Evaluating the Estrogenic Activity of Municipal Wastewater Effluents

by

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Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Brendan M. Smith

Abstract

Municipal wastewater treatment plants (MWWTPs) are point sources of environmental contamination that can cause adverse effects on fish species exposed to their effluents (MWWEs). Contaminants, such as endocrine disrupting compounds (EDCs), commonly found in MWWEs have been shown to induce many adverse reproductive effects. In the Grand River watershed of southern Ontario, three MWWTPs from the cities of Guelph, Waterloo and Kitchener have been identified to adversely alter molecular and cellular responses in fish, which have been previously associated with exposure to estrogenic EDCs. Currently these systems are undergoing process upgrades to address aging infrastructure as well as expanding capacities to serve rapidly increasing populations. Studies in this thesis were conducted to determine whether the Guelph and Waterloo MWWEs, as well as surrogate pilot plant effluents that modeled possible treatment process upgrades for MWWTPs were estrogenic *in vivo* in controlled laboratory exposures. Unfortunately, the Kitchener MWWE (conventional activated sludge and lagoon) was acutely toxic and could not be included in laboratory exposures. Rainbow trout (*Oncorhynchus mykiss*) were exposed to 20% to 90% effluent from both Guelph and Waterloo, for 2, 8 and 14 days and fish plasma was analyzed for vitellogenin (VTG) a biomarker for estrogenic exposure. These exposures indicated that neither the Guelph nor the Waterloo MWWE were estrogenic *in vivo* under the exposure conditions in this study. In contrast the total estrogenic equivalence measured using the yeast estrogen screen (YES) of the Guelph, Waterloo and Kitchener MWWEs increased respectively, inversely related to the degree of treatment. Three pilot plant effluents (Burlington MWWTP) including conventional activated sludge (CAS), nitrifying with CAS (CAS-N) and biological nutrient removal with CAS (CAS-BNR) were also tested in rainbow trout *in vivo* for plasma VTG induction. Only the CAS-BNR effluent caused weak (2-3.5 fold) induction of VTG relative to controls. Although the estrogenicity (YES) of the effluent was variable the VTG induction corresponded with the slightly higher 17β-estradiol equivalents measured in these pilot plant effluents. An effect-directed analysis

(EDA) for estrogenic substances was carried out on the Waterloo and Kitchener MWWEs and revealed that the estrogenicity (YES) of the Waterloo MWWE was associated with estrone, while the estrogenicity of the Kitchener MWWE was associated with several chemicals (estrone > 17β-estradiol > bisphenol A ~ testosterone).

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Dedication

To my friends and family, I cannot adequately express my gratitude. This last year has had its challenges. I was happy to help those of you I could, and I was most appreciative of those who helped me. You were integral to the completion of this project, thank you all.

To family, I am dedicating this thesis to you. Thank you for always being there for me. I am lucky to have you and I love you all.

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List of Abbreviations

AOP	Adverse outcome pathway
AP1	Activator protein-1
BPA	Bisphenol A
BSA	Bovine serum albumin
DES	Diethylstilbestrol
DHT	Dihydrotestosterone
E1	Estrone
E2	17β-Estradiol
E3	Estriol
EDA	Effects-directed analysis
EDC	Endocrine disrupting compound
EE2	17α-Ethynilestradiol
ER	Estrogen receptor
ERE	Estrogen response element
HOT	High pilot plant operating temperature (24ºC)
HPG	Hypothalamic pituitary gonad axis
HPLC	High performance liquid chromatography
LBD	Ligand binding domain
LOT	Low pilot plant operating temperature (12ºC)
MOA	Mode of action
mpA	Mobile phase A
mpB	Mobile phase B
MTBE	Methyl tert-butyl ether
MWWEs	Municipal wastewater effluents
MS-222	Tricaine methanesulfonate
NLS	Nuclear localization sequence
4NP	4-Nonylphenol
4OP	4-Octylphenol
OPD	*o*-Phenylenediamine
PDA	Photo diode array
SPE	Solid phase extraction
SRC	Steroid receptor coactivator
StAR	Steroidogenic acute regulatory protein
MWWTP	Municipal wastewater treatment plant
T	Testosterone
TIE	Toxicity identification evaluation
VTG	Vitellogenin
YES	Yeast estrogen screen
β-gal	β-Galactosidase

Chapter 1: Introduction

Municipal wastewater effluents (MWWEs) pose a risk to aquatic ecosystems and potentially public health. While these effluents impact multiple trophic levels, abnormalities in the reproductive systems of animals, including fish, have recently gained international attention. These effects have been attributed to a class of contaminants in the environment that disrupt the endocrine system (EDCs), including the hypothalamic pituitary gonadal axis (HPG) that controls key processes involved in growth, development and reproduction. (Tyler *et al.*, 1998). Perturbing the pathways involved in these three processes can mediate adverse outcomes that influence the performance of organisms in their environment (Ankley *et al.*, 2010). Reproductive impairment in fish exposed to MWWEs has been observed throughout the world, with the greatest prevalence associated with densely populated metropolitan areas. Many of the studies conducted in Canada, the United States of America (Nichols *et al.*, 1999, Hewitt and Servos, 2001, Vajda *et al.*, 2008, Williams *et al.*, 2009, Barber *et al.*, 2011, Garcia-Reyero *et al.*, 2011, Ings *et al.*, 2012, Tetreault *et al.*, 2012), United Kingdom (Sumpter, 1995, Sumpter, 1997, Jobling *et al.*, 1998, Jobling *et al.*, 2002, Thorpe *et al.*, 2009, Runnalls *et al.*, 2010), Germany (Daughton and Ternes, 1999, Stachel *et al.*, 2003, Grund *et al.*, 2011), Switzerland (Aerni *et al.*, 2004), Australia (Batty and Lim, 1999) and China (Ma *et al.*, 2005) have reported altered behaviour and secondary sex characteristics, as well as abnormal tissue morphology, gene expression and steroid synthesis.

Adverse responses have been observed in fish exposed to MWWEs at multiple biological levels including the organism, tissue, molecular, biochemical and genetic levels. Some MWWEs have the potential to alter fish populations by biasing sex ratios (Knorr and Braunbeck, 2002), reducing fecundity (Kang *et al.*, 2002, Brian *et al.*, 2007, Scholz and Mayer, 2008, Thorpe *et al.*, 2009) and altering energy allocation and tissue development (e.g. somatic indices) at the organism level. Some studies have identified MWWEs that have decreased the allocation of energy to

gonadal investment (i.e. decreased gonadosomatic index: GSI) (Kukkonen *et al.*, 1999, Hemming *et al.*, 2001, Tetreault *et al.*, 2011), while others have increased gonadal development (Rempel *et al.*, 2006, Barber *et al.*, 2007), and either could inhibit reproductive success. Somatic indices are strong indicators of the changes that have occurred at the tissue level of organization, however gonad histopathology has been used to reveal abnormal tissue morphologies (Blazer, 2002) in fish populations. Some studies have reported lowered sperm counts, altered motility and milt production in male fish exposed to MWWEs (Jobling *et al.*, 2002, Schoenfuss *et al.*, 2009, Tetreault *et al.*, 2011, Vajda *et al.*, 2011), whereas females have been reported to have increased follicular atresia (Jobling *et al.*, 2002). But in some circumstances, gonochoristic males have displayed both gonad morphologies. These gonads have oocytes of various stages of development in testicular tissue (ova-testes) (Jobling *et al.*, 1998, Jobling *et al.*, 2002, Seki *et al.*, 2003, Hirai *et al.*, 2006, Woodling *et al.*, 2006, Vajda *et al.*, 2008, Iwanowicz *et al.*, 2009, Tetreault *et al.*, 2011, Tanna, 2012). The presence of ova-testes in gonochoristic fish could suggest that molecular pathways controlling the differentiation or development of these tissues or cells are disrupted. The pathways that are associated with the development or differentiation of gonadal tissues reside in the HPG of the endocrine system. However, these adverse outcomes are not fully understood nor are the affects on normal spermatogenesis, oogenesis or reproducing fish.

The endocrine system is a vast network of tissues and glands that secrete hormones into the circulatory system, which alter metabolism, growth and development and reproduction. In top-down organization, the HPG axis consists of several tissues each secreting hormones that influence the reproductive system. The hypothalamus secretes gonadotropin-releasing hormone, which stimulates the release of the gonadotropins, luteinizing hormone and follicle-stimulating hormone from the anterior pituitary. These two hormones induce and enhance many cellular responses, which include gonadal development, spermatogenesis, oogenesis, and gonadal steroidogenesis. Gonadal steroidogenesis produces 3 types of gonadal steroids at different concentrations

throughout the reproductive cycles. These hormones are produced in the somatic cells and include progestogens, androgens, and estrogens. Both genders produce these hormones at different concentrations where males typically have greater concentrations of androgens, while females have greater concentrations of estrogens. Exposing males to estrogenic substances or females to androgenic substances can affect the production of these hormones and other molecular markers of impaired reproduction (Ankley *et al.*, 2001) However, not all of the reproductive mechanisms or phenotypes are fully understood, the ova-testes pathway likely shares the estrogen-agonist signaling pathway with another female reproductive pathway called vitellogenesis (Ankley *et al.*, 2010).

Vitellogenin (VTG) is an egg-yolk precursor apolipoprotein expressed in the liver of female oviparous organisms. Its expression is estrogen dependent and a substantial contributor to the rapid attainment of metabolic resources (i.e. increase liver somatic index, LSI) just prior to oocyte maturation. The circulating VTG is absorbed into developing oocytes and cleaved into lipovitellins and phosvitins, which also coincides with rapid growth of the oocyte (Wallace and Selman, 1985), to become nutrient sources for the developing embryo (Roy and Chatterjee, 1983). Pre-spawning females generally produce more circulating VTG than males, however reproductive females and produce as much as 10^6 times more VTG than males (Copeland *et al.*, 1986). Studies later confirmed that VTG was an estrogen-agonist mechanism when males were injected with the natural estrogen (17β-estradiol) and plasma VTG increased in a dose dependent manner (Covens *et al.*, 1987, Leguellec *et al.*, 1988). Interestingly, males exposed to MWWEs have also been reported to express significantly higher concentrations of VTG (Folmar *et al.*, 1996, Harries *et al.*, 1996, Harries *et al.*, 1997, Knudsen *et al.*, 1997, Harries *et al.*, 1999, Tilton *et al.*, 2002). Males exposed to MWWEs that express more VTG than reference fish suggests that MWWEs could contain substances that can activate the estrogen-signaling pathway. These compounds have since been referred to as estrogenic EDCs. As such, VTG induction in male or immature fish has been used as

an indicator of exposure to estrogenic substances at the organism level (Jobling and Sumpter, 1993, Sumpter and Jobling, 1995, Hansen *et al.*, 1998, Jobling *et al.*, 1998, Denslow *et al.*, 1999, Aerni *et al.*, 2004, Garcia-Reyero *et al.*, 2011, Kolok *et al.*, 2012), and a potential indicator for higher-order reproductive impairments including ova-testes (Jobling *et al.*, 2002).

Gene expression in the estrogen-signaling pathway can be altered by the interaction of estrogen receptors and the ligands that bind them, the ligand-receptor complex and the regulatory elements these receptors recognize. There are two estrogen receptor (ERs) isoforms that have been identified to date, ERα and ERβ (Mosselman *et al.*, 1996, Kuiper *et al.*, 1997, Pakdel *et al.*, 1997), and each isoform has up to two documented subtype variants. Currently, rainbow trout have 4 known estrogen receptors, ERα1, ERα2, ERβ1 and ERβ2 (Yamaguchi *et al.*, 2005, Leanos-Castaneda and Van der Kraak, 2007, Nagler *et al.*, 2007, Shyu *et al.*, 2011). But other fish such as fathead minnow (*Pimephales promelas*), Atlantic croaker (*Micropogonias undulatus*), Nile tilapia (*Oreochromis niloticus*) and goldfish (*Carassius auratus*) have 3 known ERs: ERα, ERβ1 and ERβ2 (Hawkins *et al.*, 2000, Filby and Tyler, 2005, Nelson *et al.*, 2007, Huang, 2012). All of these ERs share similar domain structures: the N-terminus ligand-independent region (AF-1, A/B domain), the DNA binding domain (DBD, C domain), the hinge region (D domain), the C-terminus ligand-binding domain (LBD, E domain), and the F domain (the latter two a part of AF-2) (Kumar *et al.*, 1987, Montano, 1995, Mosselman *et al.*, 1996, Gaido *et al.*, 1997, Kumar *et al.*, 2011). However, these isoforms have slightly different sequences. Generally, the ERβ is a truncated form of the longer ERα, where the C and E domains are 96% and 56% conserved but the A/B, D and F domains are not (Mosselman *et al.*, 1996). In "classical" ligand-dependent mechanisms, ERα binds most ligands with greater affinity than ERβ with some exceptions including certain phytosterols (Kuiper *et al.*, 1997, Kuiper *et al.*, 1998). These different ligand affinities could potentially infer functional differences between these two receptors in ligand-dependent mechanisms. However, certain ERs can have alternative mechanisms of activation. Both ERs can be activated by ligands,

however, while ERα is primarily activated by ligand-dependent mechanisms the ERβ can also be activated by ligand-independent mechanisms (Vivar *et al.*, 2010, Zhao *et al.*, 2010). These ERs also target different response elements and therefore genes. The primary response elements that ERα bind are the "classical" EREs (Wood *et al.*, 2001), whereas the ERβ can differentially bind multiple response elements inducing stimulating/specificity protein 1 (SP1) and activating protein 1 (AP1) elements (Paech *et al.*, 1997, Marino *et al.*, 2006, Vivar *et al.*, 2010, Levin, 2011), factors involved in cellular growth, differentiation and apoptosis (Ameyar *et al.*, 2003, Abdelrahim *et al.*, 2004, Safe and Abdelrahim, 2005). In the HPG axis of rainbow trout, ERα1 and ERβ2 mRNA are more abundant than ERα2 and ERβ1 mRNA. In the brain and pituitary ERα1 and ERβ2 mRNA are relatively equal, however the gonads express more ERα1 than ERβ2 and the liver expresses more ERβ2 than ERα1 (Nagler *et al.*, 2007). Evidentially, the ERs and their mechanisms are conserved between the genders but the genome is differentially expressed to produce dimorphic reproductive outcomes. The compounds that are found in the environment which could potentially bind these ERs and activate ligand dependent mechanisms could be introduced into the environment from several sources.

Municipal wastewater effluents are complex matrices that could contain hormonally active compounds and at the concentrations that would produce adverse outcomes (Danzo, 1997, Stahlschmidt-Allner *et al.*, 1997, Danzo, 1998, Daughton and Ternes, 1999, Ankley *et al.*, 2007). However, not every environment where these outcomes are documented produce similar responses. To date, the reproductive impairments reported in European fish (Jobling *et al.*, 1998) appear to be more abundant and severe than the effects reported in Canada (Vos *et al.*, 2000, Tetreault *et al.*, 2011). This observation could present some interpretation challenges because Canadian MWWEs have not been studied as well as their European counterparts. But, the EDCs that are found in European MWWEs (Larsson *et al.*, 1999, Johnson *et al.*, 2005) are also found in North American MWWEs (Ternes *et al.*, 1999b, Snyder *et al.*, 2001, D'Ascenzo *et al.*, 2003, Lishman *et al.*, 2006,

Togunde *et al.*, 2012). The chemical similarity would suggest that Canadian MWWEs might also have the potential to impair fish reproduction, and in these studies, estrogenic substances have been commonly found. Most of these compounds have been shown to influence reproductive adverse outcome pathways (AOPs) individually, although multiple estrogenic EDCs at various concentrations have been found in MWWEs (Ternes *et al.*, 1999b, Servos *et al.*, 2005, Lishman *et al.*, 2006, Trenholm *et al.*, 2006, Fernandez *et al.*, 2007, van der Linden *et al.*, 2008, Wang *et al.*, 2011). To date, the effects induced by low-dose exposure to EDCs including low-dose complex mixtures remain uncertain (Vandenberg *et al.*, 2012). The estrogenic EDCs that have been identified in MWWEs fall into two categories, naturally produced estrogens, and estrogen mimicking substances (xenoestrogens). These EDCs include the natural estrogens 17β-estradiol (E2), estrone (E1), estriol (E3), as well as the xenoestrogens 17α-ethinylestradiol (EE2), bisphenol A (BPA), and short-chain alkylphenols such as 4-nonylphenol (4-NP), and 4-octylphenol (4-OP), most of which can individually induce gene expression to produce various adverse effects (Guo *et al.*, 2005, Valle *et al.*, 2005, Gunnarsson *et al.*, 2007, Zhang *et al.*, 2008, Watanabe *et al.*, 2009) (Table 1.1).

The pathologies of fish exposed to MWWEs are similar to those of fish exposed to certain estrogenic substances. These include numerous shared adverse outcomes that included altered energy allocation (tissue somatic indices), histopathology, biochemical responses and gene expression. For example, fish exposed to EE2, E2 and BPA have higher LSIs as well as plasma VTG (Kosmala *et al.*, 1998, Kukkonen *et al.*, 1999) but lower GSI (Sohoni *et al.*, 2001, Andersen *et al.*, 2003, Brion *et al.*, 2004, Pawlowski *et al.*, 2004). In classical cause and effect experiments Nash *et al.* (2004) exposed zebrafish (*Danio rerio*) to 0.5 – 50 ng/L EE2 for 40 days and reported reduced reproductive success, while males exhibited ova-testes and approximately 10^4 times more plasma VTG than reference males. In an ecological perspective, Kidd *et al.* (2007) exposed a population of fathead minnows (*Pimephales promelas*) to 5 ng/L of EE2 (a major component of

contraceptive pills) over 7 years in Lake 260 of the Experimental Lakes Area of Ontario, Canada and found similar outcomes: reduced fecundity, ova-testes and elevated plasma VTG in male fish and a population collapse. These studies connect chronic low-dose exposure to environmentally relevant concentrations of a single estrogenic substance (EE2) at concentrations found in MWWEs to impair reproduction, as well as identifying potential early indicators of reproductive impairment such as histology (ova-testes) and molecular biomarkers (VTG).

Although there are several known estrogenic EDCs in MWWEs, it remains difficult to establish a direct causal link between the compounds detected in MWWEs and the biological responses observed in the receiving environment. Cause and effect linkages are generally inferred based on field observations, lab experiments and chemical profiles of the effluents. However, techniques have been developed to identify the chemicals that may be responsible for specific effects in these complex mixtures. These techniques generally involve the extraction of chemicals from a matrix, the separation of these chemicals into fractions that are tested for a specific biological response. This methodology has evolved from bio-assay directed chemical analyses (Schuetzle and Lewtas, 1986) which were used to identify carcinogens, pesticides, and diesel particulates, in a variety of matrices which included drinking water and MWWEs. Toxicity identification evaluations (TIEs) were later developed by the United States of America's Environmental Protection Agency to specifically isolate inorganic, metal and acute toxicity in complex effluents (Durhan *et al.*, 1990). Eventually, effect-directed analyses (EDAs) were developed that use specific biological endpoints mechanistically linked to effects at higher levels of organization (i.e. AOPs) to isolate organic contaminants in a variety of complex mixtures (Brack, 2003). A list of EDAs and TIEs are presented in Table 1.2, exemplifying the diversity of applications.

Table 1.1: A list of model systems used to determine the estrogenicity *in vitro* and the reproductive effects *in vivo* of estogenic substances found in municipal wastewater effluents.

Endocrine Disrupting Compound	Estrogen Response Assays (*in vitro* and *in vivo*)		Organism/Cell line	Reference
Estrone	*in vitro*	Estrogen Receptor (ER-hepatic)	rainbow trout (*Oncorhynchus mykiss*)	(Denny *et al.*, 2005)
		HGELN (HeLa derived)		
		MCF-7 (E-screen)	mammalian cells	(Gutendorf and Westendorf, 2001)
		MVLN (MCF-7 derived)		
		Vitellogenin (VTG-hepatic)	rainbow trout (*Oncorhynchus mykiss*)	(Petersen and Tollefsen, 2011)
			Atlantic Salmon	(Tollefsen *et al.*, 2003)
	in vivo	Ova-testes	Japanese medaka (*Oryzias latipes*)	(Metcalfe *et al.*, 2001)
		VTG	rainbow trout (*Oncorhynchus mykiss*)	(Thorpe *et al.*, 2003)
17β-estradiol	*in vitro*	ER (hepatic)	rainbow trout (*Oncorhynchus mykiss*)	(Denny *et al.*, 2005)
		HELN ER α/β		(Sultan *et al.*, 2001)
		HGELN (HeLa derived)	mammalian cells	(Gutendorf and Westendorf, 2001)
		MCF-7		(White *et al.*, 1994, Petit *et al.*, 1997)
		VTG		(Petit *et al.*, 1997)
		VTG (hepatic)	rainbow trout (*Oncorhynchus mykiss*)	(White *et al.*, 1994, Petersen and Tollefsen, 2011)
		Yeast Estrogen Screen (YES)	yeast hER	(Gaido *et al.*, 1997, Andersen *et al.*, 1999)
		YES	rainbow trout (*Oncorhynchus mykiss*) ER	(Petit *et al.*, 1997)
	in vivo	GFP-VTG		(Chen *et al.*, 2010)
		hatch count	Japanese medaka (*Oryzias latipes*)	(Shioda and Wakabayashi, 2000)
		Ova-testes		(Metcalfe *et al.*, 2001)
			fathead minnow (*Pimephales promelas*)	(Sun *et al.*, 2009)
		VTG	rainbow trout (*Oncorhynchus mykiss*)	(Clemens *et al.*, 1975, Flouriot *et al.*, 1997, Thorpe *et al.*, 2003, Van den Belt *et al.*, 2003b)
Estriol	*in vitro*	ER (hepatic)	rainbow trout (*Oncorhynchus mykiss*)	(Denny *et al.*, 2005)
		VTG (hepatic)	Atlantic salmon (*Salmo salar*)	(Tollefsen *et al.*, 2003)
		YES	yeast hER	(Gaido *et al.*, 1997)

Endocrine Disrupting Compound	Estrogen Response Assays (*in vitro* and *in vivo*)		Organism/Cell line	Reference
17α-ethinylestradiol	*in vivo*	GFP-VTG	Japanese medaka (*Oryzias latipes*)	(Chen *et al.*, 2010)
		Ova-testes		(Metcalfe *et al.*, 2001)
		VTG (hepatic)	rainbow trout (*Oncorhynchus mykiss*)	(Petersen and Tollefsen, 2011)
	in vitro	ER (hepatic)	rainbow trout (*Oncorhynchus mykiss*)	(Denny *et al.*, 2005)
		HGELN (HeLa derived)	mammalian cells	(Gutendorf and Westendorf, 2001)
		MCF-7 (E-screen)		(Dobbins *et al.*, 2008)
		VTG (hepatic)	rainbow trout (*Oncorhynchus mykiss*)	(Dobbins *et al.*, 2008)
			Japanese medaka (*Oryzias latipes*)	
			fathead minnow (*Pimephales promelas*)	
		YES	yeast hER	(Gaido *et al.*, 1997, Andersen *et al.*, 1999, Dobbins *et al.*, 2008)
	in vivo	GFP-VTG	Japanese medaka (*Oryzias latipes*)	(Chen *et al.*, 2010)
		Ova-testes		(Metcalfe *et al.*, 2001)
		VTG	fathead minnow (*Pimephales promelas*)	(Garcia-Reyero *et al.*, 2009)
			Japanese medaka (*Oryzias latipes*)	(Seki *et al.*, 2002)
			rainbow trout (*Oncorhynchus mykiss*)	(Pelissero *et al.*, 1993, Van den Belt *et al.*, 2003a)
4-nonylphenol	*in vitro*	ER (hepatic)	rainbow trout (*Oncorhynchus mykiss*)	(Denny *et al.*, 2005)
		HELN ER α/β	mammalian cells	(Sultan *et al.*, 2001)
		VTG (hepatic)	rainbow trout (*Oncorhynchus mykiss*)	(White *et al.*, 1994)
			Japanese medaka (*Oryzias latipes*)	(Dobbins *et al.*, 2008)
			fathead minnow (*Pimephales promelas*)	(Dobbins *et al.*, 2008)
		YES	yeast hER	(Routledge *et al.*, 1998, Sohoni and Sumpter, 1998)
		YES	rainbow trout (*Oncorhynchus mykiss*) ER	(Petit *et al.*, 1997)
	in vivo	Hatch count	Japanese medaka (*Oryzias latipes*)	(Shioda and Wakabayashi, 2000)
		Ova-testes	Japanese medaka (*Oryzias latipes*)	(Metcalfe *et al.*, 2001)
		Sex ratios and VTG	guppies (*Poecilia reticulate)*	(Cardinali *et al.*, 2004)
		VTG	fathead minnow (*Pimephales promelas*)	(Sun *et al.*, 2009)
			Japanese medaka (*Oryzias latipes*)	(Yamaguchi *et al.*, 2005)
			rainbow trout (*Oncorhynchus mykiss*)	(Van den Belt *et al.*, 2003a)

Endocrine Disrupting Compound	Estrogen Response Assays (*in vitro* and *in vivo*)		Organism/Cell line	Reference
4-octylphenol	*in vitro*	ER (hepatic)	rainbow trout (*Oncorhynchus mykiss*)	(Denny *et al.*, 2005)
		HELN ER alpha/beta	mammalian cells	(Sultan *et al.*, 2001)
		HGELN (HeLa derived)		(Gutendorf and Westendorf, 2001)
		MCF-7 (E-screen)		(White *et al.*, 1994)
		VTG (hepatic)	rainbow trout (*Oncorhynchus mykiss*)	(Petersen and Tollefsen, 2011)
		YES	yeast hER	(Routledge and Sumpter, 1997, Andersen *et al.*, 1999)
	in vivo	ER	rainbow trout (*Oncorhynchus mykiss*)	(Arukwe *et al.*, 2002)
		Ova-testes	Japanese medaka (*Oryzias latipes*)	(Knorr and Braunbeck, 2002)
		Sex ratios		
		VTG	rainbow trout (*Oncorhynchus mykiss*)	(Arukwe *et al.*, 2002, Van den Belt *et al.*, 2003a)
		Zona radiata protein		(Arukwe *et al.*, 2002)
bisphenol A	*in vitro*	HELN ER α/β	mammalian cells	(Sultan *et al.*, 2001)
		HGELN (HeLa derived)		(Gutendorf and Westendorf, 2001)
		MCF-7 (E-screen)		(Gutendorf and Westendorf, 2001, Dobbins *et al.*, 2008)
		VTG (hepatic)	rainbow trout (*Oncorhynchus mykiss*)	(Dobbins *et al.*, 2008, Petersen and Tollefsen, 2011)
		YES	yeast hER	(Gaido *et al.*, 1997, Dobbins *et al.*, 2008)
	in vivo	GFP-VTG	Japanese medaka (*Oryzias latipes*)	(Chen *et al.*, 2010)
		Hatch count		(Shioda and Wakabayashi, 2000)
		Ova-testes		(Metcalfe *et al.*, 2001)
			fathead minnow (*Pimephales promelas*)	(Sun *et al.*, 2009)
		VTG	Japanese medaka (*Oryzias latipes*)	(Yamaguchi *et al.*, 2005)
			rainbow trout (*Oncorhynchus mykiss*)	(Van den Belt *et al.*, 2003a)

GFP-VTG, green fluorescent protein tagged vitellogenin transgenic cell line; HeLa, (Henrietta Lacks) a proliferative estrogen dependent cervical cancer cell line; HELN, (HeLa Luc) transgenic human cell line; HGELN, (HeLa Gal-ER-Luc-Neo) transgenic human cell line; MCF-7, a proliferative estrogen dependent breast cancer cell line; MVLN, (MCF-7-p-Vit-tk-Luc-Neo) transgenic human cell line; Rainbow trout ER (BJ-ECZ, YEprtER ERE-*CYC*1- *lacZ*); VTG, plasma Vitellogenin; YES, Yeast Estrogen Screen hER (BJ3505 *CUP*1-hER ERE-*LacZ* or hER PGK-ERE-*LacZ*)

EDAs can be powerful tools to isolate and identify specific compounds that activate or inhibit molecular pathways from complex mixtures. At any given time an unknown number of chemicals such as personal care products, surfactants and pharmaceuticals enter MWWTPs. Some of these compounds might be relatively unaffected by the treatment process while others might undergo complex chemical or biochemical interactions before being discharged into the receiving water body. But, without much prior knowledge of the EDCs in the MWWE, EDAs can attempt to discriminate the unaltered (parental) compounds or chemical by-products that activate or inhibit a particular pathway (Ankley *et al.*, 1998, Brack, 2003, Brack *et al.*, 2007, Ankley *et al.*, 2010, Volz *et al.*, 2011). This approach has been used to identify a diverse group of contaminants, which include: phytoestrogens (Burnison *et al.*, 2003), estrogenic alkylphenols (Sheahan *et al.*, 2002), androgens (Thomas *et al.*, 2002), pyrethroid insecticides (Mehler *et al.*, 2011), and antiandrogens (Hill *et al.*, 2010). However, the first EDA to directly link an estrogenic response *in vivo* (VTG induction in male fish) to estrogenic substances in MWWEs was conducted in the United Kingdom in the 1990s (Routledge *et al.*, 1998). To do so, Routledge *et al.* (1998) used reverse-phase high pressure liquid chromatography (HPLC) to separate the chemicals by polarity, these discrete fractions were tested for active estrogenic chemicals using a yeast estrogen screen (YES) and authentic standards to identify E1, E2 and EE2 in these MWWEs (Desbrow *et al.*, 1998). These studies were the first of their kind to directly link the estrogenic substances in a MWWE to an adverse effect.

Table 1.2: The identification of agonists and antagonists using various toxicity identificaiton evaluations and effect-directed analyses assessing different modes of actions in different matrices from around the world.

Toxicological endpoints	**Location**	**Matrix**	**Authors**
Teratogens	Germany	Sediments	(Higley *et al.*, 2012)
ER and AR agonists	Belgium	Sediments	(Schmitt *et al.*, 2011)
Teratogens	Netherland	Landfill soils	(Legler *et al.*, 2011)
ER, AR and AhR agonists	Norway	Marine sediments	(Grung *et al.*, 2011)
ER agonists, H295R	Germany	Sediments	(Grund *et al.*, 2011)
AhR agonists	Germany	Suspended particulates	(Wölz *et al.*, 2010)
Antiandrogens	UK	Sediments	(Hill *et al.*, 2010)
Teratogens	Germany	Sediments	(Bataineh *et al.*, 2010)
Teratogens	Venice	Lagoon Sediments	(Picone *et al.*, 2009)
AR agonists and antagonists	Belgium	Sediments	(Weiss *et al.*, 2009)
ER agonists, VTG	USA	MWWE	(Schlenk, 2008)
ER agonists	Canada	MWWE	(Fernandez *et al.*, 2007)
V. fischeri	Germany	Drinking Water	(Scheurell *et al.*, 2007)
AhR, EROD	Germany	Sediments	(Brack *et al.*, 2005)
Estrogen agonists	Canada	Agricultural runoff	(Burnison *et al.*, 2003)
AR agonists	UK	Estuaries	(Thomas *et al.*, 2002)
Metals and ammonia	USA	Dredged sediments	(Ho *et al.*, 2002)
C. dubia and fathead minnow developmental assay	USA	Agricultural ground water	(Gustavson *et al.*, 2000)
Daphnia	China	Chemical Works effluent	(Yang *et al.*, 1999)
ER agonists	Britain	MWWE	(Desbrow *et al.*, 1998)
MFO	Canada	Lampricide formula	(Hewitt *et al.*, 1996)
Metals	US	Sediments	(Schubauer-Berigan *et al.*, 1993)

ER, estrogen receptor; AR, androgen receptor; AhR, aryl-hydrocarbon receptor; EROD, Ethoxyresorufin-o-deethylase; MFO, mixed-function oxygenase detoxification enzyme.

Recent studies in the central Grand River watershed (Figure 1.1) have reported reproductive impairment in fish exposed to three MWWEs from the city of Guelph, Waterloo and Kitchener MWWTPs (Figure 1.2). The city of Guelph MWWE altered the expression of three estrogen dependent genes in rainbow trout by inducing vitelline envelope proteins zona radiata alpha (ZRα) and VTG but suppressing ZRβ (Hyllner *et al.*, 1991, Oppen-Berntsen *et al.*, 1992, Ings *et al.*, 2012). The Guelph MWWTP is the most advanced treatment system (tertiary) of the three MWWTPs and while these estrogenic markers are sensitive there were only minor changes indicating that the Guelph MWWE was only weakly estrogenic. However, the resident benthic fish, rainbow and greenside darters (*Etheostoma caeruleum* and *E. blennioides*) exposed to the Waterloo (secondary non-nitrifying) MWWE displayed moderate ova-testes (< 10 oocytes/lobe in < 20% of the lobes) (Tanna, 2012, Tetreault *et al.*, 2012). These fish exhibited an elevated condition factor (a ratio of the overall weight to the length of the fish cubed but the GSIs and LSIs did not differ consistently from upstream references (Tanna, 2012). Downstream of the Kitchener MWWTP (also a secondary non-nitrifying plant) darters had the greatest prevalence of ova-testes (> 100 oocytes in 10% of the lobes, < 100 oocytes in 30% of the lobes, and < 10 oocytes in 30% of the lobes) and lower GSIs and LSIs for several downstream sites (Tanna, 2012). These results suggest that these effluents induce various effects in receiving environments with the Kitchener effluents appearing to be more estrogenic than the Waterloo effluent, while Guelph effluent is the least estrogenic. However, these field studies are confounded by numerous environmental factors such as stream gradients, water quality and habitat making it difficult to make direct comparisons.

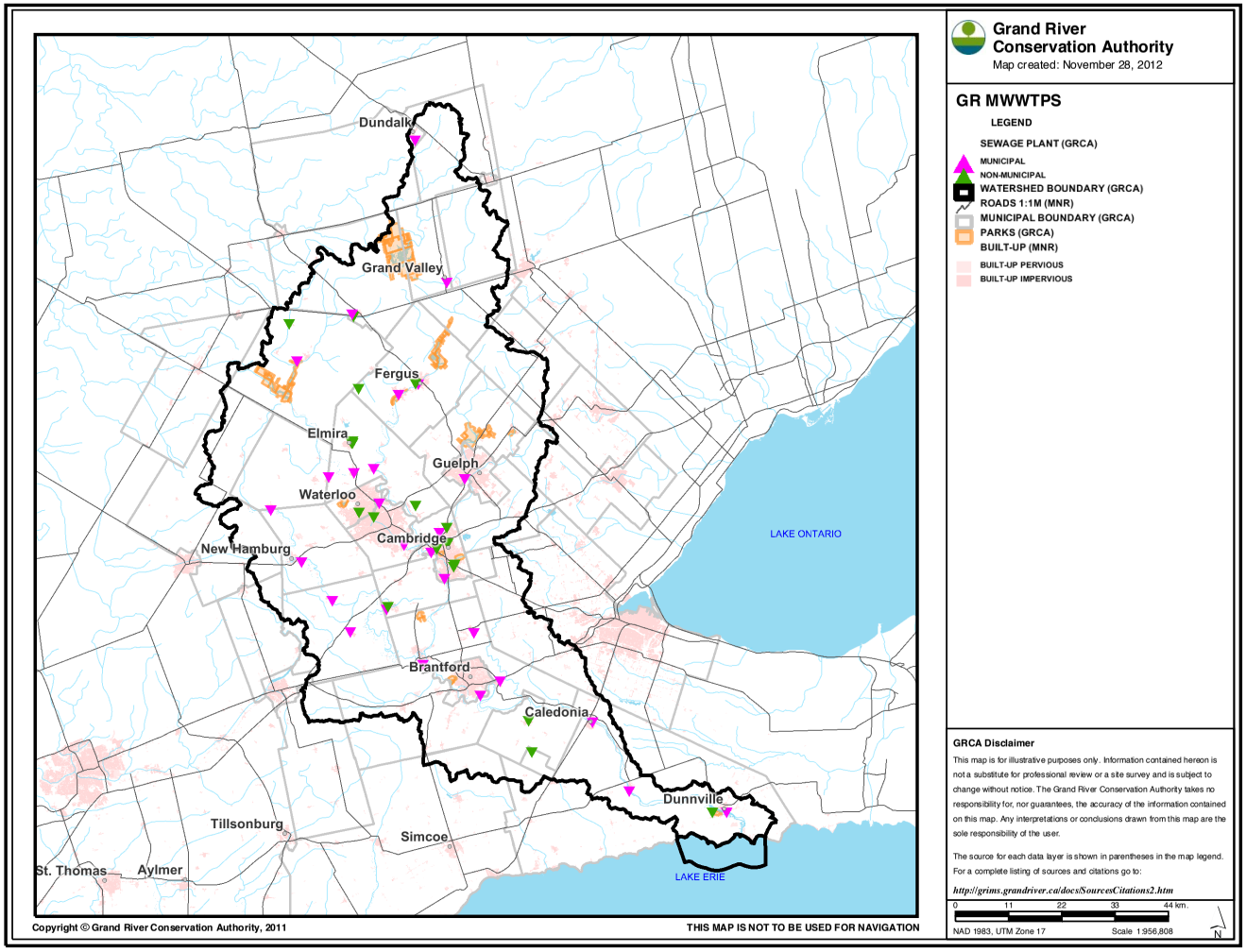


Figure 1.1: The Grand River watershed of southern Ontario, Canada (Grand River Conservation Authority, 2012).

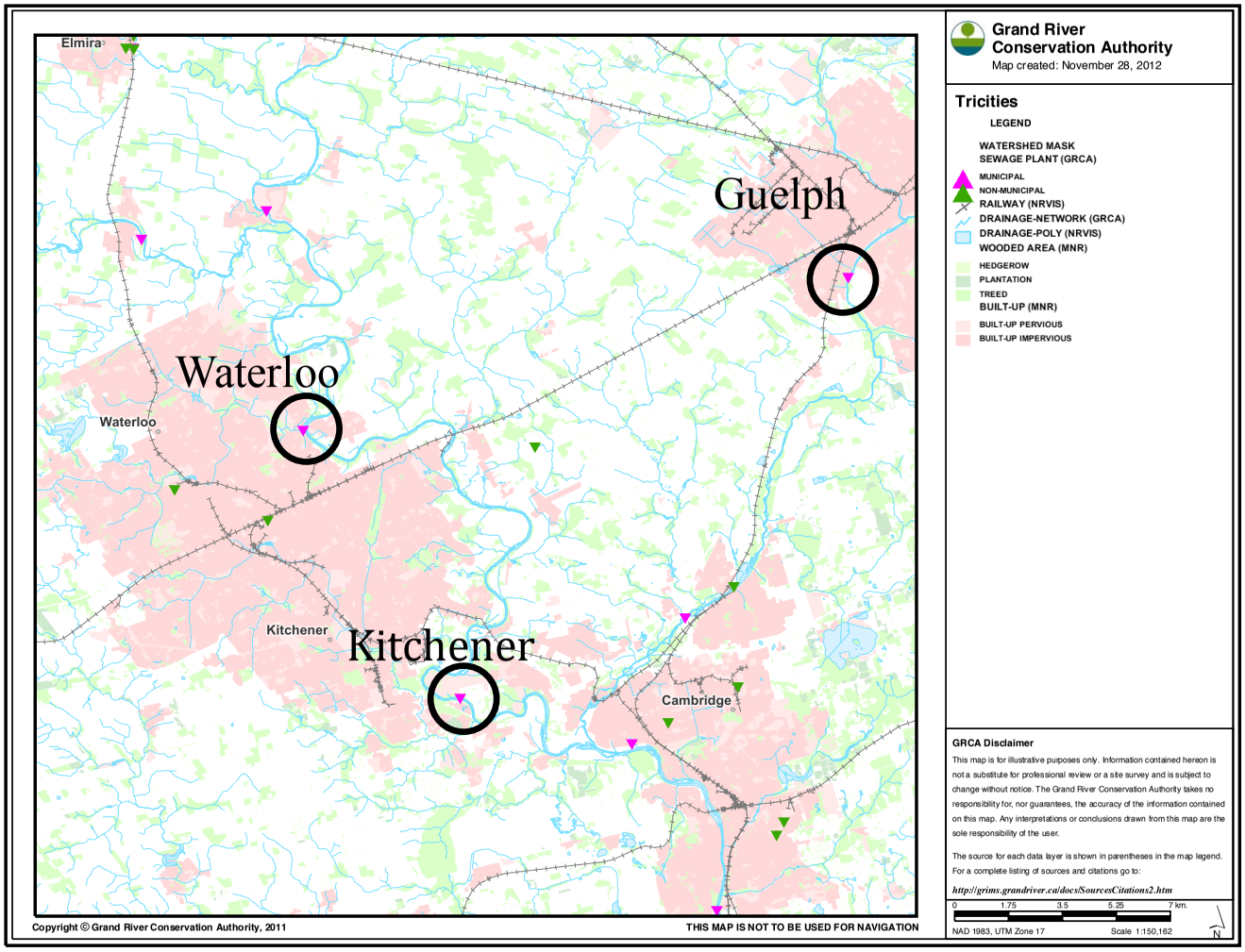


Figure 1.2: The Guelph, Waterloo and Kitchener municipal wastewater treatment plants of the central Grand River watershed (Grand River Conservation Authority, 2012).

The remediation technologies and processes implemented at these facilities (Table 1.3) could alter the chemical profile of these effluents and possibly the available EDCs. The general treatment processes of the Guelph, Waterloo and Kitchener MWWTPs include filtration, organic enrichment, sedimentation and chemical treatment before being discharged into the receiving water body. These three MWWTPs pre-filter their influent (e.g. screens) to remove large debris that is later directed to landfills for burial. Often ferrous chloride is added to this stage as a chemical treatment to precipitate phosphoric compounds in the effluent when the receiving water is sensitive to algal assimilation. After basic filtration this liquid suspension is then directed to aerobic digesters (secondary treatment) to enhance microbial growth, which assimilates organic material and flock development. The suspended flock, which is primarily microbial biomass, is then precipitated in clarifying tanks. Some of this sludge is re-introduced into the earliest processes as activated sludge (microbial biomass) while the remainder is processed as bio-solids for disposal. These processes can have variations, but all are generally referred to as activated sludge treatment. However, the effluent from this process could be further processed by advanced remediation that might include advanced filtrations, chemical treatment, membrane reactors or biological nutrient removal. For example, the Guelph MWWTP treats their effluent with extended aeration in the primary treatment process, with rotating biological columns (RBCs) and sand filtration in the tertiary treatment process. The purpose of the RBCs are to reduce the nitrogenous and phosphorus compounds in the effluent while the sand filters remove any residual fine particulate organic matter before chlorination (sodium hypochlorite) and de-chlorination (bisulphite). The final effluent from the Guelph MWWTP is then discharged into the Speed River (N43º 31’ 18”, W80º 15’ 50”). The Waterloo and Kitchener MWWTPs however, do not polish their effluents with tertiary treatment processes like the Guelph MWWTP. After secondary treatment, the Waterloo MWWTP chlorinates the effluent with sodium hypochlorite and then de-chlorinates the effluent with sodium bisulphite before discharging into the Grand River (N43º 28’ 47”, W80º 28’ 55”). At the Kitchener MWWTP, the secondary effluent is directed towards a large lagoon for particulate precipitation before it too

chlorinates and de-chlorinates its effluent prior to discharging into the Grand River (N43º 24’ 5”, W80º 25’ 18”).

The composition of the effluent and the treatment processes unique to each MWWTP can potentially alter the fate of EDCs in MWWEs and the chemicals discharged into the environment. The treatment processes at MWWTPs can improve the removal of estrogenic substances by lengthening the solids retention time and including nitrification (Servos *et al.*, 2005, Racz and Goel, 2010), but biodegradation and sorption (Khanal *et al.*, 2006) which are unique to each treatment technology and MWWTP can also affect the removal of estrogens. Upgrades at a MWWTP to include activated sludge remediation reduced the natural estrogens E1 and E2, while the xenoestrogens 4-nonylphenolethoxycarboxylic acids or 4-nonylphenolethoxylates concentrations and to a lesser extent the degradation by-products 4-NP and 4-OP were hardly affected (Barber *et al.*, 2012). However, not all MWWTPs effectively decrease the estrogenicity (Kirk *et al.*, 2002). EDCs such as E2 and E3 can be metabolically converted to E1 (Ternes *et al.*, 1999a, Weber *et al.*, 2005), while alkylphenol polyethoxylates can be degraded to short change alkylphenols such as 4-NP or 4-OP (Servos *et al.*, 2001). Therefore, the adverse effects in the environment could be to some extent predictable based on the treatment processes, technologies and operational procedures can have notable effects on the estrogenic EDCs, their conjugates and parental compounds to produce unique MWWEs that can induce adverse outcomes with different chemical profiles.

In order to assess different treatment processes within MWWTP and their linkage to adverse reproductive effects in organisms, it is desirable to control many the treatment variables under experimental conditions. At the Wastewater Technology Centre (WTC) of Environment Canada, Burlington, Ontario, independent pilot treatment plants have been designed to model the different experimental wastewater processes on a single source of municipal wastewater. These processes are also similar to the proposed process changes to be implemented at the Waterloo and

Kitchener MWWTPs in the future. These processes include the conventional activated sludge (CAS) process, nitrifying conventional activated sludge (CAS-N) and biological nutrient removal of conventional activated sludge (CAS-BNR) (Pileggi *et al.*, 2011). These different treatment trains provide a unique opportunity to evaluate the adverse reproductive effects of a single effluent (City of Burlington) treated by three different treatment technologies operated at typical temperatures simulating winter (12ºC) and summer (24ºC) conditions in Canada.

Table 1.3: General features and treatments of the Guelph, Waterloo, and Kitchener MWWTPs (2010) of the central Grand River of southern Ontario.

Treatment Plant		**Guelph**	**Waterloo**	**Kitchener**
Municipal population[a]		121,688	98,780	219,153
Treatment System		Conventional and extended aeration activated sludge (nitrifying)	Conventional activated sludge, (non-nitrifying)	
Primary Treatment		Bar screen, grit removal, primary clarifier		
Secondary Treatment		Aeration, secondary clarifier		
Advanced Treatment		Rotating Bacterial Contactors (nitrification) Sand filters	Chemical phosphorus removal	Chemical phosphorus removal
Disinfection		Sodium hypochlorite; sodium bisulphite de-chlorination		
Flow	Current Design (m^3/d)	64,000	72,730	122,7450
	Mean Daily Flow 2010 (m^3/d)	46,214	42,001	64,304
	Max. Daily Flow (m^3/d)	87,140	77,005	108,400
Ammonia	Monthly Mean 2010 (mg/L)	0.1-1.5	13.4-29.1	19.0-27.7
Future Possible Upgrades[b]		Membrane bioreactors, TP removal	BNR, UV	Nitrification, filtration, UV

[a] Statistics Canada (2011)
[b] MWWTP Final Report (August 2007)

The objectives of this thesis are to:

1. To determine if fish exposed to municipal effluents from MWWTP in the Grand River watershed (Guelph, Waterloo, Kitchener) induce an estrogenic response (plasma VTG) in fish (rainbow trout).
2. To determine if an estrogenic response (VTG induction) in fish can be altered by various effluent treatment processes and operational conditions in a pilot plant study (Burlington).
3. Develop an estrogen agonist effects-directed analysis (EDA) technique to screen effluents for estrogenic EDCs.

European MWWEs have been reported to alter fish reproduction and endocrine function. A variety of estrogenic substances have been identified in those effluents that have been shown to induce similar adverse outcomes in fish. Fish exposed to three MWWEs from the city of Guelph, Waterloo and Kitchener MWWTPs have displayed adverse reproductive effects, but environmental variables such as temperature, pH, photoperiod and habitat could have influenced the reproductive cycles in these fish. Studies of Canadian MWWEs indicate that there are estrogenic substances in these complex matrices as well. Laboratory exposures were conducted to test the estrogenicity of MWWEs in a controlled laboratory setting to reduce some of the confounding variables. The Guelph and Waterloo MWWEs were tested for their ability to induce plasma VTG in rainbow trout, a commonly used biomarker for exposure to estrogenic substances. In a previous field study of the Kitchener MWWE, 100% of the caged fathead minnows died at concentrations exceeding 50% effluent. Therefore, the Kitchener effluent was not included in the *in vivo* exposure portion of the current study. MWWE was collected from the city of Guelph, Waterloo and Kitchener MWWTPs, and the extracts were tested for estrogen agonists by calculating E2 equivalents (E2eq) using the yeast estrogen screen (YES; (Gaido *et al.*, 1997)). The YES was also used to characterize

fractions with compounds that bind the human ERα (hERα) and induce the expression of the reporter gene β-galactosidase.

The treatment processes used in wastewater treatment plants such as Guelph, Waterloo and Kitchener were primarily designed to remediate the toxicity associated with particulate organic matter, ammonia and phosphorous compounds. Therefore, it is not known if these different treatment processes will affect the estrogenicity of the final effluent. The pilot plants simulated conventional activated sludge (CAS), nitrifying-CAS (CAS-N), and biological nutrient removal (CAS-BNR) processes using the Burlington MWWTP as the source. To test the removal of estrogenicity in these processes, rainbow trout were exposed to their effluents and plasma VTG was measured *in vivo*. The Burlington WWTP pilot plant effluents were also extracted and tested in the YES. An additional component of this study was the development and application of an EDA to the Waterloo and Kitchener MWWEs to resolve the chemical profile of estrogenic substances using the YES assay. The key contaminants in these effluents were estrone (E1), 17β-estradiol (E2), 17α-ethinylestradiol (EE2), and bisphenol A (BPA) representing potential estrogenic substances in MWWEs.

Chapter 2: Methods

The methods outline the determination of plasma vitellogenin (VTG) in rainbow trout, the determination of estrogen yeast estrogen screen (YES) exposed to effluent extracts and development of an effects-directed assessment (EDA). The first section will outline the methods for the development of an indirect enzyme linked immunosorbent assay (ELISA) for vitellogenin used to detect plasma VTG from rainbow trout exposed to the Guelph, Waterloo and Burlington MWWEs. The second section outlines the methods for the yeast estrogen screen (YES), including sample collection and preparation and the chromatographic separation and fractionation (EDA). Several of the experiments and fish exposures were done in collaboration with other studies and summarized in Table 2.1.

Table 2.1: Summary of the *in vivo* experiments conducted on fish exposed to effluents and the *in vitro* experiments characterizing the effluent estrogenicity of effluent extracts.

Site/Experiment	**Laboratory *in vivo* rainbow trout exposure**	**Yeast Estrogen Screen (whole effluent)**	**Effects-directed assessment**
Guelph MWWE	2,8,14 d, 0%, 20%, 90%	Tested (Sept 2010)	NA
Waterloo MWWE	2,8,14 d, 0%, 20%, 50%	Tested (Sept 2010, Aug 2012)	Tested
Kitchener MWWE	Toxic - not tested	Tested (Sept 2010, Aug 2012)	Tested
Pilot Plants (CAS, CAS-N, CAS-BNR)	14 d, 0%, 20%, 90% (Aug) 8 d, 0%, 20% (Sept) 8 d, 0%, 20%, 50% (Nov)	Tested	NA
EE2 exposure	8 d, 0%, 20% CAS-N (Sept)	NA	NA

NA- not tested

2.1 Methods for *in vivo* analysis of MWWEs

2.1.1 Preparation of Vitellogenin Antisera

A competitive indirect ELISA was developed to quantify the circulating rainbow trout VTG. The construction of this assay involved several components: 1) VTG was induced in adult male rainbow trout with 10 mg/kg EE2 in sesame oil and their plasma was collected; 2) rainbow

trout VTG was purified; 3) purified VTG was used in an adjuvant to produce an immune response; 4) the antisera was collected and validated for its sensitivity and specificity for VTG against other EE2 induced rainbow trout.

Vitellogenin was induced in two MS-222 anesthetized male rainbow trout by injecting 10 mg/kg EE2 in sesame oil in the intraperitoneal cavity, 4 times over 2 weeks. Plasma was collected and centrifuged at 3000 G for VTG isolation by molecular exclusion and affinity using Superdex 200 from Amersham Biotech with 50 mM Tris/HCl pH 7.8 containing 5% v/v glycerol and 100 mM potassium chloride buffer. Vitellogenin was concentrated to 1.0-1.3 mg/mL in two fractions and verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

The primary antiserum for the VTG ELISA was produced using European white rabbits (4E1 and 3A2) and the inoculum consisted of 300 µg rainbow trout VTG per mL saline solution. The initial inoculation consisted of a complete adjuvant, followed by 3 separate incomplete inoculations for a total of 4 cycles. The methods for inoculation to induce the primary antisera were carried out in accordance with University of Waterloo Animal Care Committee UWSOP457 (Use of Freund's Complete Adjuvant in Rabbits) and UWSOP452 (Subcutaneous Injection in the Rabbit). At termination, blood was collected and centrifuged at 3000 G to isolate the plasma (primary antisera). Antisera was diluted 500 times with 5% bovine serum albumin in tris-HCl buffer pH 7.5 (BSA solution, see Appendix 2) and stored as working stock until analysis.

2.1.2 Fish Exposures

Previous findings in the Grand River have identified reproductive effects in fish downstream of the Guelph and Waterloo MWWTPs. To test these effluents for their estrogenicity *in vivo*, effluents were collected from these MWWTPs and fish were exposed for 14 days or less, to induce VTG in a controlled laboratory. Rainbow trout were exposed to the MWWEs collected from the Guelph and Waterloo MWWTPs and handled in accordance with University of Waterloo Animal Care Protocol AUPP 10-17. Rainbow trout were also exposed to the three effluents

produced in a pilot plant using the Burlington MWWTP as the source of primary effluents. Three treatment processes were tested simultaneously in the pilot plant: CAS, CAS-N and CAS-BNR (Pileggi *et al.*, 2011). All fish were exposed in 36 L of effluent dose within aerated (air stones) 38 L glass aquaria in 12 h of light and 12 of dark photoperiods. All tanks were chilled to approximately 12ºC by placing six aquaria per trough that contained flowing well water. All tanks contained six fish with three replicate tanks per treatment and each treatment was renewed after 48 h. Fish were not fed throughout any exposures to limit or reduce any metabolic waste products such as ammonia. Water chemistry data including pH, temperature and dissolved oxygen (mg/mL) were monitored throughout each experiment as general indicators of water quality. The fish used in these exposures were juvenile rainbow trout, however after each exposure, fish were eventually dissected and if the gonads started to differentiate (visual determination) they were noted as either female or male. However, if the gonad had not undergone differentiation they were classified as immature.

The Guelph and Waterloo effluent exposures were conducted at the Hagen Aqualab at the University of Guelph from July 14 through July 28, and August 3 through August 17, 2009. Fish were acclimated in well water for 4 days prior to exposure in a 48 h static renewal regime to both MWWEs for 2, 8 and 14 d. The Guelph and Waterloo effluent exposures contained two sets of tanks, alternating each set of tanks between renewals. Each effluent was collected just prior to the tank renewal in approximately 250 L plastic totes, previously cleaned with soap and well water, using a garden hose attached to a sump pump placed after the de-chlorination step in both MWWTPs. After transport, effluent was then chilled in the unoccupied tanks at their respective dilutions until they reached approximately 12ºC. After these tanks reached 12ºC, fish were removed from their tanks and placed in their corresponding tanks for another 48 h. The previous tanks were rinsed with well water and left to dry until the next renewal. The Guelph exposure consisted 0%, 20% and 90% effluent doses, while the Waterloo exposure consisted of 0%, 20%, 50% and 90% effluent doses. In these exposures, well water from an on-site source (Hagen

Aqualab) was used to dilute the effluent in the tanks to the desired doses. The fish in these exposures varied in sizes from 10.2-17.0 cm (8.5-42.8 g) in the Guelph MWWE exposure and 11.1-17.1 cm (11.3-42.8 g) in the Waterloo MWWE exposure, depending on the availability from the Silvercreek Aquaculture, Erin, Ontario.

The Burlington pilot plant MWWE exposures were conducted at the University of Waterloo Wet Lab from August 2010 to September 2011. Fish were acclimated in well water for 4 to 6 days prior to exposure in a 48 h static renewal regime. Effluent from the Burlington pilot plants were collected in plastic totes (approximately 250 L), previously washed with soap and well water, stored at approximately 4ºC overnight until they were picked up the next day. The effluents in these stored totes were then pumped into a second set of totes on a truck for transport using garden hoses attached to a sump pumps. Once returning to the University of Waterloo, effluents were pumped into storage totes chilled by conduction using well water for 24 h, prior to tank renewal. While tanks were being renewed, fish were placed in a bucket of well water and placed back in their tank before moving to the next tank renewal. Well water from the University of Waterloo's WetLab was used to dilute these pilot plant effluents. Fish were exposed to experimental effluents from the pilot plants that were operated at two different temperatures 12ºC (designated low pilot plant operating temperature; LOT) and 24ºC (high pilot plant operating temperature; HOT) to represent winter and summer conditions. In the first series of BPPE, fish were exposed to the LOT effluent at 0%, 20% and 90% for 14 days. In the second and third series of BPPE, fish were exposed to the LOT and HOT effluents at 0%, 20% and 50% for 8 days. Within the 8 d, HOT exposure, a positive control series was included to test whether the induction of the rainbow trout VTG was affected by experimental design. A two by two design crossed reference well water and 20% CAS-N with EE2 spiked tanks and ethanol spiked tanks (control). The EE2 positive control tanks received 100 ng/L of EE2 in absolute ethanol after tank renewals every 2 days. The fish in these exposures ranged in size: 11.5-17.4 cm (14.6-42.0 g) in the 14 d LOT, 15.2-21.2 cm (31.2-81.8 g) in the 8 d LOT and 13.4-22.4 cm (20.7-90 g) based on the availability from Silvercreek Aquaculture, Erin, Ontario.

2.1.3 Plasma Collection

At the termination of each exposure, rainbow trout were lightly anesthetized in MS-222 until they displayed disorientation by loss of equilibrium. Blood was collected from the caudal vein with 21.5 gauge syringes, previously coated with 196 USP/mL heparin. Fish were then euthanized by spinal severance. The extracted blood was stored on ice until it was centrifuged at 3000 G for 5 min to isolate the plasma. The isolated plasma was then preserved with at least 4 TIU/mL aprotinin (Parks *et al.*, 1999) and snap frozen in liquid nitrogen and stored at -80ºC.

2.1.4 Vitellogenin Analysis

To quantify the rainbow trout plasma VTG a competitive indirect ELISA was developed in-house. Briefly, purified rainbow trout VTG was irreversibly bound to the wells of a 96 well plate. The exposed rainbow trout plasma and rabbit polyclonal primary antibody developed in-house (Section 2.1.1) were added to three replicate wells for all plasma samples and left to equilibrate. The primary antibodies would bind to both the immobile and soluble rainbow trout VTG (competitive) and the soluble portion was rinsed away to leave behind the primary antibodies bound to the immobile VTG. A goat anti-rabbit secondary antibody was bought commercially and added to these wells (Sigma, A6154-5X1ML). This secondary antibody was hybridized to an enzyme that would cleave a substrate to produce a chromogenic response (indirect). The resulting color change was inversely related to the amount of plasma VTG in the sample.

The assay was conducted as follows (see Appendix 2): ion-affinity ELISA plates were used to construct the indirect competitive ELISA. First, 16 ng of rainbow trout VTG was incubated in 150 µL sodium bicarbonate buffer per well at 37ºC for 3 h. All wells were washed 5 times in excess of 150 µL per well with washing buffer (1X TBS-T buffer pH 7.5). Each well received 200 µL of blocking buffer (BSA solution) and incubated for 30 min at 37ºC, and then washed again as mentioned above. Standards were made in BSA solution and ranged from 0.29 ng/50 µL to 150 ng/50 µL rainbow trout VTG. Samples were also prepared in BSA solution starting with a 1:10 dilution in BSA solution. The primary antiserum solution was prepared by diluting working stock

to 1:600,000 in BSA solution. All wells received 50 uL of standard or diluted samples, and 100 µL of primary antiserum in BSA, except blank wells (no primary antibody), which received 150 µL of BSA solution, and primary antibody wells (zero standard) which received 50 µL of BSA solution and 100 µL of primary antiserum diluted in BSA. The total volume of each well was 150 µL. All plates were incubated over night at room temperature for no more than 24 h, to allow for equilibrium and then washed 5 times with washing buffer and incubated after it was diluted 1:2000 in secondary goat-anti rabbit antiserum in BSA solution for 2 h. After the 2 h incubation, plates washed 5 times again with washing buffer. Each well received 150 µL *o*-phenylene diamine dihydrochloride (OPD) chromogenic solution and incubated for 30 min at room temp in a dark drawer which was stopped with the addition of 50 µL 1 N H_2SO_4 to each well. All plates were read in a SpectraMax plate reader at 490 nm within 30 min of stopping the reaction. Sample VTG concentrations were calculated based on a relative standard curve (Relative OD = $(OD_{sample} - OD_{blank})/(OD_{primary\ antibody} - OD_{blank})$ using linear regression. Only data points falling within the linear range were used for regression analysis. Data points less than the assay detection limits were given a value of half the detection limit for that batch. If the data was higher than the detection limits samples were diluted again until they fell into the linear range of the assay. The assay's lower limit of detection by 99% confidence interval was 16.4 ± 3.7 ng/mL (n = 43). The assays upper limit of detection by 99% confidence interval was 2,512 ± 293 ng/mL (n = 43). Samples were considered to be within the linear portion of the assay if they fell between 20.1 ng/mL and 2,219 ng/mL. An example of a standard curve using the indirect ELISA for rainbow trout VTG is given in Figure 2.1.

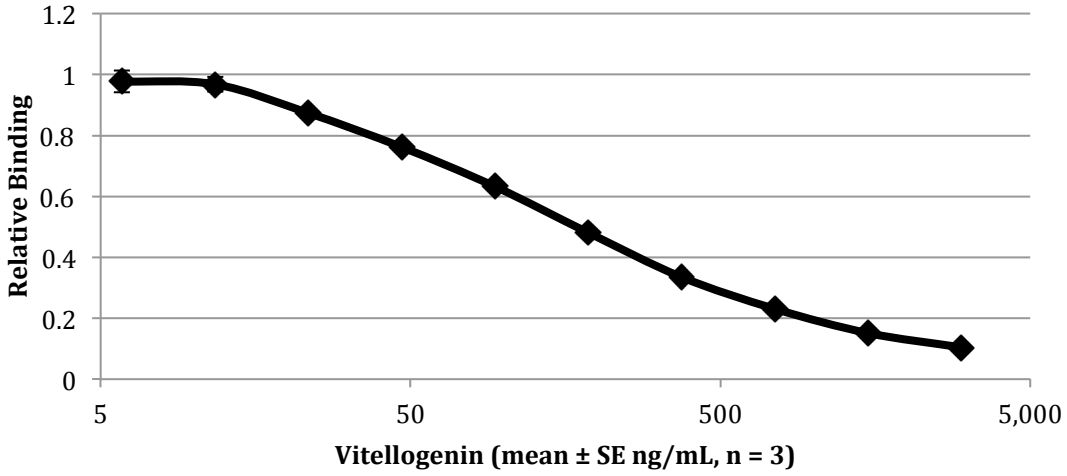


Figure 2.1: An example of a standard curve for rainbow trout plasma VTG in the indirect enzyme linked immunosorbent assay, plotting the relative binding of VTG antibody in the well verus the concentration of VTG standard (mean ± SE, n = 3).

2.1.5 Statistical Analysis

A mixed effect model analysis of variance was used to assess the response variable: rainbow trout plasma VTG on the predictor variables: gender, duration of exposure, the effluent or dose. Analysis was conducted using R version 2.14.0 (R Core Team, 2012) and lme4: Linear mixed-effects models using S4 classes, version 0.999375-42 (Bates *et al.*, 2012). All analyses were dose centered at zero percent concentration and time centered with day 2 as time zero. All data was natural logarithm transformed for data analysis, and these values are reported in the results for the comparisons of fixed effects coefficients. Graphed results are displayed as the untransformed data (mean ± the standard error of the mean (SE)), with the number of observed measurements (n) given below the independent variables in each figure.

Models were evaluated and compared by ANOVA using the maximum likelihood estimates with a significance level of $p < 0.05$ on a Chi square distribution. The optimum statistical model with the lowest Akaike's Information Criterion and Schwarz's Bayesian Criterion was chosen for statistical analysis. If significance was found within a fixed effect an all pairwise non-adjusted multiple comparisons test was conducted using multcomp: Simultaneous Inference in General Parametric Models, version 1.2-12 (Hothorn *et al.*, 2012).

2.2 Methods for effect-directed analysis for estrogenic substances

2.2.1 Chemicals

All the chemicals including the estrogen standards (Emix): E1, E2, E3, EE2, BPA, NP, OP and diethylstilbestrol (DES) as well as reagents for the yeast estrogen screen were purchased from Sigma-Aldrich.

2.2.2 Effluent Sample Collection

Effluent samples were collected in 1 L screw top dark amber glass bottles with Teflon screw-top caps, then preserved and acidified with 1.0 g/L sodium azide and 50 mg/L ascorbic acid final concentrations, and transported on ice to the laboratory. Three 1 L bottles were collected from each of the Guelph, Waterloo, Kitchener MWWTPs and the Burlington pilot plant in September 2010. Each sample set was collected, filtered and extracted within 48 h and stored at -20ºC before being tested for total estrogenicity with the YES assay. The samples for the Waterloo and Kitchener EDA were collected from each MWWTP on different days. The estrogenicity of these samples in early method development indicated that the E2eq would drop below the limit of detection (YES) in the fractions compared to the whole effluent extract from a single 1 L sample. To avoid this limitation, multiple 1 L samples were collected, extracted and combined after elution to make composites. Positive controls and samples were collected in up to five 1 L bottles. The positive controls were spiked with the Emix standards at the concentrations that would induce an EC_{50} response in the eluted fractions for each compound after the 1 L bottles were pooled. The blank samples were spiked with the Emix standards in double distilled (dd) H_2O. As such, the Waterloo EDA was a 3 L composite sample and the Kitchener EDA was a 5 L composite. All samples were suction filtered through 0.45 µm Whatmann glass fiber filter paper through a glass suction funnel within 24 h and extracted within 4 days after filtration.

2.2.3 Sample Extraction

Samples were extracted using the Waters Oasis® HLB SPE 6 cc, 500 mg cartridges (part number: 186000115). Each cartridge was individually conditioned with 5.0 mL MTBE, 5.0 mL

methanol and rinsed with 5.0 mL dd H_2O. To extract the samples two cartridges were connected in series per 1 L of filtered effluent. Samples were extracted at approximately 6.0 mL/min over approximately 3 h. After extraction, approximately 5 mL dd H_2O was used to rinse the insides of the amber collection bottles to rinse residual sample to the bottom.

Once the extraction was complete, the line was disconnected and 5.0 mL dd H_2O was added into the top cartridge and allowed to drain. The two cartridges were then disassembled and 5.0 mL dd H_2O was added to the bottom cartridge and allowed to drain. Both cartridges were dried individually through the same SPE manifold port used to extract the 1 L sample for approximately 15 min each.

2.2.4 Sample Elution

Each cartridge was eluted in sequence, with 5.0 mL of 90:10 MTBE:methanol, and then 5.0 mL of 100% methanol into a 10.mL glass test tube, at approximately 1.5 mL/min over 15 min by gravity, and then dried for another 15 min under vacuum. Each 10 mL test tube was dried under nitrogen at 300 rpm and 35ºC. Nearing completion; 1.0 mL methanol was used to rinse the wall of each test tube and combined with the eluent from the other cartridge from the same 1 L sample. Once the samples were dried they were reconstituted in 80 µL methanol and dispensed into 250 µL spring bottom glass inserts in 2.0 mL amber autosampler glass vials for HPLC separation.

2.2.5 HPLC Separation

Effluent samples were separated using a Waters Alliance 2996 with 2995 PDA and fractioned using a Gilson Fraction collector. Samples were held at 20ºC prior to injection, measured at 220 and 254 nm 2D channels, and scanned from 210-700 nm in 3D channel. Samples were separated on an Agilent Eclipse XDB-C18 4.6 x 250 mm x 5 µm (PN 990967-902) column held at 30ºC, using 80:20 water:methanol for mobile phase A (mpA), and 100% HPLC grade acetonitrile for mobile phase B (mpB) as follows: from 0-5 min at 90/10 (mpA/mpB), 15-35 min 70/30, at 60 min 10/90 until 70 min at a flow rate of 1.0 mL/min. Individual fractions were collected every 0.5

min in 80 test tubes for 40 min worth of fractions. Each effluent extract was injected twice, the first injection was used to collect the 10 – 50 min fractions, and the second injection was used to collect the 30 – 70 min fractions. The fraction tubes with overlapping elution times (30 – 50 min) were used for the second injection and provided greater sensitivity per fractions containing the most potent estrogens of interest: E2, EE2, E1. Fractions were dried under nitrogen, reconstituted in 40 µL anhydrous ethanol and stored in 200 µL flat bottom glass inserts for 2.0 mL glass amber vials until analysis by YES.

2.2.6 Yeast Estrogen Screen

The yeast cell line (YES) was constructed as outlined in (Gaido *et al.*, 1997) and kindly donated from Dr. Heidi Engelhardt, Department of Biology from the University of Waterloo. From glycerol stock, yeast cells are streaked on minimal media agar at 30ºC for ~3-4 days until colonies are clearly identifiable. A single isolated colony is selected for a second streak plate and grown at 30ºC overnight until colonies were visible. One colony was then selected for enrichment in a 50 mL centrifuge tube with 5.0 mL GOLD media, overnight at 30ºC and 300 rpm. Cells are diluted 1:10 in minimal media (6.7 g/L yeast nitrogen base without amino acids, 20 g/L dextrose, 60 mg/L leucine, 20 mg/L histidine) and incubated overnight again, and then diluted 1:1 the following morning to begin the assay that afternoon. A portion of this culture was preserved in equal volumes of culture to 30% glycerol stock. The remainder of this culture was then diluted to OD_{660} 0.03 in minimal media, and 50 µM of copper sulfate solution. Cells were dosed with sample or 17β-estradiol standard in a 1000 times dilution by adding 5 µL of sample or standard in absolute ethanol to 5.0 mL of cells in 50 mL polypropylene conical tubes (Axygen Scientific, SCT-50ML25-S). The dosed cells were incubated overnight for approximately 18 h at 30ºC, 300 rpm. The following day, the cells were diluted to OD_{660} 0.25 in microcentrifuge tubes. A 25 µL aliquot of these cell cultures were dispensed per well in triplicate on a 96 well plate and brought up to 100 µL with minimal media. Each well received 100 µL of β-galactosidase solution (β-gal) and immediately measured at

420 nm, every 15 s for 30 min. The β-galactosidase activity units were reported as E2 equivalents (E2eq) and calculated as outlined in the Yeast β-Galactosidase Microplate Assay kit, from Thermo Scientific (CAT: 75768) as the rate of chromogen production (OD_{420}) relative to the initial cell density (OD_{660}) correcting for the volume of cells [β-galactosidase activity = (1,000 * ΔOD_{420})/(t * V * OD_{660})]. Only the OD_{420} from 0.02-1.0 were used to determine the rate of chromogen production within the linear range of the kit. Whole effluent E2eq was estimated by the relative β-galactosidase induction standardized to ng/L 17β-estradiol (E2). An example of a YES (E2eq ng/L) standard curve is given in Figure 2.2. All reagents including media are given in Appendix 3. The lower limit of detection for the YES by 99% confidence interval was 3.80 ± 1.10 ng/L E2eq and the upper limit of detection by 99% confidence interval was 280 ± 68.1 ng/L E2eq (n = 14). Therefore, for a 1 L extraction: the lower limit of detection was 0.304 ± 0.088 ng/L E2eq and the upper limit of detection was 22.3 ± 5.45 ng/L E2eq (n = 3).

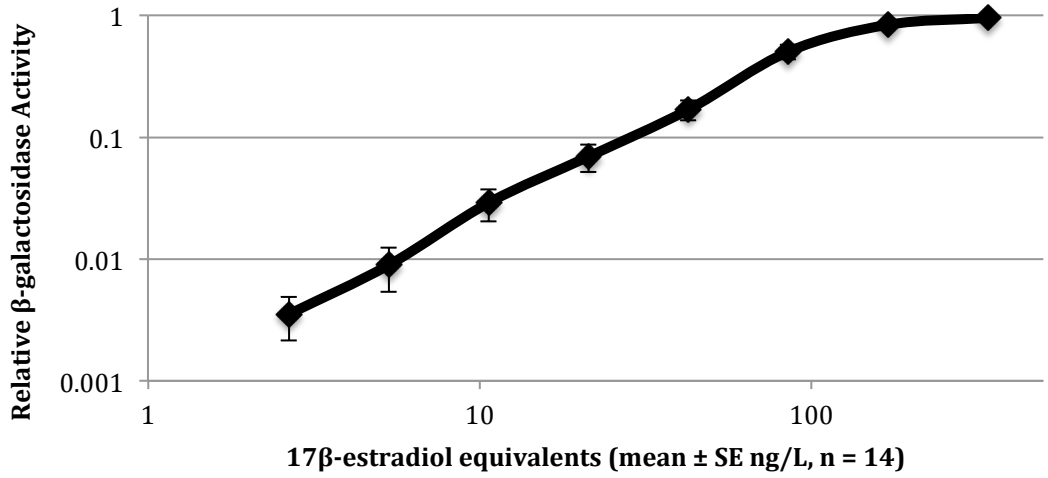


Figure 2.2: An example of 17β-estradiol equivalents in the yeast estrogen screen, plotting the relative β-galactosidase activity against the molarity of 17β-estradiol standardized by formula weight 272.38 g/mol.

2.2.7 YES Statistical Analysis

Whole effluent estrogenicity (YES) was quantified by relative β-galactosidase induction then standardized to E2eq (ng/L) and natural logarithm transformed for statistical analysis. A mixed effect model of variance was used to test for differences between extracts measuring E2eq

(ng/L) for MWWE whole extracts. Analysis was conducted using R version 2.14.0 (R Development Core Team, 2011-10-31) and lme4: Linear mixed-effects models using S4 classes, version 0.999375-42 (Bates et al., 2011). Tests were centered for Guelph MWWE when comparing the local MWWE, and for CAS when comparing the Burlington pilot plant effluents. An all pairwise non-adjusted multiple comparisons test was conducted using multcomp: Simultaneous Inference in General Parametric Models, version 1.2-12 (Hothorn, 2012) if significant differences ($p < 0.05$) were found. Graphical results are displayed as mean ± standard error of the mean (SE) of E2eq (ng/L), with number of independent observed measurements given below the figures (n).

Chapter 3: Results

3.1 Vitellogenin Results

3.1.1 Guelph Rainbow Trout Exposure

The Guelph MWWE exposure had 98% survivorship after 14 d. Male rainbow trout expressed significantly less (0.744 ± 0.197 µg/mL) VTG than females (2.07 ± 0.377 µg/mL) ($p < 0.001$), and there were differences between days ($p < 0.001$), but there were no dose effects observed within female (Figure 3.1) or male (Figure 3.2) circulating vitellogenin concentration. Over the duration of the Guelph MWWE exposure tanks were held at 11.7 ± 0.01ºC over 14 d. Female VTG concentration diminished by day 8 followed by an increase by day 14. No trends were observed for male VTG.

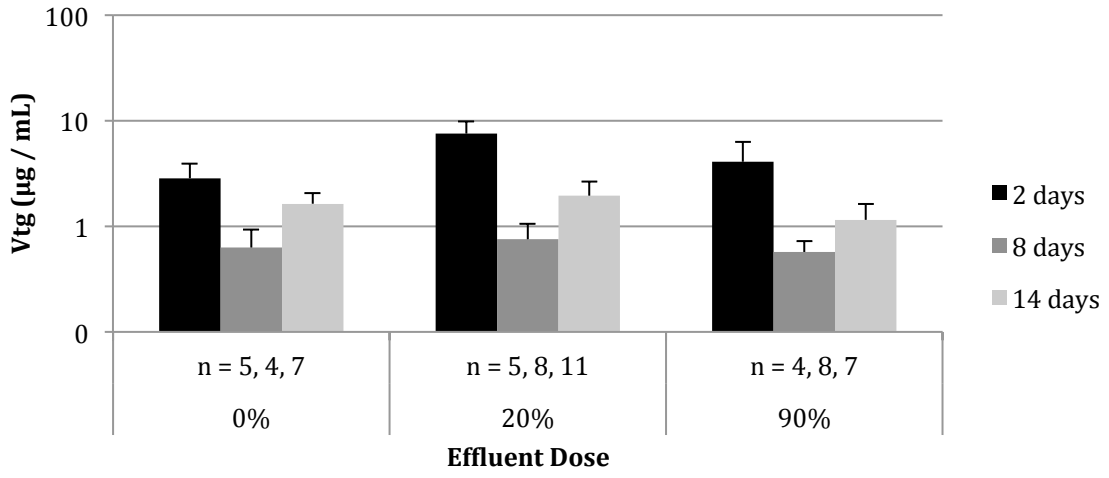


Figure 3.1: Female rainbow trout exposed to Guelph municipal wastewater effluents for 2, 8, and 14 d at 20% and 90% effluent in July 2009. No significant differences were found between doses $p \geq 0.05$.

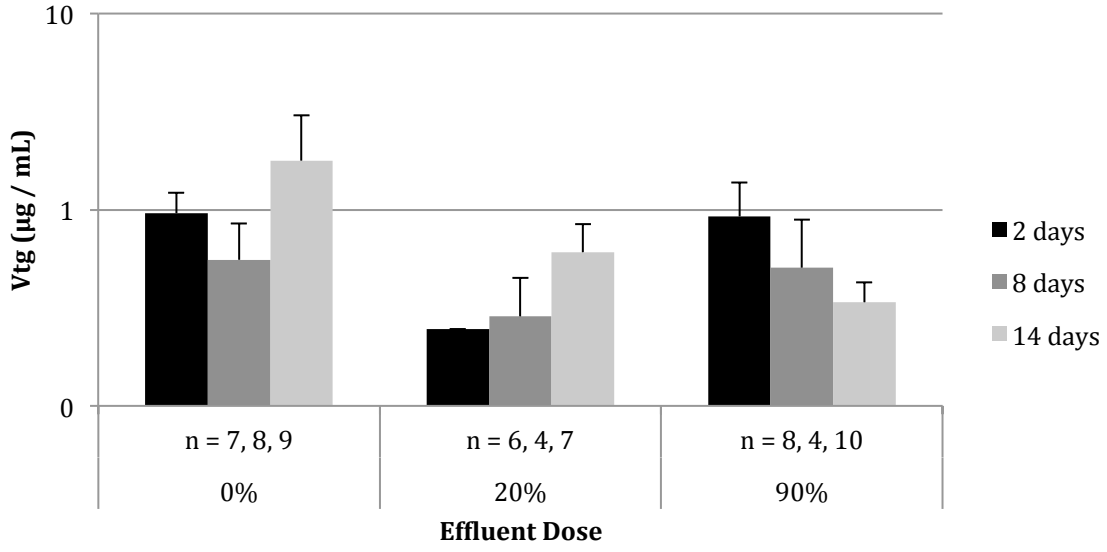


Figure 3.2: Male rainbow trout exposed to Guelph municipal wastewater effluents for 2, 8, and 14 d at 20% and 90% effluent in July 2009. No significant differences were found between doses $p \geq 0.05$.

3.1.2 Waterloo Rainbow Trout Exposure

The survivorship of the Waterloo MWWE exposure was less than the Guelph MWWE exposure with 70% survivorship by day 14, and the majority of mortalities were in the 90% effluent concentration. The whole effluent total ammonia concentration over the course of the exposure was 15.2 ± 0.3 mg/L with 1.2 ± 0.02 mg/L un-ionized ammonia (NH_3), and the un-ionized ammonia levels between renewals (48 h average), were 0.07 ± 0.01 mg/L (0%, control), 0.27 ± 0.02 mg/L (20%), 0.67 ± 0.03 mg/L (50%), 1.05 ± 0.04 (90%) and 1.21 ± 0.02 mg/L (100%). Generally, the ammonia concentrations (total and un-ionized) did not change throughout the exposures but the 50% and 90% doses were higher than desired.

The sample population of males in the Waterloo MWWE exposure 90% dose was very small, n = 5. The genders of the mortalities were not determined and therefore it is not clear whether the mortalities were male or whether this random sample had an uneven gender distribution. Although there were 18 fish per treatment (6 per tank, 3 tanks) only two males were sampled in the 90% 2 d exposure (different tanks) and three males were sampled in the 8 day 90%

dose (same tank). In addition, no males survived after 14 days in 90% effluent. Because of this distribution, the plasma VTG of the five males in the 90% dose contributed substantial variability to the analysis of the data that could not accurately measure the variance within the 2 d 90% treatment or within the repeated tank measurements. When these five fish were included in the preliminary analysis, the total tank variance accounted for 9.1% of the total variance and when these five fish were excluded from the analysis the total tank variance accounted for 9.2×10^{-11} of the total variance. Excluding these fish in the final analyses produced a more accurate statistical model to this dataset that would not bias the 90% dose for either the 2-day or 8-day cohorts and reduce the variance within tanks to more accurately reflect the true fixed effects coefficients for this specific dataset and lower the model criterion. From the reduced dataset, female rainbow trout expressed 2.00 ± 0.435 µg/mL (Figure 3.3) VTG compared to males 0.448 ± 0.066 µg/mL (Figure 3.5), $p < 0.001$. The overall circulating VTG concentration decreased significantly over time $p < 0.001$, but the dose did not significantly affect the VTG concentration $p < 0.23$. All days were significantly different from each other within females (Figure 3.4) $p < 0.0001$. However, while males exposed for 2 days were not significantly different from 8 days, $p < 0.074$, the males after 14 days were different from all other days' $p < 0.006$ (Figure 3.6).

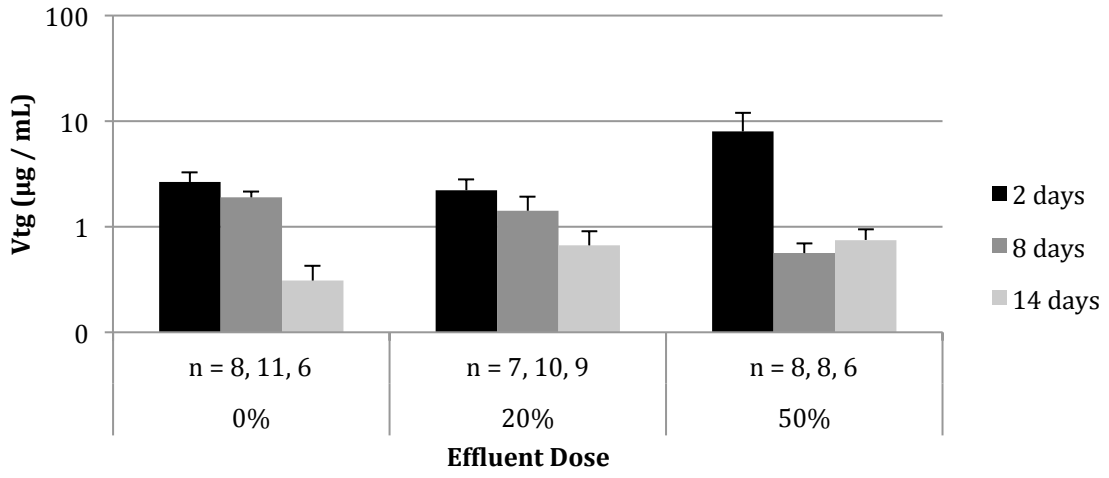


Figure 3.3: Female rainbow trout exposed to Waterloo municipal wastewater effluents for 2, 8, and 14 days at 20%, 50%, and 90% effluent in August 2009. No significant differences were found between doses $p \geq 0.05$.

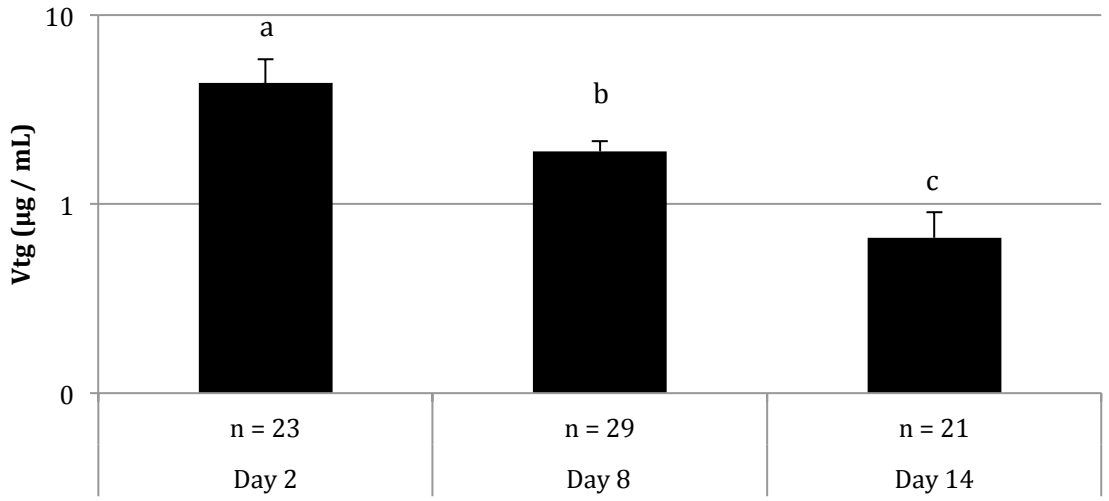


Figure 3.4: Female rainbow trout exposed to Waterloo MWWE for 2, 8, and 14 days averaged across doses within days, conducted in August 2009, $p < 0.0001$.

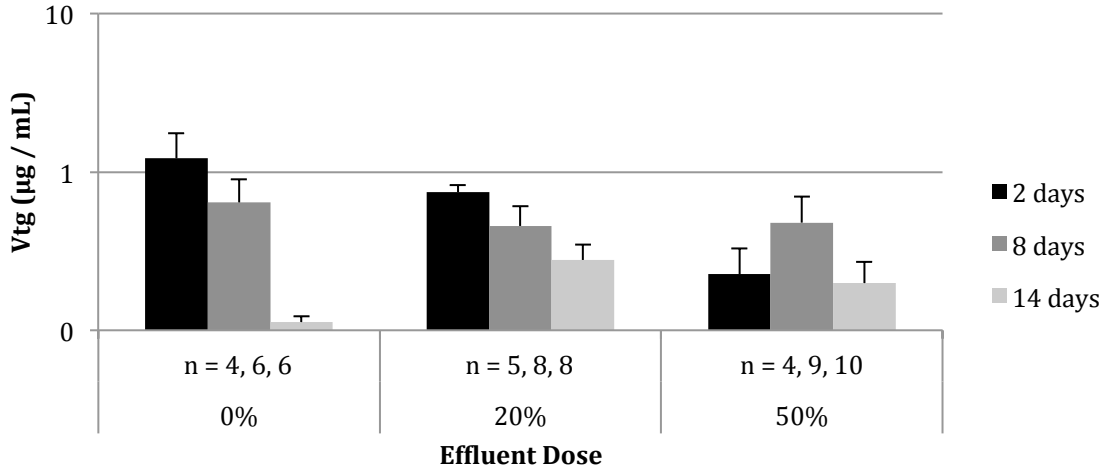


Figure 3.5: Male rainbow trout exposed to Waterloo municipal wastewater effluents for 2, 8, and 14 days at 20%, 50%, and 90% effluent in August 2009. No significant differences were found between doses $p \geq 0.05$.

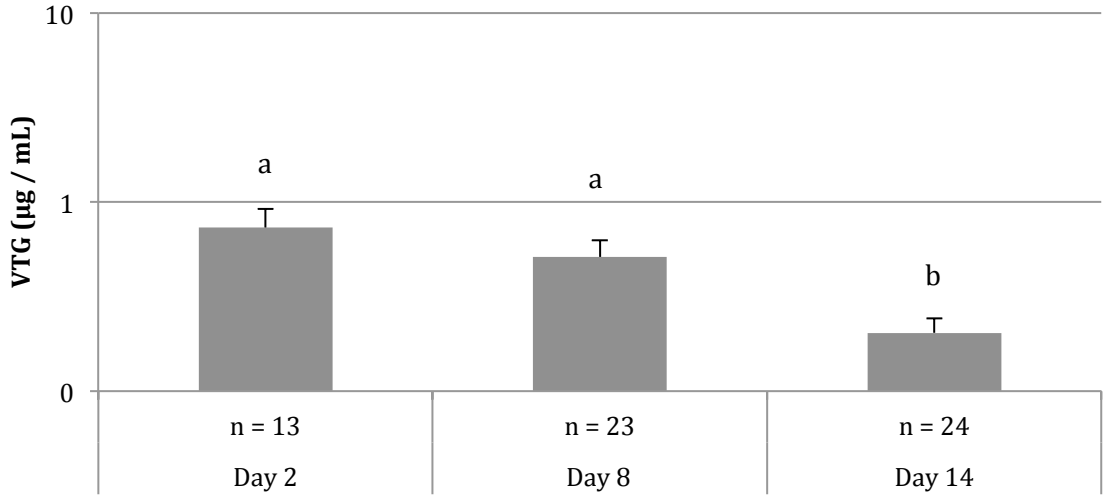


Figure 3.6: Male rainbow trout exposed to Waterloo MWWE for 2, 8, and 14 days averaged across doses within days, conducted August 2009, $p < 0.006$.

3.1.3 Burlington 14 day Rainbow Trout Exposure: Low Operating Temperatures

The Burlington pilot plant effluent 14 day low operating temperature (LOT) exposure was conducted at the University of Waterloo's Wetlab in August 2010. It consisted of two treatment concentrations, 20% and 90% effluent, for the three treatment processes: CAS, CAS-N and CAS-BNR. The 75% survivorship in the 14 day LOT controls was lower than expected, although the

CAS and CAS-N effluents inflicted 100% mortality within 14 days at 90% effluent. As a result, the between effluent comparisons could only be conducted at the 20% dose, and only CAS-BNR dose effects analysis was conducted.

The Burlington pilot plant LOT effluents had minor effects on plasma VTG induction. Neither gender responded differently to the 20% LOT effluents, although the males expressed less VTG (0.620 ± 0.110 µg/mL) than females (1.20 ± 0.224 µg/mL) $p < 0.023$ overall. Interestingly, the more advanced treatment process (20% CAS-BNR) elevated plasma VTG $p < 0.007$ (Figure 3.7). Further analysis revealed that the CAS-BNR effluent elevated male plasma VTG independent of dose $p < 0.009$, but had no affect on female plasma VTG (Figure 3.8). The unexpected mortality in the CAS and CAS-N 90% LOT effluents was unfortunate, and as a result the highest dose in subsequent exposures was reduced to 50%.

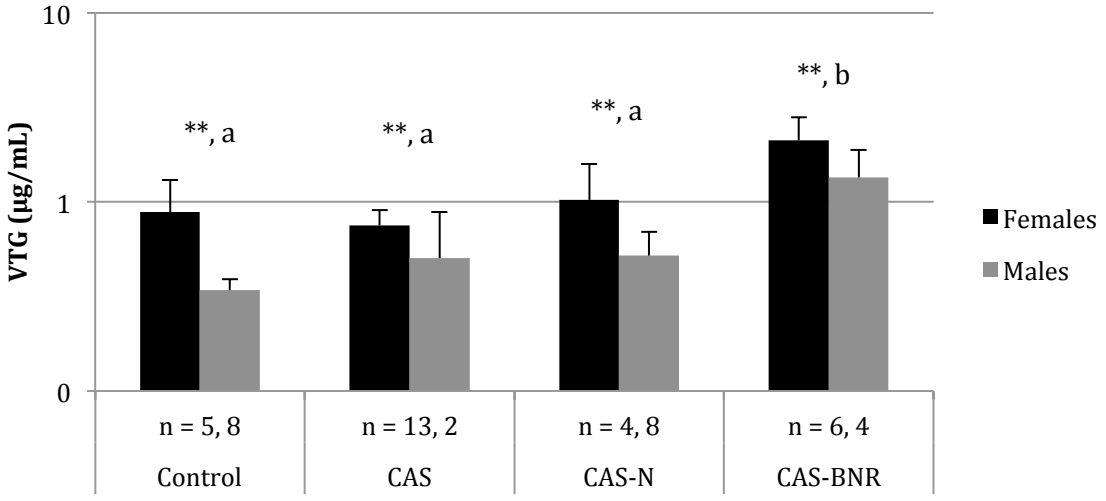


Figure 3.7: Males and females exposed to Burlington pilot plant MWWE operating at low run temperatures for 14 days at 20% concentrations. Females were not significantly different $p \geq 0.05$ (**), however CAS-BNR males (b) were significantly different from all other treatments $p < 0.007$, August 2010.

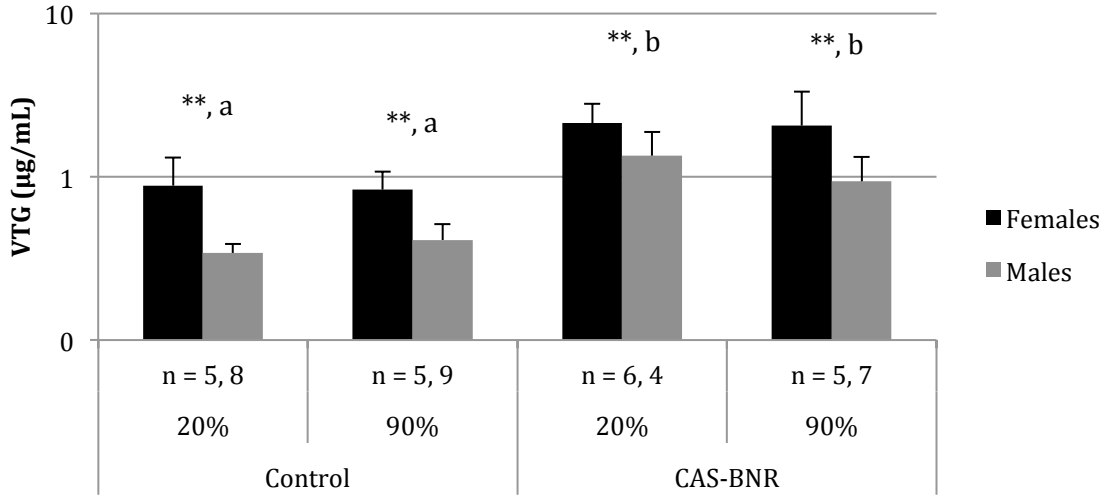


Figure 3.8: Males and females exposed to Burlington pilot plant MWWE operating at low run temperatures for 14 days at 20% concentrations, August 2010. Females were not significantly different $p \geq 0.05$ (**), however CAS-BNR males (b) were significantly different $p < 0.009$ from controls (a). No dose related effects were observed $p \geq 0.05$.

3.1.4 Burlington 8 Day Rainbow Trout Exposure: Low Operating Temperatures

The second Burlington pilot plant LOT exposure was conducted in November 2010 for 8 days and the survivorships were higher than the 14 exposures. The CAS 20% and 50% dose had 50% survivorship, while the CAS-N 20% dose had 81% survivorship and the 50% dose had 100%. The control treatments in the 8 d LOT also had greater survivorship with 91% surviving through 8 days. None of the treatment processes affected the plasma VTG after 8 days of exposure at either concentration. However, the females had significantly more circulating VTG (1.66 ± 0.238 µg/mL) than males (1.03 ± 0.176 µg/mL) $p < 0.001$ (Figure 3.9, Figure 3.10).

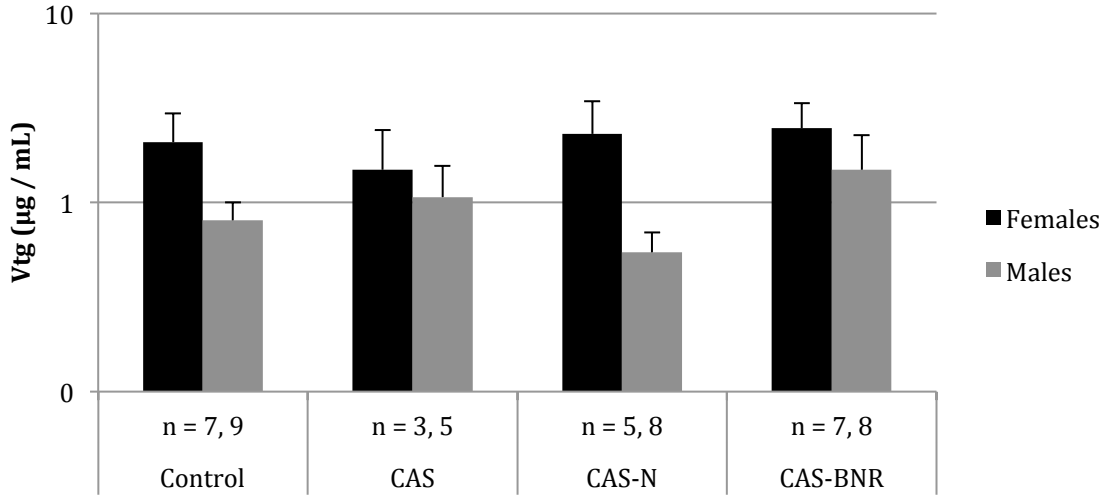


Figure 3.9: Males and females exposed to Burlington pilot plant MWWE operating at low run temperatures for 8 days at 20% concentrations, November 2010. No significant differences were found between treatments $p \geq 0.05$.

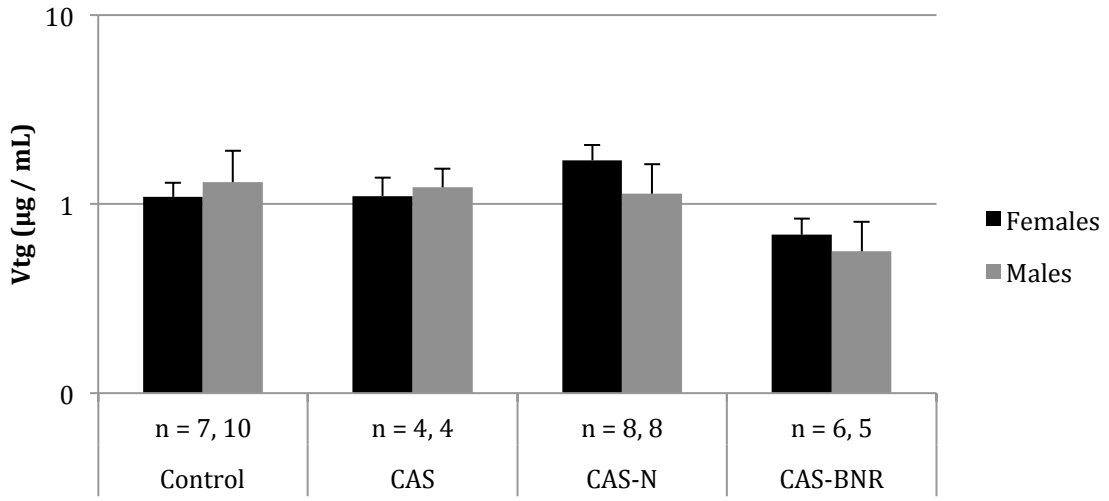


Figure 3.10: Males and females exposed to Burlington pilot plant MWWE operating at low run temperatures for 8 days at 50% concentrations, November 2010. No significant differences were found between treatments $p \geq 0.05$.

3.1.5 Burlington 8 Day Rainbow Trout Exposure: High Operating Temperatures

The final Burlington pilot plant effluent exposure using the high operating temperatures (HOT) was conducted in September 2011 and consisted of two components. The first component, tested whether the treatment types at HOT would induce VTG, and the second tested whether an

effluent (CAS-N) was suppressing VTG induction. To test for VTG suppression in the CAS-N treatment, 100 ng/L EE2 was added to 20% CAS-N and well water served as a positive control for comparison. To accommodate the effluent spikes and the positive controls, the 50% doses were not included in this component. Similar to the Waterloo MWWE exposure, there were no more than two males per any individual treatment set in the whole exposure (7 of 122 fish), and for reasons similar to those express in the Waterloo results (Section 3.1.2), males were excluded from the analyses.

None of the treatment processes affected VTG induction compared to the controls in the first experimental component. However, certain treatment processes had elevated VTG compared to others. The 20% HOT exposure did not affect the female or immature fish differently $p < 0.56$ (Figure 3.11). The CAS effluent did not alter VTG in rainbow trout compared to the control $p < 0.75$, neither did the CAS-N treatment $p < 0.069$ or the CAS-BNR $p < 0.16$. However, the VTG in fish exposed to the CAS-N effluent was greater than fish exposed to the CAS effluent $p < 0.037$, but CAS-N was not different from CAS-BNR $p < 0.094$.

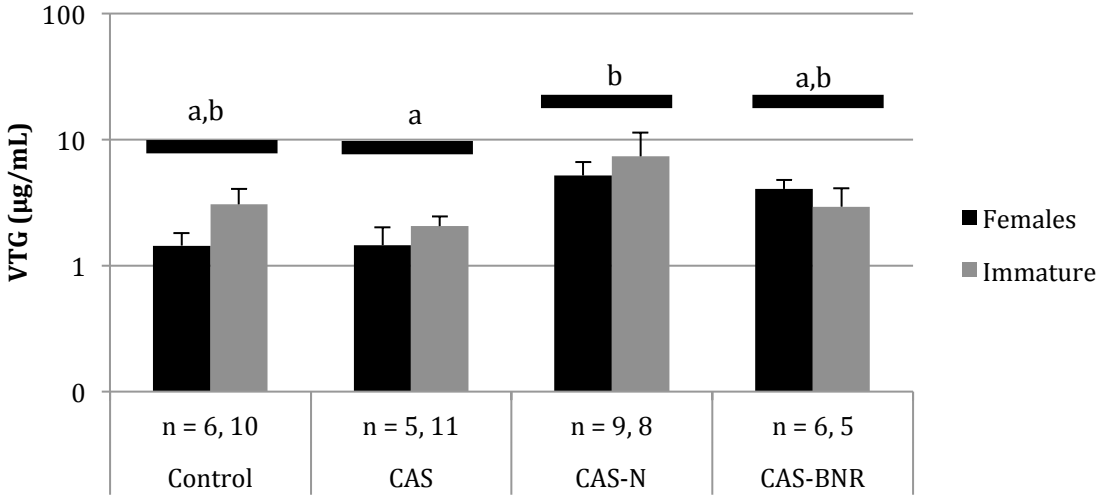


Figure 3.11: Females and immature fish exposed to Burlington pilot plant MWWE operating at high run temperatures for 8 days at 20% concentrations, September 2011. CAS-N had significantly higher VTG than CAS $p < 0.05$, but all other comparisons were $p \geq 0.05$.

All of the effluents that were tested did not induce VTG except CAS-BNR, which induced a weak response but only after 14 days. It is likely that these effluents were weakly estrogenic, but there might have been other factors in the effluent or in this experimental design that could suppress the circulating VTG concentration. To test whether these fish could induce high concentrations of plasma VTG positive control treatments of 100 ng/L EE2 were spiked into effluent and surrogate (well water) tanks (Figure 3.12). The CAS-N effluent was chosen from the Burlington pilot plant effluents because it provided a less toxic effluent than the CAS process and it presumably included more matrix effects than CAS-BNR treatment. These decisions were based on previous exposure results including survivorship and visual observation of precipitate (flock) that in the storage barrels. The positive control treatments (100 ng/L EE2) significantly induced VTG $p < 0.001$, and this observation was independent of the matrix (effluent or well water) as well as gender. Therefore, CAS-N was not likely suppressing VTG when exposed to a potent estrogen at the relatively high concentration.

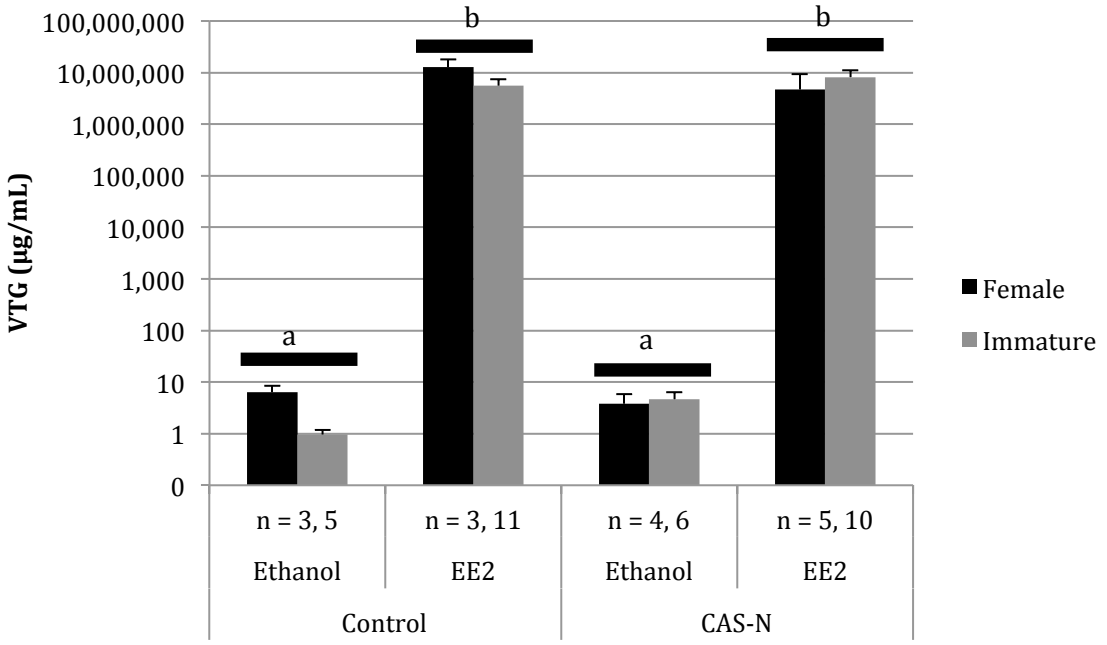


Figure 3.12: Females and immature exposed to Burlington pilot plant MWWE operating at high run temperatures for 8 days at 20% concentrations, September 2011. Negative controls were included in reference well water and CAS-N with ethanol blank spikes. Positive control spikes were also included in reference well water and CAS-N. The positive control tanks were spiked with 100 ng/L EE2 and renewed every 2 days with tank renewals. Significant differences are $p < 0.0001$.

3.2 Results of EDA

The total estrogenic equivalency (YES E2eq) of MWWE extracts collected from the Guelph, Waterloo and Kitchener MWWTPs increased respectively. The E2eq of the Guelph (0.81 ± 0.02 ng/L), Waterloo (4.32 ± 0.07 ng/L), and Kitchener (17.0 ± 0.4 ng/L) MWWEs were all significantly different from one another $p < 0.001$, and increased inversely proportional to the enhanced treatment technologies (Figure 3.13). Conversely, the total E2eq from the different Burlington pilot plant effluents increased as the remediation processes became more advanced. The total E2eq of the CAS-N (19.0 ± 11.4 ng/L E2eq) and CAS-BNR (15.5 ± 4.3 ng/L E2eq) were significantly different from the CAS (2.75 ± 0.8 ng/L E2eq), $p < 0.006$ and $p < 0.0007$ respectively. However, the E2eq YES did not differ between CAS-N and CAS-BNR $p < 0.54$ (Figure 3.14).

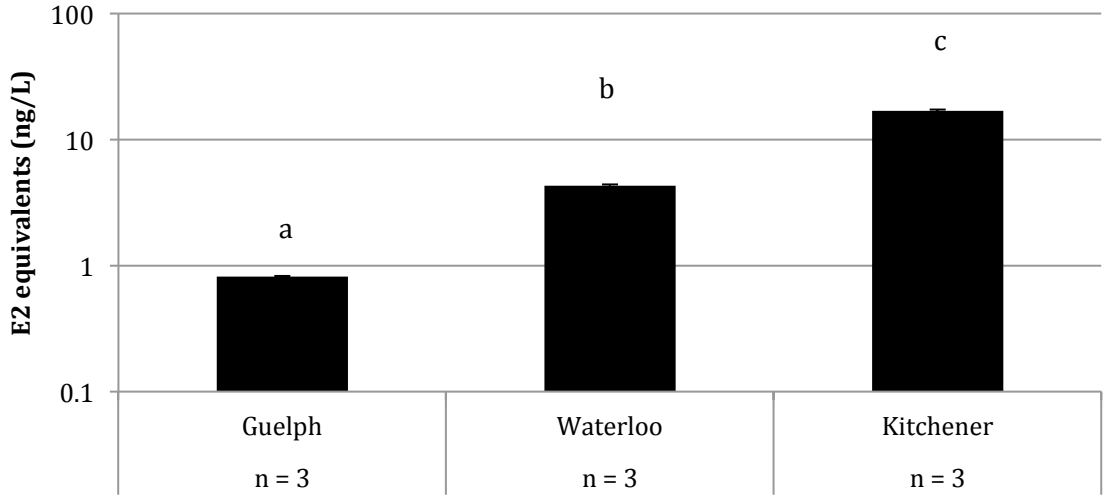


Figure 3.13: Total estrogenicity of Guelph, Waterloo and Kitchener MWWE, $p < 0.001$. Samples were collected September 14, 2010.

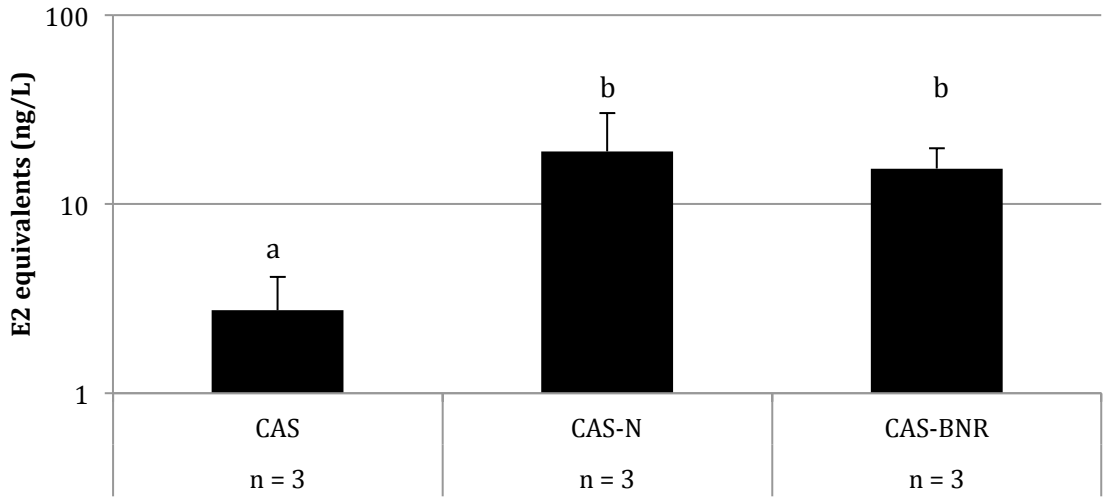


Figure 3.14: Whole effluent estrogenicity of the Burlington Pilot Plant Effluents 14 d exposure, $p < 0.001$ conducted September 15, 2010.

The initial EDAs consisted of three independent 1 L samples that were collected from the Guelph, Waterloo and Kitchener MWWTPs. These samples were spiked with DES, a potent xenoestrogen that elutes in the 44.5 min fraction as a surrogate internal spike to determine the relative retention time and shift between peaks. However neither the Waterloo nor the Kitchener effluents reproducibly produced a fraction with detectable E2 equivalents other than the DES spike,

which suggested that individual compounds were at or near the YES method detection limit. In addition, it was extremely difficult to determine the likelihood of any active fraction that could contain EE2 or E1 from the relative retention time for a DES spike because the fraction window was 30 seconds and both EE2 and E1 often eluted in sequential fractions.

To address these two limitations, the positive controls and collection volumes were adjusted. To more accurately reflect the active fractions pertaining to EE2 and E1, a set of positive control samples were spiked with all of the estrogens of interest (Emix). As well, multiple 1 L bottles were collected, and these 1 L extracts were later combined (pooled) to lower the detection limit of the assay. As such, three to five 1 L bottles of both effluent samples and dd H_2O blanks were collected. Positive controls for both effluent (matrix) and dd H_2O (non-matrix) were spiked and then pooled (composite) for both Waterloo and Kitchener MWWE EDAs respectively. These composite EDAs were only collected once (no replication) and in different months. The total YES estrogenicity of the composite samples, which were eventually fractionated, were far less than the previous total E2eq (Figure 3.15) collected in September 2011 (Figure 3.13).

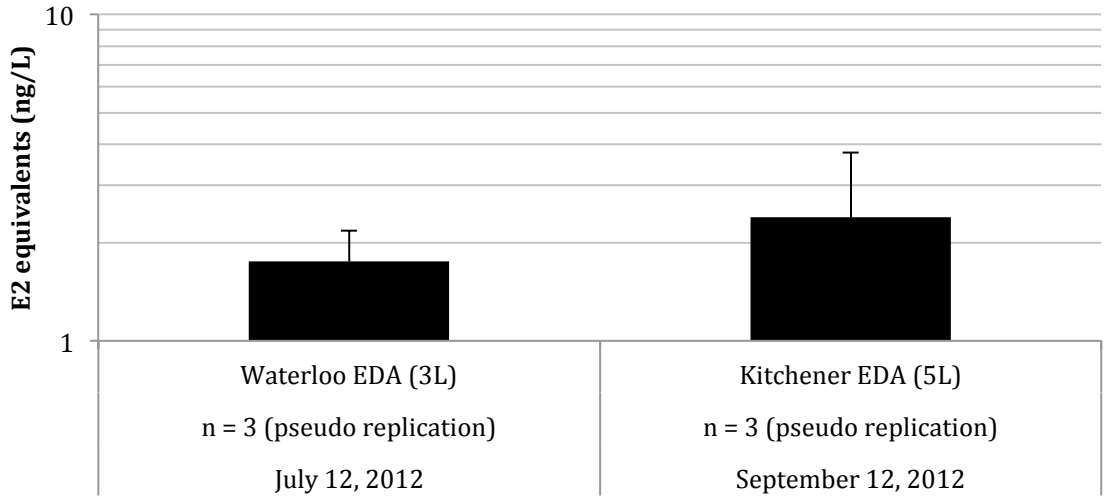


Figure 3.15: Whole effluent estrogenicity of the pooled MWWE. The Waterloo EDA consisted of 3 L and the Kitchener EDA consisted of 5 L pooled effluents. The error bars represent pseudo replication with linear dilutions of whole effluent extract. Below are the dates of effluent collection.

The total estrogenicity of the Waterloo whole effluent 3 L composite extract was approximately 1.75 ng/L E2eq, while the 5 L Kitchener composite was approximately 2.39 ng/L E2eq. After fractionating these composites, the Waterloo MWWE extract primarily consisted of E1 (Figure 3.19), while the Kitchener MWWE extract consisted of several estrogenic substances, in order of elution: BPA, E2, testosterone, EE2 and E1 (Figure 3.22).

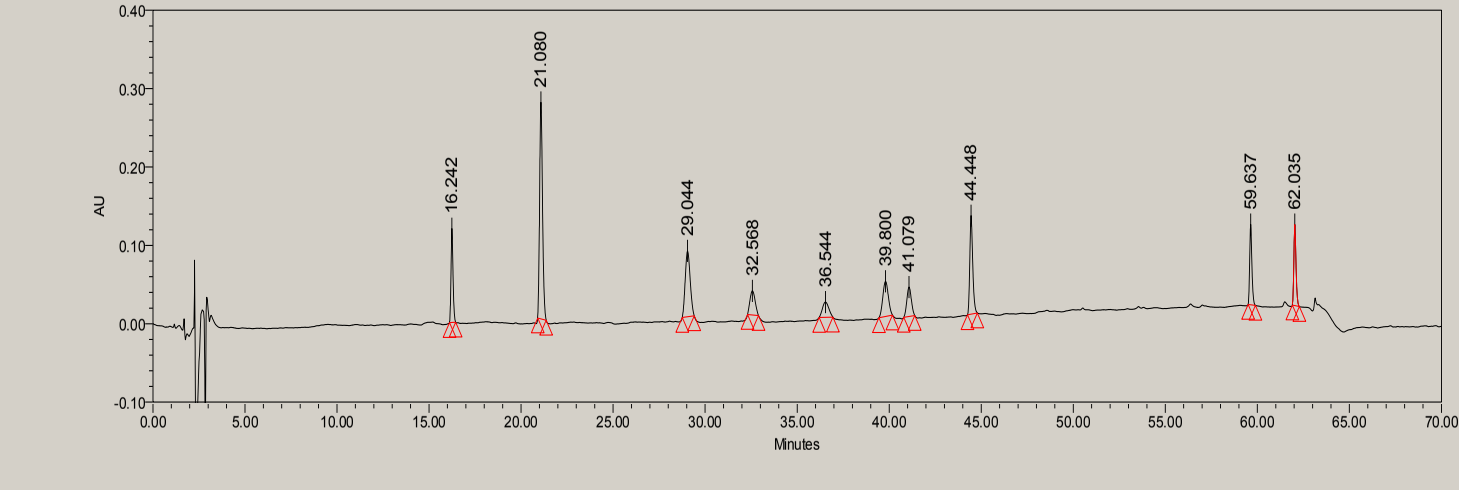


Figure 3.16: Chromatograph of Emix standards: E3, carbamazepine, BPA, E2, testosterone, EE2, E1, DES, OP and NP.

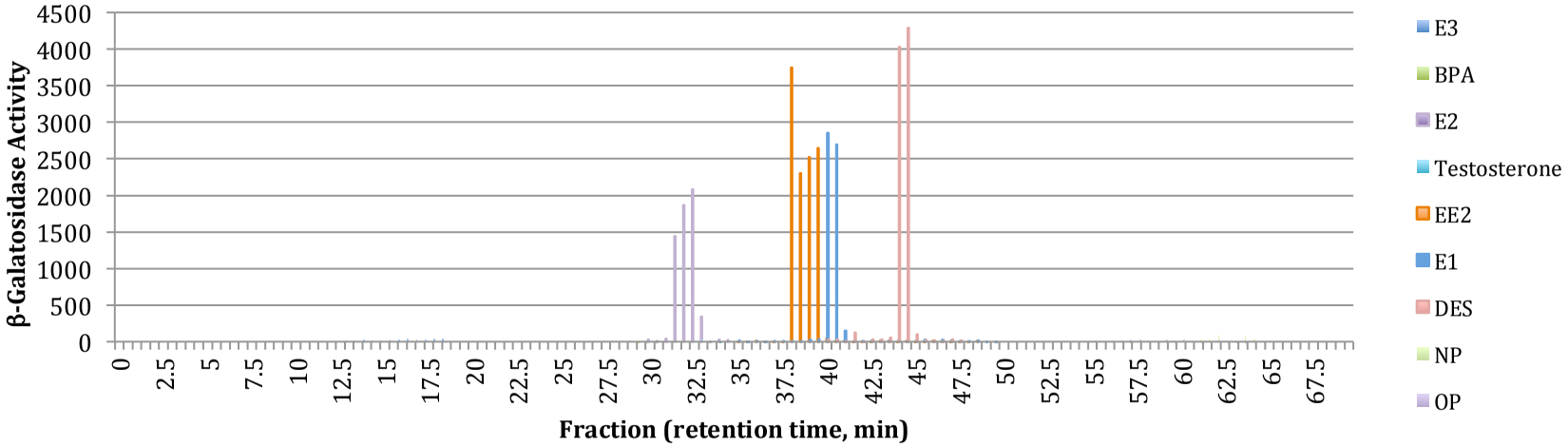


Figure 3.17: The elution of equal molar concentrations of independent Emix standards (10^{-4} M) in 20 µL injections to determine the YES activity (β-galatosidase activity) in a fractionation profile.

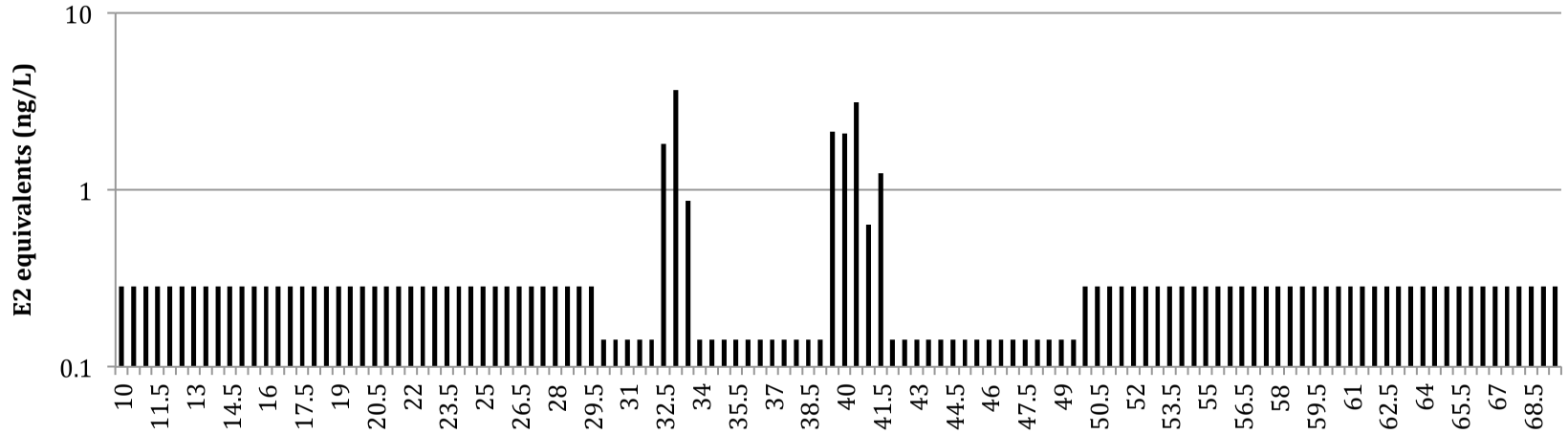


Figure 3.18: The fractionation and chemical profile of the Waterloo MWWE (3 L composite) for estrogenic substances (YES) spiked with the Emix standards.

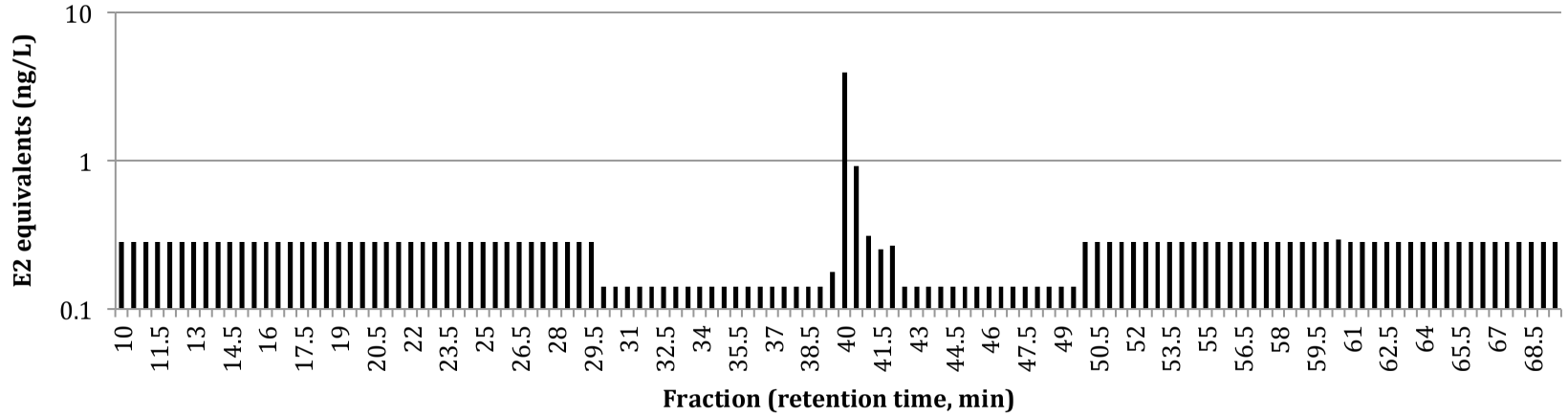


Figure 3.19: The fractionation and chemical profile of the Waterloo MWWE (3 L composite) for estrogenic substances (YES), the detectable estrogen present was estrone.

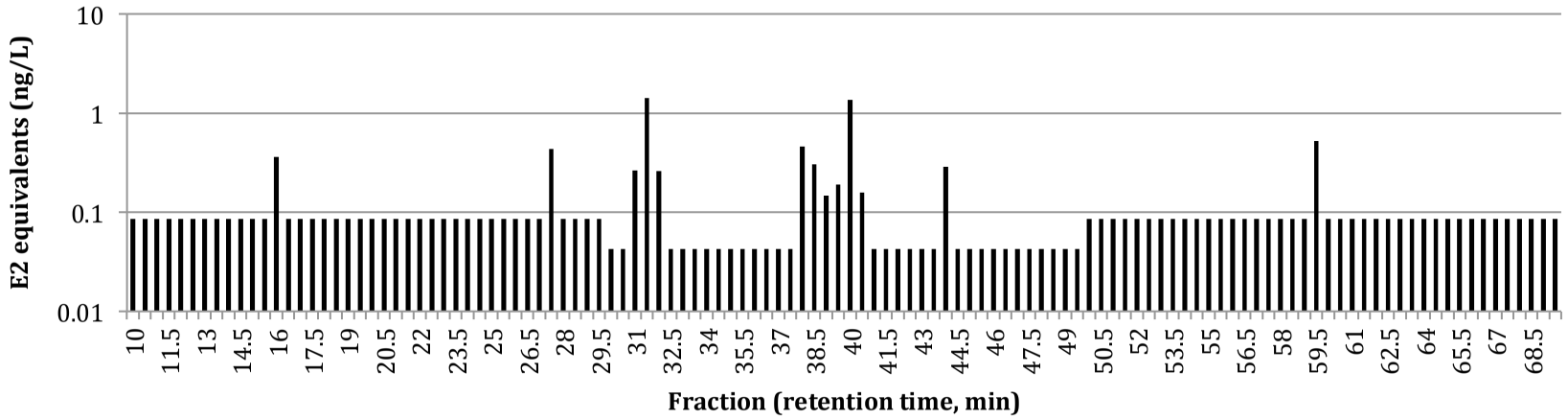


Figure 3.20: The fractionation of double distilled water (5 L composite) for estrogenic substances (YES) spiked with Emix standards for the Kitchener EDA.

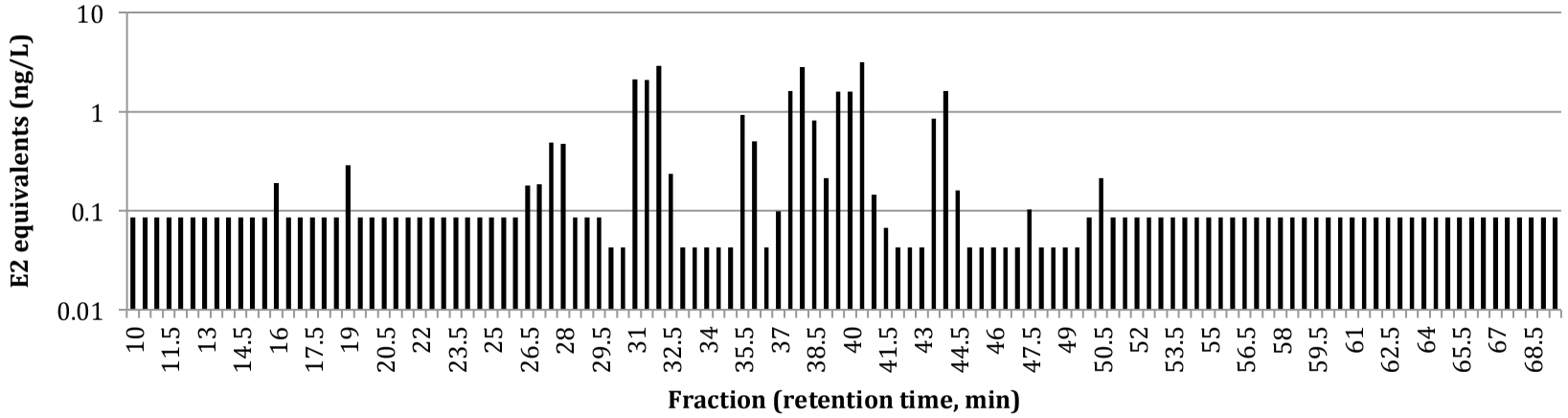


Figure 3.21: The fractionation and chemical profile of the Kitchener MWWE (5 L composite) for estrogenic substances (YES) spiked with Emix standards.

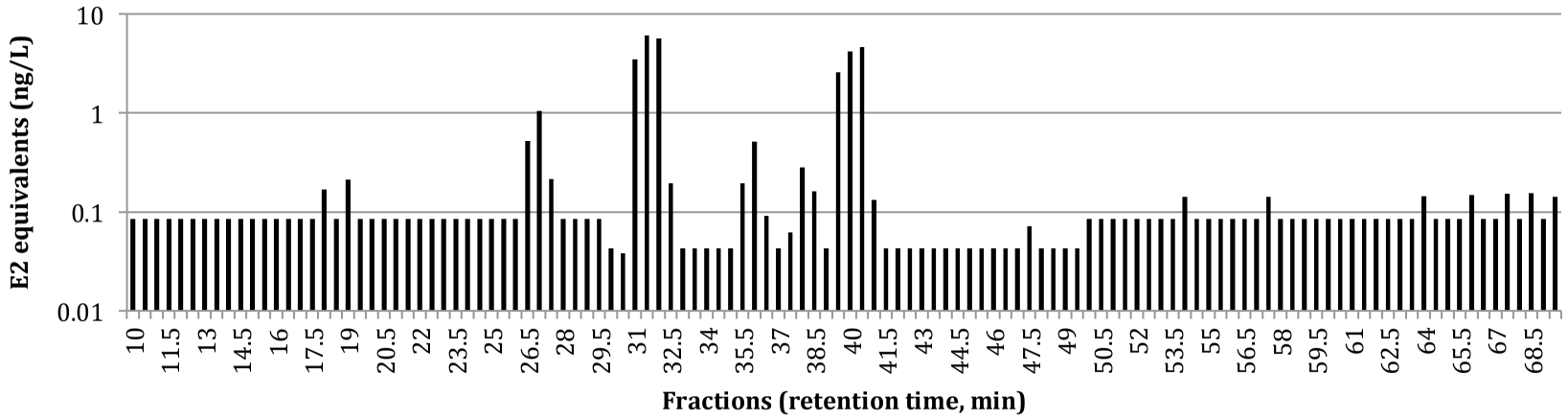


Figure 3.22: The fractionation and chemical profile of the Kitchener MWWE (5 L composite) for estrogenic substances (YES) indicating in the order of elution: bisphenol A, 17β-estradiol, testosterone, 17α-ethinylestradiol, estrone were present in this extract.

Chapter 4: Discussion

Fish exposed to MWWEs in the receiving environment within the Grand River watershed have expressed various degrees of adverse reproductive effects. These effects have included VTG induction in caged rainbow trout downstream of the Guelph effluent discharge, as well as ova-testes in male greenside and rainbow darters downstream of the Waterloo and Kitchener effluent discharges (Ings *et al.*, 2012, Tanna, 2012). These feminizing effects suggested that the Guelph, Waterloo and Kitchener MWWEs contained EDCs that induced the estrogen-signaling pathway. However these prior studies were conducted in the field and were open to confounding variables making it difficult to make direct casual inferences.

In the studies conducted from July to August 2009, effluent from the city of Guelph and Waterloo MWWTPs did not induce vitellogenin in rainbow trout and would suggest that these MWWEs do not contain sufficient estrogenic EDCs to induce an estrogen dependent *in vivo* response. Overall, female plasma VTG was greater than male plasma VTG; likely the result of higher concentrations of endogenous estrogens (not measured), however there were time dependent trends between the genders. In the Guelph effluent exposure, female plasma VTG declined by day 8 and increased slightly by day 14, while male VTG was unaffected by time. In the Waterloo effluent exposure, female plasma VTG declined linearly over time whereas male VTG declined after 14 days.

Adverse endpoints in fish downstream of MWWTPs have been observed in the Grand River and include vitellogenin and ova-testis (Ings *et al.*, 2011, Tetreault *et al.*, 2011, Tanna, 2012). The YES assay revealed that the Guelph, Waterloo and Kitchener effluent extracts contained estrogenic substances and that the total estrogenicities (E2eq) were consistent with the degree of ova-testes observed at these sites (Kitchener > Waterloo > Guelph). The E2eq in this study are consistent with the E2eq in a study in the United Kingdom, where Williams *et al.* (2009) surveyed

over 10,313 river sections impacted by 2000 MWWTPs and correlated those sites with low risk, < 1.0 ng/L E2eq (i.e. Guelph MWWE, 0.81 ± 0.02 ng/L E2eq), moderate risk, > 1.0 ng/L E2eq (i.e. Waterloo MWWE 4.32 ± 0.07 ng/L E2eq), and those at high risk, > 10.0 ng/L E2eq (i.e. Kitchener MWWE, 17.0 ± 0.4 ng/L E2eq) to contain estrogenic substances, which are linked to ova-testes. The Kitchener effluent however, was often too toxic to test *in vivo* responses but in these study it had the highest level of E2eq, and in other studies (Tanna, 2012) it had the highest prevalence of ova-testes. However, rainbow trout exposed to the Guelph and Waterloo effluents did not induce an estrogenic responses *in vivo* in the laboratory exposures under 48 h static renewals. One study reported that 2 - 5 ng/L E2eq (YES) was sufficient to increase plasma VTG by 9 fold, but 10 – 15 ng/L E2eq increased plasma VTG by 46 – 1765 fold (Thorpe *et al.*, 2009), but with comparable E2eq in the Waterloo, CAS-N and CAS-BNR effluents, only CAS-BNR induced plasma VTG, which was less than 5 fold. Besides having different matrices, renewed their effluents in a flow-through system with 75% turnover within 24 h at 15 mL/min, whereas these exposures were static renewal after 48 h. It could be possible that this discrepancy between E2eq and plasma VTG induction might be related to the exposure conditions and time.

Rainbow trout exposed to the Guelph or Waterloo effluent did not express VTG, and were therefore not estrogenic *in vivo*, however their plasma VTG generally decreased over time. There could be several factors that might limit the inference of these conclusions and could be investigated in subsequent studies, and it was not clear whether these fish could respond to an estrogenic stimulus under these experimental conditions. For example, matrix effects could have limited the bioavailability of chemicals, and resulted in a null *in vivo* response. Subsequently, rainbow trout were exposed to 100 ng/L of EE2 in similar experimental conditions, and these fish induced plasma VTG after 8 days well above the reference condition suggesting these fish were responsive to an estrogenic substances in effluent and that the matrix suppression was likely not important. However, the concentration of EE2 exceeded the concentration expected in the effluents

tested (e.g. <2 ng/L; (Ternes *et al.*, 1999b)) and further studies should include lower dose dependent concentrations of estrogens to test low dose thresholds in conjunction with each unique effluent matrix.

The VTG pathway might have been inhibited through various MOAs not tested in these studies. Allostatic load which could suppress VTG through alternative mechanisms in the HPG axis (McEwen, 1981, Leatherland *et al.*, 2010) by EDCs such as acetaminophen (Miller *et al.*, 1999), which can suppress plasma VTG expression, or prochloraz, which can suppress endogenous ERα and ERβ expression (Abdelrahim *et al.*, 2004) as well as inhibiting CYP19 (aromatase) in the brain and ovary (Ankley *et al.*, 2005). Suppression of VTG could also occur through alternative MOAs by compounds that attenuate or inhibit ER induced transcription by non-ER pathways such as the aryl hydrocarbon receptor MOA (Palumbo *et al.*, 2009, Grans *et al.*, 2010). In addition, the cumulative effects of multi-pathway suppression on VTG expression are not clear, although stress and nutrient metabolism, not measured in these exposures might have attenuated the expression of VTG (Jordan *et al.*, 2012). *In vitro* results suggest that the Waterloo effluent contains more estrogenic substances but the *in vivo* responses were indifferent from reference conditions, therefore it could be possible that the Waterloo effluent was more estrogenic than the Guelph effluent but also more suppressive.

Wild fish are continuously exposed to multiple stressors in environmental conditions and effluents with constantly changing contaminants. The Kitchener MWWTP and the Waterloo MWWTP are two point sources of contamination in the Grand River potentially discharging estrogenic EDCs. The measure of total estrogenicity (YES response) in the effluent extracts paralleled the field observations such as ova-testes in wild fish. However, wild fish in the Grand River are exposed to many other non-point sources such as agricultural and urban storm water runoff that may contain a diversity of estrogenic contaminants such as natural hormones and alkylphenols (Villeneuve *et al.*, 1997, Burnison *et al.*, 2003). Chemicals such as atrazine (Hayes *et*

al., 2011) which has weak estrogenic activity (Shyu *et al.*, 2011) and can induce ova-testes and alter gonadal hormones in fish (Rohr and McCoy, 2010).

Different treatment technologies and processes can have varying effects on the contaminants in MWWEs. The Burlington pilot plant received a single source of primary effluent from the Burlington MWWTP and treated the effluent with three different technologies (CAS, CAS-N, and CAS-BNR). The CAS, and CAS-N effluent did not affect the plasma VTG after 8 or 14 days in any of these studies at LOT or HOT. Fish exposed to the CAS-BNR effluent at LOT after 14 days had slightly higher concentrations of circulating VTG than fish exposed to either the CAS or CAS-N. However, this effect was not observed in the 8 day LOT or HOT exposures. The induction of VTG in CAS-BNR was weak and independent of dose, which could be attributable to weak xenoestrogens or EDCs with selective estrogen response mechanisms. Contaminants such as BPA or the alkylphenols can cause a relatively small increase in plasma VTG (less than 100 fold) in rainbow trout when exposed to 100 ng/L NP and 1.0 mg/L BPA, which is approximately equivalent to 20 ng/L (E2Eq) (Van den Belt *et al.*, 2003a). Surprisingly, males did not induce VTG in the shorter 8 day LOT exposure (November 2010) at 20% or 50% effluent given the weak induction after 14 days. However these two CAS-BNR exposures were conducted three months apart and poor effluent stability or influent inputs may be the result of considerable effluent variability (Pileggi *et al.*, 2011).

The plasma VTG concentrations in rainbow trout exposed to the Burlington pilot plant effluents were all low or not different from reference conditions. The 14 day CAS-BNR treatment was the only effluent exposure to show elevated levels of VTG, although it was only 2-3.5 times higher than controls. This level of VTG induction is similar to the levels observed in caged rainbow trout exposed to the Guelph effluent conducted by Ings et al. (Ings *et al.*, 2011), but still considerably less than the VTG induction reported in other MWWE studies (Sumpter and Jobling, 1995, Jobling *et al.*, 1998, Routledge *et al.*, 1998, Tilton *et al.*, 2002, Thorpe *et al.*, 2009).

Increased treatment has been shown to deconjugate estrogens and other compounds in the effluent, which may make them more bioavailable (Ternes *et al.*, 1999a). Elevated VTG in the CAS-BNR effluent may be an indication of changes in bioavailability (and extractability) of the estrogenic chemicals. Although the pilot plants all started with the same influent, the effluents demonstrated different concentrations of E2eq in their extracts. The estrogenicity of the pilot plant effluents measured with the YES assay suggested that there were low levels of estrogenic chemicals in these effluents. The CAS effluent had the lowest estrogenicity (2.75 ± 0.79 ng/L E2eq) while both CAS-N and CAS-BNR had greater E2eq (19.0 ± 11.4 ng/L E2eq and 15.5 ± 4.3 ng/L E2eq), these two effluents did not differ. In these pilot plant processes effluent processes designed to remediate nitrogenous and phosphorous compounds increased the estrogenic activities (YES). However, there appears to be considerable temporal variability in the quality and consistency of the pilot plant effluents although the CAS-BNR effluent was usually the most estrogenic. Pileggi *et al.* (2011) also measured total estrogenicity using YES and found a similar trend in August 2010, however this trend was not consistent with the total estrogenicity in November 2010, (August: CAS; 0.35 ng/L E2eq, CAS-N; 0.2 ng/L E2eq, CAS-BNR; 1.5 ng/L E2eq, November: CAS; 8 ng/L E2eq, CAS-N; 1.5 ng/L E2eq, CAS-BNR; 2.5 ng/L E2eq) (Pileggi *et al.*, 2011). Generally, CAS and CAS-N had noticeable levels of suspended solids throughout all exposures, which could have also affected the bioavailability of steroid mimicking EDCs through sorption, a primary mechanism of estrogenic removal in activated sludge systems (Racz and Goel, 2010). If there are variations in Pilot Plant effluents there may also be temporal variation in the distribution of estrogenic substances in the Guelph, Waterloo and Kitchener MWWEs. The Guelph and Waterloo *in situ* data were collected in different years than these laboratory exposures and may not be directly related. The caging exposures of rainbow trout reported by Ings *et al.* (2011) were conducted in August 2006, three years prior to these exposures and the differences could be due to simple annual or seasonal variations in the chemical profile of this effluent. Unfortunately, the annual variance and

monthly variance of estrogenic substances in the MWWEs examined in these studies are not known.

Rainbow trout have been a model organism when studying estrogenic responses such as VTG induction but alternative fish species might be more suitable for studying effluents. VTG induction is an *in vivo* biomarker that usually responds well in rainbow trout in comparison to other species (small bodied species) to low levels of estrogenic chemical exposure (Dobbins 2008). However trout are also sensitive to other effluent associated pollutants that small fish species (those typical used in the laboratory) appear to be more tolerant to, including ammonia (Hutchinson *et al.*, 2006, Dobbins *et al.*, 2008, Armstrong *et al.*, 2012). Trout are a cold-water species, thus the laboratory testing, which is conducted at lower temperatures, may alter the bioavailability of EDCs or effluent characteristics that affect biological responses. The Guelph advanced MWWTP nitrifies its effluent and the ammonia levels are much lower than in the Waterloo and Kitchener effluents that do not nitrify (at the time of this study). Effluents that do not nitrify or do so poorly such as CAS (relative to CAC-N or CAS-BNR) typically have higher levels of ammonia and its un-ionized form can be toxic to aquatic organisms. The Waterloo 50% and 90%, as well as the CAS effluent the un-ionized ammonia varied in these exposures and was at or above the concentration that would be approaching toxic levels (give the concentration and pH). Armstrong *et al.* (2012) recently showed that male fathead minnows exposed to ammonia significantly decreased egg count and suppressed VTG mRNA. Static renewal exposures (48 h) may have elevated the ammonia concentrations in these exposures to suppress plasma VTG concentrations (although fish did respond to EE2 exposure). The Waterloo, CAS and CAS-N effluents had higher than expected mortalities; stress, ammonia, altered physiology and suspended solids can alter respiration and uptake of chemicals through the gills and reduce the sensitivity of *in vivo* responses.

Classical biological indicators such as VTG induction have been suitable to test for estrogenic responses *in vivo* in simple and complex mixtures, however the mechanistic relevance of

estrogen induced VTG and ova-testes in reproductive AOPs is not certain. While estrogenic MOAs have been a large focus of AOPs in MWWEs, other steroid mimicking EDCs found in MWWEs include progestogenic, anti-estrogenic, androgenic and anti-androgenic, which could alter reproductive outcomes (Vinggaard *et al.*, 2002, Kiparissis *et al.*, 2003, Lee *et al.*, 2003, Rotchell and Ostrander, 2003, Urbatzka *et al.*, 2007, van der Linden *et al.*, 2008, Runnalls *et al.*, 2010, Perkins *et al.*, 2011, Grosse *et al.*, 2012, Petersen and Tollefsen, 2012). Although few reports have studied the synergisms of these interactions, statistical analysis of MWWEs containing EDCs indicate that de-masculinizing (anti-androgenic) compounds exacerbate feminizing (estrogenic) compounds to increase the probability of ova-testes and gonad morphology (Jobling *et al.*, 2009). Integrating several MOAs can be a great challenge when assessing *in vivo* effects within the HPG axis. Multigenerational studies with EDCs and other MOAs are also lacking. Compounds such as nonylphenol are a weak estrogen and anti-androgen that can induce VTG in parental rainbow trout without a high prevalence of ova-testes, while the next generation of offspring exhibit ova-testes (Schwaiger *et al.*, 2002). Multi-generational studies on other known EDCs are also lacking (Anway *et al.*, 2005), especially for EDCs such as neuromodulators that can alter biological pathways farther up in the HPG axis to include early sexual differentiation and development independent of hormone action (Weiss, 2011).

An effects-directed assessment (EDA) for estrogenic substances was conducted on two of the effluents to identify the chemical constituents that contribute to the E2eq. Fractionating the Waterloo and Kitchener MWWEs revealed two chemical profiles. Estrone was the only compound detected in the Waterloo MWWE, whereas the Kitchener MWWE contained several estrogenic substances, including E1 > E2 > BPA > EE2 ~ testosterone. The presence of multiple estrogenic substances such as E2 and EE2, both strong agonists in the Kitchener MWWE qualitatively suggests that this effluent could be more estrogenic than the Waterloo MWWE. However slight differences in the extraction, such as the volume of effluent may have influenced this result. The

compounds that were identified in this EDA have also been identified in UK MWWEs that induced ova-testes in Roach (Desbrow *et al.*, 1998). In addition, The Kitchener MWWE contained EDCs with multiple MOAs such as BPA, a known weak estrogen, anti-androgen (Ekman *et al.*, 2012) and thyroid receptor antagonist (Boas *et al.*, 2012), possibly suggesting the inclusion of multiple MOAs for this effluent, but also the relevance of multiple active agents and their summed effects *in vivo*.

An inherent benefit of EDAs is that they reduce the confounding effects of the matrix as a whole, therefore the summed YES E2eq in the estrogenic fractions compared to the whole effluent could suggest that there were suppressive characteristics in the effluent. The E2eq of the E1 fraction in the Waterloo effluent EDA was close to the E2eq of the whole effluent extract (4.1 ng/L vs. 1.75 ng/L E2eq) while the sum of the active E2eq fractions in the Kitchener effluent EDA (E1, E2, BPA, EE2, T) compared to the total E2eq of the whole effluent extract was much higher (44.4 ng/L vs. 2.75 ng/L). Models have been developed that try to ascertain interaction effects of multiple compounds in mixtures including concentration addition and independent action MOAs (Zhang *et al.*, 2010). It has been demonstrated that low concentrations estrogenic compounds (e.g. BPA, NP, E2) can act in an additive manner (Thorpe *et al.*, 2003), although these predictions are less reliable with matrices containing EDCs with multiple MOAs (Petersen and Tollefsen, 2012).

The EDA method developed in this study was sufficient to clearly separate and identify known estrogenic substances found in MWWEs to as low as 0.1 ng/L E2eq depending on the volume of effluent collected. However, the total estrogenic E2eq of the Kitchener MWWE and to a lesser extent the Waterloo MWWE were less than the sum of the individual estrogenic fractions. And although chromatographic separation and fractionation separates all compounds including antagonists the cumulative effects of the individual fractions may better represent the true estrogenicity at the impacted sites *in situ*. Furthermore, at relatively low concentrations, estrogen agonists tend to behave additively in trout hepatocytes inducing VTG (Petersen and Tollefsen, 2012) but *in vitro* assays such as the YES might underestimate *in vivo* effects of complex effluents

(Schlenk, 2008). In addition, many *in vitro* endpoints used in risk assessments for the screening of EDCs lack the fundamental understanding to allow extrapolation to effects at higher levels of biological organization (Judson *et al.*, 2011, Sun *et al.*, 2011, Altenburger *et al.*, 2012).

Chapter 5:Conclusions and Future Work

Effect-directed assessments could be an important tool to identify endocrine active chemicals in complex MWWEs when conducted with the goal of identifying active chemicals. Even when complex mixtures are simplified to their active chemicals, the *in vitro* effects may not accurately reflect the *in vivo* effects of the identified chemicals at similar concentrations. Currently, mixtures with relatively few agonists have been tested *in vivo* and the combined affects of natural ligands appear to act additively near their individual thresholds of activity however, the *in vivo* effects of xenoestrogen mixtures with mixed MOAs appear less predictable. Future EDAs could benefit from predictive model analyses that identify the potential for interactions of multiple EDCs *in vivo*, including agonists and antagonists. Multiple EDAs could address several known MOAs along the HPG, such as the combined effects of estrogenic substances with selective estrogenic response mechanisms for ERα and ERβ, their agonists and antagonists, antiandrogens, and aryl hydrocarbon receptor agonists and antagonists, as well as the inclusion of alternative MOAs such as neuromodulation and mechanisms directing early sexual differentiation. Ultimately, these methods can be labour intensive and there are trade-offs between the sensitivity and stochastic variability and sample reproducibility. Testing for adverse effects at higher levels of biological organization is therefore needed to better understand how these interactions translate into effects. MWWEs and EDCs can vary seasonally, daily, hydraulically, or even sporadically (Musolff *et al.*, 2009). Therefore, longitudinal studies and rapid screening techniques could identify the ideal sampling periods for EDCs unique to each MWWTP, but these screening techniques should be applied to a battery of *in vivo* EDAs. Molecular screening techniques (i.e. microarrays) with an integrated system biology approach could help narrow the direction of investigation to specific perturbed molecular pathways from which specific MOAs can be tested *in vivo*.

These studies reiterate some of the current gaps in the literature between *in vivo* and *in vitro* effects, specifically when the chemical concentrations approach the threshold for *in vivo* effects. The plasma concentrations of VTG in effluent exposed fish were dissimilar from those identified elsewhere in the world despite being associated with similar reproductive AOPs including VTG induction with comparable occurrences of ova-testes. However, different treatment processes in the pilot plant studies produced different total estrogenicities in their final effluents (YES). Intuitively, additional treatment processes should improve the effluent quality since these pilot plant effluents are primarily implemented to remediate nutrients such as ammonia to reduce effluent toxicity. However, greater estrogenicity (YES) was found in the treatment processes (CAS-N and CAS-BNR) designed to remove toxicity. Currently, there is little known about the seasonal variability of these effluents with respect to the presence of estrogenic substances, and great benefit could be obtained from routine monitoring for whole effluent estrogenicity or alternative MOAs that use multiple *in vitro* screening techniques. Although attempts have been made to simplify complex mixtures using EDAs to identifying compounds with similar MOAs, they may fail to assimilate the total effects *in vivo*, especially if compounds with multiple MOAs are identified. The Grand River MWWEs display various degrees of estrogenicity *in vitro* consistent with the occurrence of ova-testes, but the Guelph and Waterloo exposures did not indicate that these effluents could induce an *in vivo* response. Furthermore, it is not certain whether the estrogenic substances in these complex effluents are present at high enough concentrations at the critical biological stages to impact fish reproduction in the receiving environment. The failure to detect VTG induction in rainbow trout in laboratory exposures may not be directly applicable to wild fish because of numerous factors including interspecies sensitivities, exposure duration, *in vivo* suppression and matrix effects, seasonal variation, bioavailability or the exclusion of non-point sources, but future work addressing some of these uncertainties could contribute to understanding the adverse effects observed in the Grand River and other watersheds.

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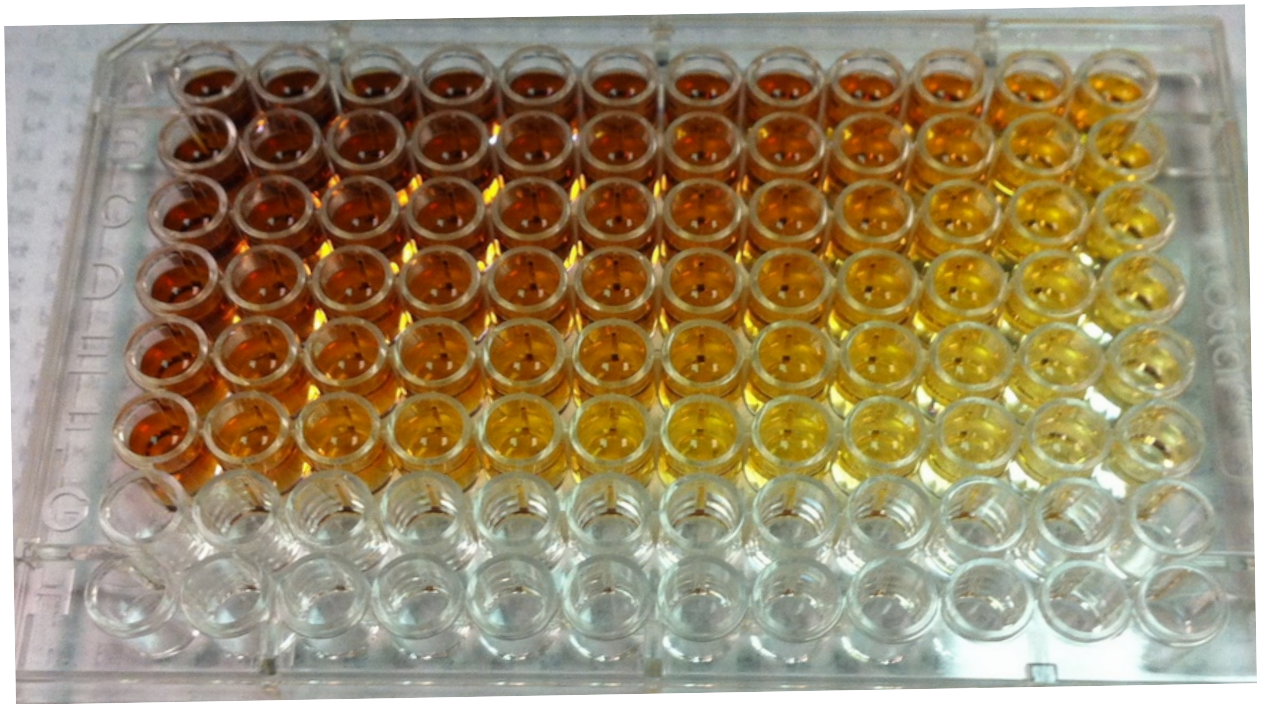
Zhao C, Dahlman-Wright K & Gustafsson JA 2010. Estrogen signaling via estrogen receptor β. *J Biol Chem,* 285**,** 39575-9.

Appendix 1: Vitellogenin Method Development and Validation

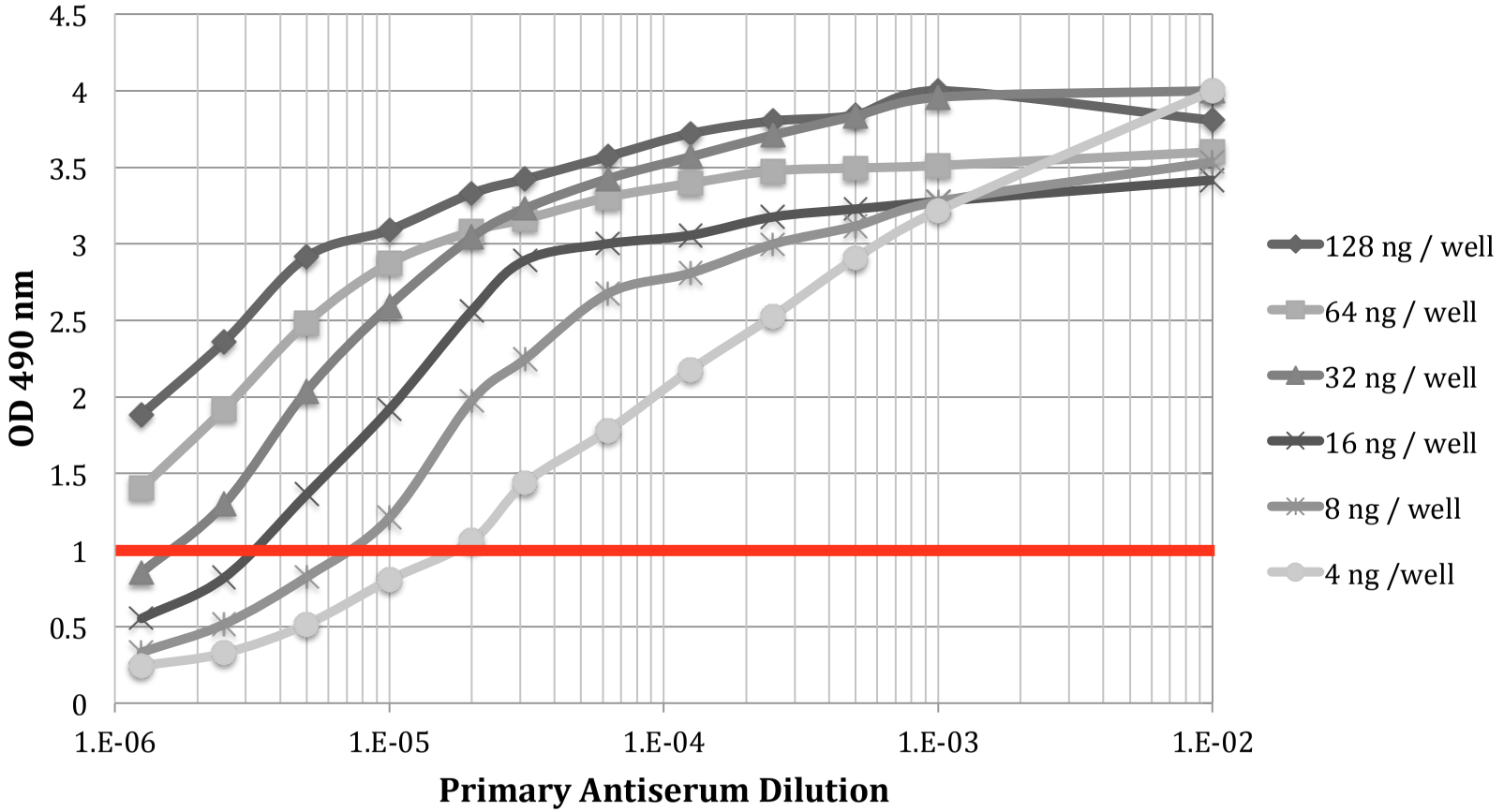
Rainbow trout VTG determination was compared to primary antiserum in a 2-factor comparison of polyclonal rabbit anti-rainbow trout VTG antibodies against rainbow trout VTG. The purpose of this cross was to identify the optimal concentrations of coating (Ag) and primary antiserum to use in the rainbow trout VTG ELISA given a sample with no VTG (maximum binding). To determine a suitable amount of rainbow trout VTG required to coat the wells, rainbow trout VTG was serially diluted from 128 ng/well to 4 ng/well, coated and blocked as outlined in the rainbow trout VTG method (Section 2.1.4). The primary antiserum was serially diluted from 100 to 800,000 times of the stock in BSA. All wells were incubated with 50 uL BSA solution and 100 uL of the primary antiserum dilutions in a 6 x 12 grid (6 VTG coating concentrations vs. 12 primary antisera dilutions, Appendix Figure 1.1). The optimal OD_{490} for this experiment should not exceed 1.5 units to minimize chromogen suppression and detection by the spectrophotometer. The wells should be coated with 4 - 32 ng/well of rainbow trout VTG and 100,000 and 800, 000 times diluted primary antiserum (Appendix Figure 1.2). The concentrations used in the analysis of these studies were arbitrarily chosen to be 16 ng/well rainbow trout VTG and 600,000 times diluted rainbow trout antiserum.

Unfortunately the primary antisera from all rabbits including rainbow trout were accidently thawed and left out for a prolonged period of time (approximately 2 weeks), which could have compromised the integrity of the antisera. A comparison of rainbow trout (rabbit 4E2) antisera (post thaw) to the working stock of the same antisera (rabbit 4E2) that was not thawed (pre thaw) is provided here to re-validate the usefulness of these samples (Appendix Figure 1.3). The reference VTG of control females from the Waterloo exposure were compared to pre thaw and post thaw antisera using plasma from 3 male rainbow trout (A1, B1, C2) induced with 10 mg/kg EE2 in sesame oil.

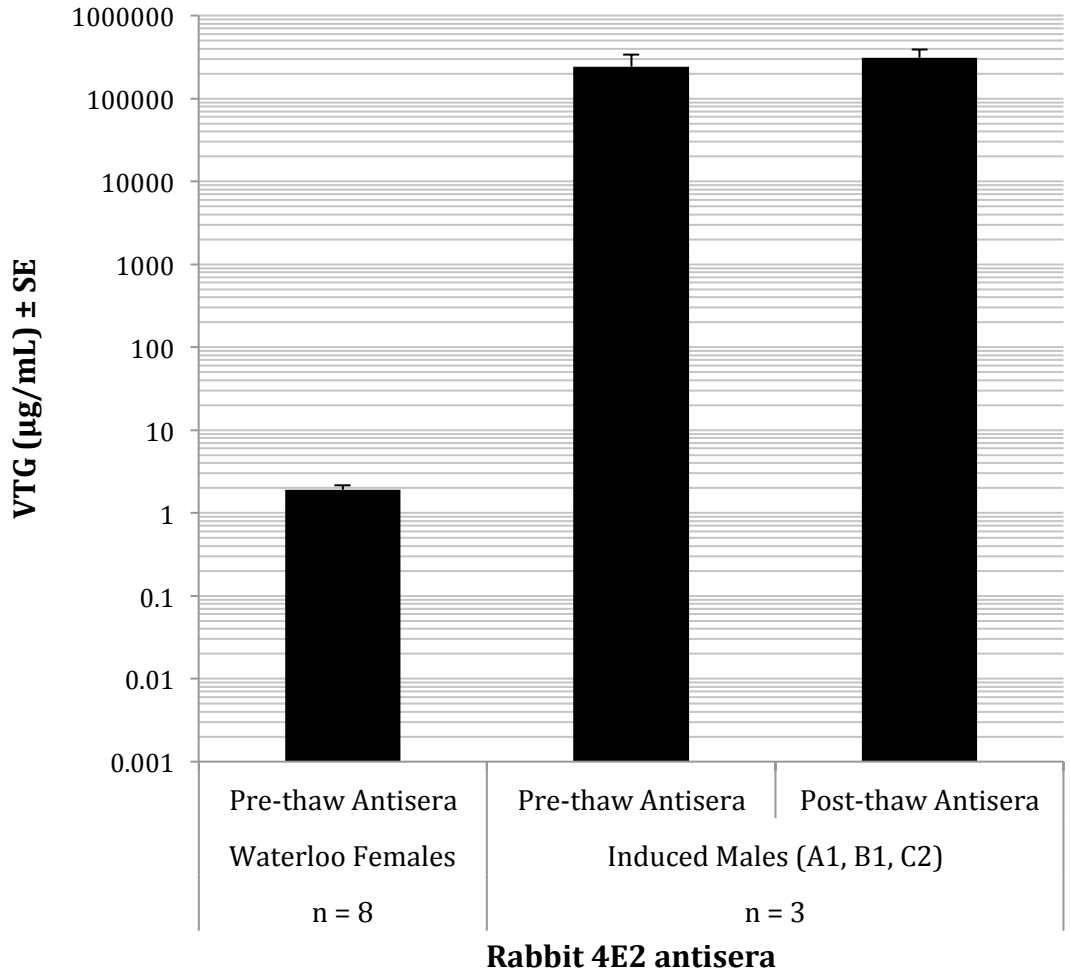
		Primary antiserum dilution (Column)											
		1	2	3	4	5	6	7	8	9	10	11	12
Row	rainbow trout VTG (ng/well)	100	1,000	2,000	4,000	8,000	16,000	32,000	50,000	100,000	200,000	400,000	800,000
A	128												
B	64												
C	32												
D	16												
E	8												
F	4												



Appendix Figure 1.1: Optimization for the coating antigen (VTG) with rabbit anti-rainbow trout antiserum.



Appendix Figure 1.2: Optimizing the concentration of antiserum to antigen coating each well.



Appendix Figure 1.3: Binding comparison of the unthawed primary antiserium to detect VTG induction in males to basal levels of VTG in females. Thawed antiserum was also compared to unthawed antiserum for its sensitivity to VTG in males induced with EE2.

Appendix 2: Supporting Information for the Vitellogenin Enzyme-linked Immunosorbant assay (ELISA)

The following solutions and procedures outline the standard operating procedure for the rainbow trout competitive indirect ELISA, developed in-house and utilized in this study. The solution used in the rainbow trout VTG ELIA were as follows: sodium bicarbonate buffer (SBB), tris-HCl buffer (TBS-T), a 5% bovine serum albumin (BSA), Ammonium acetate citric acid solution (AACA), and o-phenylene diamine (OPD). To make SBB, 4.20 g of sodium bicarbonate ($NaHCO_3$), 5.0 mg Gentamycin were added to a 1 L graduate cylinder and filled to 1.0 L with dd H_2O and corrected to pH 9.6. To this buffer, rainbow trout VTG was added, and this coating solution was dispensed to every well and incubated at 37ºC for 3 h. A 10 times more concentrated solution (10 X) stock of TBS-T was made and diluted 1:10 in dd H_2O as required. To make the 10 X TBS-T solution 12.11 g of Tris-HCl, 87.66 g NaCl, 10.00 mL Tween20 and 50.0 mg of Gentamycin were added to a 1 L graduated cylinder and brought up to 1.0 L in dd H_2O and corrected to pH 7.5. The 1 X TBS-T was used to wash the plates between incubations and also to make the BSA solution. The BSA solution was made fresh before every assay and as required. To make the 5% BSA solution 0.5 g of BSA was added to a glass cylinder and filled to 100 mL with 1 X TBS-T and corrected to pH 7.5 if required. The BSA solution was used to block the wells as well as the diluent in the samples, primary antisera and secondary antisera. To make the AACA solution, a solution of ammonium acetate was made and adjusted with a citric acid solution to pH 5.6. To make the ammonium acetate solution, 1.155 g of ammonium acetate was added to a graduate cylinder and filled to 300 mL with dd H_2O and corrected to pH 6.68 if required. To make the citric acid solution, 1.05 g of citric acid was added to a class cylinder and filled to 100 mL with dd H_2O and corrected to pH 2.63 if required. To make the AACA working solution, citric acid solution was added to the ammonium acetate solution and corrected to pH 5.6, if required. The AACA solution was then used as the diluent in the OPD solution. The OPD solution is light

sensitive and it was made as quickly as possible and as required. To make the OPD solution, a clean stir bar was added to a dispensing cup and to this, 8.0 mg of o-phenylene diamine was dissolved in 16.0 mL AACA and 8.0 µL of 30% hydrogen peroxide with gentle stirring.

To coat each wells with 16 ng of rainbow trout VTG, 1.23 µL of RBT VTG (1.3 mg/mL stock) was added to 15.0 mL of SBB pH 9.6, then 200 µL of coating solution was dispensed to each well and the plate was incubate for 3 h at 37ºC. After the coating incubation, the plate was washed by adding approximately 300 µL of 1 X TBS-T pH 7.5 using a multi-chamber pipette accessory five times. The wells were then blocked by adding 200 µL of BSA solution to each well and incubated at 37ºC for 0.5 h. The plate was washed again as described above. To make the standard curve, rainbow trout VTG (1.3 mg/mL) was diluted in BSA to a final concentration of 300 ng/50 µL and serial diluted in BSA from 150 ng/50 µL to 0.292 ng/50 µL for a total of 10 concentrations. To make the 600,000 times diluted primary antibody solution, 12.5 µL of a 500 times diluted working stock solution was added to 15.0 mL of BSA. To prepare the samples dilutions were made starting at 1:5 and often required 1:20 dilution in BSA when VTG was not induced. However, when fish were induced with EE2, their samples often required to be diluted 1:100,000 times or more in BSA to fall in the linear portion of the standard curve. Every sample, standard and control was carried out in triplicate as follows: 50 µL of diluted sample or standard concentration was added to each respective well, 50 µL of BSA was added to each positive control well, 150 µL of BSA was added to each negative control well, and 100 µL of the primary antibody solution was added to every well except the negative control wells containing 150 µL of BSA solution. The plate was then incubated over night, not exceeding 24 h at room temperature. The following morning the plates were washed again with 1X TBS-T as described above. Goat anti-rabbit secondary antibody (Sigma, A6154-5X1ML) was diluted to 1:10 in BSA and stored at -20ºC. To make the secondary antibody solution, 150 µL of goat-anti rabbit antibodies (working stock) was added to 15.0 mL of BSA. Each well then received 150 µL of secondary antibody solution and

the plate was incubated at 37ºC for 2 h. After the secondary antibody incubation the plate was washed again as described. In the final step of this assay 150 µL of the OPD solution was dispensed into each well and the plate was incubated in a dark drawer for 0.5 h. To stop the reaction 50 µL of 5 M sulfuric acid (H_2SO_4) was added to each well. Within 20 min of stopping the reaction the optical density of each well was measured at 490 nm (OD_{490}).

Appendix 3: Supporting information for the yeast estrogen screen (YES)

In this section, the reagents for the media and solutions used in the YES assay (section 2.2.6) will be discussed in detail. A list of the reagents and materials for the YES assay are included in Appendix Table 3.1. Also, all glassware was cleaned with soap and water and then autoclaved for 30 min prior to use and as directed. To maintain sterility, solutions were made on a bench space with access to both a water syphon and tap to operate the suction filters and a Bunsen burner for aseptic technique. The assay however, was conducted in a laminar flow hood to provide greater sterile working area.

Many of the solutions used throughout this assay require suction filtration through a 0.2 µm screw top filter into a glass screw top container. These solutions were made next to a Bunsen burner, assuring the flame plume reached the screw top filter without melting the filter. The solutions made in this assay include: amino acid solutions, GOLD media, 20% dextrose, yeast nitrogen base (YNB), minimal media, growth media, and 10 mM copper II sulfate (Cu_2SO_4) solution. The amino acid solutions are used in the GOLD media as enrichment nutrients, while L-lysine and L-histidine are supplemental in the minimal media. To make the amino acid solutions refer to Appendix Table 3.2 for the mass of the reagents, the sterilization procedure and the storage requirements. Each solution is made individually and stored individually. In a graduated glass cylinder add the amount (g) per 400 mL dd H_2O to a glass screw top bottle and follow the sterilization procedure. For autoclaved amino acid solutions autoclave for 30 min, and for L-tryptophan, autoclave the glass vessel prior to making the L-tryptophan stock and then assemble a 0.2 µ suction filter. Pour the L-tryptophan solution through the suction filter. Follow the storage instructions for the amino acid solutions; some require storage at 4ºC, while others can be stored at room temperature (RT). To make the GOLD stock media, assemble a 0.2 µm suction filter to an

autoclaved screw top bottle. To make the GOLD stock solution, assemble a 0.2 µm suction filter to an autoclaved glass screw top container and add the volume for GOLD stock media (mL) as outlined in Appendix Table 3.1. The total volume for the GOLD stock media should be 600 mL.

Appendix Table 3.1: The list of the reagents, their suppliers and catalogue numbers used in the yeast estrogen screen.

Amino acid and nucleic bases	**Supplier**	**Catalogue number**
Adenine Sulfate	Sigma	A8751-25g
L-Histidine-HCl	Sigma	H5659-25g
L-Arginine-HCl	Sigma	A6969-25g
L-Methionine	Sigma	M5308-25g
L-Tyrosine	Sigma	T8566-25g
L-Isoleucine	Sigma	I7403-25g
L-Lysine-HCl	Sigma	L8662-25g
L-Phenylalanine	Sigma	P5482-25g
L-Glutamic Acid	Sigma	G8415-100g
L-Aspartic Acid	Sigma	A7219-100g
L-Valine	Sigma	V0513-25g
L-Threonine	Sigma	T8441-25g
L-Serine	Sigma	S4311-25g
L-Leucine	Sigma	L8912-25g
L-Tryptophan	Sigma	T8941-25g
Uracil	Sigma	U1128-25g
Growth Media Components		
Yeast Nitrogen Base without amino acids	Sigma	Y0626-250g
Dextrose	Sigma	G5400-250g
Bactoagar (500 g)		CA95026-642
Assay Components		
Copper (II) Sulfate pentahydrate	Sigma	C8027-500G
β-Galactosidase Assay Kit	Pierce	75768
Miscellaneous		
Disposable 1.0 mL Cuvettes	VWR	97000-586
Petri Plates 100x15	Fisher	08-757-13
Absolute Ethanol	Commercial Alcohols	
0.2 µm Screw top filters 150 mL	Fisher	09-741-09
Test Tube Enclosures	Fisher / VWR	14-957-92C

Appendix Table 3.2: The amino acids and nucleic acid solutions for the YES and GOLD media solution

Compounds	Amount (g) per 400 mL dd H_2O	Sterilization procedure	Storage Temperature	Volume for GOLD stock media (mL)
Adenine Sulfate	0.48	Autoclave	RT	50
L-Histidine-HCl	0.96	Autoclave	4°C	25
L-Arginine-HCl	0.96	Autoclave	4°C	25
L-Methionine	0.96	Autoclave	4°C	25
L-Tyrosine	0.36	Autoclave	RT	100
L-Isoleucine	1.44	Autoclave	4°C	25
L-Lysine-HCl	1.44	Autoclave	4°C	25
L-Phenylalanine	1.2	Autoclave	RT	50
L-Glutamic Acid	2.4	Autoclave	RT	50
L-Aspartic Acid	1.6	Autoclave	RT	75
L-Valine	7.2	Autoclave	4°C	25
L-Threonine	9.6	Autoclave	4°C	25
L-Serine	1.8	Autoclave	4°C	25
L-Leucine	1.44	Autoclave	RT	25
L-Tryptophan	1.92	Screw top filter	4°C	25
Uracil	0.96	Autoclave	RT	25

The screw top filter refers a 0.2 µm suction filter; RT, room temperature (approximately 20ºC).

The dextrose and YNB solutions are components of the minimal media and growth media, while the GOLD stock is an enrichment component of the growth media. To make the 20% dextrose solution, 200 g of dextrose was slowly added to approximately 800 mL of vigorously stirring dd H_2O in a 1 L graduated cylinder until all 200 g was completely dissolved and filled to 1.0 L with dd H_2O. This solution was filter sterilized with a 0.2 µ screw top filter into a 1 L bottle and stored at 4ºC. To make a 10X stock solution of YNB, 67 g of YNB without amino acids was added to a graduated cylinder and filled to 1.0 L with dd H_2O and stirred until dissolved. The YNB solution was filter sterilized with a 0.2 µm screw top filter and stored at 4ºC. To make the minimal media, a 0.2 µ filter was assembled to a 1 L bottle. To this assembly 100 mL of 10X YNB, 100 mL of 20% dextrose, 10.0 mL L-histidine, 10.0 mL L-lysine and 780 mL of dd H_2O were suction filtered and stored at 4ºC until required. To make the growth media, a screw top filter was attached to a 1 L glass bottle to which 60 mL of 10X YNB, 60 mL of 20% dextrose, 110 mL of GOLD stock media and 370 mL of dd H_2O was suction filtered and stored at 4ºC until required.

To initiate the assay, cells were grown on a selection agar plates to isolate a single colony of yeast cells. To make the selection plates, 10 mL of 1X YNB, 2 g of Bactoagar and 78 mL of dd H_2O were added to a glass screw top container and autoclaved for 30 min. Once the glass screw top container cooled to touch, 1 mL of L-histidine, 1 mL of L-lysine and 20% dextrose solution were added, gently swirled and poured immediately with enough agar media to coat the bottom of the petri plate. These plates were left to solidify under sterile conditions in the laminar flow hood. After the plates had solidified they were inverted and placed in sealable plastic bags and stored at 4ºC until required.