B. D. L., Sanderson, P. M., White, P. A., Blais, J. M., 2010. Plasma vitellogenin in male teleost fish from 43 rivers worldwide is correlated with upstream human population size. *Environmental Pollution* 158, 3279-3284.
- Della Greca, M., Pinto, G., Pistillo, P., Pollio, A., Previtera, L., Temussi, F., 2008. Biotransformation of ethinylestradiol by microalgae. *Chemosphere* 70, 2047-2053.
- Dell'Angelica, E.C., Bonifacino J.S., 2000. Staining protein in gels, in: J. S. Bonifacino, et al. (Eds.), *Current Protocols in Cell Biology*, John Wiley & Sons Inc., New York, pp. 6.1 - 6.14.
- DiLiberti, C.E., O'Leary, C.M., Hendy, C.H., Waters, D.H., Margolis, M.B., 2011. Steady-state pharmacokinetics of an extended-regimen oral contraceptive with continuous estrogen. *Contraception* 83, 55-61.
- Dionex Corporation. 2004. Rapid determination of fat in meat using accelerated solvent extraction (ASE). Application note 334.
- Doyle, J.R., Al-Ansari, A.M., Gendron, R.L., White, P.A., Blais, J.M., 2011. A method to estimate sediment ingestion by fish. *Aquatic Toxicology* 103, 121- 127.

- Dussault, E.B., Balakrishnan, V.K., Borgmann, U., Solomon, K.R., Sibley, P.K., 2009. Bioaccumulation of the synthetic hormone 17alpha-ethinylestradiol in the benthic invertebrates *Chironomus tentans* and *Hyalella azteca*. *Ecotoxicology and Environmental Safety* 72, 1635-1641.
- Extension Toxicology Network (EXTOXNET), 1996. Pesticide Information Profiles. <<http://extoxnet.orst.edu/pips/tributyl.htm>> (cited February 24 2012).
- Falconer, I.R., Chapman, H.F., Moore, M.R., Ranmuthugala, G., 2006. Endocrine-disrupting compounds: a review of their challenge to sustainable and safe water supply and water reuse. *Environmental Toxicology* 21, 181-191.
- Fernandez, M.P., Ikonomou, M.G., Buchanan, I., 2007. An assessment of estrogenic contaminants in Canadian wastewaters. *Science of the Total Environment* 373, 250-269.
- Fernández, N., Diez, M.J., Terán, M.T., García, J.J., Calle, A.P., Sierra, M., 1998. Influence of two commercial fibers in the pharmacokinetics of ethinylestradiol in rabbits. *Journal of Pharmacology and Experimental Therapeutics* 286, 870-874.
- Flores-Valverde, A.M., Horwood, J., Hill E.M., 2010. Disruption of the steroid metabolome in fish caused by exposure to the environmental estrogen 17alpha-ethinylestradiol. *Environmental Science and Technology* 44, 3552-3558.
- Gabet-Giraud, V., Miege, C., Herbreteau, B., Hernandez-Raquet, G., Coquery, M., 2010. Development and validation of an analytical method by LC-MS/MS for the quantification of estrogens in sewage sludge. *Analytical and Bioanalytical Chemistry* 396, 1841-51.

- Gallagher, S.R., 2007. One-dimensional SDS gel electrophoresis of proteins, in: J. S. Bonifacino, et al. (Eds.), *Current Protocols in Cell Biology*, John Wiley & Sons Inc., New York. pp. 1.1 -1.38.
- Gibson, R., Smith, M.D., Spary, C.J., Tyler, C.R., Hill, A.M., 2005. Mixtures of estrogenic contaminants in the bile of fish exposed to wastewater treatment works effluents. *Environmental Science and Technology* 39, 2461-2471.
- Giese, R.W., 2003. Measurement of endogenous estrogens: analytical challenges and recent advances. *Journal of Chromatography A* 1000, 401-412.
- Gobas, F.A.P.C., Morrison, H. A., 2000. Bioconcentration and biomagnification in the aquatic environment, in Boething R.S., Mackay, D., *Handbook of property estimation methods for chemicals: Environmental and health sciences*, CRC Press, Florida. pp. 9.189 – 9.227.
- Gross-Sorokin, M.Y., Roast, S.D., Brighty, G.C., 2006. Assessment of feminization of male fish in English rivers by the environment agency of England and Wales. *Environmental Health Perspectives* 114, 147-151.
- Hansen, M., Björklund, E., Halling-Sørensen, B. Determination of steroids in manure and soil. Poster session presented at: SETAC Europe. 20th Annual Meeting of the Society of Environmental Toxicology and Chemistry Europe; 2010 May 23-27; Seville, Spain.
- Healy, D.F., Chambers, D.B., Rachol, C.M., Jodoin, R.S., 2007. Water quality of the St. Clair River, Lake St. Clair, and their U.S. tributaries, 1946-2005. U.S. Geological Survey, Scientific investigations report 2007-5172.

- Hemmer, M.J., Bowman, C.J., Hemmer, B.L., Friedman, S.D., Marcovich, D., Kroll, K.J., Denslow, N.D., 2002. Vitellogenin mRNA regulation and plasma clearance in male sheepshead minnows, (*Cyprinodon variegatus*) after cessation of exposure to 17 β -estradiol and p-nonylphenol. *Aquatic Toxicology* 58, 99-112.
- Holthaus, K. I. E., Johnson, A. C., Jürgens, M. D., Williams, R. J., Smith, J. J. L., Carter, J. E., 2002. The potential for estradiol and ethinylestradiol to sorb to suspended and bed sediments in some English rivers. *Environmental Toxicology and Chemistry* 21, 2526-2535.
- Houde, M., Muir, D.C.G., Kidd, K.A., Guildford, S., Drouillard, K., Evans, M.S., Wang, X., Whittle, D.M., Haffner, D., Kling, H., 2008. Influence of lake characteristics on the biomagnification of persistent organic pollutants in lake trout food-webs. *Environmental Toxicology and Chemistry* 27, 2169-2178.
- Howard, P.H., Muir, D.G.G., 2011. Identifying new persistent and bioaccumulative organics among chemicals in commerce II: pharmaceuticals. *Environmental Science and Technology* 45, 6938-6946.
- Ikemoto, T., Tu, N.P.C., Watanabe, M.X., Okuda, N., Omori, K., Tanabe, S., Tuyen, B.C., Takeuchi, I., 2008. Analysis of biomagnification of persistent organic pollutants in the aquatic food-web of the Mekong Delta, South Vietnam using stable carbon and nitrogen isotopes. *Chemosphere* 72, 104-114.
- Indrasena, W.M., Henneberry, K., Barrow, C.J., Kralovec, J.A., 2005. Qualitative and quantitative analysis of lipid classes in fish oils by thin-layer chromatography with an iatroscan flame ionization detector (TLC-FID) and liquid chromatography

- with an evaporative light scattering detector (LC-ELSD). *Journal of Liquid Chromatography and Related Technologies* 28, 2581-2595.
- Jin, S., Yang, F., Liao, T., Hui, Y., Xu, Y., 2008. Seasonal variations of estrogenic compounds and their estrogenicities in influent and effluent from a municipal sewage treatment plant in China. *Environmental Toxicology and Chemistry* 27, 146-153.
- Jobling, S., Beresford, N., Nolan, M., Rodgers-Gray, T., Brighly, G.C., Sumpter, J.P., Tyler, C.R., 2002a. Altered sexual maturation and gamete production in wild roach (*Rutilus rutilus*) living near rivers that receive treated sewage effluent. *Biology of Reproduction* 66, 272-281.
- Jobling, S., Coey, S., Whitmore, J.G., Kime, D.E., Van Look, K.J., McAllister, B.G., Beresford, N., Henshaw, A.C., Brighly, G., Tyler, C.R., Sumpter, J.P., 2002b. Wild intersex roach (*Rutilus rutilus*) have reduced fertility. *Biology of Reproduction* 67, 515-524.
- Jobling, S., Tyler, C.R., 2003. Endocrine disruption, parasites and pollutants in wild freshwater fish. *Parasitology* 126, 103-108.
- Jobling, S., Nolan, M., Tyler, C.R., Brighly, G., Sumpter, J.P., 1998. Widespread sexual disruption in wild fish. *Environmental Science and Technology* 32, 2498-2506.
- Jürgens, M.D., Holthaus, K.I., Johnson, A.C., Smith, J.L., Hetheridge, M., Williams, R.J., 2002. The potential for estradiol and ethinylestradiol degradation in English rivers. *Environmental Toxicology and Chemistry* 21, 480-488.
- Kavanagh, R.T., Balch, C.D., Kiparissis, Y., Nimi, A.J., Sherry, J., Tinson, C., Metcalfe, C.D., 2004. Endocrine disruption and altered gonadal development in white perch

- (*Monroe Americana*) from the lower great lakes region. Environmental Health Perspectives 112, 898-902.
- Keriko, J.M., Chege, C.W., Magu, M.M., Mwachiro, E.C., Murigi, A.N., Githua, M.N., Kareru, P.G., 2010. Fish lipid contents and classes of selected fish species found in Lake Naivasha (Kenya) and the fish feeding habits of the lake's inhabitants. African Journal of Pharmacy and Pharmacology 4, 745-753.
- Khanal, S.K., Xie, B., Thompson, M.L., Sung, S., Ong, S.K., Van Leeuwen, J., 2006. Fate, transport, and biodegradation of natural estrogens in the environment and engineered systems. Environmental Science and Technology 40, 6537-6546.
- Kidd, K.A., Blanchfield, P.J., Mills, K.H., Palace, V.P., Evans, R.E., Lazorchak, J.M., Flick, R.W., 2007. Collapse of a fish population after exposure to a synthetic estrogen. Proceedings of the National Academy of Sciences of U S A. 104, 8897-8901.
- Kleywegt, S., Smyth, S-A., Parrott, J., Schaefer, K., Lagacé, E., Payne, M., Topp, E., Beck, A., McLaughlin, A. and K. Ostapyk (eds). 2007. Pharmaceuticals and personal care products in the Canadian environment: Research and policy directions. NWRI scientific assessment report series No.8. 53 p.
- Kolodziej, E. P., Harter, T., Sedlak, D. L., 2004. Dairy wastewater, aquaculture, and spawning fish as sources of steroid hormones in the aquatic environment. Environmental Science and Technology 38, 6377-6384.
- Kolodziej, E.P., Gray, J.L., Sedlak, D.L., 2003. Quantification of steroid hormones with pheromonal properties in municipal wastewater effluents. Environmental Toxicology and Chemistry 22, 2622-2629.

- Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton, H.T., 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999-2000: a national reconnaissance. *Environmental Science and Technology* 36, 1202-1211.
- Korte, J.J., Kahl, M.D., Jensen, K.M., Pasha, M.S., Parks, L.G., LeBlanc, G.A., Ankley, G.T., 2000. Fathead minnow vitellogenin: complementary DNA sequence and messenger RNA and protein expression after 17 β -estradiol treatment. *Environmental Toxicology and Chemistry* 19, 972-981.
- Kuch, H.M., Ballschmiter, K., 2001. Determination of endocrine-disrupting phenolic compounds and estrogens in surface and drinking water by HRGC-(NCI)-MS in the picogram per liter range. *Environmental Science and Technology* 35, 3201-3206.
- Kuster, M., Lòpez de Alda, M.J., Barcelo, D., 2004. Analysis and distribution of estrogens and progestogens in sewage sludge, soils and sediments. *Trends in Analytical Chemistry* 23, 790-798.
- Lai, K.M., Scrimshaw, M.D., Lester, J.N., 2002. Prediction of the bioaccumulation factors and body burden of natural and synthetic estrogens in aquatic organisms in the river systems. *Science of the Total Environment* 289, 159-168.
- Länge, R., Hutchinson, T.H., Croudace, C.P., Sigmund, F., Schweinfurth, H., Hampe, P., Panter, G.H., Sumpter, J.P., 2001. Effects of the synthetic estrogen 17 α -ethinylestradiol on the life-cycle of the fathead minnow (*Pimephales promelas*). *Environmental Toxicology and Chemistry* 20, 1216-1227.

- Larsson, D.G.J., Adolfsson-Erici, M., Parkkonen, J., Pettersson, M., Berg, A.H., Olsson, P-E., Förlin, L., 1999. Ethinyloestradiol - an undesired fish contraceptive? *Aquatic Toxicology* 45, 91-97.
- Larsen, M.G., Hansen, K.B., Henriksen, P.G., Baatrup, E., 2008. Male zebrafish (*Danio rerio*) courtship behaviour resists the feminising effects of 17 alpha-ethinyloestradiol - morphological sexual characteristics do not. *Aquatic Toxicology* 87, 234-244.
- LeGuellec, K., Lawless, K., Valotaire, Y., Kress, M., Tinniswood, M., 1988. Vitellogenin gene expression in male rainbow trout (*Salmo gairdinar*). *General and Comparative Endocrinology* 71, 359-371.
- Liebig, M., Egeler, P., Oehlmann, J., Knacker, T., 2005. Bioaccumulation of ¹⁴C-17a-ethinyloestradiol by the aquatic oligochaete *Lumbriculus variegatus* in spiked artificial sediment. *Chemosphere* 59, 271-280.
- Lien, G.J., McKim, J.M., 1993. Predicting branchial and cutaneous uptake of 2,2',5,5'-tetrachlorobiphenyl in fathead minnows (*Pimephales promelas*) and Japanese medaka (*Oryzias latipes*); rate limiting factors. *Aquatic Toxicology* 27, 15-32.
- Lishman, L., Smyth, S.A., Sarafin, K., Kleywegts, S., Toito, J., Peart, T., Lee, B., Servos, M., Beland, M., Seto, P., 2006. Occurrence and reductions of pharmaceuticals and personal care products and estrogens by municipal wastewater treatment plants in Ontario, Canada. *Science of the Total Environment* 367, 544-558.
- Liu, J., Pan, X., Huang, B., Fang, F., Wang, Y., Gao, J., 2011b. An improved method for simultaneous analysis of steroid and phenolic endocrine disrupting chemicals in biological samples. *International Journal of Analytical Chemistry*, (iFirst), 1-15.

- Liu, J., Wang, R., Huang, B., Lin, C., Wang, Y., Pan, X., 2011a. Distribution and bioaccumulation of steroidal and phenolic endocrine disrupting chemicals in wild fish species from Dianchi Lake, China. *Environmental Pollution* 159, 2815-2822.
- Lye, C.M., Frid, C.L.J., Gill, M.E., McCormick, D., 1997. Abnormalities in the reproductive health of flounder (*Platichthys flesus*) exposed to effluent from a sewage treatment works. *Marine Pollution Bulletin* 3, 34-41.
- Ma, T.W., Wang, Z.J., Gong, S.J., 2007. Comparative sensitivity in Chinese rare minnow (*Gobiocypris rarus*) and Japanese Medaka (*Oryzias latipes*) exposed to ethinylestradiol. *Journal of Environmental Science and Health. Part A, Toxic/Hazardous Substances & Environmental Engineering* 42, 889-894.
- Mackay, D., 2001. Multimedia environmental models: The Fugacity Approach, Second Edition, CRC Press, Florida, pp. 3.44 – 3.48.
- Mackie, R.I., White, B.A., Bryant, M.P., 1990. Lipid metabolism in anaerobic ecosystems. *Microbiology* 17, 449 - 479.
- Marlatt, V.L., Martyniuk, C. J., Zhang, D., Xiong, H., Watt, J., Xia, X., Moon, T., Trudeau, V.L., 2008. Auto-regulation of estrogen receptor subtypes and gene expression profiling of 17 β -estradiol action in the neuroendocrine axis of male goldfish. *Molecular and Cellular Endocrinology* 283, 38-48.
- Martin, R.C., Dixon, D.G., R. Maguire, J., Hodson, P.V., Tkacz, R.T., 1989. Acute toxicity, uptake, depuration and tissue distribution of tri-n-butyltin in rainbow trout, *Salmo gairdneri*. *Aquatic Toxicology* 15, 37 – 52.
- Mayne, G., 2003. St. Clair RAP progress report: George et al. (Eds), Volume 1.

- McDaniel, T.V., Martin, P.A., Struger, J., Sherry, J., Marvin, C.H., McMaster, M.E., Clarence, S., Tetreault, G., 2008. Potential endocrine disruption of sexual development in free ranging male northern leopard frogs (*Rana pipiens*) and green frogs (*Rana clamitans*) from areas of intensive row crop agriculture. *Aquatic Toxicology* 88, 230-242.
- Metallinou, C., Asimakopoulos, B., Schröer, Nikolettos, N., 2007. Gonadotropin-releasing hormone in the ovary. *Reproductive Sciences* 14, 737-749.
- Medberry, S., Gallagher, S., 2002. Digital electrophoresis analysis, in: J. S. Bonifacino, et al. (Eds.), *Current Protocols in Cell Biology*, John Wiley & Sons Inc., New York. pp. 9.1-9.14
- Metcalf, C.D., Metcalfe, T.L., Kiparissis, Y., Koenig, B.G., Khan, C., Hughes, R.J., Croley, T.R., March, R.E., Potter, T., 2001. Estrogenic potency of chemicals detected in sewage treatment plant effluents as determined by in vivo assays with Japanese medaka (*Oryzias latipes*). *Environmental Toxicology and Chemistry* 20, 297-308.
- Mills, L.J., Chichester, C., 2005. Review of evidence: Are endocrine-disrupting chemicals in the aquatic environment impacting fish populations? *Science of the Total Environment* 343, 1-34.
- Mimeault, C., Woodhouse, A.J., Miao, X.S., Metcalfe, C.D., Moon, T.W., Trudeau, V.L., 2005. The human lipid regulator, gemfibrozil bioconcentrates and reduces testosterone in the goldfish, *Carassius auratus*, *Aquatic Toxicology* 73, 44-54.
- Nash, J.P., Kime, D.E., Van der Van, L.T.M., Wester, P.W., Brion, F., Maack, G., Stahlschmidt-Allner, P., Tyler, C.R., 2004. Long-term exposure to

- environmental concentrations of the pharmaceutical ethinylestradiol causes reproductive failure in fish. *Environmental Health Perspectives* 122, 1725-1733.
- Neef, G., 1999. Steroidal estrogens. P. 17 - 41. In M. Oettel and E. Schillinger (eds.). *Estrogens and Antiestrogens I. Handbook of Experimental Pharmacology* 135. Springer-Verlag Berlin 135.
- Newman, M.C., Unger, M.A., 2003. *Fundamentals of Ecotoxicology*, Second Edition. Ann Arbor Press, Chelsea, ISBN 1-56670-598-3.
- Notelovitz, M., 2006. Clinical opinion: the biologic and pharmacologic principles of estrogen therapy for symptomatic menopause. *MedGenMed* 8, 85. Available at: www.medscape.com/viewarticle/523196. Accessed July 28, 2011.
- Örn, S., Yamani, S., Norrgren, L., 2006. Comparison of vitellogenin induction, sex ratio, and gonad morphology between zebrafish and Japanese medaka after exposure to 17 α -ethinylestradiol and 17 β -trenbolone. *Archives of Environmental Contamination and Toxicology* 51, 237-243.
- Parrot, J.L., Blunt, B., 2005. Life-cycle exposure of fathead minnows (*Pimephales promelas*) to an ethinylestradiol concentration below 1 ng/L reduces egg fertilization success and demasculinizes males. *Environmental Toxicology* 20, 131-141.
- Paterson, G., Metcalfe, C.D., 2008. Uptake and depuration of the anti-depressant fluoxetine by the Japanese medaka (*Oryzias latipes*). *Chemosphere* 74, 125-130.
- Peck, M., Gibson, R.W., Kortenkamp, A., Hill, E.M., 2004. Sediments are major sinks of steroidal estrogens in two United Kingdom rivers. *Environmental Toxicology and Chemistry* 23, 945-952.

- Peng, X., Wang, Z., Yang, C., Chen, F., Mai, B., 2006. Simultaneous determination of endocrine-disrupting phenols and steroid estrogens in sediment by gas chromatography-mass spectrometry. *Journal of Chromatography A* 1116, 51-56.
- Peterson, B.J., Fry, B., 1987. Stable isotopes in ecosystem studies. *Annual Review of Ecology and Systematics* 18,293-320.
- Pereira, R.O., Postigo, C., de Alda, M.L., Daniel, L.A., Barceló. D., 2011. Removal of estrogens through water disinfection processes and formation of by-products. *Chemosphere*. 82, 789-799.
- Pérez-Campos, E.F., 2010. Ethinylestradiol/dienogest in oral contraception. *Drugs* 70, 681-689.
- Phillips, B., and Harrison, P., 1999. Overview of the endocrine disruptors issue, in Hester, R., E., and Harrison, R., M., (eds) *Endocrine Disrupting Chemicals*, Cambridge: The Royal Society of Chemistry, 14.
- Prescription drug info., 2011. The people's medicine community (top 200 prescription drugs). < http://www.prescriptiondrug-info.com/top_prescription_drugs.asp> (cited June 21 2011).
- Purdom, C.E., Hardiman, P.A., Bye, V.J., Eno, N.C., Tyler, C.R., Sumpter, J.P., 1994. Estrogenic effects of effluents from sewage treatment works. *Chemistry and Ecology* 8, 275-285.
- Qiao, P., Gobas, F.A., Farrell, A.P., 2000. Relative contributions of aqueous and dietary uptake of hydrophobic chemicals to the body burden in juvenile rainbow trout. *Archives of Environmental Contamination and Toxicology* 39, 369-377.

- Robinson, B. J., Hellou, J., 2009. Biodegradation of endocrine disrupting compounds in harbour seawater and sediments. *Science of the Total Environment* 21, 5713-5718.
- Robinson, B.J., Hui, J.P., Soo, E.C., Hellou, J., 2009. Estrogenic compounds in seawater and sediment from Halifax Harbour, Nova Scotia, Canada. *Environmental Toxicology and Chemistry* 28, 18-25.
- Robinson, C.D., Brown, E., Craft, J.A., Davies, I.M., Moffat, C.F., Pirie, D., Robertson, F., Stagg, R.M., Struthers, S., 2003. Effects of sewage effluent and ethynyl oestradiol upon molecular markers of oestrogenic exposure, maturation and reproductive success in the sand goby (*Pomatoschistus minutus*, Pallas). *Aquatic Toxicology* 62, 119-134.
- RoC. 2011. Estrogens, Steroidal. Report on Carcinogens 12th edition. U.S. Department of Health and Human Services National Toxicology Program. P 184-188.
- Rodgers-Gray, T.P., Jobling, S., Kelly, C., Morris, S., Brighty, G., Waldock, M.J., Sumpter, J.P., Tyler, C.R., 2001. Exposure of juvenile roach (*Rutilus rutilus*) to treated swage effluents induce dose-dependant and persistent disruption in gonadal duct development. *Environmental Science and Technology* 35, 462-470.
- Runnqvist, H., Bak, S.A., Hansen, M., Styrisshave, B., Halling-Sørensen, B., Björklund, E., 2010. Determination of pharmaceuticals in environmental and biological matrices using pressurised liquid extraction are we developing sound extraction methods? *Journal of Chromatography A* 1217, 2447-2470.
- Saravanabhavan, G., Helleur, R., Hellou, J., 2009. GC-MS/MS measurement of natural and synthetic estrogens in receiving waters and mussels close to a raw sewage ocean outfall. *Chemosphere* 76, 1156-1162.

- Santos, I.R., Costa, R.C., Freitas, U., Fillmann, G., 2008. Influence of effluents from a wastewater treatment plant on nutrient distribution in a coastal creek from southern Brazil. *Brazilian Archives of Biology and Technology* 51, 153-162.
- Sawan, S. P., James, T. L., Gruenke, L. D., Craig, J. C., 1979. Proton NMR assignments for cholesterol. Use of deuterium NMR as an assignment aid. *Journal of Magnetic Resonance* 35, 409-413.
- Schultz, I.R., Orner, G., Merdink, J.L., Skillman, A., 2001. Dose-response relationships and pharmacokinetics of vitellogenin in rainbow trout after intravascular administration of 17 α -ethynylestradiol. *Aquatic Toxicology* 51, 305-318.
- Scott, W.B., Crossman, E.J., 1973. *Freshwater fishes of Canada*. Ottawa: Fisheries research board of Canada. Bulletin 184.
- Servos, M.R., Bennie, D.T., Burnison, B.K., Jurkovic, A., McInnis, R., Neheli, T., Schnell, A., Seto, P., Smyth, S.A., Ternes, T.A., 2005. Distribution of estrogens, 17 β -estradiol and estrone, in Canadian municipal wastewater treatment plants. *Science of the Total Environment* 336, 155-70.
- Simpson, M.G., Parry, M., Kleinkauf, A., Swarbreck, D., Walker, P., Leah, R.T., 2000. Pathology of the liver, kidney and gonad of flounder (*Platichthys flesus*) from a UK estuary impacted by endocrine disrupting chemicals. *Marine Environmental Research* 50, 283-287.
- Skillman, A.D., Nagler, J.J., Hook, S.E., Small, J.A., Schultz, I.R., 2006. Dynamics of 17 α -ethynylestradiol exposure in rainbow trout (*Oncorhynchus mykiss*): absorption, tissue distribution, and hepatic gene expression pattern. *Environmental Toxicology and Chemistry* 25, 2997-3005.

- Socorro, S., Power, D.M., Olsson, P.E., Canario, A.V.M., 2000. Two estrogen receptors expressed in the teleost fish, *Sparus aurata*: cDNA cloning, characterization, and tissue distribution. *Journal of Endocrinology* 166, 293-306.
- Soto, A. M., Calabro, J. M.; Prechtel, N. V., Yau, A. Y., Orlando, E. F., Daxenberger, A., Kolok, A. S., Guillette, L. J., le Bizec, B., Lange, I. G., Sonnenschein, C., 2004. Androgenic and estrogenic activity in water bodies receiving cattle feedlot effluent in eastern Nebraska, USA. *Environmental Health Perspectives* 112, 346-352.
- Spacie, A., Landrum, P.F, Levesee, G.J., 1983. Uptake, depuration, and biotransformation of anthracene and benzo[a]pyrene in bluegill sunfish. *Ecotoxicology and Environmental Safety* 7, 330 – 341.
- Sumpter, J.P., Jobling, S., 1995. Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. *Environmental Health Perspectives* 103, 173-178.
- Sumpter, J.P., Johnson, A.C., 2009. 10th anniversary perspective: reflections on endocrine disruption in the aquatic environment: from known knowns to unknown unknowns (and many things in between). *Journal of Environmental Monitoring* 10, 1476-1485.
- Swanson, H., Gantner, N., Kidd, K.A., Muir, D.C., Reist, J.D., 2011. Comparison of mercury concentrations in landlocked, resident, and sea-run fish (*Salvelinus* spp.) from Nunavut, Canada. *Environmental Toxicology and Chemistry* 30, 1459-1467.
- Trudeau, V.L., 1997. Neuroendocrine regulation of gonadotrophin II release and gonadal growth in the goldfish, *Carassius auratus*. *Reviews of Reproduction*, 2, 55-68.

- Trudeau, V.L., Metcalfe C.D., Mimeault C., Moon T.W., 2005. Pharmaceuticals in the environment: Drugged fish? *Biochemistry and Molecular Biology of Fishes*. Vol. 6 (Chapter 17), 475-493.
- Tyler, C.R., Jobling, S., Sumpter, J.P., 1998. Endocrine disruption in wildlife: A critical review of the evidence. *Critical Reviews in Toxicology* 28, 319-361.
- U. S. EPA, 1998. Endocrine Disruptors Screening and Testing Advisory Committee, Final Report, available online at <http://www.epa.gov/endo/pubs/edstac/chap3v14.pdf>
- U. S. EPA, 1994. Gel-permeation clean-up available online at <http://www.epa.gov/waste/hazard/testmethods/sw846/pdfs/3640a.pdf>
- van Aerle, R., Nolan, T.M., Jobling, S., Christiansen, L.B., Sumpter, J.P., Tyler, C.R., 2001. Sexual disruption in a second species of wild cyprinid fish (the gudgeon, *Gobio gobio*) in the United Kingdom freshwaters. *Environmental Toxicology and Chemistry* 20, 2842-2847.
- Van den Belt, K., Berckmans, P., Vangenechten, C., Verheyen, R., Witters, H., 2004. Comparative study on the in vitro/in vivo estrogenic potencies of 17beta-estradiol, estrone, 17alpha-ethynylestradiol and nonylphenol. *Aquatic Toxicology* 66, 183-195.
- Vosges, M., Braguer, J.C., Combarous, Y., 2008. Long-term exposure of male rats to low-dose ethinylestradiol (EE2) in drinking water: effects on ponderal growth and on litter size of their progeny. *Reproductive Toxicology* 25, 161-168

- Wang, S., Huang W., Fang, G., Zhang, Y., Qia, H., 2008. Analysis of steroidal estrogen residues in food and environmental samples. *International Journal of Environmental Analytical Chemistry* 88, 1-25.
- Wierman, M.E., 2007. Sex steroid at target tissues: mechanism of action. *Advances in Physiology Education* 31, 26-33.
- Wise, A., Brien, K., Woodruff, T., 2011. Are oral contraceptives a significant contributor to the estrogenicity of drinking water? *Environmental Science and Technology* 45, 51-60.
- Wood, J.R., Strauss, J.F.^{3rd}, 2002. Multiple signal transduction pathways regulate ovarian steroidogenesis. *Reviews in Endocrine and Metabolism Disorders* 3, 33-46.
- Wyn, B., Kidd, K.A., Burgess, N.M., Curry, R.A., Munkittrick KR, 2010. Increasing mercury in yellow perch at a hotspot in Atlantic Canada, Kejimikujik National Park. *Environmental Science and Technology* 44, 9176-9181
- Xu, H., Yang, J., Wang, Y., Jiang, Q., Chen, H., Song, H., 2008. Exposure to 17 alpha-ethynylestradiol impairs reproductive functions of both male and female zebrafish (*Danio rerio*). *Aquatic Toxicology* 88, 1-8.
- Xu, Y., Luo, F., Pal, A., Gin, K. Y., Reinhard, M., 2011. Occurrence of emerging organic contaminants in a tropical urban catchment in Singapore. *Chemosphere* 83, 963-969.
- Ying, G.G., Kookana, R.S., 2003. Degradation of five selected endocrine-disrupting chemicals in seawater and marine sediment. *Environmental Science and Technology* 37, 1256-1260.

- Ying, G.G., Kookana, R.S., Dillon, P., 2003. Sorption and degradation of selected five endocrine disrupting chemicals in aquifer material. *Water Research* 37, 3785-3791.
- Zha, J.M., Wang, Z.J., Wang, N., Ingersoll, C., 2007. Histological alternation and vitellogenin induction in adult rare minnow (*Gobiocypris rarus*) after exposure to ethynylestradiol and nonylphenol. *Chemosphere* 66, 488-495.
- Zhang, Z, Rhind, S.M., Kerr, C., Osprey, M., Kyle, C.E., 2011. Selective pressurized liquid extraction of estrogenic compounds in soil and analysis by gas chromatography-mass spectrometry. *Analytica Chimica Acta* 685, 29-35.

Appendix

Appendix A

List of publications and presentations

Peer-reviewed publications

Al-Ansari, A. M., Saleem, A., Kimpe, L.E., Sherry, J.P., McMaster, M.E., Trudeau, V.L., Blais, J.M., 2010. Bioaccumulation of the pharmaceutical 17 α -ethinylestradiol in shorthead redhorse suckers (*Moxostoma macrolepidotum*) from the St. Clair River, Canada. *Environmental Pollution* 158, 2566-2571.

Al-Ansari, A. M., Saleem, A., Kimpe, L.E., Trudeau, V.L., Blais, J.M., 2011. The development of an optimized sample preparation for trace level detection of 17 α -ethinylestradiol and estrone in whole fish tissue. *Chromatography B* (in press).

Doyle, J.R., **Al-Ansari, A.M.**, Gendron, R.L., White, P.A., Blais, J.M., 2011. A method to estimate sediment ingestion by fish. *Aquatic Toxicology* 103, 121- 127.

Oral presentations

Ahmed Al-Ansari, Ammar Saleem, Linda Kimpe, Jim Sherry, Mark McMaster, Vance Trudeau, Jules Blais. 17 α -ethinylestradiol (EE2) bio-accumulates in Shorthead Redhorse Sucker (*Moxostoma macrolepidotum*). 62^{ed} Canadian Conference for Fisheries Research and Society of Canadian Limnologists, Ottawa, Ontario, January 9-11, 2009.

Ahmed Al-Ansari, Ammar Saleem, Linda Kimpe, Jim Sherry, Mark McMaster, Vance Trudeau, Jules Blais. Bioaccumulation of the Pharmaceutical Ethinylestradiol in Shorthead Redhorse Sucker (*Moxostoma macrolepidotum*) from the St. Clair River, Canada. SETAC Europe 20th Annual Meeting, Seville, Spain, May, 23-27, 2010.

Ahmed Al-Ansari, Ammar Saleem, Linda Kimpe, Jim Sherry, Mark McMaster, Vance Trudeau, Jules Blais. Bioaccumulation of ethinylestradiol in wild fish. 7th Annual CEMN Partners' Meeting, Lakefield, Ontario, June 16-18, 2010.

Poster presentations

Ahmed Al-Ansari, Ammar Saleem, Linda Kimpe, Jim Sherry, Mark McMaster, Vance Trudeau, Jules Blais. 17 α -ethinylestradiol (EE2) bioaccumulates in Shorthead Redhorse Sucker (*Moxostoma macrolepidotum*). SETAC North American 29th Annual Meeting, Tampa, FL, November 16-20, 2008.

Ahmed Al-Ansari, Ammar Saleem, Linda Kimpe, Vance Trudeau, Jules Blais. Development of a sample preparation procedure for the determination of 17 α -ethinylestradiol in whole fish tissues. SETAC North American 32nd Annual Meeting, Boston, MA, November 13-17, 2011.

Appendix B

In cell clean up experiments

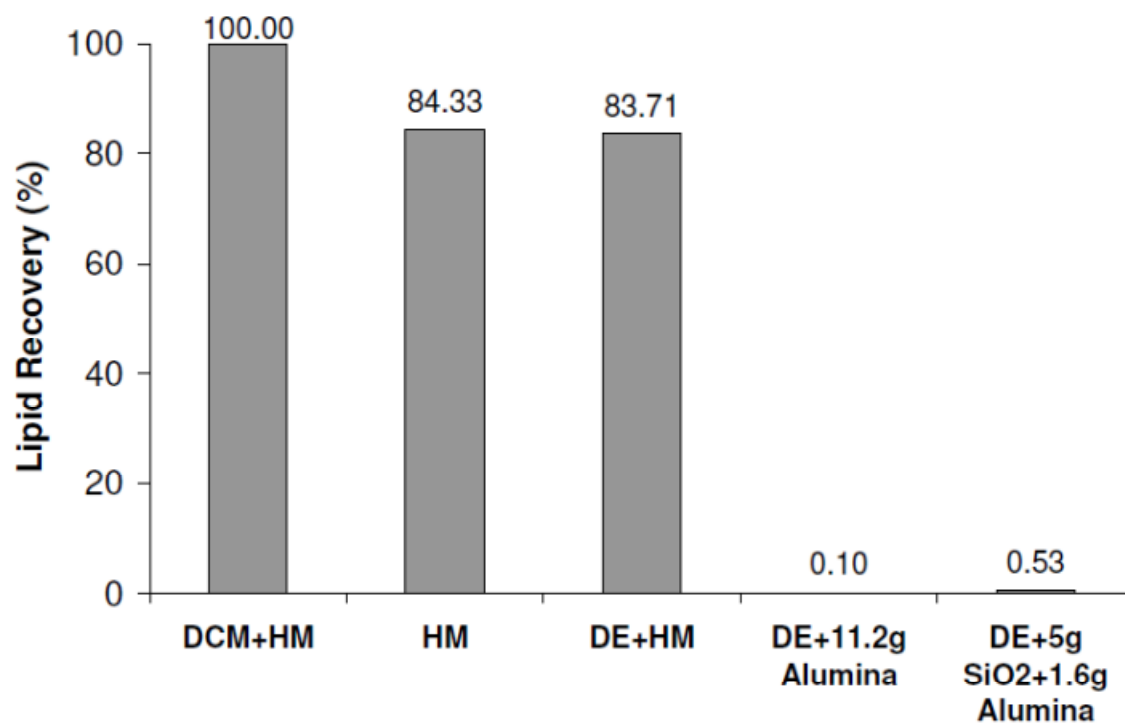


Figure B1: Lipid recovery of a known lipid content ShRHS sample (0.218 g/g) extracted by dichloromethane (DCM) with cells filled with hydromatrix (HM). Hexane flush with a cell filled with HM and filled with HM plus (4 g) diatomaceous earth (DE). The lipid retention capacity of the following packing: alumina (11.2 g) versus silica (SiO₂) (5.0 g) with alumina (1.6 g).

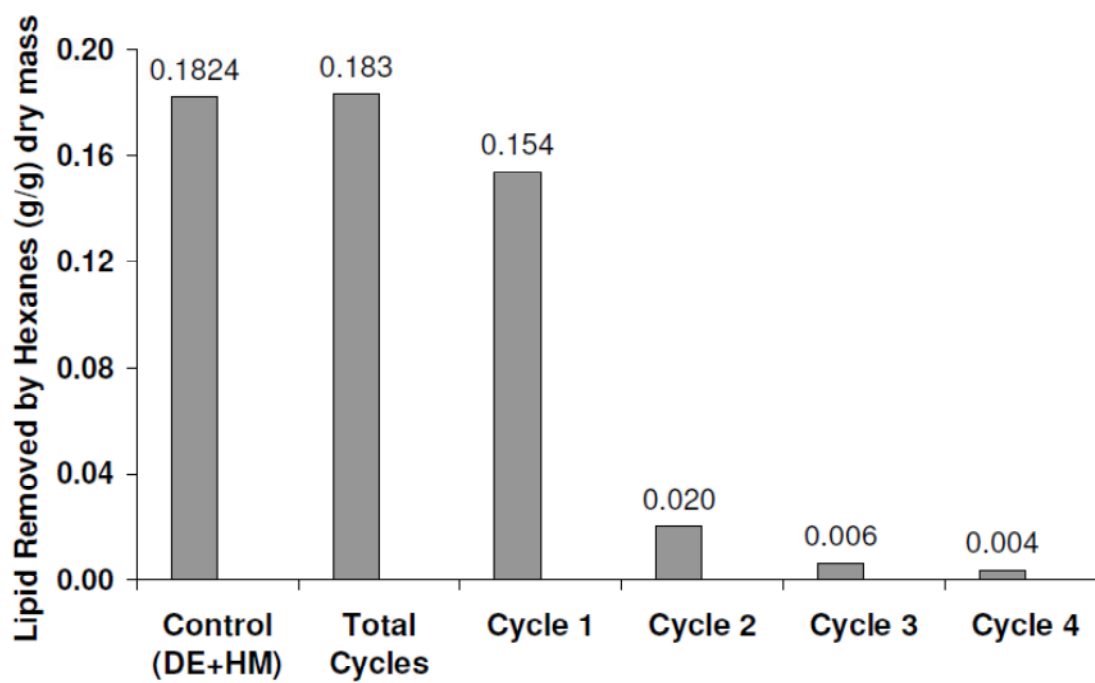


Figure B2: Optimization of the number of ASE cycles for total lipid removal from the sample during the hexane flush (step 1) extraction.

Appendix C

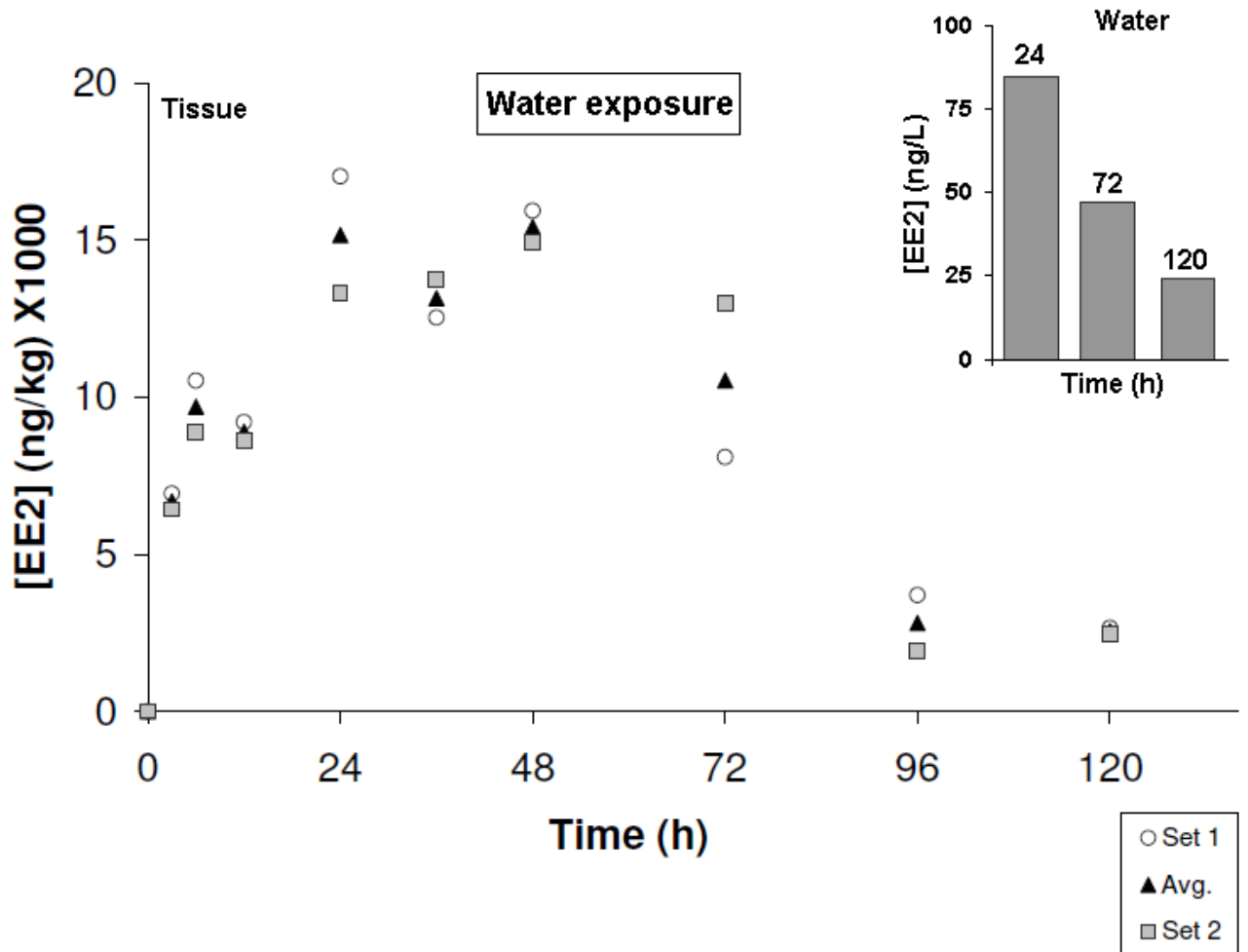


Figure C1: EE2 uptake curve obtained from (2 g) whole tissue goldfish extracts after a flow-through exposure to a nominal of 273 ng/L. Duplicate samples were removed from the 70-L exposure tank. The bar figure shows levels of EE2 in 1 L water samples showing a decline in EE2 concentration overtime. This experiment was redesigned as illustrated in Chapter 5 to stabilize EE2 water levels during the whole time course and to determine whether EE2 decline was caused by metabolism. Sample preparation was carried out according to methods described in Chapter 5.

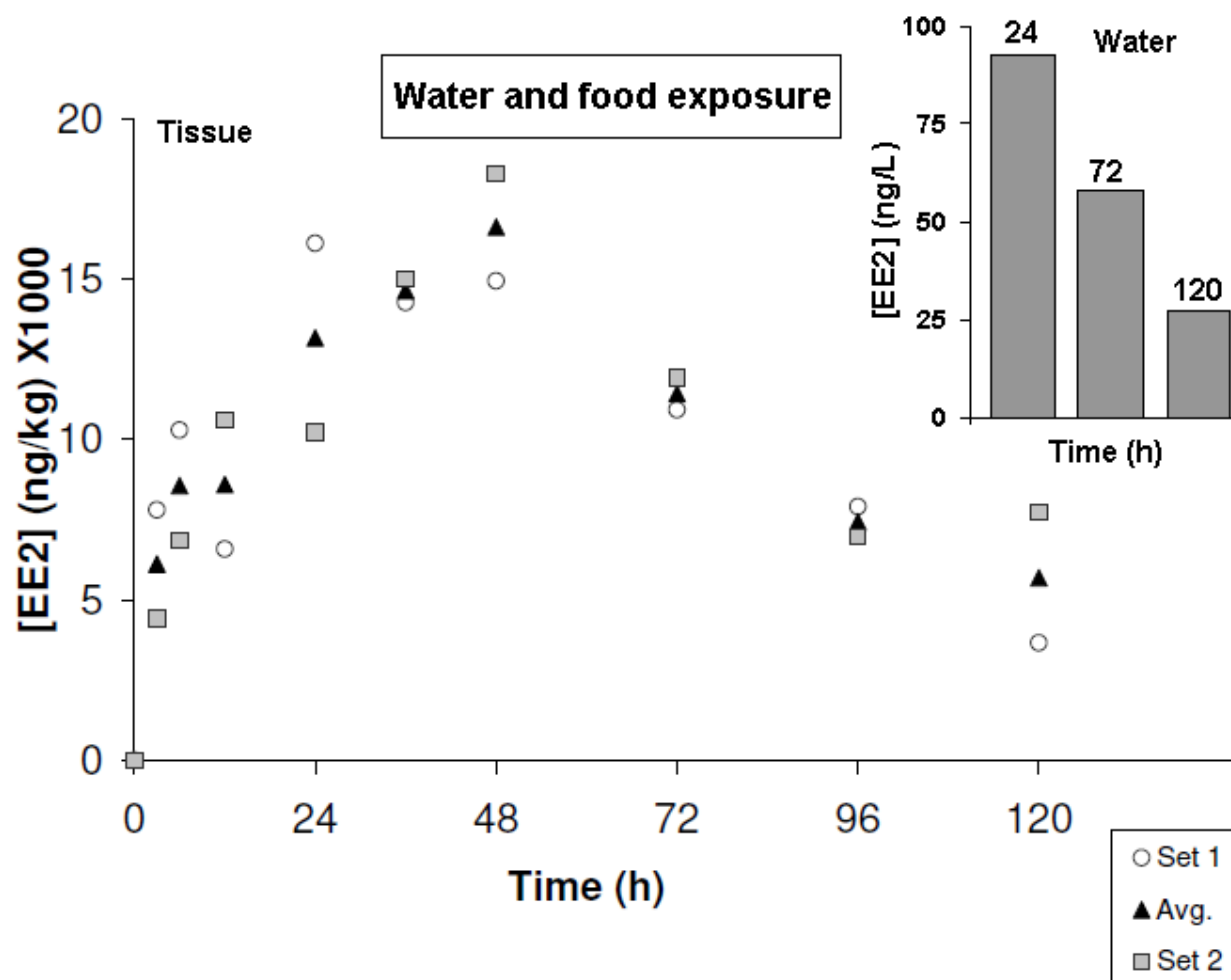


Figure C1: EE2 uptake curve obtained from (2 g) whole tissue goldfish extracts after a flow-through exposure to a nominal of 273 ng/L plus 50 ng/g food. Duplicate samples were removed from the 70-L exposure tank. The bar figure shows levels of EE2 in 1 L water samples showing a decline in EE2 concentration overtime. This experiment was redesigned as illustrated in Chapter 5 to stabilize EE2 water levels during the whole time course and to determine whether EE2 decline was caused by metabolism. Sample preparation was carried out according to methods described in Chapter 5.

Appendix D

This appendix provides some useful tips and visual illustrations in the dissection of goldfish to minimize the loss of body fluids if surgical removal of the gastrointestinal tract (GI) is required.

Picture #1

■ Blood drainage



Picture #2

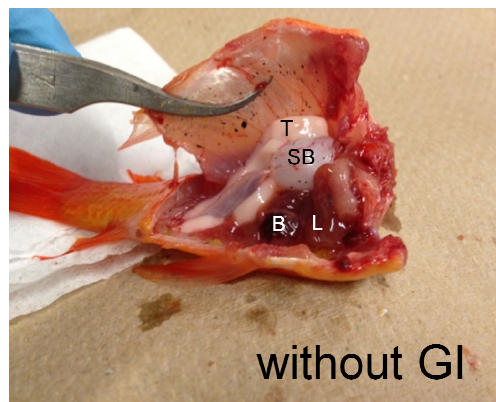
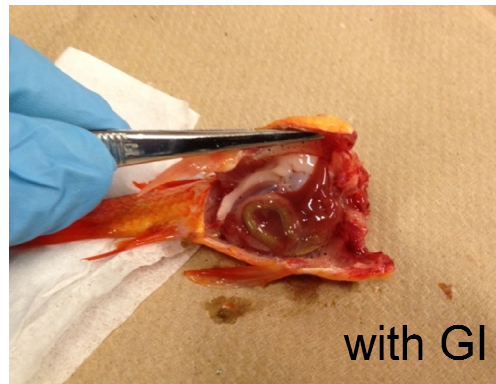
■ No bleeding



- 1- Once the fish is sacrificed by the trans-spinal sectioning, it should be placed vertically on ice as shown the picture above for minimum 10 min to allow blood drainage and coagulation
- 2- The head should be separated from the body and left in the 50 mL falcon tube
- 3- Performing step 2 carefully results in no bleeding as shown in picture 2

Picture #3

■ **Careful dissection**



Picture 3 shows a goldfish samples before (above) and after (below) the surgical removal of the GI tract. It also shows that the reminder organs such as the liver (L), testes (T), swimming bladder (SB), and bile (B) are still intact.

Appendix E

This appendix provides some useful tips and visual illustrations in some critical steps during sample preparation to insure a successful detection of EE2.

The cholesterol removal step

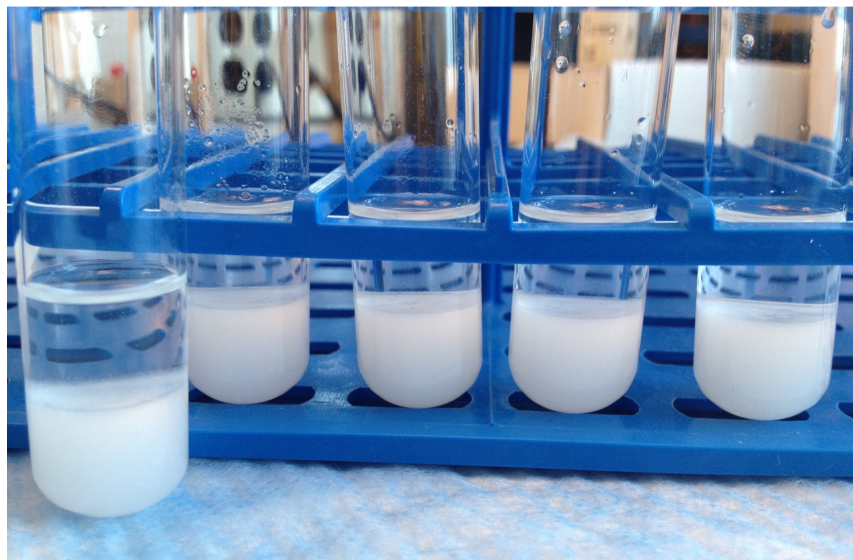


The two pictures above show the freezing step of the a fish sample (2 g dw) in order to remove cholesterol

- 1- Make sure to perform this step twice where the sample should be frozen at -20°C for minimum 1 h.
- 2- The required spinning speed is 4000 rpm at the minimum temperature of the centrifuge for 15 min. However, reduction of this speed to 3000 or 2000 rpm should work as well.
- 3- Careful removal of the clear supernatant to a clean vial with a pasture pipette is double however, the disturbance of the cholesterol pellet could easily occur. Therefore, (if needed) this step could be replaced by a filtration step with 0.25 micron (PTFE) syringe filters
- 4- A hexane (2 mL) wash step is usually applied twice to insure a complete removal of cholesterol or other non-polar compounds. During this step, some emulsion

may form which makes the phase separation between the polar layer (below) containing EE2 and the non-polar layer (waste) somewhat difficult, to solve this problem a quick spinning (2 - 5min) at 2000 rpm would help.

The derivatization step



The derivative of EE2 to be detected by the GC/MS step is found in the clear upper non-polar phase as shown in the picture above. However, this phase may become somewhat cloudy and if taken analyzed without purification the detection of EE2 could be impossible. To solve this problem a quick spinning (2 - 5min) at 2000 rpm would help.