DEVELOPMENT OF A CHEMICAL ANALYSIS PROTOCOL AND APPLICATION WITH WHOLE ESTROGENIC AND ANDROGENIC ASSAYS TO ASSESS ENDOCRINE DISRUPTOR ACTIVITY DURING WASTEWATER AND SLUDGE TREATMENT PROCESSES

by

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ABSTRACT

Wastewater and sludge treatment can decrease or increase estrogenic activity through degradation and transformation processes. Endocrine disrupting chemicals (EDCs) tend to adsorb to solids and partition into sludge during the wastewater treatment process.

Analytical procedures to detect EDCs in sludge media can be time / labour intensive and require expensive analytical instrumentation. As a result, little information is available on EDC content or fate in municipal sludge. A gas chromatography-mass spectrometry (GC-MS) chemical analysis procedure to detect estrogens in mixed and digested sludges without freeze-drying prior to extraction could not be located in the literature. Therefore, GC-MS chemical analysis protocols were developed for detection of estrone (E1) and 17β-estradiol (E2) in municipal sludges that was also compatible with bioluminescent yeast assays. This protocol is elucidated and summarised for consideration and use by other researchers and the user community.

Municipal trickling filter / solids contact wastewater treatment processes were examined for reduction of E1 and E2; whole estrogenic and androgenic activity; and toxic luminescence inhibition. Conventional heat and combinations of microwave irradiation and oxidation treatments were applied to municipal sludge and evaluated using the same methods.

The specified wastewater treatment plant reduced total E1 and E2 by 54%; estrogenic activity by 27%; and androgenic activity by 38%. The most potent estrogen, E2, was reduced by 69% and E1 was reduced by 26%. More importantly, the increased ratio of E1 to E2 from 0.6

(influent) to 1.4 (pre-chlorinated effluent), indicated E2 was biologically degraded to the less estrogenic E1.

Mesophilic (35–40 °C) sludge digestion reduced E1 by 12%, E2 by 63%, whole estrogenic activity by 73% and androgenic activity by 81%. The digestion process reduced toxicity to the yeast strain, BLYR, by threefold.

Overall, microwave irradiation was more effective than conventional heating in reducing concentrations of E1 and E2 in mixed and digested sludges. Oxidative (H_2O_2) treatments did not reduce E1, E2, estrogenic or androgenic activity. The treatment plant reduced E1, E2, estrogenic activity and androgenic activity in the wastewater stream. The anaerobic mesophilic sludge digestion process reduced E1, E2, BLYR toxicity, and whole estrogenic and androgenic activity.

PREFACE

This dissertation is original, unpublished, independent work by the author, H. Slater.

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LIST OF ABBREVIATIONS AND TERMINOLOGY

APCI	Atmospheric pressure chemical ionization
AS	Activated sludge
ASE	Accelerated solvent extraction
BCF	Bioconcentration factor
BLYAS	Bioluminescent Yeast Androgen Screen
BLYES	Bioluminescent Yeast Estrogen Screen
BLYR	Bioluminescent Yeast Reporter
BMP	Biochemical methane potential
BOD	Biochemical oxygen demand
BPA	Bisphenol A
	2,2-bis-(4-hydroxy-phenyl)propane
	4,4-isopropylidenediphenol
BSA	N,O-bis(trimethylsilyl)acetamide
BSTFA	N,O-Bis(trimethylsilyl)trifluoroacetamide
$CaCO_3$	Calcium carbonate
CH_4	Methane
COD	Chemical oxygen demand
DCM	Dichloromethane
DCDMS	Dichlorodimethylsilane (aka DMDCS)
DMDCS	Dimethyldichlorosilane (aka DCDMS)
Di,a	Air diffusivity
Di,w	Water diffusivity

DS	Digested sludge
DTE	Dithioerytrol
dw	dry weight
E1	Estrone
E2	17β-estradiol
E3	Estrone
EE2	17α-ethinyl estradiol
EDC	Endocrine disrupting chemical
EDCs	Endocrine disrupting chemicals
ED	Endocrine disruptor
EEQ	17β-estradiol equivalent
Eff	Effluent
ELISA	Enzyme-linked immunosorbent assay
Eluant	Eluent
Eluate	The solution of solvent and dissolved matter resulting from elution
Eluent	"carrier" portion of the chromatography mobile phase
Elution	Process of extracting one material from another by washing with a solvent
EPS	Exocellular polymeric substances
ESI	Electrospray ionization
“g”	Standard unit for acceleration due to gravity on earth surface
GC	Gas chromatography
GC-MS	Gas chromatography paired with mass spectrometry
GC-MS/MS	Gas chromatography paired with tandem mass spectrometry

GPC	Gel permeation chromatography
H_2O_2	Hydrogen peroxide
-HCl	Hydrochloride salt
HCl	Hydrochloric acid
HFBAA	Hepatafluoric butyric acid anhydride (derivatization agent for GC)
HLC	Henry's law constant
Inf	Influent
Kd	Soil-water partition coefficient for inorganic constituents
Koc	Organic carbon normalized soil-water partition coefficient for organic compounds
Kow	Octanol-water partition coefficient (also used to calculate Koc)
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification for method
MCRT	Mean cell retention time
MDL	Method detection limit
MELN	Estrogen-responsive reporter Mcf-7EreLucNeo cell lines
MeOH	Methanol
Mo	Methoximation
MOX	Methoxamine
MS	Mixed sludge
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MSTFA	N-Methyl-N-(trimethylsilyl) trifluoroacetamide

MW	Microwave
MWWTP	Municipal wastewater treatment plant
NCI	Negative chemical ionization (mass spectrometry)
NP	Nonylphenol
OP	Octylphenol
PS	Primary sludge
PT	Primary treatment plant
RCF	Relative centrifugal force
Recovery	Percent recovery of target compounds for method
Rho (ρ)	Spearman's rank correlation coefficient
RRF	Relative response factor
RSD	Relative standard deviation
S	Water solubility
SC	Solids contact
SE	Solvent extraction
Silica	Silica gel
Silica gel	Chromatography column solid phase
Silicic acid	Silica gel
SPE	Solid phase extraction
Si	Silylation derivatization step
SRT	Solids retention time
SS	Suspended solids
STP	Sewage treatment plant

TF	Trickling filter
TMCS	Trimethylchlorosilane
TMS	Trimethylsilyl
TMSI	Trimethylsilylimidazole
TS	Total solids
TSS	Total suspended solids
TT	Testosterone
t-	Tertiary or tert (abbreviated)
WAS	Waste activated sludge
WW	Wastewater
WWTP	Wastewater treatment plant

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1. INTRODUCTION AND BACKGROUND

Natural estrogens and other substances demonstrating estrogenic activity are classified as endocrine disruptors and emerging contaminants. Wastewater discharges are thought to be the most important contributor of endocrine disruptors to the environment. This project assessed the ability of a municipal wastewater treatment plant to remove estrogenic activity from the wastewater stream. Using an autobioluminescent yeast screen assay, effluents from various stages of the municipal wastewater treatment process were examined in terms of estrogenic activity.

Wastewater and sludge treatment can decrease or increase estrogenic activity through degradation and transformation processes. Endocrine disrupting chemicals (EDCs) tend to adsorb to solids and partition into sludge during the wastewater treatment process. Steroidal estrogens have the highest estrogenic activity of known EDCs in wastewater and estrone (E1), 17β-estradiol (E2), and 17α-ethinyl estradiol (EE2) are thought to be the priority EDCs to control in municipal wastewater treatment plants (Sun et al., 2013).

A chemical analysis for detecting estrogenic substances in mixed and digested sludge with 2–4% solids using GC-MS, liquid-liquid extractions (e.g. without freeze-drying sludges) and a simple clean up procedure could not be located in the literature. Therefore a method of preparing mixed and digested sludge samples for analysis of 17β-estradiol (E2) using gas chromatography followed by mass spectrometry (GC-MS) was developed. In order to directly compare the results from chemical (GC-MS) and biological (whole estrogenic and androgenic) analysis, the sample preparation should be the same (ideally) or very similar. The protocol for

preparing samples to minimize toxic effects to the yeast strains used for biological analysis, also produced satisfactory recovery of E2 by GC-MS. The indicator, E2, was selected because it is a potent estrogen found in municipal wastewater and used as a standard for estrogenic activity in many common biological assays, including the yeast estrogenic screen and bioluminescent yeast screen (BLYES) assays (Sanseverino et al, 2005; Routledge et al, 1998; Sun et al., 2013; Routledge and Sumpter, 1996; Matsui et al., 2000). The developed method for analysis of E2 in municipal sludge by GC-MS, was suitable for both BLYES and bioluminescent yeast androgenic screen (BLYAS) analysis.

Preliminary testing confirmed mixed sludge was too toxic to obtain reliable concentrations of estrogenic activity using the autobioluminescent yeast estrogen screen. Mixed sludge was more toxic, as measured by luminescence inhibition to the bioluminescent yeast reporter strain (BLYR), than sludge collected at the end of the mesophilic anaerobic digestion process. An analytical method to reduce this toxicity in order to reliably measure the estrogenic activity in the mixed sludge was developed. In addition, the steps in the MWWTP trickling filter-solids contact wastewater and sludge treatment processes responsible for reducing toxic effects to the autobioluminescent yeast reporter were identified.

Standard treatment of wastewater involves separating liquid from solid wastes. Liquids, called effluent, are usually discharged to an aqueous environment (e.g. rivers, lakes, oceans). Solids removed in wastewater treatment plants include screenings, grit, scum, solids (sludge) and biosolids. Screenings and grit contain larger solids that are removed as they enter the treatment

plant to protect equipment and improve wastewater treatment. The term sludge is used with a process descriptor such as primary sludge, waste-activated sludge, and secondary sludge.

Sludge and biosolids produced by wastewater treatment operations are usually in a liquid or semi-liquid form, typically containing 0.25 to 12 percent solids by weight depending how the sewage sludges are processed. Biosolids are sludge that has undergone further treatment with processes such as stabilization (e.g. pH ≥12 and heat treatments), and composting so they have beneficial uses as fertilizers, soil conditioners, etc. Solids and biosolids are stabilized to:

1) reduce solids volume for ultimate disposal
2) reduce pathogens
3) eliminate offensive odors,
4) inhibit, reduce, or eliminate the potential for putrefaction and
5) in the case of anaerobic stabilization, off gas energy production from methane.

1.1 ENDOCRINE DISRUPTORS

Endocrine systems release hormones that act as chemical messengers, coordinating and regulating communication among cells. These messengers interact with receptors in cells to trigger responses and prompt normal biological functions such as growth, embryonic development and reproduction.

Endocrine disrupting chemicals (EDCs) are substances that interfere with the normal communication between the messenger and the receptor in the cell, so that the chemical message

is not interpreted properly. The specific mechanisms by which EDCs disrupt the endocrine systems are very complex, and not yet completely understood. Endocrine disrupting chemicals can interfere with normal cellular functions by (Environment Canada, 2002):

- acting like a natural hormone and binding to a receptor. This causes a similar response by the cell, known as an agonist response.
- binding to a receptor and preventing a normal response, known as an antagonistic response
- interfering with the way natural hormones and receptors are synthesized or controlled

Since the endocrine system plays a critical role in normal growth, development and reproduction, even small disturbances in endocrine function may have profound and lasting effects. This is especially true during highly sensitive prenatal periods, such that small changes in endocrine status may have delayed consequences that are evident much later in adult life or in a subsequent generation. At least four major categories of adverse biological effects may be linked to exposure to EDCs: cancer, reproductive and developmental alterations, neurological and immunological effects. Endocrine systems that may be involved include the thyroid, adrenal, pituitary, and gonadal (Environment Canada, 2002).

The US Environmental Protection Agency considers EDCs a serious problem because of the potential global scope, the possibility of serious problems in humans and wildlife, and the persistence of some suspected EDCs in the environment. There is evidence that domestic animals

and wildlife have suffered adverse consequences from exposure to EDCs in the environment such as (Environment Canada, 2002):

- Deformities and embryo mortality in birds and fish caused by exposure to industrial chemicals and organochlorine insecticides
- Impaired reproduction and development in fish exposed to effluents from pulp and paper mills
- Abnormal reproduction in snails exposed to antifouling substances applied to the exteriors of ships
- Depressed thyroid and immune functions in fish-eating birds
- Feminization of fish near municipal effluent outlets

These problems have been identified primarily in species exposed to relatively high concentrations of organochlorine pesticides, PCBs, dioxins, as well as synthetic and plant-derived estrogens (U.S. Environmental Protection Agency, 2006). The potential for additive and /or synergistic effects from exposure to multiple EDCs is also a concern (Sumpter and Jobling, 1995). Whether similar effects are occurring in the general human population from exposures to ambient environmental concentrations is unknown. Reported increases in incidences of certain cancers (breast, testes, prostate) may be related to endocrine disruption (U.S. Environmental Protection Agency, 2006). Documented cases of adverse reproductive outcomes in individuals (or their offspring) exposed accidentally to high doses of EDCs reported effects such as (National Science and Technology Council, 1996):

- Shortened penises in offspring of women exposed to dioxin contaminated rice oil (Yucheng, China)
- Reduced sperm count in workers exposed to kepone at a pesticide factory (Hopewell, Virginia, USA)
- High ratio of female to male births for women who were pregnant and living near a pesticide plant when it exploded in 1976 (Seveso, Italy)

All the above incidences or cases are related to very high exposure levels to EDCs and are not directly relevant to municipal wastewater treatment processes.

1.2 ENDOCRINE DISRUPTORS IN THE ENVIRONMENT

Wastewater effluents and stormwater runoff are thought to be the major sources of EDCs discharged to the aquatic environment (Desbrow et al., 1998; Boyd et al., 2003; Environment Canada, 2002). There has been a growing concern about the presence of EDCs in the aquatic environment and studies have documented a wide variety of EDCs in surface waters (Sumpter and Jobling, 1995; Environment Canada, 2002).

Kolpin et al. (2002) found organic wastewater contaminants in 80% of 139 streams sampled in the US. The most frequently detected compounds were coprostanol (fecal steroid), cholesterol (plant and animal steroid), N,N-diethyltoluamide (insect repellant), caffeine (stimulant), triclosan (antimicrobial disinfectant), tri(2-chloroethyl)phosphate (fire retardant),

and 4-nonylphenol (nonionic detergent metabolite). All thirty-three of the suspected hormonally active compounds (EDCs) they analyzed for in the stream samples were detected with varying frequency and concentration (See Table 1-2).

Concentration levels of EDCs in nanograms per liter have been reported in WWTP effluent and river water (Kolpin et al., 2002; Desbrow et al., 1998; Fernandez et al., 2007). This is a concern, since laboratory studies have shown that some EDCs can be potent and exert estrogenic effects at concentrations as low as 1 ng/L in water (Routledge et al., 1998; Purdom et al., 1994). In the aquatic environment, exposure of organisms to EDCs has been linked to endocrine effects in male fish such as vitellogenin induction and feminized reproductive organs (Routledge et al., 1998; Purdom et al., 1994; Sumpter and Jobling, 1995).

Human estrogens are major causative substances in terms of estrogenic activity in municipal sewage and treated effluent as measured by yeast estrogen screening. Of the natural human estrogens, estrone (E1), 17β-estradiol (E2), and estriol (E3) are the primary contributors to estrogenic activity in domestic wastewater (Sun et al., 2013). In addition, the synthetic estrogen, 17α-ethinyl estradiol (EE2) is the predominant ingredient in birth control medication and is considered more estrogenically potent than the most estrogenically potent natural estrogen, E2 (Sun et al., 2013). A list of relative estrogenic potencies of these estrogens and their conjugates can be found in Appendix B.

Endocrine disruptors have a log k_{ow} range of 3.1–7 (Tan et al., 2008). This indicates that EDCs tend to be lipophilic and should mostly adsorb onto organic matter such as sludge. This is

supported by the detection of high concentrations of estrogens in water from sewage sludge dewatering processes (Matsui et al., 2000). It follows that soil may be contaminated with EDCs from land application of digested sludge and EDCs in runoff from these lands could reach the aquatic environment.

GuangGuo and Kookana (2005) studied the soil sorption of seven EDCs (E1, E2, E3, EE2, BPA, 4-t-OP, and 4-n-NP), biodegradation of five EDCs (E2, EE2, BPA, 4-t-OP, and 4-n-NP) and the biotransformation of E2 and E1 in loam soils. Their findings included:

1. Alkylphenols (4-t-OP and 4-n-NP) had the strongest sorption, followed by estrogens (EE2 > E2 > E1 > E3) and finally BPA. (Four soil types ranging from sandy to loam)
2. All five EDCs degraded rapidly under aerobic soil conditions (within 7 days)
3. Little or no degradation of EE2, E1, E3, and BPA under anaerobic soil conditions
4. Half-life of E2 under anaerobic soil conditions was 24 days
5. E2 was biotransformed to E1 under both anaerobic and aerobic conditions
6. The authors opined, while EDCs will likely degrade in aerobic soils within 7 days, they may persist in anaerobic soils, adversely affecting soil, groundwater and surface water quality.

A large number of organic wastewater compounds (82 out of 95) were detected at least once and one or more were found in 80% of 139 streams sites considered susceptible to contamination (e.g. high urbanization or agricultural influences) (Kolpin et al., 2002). Although median detectable concentrations of all target compounds were generally low (<1 μg/L) even

low-level exposure (<0.001 μg/L), to select hormones can illicit deleterious effects in aquatic species (Kolpin et al., 2002). The authors concluded that, "when toxicity is considered, measured concentrations of reproductive hormones may have greater implications for health of aquatic organisms than measured concentrations of non-prescription drugs".

Triclosan (TCS) is a polychlorinated aromatic antimicrobial used in many household products including soap, toothpaste and cosmetics. The United States Geological Survey (USGS) calls triclosan one of the top five microcontaminants in rivers. It is known to persist in the environment, bioaccumulate in fish and human milk, and is thought to be an endocrine disruptor (Zorrilla et al., 2009; Crofton et al., 2007) and cause cross-resistance to clinically important antibiotics (Yazdankhah et al., 2006).

Heidler and Halden (2005) studied an activated sludge wastewater treatment plant and found 98% removal efficiency for triclosan from the aqueous stream. However, a significant amount of the antimicrobial withstood aerobic and anaerobic degradation and accumulated in the sludge component. The concentration of triclosan found in digested sludge was four orders of magnitude higher than in the influent. A mass balance calculation showed a significant fraction of triclosan (~57%) partitions into and persists in biosolids. They concluded "the widespread land application of municipal biosolids as agricultural fertilizer represents an important, but currently underappreciated pathway for re-entry of triclosan into the environment".

1.3 TARGET COMPOUNDS

A chemical analysis was developed for this research project to target the following compounds; estrone (E1), 17β-estradiol (E2), 17α-ethinyl estradiol (EE2), estriol (E3) and testosterone (TT). Matsui et al. (2000) found E1 and E2 were the highest concentrations of estrogens in domestic wastewater and that E2 was responsible for 34% of the whole estrogenicity of raw sewage and almost 100% in the final effluent. Steroidal estrogens have the highest estrogenic activity of known EDCs in wastewater and estrone (E1), 17β-estradiol (E2), and 17α-ethinyl estradiol (EE2) are thought to be the priority EDCs to control in municipal wastewater treatment plants (Sun et al., 2013).

The natural estrogen, 17β-estradiol, and the most common estrogen in birth control formulations, 17α-ethinyl estradiol, are considered the most potent estrogens in municipal wastewater and sludge treatment processes. Yeast estrogen screening assays use E2 as the primary standard to measure estrogenic activities. Most of the estrogenic activity in municipal wastewaters, as measured by yeast estrogen screening, is thought to be due to E1 and E2 concentrations (Sun et al., 2013).

The natural estrogens, estrone, 17β-estradiol, and estriol, are generally quickly and well absorbed from the gastrointestinal tract, with little difference in uptake between estrone, estradiol and estriol. They are inactivated by the liver. Few studies have addressed the accumulation and storage of estradiol, estrone and estriol after exogenous administration. All three are distributed to various target and non- target organs through the systemic circulation, but are also produced locally and accumulate in target tissues particularly rich in fat (National Center for

Biotechnology Information, CID=5870). Estrogens and their metabolites are excreted mainly in urine; however, small amounts are also present in feces.

Table 1-1 lists the physical properties important to the environmental fate of the targeted compounds in the developed chemical analysis; estrone, 17β-estradiol, 17α-ethinyl estradiol (EE2), estriol and testosterone (TT). Hydrolysis is not expected to be an important environmental fate process since these compounds lack functional groups that hydrolyze under environmental conditions (National Center for Biotechnology Information CID=5991). A laboratory study by Ying and Kookana (2005) showed these four estrogens were degraded rapidly in the soil, within 7 days, under aerobic conditions and suggested they would not persist in well-aerated soils. However, under anaerobic conditions in the soil, little or no degradation was noted and the authors opined estrogens persisting in anaerobic soils may affect soil and groundwater quality and the ecosystem.

If released into water, estrone is expected to adsorb to suspended solids and sediment and volatilization from moist soil or water surfaces is not expected to be an important fate process. The potential for bioconcentration in aquatic organisms is moderate (National Center for Biotechnology Information, CID=5870). Sorption of estrone is dependent on soil organic carbon content (Casey et al., 2005; Ying and Kookana, 2005; National Center for Biotechnology Information, CID=5870). C14-labeled estrone, present in soil at 0.1 mg/kg, reached 2.0–17.4% mineralization in 21 days using natural soils, indicating that biodegradation may not be an important environmental fate process in soil (National Center for Biotechnology Information, CID=5870).

Table 1-1: Physical properties of target compounds, estrone, 17β-estradiol, 17α-ethinyl estradiol, estriol, and testosterone[1]

Hormone	**Molecular weight and formula**	**Estrogenic activity potency factor**	**Soil organic carbon-water partitioning coefficient (log K_{oc})**	**Octanol-water partitioning coefficient (log K_{ow})**	**Topological polar surface area (polarity)**	**Solubility in water (mg/L)**	**Bio-concentration factor (BCF)**	**Henry's Law Constant (atm-m^3/mole)**
Estrone (E1)	270.36608 $C_{18}H_{22}O_2$	0.135	3.69 [(2)]	3.13	37.3	30 @ 25 °C	54 Moderate	3.8 X 10^{-10}
17β-estradiol (E2)	272.38196 $C_{18}H_{24}O_2$	1.0	3.52 [(2)]	4.01	40.46	3.90 @ 27 °C	200 High	3.6 X 10^{-11}
17α-ethinyl estradiol (EE2)	296.40336 $C_{20}H_{24}O_2$	1.36	3.28 [(2)]	3.67	40.5	11.3 @ 27 °C	110 high	7.9 X 10^{-12}
Estriol (E3)	288.38136 $C_{18}H_{24}O_3$	2.8 X 10^{-11}	3.68 [(2)]	2.45	60.7	27.3 @ 25°C	19 and 50 Low to moderate	1.3 X 10^{-12}
Testosterone (TT)	288.42442 $C_{19}H_{28}O_2$	1.32 X 10^{-5}	3.34	3.32	37.3	23.4 @ 25°C	72 Moderate	3.5 X 10^{-9}

[1]Source (unless otherwise stated): Hazardous Substances Data Bank, ToxNet: Toxicology Data Network, U.S. National Library of Medicine
[2] Ying et al., 2002

17β-estradiol is the most potent form of mammalian estrogenic steroids having the greatest physiological activity of any naturally occurring estrogen. In humans, it is produced primarily by cyclic ovaries, placenta, and the adipose tissue of men and postmenopausal women. If released to soil, 17β-estradiol will have almost no mobility and volatilization from moist soil and water surfaces is not expected to be an important fate process (National Center for Biotechnology Information, CID=5757). Ying and Kookana (2005) noted very slow degradation of E2 in soils under anaerobic conditions and calculated a half-life of 24 days. However, degradation of E2 was expected to occur quite rapidly (less than 7 days) in soils under aerobic conditions. Estradiol was found to be biotransformed to E1 under both aerobic and anaerobic conditions (Ying and Kookana, 2006). Potential for bioconcentration in aquatic organisms is high, provided the compound is not metabolized by organisms. Monitoring data indicate that the general population may be exposed to estradiol at well below the therapeutic dose via ingestion of drinking water and dermal contact with contaminated sediments (National Center for Biotechnology Information, CID=5757). The 17-alpha-isomer of estradiol binds weakly to estrogen receptors and exhibits little estrogenic activity in estrogen-responsive tissues and is considered relatively inactive.

17α-ethinyl estradiol has high estrogenic potency when administered orally and is often used as the estrogenic component in oral contraceptives. If released to soil, 17α-ethinyl estradiol is expected to have low mobility and volatilization from moist soil surfaces not expected to be an important fate. 17α-ethinyl estradiol has been classified as not readily biodegradable using a sewage inoculum, indicating that biodegradation may not be an important environmental fate process. While little to no degradation was observed under anaerobic conditions, 17α-ethinyl

estradiol was degraded rapidly in soils within 7 days under aerobic conditions (Ying and Kookana, 2005). If released into water, 17α-ethinyl estradiol is adsorbed to suspended solids and sediment and volatilization from water surfaces is not expected to be an important fate process. Potential for bioconcentration in aquatic organisms is high, provided the compound is not metabolized by organisms. (National Center for Biotechnology Information, CID=5991).

Production and use of estriol in human and veterinary medicine may result in its release to the environment through various waste streams. It is usually the predominant estrogenic metabolite found in urine. If released to soil, estriol is expected to have moderate mobility. Volatilization from moist soil, dry soil or water surfaces is not expected to be an important fate process. If released into water, estriol is adsorbed to suspended solids and sediment. Volatilization from water surfaces is not expected to be an important fate process based upon this compound's estimated Henry's Law constant (Table 1-1). Estimated bioconcentration factor (BCF) values of 19 and 50 suggest the potential for bioconcentration in aquatic organisms is low to moderate. (National Center for Biotechnology Information, CID=5756).

Testosterone is a principal hormone of the testes and its production and use as a male hormone, steroid and a performance enhancement drug in athletes may result in its release to the environment through various waste streams. If released to soil, testosterone is expected to have slight mobility. Volatilization from water and moist or dry soil surfaces is not expected to be an important fate process. A sewage treatment plant removed 58–65% of testosterone from the influent, with 95% removal reported for the aqueous phase of treatment suggesting biodegradation may be an important environmental fate process (National Center for

Biotechnology Information, CID=6013). If released into water, testosterone is expected to adsorb to suspended solids and sediment. The potential for bioconcentration in aquatic organisms is moderate.

1.4 OCCURRENCE AND WWTP REMOVAL

Women can excrete around 7-8 μg of estrone; 2.4-3.0 μg of 17β-estradiol; and 4.6-4.8 μg of estriol in 1-2 L urine/day (Adlercreutz et al., 1986; Matsui et al., 2000) and a greater quantity are excreted in an inactive form such as glucuronide and sulfate conjugates (Matsui et al., 2000). In addition, 0.5 μg of estrone, 0.4 μg of 17β-estradiol and 1.25 μg of estriol are excreted in the feces per day (Adlercreutz et al., 1994). While men can excrete these estrogens in similar ratios, they are excreted in smaller quantities (Matsui et al., 2000). Debrow (1998) isolated 17β-estradiol (range 1–48 ng/L: mean 11 ng/L) and estrone (1–76 ng/L: mean 17 ng/L) and 17α-ethinyl estradiol (range 0–7 ng/L: mean 0.6 ng/L) in effluents from seven sewage-treatment works (STW). Although the concentration of 17α-ethinyl estradiol (EE2) was generally below the limit of detection, it was positively identified in three of the effluent samples. These authors note the ratio of the levels of estrone to estradiol reported in urine (3.5 parts E1:1 part E2) is fairly similar to the ratio they observed in the effluent (1.5 parts E1:1 part E2).

Natural steroids in the urine are primarily inactive glucuronide or sulfated conjugates while those in feces occur mainly as unconjugated forms (Adlercreutz and Järvenpää, 1982). In the gut flora, *Escherichia coli* produces high levels of the enzyme β-glucuronidase that quantitatively hydrolyze different classes of steroid glucuronides at a very high rate (Dray et al.,

1972). Since feces have high levels of *E. coli*, WWTPs would also foster a large population of bacteria capable of deconjugating estrogens at a rapid rate, during the sewage-treatment process.

Conjugated estrogens are excreted and may be cleaved during wastewater treatment, into the more estrogenically potent unconjugated forms, resulting in an initial increase in estrogenicity. Adler et al. (2001) showed that approximately 50% of the estrogens in the WWTP influent were conjugated estrogens. Gomes et al. (2005) examined the relationship between free and conjugated forms of estrone, estriol and 17α-ethinyl estradiol in raw municipal sewage and the final WWTP effluent. The free forms of estrone, 17β-estradiol, and estriol were detected in both raw and final effluents. However, estrone-3 sulphate was the only conjugate detected in the effluent due to the recalcitrant nature of the sulphate moiety (Gomes et al., 2005). Since conjugated estrogens can be quite persistent in the WWTP, transformation of the conjugated estrogen to the free estrogen may occur too late in the treatment process for degradation of the unconjugated estrogen to take place. Fernandez et al. (2009) analyzed sludge extracts only for free steroidal estrogens, stating their conjugates were too polar to be adsorbed by sludge. Studies often don't examine wastewater and sludges for both free estrogens and their conjugates when evaluating removal efficiencies in wastewater treatment plants. Hence, an information gap exists, and the importance of conjugated forms is not yet clear.

Researchers have reported wide ranging EE2 concentrations in domestic wastewater treatment plants for wastewater (Tabak et al., 1981; Debrow et al., 1998; Terns et al., 1999; Servos et al., 2005; Ifelebuegu, 2011; and Sim et al, 2011) and sludge (Ternes et al., 2002; Ifelebuegu, 2011; Sim et al, 2011) (Table 1-3). No EE2, either in the free or conjugated forms

was observed in any of the influent or effluent wastewater samples in a study by Gomes et al.(2005). While EE2 remains the predominant ingredient in birth control pills overall, combinations of progesterone and estrone as well as dosage influence this rapidly changing and competitive market. Birth control prescribing practices have changed over the years and may vary greatly geographically. Combination treatments vary ingredients and low dosage administration (e.g. multiphase, low estrogen and progesterone-only pills; intrauterine devices; and dermal implants) can impact the uptake and elimination rates in the body. This can directly impact the occurrence of EE2 in domestic wastewaters.

17β-estradiol may be removed through microbial degradation in activated sludge and biofilm systems with estrone as the primary biodegradation intermediate of E2. The frequent detection of phylogenetically diverse E2 degrading bacteria in engineered systems suggests that E2 degrading bacteria might be enriched in engineered water /wastewater treatment systems and a fraction of these are capable of degrading E1 (Li, 2011).

Nitrification during wastewater treatment processes may play a significant role in removing estrogens from wastewater. Moschet and Hollender (2009) have summarised research studies that determined the half-lives of E1, E2 and EE2 degradation in aerobic and denitrifying tanks in wastewater treatment plants. While it is well established that nitrifying sludge and ammonia oxidizing bacteria can degrade 17β-estradiol (E2), estrone (E1), 17α-ethinyl estradiol (EE2), and estriol (E3) (Li, 2011), little is known of the individual species responsible for biodegradation of these estrogens. Yi and Harper (2007) investigated nitrification and biotransformation of 17α-ethinylestradiol (EE_2) using enriched cultures of autotrophic ammonia-

oxidizers and concluded EE2 biotransformation can be co-metabolically mediated under WWTP operating conditions that allow for enrichment of nitrifiers.

Heterotrophic bacteria populations can be selectively enriched by adjusting operating conditions such as food-to-mass ratio, dissolved oxygen content and solids retention time. Zeils et al. (2014) found selectivity of the microbial population determines the biodegradation kinetics of EE2 and the first-order biodegradation kinetics imply improved removals in reactors with staged or plug-flow designs. The same study reports estrogen removal efficacies were directly correlated with the influent concentration.

The synthetic estrogen, EE2 is much more persistent than E2 in the WWTP because of the ethinyl group at the C-17 which hinders the oxidation of the hydroxyl group (Moschet and Hollender, 2009). However, Ren et al., (2006 and 2007c) reported that *Sphingobacterium sp. JCR5* can degrade EE2 in the wastewater treatment plant under aerobic conditions. The same studies reported degradation of E1, E2, and E3 in wastewater treatment by *Sphingobacterium sp. JCR5.* Yoshimoto et al. (2004) identified four strains of Rhodococcus which specifically degraded estrogens: 17β-estradiol, estrone, estriol, and 17α-ethinyl estradiol. These strains also reduced 17β-estradiol to 1/100 of the estrogenic activity (measured with human breast cancer-derived MVLN cells) within 24 hours, suggesting these strains degrade 17β-estradiol into substances without estrogenic activity.

Fractionation of effluents from wastewater treatment plants revealed natural estrogens 17β-estradiol (E2), estrone (E1) and the synthetic estrogen 17α-ethinyl estradiol (EE2) used in

oral contraceptives as the compounds mainly responsible for the estrogenic activity measured in recombinant yeast assays (Nakada et al., 2004; Desbrow et al., 1998). Based on the concentration and relative potency, Nakada (2004) found the natural estrogens E1 and E2 represented more than 98% of the total 17β-estradiol equivalent concentration (EEQ) in STP effluent, while the contribution of phenolic compounds to total EEQ was less than 2%. When preparatory column elutions were fractionated, E1 and E2 represented 66 to 88% of the total estrogenic activities estimated from the bioassay data. Nakada (2004) concluded E1 and E2 were the dominant environmental estrogens in the STP effluent, but a significant contribution to estrogenic activities stems from unidentified components in the effluents.

Ultra-trace analysis was used in three separate studies to determine the concentration of ten endocrine disrupting chemicals and testosterone in wastewater (influent and effluent) and streams (Table 1-2). While nonylphenol and bisphenyl A were frequently detected in wastewater effluent in high concentrations, the more estrogenically potent E1, E2 and EE2 would have posed more of a risk to the receiving environment. Testosterone was rarely present in wastewater samples and in low concentrations when it was detected in effluent. In addition, 33 of the 95 target organic wastewater compounds were known or suspected to exhibit at least weak hormonal activity with the potential to disrupt normal endocrine function and the maximum total concentration of hormonally active compounds was 57.3 μg/L (Kolpin et al., 2002).

Table 1-2: Ultra-trace analysis concentration and detection frequency of endocrine disrupting chemicals in streams and municipal wastewater treatment plant influent and effluent

Chemical	**Average method detection limit (ng/L)[1]**	**Range of method detection limits (ng/L)[1]**	**Detection frequency (percent of wastewater samples)**	**Range (average) in wastewater influent (ng/L)[2]**	**Range (average) in wastewater effluent (ng/L)[2]**	**Detection frequency in streams (percent of stream samples)[3]**	**Median (maximum) in streams (ng/L)[3]**
Bisphenol A	2.1	1.7–2.4	100	0–590 (265)	11–054 (865)	41.2	0.14 (12)
17α-Ethinylestradiol	7.1	6.1–9.0	26	0–2 (0)	0–178 (12)	15.7	0.073 (0.831)
17α-Estradiol	6.9	4.5–11	33	0–1	0–38 (3.8)	5.7	0.03 (0.074)
17β-Estradiol	7.1	1.6–12	80	0–10 (3)	0–158 (20)	10.6	0.16 (0.2)
Estriol	1.5	1.1–2.3	46	0–22 (2)	0–29 (4)	21.4	0.019 (0.051)
Estrone	7.6	5.0–11	67	0–33 (13)	0–147 (24)	7.1	0.027 (0.112)
d-Equilenin	17	4.1–31	15	0–1 (0)	0–13 (2)		
Equilin	18	9.3–28	4	0	0–207 (7)	1.4	0.147 (0.147)
(-)-Norgestrel	84	74–98	5	0–48 (2)	0–126 (6)		
Nonylphenol	172	115–219	100	2553–41,207 (14,630)	1592–90,043 (9975)	50.6	0.8 (40)
Testosterone	33	22–41	6	0–95 (27)	0–21 (1)	2.8	0.116 (0.214)

1. Ikonomou et al. (2008)
2. Fernandez et al. (2007)
3. Kolpin et al. (2002)

Kolpin et al. (2002) recommend research on the toxicity of organic wastewater contaminants include not only the individual chemicals, but also mixtures of these compounds, as select chemical combinations can exhibit additive or synergistic toxic effects. This echoes the recommendations of environmental protection agencies in Canada and United States to use both chemical analysis and bioassays to determine the presence of endocrine disruptors (Environmental Protection Agency, 2013; Servos et al., 2001).

Matsui et al. (2000) found E1 and E2 were the highest concentrations of estrogens in domestic wastewater and that E2 was responsible for 34% of the whole estrogenicity of raw sewage and almost 100% in the final effluent. Steroidal estrogens have the highest estrogenic activity of known EDCs in wastewater and estrone (E1), 17β-estradiol (E2), and 17α-ethinyl estradiol (EE2) are thought to be the priority EDCs to control in municipal wastewater treatment plants (Sun et al., 2013).

Ying et al. (2009) surveyed the occurrence of estrogens (estrone, E1; 17β-estradiol, E2; 17α-ethinyl estradiol, EE2) in effluents from five wastewater treatment plants and their receiving waters using both chemical analysis and bioassays. The estrogen levels in WWTP effluent varied from 9.12 to 32.22 ng/L for E1, from 1.37 ng/L to 6.35 ng/L for E2 and from 0.11 ng/L to 1.20 ng/L for EE2. No significant differences ($p < 0.05$) in the concentrations of the selected estrogenic compounds were found for the effluents from the five sewage treatment plants. The estrogens were found in the receiving waters at lower concentrations due to dilution of effluents in the rivers. They calculated in vitro EEQ values (estrogen equivalents) in the receiving river waters downstream of the effluent discharge points ranged from 1.32 to 11.79 ng/L, while the in

vivo EEQ values (vitellogenin response in rainbow trout) ranged from 2.48 to 21.18 ng/L. The three estrogens (E1, E2, EE2) accounted for the majority of the EEQ in the water samples (Ying et al., 2009).

Ying et al. (2008) investigated the fate and removal of E1, E2 and EE2 in four South Australian sewage treatment plants with differing treatment technologies. The concentrations in the effluent from the two-year survey were similar to those reported in other studies. Estrone had the highest concentrations among the three estrogens, ranging between 13.3 and 39.3 ng/L, whereas the concentrations for E2 and EE2 varied between 1.0 and 4.2 ng/L and between 0.1 and 1.3 ng/L, respectively. While removal of E2 ranged between 47 and 68% at the four plants, both E1 and EE2 were more persistent during treatment (Ying et al., 2008).

A survey of 18 Canadian wastewater treatment plants (Servos et al., 2005) found mean concentrations of 17β-estradiol and estrone in the influent was 15.6 ng/L (range 2.4–26 ng/L) and 49 ng/L (range 19–78 ng/L). Secondary treatment (not including trickling filter/solids contact) reduced 17β-estradiol by 75–98% with mean concentrations in the final effluent of 1.8 ng/L (range 0.2–14.7 ng/L) (see Table 1-3). Removal of estrone by secondary treatment was more complex with final effluent concentrations ranging from 1–96 ng/L with a mean of 14.0 ng/L. Removal of estrogenic activity, measured as percent Yeast Estrogen Screen response, was equally complex with a mean of 79% (range ND–145%) in influent and a mean of 50% (range ND–160%) in the final effluent. The addition of advanced treatment (filtration or phosphorous removal) also did not have an apparent effect on increasing the removal of estrogens in the Canadian plants studied.

Drewes et al. (2005) used the E-screen assay to assess estrogenic activity in a study of seven secondary wastewater treatment plants across the U.S.. They found E2, E1, E3, and TT were prevalent in all primary effluent samples in concentrations greater than 7, 26, 138, and 19 ng/L, respectively. Estrone, the metabolite of E2, exhibited the highest concentration of all steroidal hormones targeted in secondary treated effluents. During secondary treatment, the total estrogenic activity was reduced by 96%; average removal efficiencies of E2/E3, testosterone and estrone were 98%, 95%, and 85%, respectively (Drewes et al., 2005).

Servos et al. (2005) examined estrogen removal in a tricking filter / solids contact domestic wastewater treatment plant and a primary treatment plant in Canada and found both treatment systems were ineffective at removing estrogens or estrogenic activity. In fact, they reported the trickling filter / solids contact (n = 2) and primary treatment plants (n = 3) had a mean percent increase in 17β-estradiol (18.5% and 1.0%), estrone (62.4% and 28.6%) and YES response (62% and 10%), respectively. However, Ternes et al. (1999), in a more detailed examination (n = 6 composite daily samples) of a tricking filter / solids contact domestic wastewater treatment plant in Brazil, determined this system to be effective for percent removal of E1 (67%), E2 (92%) and EE2 (64%) from the wastewater stream.

Table 1-3 summarizes a selection of studies examining the concentrations of estrogens E1, E2, EE2 and/or estrogenic activity in sludge and wastewater samples with high solids content. Influent, activated sludge, mixed sludge, digested sludges and even primary treatment effluent have higher solids content than effluent from secondary wastewater treatment plants. Many studies have determined estrogen concentrations in effluent samples without examining

influent or sludge samples. This is likely due to the difficulty in conducting laboratory analysis for estrogens in samples with high solids content.

Table 1-3: Occurrence of estrogens and estrogenic activity during sludge digestion and domestic wastewater treatment by activated sludge (AS) or tricking filter / solids contact (TF/SC)

Media	**Target compound**	**Concentration range**	**Reference**
Effluent	E1 E2 EE2	ND–70 ng/L (LOD = 1 ng/L) ND–64 ng/L (LOD = 1 ng/L) ND–42 ng/L (LOD = 1 ng/L)	Ternes et al. (1999)
Activated sludge	E1 E2 EE2	ND–37 ng/g (LOQ = 2 ng/g) 5–17 ng/g ND–4 ng/g (LOQ = 2 ng/g)	Ternes et al. (2002)
Digested sludge	E1 E2 EE2	ND–16 ng/g 9–49 ng/g 2–17 ng/g	Ternes et al. (2002)
Influent	E1 E2 EEQ	19–78 ng/L (mean 49 ng/L) 2.4–26 ng/L (mean 15.6 ng/L) ND–145 ng/L (mean 79 ng/L)	Servos et al. (2005)
Secondary, tertiary, and lagoon effluent	E1 E2 EEQ	1–6 ng/L (mean 17 ng/L) 0.2–14.7 ng/L (mean 1.8 ng/L) ND–106.0 ng/L (mean 50.1 ng/L)	Servos et al. (2005)
Influent	EEQ	1–185 ng/L	Fernandez et al. (2009) (2007)
Secondary effluent	EEQ	1–23 ng/L (AS) 1–191 ng/L (TF/SC)	Fernandez et al. (2009) (2007)
Digested sludge (anaerobic RT=1 month)	E1 E2 E3	0.056±0.04 ng/g 0.155±0.06 ng/g N/A	Fernandez et al. (2009)
Influent	E1 E2 E3 TT	0–71 ng/L 0–10 ng/L 0–171 ng/L 0–95 ng/L	Fernandez et al. (2009) (2007)
Secondary effluent	E1 E2 E3 TT	0–18 ng/L (AS); 0–143 ng/L (TF/SC) ND (AS); 0–25 ng/L (TF/SC) ND (AS); 0–8 ng/L (TF/SC) ND (AS); 0–1 (TF/SC)	Fernandez et al. (2009) (2007)
Influent	E1 E2 EE2	64.5 (± 18.7)–119.3 (± 30.0) ng/L 15.7 (± 4.7)–82.6 (± 23.4) ng/L ND–1.5 (± 1.1) ng/L	Ifelebuegu (2011)
Waste activated sludge	E1 E2 EE2	88.1 (± 16.2)–140.3 (± 28.2) ng/g 23.1 (± 7.5)–79.6 (± 18.1) ng/g 0.5 (± 0.2)–1.8 (± 0.7) ng/g	Ifelebuegu (2011)
Primary sedimentation	E1 E2 EE2	70.1 (± 6.7)–121.1 (± 14.1) ng/L 20.7 (± 5.9)–72.6 (± 19.4) ng/L 0.4 (± 0.2)–1.3 (± 0.4) ng/L	Ifelebuegu (2011)

Media	Target compound	Concentration range	Reference
Secondary effluents (five various treatments)	E1	8.6 (± 1.2)–9.1 (± 18.9) ng/L (E1 removal = 21–24%)	Ifelebuegu (2011)
	E2	0.9 (± 0.6)–20.9 (± 4.3) ng/L (E2 removal = 18–32%)	
	EE2	0.3 (± 0.2)–0.8 (± 0.3) ng/L (EE2 removal = 10–15%)	
Primary sludge	E1	87.94 (± 5.3)–117.86 (± 11.2) ng/g	Ifelebuegu (2011)
	E2	3.76 (± 0.7)–15.65 (± 2.4) ng/g	
	EE2	ND–1.89 (± 0.4) ng/g	
Digested sludge (anaerobic, mesophilic, 12.5–13 d SRT)	E1	67.11 (± 5.8)–80.54 (± 8.7) ng/g (E1 removal = 21–24%)	Ifelebuegu (2011)
	E2	2.55 (± 0.2)–7.08 (± 1.1) ng/g (E2 removal = 18–32%)	
	EE2	1.48 (± 0.1)–61 (± 0.3) ng/g (EE2 removal = 10–15%)	
Primary effluent	E1	26.3–80.3 ng/L	Drewes et al. (2005)
	E2	7.0–24.5 ng/L	
	EE2	< 0.7–14.4 ng/L	
	E3	138–381 ng/L	
	TT	19.4–143 ng/L	
Secondary effluent	E1	< 1–50.4 ng/L	Drewes et al. (2005)
	E2	< 1–6 ng/L	
	EE2	< 0.7–4.1 ng/L	
	E3	< 2–4.9 ng/L	
	TT	< 1–4.9 ng/L	
Primary effluent	EEQ (E-screen)	17.4–94.7 ng/L	Drewes et al. (2005)
Secondary effluent	EEQ (E-screen)	0.18–7.91 ng/L	Drewes et al. (2005)
Influent	E1	ND–52 ng/L	Sim et al. (2011)
	E2	ND–17 ng/L	
	EE2	ND	
	E3	46–1130 ng/L	
Effluent	E1	ND–79 ng/L	Sim et al. (2011)
	E2	ND	
	EE2	ND	
	E3	ND–273 ng/L	
Digested sludge (dewatered)	E1	ND–351 ng/g	Sim et al. (2011)
	E2	ND–202 ng/g	
	EE2	ND	
	E3	ND–79.8 µg/g	

Ternes et al. (1999) found activated sludge treatment to be very effective at removing E1 (83%), E2 (99.9%) and EE2 (78%). They also demonstrated that an increase in solids retention time of 6 days to 11 days in an activated sludge system, improved removal for E2 and E1 by 21% and 30%, respectively. In addition, a higher MLSS could increase estrogen partitioning and biodegradation, resulting in increased removal. Khanal (2006) reported that estrogen removal increased significantly when MLSS was increased from 1000 to 10,000 mg/L. They suggested a higher MLSS could increase the estrogen partition coefficient and biodegradation constant and result in increased removal. Therefore, if the reduction of estrogens, estrogenic activity and androgenic activity is to be considered, when designing an activated sludge wastewater treatment, the effects of increasing mixed liquor suspended solids (MLSS) and retention time should be examined.

Holbrook et al. (2002) observed a correlation between the estrogenicity (YES assay) of mixed liquor suspended solids and aerobic sludge age and suggested wastewater treatment facilities can be designed and operated to enhance the sorption and removal of estrogenic compounds from the liquid phase. If the wastewater treatment system is to be redesigned and the reduction of estrogens, estrogenic activity and androgenic activity is considered, the effects of increasing mixed liquor suspended solids (MLSS) and retention time should be examined. Overall, secondary wastewater treatment plants can be very effective at removing estrogens and estrogenic activity. Activated sludge treatment with nutrient removal had high removal rates and increased retention times appeared to increase estrogen removal (Terns et al., 1999; Servos et al. 2005).

Final treatment of effluent with chlorine or ozone, just prior to discharge from the wastewater treatment plant, may affect estrogenic activity levels. Alum et al. (2004) found ozone and chlorine to have comparable residual potential estrogenic values for equivalent molar dosages with 99% loss of the parent compounds, BPA, E2, and EE2. They anticipated a 99% transformation in less than two seconds with an ozone concentration of 30 M. Transformation reached a stabilized estrogenic level in 10 min for ozone, but took more than 120 min for chlorination. Potential estrogenic value was measured using E-screen (human breast cancer cell line MCF-7). A residual estrogenic response may be present after chlorination and ozonation due to oxidation by-products. Both chlorination and ozonation removed 75% to 99% of the test EDCs in distilled water. Increasing contact time and chlorination dose improved EDC removal. Oxidative treatments may be an effective method to reduce estrogens and further research into practical application is required

Li (2011) reported concentration profiles of E2 in aqueous, solid and mixed liquor revealed removal was achieved by sorption onto solid and subsequent biodegradation by the microorganisms. In addition, Li (2011) found adsorption to solids was mildly competitive between E1 and E2. Primary and mixed sludges are particularly difficult to process for laboratory analysis of estrogens due to the high solids content and variable texture / composition. Although Ternes et al. (2002) and Sim et al. (2011) reported estrogen concentrations in digested sludge, Ifelebuegu (2011) was one of the few researchers to compare estrogen concentrations in both primary and digested sludges from municipal wastewater treatment plants (Table 1-3).

Ifelebuegu (2011) found wastewater removal efficiencies of E1, E2 and EE2 ranged from 41 % to 100 % and were dependent on the type of wastewater treatment system. Removal efficacies for the wastewater treatment systems examined from most to least effective were: activated sludge with biological nutrient removal > activated sludge > oxidative ditch > biological filtration > rotating biological contact. Primary mechanisms of removal from the wastewater stream were biodegradation and sorption unto sludge biomass (Ifelebuegu, 2011).

Adsorption to sewage sludge is expected based on the distribution coefficients for estrogens, and is considered an important removal pathway in municipal wastewater treatment plants, but it does not eliminate estrogens. The extent to which estrogen adsorbs to sludge is unknown. While several studies have < 10% of the total estrogen is adsorbed to sludge during wastewater treatment (Anderson et al., 2003; Janex-Habibi et al, 2009; Joss et al, 2004; Muller et al, 2008), other research has shown that sludge has a high capacity to adsorb estrogen (Clara et al., 2004; Suzuki and Maruyama, 2006; Ren et al, 2007). Janex-Habibi et al., (2009) found only 10% of the estrogens were adsorbed to the solid phase while Suzuki and Maruyama, (2006) showed 30% adsorbed to the solid phase. Also, other authors expect that estrogens will show a preference for partitioning to the solid phase based on the Kow partition coefficients (Gomes et al., 2004; Khanal et al., 2006).

Endocrine disruptors have demonstrated a high affinity to anaerobically digested sludge. For example, Ivashechkin (2004) demonstrated 75% of BPA was sorbed onto sludge. However, BPA (pKa = 10.3) was desorbed from sludge when the pH was raised to 12.4 (Ivashechkin et al., 2004). The same desorption pattern is expected in phenolic EDCs with a similar pKa

(nonylphenol, 17β-estradiol, estriol, estrone, 17α-ethinyl estradiol). Therefore, the practice of alkaline stabilization of dewatered sludge increases the risk of environmental contamination for land applications. If the pH is raised prior to dewatering, much of the EDC content should be removed with the supernatant; this potentially could be treated before being discharged to the environment. The degree of EDC removal during the sludge dewatering processes is unknown and may not be adequate even at pH > 13. Hence, it is important to investigate sludge pre-treatment processes in terms of EDC removal efficiencies.

Anaerobic digestion is one of the most common processes for sludge stabilization in wastewater treatment plants. Estrone can be converted to 17β-estradiol during anaerobic digestion (de Mes et al., 2008). Under laboratory conditions, decrease of total estrogen concentrations (total E1 and E2) during anaerobic digestion was not observed by Sarkar (2013) or de Mes et al. (2008). However, the ratio of E1 to the more potently estrogenic E2 did vary with anaerobic digestion.

Limited research has been performed on the fate of E1, E2 and EE2 under anaerobic conditions and reported results are contradictory. Ifelebuegu (2011) targeted endocrine disrupting chemicals that included E1, E2, EE2 and found they persisted in the anaerobic sludge digestion process with percentage removals ranging from 10% to 48 %. The authors suggested these compounds may persist in the environment under anoxic/anaerobic conditions. Carballa et al. (2006) revealed around 85% removal of E1, E2 and EE2 in a continuous sludge digestion experiment under both mesophilic and thermophilic conditions, whereas Czajka and Londry

(2006) did not observe any reduction of the sum of E1, E2 and EE2 in an experiment with a duration of three years with sludge and sediments under anaerobic conditions.

Nonylphenol (NP) is commonly found in sewage sludge. It is not removed with anaerobic sludge digestion and its estrogenic activity is not reduced (Hernandez-Raquet et al., 2007). The same study showed estrogenic potency and NP was greatly reduced in aerobic sludge digestion (90% and 100%) and aerobic post-treatment of anaerobically pre-digested sludge (91% and 98%), respectively.

The estrogenic activity of the sludge measured by YES bioassay has been observed to increase during anaerobic digestion due to reduction of E1 to more estrogenic E2 (Sarkar, 2013). No estriol (E3) was detected in the sludge during anaerobic digestion and most of E1 and E2 partitioned onto the solid phase and remained there during digestion (Sarkar, 2013). Holbrook et al. (2002) also found that estrogenic activity, as measured by YES assay, almost doubled during mesophilic aerobic and anaerobic digestion, but concluded 51–67% of the estrogenic activity contained in the influent wastewater was either biodegraded during the wastewater or sludge treatment processes or was unavailable to the extraction/detection procedure.

de Mes et al. (2008) reported no substantial decline in the total of E1 and E2 observed over 205 days of anaerobic sludge digestion in pilot scale batch experiments. On the other hand, Carballa et al. (2006) found that more than 85% of estrogens (E1 and E2) can be removed using either mesophilic (37 °C) or thermophilic (55 °C) anaerobic digestion in pilot plants fed mixed sludge collected from a municipal wastewater treatment plant. These studies indicate that there

are conflicting results for anaerobic digestion. Due to these contradictory results, there is a need for further research in this particular field.

Primary mechanisms of removal from the wastewater stream are thought to be biodegradation and sorption unto sludge biomass. In light of an increasing trend for land application of biosolids, as opposed to landfilling, it will become even more important to monitor estrogens in wastewater sludge.

1.5 CHEMICAL ANALYSIS

Analysis of estrogenic compounds in aqueous samples is well documented in the literature, unlike analysis in solid samples (e.g. sediments and sludge). Aqueous samples are mostly analyzed by LC-MS/MS or GC-MS/MS, while, for solid samples, GC-MS or GC-MS/MS are generally used. Limits of detection (LODs) are under 1 ng/L for river waters, in the range of 1 ng/L for wastewaters, and 0.2–5 ng/g for sediments and sludge (Gabet et al., 2007).

High solids in wastewater samples greatly increase the complexity of extraction and clean up procedures in analytical procedures to detect estrogens. In terms of increasing solids content and analytical complexity: Effluent < Influent < activated sludge < digested sludge < mixed sludge. A selection of published analytical protocols for detecting estrogens in municipal sludges and wastewater with high solids content are listed in Table 1-4.

Table 1-4: Analysis methods for detection of estrogens [estrone (E1), 17β-estradiol (E2), 17α-ethinyl estradiol (EE2), and /or estriol (E3)], testosterone and estrogenic activity in influent (Inf), effluent (Eff), activated sludge (AS), primary sludge (PS), and digested sludge (DS) media

Reference	**Media**	**Target compounds**	**Sample size**	**LOQ**	**Recovery**	**Extraction**	**Clean up**	**Detection**
Ternes et.al. (2002)	AS, DS	E1, E2, EE2	0.5 g	2 ng/g	> 70%	Freeze dry SE	GPC Silica	GC-MS/MS
Fernandez et al. (2009)	DS	E1, E2, EE2, E3	0.2 g dry wt (2 g wet wt)		> 80%	ASE	Florosil	HPLC-MS/MS Yeast strain BY4741
Fernandez et al. (2009)	Inf, Eff,	E1, E2, EE2, E3	50 ml, 100 ml		> 80%	2.8 um filter MeOH rinsed	Florosil (Inf only) SPE Oasis HLB	HPLC-MS/MS Yeast strain BY4741
Ifelebuegu (2011)	Inf Eff	E1, E2, EE2	Not reported	Not reported		Solvent extraction	GPC SPE	LC – MS/MS
Ifelebuegu (2011)	DS PS	E1, E2, EE2	2.5 g dw	Not reported		Freeze dry SE	SPE	LC – MS/MS
Drewes et al. (2005)	Inf Eff	E1, E2, E3, EE2, TT	1 L	0.15 – 1.5 ng/L		Filtered pH< 2	SPE	HFBAA GC-NCI-MS E-screen
Esperanza et al. (2007) (pilot plant)	centrifuged solids only: PS DS	E1, E2, EE2	5 g	1 ng/g		pH 2.5 Centrifuged Freeze dry SE	SPE HPLC	GC-MS
Muller et al. (2008)	WAS DS	E1, E2, E3, EE2	1 g	(ng/g) LC-MS/MS E1=0.3 E2=10 E3=6 EE2=10 GC/MS E1=1-2 E2=1-2 E3= ~1 EE2=1-3	(ng/g) LC-MS/MS Not reported GC/MS E1=91 E2=101 E3=132 EE2=101	pH 3-5 Freeze dry ASE SPE	LC-NH_2	GC-MS and LC-MS/MS MELN

ASE = accelerated solvent extractor
GC-MS = gas chromatography coupled with a mass spectrometer
GPC = gel permeation chromatography
HPLC = high performance liquid chromatography
LC-MS/MS = liquid chromatography coupled with tandem mass spectrometers
MELN = estrogen-responsive reporter Mcf-7EreLucNeo cell lines
SE = solvent extraction
SPE = solid phase extraction (usually SPE cartridge)

Due to the complexity of analysing samples with high solids content, separate laboratory protocols are typically used to determine concentrations of estrogens in wastewater and sludge samples. Wastewater analysis for estrogens is relatively simple in comparison to the complex protocols for municipal sludges. For example: Drewes et al. (2005) filtered wastewater prior to solid phase extraction (Table 1-4). Ifelebuegu (2011) also used separate laboratory extraction methods for wastewater and sludge. Target analytes in wastewater were separated with gel permeation chromatography prior to SPE clean up and analysis by LC-MS/MS. The range of concentrations of E1, E2, E3, and EE2 the authors found in influent, effluent, mixed and digested sludge can be found in Table 1-3.

Comparison of estrogen concentrations in mixed and digested sludges is difficult due to differences in sludge treatment systems, wastewater sources and analytical strategies. A variety of methods have been used to extract estrogen from sludge, including sonication, shaking, solid-phase extraction and vortexing (Esperanza et al., 2007; Gabet-Giraud et al., 2010; Muller et al., 2008; Ifelebuegu, 2011; and Ternes et al, 2002) and method recoveries vary for the method and solvents used. Also some analytical methods quantify natural estrogens separately or combined and the reporting limits are not always included in the published studies.

Ternes et al. (2002) were the first researchers to publish a method to detect estrogens at environmentally relevant concentrations in municipal sludges. Sludges were freeze dried prior to solid-liquid ultrasonic extractions using solvents miscible with water such as methanol and acetone. Laboratory analysis of sludges frequently involves freeze-drying samples as some solvents suitable for extracting estrogens (e.g. methanol and acetone) are miscible in water and

cannot be used for liquid-liquid extractions (Ifelebuegu, 2011; Sim et al., 2011). Dewatered digested sludge can be freeze-dried in less time than mixed or digested sludge and is frequently analyzed for estrogens with similar extraction procedures to Ternes et al. (2002).

Sim et al. (2011) analysed influent, effluent and sludge from 12 municipal wastewater treatment plants and 4 livestock wastewater treatment plants for five estrogens using liquid chromatography coupled with tandem mass spectrometers (LC-MS/MS). Concentrations of E1, E2, and E3 found in influent, effluent, and sludge from the twelve municipal wastewater treatment plants are summarized in Table 1-3. Although more frequently detected in wastewater and sludge samples from the four livestock treatment plants, concentrations of E1, E2 and E3, when detected, were similar to concentrations from the municipal wastewater treatment plants summarized in Table 1-3. Sim et al. (2011) reported recoveries the target estrogens ranged from 70% to 110% in both wastewater and sludge samples. Like Ternes et al. (2002), recoveries of target estrogens in sludge were determined by spiking estrogen standards onto sludge after samples were freeze-dried.

In a pilot plant study, Esperanza et al. (2007) examined estrogen (E1, E2, EE2) in primary and secondary sludges. Sludges were dewatered by settling and centrifuged (2500 rpm, 7 min) and the water phase discarded without examination for estrogens or other components. Dewatered sludges were freeze-dried and extracted by tumbling with methanol at 35 °C for 2, 4, and 4 hours for a total of three extractions. Clean up by SPE and HPLC was followed by derivatization and analysis by GC-MS.

Using a method developed by Patrolecco et al (2004), Ifelebuegu (2011) extracted freeze-dried sludge and analysed it by LC-MS/MS (Table 1-4). The method of determining recoveries for target compounds was not described and recoveries were not stated in this paper. While most researchers freeze-dry sludge prior to extracting with a polar solvent, Fernandez et al. (2009) incorporated the extraction of wet (digested) sludge in the laboratory protocol.

Fernandez et al. (2009) filtered wastewater and eluted with methanol during solid phase extraction using an Oasis HLB solid phase extraction (SPE) cartridge. However, wet sludge samples were extracted with an accelerated solvent extractor (ASE) (75 °C; 5 min; 100 bar; 3 cycles) and Florisil chromatography column for clean up prior to separation and detection with liquid chromatography coupled with tandem mass spectrometers (Table 1-4).

Fernandez et al. (2009, 2007) analysed wastewater and sludge samples from domestic WWTPs for estrogenic activity with an E-screen assay and estrogens with separate analytical protocols for wastewater and sludge samples. Toxicity to E-screen yeast was measured by applying a series of E2 standards. This evaluated the concentration of E2 that may be toxic to the yeast used in the E-screen assay but did not address toxicity of sludge and wastewater extracts.

Fernandez et al. (2009) did not freeze dry sludge as part of the analytical protocol and reported recoveries of >80% for estrogens in wastewater and sludge samples. Instead, they liquid–liquid extracted sludge (9% solids) with methanol:DCM (30:70) in an accelerated solvent extractor. Despite reporting recoveries of >80% for estrogens, the concentrations of E1 and E2 found in the anaerobic digested sludge (authors do not state if system was mesophilic or

thermophilic) was much less than found by Ternes et al. (2002); Sim et al., (20ll); and Ifelebuegu, (2011) (see Table 1-3).

Laboratory extraction protocols for determining estrogens are simpler for influent and effluent (e.g. SPE cartridge), than for sludges and wastewaters with higher solids content (e.g. freeze drying and solid-liquid extraction prior to SPE cartridge). With minor adjustments, protocols for clean up of sludge extracts will usually work for wastewaters but, due to the complex mixture of compounds in sludge, clean up protocols for wastewater are not typically applied to sludge. This is illustrated by laboratory protocols used by Muller et al. (2008) for determining estrogens in secondary and dewatered digested sludges. Sludges were freeze dried and extracted using accelerated solvent extraction, prior to treatment with the laboratory protocol used for influent and effluent wastewaters (SPE extraction and LC-NH2 separation). Wastewater and sludge cleaned and separated extracts were derivatized and analyzed by GC-MS and LC-MS/MS. Utilizing similar process trains for sludge and wastewater would be advantageous in comparing analytical data, since differences would be minimized, in terms of interaction and removal of interfering compounds in the complex media throughout the laboratory protocols.

1.5.1 Silanization

The surface of laboratory glassware is slightly acidic and can adsorb some analytes. In low level analyses, losses of target analytes can be significant. To prevent sample loss through adsorption, glassware used in low level analyses is usually silanized. Silanization masks the polar Si-OH groups on the glass surface by chemically binding a non-adsorptive silicone layer to

the surface, in effect "derivatizing" the glass. The most common silanization procedure for laboratory glassware doesn't incorporate pre-rinse step(s) and glassware is treated with 5–10% dimethyldichlorosilane (DCDMS) in toluene for 30 minutes. The deactivated glassware is rinsed with toluene, then immediately thereafter with methanol (Sigma-Aldrich, 1997).

Ikonomou et al. (2007) pre-rinsed laboratory glassware with acetone, hexane, and DCM prior to silanization with 5% DCDMS in DCM and rinsed with DCM. The deactivated glassware was baked overnight at 325 °C. Before use, the glassware was rinsed with: 1) DCM; 2) hexane; and 3) acetone, for a total of three post-silanization rinses. However, DuPont Co. (1997) laboratory protocols for GC-MS detection of pesticides require no pre-rinsing step. Laboratory glassware was filled with 8% DCDMS in toluene for 1–2 minutes, then rinsed with: 1) toluene; 2) methanol; 3) water; and 4) acetone, before air drying.

Adsorption can also be reduced by adding a compound that competes for the adsorptive sites on the glass surface. A small amount, often less than 1%, of an alcohol added to the solvent significantly reduces adsorption losses (Sigma-Aldrich, 1997). In laboratory protocols for detection of environmental contaminants in blood matrices, the Centres for Disease Control and Prevention (2002) incorporated a pre-silanization rinse with acetone before drying in an oven at 130°C for 10 minutes. The dried glassware was filled with 10% DMCS in toluene and allowed to stand for 10 minutes. There are five post-silanization rinses in this protocol: 1) toluene; 2) methanol and allowed to stand for 5 minutes; 3) methanol; 4) toluene; and 5) acetone.

1.5.2 Methanol Addition and pH Adjustment

Many researchers add a preservative such as formaldehyde (1% v/v) or adjust pH to 2.5 – 5 to inhibit microbial activity in wastewater and sludge samples soon after collection and before samples storage (Nakada et al., 2004; Esperanza et al., 2007; Muller et al., 2008; Sim et al., 2011). Microbial activity is high in domestic wastewaters and sludges and microbial degradation of estrogens in the samples can potentially cause erroneous results. Since solubility of estrogens increase with pH it is thought raising pH in municipal sludge post-digestion treatments may increase partitioning from the solid to liquid phase and leach estrogens during land applications of treated biosolids. Hence, researchers have reported on the effects of raising pH on the solubility of estrogens to examine the fate of estrogens in the environment (Shareef et al., 2006). However, examination of lowering the pH and extraction efficacy of estrogens in domestic sludge and wastewaters could not be found in the published literature.

Methanol and ethanol are frequently used to break up emulsions (Milkshake, 2008), and wastewaters with high solids content and wet sludges would be expected to form emulsions during liquid-liquid solvent extraction protocols. However, an examination of the effect of methanol addition to sludge and wastewater samples prior to solvent extraction of estrogens, also could not be found in the published literature.

1.5.3 Extraction

Prior to extraction, sediment and sludge samples are, in most cases, freeze-dried. Several extraction techniques have been used to detect estrogens in sludge: sonication; microwave-assisted extraction; accelerated solvent extraction (ASE); or, Soxhlet extraction. Soxhlet

extraction, which is time consuming and has high solvent consumption, is used less and less. The extraction step is usually performed with MeOH (pure, mixed with another solvent, or followed by acetone extraction). When sludge is freeze dried, extractions for estrogenic compounds are typically performed with polar solvents, such as methanol, that induce coextraction of many interferents and result in heterogeneous extracts (Marti, 2012). To solve this problem, some authors centrifuged the extract to remove suspended particles before purification (Marti, 2012).

Esperanza et al. (2007) centrifuged sludge samples and discarded the water phase before extracting estrogens from the solid phase. Only one published study was located in the literature that examined estrogens in the liquid and solid phases of municipal wastewaters or sludge. Marti (2012) centrifuged municipal sludges prior to extraction and reported estrogen concentrations in the water (ng/L) and solid phases (ng/g) of the sludges as the combined total E1+E2+E3 using a combination E1, E2, and E3 ELISA kit for detection and quantification. Percent partitioning of estrogens (E1+E2+E3) between water and solid phases of primary and digested sludges was not reported. Research examining the partitioning of individual steroidal hormones in the water and solid phases of municipal sludges could not be found in the published literature.

In the first published laboratory protocol for detecting environmentally relevant concentrations of estrogens, Ternes et al. (2002) freeze dried municipal sludge, solvent extracted ultrasonically, separated target estrogens by preparatory permeation chromatography (GPC) and cleaned up with silica. Extracts were derivatized with N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA)/ trimethylsilylimidazole (TMSI)/dithioerytrol (DTE) prior to

separation and detection by gas chromatography coupled with tandem mass spectrometry (GC MS/MS).

Using a method developed by Patrolecco et al. (2004), Ifelebuegu (2011) extracted freeze-dried sludge with a non-ionic surfactant (Tween 80), mixed at 300 rpm and centrifuged at 4000 rpm. Supernatant was loaded to SPE cartridges (LC18 on a vacuum manifold) and eluted with acetone, nitrogen dried, reconstituted with methanol:water (55:45) and analysed by LC-MS/MS. The method of determining recoveries for target compounds was not described and recoveries were not stated in this paper.

Sim et al. (2011) used separate laboratory protocols for extracting estrogens from wastewater and sludge (Table 1-3). Wastewater was filtered before loading to SPE (Strata C18E) cartridges and eluted with methanol similar to Fernandez (2009). However, sludge was freeze-dried and ultrasonically extracted with ammonium acetate and methanol (10:90 v/v). Samples were ultrasonicated for 30 minutes and mixed at 200 rpm for 30 minutes, centrifuged at 3000 rpm for 15 minutes. Extractions were combined, evaporated to 15 ml, reconstituted to 250 ml with water and processed as wastewater samples by SPE cartridges.

Soxhlet apparatus are typically very effective for solid-liquid extractions with organic solvents but can be labour intensive and consume valuable time and fume hood space. Luque de Castro and García-Ayuso (1998) stated that shaking extraction and stirring extraction methods have been compared with their Soxhlet counterparts and the former were, in general, less effective than Soxhlet extractions.

However, Jenkins and Grant (1987) compared four extractions techniques, Soxhlet, ultrasonic bath, mechanical shaker, and homogenizer-sonicator and found that the extraction results obtained with the shaker were not significantly different from the other three methods at the 95% confidence level. Overall they preferred the sonic bath, based on excellent performance with both soil matrices and the four analytes tested, as well as apparatus and solvent cost, convenience, and sample size (Jenkins and Grant, 1987). Although Clarke et al. (1991) favoured Soxhlet over jar shaker, both solid-liquid extraction methods, as performed by the same laboratory and analyzed by low resolution mass spectrometer, gave similar results for concentrations of polychlorinated dibenzodioxins and polychlorinated dibenzofurans in sediments and sludges. A comparison of Soxhlet, shaker, sonication and vortex found no significant difference between the four methods for greater than 95% recovery of 1.0 to 50 µg pentachlorophenol per gram soil.

In conventional Soxhlet, the sample is placed in a thimble-holder, and during operation gradually filled with condensed fresh solvent from a distillation flask. When the liquid reaches the overflow level, a siphon aspirates the solute from the thimble-holder and unloads it back into the distillation flask, carrying the extracted analytes into the bulk liquid. This operation is repeated until complete extraction is achieved (Luque de Castro and García-Ayuso, 1998). The advantages and disadvantages of the Soxhlet extraction method are summarized below.

Advantages

- Simple methodology — little training required
- Can extract large sample mass
- Little or no matrix effects
- Continuous renewed solvent extraction

Disadvantages

- Time consuming separating and freeze drying solids
- Time consuming extraction
- Large amount of solvent
- Space consuming in solvent exhaust hood
- No agitation to expedite process
- Limited by extractant
- Potential thermal decomposition of thermoliable analytes

Ultrasound-assisted extraction is, together with Soxhlet, the most accepted conventional leaching technique. Ultrasound-assisted methods are usually developed in a discontinuous, batch mode, and the shortening of the extraction time (with respect to that in the absence of ultrasound), is due to an increase of both pressure (which favours penetration and transport), and temperature (which improves solubility and diffusivity), both by increasing mass transport and displacing the partitioning equilibrium. Two major shortcomings of the ultrasound-assisted extraction are: (i) its inability to renovate the solvent during the process, which causes its efficiency to be a function of the partition constant, and (ii) the danger of both loss and/or contamination of the extracted species during manipulation (Luque de Castro and Garcia-Ayuso, 1998).

Ternes et al. (2002) determined recoveries of >70% for estrogens by spiking freeze dried sludges. Estrogens in methanol were stirred into the freeze-dried sludge and dried in a fume hood for 14 hours. Therefore, these estrogens spiked onto the freeze-dried sludge may have been easier to extract than estrogens in the unprocessed sludge samples. In addition, losses of estrogens in sludge during freezing, time in freeze-dryer, and drying after spiking in fume hood were not determined.

Fernandez et al. (2009) was one of the few researchers to examine municipal sludge for estrogen concentrations without freeze drying the samples first (Table 1-4). Using a Dionex ASE 200 accelerated solvent extractor, they extracted wet sludge (9% solids content) in Hydromatrix (an inert diatomaceous earth sorbent) with 30:70 methanol/dichloromethane (DCM) using 100 bar pressure at 75 °C. Fernandez et al. (2009) reported recoveries of >80% for estrogens in wastewater and sludge samples. However the concentrations of estrogens found in the anaerobic digested sludge (authors do not state if system was mesophilic or thermophilic) was much less than found by Ternes et al. (2002) and more recently Sim et al., (20ll) and Ifelebuegu, (2011) (see Table 1-3).

1.5.4 Chromatography

Silica (SiO_2), also known as silicic acid and silica gel, is a regenerative silica adsorbent with weakly acidic properties. It is produced from sodium silicate and sulfuric acid. Silica can be used in column chromatography for the separation of analytes from interfering compounds of a different chemical polarity. It may be used activated, after heating to 150–160 °C, or deactivated with up to 10% water. Florisil and silica can be deactivated with acid instead of water.

Florisil, a synthetic magnesium-silica, is less polar and less acidic than silica. Due to its low polarity it's useful for very polar compounds which would "stick" too strongly to silica and very non-polar compounds which would not be retained at all. Carroll (1961) found Florisil had definite advantages over silicic acid (silica) for the separation of lipid classes by column chromatography and separations could be achieved in much shorter times with smaller volumes of eluting solvents.

Carroll (1961) proposed highly active Florisil absorbs moisture from the atmosphere, eventually reaching equilibrium, unless it is kept under anhydrous conditions and suggested Florisil be stored in the hydrated state or maintain the highly active Florisil under anhydrous conditions in order to ensure consistent chromatographic results. Newly activated Florisil, deactivated with 7%, water gave similar lipid class elutions as Florisil stored in Carroll's laboratory for years.

The degree of Florisil hydration affects its chromatographic properties and adsorption affinity for a wide variety of compounds. Separation of desired compounds may be facilitated by using the appropriate degree of hydration. Nakada et al. (2004) used a preparatory chromatography column containing 5% H_2O deactivated silica gel to purify and fractionate midpolar to polar compounds in wastewater samples. Deactivation of Florisil with 7% distilled water was preferred over 6% for separation of cholesterol in liver and blood samples although the exact degree of hydration did not seem to be important (Carroll, 1961). Since steroid estrogens are cholesterol derivatives, E1, E2, E3, and EE2 have molecular structures which are

very similar to cholesterol. Therefore, using 7% distilled water for separation of estrogens may be a good option in preparatory chromatography columns packed with Florisil.

Fine fractionation improves the reliability and sensitivity of the recombinant yeast assays (Nakada et al., 2004) and improves clean up for GC-MS analysis. Nakada et al. (2004) fractionated wastewater effluent eluates from silica gel preparatory chromatography columns and analyzed for estrogenic activity using a recombinant yeast assay. After applying less polar eluent mixtures, 75–20% hexane in DCM and 100% DCM, to the preparatory silica gel chromatography columns, Nakada (2004) noted E1 and E2 eluted with a polar fraction of 30% acetone in DCM. Estrone and E2 and represented 66 to 88% of the total estrogenic activities estimated from the bioassay data. Nakada (2004) concluded E1 and E2 were the dominant environmental estrogens in the STP effluent, but a significant contribution to estrogenic activities stems from unidentified components in the effluents.

1.5.5 Derivatization

An ideal derivatizing procedure should improve chromatographic separation, be reproducible, efficient, and nonhazardous. Three derivatization reactions are commonly used for gas chromatography: silylation, acylation, and alkylation. Silylating reagents target active hydrogens on the molecule; acylating reagents react with highly polar functional groups; alkylating derivatization agents target active hydrogens on amines and acidic hydroxyl groups.

Multiple step procedures may be necessary to derivatize compounds with several different functional groups such as androsterone. However, just as every coin has two sides, the

introduction of a derivatization procedure has disadvantages (e.g., column damage, time taken, formation of unexpected derivatives and multiple derivatives, signal overlapping, and introduction of additional substances).

Silylation is most commonly used for conversion of mixtures of related compounds into derivatives capable of separation and analysis by gas chromatography/mass spectrometry. Derivatives, ideally, should be less polar, more volatile and more thermally stable to improve gas chromatographic separation. The introduction of one or more silyl group(s) on –OH, –SH, and –NH groups can enhance mass spectrometric properties by producing characteristic ions of use in trace analyses.

N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) is the preferred reagent for trimethylsilylation due to high reactivity with polar organic compounds, readily replacing active hydrogens with a $–Si(CH_3)_3$ (trimethylsilyl) group (TMS). One of the advantages of TMS derivatives over other derivatives is their thermal stability. They are routinely used at column and injector temperatures of 300 °C and temperatures of 350 °C have been used successfully (ThermoScientific, 2008). Although TMS derivatives are thermally stable, they are more susceptible to hydrolysis than the parent compounds (ThermoScientific, 2008).

The TMS reagents themselves are also quite thermally stable; however, the more reactive silyl donors such as BSTFA and N,O-bis(trimethylsilyl)acetamide (BSA) will decompose at elevated temperatures, especially in the presence of metals. Care must be used when temperatures above 75°C are needed for a derivatization procedure, as decomposition of these

reagents can be significant at these temperatures (ThermoScientific, 2008). On the other hand, BSTFA and its by-products (trimethylsilyltrifluoroacetamide and trifluoroacetamide) are more volatile than many other silylating reagents, causing less chromatographic interference. Good chromatographic separations can be obtained with BSTFA, as the by-products from this reagent usually elute with the solvent front (ThermoScientific, 2008; Sigma-Aldrich 1997).

The silylation reagent most frequently used in derivatization techniques is BSTFA with 1% trimethylchlorosilane (TMCS). The use of TMCS produces an increase in the derivatization yield, especially for the compounds with multiple hydroxyl groups (i.e., 17β-estradiol (E_2) and estriol (E_3)). Since the exact concentration of TMCS is seldom critical, either BSTFA or BSTFA + 1% TMCS can be used in most cases (ThermoScientific, 2008).

ThermoScientific (2008) protocols for heat silylation with BSTFA + TMCS recommend time-temperature derivatization at 60 °C for 15 minutes for reactions with or without solvent. Sigma-Aldrich (1997) silylation with BSTFA + TMCS protocols state derivatization times vary widely, depending upon the specific compound(s) being derivatized. Many compounds are completely derivatized as soon as they dissolve in the reagent, while those with poor solubility may require warming. A few compounds will require heating at 70°C for 20–30 minutes (Sigma-Aldrich Co., 1997).

Detecting trace amounts of 17β-estradiol with gas chromatography followed by mass spectrometry (GC-MS) is difficult due to the relatively low volatility of natural estrogens. Volatility can be increased by derivatization of the functional group with silylation agents

creating a trimethylsilyl (TMS) derivative. These derivatization techniques involved replacement of the acidic hydrogen in the alcohol groups of 17β-estradiol (–OH) with an alkylsilyl group (–OTMS).

Since some of the target compounds (i.e. estrone, testosterone) also contain a carbonyl group, another derivatizing reagent may improve chromatographic peak shape. Methoxyamine will react with the carbonyl group (C=O) forming an oxime derivative (CH_3ON). Oxime derivatives can not only improve chromatographic performance, but also alter GC separations (Sellers, 2010).

During the derivatization procedure, some factors (e.g., types and amounts of derivatization reagent, reaction time, and temperature) will significantly affect the sample profiles and lead to multi-peak phenomena. Xu et al., (2010) reported "incomplete derivatization for compounds with multi-function groups and geometrical conversions can give rise to multi-peaks". In order to reduce or to eliminate conversion reactions during silylation, methoxamine hydrochloride may be used first for the oximation reaction prior to the silylation reaction (Xu et al., 2010). For example, by introducing an oximation step prior to silylation, cyclization of sugars is inhibited, resulting in fewer peaks per sugar (Pasikanti et al., 2008).

Methoxamine (MOX) reagent is useful for preparing oximes of steroids and ketoacids prior to silylation. A mixture of 2% methoxyamine·HCl (M.W. 83.51) in pyridine can derivatize carbonyl groups and help prevent formation of multiple derivatives during silylation. Pyridine

serves as catalyst in the methoximation procedure which protects carbonyl moieties. It does not seem to be replaceable by other aprotic polar solvents (Fiehn, 2006).

Temperatures and times of derivatization steps can be kept flexible, because they present a compromise between completeness of reaction, time and effort needed to perform the reactions, and breakdown of certain compounds (Fiehn, 2006). Bowden et al. (2009) found steroid derivatization using BSTFA/TMCS was most successful at producing the highest relative response factor (RRF) values in the range of 55–70 °C for 15–30 min. A wide variation of time and temperature combinations has been used to create methoxyamine derivatives. For example, the most commonly used derivatization procedure for urine samples, following extraction, is where the dried extract is dissolved in pyridine, while oximation is carried out using methoxamine hydrochloride (28–37 °C, up to 120 min) followed by trimethylsilyl (TMS) derivatization (Pasikanti et al., 2008). For the detection of opiates, Dietzen et al. (1995) prepared methoxime derivatives by adding 100 µl 0.5% (w/v) methoxyamine-HC1 in pyridine to dried samples and incubating for 30 min at 75 °C. A wide range of compounds (amines, amino acids, organic acids, alcohols, and xanthines) in a cerebrospinal fluid matrix were derivatized by adding 100 µl methoxyamine (60 min, 40°C) and subsequently, 50 µl MSTFA (30 min, 40°C) (Pacchiarotta et al., 2010).

Testosterone contains a hydroxyl group and a carbonyl group and exhibits poor peak shape and poor separation if analyzed underivatized by GC. A silylation reagent will react with the hydroxyl group to create a TMS derivative but because testosterone also contains a carbonyl group, another derivatizing reagent is needed to improve chromatographic peak shape.

Methoxyamine will react with the carbonyl group forming an oxime derivative (CH_3ON) and improve chromatographic performance (Sellers, 2010). The formation of two chromatographic peaks when testosterone is derivatized using silylation and oxamination-silylation is likely due to a stereoisomer of derivatized testosterone (Danaceau et al., 2008; Bowden et al., 2009).

During GC-MS analysis, thermal degradation of components can occur in the following parts with high temperatures leading to multi-peaks for one compound in the GC-MS analysis. (Xu et al., 2010):

(i) the injection port (usually 200–250 °C);

(ii) column (temperature program 50–300 °C);

(iii) mass-spectrometer ion source (>200 °C); and transfer line (>200 °C).

1.5.6 Separation/Detection/Identification

A gas chromatograph (GC) is used to separate complex chemical mixtures into individual components that can then be identified and quantified by the mass spectrometer (MS). Analytes of interest are usually extracted from the sample into a liquid solvent phase and may also be derivatized for better detection. This extract is then injected into the GC where it is carried through the separation column by an inert carrier gas such as helium. The analytes in the mixture are separated from one another by their interaction between the stationary phase coated on the inside wall of the column and the carrier gas. Analytes that react very little to the stationary phase move through the column quickly and will exit into the mass spectrometer before those analytes having longer interaction and retention times.

In the mass spectrometer analytes are bombarded with electrons to form ionized fragments. Charged particles are detected and signal processing results are displayed as relative ion abundance spectra as a function of the mass-to-charge ratio. This mass spectrum is a graph showing the abundance of each ionized mass fragment forming a peak. Mass fragments belonging to individual analytes will form peaks at a particular retention time. The atoms or molecules can be identified by correlating known masses to the identified masses or a characteristic fragmentation pattern. These characteristic mass spectral fragmentation patterns can be searched against libraries of EI spectra to achieve identification.

Typically two to four ions are monitored per compound and the ratios of those ions will be unique to the analyte of interest. Because unwanted ions are being filtered, the selectivity is greatly enhanced providing an additional tool to eliminate difficult matrix interferences (ALS Environmental).

Gas chromatography, coupled with a single mass spectrometer (GC-MS), is an excellent technique for detection and quantification of analytes in complex mixtures. When gas chromatography is coupled with two mass spectrometers (GC-MS/MS) in sequence, tandem mass spectrometers, selectivity is greatly enhanced. Therefore, improved detection and quantification limits can be achieved by using a GC-MS/MS. Two requirements of GC-MS and GC-MS/MS for detecting and quantifying estrogens are that the sample must be in an organic injection solvent and derivatization is necessary for environmental samples to improve ionization, volatility, and chromatogram peak shape. These two requirements can be avoided with the use of liquid chromatography (LC), coupled with tandem mass spectrometers (MS/MS).

Some of the research studies in Table 1-4 used LC-MS/MS to take advantage of improved detection limits (MS/MS vs MS), faster analytical run times, and reduced sample preparation and handling (LC vs GC).

Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are the two ionization techniques most commonly used in LC/MS. Unlike electrospray ionization (ESI) used in GC-MS, ion suppression can occur with both ESI and APCI so co-eluting compounds may be underestimated or not detected at all. Therefore, for complex samples, such as municipal sludge, greater separation (more intensive clean up) techniques must be employed in the laboratory protocol for reliable results using LC-MS. Incorporating satisfactory separation techniques in laboratory protocols for LC-MS analysis of estrogens in the complex high-solids-content sludge matrix, can be as, if not more, time consuming than incorporating derivatization or an organic injection solvent as required in GC analysis. Common separation techniques for LC-MS analysis of estrogens in municipal sludge have been based on freeze-drying sludge samples and extracting with polar solvents (solid-liquid extraction) prior to clean up with various solid phase extraction methods.

While both GC-MS and LC-MS are suitable for separation, detection and quantification of steroids, the cost of GC-MS systems is substantially less than LC-MS. In addition, there are no spectral libraries for LC-MS identification. Instead, the mass of an identifying molecular ion, usually present in LC-MS analysis, can be searched in a database. The use of accurate-mass time of flight mass spectrometers with LC has enabled the calculation of an empirical formula from the molecular ion. Of course, this further increases the cost of LC-MS systems.

In the first published laboratory protocol to detect estrogens in municipal sludge, Ternes et al. (2002) used GC-MS/MS and several subsequent studies used LC-MS/MS (see Table 1-4). Muller et al. (2008) analyzed waste activated sludge using GC-MS and LC-MS/MS and found the LC-MS/MS technique could not confirm the presence of E2 and EE2 because of insufficient sensitivity. If suitable detection limits can be achieved with a GC-MS, the cost savings to smaller laboratories with limited research funding would be well worth the effort of incorporating a derivatization step and organic injection solvent into the laboratory protocol for analyzing estrogen concentrations in municipal sewage sludge.

1.6 WHOLE ESTROGENIC ACTIVITY ANALYSIS WITH YEAST BIOASSAYS

Municipal sludge is a difficult matrix for laboratory analysis of estrogenic substances. Few laboratories currently carry out this analysis without first drying the mixed and digested sludge samples. Aside from the concern of losing estrogenic activity in liquid vs. solid phases, the method must also be suitable for biological testing. Yeast estrogen screen (YES) assays are very sensitive to toxins within the sample matrix. Therefore, sample extraction procedures must remove substances toxic to the yeast strains, while leaving as much of the estrogenic material as possible.

A *Saccharomyces cerevisiae* strain, capable of autonomous bioluminescence, was engineered to detect estrogenic, androgenic, and toxic activities using a bioluminescence yeast estrogen receptor (BLYES), bioluminescence yeast androgen receptor (BLYAS) and bioluminescence yeast reporter (BLYR) by Sanseverino et al. (2005) at The Center for

Environmental Technology at the University of Tennessee. Specifically, three *S. cerevisiae* strains were developed to produce a measurable bioluminescent signal in response to chemicals with estrogenic (*S. cerevisiae* BLYES), androgenic (*S. cerevisiae* BLYAS), or toxic activities (*S. cerevisiae* BLYR) (Sanseverino et al., 2005; Eldridge et al., 2007). These bioluminescent bioreporter strains may also be used for monitoring of waterways, wastewater treatment plant influents and effluents, runoff from farms, etc. When used as a Tier I screening tool, four outcomes were determined to be (Center for Environmental Biotechnology, 2013):

- *Chemical is hormonally active*. These chemicals induce bioluminescence in BLYES and BLYAS, produce a sigmoidal curve, and exhibit no toxicity.
- *Chemical is toxic*. These chemicals result in a decrease in bioluminescence in the constitutive strain BLYR. Generally, an IC_{50} cannot be determined from this data, but an IC_{20} can be calculated. The IC_{20} is defined as the concentration at which bioluminescence is reduced by 20 percent.
- *Chemical is not hormonally active and not toxic*. There is no increase in bioluminescence in the BLYES and BLYAS strains and no decrease in bioluminescence in the BLYR strain.
- *Chemical has hormonal activity but an EC_{50} cannot be calculated*. Limited bioluminescence (no sigmoidal curve) is observed in BLYES and BLYAS. Bioluminescence may be hampered due to a chemical's toxicity, uptake by the cells or concentration range.

A comparison of BLYES to the colorimetric-based estrogenic assay with the yeast lacZ reporter strain (YES) found the lower (4.5×10^{-11} M) and upper limits (2.8×10^{-9} M) of detection (17β-estradiol) were similar as were the 50% effective concentrations (EC_{50}) for YES [$(4.4 \pm 1.1) \times 10^{-10}$ M] and BLYES [$(2.4 \pm 1.1) \times 10^{-10}$] (Sanseverino, 2005). The BLYES screen consistently detected estrogenic potencies at 5- to 10-fold lower levels than those attained in the YES assay (Sanseverino, 2004). The YES assay requires a minimum of 3 days for results (red colour measured by absorbance at 540 nm wavelength) while BLYES luminescence can be observed in 1 hour and reaches a maximum in 6 hours (Sanseverino et al., 2005). The BLYES luminescence signal in an environmental sample is compared to the corresponding luminescent intensity in a standard curve of BLYES in a series of 17β-estradiol dilutions. Estrogenic activity in the environmental sample is measured as the concentration of 17β-estradiol from the standard curve and expressed as estradiol equivalent (EEQ) to 17β-estradiol concentrations.

The bioluminescence yeast androgen screen (BLYAS) assay created by Eldridge et al. (2007) at The Center for Environmental Biotechnology at the University of Tennessee has a response time of 3 to 4 hours and a lower limit of detection (testosterone) of 2.5×10^{-10} M (68 ng/L). In contrast, the yeast androgen screen (YAS) developed by Purvis et al. (1991) requires 3 to 5 days for colour development and mammalian cell-based androgen reporters require at least 24 hours for luminescence detection (Eldridge et al., 2007).). The BLYAS luminescence signal in an environmental sample can be compared to the corresponding luminescent intensity in a standard curve of BLYAS in a series of dihydrotestosterone (DHT) or testosterone (TT) dilutions. When androgenic activity in the environmental sample is measured as the

concentration of testosterone from the standard curve and expressed as testosterone equivalent (TEQ) to testosterone concentrations.

The bioluminescent yeast reporter (BLYR) assay produces a measurable auto bioluminescent signal that does not increase in the presence of estrogenic or androgenic activities. However, the toxic activities of environmental samples can be measured by the inhibition of luminescence when BLYR reacts with the sample. Toxic responses (IC_{20} and IC_{50}) can be determined by calculating the concentration of chemical that inhibits BLYR luminescence by 20% and 50% (less the BLYR background bioluminescence). The background bioluminescence is determined as the luminescence measured from BLYR in methanol blanks.

With respect to colorimetric detection, luminescence is more sensitive, has a larger dynamic range, and does not require the addition of a stopping reagent (PerkinElmer, 2013). Luminous intensity of the sample is directly related to the biological interaction of BLYES with estrogenic activity, BLYAS with androgenic activity and indirectly related to BLYR with toxic activity. The luminescence plate reader expresses photometric measurement of this luminous intensity per unit area of light as candela per square meter (cd/m^2).

The method of detecting estrogenic activity by using estrogen-responsive reporter Mcf-7EreLucNeo cell lines (MELN), may be more sensitive than YES or BLYES. However, when using MELN or YES assays, there is no concurrent protocol for measuring toxic activity that may originate from wastewater and sludge samples and inhibit assay results. In waste activated and dewatered digested sludges, Muller et al. (2008) detected no estrogenic activity using the

MELN assay, even though chemical analysis revealed the presence of low levels of E1 (confirmed by both GC-MS and LC–tandem MS) and of E2 and EE2 (as determined by GC-MS but not confirmed by LC–tandem MS). Moreover, some inhibition of MELN cells was observed when testing sludge samples—behavior that could be explained by the presence of inhibitory compounds in complex matrices, such as sludge. Although samples were diluted to below the 17β-estradiol EC_{50}, inhibitory assay responses were noted and there was no protocol, such as the concurrent use of BLYR with the BLYES assay, for measuring and correcting for the inhibition.

1.7 SLUDGE TREATMENT

Anaerobic sludge digestion is often applied to waste sludge to reduce the mass of solids for disposal, to reduce the pathogen content and to generate biogas for energy recovery (Eskicioglu et al., 2007a). Although anaerobic digestion is among the oldest processes used for the stabilization of solids and biosolids, it continues to be the dominant process for stabilizing sludge. Its popularity is due to the current emphasis on energy conservation /recovery and the desirability of obtaining beneficial use of wastewater biosolids. Most anaerobic digestion systems are designed to operate in the mesophilic temperature range (35–40 °C). Other systems are designed to operate in the thermophilic temperature range (52–60 °C) or a combination of mesophilic and thermophilic digestion in separate stages (Mavinic, 2014). The bacteria involved in anaerobic digestion, especially methane formers, are sensitive to changes in temperature. Generally, temperature changes greater than 1°C /d affect process performance, thus, variation of less than 0.5 °C/d is recommended (WEF, 1998). Carballa et al. (2006) reported that E1 and E2 concentrations were reduced by 85% under mesophilic (37 °C and 10 day SRT) and thermophilic (55 °C and 6 day SRT) conditions for anaerobic digestion.

The production of large volumes of sludge as an end-product from the activated sludge biological wastewater treatment process poses one of the biggest challenges to the wastewater treatment industry. Significant factors in the dewaterability and digestibility of activated sludge are the cellular material (microbial cells) and exocellular polymeric substances (EPS). Microbial cell walls are physical and chemical barriers to exoenzyme degradation and, hence, resistant to direct anaerobic degradation. It has been suggested that divalent cations bind to negative sites on EPS to increase floc strength and size (Andreadakis, 1993; Park et al., 2006). To improve biodegradability during anaerobic digestion, pre-treatment should concentrate on enhancing disintegration of the sludge floc structure and microbial cell walls.

Mechanical pre-treatment methods such as high pressure homogenizer and ball milling have resulted in increased polymer demand for sludge dewatering after anaerobic digestion (Muller et al., 1998) and no pathogen reduction based on total and fecal coliform (Muller et al., 2003). In a bench scale pilot plant study, Carballa et al. (2006) examined alkaline (pH 12) and thermal (autoclave 130 °C) pre-treatments of domestic sludge with mesophilic (37 °C) anaerobic sludge digestion (SRT of 10 and 20 days) and thermophilic (55 °C) anaerobic sludge digestion (SRT of 6 and 10 days) with respect to spiked concentrations of personal care products and estrogens. While E1 and E2 concentrations were reduced by >85% in mesophilic and thermophilic anaerobic digestion and EE2 was reduced by 85% (mesophilic) and 75% (thermophilic) anaerobic digestion, no reduction was associated with pre-treatments, SRT or temperature.

The handling and disposal of sludge residuals has significant social, environmental, and economic implications (Wong et al., 2006a). Sludge management and disposal can consume from 30 to 60 percent of a wastewater treatment operation and maintenance budget (US Office of Technology Assessment, 1991). Sewage sludge disposal methods include incineration, landfill, land application (fertilizer, soil conditioners), and ocean disposal. All of these disposal methods are associated with environmental, and in most cases public health, concerns.

1.8 MICROWAVE IRRADIATION

Microwaves referred to in this work (1000 watts) will heat only sludge constituents that are capable of absorbing electromagnetic radiation with an oscillation frequency of 2450 MHz (similar to a household microwave). More uniform heating and precise temperature control are the primary advantages of the microwave process. Non-ionizing electromagnetic radiation from the microwave produces changes in sludge at a molecular level in two ways:

1. Thermal effects – Like conventional heating, ions are accelerated and collide with other molecules. This is the predominant mechanism.
2. Athermal effects –the alternating electric field will cause a rapid alignment and realignment of molecular dipoles within a polar solvent. It has been suggested microwaves athermally induce different biological effects by changing microbial structures (differentially partitioning ions; altering the rate and/or direction of biochemical reactions) (Banik et al., 2003; Samarketu et al., 1996; Porcelli et al., 1997).

Eskicioglu et al. (2007b) evaluated the athermal effects of microwave irradiation on WAS floc disintegration and anaerobic digestion by comparing conventional and microwave heating methods at pretreatment temperatures of 50, 75, and 96^{0}C. Both microwave and conventionally heated WAS had similar particulate COD and biopolymer (protein and polysaccharide) solubilization and no discernable microwave athermal effect was noted for the COD solubilization of WAS. However, biochemical methane potential tests showed the microwave pretreated WAS consistently produced higher biogas than conventionally heated WAS, indicating the microwave athermal effect had a positive impact on the mesophilic anaerobic biodegradability of WAS. In a temperature range of 50–96 °C, there was a linear relation between microwave irradiation temperature and level of hydrolysis in the mixed sludge (Eskicioglu et al., 2007a).

Pretreatment of sludge by microwave processing has shown to improve anaerobic digestion. Eskicioglu et al. (2007a) found waste activated sludge microwaved to 96 ^{0}C, produced 15–20% more biogas and 3.2–3.6 fold increase in soluble to total chemical oxygen demand (SCOD/COD). They also noted dewaterability of the microwaved sludge was enhanced after anaerobic digestion. Similarly, Hamid and Eskicioglu (2013) found microwave pretreatment of municipal sludge increased organic removal and methane production rates during mesophilic (37 °C) digestion, especially at shorter SRTs (5 and 10 days).

In a bench scale study, Hamid and Eskicioglu (2013) examined the effects of microwave pretreatment on mesophilic and thermophilic digestion. Municipal sludge cake (17.5% solids) was either used as a control or microwave irradiated at temperatures of 80, 120, and 160 °C and

mixed with landfill leachate and tap water to a typical feed concentration of 3.4% solids for the mesophilic and thermophilic digesters. Evaluated parameters included E1 and E2 that were detected in sludge supernatant but not in whole sludge samples. The supernatant of the influent feed mixture demonstrated an increase in E2 and testosterone and a decrease in E1 at a microwave irradiation temperature of 80 °C. In fact, E2 was the only hormone showing consistent release with increasing microwave pretreatment temperature. However, concentrations in the effluent supernatants indicated accumulation of E1 and removal of E2 in both control and pretreated digesters. They concluded that at higher SRTs, conventional thermophilic digestion performs better in terms of hormone removal and pretreatment may be advantageous only with mesophilic conditions in anaerobic digesters.

1.9 OXIDATIVE TREATMENT

Ozone treatment has been effective at reducing estrogens in drinking water (Huber et al., 2004) and municipal wastewater (Baig et al., 2008; Nakada et al., 2007). Advanced Oxidation Processes (AOPs) can be utilized in wastewater treatment for: overall organic content (COD) reduction, specific pollutant destruction, sludge treatment, increase of bioavailability of recalcitrant organics, and color and odor reduction (Bergendahl and O'Shaughnessy, 2006).

Nakada et al. (2007) investigated the removal efficiencies of twenty-four pharmaceuticals, personal care products and EDCs during ozonation of municipal sewage treatment plant effluent. The target EDCs were 3 phenolic endocrine-disrupting chemicals [nonylphenol (NP), octylphenol (OP), bisphenol A (BPA)] and 3 natural estrogens [17β-

estradiol, estrone, estriol). Ozonation removed significant quantities of the natural estrogens: approximately 80% of the 17 β-estradiol and greater than 50% of Estrone and Estriol.

Deborde et al. (2005) investigated the ozone-induced oxidation of six EDCs 4-n-nonylphenol, bisphenol A, 17α-ethinyl estradiol, 17β-estradiol, estrone, and estriol) over a pH range of 2.5 – 10.5 at 20 ± 2 ^{0}C and in the presence of tert-butyl alcohol. Ozone reaction rates were pH dependent and increased with pH. Ozone reacted with ionized endocrine disruptors 10^{4}–10^{5} times faster than with neutral EDCs. At pH > 5, ozone reacted to the greatest extent with dissociated EDC forms. They suggested molecular ozone attacks structures with a high electron density, such as C=C (carbon double bonds), activated aromatic systems, and non-protonated amines, but not aromatic rings with ethinyl, amide, or carboxyl groups. In drinking water treatment conditions and at pH = 7 and 20 ±2 °C, O_3 exposures of only $\sim 2 \times 10^{-3}$mg min L^{-1} were calculated to achieve ≥95% removal efficiency for all six EDCs studied (Deborde et al., 2005).

Sludge treatments can enhance the sludge digestion process by increasing the rate of cell hydrolysis to reduce volume and mass, increase biogas and methane (CH_4), and produce more stabilized biosolids. While thermal treatment alone did not increase solids destruction, the addition of H_2O_2 as an oxidative treatment for mixed sludge at 90 °C had a synergistic effect when both treatments were combined and enhanced removal of COD and VSS (Rivero et al., 2006).

Hydrogen peroxide reaction chemistry is complex, but potentially capable of degrading a wide range of organic contaminants depending on conditions (pH, availability of organic compounds, concentration, etc.). pH has a strong effect on hydrogen peroxide chemistry and effectiveness. pH impacts catalyst solubility and reactivity towards hydrogen peroxide, as well as the radicals formed and the degradation of target contaminants. Radicals known to play significant roles in hydrogen peroxide chemistry include the hydroxyl radical ($OH^{\bullet}$) (SRP = 2.59v; pH < 11.9), superoxide radical ($O_2^{\bullet -}$) (SRP = -0.33v; pH <4.8), and perhydroxyl radical ($HO_2^{\bullet}$) (SRP = 1.495v; pH < 4.8). Hydrogen peroxide (H_2O_2) (SRP = 1.776v; pH < 11.6) and solvated electrons (e^-) (SRP = -2.77v; pH >7.85) also play a significant role degradation of organic material. The superoxide radical has recently been implicated as a major reactive species, particularly when H_2O_2 concentrations are high (e.g., 3.5–35 g/L). (Petri et al., 2011)

Hydroxyl radicals ($OH^{\bullet}$) are extremely reactive; concentrations in aqueous systems tend be very low even during AOPs because they are consumed nearly instantaneously. Despite the strong standard reduction potential of the $OH^{\bullet}$ radical, different organic compounds will react with hydroxyl radicals at differing kinetic rates, depending on their affinity for the oxidant (Petri et al., 2011). Municipal sludges are a concentrated and complex mix of compounds, including contaminants and natural organic matter, as well as inorganic minerals and dissolved solutes. Competition between all of these constituents for hydroxyl radicals and whether the target contaminant's rate of reaction with hydroxyl radicals is competitive with that of the other constituents in solution, impacts the extent to which hydroxyl radicals will degrade a particular contaminant.

Microwave heating with hydrogen peroxide can significantly reduce the quantity of sludge (Wong et al., 2006). The microwave heating process was also found to limit microbial activity (Liao et al., 2005) and, with hydrogen peroxide, the pasteurization or sterilization (depending on time-temperature application) of pathogens in the solution can be achieved (Wong et al., 2006).

Fenton's reagent is a solution of hydrogen peroxide and an iron catalyst that is commonly used to oxidize organic compounds in water waters. Li and Zhang (2013) demonstrated removal efficiencies of E1, E2, EE2, and E3 were 70%, 90%, 84% and 98%, respectively, with Fenton treatment (application of Fenton's reagent) to waste activated sludge. Based on both the removal of estrogens and the solubilization of WAS, the recommended reaction conditions were: H_2O_2 = 15.62 mmol g^{-1}; initial pH = 3, reaction time = 60 min; and a Fe(II) to H_2O_2 molar ratio = 0.167.

2 OBJECTIVES

This work was comprised of three major objectives:

1) To develop a GC-MS laboratory protocol for chemical analysis of estrogens in municipal sludges and wastewaters with high solids content that did not incorporate freeze-drying as part of the extraction protocol. In order to compare estrogen concentrations and whole estrogenic activity, this analytical protocol should be suitable for use with a yeast estrogen screen assay.

2) To examine estrogens and estrogenic activity in municipal wastewater and sludge treatment processes using whole estrogenic activity and the laboratory protocol developed in the first objective.

3) To research and demonstrate the ability of microwave irradiation, with and without oxidative treatment, to reduce the concentrations of estrogen and estrogenic activity in municipal mixed and digested sludges.

2.1 LABORATORY ANALYSIS

This author originally proposed this project with the chemical analysis being conducted in a government laboratory. However, the government laboratory was unable develop a chemical analysis to detect estrone (E1) and 17β-estradiol (E2) in mixed and digested sludge samples, due to the high solids content. Therefore, this author proposed to develop a GC-MS chemical analysis protocol to detect E1 and E2 in wastewater and sludge samples, without freeze-drying

samples prior to extraction, and use this analysis to evaluate sludge treatment systems and processes.

The first objective of this work was to develop a chemical analysis for detecting estrogens, in particular 17β estradiol (E2) and estrone (E1), in mixed and digested sludge with 2–4% solids without freeze-drying samples and using gas chromatography coupled with mass spectrometry. Municipal sludge is a difficult matrix for laboratory analysis of estrogenic substances. Most laboratories carry out this analysis by first freeze drying mixed and digested sludges. Aside from addressing the concern of losing estrogenic activity in liquid vs. solid phases, this protocol must also be suitable for biological testing. Collection, storage, extraction and clean up steps should be similar for the chemical and whole estrogenic analysis for a more accurate comparison of sample values and evaluation of system efficacy and treatment performance.

In order to directly compare the results from chemical (GC-MS) and biological (BLYES) analysis, the sample preparation should be the same (ideally) or very similar. Whole estrogenic assays, such as yeast estrogenic screening (YES) assays, can be very sensitive to toxins within the sample matrix. Therefore, the developed extraction protocols aimed to remove substances toxic to the yeast strains, while leaving as much of the estrogenic material as possible.

This author originally proposed this project with Simon Fraser University carrying out the YES assay. Although SFU was able to overcome the toxicity issues with mixed sludge, the YES assay was not suitable for this project due to the limited number of samples that could be

processed per sampling event. A large number of samples were collected during each sampling event with three replicates per treatment or process evaluated, not including quality control samples (e.g. method blanks, duplicate samples, etc.).

Bioluminescence Yeast Estrogenic Screening (BLYES) and Bioluminescence Yeast Androgenic Screening (BLYAS) were used to determine whole estrogenic activity and whole androgenic activity, respectively, in the mixed and digested sludge samples. A third strain, Bioluminescence Yeast Receptor (BLYR), was run concurrently with the BLYES and BLYAS assays to detect toxic activities and aid in the interpretation of whole estrogenic and androgenic yeast luminescence data.

The indicator E2, was selected because it is a potent estrogen found in municipal wastewater and used as a standard for estrogenic activity in many common biological assays, including the YES assay. Laboratory protocols developed for analysis of municipal sludge for E2 by GC-MS were also suitable for detection of E1 and whole estrogenic analysis.

2.2 EXAMINATION OF MWWTP

The second objective was to examine estrogens, in particular E1 and E2; estrogenic activity, and androgenic activity, throughout municipal secondary wastewater treatment processes and mesophilic (35–40 °C) anaerobic sludge digestion, using the above auto bioluminescent yeast assays and developed chemical analysis protocols.

2.3 INNOVATIVE SLUDGE TREATMENT

The third objective of this project was to research and demonstrate the ability of an innovative new technology using microwave irradiation to reduce endocrine disrupting chemicals (EDCs) in municipal sewage sludge. Microwave technology was used with pre- and post-digested sludge from a municipal sewage treatment plant utilizing conventional sludge digestion (mesophilic, anaerobic). The focus of this objective was on bench scale research and demonstration of microwave technology to reduce EDCs in municipal sewage sludge. Few, if any, studies have been published on EDC removal in municipal sludge using microwave technology. The microwave application experiments were carried out as a pretreatment and post-treatment to mesophilic anaerobic sewage sludge digestion.

Overall, the primary focus of this research was to demonstrate the potential of sludge treatments to reduce endocrine disrupting chemicals through the examination of mesophilic anaerobic municipal digestion in a municipal secondary wastewater treatment plant and the use of microwave technology for sludge treatment, with and without the addition of hydrogen peroxide. Detection of EDCs was carried out using both chemical analysis and whole estrogenic and androgenic auto bioluminescent yeast screening assays.

3 METHODOLOGY

Municipal sludge is a difficult matrix for laboratory analysis of estrogenic substances. Few laboratories carry out this analysis without first drying the mixed and digested sludge samples. Aside from the concern of losing estrogenic activity in liquid vs. solid phases, this chemical analysis must also be suitable for biological testing (e.g. removal of toxic activity while retaining estrogenic activity in the sludge extracts). In order to directly compare the results from chemical (GC-MS) and biological (BLYES) analysis, the sample preparation must be the same (ideally) or very similar. Yeast estrogen screen (e.g. YES and BLYES) assays are very sensitive to toxins within the sample matrix. Therefore, sample extraction and clean up procedures must remove substances toxic to the yeast strains, while leaving as much of the estrogenic material as possible.

3.1 CHEMICAL ANALYSIS

The targeted compounds in this laboratory protocol were the most potent estrogen, 17β-estradiol (E2), as well as estrone (E1), 17α-ethinyl estradiol (EE2), estriol (E3), and testosterone (TT). The whole estrogenic, androgenic and toxicity analysis used E2 and TT, respectively, as standards for auto bioluminescent yeast screen assays. Although other estrogenic and androgenic compounds can be used to examine whole estrogenic activity, E2 and TT (and dihydrotestosterone) are the most commonly used standards for calculating equivalency concentrations in sludge and wastewater treatment processes.

The optimized chemical analysis protocol for municipal mixed and digested sludges was applied to wastewaters with simple modifications to the extraction step as described in Chapter 3

Methodology, Section 3.1.5.2 Wastewater extraction. The modified procedure provided excellent recoveries of E1 and E2 from wastewaters and no further optimization experiments were considered necessary.

An overview of the laboratory protocol for determination of estrogens in mixed and digested municipal sludge samples is shown in Figure 3-1. The sections of the methodology chapter follow the sequence of this laboratory protocol with sections added at the end of Chapter 3 on method recoveries and quality control procedures. The optimized protocol is presented in the first sections describing extraction, chromatography and derivatization steps and subsequent sections describe experiments designed to develop the method and optimize the laboratory protocol in the following sections.

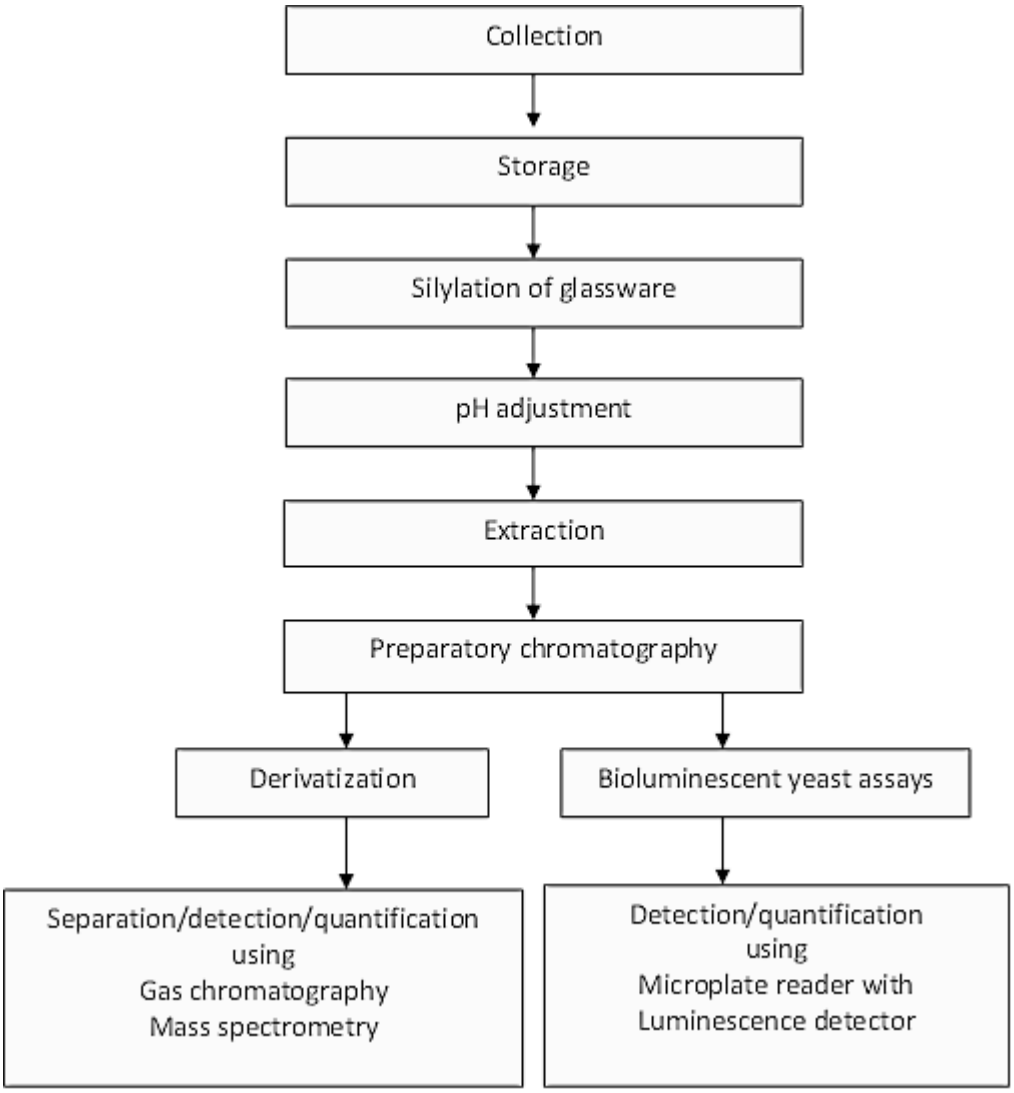


Figure 3-1: Overview of the laboratory protocol for whole estrogenic, whole androgenic and gas chromatography-mass spectrometer analysis of targeted estrogens and androgens in municipal mixed and digested sludge samples.

3.1.1 Collection

Mixed and digested sludge grab samples were obtained from a local domestic wastewater treatment plant with mesophilic (35–40 °C) anaerobic sludge digestion. This MWWTP produced Class B biosolids suitable for recycling to land. Sludge grab samples were obtained immediately prior to the sludge digester (mixed sludge influent to the digesters) and immediately after sludge digestion (digested sludge effluent from the digesters). Grab samples of mixed and digested sludge were collected between 9:00 and 13:00 after routine daily plant maintenance had been completed. Both mixed and digested sludge were collected at the same time.

Mixed sludge had approximately 4% solids, a pH of 6.0, and was a mixture of raw primary sludge and secondary sludge (Figure 3-2). Primary sludge from primary sedimentation tanks was concentrated by gravity thickener and screened in a sludge screen. Secondary sludge from the mixed liquor channel was thickened by dissolved air flotation thickener. The thickened primary and secondary sludges were mixed in a sludge blending tank. The resulting mixed sludge (average 4.1% total solids, pH of 6.0 and 88.1% volatile solids) was comprised of approximately 65% primary sludge and 35% secondary sludge.

The digested sludge had approximately 1.5% solids and a pH of 8.0 and was collected after mesophilic anaerobic digestion. An average 29 day hydraulic retention time in the mesophilic digesters reduced volatile solids by 60%. The digested sludge supernatant had bicarbonate alkalinity concentrations between 3,780 and 4,720 mg/l, as $CaCO_3$.

Table 3-1: Sampling locations within a municipal wastewater treatment plant with tricking filter/solids contact and mesophilic anaerobic sludge digestion.

Set #	Point within MWWTP system where grab samples were collected
MS	Mixed sludge – collected prior to digesters. A combination of sludge from primary and secondary (after trickling filter tank) clarifiers
DS	Digested sludge – collected from sludge digesting tanks after mesophilic digestion process has been completed
WW-1	Influent after bar screening
WW-2	After primary settling tank
WW-3	After trickling filter
WW-4	After solids contact tank
WW-5	After secondary clarifier
WW-6	Final effluent (chlorinated/dechlorinated June-September)

Three one-liter grab samples were collected from each of six locations within the municipal wastewater treatment plant to evaluate wastewater treatment processes. Grab samples were collected at key treatment points, described in Table 3-1, throughout a local municipal secondary wastewater treatment plant, using trickling filter/solids contact technology (Figure 3-2). Wastewater grab samples were collected between 9:00 and 12:30 after daily routine plant maintenance had been completed. Mixed and digested sludge grab samples were collected at the same time as the wastewater samples. Influent and effluent had average total suspended solids of 200 mg/l and 5 mg/l and a pH of 7.0 and 7.5, respectively.

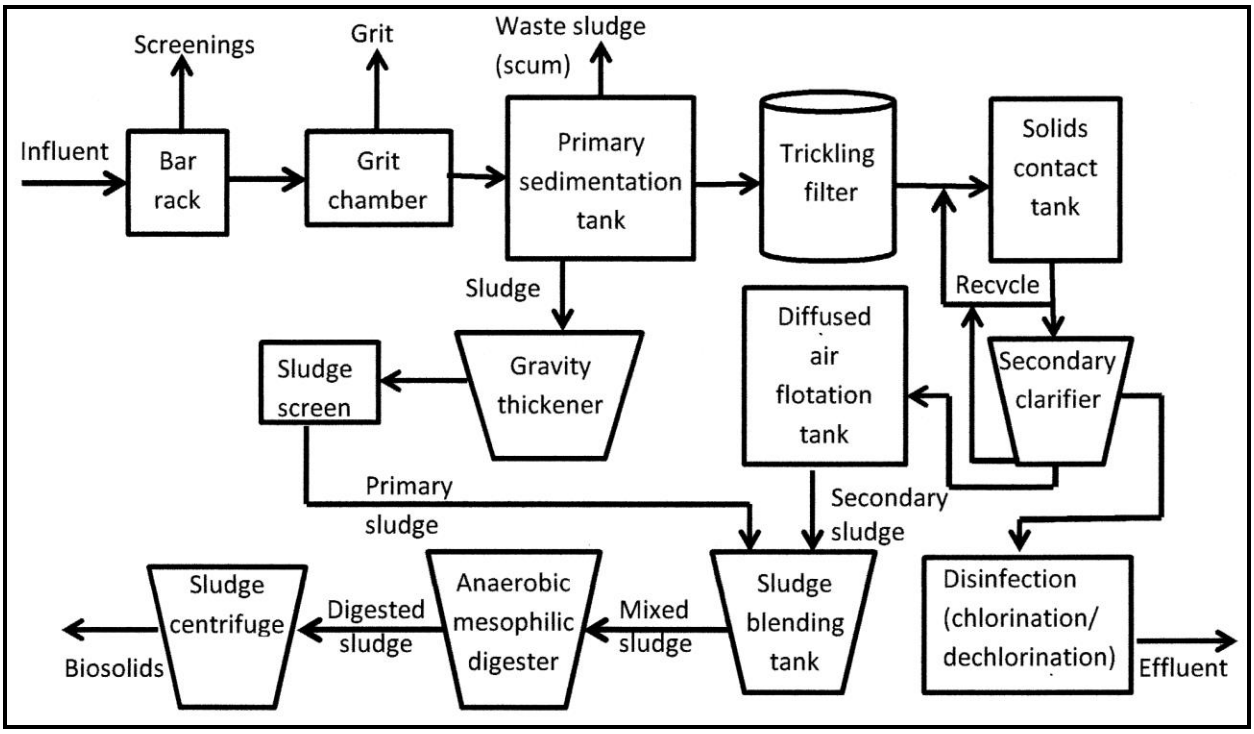


Figure 3-2: Typical secondary wastewater treatment plant using trickling filter/solids contact and anaerobic mesophilic sludge digestion technologies (Tchobanoglous et al., 2003; Metro Vancouver, 2013)

3.1.2 Storage

Sludge (1 litre) and wastewater (8–12 litre) samples were transported to the laboratory within 1 hour of collection and refrigerated (2–4 °C). Samples were processed for extraction within twenty-four hours and extracted within forty-eight hours of collection, unless otherwise stated. If sample extracts required storage between processing steps, extracts were refrigerated for 12 hours or less prior to clean up; underivatized (cleaned and N_2 dried) and derivatized sample extracts were frozen at -27 °C until derivatized and /or GC-MS analysed.

Mixed and digested sludges were processed after two weeks of refrigerated storage at 2–4 °C, to examine the effects of refrigerated storage on levels of estrone and 17β-estradiol naturally present in sludge.

3.1.3 Silylation

All glassware was cleaned, baked at 540 °C for four hours and let cool to room temperature. Glassware was treated with 5% dichlorodimethylsilane (DCDMS) in toluene for 30 minute contact time, rinsed with toluene, methanol, and acetone, then dried in a muffle furnace until the temperature reached 200 °C and cooled overnight. Cleaned and silylated glassware was covered with aluminum foil and stored to protect from contamination.

Optimization of the silylation procedure involved comparing five methods of silylation. HACH test tubes were washed in a laboratory glasswasher three-hour cycle, gently dried at 150 °C in a muffle furnace and cooled overnight. Three replicates of two sets (10 ng and 100 ng E2) were prepared for each of five silylation methods that varied pre-rinse techniques and silylation agent solvents (see Table 3-2). All silylation mixtures were dichlorodimethylsilane (DCDMS) in either dichloromethane (DCM) or toluene. After silylation, all glassware was rinsed with toluene, methanol, and finally acetone before drying in a muffle furnace at 200 °C and left to cool overnight.

Table 3-2: Five silylation methods varying pre-treatment rinse and silylation agents

Sample set	Pre-treatment rinse solution(s)	Silylation agent
# 1	1) Acetone 2) Toluene	5% DCDMS in toluene
#2	Toluene	5% DCDMS in toluene
#3	DCM	5% DCDMS in DCM
#4	No pre-treatment rinse(s)	5% DCDMS in DCM
Control	No pre-treatment rinse(s)	5% DCDMS in toluene

One set of three replicates for each treatment contained 10 ng and the other 100 ng 17β-estradiol in 2 ml methanol. These were vortexed and frozen for one week before being N_2 dried, derivatized and analyzed by GC-MS in one millilitre carrier solvent.

3.1.4 pH Adjustment and Methanol Addition

In an attempt to improve recoveries during extraction, the effects of adding 10% MeOH and/or lowering the pH of sludge samples was explored. Twelve 20 ml digested sludge samples were divided into four treatment sets of three replicates. The four treatments were applied prior to extraction with DCM.

1) Controls.
2) Addition of 10% MeOH v/v and mixed by hand shaking.
3) Addition of 10% MeOH v/v, mixed by hand shaking and pH adjusted from 8.0 to 5.5 with 4N HCl
4) Adjustment of pH from 8.0 to 5.5 with 4N HCl

All digested sludge samples were unspiked, since it was important that the extraction method efficacy would be suitable for environmentally relevant concentrations of E2 in wastewater and sludge matrices. Samples were then processed as described in the optimized protocol (Section 3.1.11). When calculating recoveries in unspiked samples, the treatment set producing the highest average concentration (n = 3) was assumed to represent 100% recovery and other treatment sets were assessed as a percentage of this recovery.

The effects of further lowering the pH in digested sludge to 4.0, with and without the addition of 10% methanol, was examined in a separate experiment. This experiment also looked at the effects of lowering pH with and without the addition of 10% MeOH prior to extraction of mixed sludge samples to improve recoveries of environmentally relevant concentrations of E2. Six 20 ml digested sludge samples were split into two treatment sets of three replicates and nine 20 ml mixed sludge samples were split into three treatment sets of three replicates, for a total of five treatment sets as follows:

1) Digested sludge — pH lowered from 8.0 to 5.5 with 4N HCl, 10% MeOH (v/v) added and hand shaken to mix.
2) Digested sludge — pH lowered from 8.0 to 4.0 with 4N HCl, 10% MeOH (v/v) added and hand shaken.
3) Mixed sludge — control (no treatment)
4) Mixed sludge — pH lowered from 6.0 to 4.0 with 4N HCl and hand shaken
5) Mixed sludge — pH lowered from 6.0 to 4.0 with 4N HCl, 10% MeOH (v/v) added and hand shaken to mix.

Samples were further processed as described in the optimized protocol in Section 3.1.11 of this chapter.

To examine the effects of further lowering the pH for recovery of E2 in spiked mixed sludge samples, nine 20 ml mixed sludge samples were split into three treatment sets of three replicates as follows:

1) pH 6.0—this control set of three replicates of 20 ml mixed sludge was homogenized
2) pH 4.0—pH was adjusted from 6.0 to 4.0 with 4M HCl and 10% methanol added. Mixing was done by hand shaking after each addition and pH was readjusted after 15 minutes (pH bounce due to low alkalinity) to attain a final pH of 4.0.

3) pH 2.0—pH was adjusted from 6.0 to 2.0 with 4M HCl and 10% methanol added. Mixing was done by hand shaking after each addition and pH was readjusted after 15 minutes (pH bounce due to low alkalinity) to attain a final pH of 2.0.

Samples were then processed as described in the optimized protocol (Section 3.1.11).

3.1.5 Extraction

Extraction procedures for mixed and digested sludges must reduce compounds in complex mixtures that complicate detection and identification of target compounds in GC-MS and LC-MS laboratory analyses. While most methods freeze dry municipal sludge samples prior

to extraction, the extraction method described in Section 3.1.5.1 utilizes wet sludge samples for a laboratory protocol to detect and quantify estrogens and estrogenically active components from sludge and wastewater, respectively. The extraction procedure described in 3.1.5.2 for wastewater with high solids content is a liquid-liquid extraction method that incorporates many of the components from Section 3.1.5.1 to extract estrogens (E1, E2, and EE2), testosterone, estrogenic activity and androgenic activity. Subsections 3.1.5.3 and 3.1.5.4 describe the extraction experiments that contributed to the development or optimization of the extraction methods for sludge (Section 3.1.5.1) and wastewater (Section 3.1.5.2). Standards for target analytes (E1, E2, EE2, E3, TT) and internal standard (deuterated 17β-estradiol) were obtained from Sigma Aldrich (see Appendix E-1). Dichloromethane was analytical grade, ACS certified and obtained from Sigma Aldrich. All other solvents used in the extraction protocols were HPLC grade, ACS certified and obtained from Fisher Scientific (see Appendix E-1).

3.1.5.1 SLUDGE EXTRACTION

Prior to extraction, 20 ml sludge samples were homogenized with 10% methanol in a tissue grinder/homogenizer (Brinkman Homoginizer, Polytron) and pH adjusted to 4.0 with 4 M hydrochloric acid (HCl). Sludge and wastewater pH was determined using a pH meter (Beckman ϕ44). Due to the low alkalinity, a pH bounce in mixed sludge samples was significant. While all mixed sludge samples had a final pH between 3.5 and 4.5, the pH was adjusted a second time, approximately 10-20 minutes after the first pH adjustment. Digested sludge samples, with a bicarbonate alkalinity range of 3780–4720 mg/L (Metro Vancouver, 2013), only required one pH adjustment to 4.0.

If samples were spiked to determine recovery values, 500 ng of E1, E2, EE2, E3 and TT were added to each 20 ml sludge sample after pH adjustment and prior to extraction procedures, with the exception of experiments determining losses of target compounds during extraction, clean up, and derivatization steps. Increasing concentrations of these standards (10–750 ng in 20 ml sludge) were also added before extractions to produce standard curves in mixed and digested sludge media. When determining method recoveries, an equal quantity of a surrogate standard (200 or 500 ng deuterated 17β-estradiol) was added to all sludge samples prior to extraction.

Ten millilitres of dichloromethane (DCM) were added to 20 ml aliquots of sludge and contact with solvent enhanced by mechanical shaker (Burrell Wristaction Shaker E23) and ultrasonic bath (Fisher Scientific FS220H). Because DCM would separate from the sample solution during mechanical shaking and ultrasonic bath treatments, hand shaking was incorporated into the procedure to mix the samples before and after placement on the wrist shaker and in the ultrasonic bath. Therefore, after each of the three extraction solvent additions, samples were hand shaken to mix, mechanically shaken for 20 minutes, hand shaken to mix, then placed in ultrasonic bath at 30 °C for 20 minutes, and hand shaken once again to mix. Samples were then centrifuged at 2750 RPM (RCF = 1730 x g) for 15 minutes and the DCM subnatant pipetted into rotary evaporator flasks. Extraction with 10 ml DCM was repeated two times for a total of three extractions. The subnatant from the three extractions was reduced by rotary evaporation to less than 1 ml (almost dry) per sample.

3.1.5.2 WASTEWATER EXTRACTION

One litre wastewater aliquots were pH adjusted to 4.0 with 4 M HCl, then hand shaken to mix in a 2 litre separatory funnel with 10% methanol. An equal quantity of internal standard, deuterated 17β-estradiol was added to all samples. Spiked samples had 500 ng of E1, E2, EE2, E3 and TT added, before mixed by hand shaking in the 2 litre separatory funnel.

Three hundred millilitres DCM was added to the prepared wastewater sample, hand shaken for 15 minutes and let settle an additional 15-20 minutes to separate into water and DCM layers. The bottom DCM layer was funnelled off into a rotary evaporator flask. Two more extractions with 300 ml DCM were carried out for a total of three extractions per wastewater sample.

If the wastewater sample had higher a solids content (e.g. a sample collected after solids contact but before settling tanks) an emulsion of water, DCM, and solids was sometimes formed. Salt was unsuccessful in breaking up the emulsion and, therefore, omitted from the procedure when evaluating sludge and wastewater samples for target compounds. If separation into water and DCM layers was insufficient and this emulsion formed, the emulsion was broken mechanically by first draining into a beaker, then pouring into a second separatory funnel, where layering into water and DCM usually occurred within 15–30 minutes. If the emulsion was still present, it was returned to the wastewater sample supernatant in the first separatory funnel. An additional 200 ml DCM was poured into the wastewater sample and hand shaken for five minutes, before letting the mixture separate into layers. If the solution didn't layer into supernatant and a DCM subnatant within 15 minutes, this emulsion was always mechanically

broken by draining the emulsion from the first separatory funnel into a beaker, then pouring it into the second separatory funnel and repeating the mechanical separation process once again.

The lower DCM layer in each of the three extractions was drained into a labelled rotary evaporator flask. Extractions were reduced to less than 1 ml (almost dry) by rotary evaporation (Heidolph Laborota 4000) at 40 °C.

3.1.5.3 COMPARISON OF FIVE EXTRACTION METHODS

Five extraction methods using Soxhlet, wrist shaker, and shaker–ultrasonic bath apparatus and three extraction solvents were compared for efficacy in extracting E2 from mixed and digested domestic sewage sludge. Eleven sets of three replicate 20 ml samples of domestic mixed (6) and domestic (5) sewage sludge were homogenized, centrifuged for 20 minutes at 2200 RPM, and water portion removed. Seven of these treatment sets were frozen for 20 hours at -28 °C and placed in a manifold freeze dryer (Ilshin TFD5505) for 48 hours under vacuum. The water portion of the centrifuged samples were analyzed for E2 to determine percent E2 in the water portion of mixed and digested sludge samples. A flow chart of the five extraction methods used for the mixed and digested sludges is shown in Figure 3-3. These five extraction methods were applied to the eleven treatment sets as follows:

1) Soxhlet—Soxhlet sleeves (43 mm X 123 mm) were cleaned with four DCM solvent washings (2–3 hours) in a Soxhlet apparatus and N_2 dried under a laboratory solvent exhaust hood. Three freeze dried replicates (1 set) of mixed sludge (average = 0.766 g) were placed in the DCM washed sleeve and Soxhlet extracted with DCM for 18 hours.

2) Wrist shaking (Burrell wristaction shaker, model: E23) with DCM—Ten millilitre of DCM was added to two sets of freeze dried mixed (1 set; n=3; average weight = 0.745 g) and digested sludge (1 set; n=3; average weight = 0.256 g) and wrist shaken for 20 minutes. Solvent was poured into separate rotary evaporator flasks for each replicate sample. Extraction with 10 ml of DCM was repeated two more times for a total of three extractions.

3) Wrists shaking with acetone—Three extractions using 10 ml acetone were conducted on two sets of mixed (1 set; n=3; average weight = 0.713 g) and digested sludge (1 set; n=3; average weight = 0.267 g) as for the "wrist shaking with DCM" method above.

4) Wrists shaking with 30% acetone in DCM—Three 10 ml extractions using 30% acetone in DCM were conducted on two sets of mixed (1 set; n=3; average weight = 0.699 g) and digested sludge (1 set; n=3; average weight = 0.270 g) as per the "wrist shaking with DCM" method above.

5) Wrist shaking/ultrasonic with DCM—Wet solids from four sets of mixed (one spiked with 1 µg E2 and one unspiked) and digested (one spiked with 1 µg E2 and one unspiked) sludge had 10 ml DCM added and placed on the wrist shaker for 20 minutes. Separation of the DCM layer from the wet solids occurred during wrist shaking so samples were hand shaken to thoroughly mix each sample before placement in the ultrasonic bath (Fisher Scientific FS220H) at 30 °C for 20 minutes. Since separation of

the DCM and solid/water phases also occurred during ultrasonic treatment, hand shaken once again to thoroughly mix samples prior to centrifuging at 2750 rpm (RCF = 1730 ‘g’) for 15 minutes. The DCM layer was placed into a separate rotary evaporation flask for each sample. This extraction method was repeated two more times, for a total of three extractions with 10 ml DCM.

All sample extracts were reduced by rotary vacuum evaporation (Heidolph rotary evaporator Laborota 4000) to 1 ml for preparatory chromatography, followed by derivatization, as described in Sections 3.1.6.1 and 3.1.7.1, respectively.

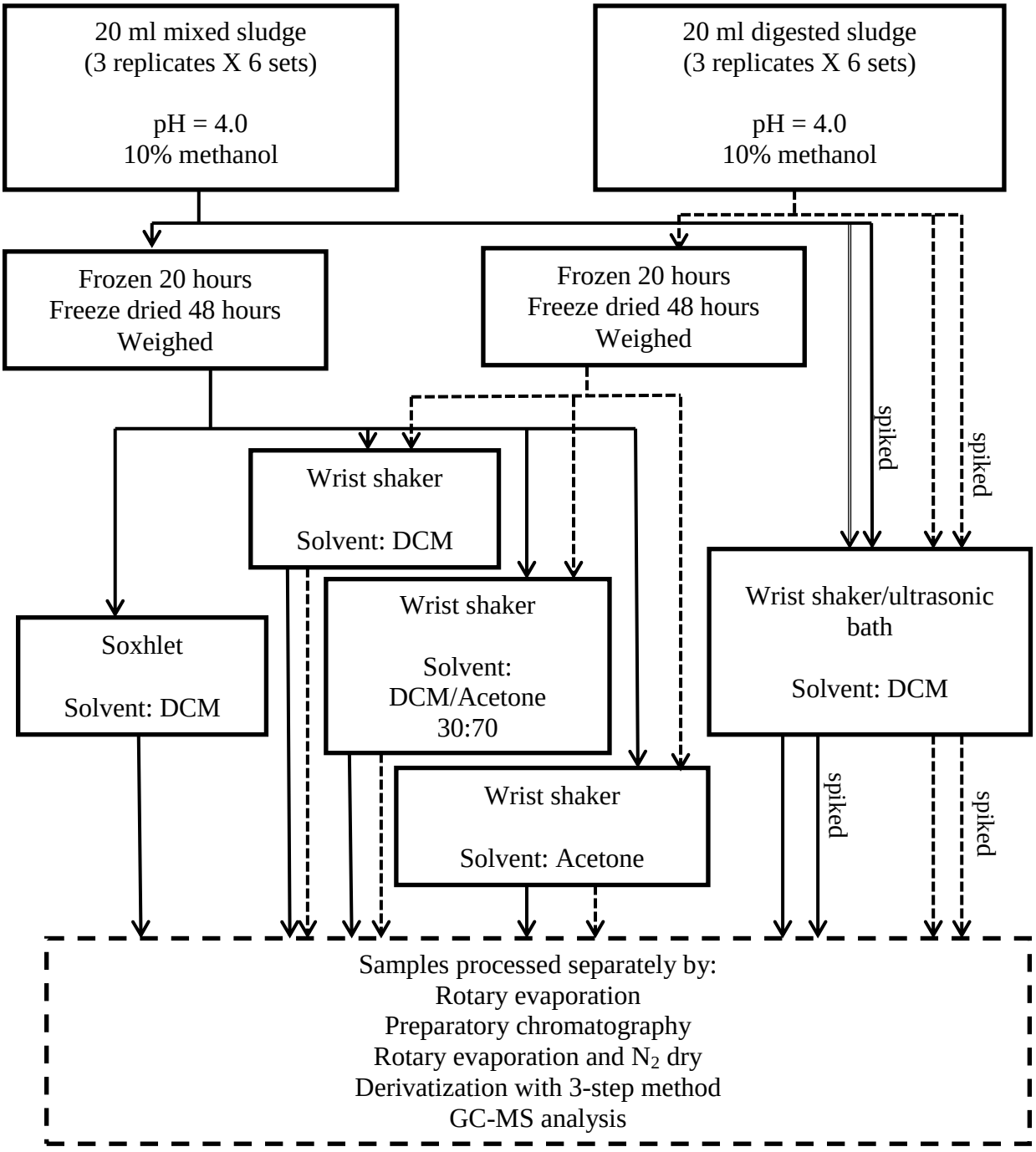


Figure 3-3: Five extraction methods for mixed and digested sludges used dichloromethane (DCM) solvent with Soxhlet, wrist shaker and a combination wrist shaker/ultrasonic bath apparatus. Extraction solvents DCM, acetone and a mixture of acetone-DCM (30:70) were used for the wrist shaker method

3.1.5.4 EXTRACTION SOLVENTS

Acetone is too miscible with wet samples to accommodate the separation required for these extraction methods. To examine if acetone mixtures can recover E2 more efficiently than DCM, freeze dried mixed and digested sludge solids were extracted with three extraction solvent mixtures; 1) DCM, 2) 30% acetone in DCM, 3) acetone. Three replicates per treatment set were extracted using the wrist shaker methods described for treatment sets 2–4 in the previous section. All sample extracts were reduced to 1 ml by rotary evaporation for preparatory chromatography, followed by derivatization as described in as described in the optimized protocol in Section 3.1.11.

Chloroform was also used as the extraction solvent for six 20 ml aliquots of mixed (3) and digested (3) sludge samples to determine if it would provide better recoveries for estrone and 17β-estradiol. Extractions were performed as described above for the DCM extractions.

3.1.6 Chromatography

Mixed and digested sludges are complex mixtures of compounds that complicate detection and identification of target compounds in GC-MS and LC-MS laboratory analyses. Clean up procedures must be incorporated into laboratory protocols for detection of environmentally relevant concentrations of estrogens in municipal sludges and wastewaters with high solids content. Section 3.1.6.1 describes the chromatography method used for evaluating wastewater and sludge treatment processes through the detection of estrogens, E1, E2, EE2 and TT and whole estrogen and androgenic activity in municipal sludge and wastewater samples.

Subsections 3.1.6.2 to 3.1.6.9 describe the chromatography experiments that contributed to the development or optimization of the chromatography method in Section 3.1.6.1.

3.1.6.1 SLUDGE AND WASTEWATER CHROMATOGRAPHY

Preparative chromatography columns were silanized 100 ml glass columns with built in glass frit (coarse porosity fritted disc sealed in bottom of column) for stationary phase support (see Figure 3-4).

Preparatory chromatography columns for 20 ml mixed and digested sludge extracts and 1 L wastewater samples contained stationary phase media obtained from Thermo Fisher Scientific (Appendix E).

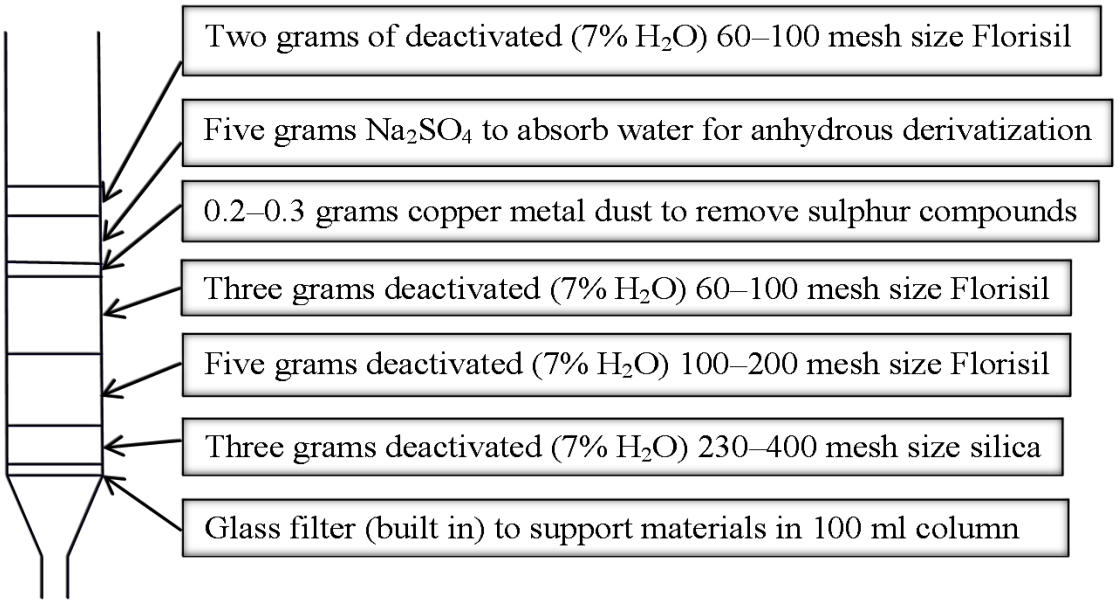


Figure 3-4: Preparatory chromatography column packing materials for separation of natural estrogens from extracts of 20 ml aliquots of municipal mixed and digested sludges

Florisil and silica were baked for 16 hours at 160 °C, cooled in a desiccator to room temperature, and deactivated with 7% distilled water. The above five components were layered in the column with the finest mesh sizes (230–400 mesh SiO_2) at the bottom and larger particle sizes (Na_2SO_4) at the top as shown in Figure 3-4. The column was conditioned with 20 ml DCM and the eluate discarded.

Extracts of DCM from sludge and wastewater samples were rotary vacuumed to less than one ml (almost completely dry). Sample from the rotary evaporator flask was pipetted onto the top of the preparatory chromatography column. The rotary flask was rinsed three times with 3 ml hexane and the rinsate added to the column. Flasks were then rinsed with 20 ml DCM three times for a total of 60 ml DCM and rinsate added to the column each time. Fifty millilitres of 30% acetone in DCM was used to elute the target compounds from the preparatory chromatography column. Where specified, to improve recovery of E3, a final elution of 40 ml 70% acetone in DCM was added to the above elution series.

All DCM and the first 10 ml of 30% acetone (in DCM) were discarded when eluted from the column. The next eluate of 40 ml of 30% acetone in DCM (and 40 ml 70% acetone in DCM when targeting E3) was collected in a rotary evaporating flask for analysis of the target compounds: E1, E2, EE2, and TT. The elution was evaporated by rotary vacuum to 1–2 ml, transferred to a HACH test tube, placed in a block heater at 50 °C, and dried under a gentle N_2 stream. Derivatization was as per Section 3.1.7.1 Sludge and wastewater derivatization, and GC-MS separation, detection and quantification as per Section 3.1.8.

3.1.6.2 ACID DEACTIVATION OF COLUMN SOLID PHASES

The effect of acidification of preparatory chromatography materials on the recovery of E2 in mixed and digested sludges was examined by comparing Florisil and silica deactivated with either 7% HCl or 7% H_2O (Table 3-3). Florisil and silica were baked for 16 hours at 160 °C and stored in an air tight container until deactivated by acid or water. Solid phases were acid deactivated by adding 7g HCl (48 ml of 4M HCl) per 100 g Florisil and silica, mixing thoroughly and drying in a muffle furnace at 100 °C. Deactivation with 7% distilled water (7g H_2O /100g solid phase) in Erlenmeyer flask and mechanically rotated until thoroughly mixed. Preparatory chromatography columns with a combination of Florisil and silica stationary phases were packed as shown in Figure 3-4 and described above. Preparatory chromatography columns with only silica as the stationary phase were packed, as shown in Figure 3-4, but substituting 230–400 mesh silica for Florisil layers. All columns were conditioned with 20 ml DCM and this eluate discarded.

Table 3-3: Acid and water deactivated Florisil and silica packed preparatory chromatography columns for detection of 17β-estradiol (E2) in extracts from 20 ml aliquots of spiked (100 ng) and unspiked mixed (MS) and digested (DS) municipal sludge

Column #	Extract from 20 ml sludge	Deactivated Floricil and silica with 7%	Packing materials
1-3	Mixed sludge with 100 ng E2	H2O	Silica/Florisil
4-6	Digested sludge with 100 ng E2	H2O	Silica/Florisil
7-9	Mixed sludge with 100 ng E2	HCl	Silica/Florisil
10-12	Digested sludge with 100 ng E2	HCl	Silica/Florisil
13-15	Mixed sludge with 100 ng E2	HCl	Silica
16-18	Digested sludge with 100 ng E2	HCl	Silica
19-21	Mixed sludge with 100 ng E2	H2O	Silica
22-24	Digested sludge with 100 ng E2	H2O	Silica
25-27	Mixed	H2O	Silica/Florisil
28-30	Digested	H2O	Silica/Florisil

Mixed and digested sludge 20 ml samples were pH adjusted to 3.0 and 4.0, respectively, 10% methanol added and hand shaken to mix. Spiked samples had 100 ng added to each 20 ml sample and hand shaken to mix. Sludge samples were extracted three times by adding 10 ml chloroform and mixing by hand shaking, mechanically extracted by wrist shaker for 10 minutes, and centrifuged for 15 minutes, before the supernatant was removed to rotary flasks for individual samples. This extraction process was repeated two more times for a total of three extractions.

Sludge extracts were reduced to 0.5–1 ml by rotary evaporation and sample placed on preparatory chromatography columns, as described in Table 3-3. Flasks were rinsed with 2–3 ml hexane three times and rinsate placed on column over sample. Flasks were rinsed with three 20 ml DCM and used as eluate for sample. The initial clean up elution of 60 ml DCM was discarded as was the first 10 ml of 50 ml of 30% acetone in DCM used to elute E2 from the sample in the chromatography column. Although usually discarded, these initial clean up elutions from columns packed with silica (acid and water activated) were analyzed for E2; since the elution fractions from columns packed with only silica had not been examined previously and colour was eluted in these fractions from the water activated silica packed columns, it was deemed to be a worthwhile extra task. The last 40 ml of the elution with 50 ml 30% acetone in DCM was collected and analyzed for recovery of 17β-estradiol from the mixed and digested sludge samples.

Nitrogen drying was as per Section 3.1.6.1 Sludge and wastewater chromatography; derivatization was as per Section 3.1.7.1 Sludge and wastewater derivatization; and GC-MS separation, detection and quantification as per Section 3.1.8.

3.1.6.3 WATER DEACTIVATION OF COLUMN SOLID PHASES

All Florisil and silica materials were baked at 160 °C for 16 hours and stored in a desiccator until use or deactivation with distilled water. Nine 100 ml preparatory chromatography columns were packed with sodium sulphate (5 g), copper metal (0.2–0.3 g), 60–100 mesh and 100–200 mesh Florisil (5 g each) and 230–400 mesh silica (3 g) as shown in (Figure 3-4). Six of these preparatory chromatography columns were prepared with Florisil and silica deactivated with 5% distilled H_2O and the other three columns prepared with activated Florisil and silica (baked and stored in desiccator until use).

3.1.6.3.1 Activated versus deactivated Florisil and silica

Activated versus deactivated Florisil (5% H_2O) and silica in the above preparatory chromatography design were compared for recovery of 20 µg 17β-estradiol spiked into mixed sludge extracts. Six of nine extracts from 20 ml mixed sludge samples were spiked with 20 µg of 17β-estradiol. The three unspiked samples were used as replicate controls to obtain a baseline estimate of E2 in the mixed sludge. The six spiked samples were split into two sets of three replicates.

Three spiked samples and the three control samples were subject to clean up by the chromatography columns with deactivated Florisil and silica as the stationary phase while the

remaining three spiked samples were subject to clean up by the chromatography columns with activated Florisil and silica. Column conditioning, elution, N_2 drying was as per Section 3.1.6.1 Sludge and wastewater chromatography; derivatization was as per Section 3.1.7.1 Sludge and wastewater derivatization; and Section 3.1.8 GC-MS separation, detection and quantification.

3.1.6.3.2 Florisil and silica deactivated with 5% versus 7% water

This work was continued by following this experimental procedure but comparing Florisil and silica deactivated with either 5% or 7% distilled water. Florisil and silica materials in the above preparatory chromatography design were deactivated with either 5% or 7% water and compared for recovery of 20 µg 17β-estradiol spiked into mixed sludge extracts.

Six of nine extracts from 20 ml mixed sludge samples were spiked with 20 µg of 17β-estradiol. Three replicate unspiked samples were used as controls to obtain a baseline estimate of E2 in the mixed sludge. The six spiked samples were split into two sets of three replicates.

Three replicate spiked samples and the three replicate control samples were subject to clean up by the chromatography columns with Florisil and silica deactivated with 5% distilled water as the stationary phase while the remaining three replicate spiked samples were subject to clean up by the chromatography columns with Florisil and silica deactivated with 7% distilled water.

Column conditioning, elution, N_2 drying was as per Section 3.1.6.1 Sludge and wastewater chromatography; derivatization was as per Section 3.1.7.1 Sludge and wastewater derivatization; and GC-MS separation, detection and quantification as per Section 3.1.8.

3.1.6.4 COLUMN CONDITIONING

Six of nine extracts from 20 ml mixed sludge samples were spiked with 20 µg of 17β-estradiol, split into two sets of three replicates and put through chromatography columns described in the previous section but conditioned with either hexane or dichloromethane. Three unspiked samples were used as replicate controls to obtain a baseline estimate of E2 in the mixed sludge and put through three preparatory chromatography columns conditioned with DCM. Analyte elution and N_2 drying was as per Section 3.1.6.1 Sludge and wastewater chromatography; derivatization was as per Section 3.1.7.1 Sludge and wastewater derivatization; and Section 3.1.8 GC-MS separation, detection and quantification.

3.1.6.5 INITIAL CLEAN UP VOLUME

The initial clean up volume elution of sample with 60 ml DCM in this preparatory chromatography method is discarded. To determine if this discarded initial elution could be reduced to 20 ml DCM, twelve extracts from 20 ml unspiked mixed (6) and digested (6) sludge samples were split into four sets of triplicate samples for the preparatory chromatography protocol described in Section 3.1.6.1, using either 20 ml or 60 ml DCM for the initial elution (as shown in Table 3-4).

Table 3-4: Dichloromethane (DCM) versus chloroform ($CHCl_3$) in elution solvent mixtures and variation of initial elution volume 20 ml versus 60 ml in preparatory chromatography column elution of estrogenic activity in mixed (MS) and digested (DS) sludges

Elution solvent mixtures				
Conditioning solvent	1^{st} elution	2^{nd} elution	MS (n)	DS (n)
DCM	20 ml DCM	50 ml 30% acetone in DCM	3	3
$CHCl_3$	20 ml $CHCl_3$	50 ml 30% acetone in $CHCl_3$	3	3
DCM	60 ml DCM	50 ml 30% acetone in DCM	3	3

Chromatography column elutions and N2 drying was as per Section 3.1.6.1 Sludge and wastewater chromatography; derivatization was as per Section 3.1.7.1 Sludge and wastewater derivatization; and GC-MS separation, detection and quantification as per Section 3.1.8.

3.1.6.6 PRIMARY ELUTION SOLVENT

To determine if chloroform ($CHCl_3$) can be substituted as the primary elution solvent for this preparatory chromatography method, an additional six extracts from 20 ml unspiked mixed (3) and digested (3) sludge samples were placed in six preparatory chromatography columns (as shown in Figure 3-4). Columns were conditioned with $CHCl_3$ and samples were eluted with $CHCl_3$ substituted as the primary solvent for the elution mixtures and 20 ml $CHCl_3$ as the initial elution volume (Table 3-4).

Samples were nitrogen dried as per Section 3.1.6.1 Sludge and wastewater chromatography; derivatization was as per Section 3.1.7.1 Sludge and wastewater derivatization; and GC-MS separation, detection and quantification as per Section 3.1.8. Results were compared to the digested and mixed sludge extracts eluted with DCM as the primary solvent and the first elution volume as 20 ml described in Section 3.1.6.5.

3.1.6.7 ELUENT SOLVENT RATIOS

In this research, the eluate from the preparatory chromatography columns was collected in 20 ml fractions, in order to determine the efficacy of two eluents (solvent mixtures) to separate and recover 17β-estradiol in 20 ml samples of mixed and digested sludge from a domestic wastewater treatment plant. Preparatory chromatography columns were packed as shown in Figure 3-4. Columns were conditioned with 20 ml of DCM prior to adding the extracted sample (reduced by rotary evaporated to 1–2 ml) and 6–8 mls hexane rotary flask rinsate.

Two acetone:DCM eluent mixtures were compared to optimize elution of E2 in mixed sludge extract from a preparatory chromatography column (Figure 3-4). Twelve mixed sludge samples were pH adjusted to 4.0, spiked with 1 mg E2, extracted with DCM as described above, rotary evaporated to 1 ml, and subjected to preparatory chromatography using one of two elution methods utilizing different acetone in DCM eluent mixtures. Samples were washed from the rotary flask with 6 ml hexane and placed on the preparatory chromatography column. Elution 1 was comprised of 60 ml DCM, 60 ml 20% acetone in DCM, and 40 ml of 30% acetone in DCM. Elution 2 was comprised of 60 ml DCM, 60 ml 30% acetone in DCM, and 40 ml of 70% acetone in DCM. Eluate was collected in 20 ml aliquots and analysed for percent recovery of E2 from the spiked samples. Eluent mixtures in 20 ml aliquots for the two methods, elution 1 and elution 2, are detailed in Table 3-5.

Table 3-5: Solvent mixture eluates analyzed in 20 ml aliquots for two preparatory chromatography column elution protocols to recover estrogenic activity from mixed and digested sludge extracts

20 ml aliquots of eluate	**Elution 1**	**Elution 2**
	Eluent	Eluent
1	DCM	DCM
2	DCM	DCM
3	DCM	DCM
4	Acetone:DCM (2:8)	Acetone:DCM (3:7)
5	Acetone:DCM (2:8)	Acetone:DCM (3:7)
6	Acetone:DCM (2:8)	Acetone:DCM (3:7)
7	Acetone:DCM (3:7)	Acetone:DCM (7:3)
8	Acetone:DCM (3:7)	Acetone:DCM (7:3)

Rotary evaporation of the 20 ml aliquots and nitrogen drying was as per Section 3.1.6.1 Sludge and wastewater chromatography; derivatization was as per Section 3.1.7.1 Sludge and wastewater derivatization; and GC-MS separation, detection and quantification as per Section 3.1.8.

3.1.6.8 FRACTIONATION OF ELUENT MIXTURES

Elution mixture 2 (Table 3-5) efficacies for E1, EE2, E3 and TT in addition to E2 were examined by comparing recoveries in triplicate spiked and unspiked mixed and digested sludge samples. Triplicate mixed and digested sludge samples were spiked with 500 ng of each of the target compounds prior to extraction. An internal standard, 17β-estradiol 2D (E2dd) was added prior to derivatization. These sludge samples were extracted with DCM, rotary evaporated to 0.5 – 1 ml, flasks were rinsed three times with 2 ml hexane and rinsate/sample mix was added to the top of preparatory chromatography columns and eluted with elution mixture 2 as described in

Section 3.1.6.1. Eluate was collected in three aliquots of 1) 60 ml DCM; 2) 60 ml 3:7 acetone: DCM; and 3) 40 ml of 7:3 acetone: DCM.

Eluate aliquots were rotary evaporated and N_2 dried as per Section 3.1.6.1 Sludge and wastewater chromatography; derivatization was as per Section 3.1.7.1 Sludge and wastewater derivatization; and GC-MS separation, detection and quantification as per Section 3.1.8. Percent recoveries of E1, E2, EE2, E3 and TT for each of the three eluate aliquots were estimated by subtracting concentrations in unspiked mixed and digested sludge from concentrations found in spiked samples.

3.1.6.9 ADDITIONAL ELUTION STEP

The final elution with 40 ml of 70% acetone in DCM was carried out during the protocol in Section 3.6.8 to determine if the eluent, 30% acetone in DCM, used to elute 17β-estradiol from the column was adequately polar to capture not only 17β-estradiol but also estrone, estriol, 17α-ethinyl estradiol, deuterated 17β-estradiol and testosterone (please see Table 4-14 in the Results and Discussion chapter for further discussion).

Rotary evaporation of the 20 ml aliquots and nitrogen drying was as per Section 3.1.6.1 Sludge and wastewater chromatography; derivatization was as per Section 3.1.7.1 Sludge and wastewater derivatization; and GC-MS separation, detection and quantification as per Section 3.1.8.

3.1.7 Derivatization

An equal quantity of internal standard (200 or 500 ng), deuterated 17β-estradiol, was added to all sludge and wastewater samples prior to derivatization steps. Deuterated 17β-estradiol and all standards for the target analytes (E1, E2, EE2, E3, and TT) were purchased from Sigma Aldrich (see Appendix E-1).

Detecting trace amounts of 17β-estradiol with gas chromatography, followed by mass spectrometry (GC-MS), is difficult due to the relatively low volatility of natural estrogens. Volatility can be increased by derivatization of the functional group with silylation agents. Derivatization agents, N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) and methoxamine hydrochloride (MOX HCl) were obtained from Thermo Scientific. Anhydrous pyridine, a reaction catalyst, was purchased from Sigma-Aldrich.

Subsection 3.1.7.1 describes the derivatization method used in the laboratory protocol to evaluate sludge and wastewater treatment processes by assessing concentrations of estrogens (E1, E2, and EE2), testosterone, whole estrogenic and androgenic activity in municipal sludge and wastewater. Subsequent sections in this chapter describe experiments conducted to develop and optimize the derivatization method in Section 3.1.7.1.

3.1.7.1 SLUDGE AND WASTEWATER DERIVATIZATION

Chromatography elutions for each sludge and wastewater sample to be analyzed by GC-MS were reduced to 1 ml by rotary evaporation and transferred by pipette to a HACH test tube. Rotary flasks were rinsed 3X with 2 ml DCM and rinsate added to the HACH test tube. Samples

were N_2 dried in a block heater at 50 °C until completely dry. Samples were derivatized using a three-step method:

1) One hundred microliters BSTFA with 1% TMCS and 100 µl pyridine were added to each sample, HACH tube was tightly capped and vortexed until thoroughly mixed. Samples were heated in a block heater at 70 °C for 15 minutes, cooled for 15 minutes at room temperature, vortexed to mix thoroughly and N_2 dried at 50 °C.

2) Seven hundred µl 2% methoxyamine in anhydrous pyridine was added to the N_2 dried samples and heated in a block heater at 70 °C for 60 minutes, cooled for 15 minutes at room temperature and vortexed to mix thoroughly.

3) One hundred microliters BSTFA with 1% TMCS and 200 µl pyridine were added and samples were heated to 70 °C in a block heater for 15 minutes, cooled to room temperature, vortexed to thoroughly mix and transferred, with silylated disposable glass transfer pipettes, to a GC vial, capped and labeled for GC-MS analysis.

3.1.7.2 SILYLATION AGENT RATIO

The silylation reagent used in these derivatization techniques is N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), with 1% trimethylchlorosilane (TMCS). Since the exact concentration of TMCS is seldom critical, either BSTFA or BSTFA + 1% TMCS can be used as the lower component in most cases (Thermo Scientific, 2008). These derivatization techniques involved replacement of the acidic hydrogen in the alcohol groups of 17β-estradiol

with an alkylsilyl group. The introduction of this silyl group can enhance mass spectrometric properties by producing characteristic ions of use in trace analyses.

Six BSTFA based trimethylsilylation derivatization agent mixture ratios (Table 3-6) for detection of 17β-estradiol by GC-MS were compared; using mixed and digested sludge matrices. Extractions from 20 ml aliquots of 1) mixed sludge (18 samples) and 2) digested sludge (18 samples) were spiked to 2.5 µg/L 17β-estradiol after preparative chromatography. Six BSTFA based derivatization procedures were replicated three times with both mixed and digested sludge extracts, for a total of six samples for each procedure. All samples were derivatized for 15 minutes at 60 °C, cooled to room temperature, N_2 dried, reconstituted with 1 ml toluene and vortexed.

Table 3-6: Derivatization agent mixtures for GC-MS detection of 17β-estradiol (E2) in municipal mixed sewage sludge

Method abbreviation	**Mixture (100 ul)**	**Derivatization agents**
90-10T	BSTFA:TMCS (90:10)	N,O-bis(trimethylsilyl)trifluoroacetamide + trimethylchlorosilane
70-30	BSTFA: pyridine (70:30)	N,O-bis(trimethylsilyl)trifluoroacetamide + pyridine
90-10D	BSTFA:DCDMS (90:10)	N,O-bis(trimethylsilyl)trifluoroacetamide + dichlorodimethylsilane
50-50	BSTFA: pyridine (50:50)	N,O-bis(trimethylsilyl)trifluoroacetamide + pyridine
Control	none	none
BSTFA	100	N,O-bis(trimethylsilyl)trifluoroacetamide

3.1.7.3 SILYLATION TIME-TEMPERATURE

The TMS reagents are thermally stable at GC column and injector temperatures of 300 °C. However, decomposition of BSTFA can be significant at temperatures above 75 °C, especially in the presence of metals (Thermo Scientific, 2008). Therefore all derivatization and N_2 drying temperatures were below 75 °C.

To test the effect of time on trimethylsilylation of E2 in unspiked MS samples, six extracts from 20 ml samples of unspiked MS were derivatized with 50 µl BSTFA (with 1% TMCS) + 50 µl pyridine at 70 °C for either 15 minutes (3 samples) or one hour (3 samples).

In an effort to optimize trimethylsilylation derivatization of E2 in domestic sludge extracts, three variations of the time-temperature relationship were examined. Three replicates each of 20 ml samples of unspiked MS; MS spiked with 1 mg/L E2; unspiked DS; and DS spiked with 1 mg/L E2 (total of 12 samples per treatment) were derivatized using 50 µl BSTFA with 1% TMCS + 50 µl pyridine and subjected to one of the following time-temperature treatments:

1) 70 °C for 15 minutes
2) 70 °C for seven hours
3) 60 °C for one hour

3.1.7.4 OXIMATION-SILYLATION AND PRE-SILYLATION

To determine if a two-step, oximation-silylation, or a three step, silylation-oximation-silylation, derivatization procedures was appropriate for other sex hormones, four other steroids,

estrone, 17α-ethinyl estradiol, estriol, and testosterone were targeted in addition to 17β-estradiol. In addition to the silylation reagent BSTFA with 1% TMCS, another derivatization agent, methoxyamine, was added in an attempt to form an oxime derivative (CH_3ON) with the carbonyl group in estrone and testosterone and improve chromatographic performance.

Twenty-one samples with 500 ng each of estrone, 17β-estradiol, 17α-ethinyl estradiol, estriol, testosterone and an internal standard (deuterated 17β-estradiol) were prepared. These were split into seven sets of three replicates and used to compare seven methods varying derivatization agent dosages for one-step (silylation), two-step (oxyamination-silylation) and three-step (silylation-oxyamination-silylation) derivatization at 60 °C under varying time conditions, as detailed in Table 3-7. Pyridine was added to make up volume to 1 ml and vortexed prior to transferring sample from HACH tube to GC vial, capped and labeled for GC-MS analysis.

Table 3-7: Seven methods for derivatizing estrone (E1), 17β-estradiol (E2), 17α-ethinyl estradiol (EE2), estriol (E3), and testosterone (TT) using combinations of silylation and oximation at 60 °C while varying derivatization time

Method		**Derivatization agent(s)**		**Derivatization treatment**	
Sample set	Step	Volume (µl)	Agent	Time (min)	Temperature(°C)
1–Si	1	100 100	BSTFA+1%TMCS Pyridine	15	60
2-MoSi + 2 method blanks	1	200 700	10% Methoxyamine Pyridine	30	60
	2	100	BSTFA+1%TMCS	15	60
3-MoSi	1	200 600	10% Methoxyamine Pyridine	30	60
	2	100	BSTFA+1%TMCS	15	60
4-SiMoSi	1	100 100	BSTFA+1%TMCS Pyridine	15	60
	2	200 500	10% Methoxyamine Pyridine	30	60
	3	100	BSTFA+1%TMCS	15	60
5-MoSi	1	200 600 100	10% Methoxyamine Pyridine BSTFA+1%TMCS	30	60
6-SiMoSi	1	100 100	BSTFA+1%TMCS Pyridine	15	60
	2	200 500	10% Methoxyamine Pyridine	30	60
	3	100	BSTFA+1%TMCS	15	60
7-MoSi + 2 method blanks	1	200 700	10% Methoxyamine Pyridine	45	60
	2	100	BSTFA+1%TMCS	15	60

3.1.7.5 TIME-TEMPERATURE

Temperatures and times of derivatization steps can be kept flexible, because they present a compromise between completeness of reaction, time and efforts needed to perform the reactions, and breakdown of certain compounds (Fiehn, 2006).

Comparison of time-temperature relationships for two-step (oximation-silylation) and three-step (silylation-oximation-silylation) derivatization methods were compared to optimize detection of estrone and 17β-estradiol by GC-MS, although other compounds (estriol, 17α-ethinyl estradiol and testosterone) were targeted as well. All samples contained 500 ng of each of the target compounds. These twelve treatments are detailed in Table 3-8. All samples were cooled to room temperature (20 °C) after each treatment and prior to the next treatment or nitrogen drying at 50 °C. Three treatment sets were stored at -28 °C for two weeks prior to being run on the GC-MS.

Table 3-8: Twelve derivatization methods varying time and temperature during oximation and trimethylsilylation of estrone, 17β-estradiol, 17α-ethinyl estradiol, estriol, and testosterone

	70 µl BSTFA 30 µl pyridine			200 µl 10% methylamine + 700 µl pyridine			100 µl BSTFA			Toluene added as carrier solvent	Storage at -28°C for 2 weeks
Set #	Temp °C	Time (min)	N_2 dry	Temp °C	Time (min)	N_2 dry	Temp °C	Time (min)	N_2 dry		
1				70	15		70	15			
2				70	60		60	15			
3				70	60		70	60			
4				60	15		60	15			
5				60	60		70	15			
6				60	60		60	15			
7				70	15	yes	70	15		900 µl	
8				70	15	yes	70	15	yes	1 ml	
9				20	15		20	15			yes
10	70	15	yes	70	15		70	15			yes
11	70	15	yes	70	15		20	15			yes
12				70	60		70	60			yes

To optimize time-temperature for the three-step derivatization method, five treatment sets of three replicates, compared silylation, methoximation-silylation, and three time-temperature variations for pre-silylation-methoximation-silylation, as detailed in Table 3-9. All samples

contained 1 µg of estrone, 17β-estradiol, 17α-ethinyl estradiol, estriol, and testosterone. These samples differed from the above experiment as all samples also contained 500 ng of 2-deuterated 17β-estradiol (E2dd) as a quality control check for E2 derivatization results.

Table 3-9: Three time-temperature variations for a silylation-methoximation-silylation (SMS) derivatization method compared to silylation (Si) and methoximation-silylation (Mo-Si) methods

Set #	70 µl BSTFA 30 µl pyridine			200 µl 10% methylamine + 700 µl anhydrous pyridine		100 µl BSTFA		GC carrier solvent added to make up a one millilitre volume
n=3	Temp °C	Time (min)	N_2 dry	Temp °C	Time (min)	Temp °C	Time (min)	
1-Si	70	15						+ 900 µl pyridine
2-Mo-Si				70	30	70	15	
3-SMS	70	15	yes	70	30	70	15	
4-SMS	70	15	yes	70	60	70	15	
5-SMS	70	15	yes	70	30	70	30	

To determine if N_2 drying at a higher temperature decreases recovery of E2 in sludge matrices, six mixed sludge samples were derivatized using 70 µl BSTFA + 30 µl pyridine and heated at 70 °C for 15 minutes. After cooling to room temperature, three samples were N_2 dried at 50 °C and the remaining three samples were N_2 dried at 37 °C, before reconstituting samples in one millilitre toluene.

3.1.7.6 PRE-SILYLATION IN MIXED SLUDGE

To determine how the three and two step derivatization methods would perform in the detection of the target compounds in sludge media, six extracts from 20 ml of mixed sludge were spiked with standard mixtures of estrone, 17β-estradiol, 17α-ethinyl estradiol, estriol, and testosterone. Three of these extracts were derivatized by the two-step method (methoximation-silylation), and the other three by the three-step method (silylation-methoximation-silylation).

A mixture of 2% methoxyamine·HCl (M.W. 83.51) in pyridine was applied to three replicates of 5 µg mixtures of 17β-estradiol, 17α-ethinyl estradiol, estrone, estriol, and testosterone using one of two derivatization methods at 70 °C (see Table 3-10):

1) Methoximation before silylation (two step method)

2) Methoximation between two silylation steps (three step method)

Table 3-10: Silylation and oximation derivatization steps

Derivatization step	Derivatization agent	Time (minutes)	Temperature (°C)
initial silylation (three step derivatization method only)	100 µl bis(trimethylsilyl)trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS) and 100 µl pyridine	15	70
oximation	700 µl 2% methoxyamine hydrochloride in pyridine	30	70
final silylation	100 µl BSTFA + 1% TMCS	15	70

3.1.8 Separation/Identification/Quantification

Gas chromatography/mass spectroscopy–selective ion monitoring (GC/MS-SIM) was used to separate, identify and quantify the target compounds. Quantitation was based on peak abundance of the quantitation ion in the mass spectrum (intensity versus mass-to-charge ratio). At least three molecular ions in the base peaks for each of the target compound derivatives were monitored to obtain maximum detection sensitivity and specificity. The ions with the highest molecular weight and strong well shaped peaks were used as the quantitation ions (see Table 3-11).

Table 3-11: Quantitation ions used to determine GC-MS peak abundance for hormone derivatives

Hormone	Abbreviation	Quantitation ion	Molecular ion 1	Molecular ion 2
Estrone	E1	342	257	327
17β-estradiol	E2	416	285	401
17β-estradiol 2D	E2dd	418	287	403
17α-ethinyl estradiol	EE2	425	285	440
Estriol	E3	504	345	311
Testosterone	TT	360	345	270

A Hewlett Packard HP6890 series GC system was coupled with a Hewlett Packard 5973 Mass Selective Detector (electron ionization). It is a high performing mass selective detector with a high energy dynode (HED) electron multiplier detector. The GC-MS was operated in scan mode to identify monitoring ions, retention time, intensity and peak shape of derivatized analytical standards then operated in selected ion monitoring (SIM) mode for all laboratory analytical protocols. Table 3-12 shows the SIM program time-temperature settings used for GC operation. The silylated injection port liner was replaced, needle cleaned, septum replaced, O-ring checked and column trimmed prior to every experimental run. One µl of the 1 ml sample/standard/blank in the GC-MS vials in the auto-sampler was injected into 280 °C splitless inlet and carried through the ~18 m Agilent capillary (phenyl arylene polymer) column with helium as the carrier gas.

The TMS reagents are thermally stable at GC column and injector temperatures of 300-350 °C. A bake out program of 280 °C for one hour was used prior to all sample runs to burn off any contaminants added during instrument maintenance. The mass spectrometer was tuned (MS tune program) and the output of a known standard, checked prior to every run. The gas chromatography program time-temperature settings are detailed in Table 3-12.

Table 3-12: Gas chromatography time-temperature program settings

Oven ramp	Ramp (°C /min)	Hold temperature (^{o}C)	Hold time (min)	Run time
Initial		95	1.00	1.00
Ramp 1	15.00	290	1.00	15
Ramp 2	5.00	310	8.00	29

Method detection limits and limits of quantification for each of the target compounds were determined by spiking method reagent blanks with a series of standard dilutions. Concentrations of 1.0, 5.0, 10, 25 and 100 ng of estrone, 17β-estradiol, 17α-ethinyl estradiol, estriol, and testosterone were prepared in method blanks and analyzed by GC-MS in one millilitre derivatization agent. Seven replicates of the 1.0 and 5.0 ng concentrations were prepared for all target compounds, except 17β-estradiol, and seven replicates of a 10.0 ng solution of testosterone was also prepared. Further dilutions of 0.05, 0.1, 0.5, 1.0 and 5.0 ng E2/ml derivatizing agent were prepared from method blanks as above and analyzed by GC-MS. Finally, seven 0.5 ng/ml and 1.0 ng/ml dilutions of E2 were prepared in method blanks.

GC-MS data from all sets of seven replicates were used to validate the calculated analytical limits for this method using laboratory accepted standards (Ripp, 1996; American Public Health Association, 2005). The method detection limits were approximately 3X the instrument noise, the distance between the maximum and minimum baseline response on the chromatograph. While instrument noise is measured directly from the GC-MS chromatogram, two tests were carried out to validate the calculated detection limits.

The signal to noise (S/N) ratio is a useful test to validate the detection limit, with the S/N ratio ideally falling within 2.5 to 10 X instrument noise. The S/N ratio was calculated as the average of seven replicates divided by the sample standard deviation of the replicates.

The second validation method was by serial dilutions (Ripp, 1996; American Public Health Association, 2005), in which analytical standards were prepared: 1) at a level significantly higher than the MDL and 2) successive dilutions of the standard down to and below the MDL. The dilutions of the analytes were used as a practical check to confirm that they could be detected at the MDL concentrations and the calculated MDL was correct.

3.1.9 Recoveries

To determine method recoveries, a surrogate (or system monitoring compound), deuterated 17β-estradiol (E2dd), was added to samples prior to extraction procedures. The deuterated E2 standard was very expensive ($400 / 5 mg). It was beyond the resources of this research project budget to purchase another surrogate and/or internal standard. Therefore, during the recovery experiments only, the use of E2dd as an internal standard was discontinued; smaller batches run, and instrument performance well evaluated with several target compound standards and reagent blanks run at the beginning and end of the run, as well as repeated between samples throughout the run.

Losses of E2 throughout the method was evaluated by spiking three replicates of three sets of 20 ml mixed and digested sludge and two sets of one litre influent and effluent wastewater samples, for a total of twenty-four spiked samples and three replicates of the control sample sets for each of the four matrices (12 unspiked samples). One µg E2 was spiked into one set of each of the four matrices just prior to: 1) extraction; 2) preparatory chromatography (mixed and digested sludges only); or 3) derivatization steps. Spiking just before the optimized three step derivatization method described above was assumed to represent 100% of E2 in the

sample. To determine losses of E2 throughout the phases of the chemical analysis, concentrations of E2 recovered from samples spiked prior to extraction or chromatography were compared to those spiked immediately prior to derivatization.

3.1.10 Quality Control

Maintenance on the GC-MS was performed as recommended by the manufacturer prior to every sample run (Agilent Technologies, 2001) including column trimming, needle cleaning, new septum and silanized injection port liner, and one hour bake program at 280 °C.

A set of at least five standards in toluene or derivatizing agent, of concentrations relevant to the experiment, were run before and after the samples as well as between every 3-6 samples depending on samples size for each experimental treatment. Sample results may have been adjusted if the standard repeated throughout the run showed a variation greater than 10% and internal standard variation justified an adjustment to peak abundance values.

Two reagent blanks of toluene and derivatization agent were run at the beginning and two at the end of batch runs and one of each reagent blank was tested repeatedly between sample treatments (3-6 samples). At least two method blanks were prepared and run with each sample batch.

Deuterated 17β-estradiol (E2dd) was added to each sample, prior to derivatization, as an internal standard and peak abundance for sample sets may have been adjusted, if variation in concentration throughout the run indicated variance in system performance.

To justify the use of standard curves, based on standards in derivatizing agent to determine concentrations of target compounds in sludge, standard curves were created from standards in mixed and digested sludge media. Twelve 20-ml aliquots of mixed (6) and digested (6) sludge were extracted as per Sections 3.1.5.1 sludge extraction and 3.1.5.2 wastewater extraction; cleaned up as per Section 3.1.6.1 Sludge and wastewater chromatography; derivatized as per Section 3.1.7.1 Sludge and wastewater derivatization; and GC-MS separated, detected and quantified as per Section 3.1.8.

Immediately prior to derivatization, standards for E1, E2, EE2, E3, and TT were added to the eluted solvents from the preparatory chromatography step after reduction by rotary vacuum and prior to N_2 drying. For mixed and digested sludge each set of six consisted of: 0, 10, 50, 100, 250, and 500 ng of each hormone in one millilitre of derivatizing agent. In addition to the above five hormones, 200 ng of an internal standard, deuterated 17β-estradiol (E2dd), was added to each vial. This experiment was repeated six months later, to confirm method repeatability.

3.1.11 Optimized Chemical Analysis Protocol

A summary of the optimized final laboratory protocol for analysis of estrone, 17β-estradiol, 17α-ethinyl estradiol, estriol and testosterone in mixed and digested sludge and wastewaters, with high solids content, is outlined in Figure 3-5. Domestic mixed and digested sludges and wastewater samples were collected from a municipal wastewater treatment plant with anaerobic mesophilic sludge digestion, pH adjusted to 4.0, homogenized with 10% methanol, and stored at 4 °C until extraction, as per the laboratory protocols described in

Sections 3.1.5.1 Sludge extraction and 3.5.2 Wastewater extraction. Preparatory chromatography clean up protocols were conducted as described in Section 3.1.6.1. Extracts were rotary evaporated to 1 ml, placed with hexane on a preparatory chromatography column packed with Florisil and silica, and eluted with DCM and acetone: DCM (30:70). Extracts are rotary evaporated to 1 ml and completely dried under a gentle N_2 stream. Sludge and wastewater samples were derivatized at 70 °C, using the three-step method in Section 3.1.7.1. Anhydrous sludge and wastewater samples were initially silylated with BSTFA+1% TMCS; oxyamination with 2% methoxyamine-HCl; and silylated once again with BSTFA+1% TMCS using pyridine as catalyst and solvent for the derivatizing agents.

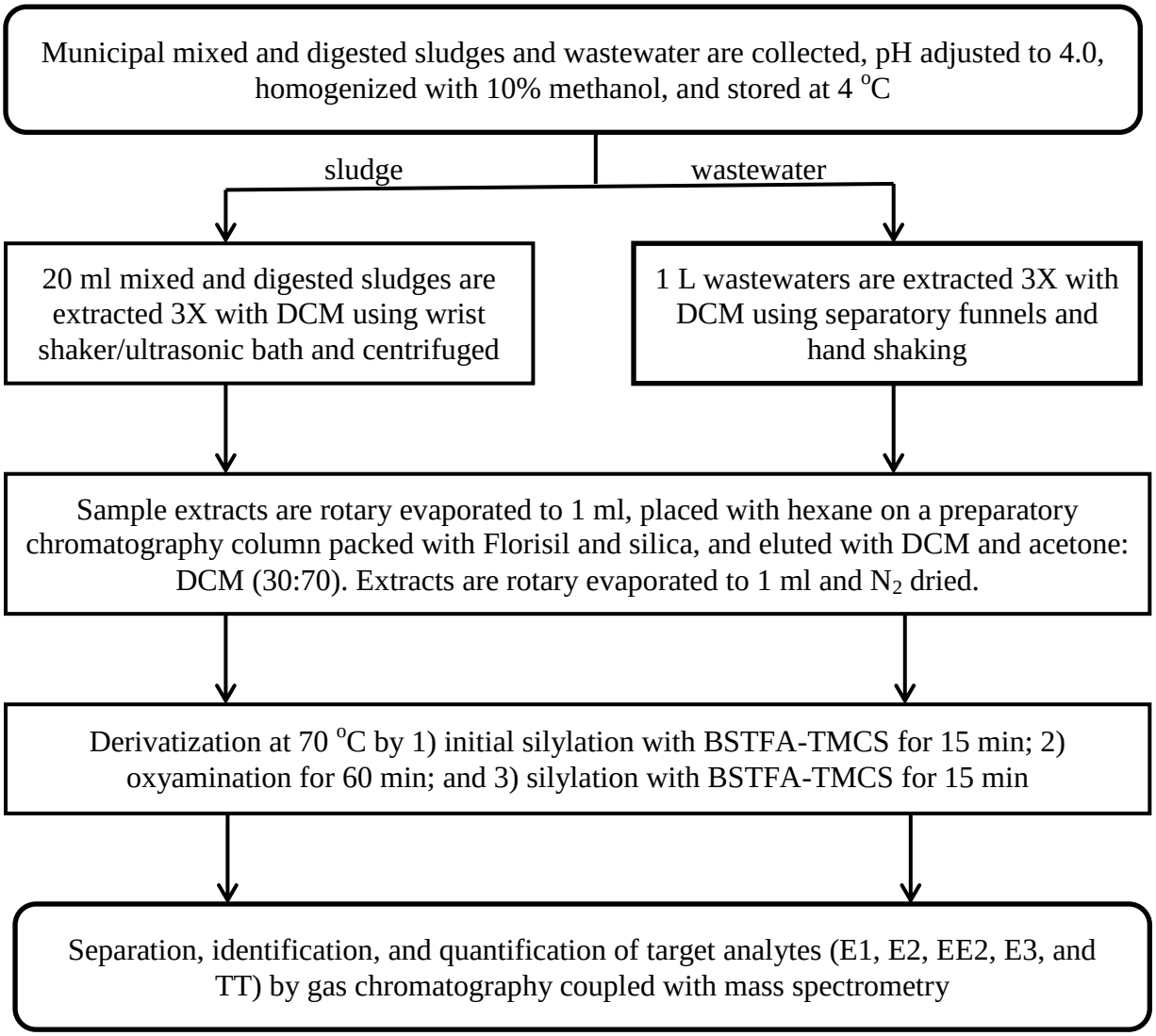


Figure 3-5: GC-MS laboratory analytical protocol for determination of estrone, 17β-estradiol, 17α-ethinyl estradiol, estriol, and testosterone in mixed and digested sludges and wastewaters with high solids content.

3.2 BIOLOGICAL ASSAYS

Estrogenic, androgenic, and toxic activities were performed using bioluminescence yeast estrogen screen (BLYES), bioluminescence yeast androgen screen (BLYAS) assays and bioluminescence yeast reporter for toxicity (BLYR), respectively. A *Saccharomyces cerevisiae* strain, capable of autonomous bioluminescence, was engineered to produce BLYES, BLYAS, and BLYR by The Center for Environmental Technology at the University of Tennessee and very generously supplied to the University of British Columbia, free of charge, for the purpose of conducting this study.

3.2.1 Collection and Storage

Yeast strains BLYES, BLYR, and BLYAS were received from the University of Tennessee streaked on agar plates with extra plates for streaking with fresh culture at a later date. Plates were stored at 4 °C in UBC Professor Don Mavinic's Environmental Engineering laboratory in the Civil Engineering and Mechanical Engineering (CEME) building, but culturing from these plates and streaking of new agar plates was conducted in a sterile laminar flow hood at UBC Professor Louise Craig's laboratory in the Biological Engineering building. Flame sterilized non-disposable inoculating loops were used to streak new plates and transfer yeast strains from the agar plates to the growth media.

Yeast strains were grown to 1.0 optical density (OD_{600}) in modified (YMM leu^-, ura^-) media at 29 °C in a shaker incubator at 225 rpm. Optical density readings were either by a Biochrom Ultrospec 1000 UV/visible spectrophotometer in UBC Professor Susan Baldwin's laboratory in the Biological Engineering building or a HACH DR 2800 in the Environmental

Engineering laboratory. Five hundred µl of 1.0 OD_{600} culture was transferred to a two millilitre cryogenic vial with 500 µl 40% glycerol to provide osmotic support and prevent cell damage. Cryogenic vials were stored at -80 °C in a freezer in the Louise Craig laboratory.

Fresh yeast strain cultures were grown by adding one vial of cryogenically frozen BLYES, BLYR or BLYAS to 30 mls of YMM (leu^-, ura^-) media in a 250 ml Erlenmeyer flask and incubated at 29 °C (28 °C–30 °C) for 20–24 hours in a shaker incubator at 225 rpm until 1.0 OD_{600} (range 0.9–1.1 OD_{600}). These cultures were grown in shaker incubators located in the Susan Baldwin laboratory in the Biological Engineering building or the clean water Environmental Engineering laboratory in the CEME building.

3.2.2 Culturing and Microplating

Sludge and wastewater samples and/or extracts were not spiked with any of the target compounds, internal or surrogate standards. Samples were prepared as per the laboratory analysis protocols described in Chapter 3, Section 3.1 Chemical Analysis: subsections: 3.1.1 Collection; 3.1.2 Storage; 3.1.4 pH adjustment; 3.1.5.1 Sludge extraction; 3.1.5.2 Wastewater extraction; and 3.1.6.1 Sludge and wastewater chromatography. They were dried under a gentle stream of nitrogen after preparatory chromatography, sealed and frozen at -27 °C until yeast cultures were ready to be plated. Samples were then reconstituted/diluted with methanol to 2 mls and vortexed three times to thoroughly mix.

Standards, blanks, and samples (20 µl per well) were plated on corning flat-bottom 96 well microplates with 300 µl well capacity at room temperature (Figure 3-6) and dried at 25 °C

in a dark warming oven. Yeast cultures (200 µl per well) were applied to each 96 well plate and incubated at 30 °C for 6 hours, without light. Incubated samples were transferred from the plate incubator in the Environmental Engineering laboratory to the luminescent plate reader in UBC Professor Steve Hallam's laboratory in the Life Science building, via a box with an electrical warmer (to ensure plated samples were at 30 °C and without exposure to light). Plated samples were stored in a walk-in incubator at 30 °C until they were run in the luminescence plate reader.

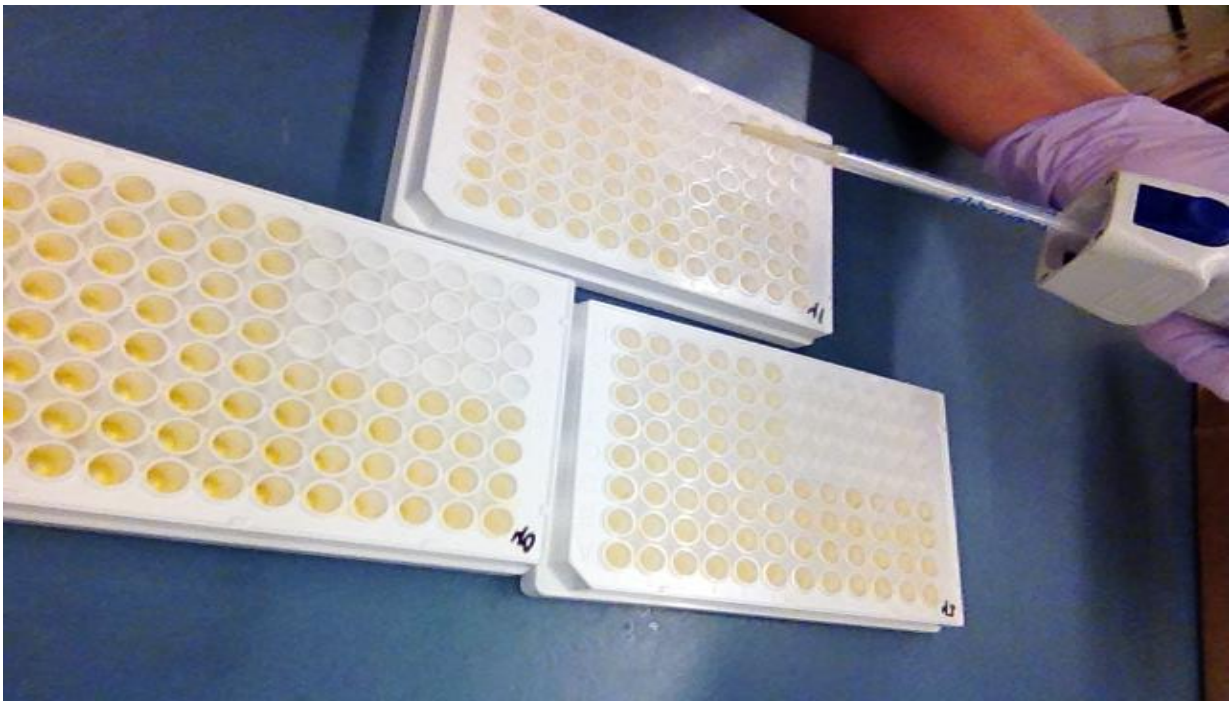


Figure 3-6: Pipetting sludge samples and autobioluminescent yeast into 300 ul well capacity microplates

The bioluminescent yeast reporter, BLYR, measures toxicity to the yeast. Toxicity is expressed as the inhibition of BLYR luminescence. Efficacy of the yeast assay in determining concentrations of estrogenic and androgenic activity has an indirect relationship with inhibition of the luminescence signal. Percent luminescence inhibition is obtained by comparing

luminescence from BLYR exposure to blanks, methanol, or water to BLYR luminescence expressed by exposure to the wastewater or sludge extracts.

Inhibitory concentrations (IC_{20} and IC_{50}) can be calculated for chemicals by charting a curve with increasing concentrations of a chemical. However, no one chemical (or synergistic / additive / antagonistic effects) could be identified within the complex wastewater and sludge mixtures as responsible for inhibiting luminescence in the yeast. Therefore, the IC_{20} and IC_{50} were defined in terms of sample size and dilutions required to produce 20% inhibition and 50% inhibition of the BLYR luminescent signal.

3.2.3 Quality Control

Whole estrogenic assay (BLYES) detection limits (17β-estradiol standards) were approximately 12 ng/L to 650 ng/L E2 equivalents. Assays with strains BLYES and BLYAS were characterized using 17β-estradiol (E2) and testosterone (TT) as standards. A set of standards was plated four times; E2 for BLYES and TT for BLYAS during each experiment to ensure differences in position on the well plate and time of reading could be detected for each run. Two standard plates (one set of TT standards and one set of E2 standards) were run prior to and after each experiment run on the luminescence reader.

To ensure that no cross-contamination had occurred, at least one set of E2 and TT standards were run with BLYES, BLYAS, and BLYR. Two 96 well plates contained distilled water (20 µl per well) and two plates were ethanol (20 µl per well). One of each set was plated with BLYES (200 µl per well) and the other with BLYAS (200 µl per well) to determine

uniformity of luminescence across the plate and establish a baseline reading with these blanks. These plates were placed in the luminescence reader at the beginning and end of each run.

3.2.4 Detection

Luminescence was detected with a Thermo Scientific Varioskan Flash Spectral Scanning Multimode reader in Steve Hallam's Laboratory, Department of Microbiology and Immunology, Life Science Center at UBC. This luminescence plate reader was operated with Thermo Scientific Skanit 2.4.3 software and was set for normal luminometric optics; dynamic range autorange; lagtime 2s; measurement time 200 ms; and 30 °C while reading plate luminescence. Although a Rapidstak microplate stacker was available, it was not used for this study, since it was not at the desired incubation temperature and manual delivery to the plate reader reduced light exposure.

Luminescence was expressed as a number (cd/m^2) per plate well in the Varioskan software and had to be formatted as an Excel chart. These luminescent numbers were manually compared to an average of four standard curves for E2 in BLYES or TT in BLYAS, to express average sample activity values as concentrations of estradiol (E2) equivalents (EEQ) or androgenic (TT) equivalents (TEQ), respectively. Percent toxicity was based on inhibitory effects and measured by comparison of BLYR luminescence activity with samples to the average of BLYR luminescence activity in methanol and water.

3.3 ADVANCED SLUDGE TREATMENTS

Two types of sewage sludge, mixed sludge collected prior to and digested sludge collected after mesophilic anaerobic digestion, were treated as follows:

1) Microwave heating to 60 ^{0}C, 80^{0}C, and 100 ^{0}C
2) Microwave heating with hydrogen peroxide
3) Hydrogen peroxide (no heat application)
4) Conventional heating to 60 ^{0}C, 80^{0}C, and 100 ^{0}C

3.3.1 Microwave Irradiation

A batch process, microwave digestion system (Ethos TC Digestion Labstation 5000, Milestone Inc., USA) with dual independent magnetrons with a rotating microwave diffuser for homogeneous microwave distribution (Figure 3-7), was used to evaluate the effects of microwave irradiation on estrone, 17β-estradiol, 17α-ethinyl estradiol, estriol, and testosterone in mixed and digested sludges. The microwave digestion system delivered 1000 W power (2.45 GHz) and had a maximum capacity of 12–100 ml vessels per run at a temperature of 220^{0}C and pressure of 30 bar (435 psig) (Wong et al., 2006). Although the microwave vessels have a 100 ml capacity, sample volumes were 30–35 ml per vessel to accommodate pressure changes during the heating process. An independent system controller provides real-time temperature control.

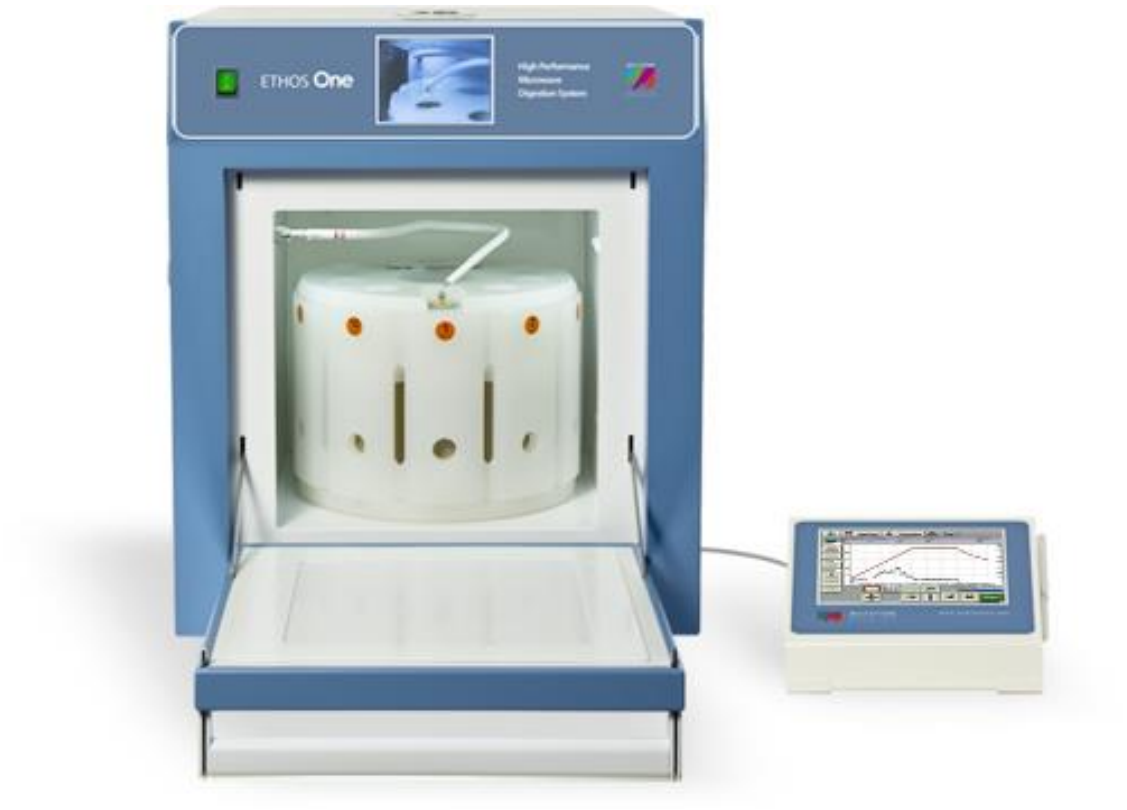


Figure 3-7: Batch digestion process laboratory microwave with high pressure vessels and real time temperature control

Municipal wastewater treatment plant mixed and digested sludges were treated either as controls (20 °C) or by closed vessel microwave heating to 60 °C, 80 °C, or 100 °C (Table 3-13). Samples were cooled below 50 °C, before they could be removed from the microwave.

Table 3-13: Microwave digestion operating program parameters for heat treatment of mixed and digested sludge at 60 °C, 80 °C, and 100 °C

Temperature (°C)	Ramp time (min)	Hold time (min)	Cooling time (min)
20 = Control samples	0	0	0
60	2	5	10
80	3	5	15
100	4	5	20

Within 1 hour of microwave treatment, samples were homogenized with 10% methanol, pH adjusted to 4 and within 8 hours they had been solvent extracted three times with dichloromethane using the ultrasonic bath / wrist shaker method described in Section 3.1.5.1 Sludge extraction. Extractions from each sample were reduced by rotary evaporation and stored at 4 °C overnight. Preparatory chromatography clean up for all samples was as described in the chromatography Section 3.1.6.1 Sludge and wastewater chromatography. Three 10 ml replicates of each treatment set, for both mixed and digested sludges, were set aside for whole estrogenic and androgenic yeast screening analyses.

Three replicates of cleaned up extracts, from 20 ml mixed and digested sludge samples, for each of the control and microwave treatment sets were derivatized using the pre-silylation-oxyamination-silylation steps described in the derivatization Section 3.1.7.1 Sludge and wastewater chromatography. An internal standard, 500 ng deuterated 17β-estradiol (E2dd), was added to each sample prior to derivatization. Concentration of estrone, 17β-estradiol, 17α-ethinyl estradiol, estriol and testosterone in the derivatized samples was quantified by GC-MS, as described in Section 3.1.8 Separation, detection and identification. The ratio of all three monitoring ions for each compound is unique to the retention time and was used with manual integration to determine analyte concentrations in mixed and digested sludges.

3.3.2 Hydrogen Peroxide Additions

The effect of hydrogen peroxide, with microwave digestion, on 17β-estradiol found in mixed and digested sludge was examined. Hydrogen peroxide (30% concentration = 1.135 g/cm^3) additions were by weight and based on a solids content of 1.5% total solids (TS) for

digested sludge and 4.0% TS for mixed sludge. Treatments of 0.5 %, 1.0 %, and 1.5 % H_2O_2 were applied in combination with microwave digestion at 60 °C, 80 °C, or 100 °C (Table 3-14). Microwave and microwave operating parameters for the 60 °C, 80 °C, and 100 °C treatments were as described in Section 3.3.1 Microwave irradiation.

Each of the treatment sets consisted of three 20 ml replicates of mixed sludge and three 20 ml replicates of digested sludge, for chemical analysis, and three 10 ml replicates of mixed sludge and three 10 ml replicates of digested sludge, for whole estrogenic analysis. Due to excess foaming with increasing H_2O_2 additions, each treatment (Table 3-14) consisted of 90 ml of mixed or digested sludge placed in an Erlenmeyer flask, 0.5%, 1.0%, or 1.5% H_2O_2 by weight added, mixed thoroughly, equal aliquots poured into three microwave vessels and vessels pressure sealed.

Table 3-14: Microwave digestion treatments with hydrogen peroxide additions to mixed and digested municipal sludges

Microwave (°C)	0.5 % g/g H_2O_2	1.0 % g/g H_2O_2	1.5 % g/g H_2O_2
Control	—	—	—
Control	X	—	—
Control	—	X	—
Control	—	—	X
60	—	—	—
60	X	—	—
60	—	X	—
60	—	—	X
80	—	—	—
80	X	—	—
80	—	X	—
80	—	—	X
100	—	—	—
100	X	—	—
100	—	X	—
100	—	—	X

Within 8 hours of microwave treatment, samples were homogenized with 10% methanol, pH adjusted to 4, and solvent extracted three times with dichloromethane using the ultrasonic bath / wrist shaker method described above. Extractions from each sample were reduced by rotary evaporation and stored at 4 °C overnight. Preparatory chromatography clean up for all samples was as described in the chromatography Section 3.1.6.1 Sludge and wastewater chromatography. Three 10 ml replicates of each treatment set for both mixed and digested sludges were set aside for whole estrogenic analyses.

Three replicates of 20 ml mixed and digested sludge samples for each of the control, microwave digestion and microwave- H_2O_2 digestion treatment sets were derivatized using the pre-silylation-oxyamination-silylation steps described in the derivatization Section 3.1.7.1 Sludge and wastewater derivatization. An internal standard, 500 ng deuterated 17β-estradiol (E2dd), was added to each sample prior to derivatization.

Concentration of estrone, 17β-estradiol, 17α-ethinyl estradiol, estriol and testosterone in the derivatized samples was quantified by GC-MS as described in the detection Section 3.1.8. The ratio of all three monitoring ions for each compound is unique to the retention time and was used with manual integration, to determine analyte concentrations in mixed and digested sludges.

3.3.3 Athermal Effects

The athermal effects of microwave irradiation on 17β-estradiol and whole estrogenic activity in mixed and digested sludge was examined by comparing conventional heating (water bath) with microwave heating at 60 °C, 80 °C, and 100 °C. This laboratory batch process

microwave unit, with 1000W delivered power, was equipped with a thermocouple probe within the pressure sealed vessels.

Treatments of 20 °C, 60 °C, 80 °C, and 100 °C using 1) microwave irradiation and 2) conventional heat (water bath) were applied to three 20 ml replicates of mixed and digested sludges for chemical analysis and three 10 ml replicates of mixed and digested sludges for whole estrogenic activity assessment. Microwave operating parameters for MW heat treatments of 60 °C, 80 °C, and 100 °C were as listed in Table 3-13.

The electronic temperature-controlled water bath on a laboratory rotary evaporation apparatus was used to conventionally heat mixed and digested sludges to the target temperatures. A thermocouple probe was placed through the septum of one of the sludge sample containers during conventional heating in the water bath to track sample heating. Time required to heat samples to the target temperature, hold time and cooling times for the conventional heat treatments are shown in Table 3-15.

Table 3-15: Parameters for three conventional heat treatments of mixed and digested sludge in a water bath

Sample Temperature (°C)	Time to heat sludge (min)		Hold time (min)	Cooling time (min)
	Digested	Mixed		
20			30	
60	7	9	5	10
80	12	15	5	15
100	20	23	5	20

Within 8 hours of microwave or conventional heat treatment, samples were homogenized with 10% methanol, adjusted to pH 4 and solvent extracted three times with dichloromethane

using the ultrasonic bath / wrist shaker method. Extractions from each sample were reduced by rotary evaporation to approximately 1 ml, sealed and stored at 4 °C overnight. Preparatory chromatography clean up for all samples was as described in the chromatography chapter, Section 3.1.6.1 Sludge and wastewater chromatography. Three 10 ml replicates of each treatment set for both mixed and digested sludges were set aside for whole estrogenic analyses.

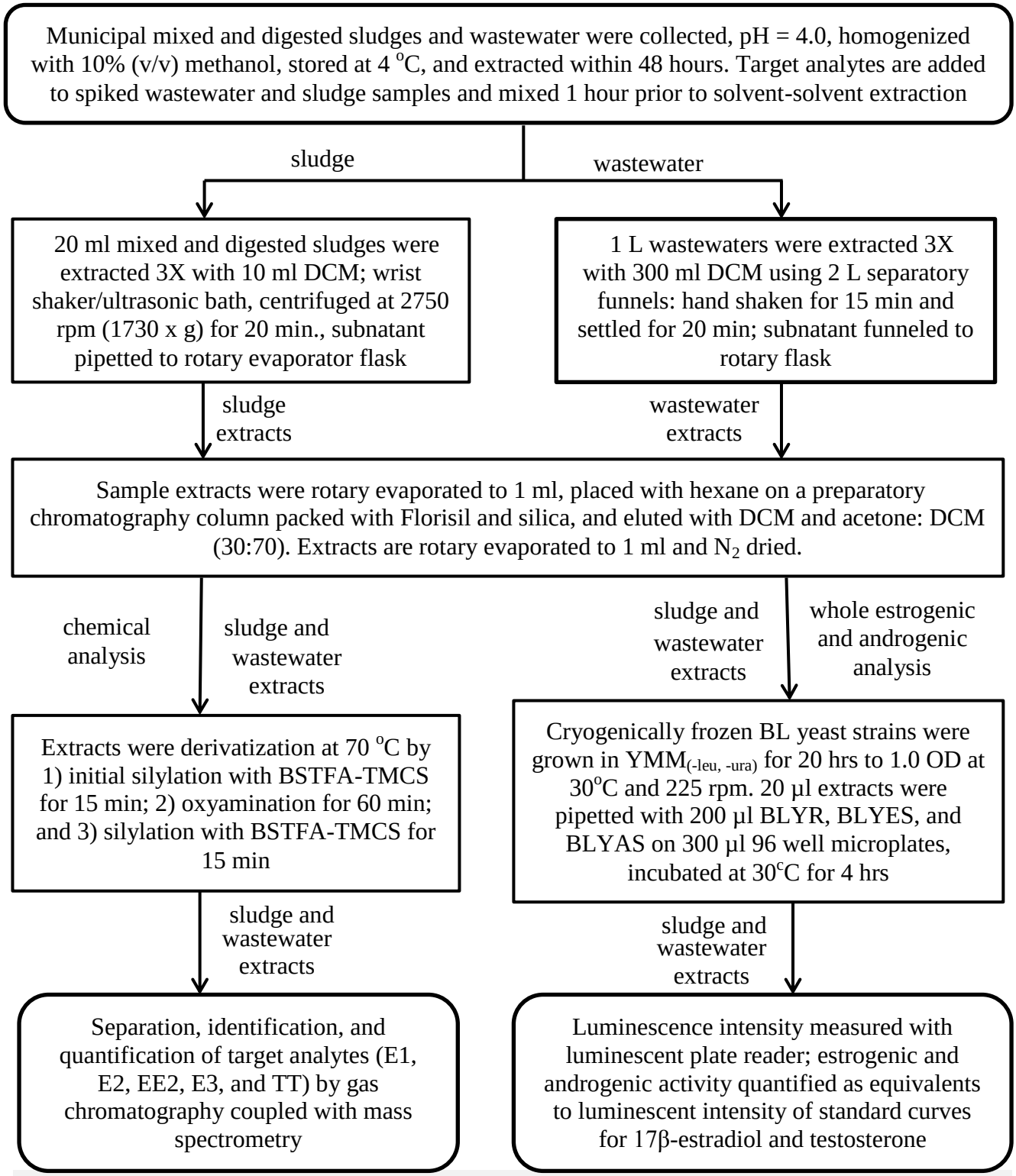
Three replicates of 20 ml mixed and digested sludge samples for each of the control, microwave, and conventional heat treatment sets were derivatized using the pre-silylation-oxyamination-silylation steps described in the derivatization Chapter 3, Section 3.1.7.1. An internal standard, 500 ng deuterated 17β-estradiol (E2dd), was added to each sample prior to derivatization. Concentration of estrone, 17β-estradiol, 17α-ethinyl estradiol, estriol and testosterone in the derivatized samples was quantified by GC-MS, as described in Section 3.1.8. The ratio of all three monitoring ions for each compound is unique to the retention time and was used with manual integration, to determine analyte concentrations in mixed and digested sludges.

4 RESULTS AND DISCUSSION

Given the increasing concern about endocrine disrupting compounds (EDCs), and the fact that the quantity of estrone (E1) discharged into receiving waters can be more than 10 times that of 17β-estradiol (E2) (Sarkar, 2013), these two estrogenic compounds, E1 and E2, were monitored in the wastewater treatment processes. While estrone is an intermediate by-product of E2 degradation during wastewater treatment, it can also be anaerobically transformed to E2 during anaerobic digestion (de Mes et al., 2008; Sarkar, 2013). Testosterone (TT) and 17α-ethinyl estradiol (EE2) were detected sporadically and in too few wastewater and sludge samples to evaluate removal patterns in the municipal wastewater treatment plant. Therefore, total concentration of both E1 and E2, as well as the ratio of E1 to E2, were used to evaluate their presence in sludge and wastewater treatment processes.

Bioluminescence Yeast Estrogen Screen (BLYES) and Bioluminescence Yeast Androgen Screen (BLYAS) were used to determine whole estrogenic activity in the mixed and digested sludge samples. The BLYES detects whole estrogenic activity and BLYAS detects whole androgenic activity through auto-bioluminescence. To detect toxic effects, Bioluminescence Yeast Receptor (BLYR) was run concurrently with the BLYES and BLYAS assays.

The chemical analysis developed for detection of estrogens, E1 and E2, in municipal sludges and wastewaters with high solids content was also compatible for use with the whole estrogenic analysis with the bioluminescent yeast assays. The laboratory protocol for the chemical and whole estrogenic analyses used to evaluate wastewater and sludge is summarized in Figure 4-1.



Acronyms: OD = optical density; $YMM_{(-leu, -ura)}$ = bioluminescent yeast growth media; BSTFA = N,O-Bis(trimethylsilyl) trifluoroacetamide; TMCS = trimethylchlorosilane; DCM = dichloromethane

Figure 4-1: Summary of laboratory protocols for analysis of: estrone (E1), 17β-estradiol (E2), 17α-ethinyl estradiol (EE2), estriol (E3), testosterone (TT) with GC-MS; and whole estrogenic and androgenic activities with bioluminescent yeast: reporter (BLYR), estrogen receptor (BLYES) and androgen receptor (BLYAS) assays, in municipal wastewaters and mixed and digested sludges

4.1 CHEMICAL ANALYSIS

A GC-MS analysis to detect estrogenic substances in mixed and digested sludge with 2–4% solids, without freezing-drying the sludge samples prior to extraction, could not be located in the literature for this project. Therefore, a method of preparing mixed and digested sludge samples for analysis of E2 using GC-MS was necessary. The estrogen, 17β-estradiol (E2), was selected because it is estrogenically potent, found in municipal wastewater and is used as a standard for estrogenic activity in many common biological assays, including the BLYES assay. A summary protocol for use with BLYES, BLYAS and BLYR was developed and is provided in Chapter 4, Biological Assay.

Municipal sludge is a difficult matrix for laboratory analysis of estrogenic substances. Few laboratories currently carry out this analysis without first drying the mixed and digested sludge samples. Extraction and clean up protocols are more complex for sludge and wastewaters with high solids content than for wastewater effluent and influent samples. When targeting specific chemical compounds, such as estrogens, in sludge media, estrogenic activity may be lost in the complex extraction and clean up protocols. Chemical analysis methods for detection of estrogens in sludge media should also be capable of recovering estrogenic activity if a whole estrogenic analysis is to be paired with the chemical analysis. In order to directly compare the results from chemical (GC-MS) and biological (BLYES) analysis, the sample preparation must be the same (ideally) or very similar. Therefore, this chemical analysis protocol, developed for detection of E1 and E2 in municipal mixed and digested sludge by GC-MS, was also suitable for recovery of estrogenic activity for the BLYES analysis.

The optimized chemical analysis protocol for municipal mixed and digested sludges was applied to wastewaters with simple modifications to the extraction step as described in chapter 3 Methodology, Section 3.1.5.2 Wastewater extraction. The modified procedure provided excellent recoveries of E1 and E2 from wastewaters and no further optimization experiments were considered necessary.

4.1.1 Sample Storage

A loss of estrogens during sludge sample storage was a concern due to biological and chemical degradation. To assess the effects of sludge sample storage in terms of target analyte loss, estrogen concentrations were compared at time of collection and after two weeks of refrigerated storage. After two weeks of refrigerated storage at 2–4 °C, unspiked controls of mixed and digested sludges had losses of estrone (20%; 18%) and 17β-estradiol (23%; 33%), respectively. Therefore, all mixed and digested sludge samples were extracted within 24 hours of collection.

Derivatized standards and media samples (in toluene, derivatization agents, and N_2 dried) stored at -28 °C for two weeks did not demonstrate a loss in target analytes, estrone and 17β-estradiol.

4.1.2 Silylation of Glassware

In trace analysis, it is important to neutralize the active sites on glassware surfaces to prevent target analytes from binding to glass surfaces. Glassware silylation methods utilize various solvents and silylation agents to prevent steroidal compounds from binding to active sites

on laboratory glassware (Sigma-Aldrich, 1997a; Sigma-Aldrich, 1997b; Ikonomou et al., 2008; Thermo-Scientific, 2008). To optimize the silylation procedure for glassware used in this research for recovery of estrogens, five variations to pre-treatment rinses and silylation agent solvents were compared against a method with no pre-treatment rinses and 5% dichlorodimethylsilane (DCDMS) in toluene as the silylation agent, labelled the control method. Silylation method performance was based on detection of E2 in methanol, after storage for a week, in triplicate sets of test tubes silylated using one of the five methods outlined in Table 4-1.

Table 4-1: Five silylation methods for glassware compare pre-treatment rinses and silylation agent solvents by GC-MS peak abundance for 17β-estradiol (E2) in methanol after storage in the silanized glassware at -27 °C for one week

Sample sets (n=3)	Pre-treatment rinse solution(s)	Silylation agent	Detection of 10 ng E2/ ml solvent		Detection of 100 ng E2/ ml solvent	
			% E2	% RSD[3]	% E2	% RSD
# 1	Acetone (1st rinse) Toluene (2nd rinse)	5% DCDMS[2] in toluene	57	24	68	12
#2	Toluene	5% DCDMS in toluene	46	14	94	23
#3	DCM[1]	5% DCDMS in DCM	93	45	94	15
#4	None	5% DCDMS in DCM	88	29	99	16
Control	None	5% DCDMS in toluene	100	24	100	10

1. DCM = dichloromethane
2. DCDMS – dichlorodimethylsilane
3. RSD = relative standard deviation

The results (as shown in Table 4-1) clearly show no pre-treatment rinses were necessary, as long as the glassware had been thoroughly cleaned. Poorer performance with methods incorporating a pre-treatment solvent rinse may be caused by incomplete silylation, if the silylation agent is diluted by rinse solvents left in the glassware. Silylation is sensitive to water and any water present in pre-treatment rinses, such as acetone, could result in reduced silylation of the glassware. This would be especially noticeable with lower concentrations of the target analyte.

Using toluene, instead of DCM, as a solvent for the silylation agent, DCDMS, may improve silylation when glassware is not pre-rinsed. Although this method appeared to improve detection of the target analyte, the percent difference in peak abundance was less than the percent relative standard deviation (% RSD). When a pre-rinsing step was incorporated into the silanization protocol, DCM outperformed toluene for recovery of lower concentrations of 17β-estradiol. However, the results, shown in Table 4-1, indicate the use of toluene as the primary solvent and eliminating the solvent pre-rinsing step(s) improved percent recovery of 17β-estradiol (% E2) with lower percent relative standard deviation.

Sigma-Aldrich (1997) states the most common silanization procedure is to treat the glassware with 5–10% DCDMS in toluene for 30 minutes, rinse the deactivated glassware with toluene, then immediately thereafter with methanol. However, Ikonomou et al., (2007) incorporated three solvent pre-rinses prior to silanization of laboratory glassware with 5% DCDMS in DMC. Since there was no measurable loss of 17β-estradiol, a solvent pre-rinse step was not incorporated into the laboratory glassware protocol for this research project.

Based on the results shown in Table 4-1, the laboratory protocol for silanizing glassware, described in Chapter 3 Methodology, Section 3.1.3 silylation, utilized 5–7% DCDMS in toluene with no pre-rinse step(s).

4.1.3 pH Adjustment and Methanol Addition

Wastewater and sludges are complex mixtures that are well known to form emulsions during solvent extractions making the separation into water and solvent phases very difficult. Two of the methods recommended to help break up emulsions are lowering the pH and adding ethanol (Milkshake, 2008). Since methanol was more cost effective and easier to obtain, it was often substituted for ethanol in the chemical and whole estrogenic analysis protocols. The practice of methanol or ethanol addition to sludge and wastewater samples, prior to solvent extraction of estrogens, could not be found in the published literature.

It is common to adjust pH to 2.5–5 to inhibit microbial activity in wastewater and sludge samples soon after collection and before samples storage (Nakada et al., 2004; Esperanza et al., 2007; Sim et al., 2011). However, examination of lowering the pH and extraction efficacy of estrogens in domestic sludge and wastewaters could not be found in the published literature.

Decreasing pH of sludge samples improved recoveries of 17β-estradiol (Table 4-2) perhaps by helping to break emulsions in the sludge matrix and release the compound from the solid/water/solvent emulsion into the solvent phase. Adding 10% methanol to digested sludge, without lowering the pH did not improve recoveries of E2 (Table 4-2). However, lowering the pH, from 8.0 to 5.5 doubled the recovery of E2 from unspiked digested sludge. By adding 10% MeOH and lowering pH to 5.5, the recovery of E2 increased by more than 300% over the control (untreated digested sludge) sample. A further 15% increase in recovery of E2 was noted when pH = 4.0 and 10% MeOH added prior to extraction procedures (Table 4-2).

Similarly, recoveries of E2 were not improved with the addition of 10% MeOH to mixed sludge sample. However, lowering the pH of mixed sludge from 6.0 to 4.0 increased recoveries of E2 by 35%. No further improvements to E2 recoveries were noted when an addition of 10% MeOH accompanied a pH adjustment from 6.0 to 4.0 (see data table in Appendix D-1). Further lowering the pH of mixed sludge from 6.0 to 2.0 did not improve recoveries of E2 in spiked samples (Table 4-2).

Table 4-2: Effect of pH adjustment and methanol addition prior to solvent extraction on recovery of 17β-estradiol (E2) in spiked and unspiked mixed (MS) and digested (DS) sludges

Treatment prior to extraction (n = 3)			E2 recovery in sludge*		percent relative standard deviation
Spiked	pH	Methanol	Mixed	Digested	
—	8.0	—	—	25	2.2
—	8.0	10%	—	23	33
—	6.0	—	65	—	4.1
5 µg	6.0	—	69	—	12
—	5.5	—	—	49	7.3
—	5.5	10%	—	84	5.8
—	4.0	—	97	—	11
—	4.0	10%	100	100	7.3 (MS); 9.6 (DS)
5 µg	4.0	10%	95	—	19
5 µg	2.0	10%	85	—	11

*Recoveries of E2 in unspiked samples assume treatment with highest unspiked E2 represents 100% recovery.

Since the addition of 10% MeOH did not appear to negatively affect E2 recoveries in mixed sludge and appeared to improve recoveries of E2 in digested sludge, all wastewater and sludge samples were subsequently pH adjusted to 4.0 and 10% MeOH added, prior to DCM extraction.

4.1.4 Solvent Extraction

Most researchers have freeze dried municipal sludge samples prior to extraction (Table 1-4). However, some extraction methods require separation of solid and liquid portions of the

wastewater and sludge samples. Although it is not uncommon to discard filtered wastewater solids prior to solvent extraction of the water phase, evaluation of estrogen partitioning into the solid and liquid phases of wastewaters is rarely carried out as part of the laboratory protocol. One study was found in the published literature, Esperanza et al. (2007), that reported partitioning of estrogens (E1, E2, EE2, and E3) and androgens including testosterone in water and solid phases of wastewater and sludge from pilot plants with aerobic and anaerobic digestion. Separation of sludges into water and solid phases can be advantageous in that they have high solids content and are difficult matrices to process whole for chemical analysis. Previous examination of estrogen partitioning between the water and solid phases of municipal WWTP sludges could not be found in the published literature.

Therefore, the partitioning of E2 in the water and solid phases of mixed and digested sludges was examined in spiked and unspiked samples. Samples were centrifuged at 2750 rpm (1750 x g) for 15 minutes to separate the aqueous and solid phases. The aqueous phase was extracted as per the protocol for wastewater analysis (Section 3.1.5.2) while the solids portion was extracted as per the protocol for whole sludge sample analysis (Section 3.1.5.1). Remaining analysis was carried out as per Sections 3.1.6.1, 3.1.7.1, and 3.1.8. The percent of total E2 found in centrifuged solid and liquid layers of mixed and digested sludge samples was determined to be much higher in the solid (74–95%) versus liquid (5–26%) portions (Table 4-3).

The partitioning of 92% E2 in the solid phase of anaerobically digested sludge closely agrees with that found by Esperanza et al. (2007) of 89% E2 in the solid phase. However, the partitioning of 95% E2 in the solid phase of the mixed sludge feeding into the anaerobic digester

varies greatly with the 18% E2 in the solid phase reported by Esperanza et al. (2007). This difference in E2 partitioning is likely due to the difference in sludge quality from the pilot plant in the study by Esperanza et al. (2007) and the full scale municipal wastewater treatment plant employed in this study.

Table 4-3: Percent 17β-estradiol (E2) in the liquid and solid layers of centrifuged 20 ml of spiked (1 µg) and unspiked mixed (4% solids) and digested (1.5% solids) domestic WWTP sludge

Centrifuged Sludge (20 ml) (n = 3)	Liquid portion			Solid portion		
	E2 (ng)	% RSD[1]	% E2 in liquid	E2 (ng)	% RSD[1]	% E2 in solids
Mixed	2	101	5	38	17	95
Mixed + 1 µg E2	167	9	20	848	19	80
Digested	1	57	8	12	30	92
Digested + 1 µg E2	217	9	26	816	17	74

1. RSD = relative standard deviation

Unspiked samples showed greater partitioning of E2 between liquid and solid layers with only 5–8 % of E2 in the liquid portion of the sample. Lower partitioning in spiked samples may be due to the saturation of E2 in solids (partitioning coefficient) and/or the short contact time between the sample spike of 1 ug E2 (one hour prior to extraction procedures). Spiked E2 may not adsorb to the solids in the same manner as E2 found in raw sewage or during wastewater and sludge treatment processes.

Ternes et al. (2002) expressed concern that adsorption of spiked estrogens to solids in freeze dried samples may not be representative of environmental samples and may influence the reported method recoveries (Ternes et al. 2002). If spiked estrogens do not adsorb to solids to the same degree as environmental samples, they will likely be more available to solvent extraction than estrogens adsorbed to solids in municipal wastewater treatment plant sludges. Therefore, spiked estrogens in the sludge matrix would be solvent extracted with greater ease than estrogens

more tightly bound to the solid phase and method recoveries, calculated from spiked sludge matrices, may be overestimated and environmental concentrations underestimated.

Since all wastewater samples were adjusted to pH 4.0 and 10% methanol added, it is difficult to assess if this reduced emulsion formation during solvent extraction. Only wastewater samples with high solids content [e.g. solids contact MLSS (1200 – 1450 mg/L] formed emulsions that prevented separation of the solvent phase. Methanol addition, lowering the pH to 2.0, and/or adding brine did not break up the emulsion. However, if an emulsion did form, it was broken by simply adding more DCM and pouring into another container.

4.1.4.1 EXTRACTION METHODS

A variety of extraction methods were compared for efficacy in extracting E2 from mixed and digested domestic sewage sludge. Soxhlet extraction is a proven method, but was not practical; due to limited fume hood space in the UBC Environmental Engineering Laboratory and the long extraction times required for this method, only four samples could be extracted at a time. Therefore, two other extraction methods were compared to Soxhlet extractions using mixed sludge, as it is a difficult media to process for detection of E2. Assuming the Soxhlet extraction recovered 100% of E2 in the freeze dried solids of 20 ml mixed sludge, the wrist shaker extraction recovered approximately 50%, while the combination of wrist shaker / ultrasonic bath treatment produced similar results to the Soxhlet (Table 4-4) when using DCM as the extraction solvent.

Table 4-4: Comparison of Soxhlet, wrist shaker, and combination wrist shaker/ultrasonic bath for extraction of E2 from 20 ml mixed sludge solids with dichloromethane

Extraction (n=3)	Solids	E2 (ng)	Range E2 (ng)	E2 recovered (ng/g solids)*	Range E2 (ng/g solids)*	% RSD
Soxhlet	freeze dried	37	20-41	49	26-54	43
Wrist shaker	freeze dried	14	11-17	19	15-23	26
shaker/ultrasonic	centrifuged	37	35-44	41*	39-49*	17
* wet weight of centrifuged solids for shaker/ultrasonic method under-reports recovery of E2 as ng/g due to higher water content and lower E2 in water portion of sample (5% of total E2)						

The superior E2 recoveries for the combination wrist shaker / ultrasonic bath method may be due to increased sample contact time with the solvent for each extraction (Llewellyn et al., 2004). Contact time was increased, not only by requiring time on two sets of apparatus (wrist shaker and ultrasonic bath), but also transfer time between each treatment and time during the more difficult solvent removal (pipetting subnatant of centrifuged wet sludge), for a total of over four hours for the three extractions. In addition, the increased pressure and heat of the ultrasonic bath extraction has been reported to improve recovery of many target analytes (Jenkins and Grant, 1987; Luque de Castro and Garcia-Ayuso, 1998).

The wrist shaker / ultrasonic bath method was preferred for the following reasons:

1) Required less fume hood space
2) Recoveries of E2 were similar to Soxhlet
3) Separation of sample into solid-liquid portions not required
4) Freeze drying not required
5) Can process 28–30 samples per batch versus 4 for Soxhlet

Therefore, the wrist shaker / ultrasonic bath method was used, thereafter, for extracting wastewater and sludge to evaluate wastewater treatment processes.

4.1.4.2 EXTRACTION SOLVENTS

Since DCM is not miscible with water, it separates well from the aqueous fraction during extractions of estrogens in wastewaters and wet sludges. Wet mixed and digested sludges were extracted with dichloromethane in the laboratory protocol used for evaluating wastewater and sludge treatment in this research (Figure 4-1). However, laboratory protocols that freeze-dry sludges prior to extracting estrogens, generally use more polar solvents than DCM (Ternes et al., 2002; Muller et al., 2008; Anderson et al., 2003; Joss et al., 2004; Sim et al., 2011).

In an effort to improve wrist shaker method recoveries of E2 in freeze-dried mixed and digested sludges, more polar extraction solvents were compared to recoveries with 100% DCM in both mixed and digested sludge samples. Acetone mixtures can be used as extraction solvents for freeze dried sludge solids but not wet solids, because acetone is miscible with water in the sample; this prevents the separation necessary for this extraction process. Wrist shaker extractions, when DCM extraction solvent was substituted for 100% acetone or 30% acetone in DCM, did not demonstrate improved recoveries of E2 in freeze dried solids from 20 ml mixed sludge (Table 4-5) and digested sludge (Table 4-6) samples.

Table 4-5: Comparison of three extraction solvents for wrist shaker extraction method recoveries of 17β-estradiol in freeze dried solids from 20 ml mixed sludge samples

Extraction solvent (n = 3)	MS solids (g)	E2 (ng)	Range (ng)	E2 (ng/g solids)	Range E2 (ng/g solids)	% RSD
DCM	0.7450	14	11-17	19	15-23	26
30% acetone in DCM	0.6989	13	ND-22	18	ND-31	89
Acetone	0.7126	15	13-17	21	18-24	15

Table 4-6: Comparison of three extraction solvents for wrist shaker extraction method recoveries of 17β-estradiol in freeze dried solids from 20 ml digested sludge samples

Extraction solvent (n = 3)	DS solids (g)	E2 (ng)	Range (ng)	E2 (ng/g solids)	Range E2 (ng/g solids)	% RSD
DCM	0.2560	12	ND-22	47	ND-86	173
30% acetone in DCM	0.2700	15	10-26	56	37-96	47
Acetone	0.2670	12	ND-29	45	ND-108	128

When freeze dried mixed and digested sludge samples were extracted with 100% DCM; 30% acetone in DCM; and 100% acetone, some samples had non-detectable concentrations of E2. Non-detectable concentrations were assigned a zero value when calculating the percentage relative standard deviation (% RSD) of the three replicates samples for each treatment. Non-detectable values would increase the % RSD for the sample treatment set. This was not a concern for wet samples, extracted with DCM using wrist shaking/ultrasonic extraction apparatus, because E1 and E2 were detectable in all replicates of unspiked mixed and digested sludges.

The most common laboratory protocols for examining estrogens in municipal sludge freeze dry sludge samples and extract with solvents more polar than DCM, such as acetone and methanol with a polarity of 5.1 (Table 1-4). While it is more convenient to work with freeze dried sludge, it can take considerable time to freeze dry samples. Dichloromethane (polarity = 3.1) and chloroform (polarity = 4.1) separate from the water phase and are suitable to extract estrogens from wet sludge samples. When chloroform was substituted for dichloromethane as an extraction solvent for mixed and digested sludges, losses of estrone (27% and 3%) and 17β-estradiol (27% and 25%), respectively, were accompanied by percent RSDs (n=3) in the replicates ranging from 5–43% for DCM and 12–61% for chloroform extractions. Dichloromethane appeared to perform slightly better as an extraction solvent in terms of percent recovery of E1 and E2 from mixed and digested sludges. However, the inter-sample % RSDs

(n=3) could be larger than the percent losses of E1 and E2 when chloroform was substituted for DCM as shown in Table 4-7. Therefore, despite appearances, dichloromethane may not provide better recoveries.

Table 4-7: Dichloromethane and chloroform as solvents for extraction of estrone (E1) and 17β-estradiol (E2) in 20 ml aliquots of municipal mixed and digested sludges and percent loss of E1 and E2 when substituting chloroform for dichloromethane.

Target compounds in 20 ml sludge	Dichloromethane (n=3)				Chloroform (n=3)					
	Mixed sludge		Digested sludge		Mixed sludge			Digested sludge		
	Average (ng)	% RSD	Average (ng)	% RSD	Average (ng)	% RSD	Loss %	Average (ng)	% RSD	Loss %
Estrone	66	14	90	5	48	61	27	87	12	3
17β-estradiol	52	11	7	43	38	60	27	5	20	25

One liter of influent and 1L effluent was extracted with either dichloromethane or chloroform in 2 L separatory funnels in laboratory fume hoods. Emulsions formed during hand shaking in the separatory funnel disappeared quickly during settling and both extraction solvents separated from the water phase within 15 minutes. Little to no difference in recovery of E2 was noted when chloroform was substituted for dichloromethane in extractions of influent and effluent wastewaters (Table 4-8).

Table 4-8: Dichloromethane and chloroform as solvents for extraction of 17β-estradiol (E2) from one litre influent and effluent wastewater samples by hand shaking in separatory funnels

1 L wastewater (n = 3)	Extraction solvent	17β-estradiol (ng/L)	% relative standard deviation
Influent	dichloromethane	16	12
Effluent	dichloromethane	4	26
Influent	chloroform	17	10
Effluent	chloroform	3	42

Although, dichloromethane appeared to provide slightly better recovery of E1 and E2 in sludge and no difference in recovery of these target compounds in wastewater, there was no clear

disadvantage to using DCM as the extraction solvent. In addition, dichloromethane was preferred for ease of use and personal safety while extracting wastewater samples by hand shaking in 2-litre separatory funnels under the solvent exhaust hoods. Hence, dichloromethane was used as the extraction solvent for all experiments.

4.1.5 Preparatory Chromatography

The optimized design of the preparatory chromatography column is shown in Figure 3-4. Packing materials were a mixture of silica-Florisil with a sodium sulphate layer for removing water and a copper layer to remove sulphates (as $CuSO_4$) from the eluate. Solid phases, solvent conditioning, elution mixtures and volumes for the preparatory chromatography protocol (described in Methodology Chapter 3; Chromatography Section 3.1.6; subsection 3.1.6.1 Sludge and wastewater chromatography) were optimized as described in the following sections.

4.1.5.1 ACID DEACTIVATION

There is a critical need for clean up steps in the analysis of trace contaminants in environmental samples, especially if concentrations are low. The presence of higher levels of other substances in that co-elute can cause analytical interference and make quantification difficult. When processing biota samples, it is necessary to remove lipids from the extracts. Common clean up techniques for removing fats from environmental samples include impregnating silica with alumina, Florisil and/or sulphuric acid (Quevauviller et al., 2011). It is often necessary to combine clean up applications such as sulphuric acid, Florisil and silica to remove lipids from environmental samples.

One of the techniques used to remove lipids from complex matrices was acid deactivation of silica in preparatory chromatography columns for the analysis of PCBs (Quevauviller et al., 2011). Deactivation of the preparatory chromatography columns solid phase (column design shown in Figure 3-4) with acid or water was compared by percent recovery of 17β-estradiol in spiked mixed and digested sludge samples. In addition, preparatory chromatography columns, packed with silica or a combination of silica/Florisil, were compared by percent recovery of E2 in sludges (Table 4-9).

Preparatory chromatography columns were packed with silica or a combination of Florisil + silica of various mesh sizes and deactivated with either 7% water or acid (Table 4-9). Silica chromatography columns took twice as long to elute solvents and sample than the combination Florisil-silica columns due to the overall smaller particle size (230–400 mesh) of the solid phase. Initial elutions (60 ml DCM + 10 ml 30% acetone in DCM) from the chromatography columns with acid-deactivated silica had no detectable 17β-estradiol.

All elutions from columns with acid deactivated silica and Florisil-silica packing had no detectable E2 concentrations. None of the elution fractions from water or acid deactivated silica chromatography columns had eluted E2 from mixed sludge. Two of the three water deactivated silica columns eluted detectable E2 for digested sludge, but recoveries were one-third of E2 recoveries from digested sludge using water-deactivated Florisil-silica chromatography columns (Table 4-9).

Eluates from water-deactivated Florisil-silica chromatography columns were very clear with a light amber tint and dried completely by N_2 with no residue. However, eluates from acid-deactivated silica and silica-Florisil columns co-eluted with coloured compounds resulting in a dark brown/green/black eluate. The dark eluate contained a gel-like substance that was difficult to N_2 dry and may have interfered with derivatization of the analytes.

The chromatography columns packed with water deactivated silica did not perform as well as columns with water-deactivated Florisil-silica. Silica columns co-eluted coloured compounds resulting in a clear green tinted eluate with a gel-like substance that was difficult to N_2 dry and may have interfered with derivatization. Clean up of mixed sludge extracts with silica columns was unsuccessful in that no E2 could be detected, while Florisil-silica columns recovered 72% of E2 from mixed sludge extracts. While clean up of digested sludge extracts with silica columns resulted in almost 60% recovery of E2, clean up with Florisil-silica columns resulted in recoveries of 99% for digested sludge extracts.

Table 4-9: Comparison of acid (HCl) and water (H_2O) deactivation of silica and Florisil/silica packing materials in preparatory chromatography columns for the recovery of 17β-estradiol (E2) from mixed (MS) and digested (DS) sludge samples with relative standard deviations (% RSD).

20 ml sludge (n = 3)	Deactivated with	Packing materials	% RSD	E2 (ng)	% recovery
MS+100 ng E2	H2O	Silica/Florisil	3	94	72
DS+100 ng E2	H2O	Silica/Florisil	17	107	99
MS+100 ng E2	HCl	Silica/Florisil	—	ND	ND
DS+100 ng E2	HCl	Silica/Florisil	—	ND	ND
MS+100 ng E2	HCl	Silica	—	ND	ND
DS+100 ng E2	HCl	Silica	—	ND	ND
MS+100 ng E2	H2O	Silica	—	ND	ND
DS+100 ng E2	H2O	Silica	87	65	59
MS	H2O	Silica/Florisil	12	13	—
DS	H2O	Silica/Florisil	28	4	—

Silica preparatory chromatography columns and acid deactivation of the solid phase in chromatography columns may work with other sample clean up and derivatization methods for chemical analysis of E2. However, they were unsuccessful in adequately recovering E2 from mixed and digested sludge, using this method. Eluted compounds may have interfered with the silylation derivatization used in this chemical analysis. Acid deactivation of Florisil and silica produced darkly coloured samples with compounds that resisted N_2 drying before (digested sludge) or after (mixed sludge) derivatization. In addition, overall recoveries could have been affected by extraction procedures which had been optimized (e.g. increased heat, pressure, and contact time) after this experiment and before evaluation of WWTP processes for wastewater and sludge and microwave treatments.

Overall, preparatory chromatography columns packed with a combination of silica-Florisil of various mesh sizes (as shown in Figure 3-4) greatly improved recoveries of E2 in mixed and digested sludges over silica columns or columns packed with acid-deactivated silica-Florisil materials. Therefore, water-deactivated silica-Florisil preparatory chromatography columns were used to evaluate wastewater and sludge process technologies in this research project.

4.1.5.2 WATER DEACTIVATION

Nakada et al. (2004) used both deactivated (5% water) and activated silica gel columns to clean up wastewater effluent extracts to recover various EDCs. Using chemical analysis and recombinant yeast assays, E1 and E2, as well as the bulk of the estrogenic activity, was eluted from the deactivated (5% water) silica column. Results comparing activated and deactivated (5%

and 7%) Florisil-silica chromatography columns (Table 4-10) agreed with Nakada et al. (2004) partitioning of E2 from deactivated and activated silica columns.

Recoveries of 17β-estradiol in mixed sludge extracts improved by 53% when Florisil and silica preparatory chromatography columns were deactivated (83% recovery) with 5% H_2O compared to activated (30% recovery) packing materials.

Table 4-10: Comparison of two conditioning solvents and activated versus deactivated Florisil and silica in preparatory chromatography columns for recovery of 20 µg 17β-estradiol in mixed sludge extracts with percent relative standard deviations (% RSD)

Florisil and silica (n = 3)	Column conditioned with	17β-estradiol recovery (µg)	Average % recovery	% RSD
Activated	Hexane	5.2	26	3.0
Activated	DCM	6	30	3.3
Deactivated (5% H_2O)	Hexane	9.7	48.5	51.0
Deactivated (5% H_2O)	DCM	16.7	83.5	8.4
Deactivated (7% H_2O)	DCM	17.5	87.7	5.2

There appeared to be a slight increase in recoveries when Florisil and silica were deactivated with 7% H_2O instead of 5% H_2O but inter-sample percent RSDs were higher than the increase in recoveries of 17β-estradiol (Table 4-10). Therefore, Florisil and silica stationary phases of the preparatory chromatography columns were deactivated with 7% H_2O for all wastewater and sludge matrices.

4.1.5.3 COLUMN CONDITIONING

Nakada et al. (2004) eluted wastewater effluent extracts in silica gel preparatory chromatography columns with increasing concentrations of DCM in hexane (25% to 80%) before eluting E1 and E1 with 30% acetone in DCM. Solvent conditioning of the columns was not reported. A modification of this method was to condition the deactivated (7% water) Florisil-

silica chromatography column with 20 mls hexane (instead of DCM) prior to sample placement and initial elution with DCM. However, recoveries of 17β-estradiol in mixed sludge extracts almost doubled when columns were conditioned with 20 mls of DCM (84%) instead of hexane (49%) (Table 4-10). Therefore preparatory chromatography columns were conditioned with DCM in our optimized laboratory protocol.

4.1.5.4 INITIAL CLEAN UP VOLUME

When the initial elution 60 ml volume of the primary solvent for the elution mixtures was reduced to 20 ml, recoveries of 17β-estradiol were reduced by 57% in mixed and 45% in digested sludge matrices (Table 4-11). Therefore, the volume for the initial clean up elution in this preparatory chromatography method was 60 ml for wastewater and sludge process evaluations.

Table 4-11: Preparatory chromatography comparing dichloromethane (DCM) versus chloroform ($CHCl_3$) as the primary elution solvent mixtures to elute 17β-estradiol (E2) from extracts of 20 ml aliquots of municipal mixed (MS) and digested (DS) sludges. Initial elution volumes 20 ml and 60 ml DCM were also compared for recovery of E2.

Preparatory chromatography elution mixtures (n = 3)			MS (20 ml)		DS (20 ml)	
Solvent	1st elution	2nd elution	E2 (ng)	% RSD[1]	E2 (ng)	% RSD1
DCM	20 ml	50 ml 30% acetone in DCM	12.3	14.8	4.6	18.5
$CHCl_3$	20 ml	50 ml 30% acetone in $CHCl_3$	11.8	5.7	3.9	27.5
DCM	60 ml	50 ml 30% acetone in DCM	21.4	4.0	10.2	21

[1] RSD = relative standard deviation

4.1.5.5 PRIMARY ELUTION SOLVENT

Preparatory chromatography of mixed and digested sludge samples showed a slight increase in recoveries of E2 when using DCM over using chloroform ($CHCl_3$), as the primary solvent for all elutions (Table 4-11). Because inter-sample percent RSDs were larger than the

percent difference in recoveries of E2 from sludge extracts, efficacies of DCM and CHCl3 as primary elution mixtures were considered relatively equal. Dichloromethane was used as the primary solvent for all elution mixtures in this preparatory chromatography method.

4.1.5.6 ELUENT SOLVENT RATIOS

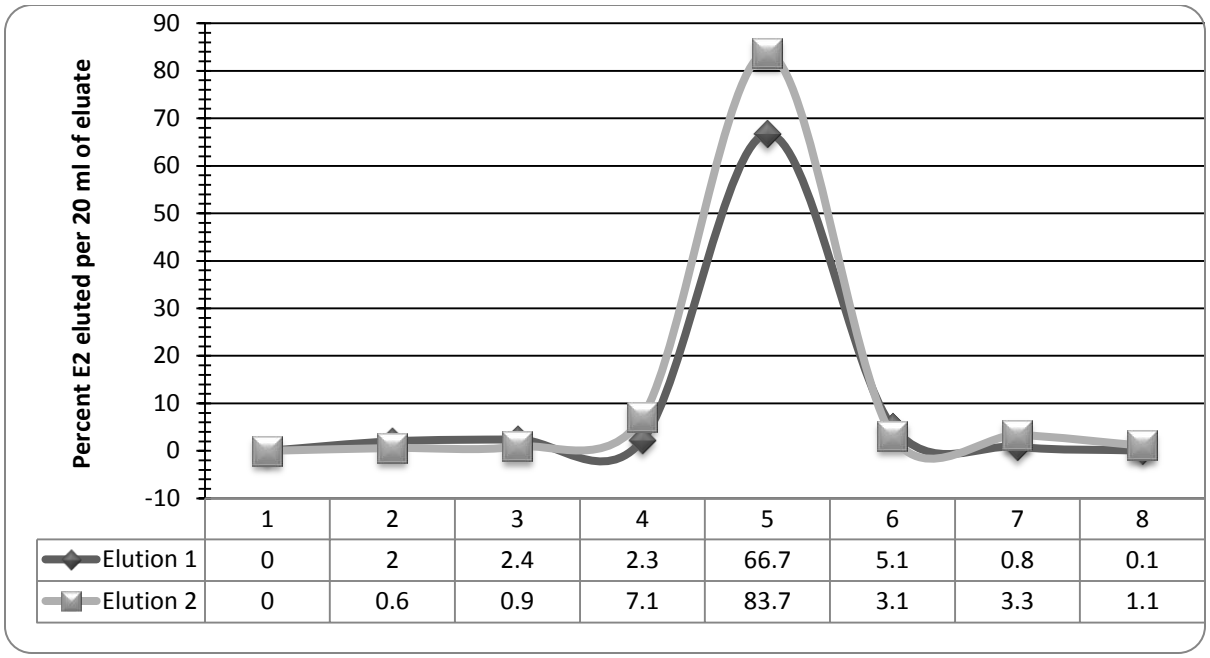
Two acetone-DCM eluent mixtures were compared to optimize elution of E2 in mixed sludge extract from a preparatory chromatography column (Table 4-12). Twelve mixed sludge samples were pH adjusted to 4.0, spiked with 1 µg E2, and subjected to preparatory chromatography using one of two elution methods utilizing different acetone in DCM eluent mixtures. Eluent mixtures for the two methods, elution 1 and elution 2, are detailed in Table 4-12. Eluate was collected in 20 ml aliquots and analysed for percent recovery of E2 from the spiked samples. Elution method 1 yielded E2 recoveries of 55-109% with an RSD of 34% (n=3), whereas elution method 2 had recoveries of 95–104 % of E2, with an RSD of 4% (n=3) (Figure 4-2).

Table 4-12: Composition of 20 ml aliquots of eluate solvents for two methods of eluting 17β-estradiol (E2) from a preparatory chromatography column

20 ml aliquots of eluate	**Elution 1**		**Elution 2**	
	Eluent	% recovery of E2	Eluent	% recovery of E2
1	DCM	0	DCM	0
2	DCM	2	DCM	0.6
3	DCM	2.4	DCM	0.9
4	Acetone:DCM (2:8)	2.3	Acetone:DCM (3:7)	7.1
5	Acetone:DCM (2:8)	66.7	Acetone:DCM (3:7)	83.7
6	Acetone:DCM (2:8)	5.1	Acetone:DCM (3:7)	3.1
7	Acetone:DCM (3:7)	0.8	Acetone:DCM (7:3)	3.3
8	Acetone:DCM (3:7)	0.1	Acetone:DCM (7:3)	1.1

Elution method 2 consistently eluted more E2 from mixed sludge extracts than Elution method 1. Elution method 2 eluted 90% of E2 spiked to the mixed sludge extracts in the first 60 ml of the acetone: DCM eluate mixture, while elution method 1 eluted only 74% of E2 in these eluate aliquots. Therefore, the preparatory chromatography elution protocol was optimized use elution method 2 solvent mixtures and volumes.

The preparatory chromatography protocol for the elution of estrogens collected 40 mls of the first 50 mls of the acetone: DCM elution mixture. The first 60 ml DCM elution was discarded as it contained only 1.5% of E2. The first 10 ml of acetone: DCM (3:7) was discarded as most of the 7% found in the 20 ml aliquot was assumed to be in the second half of this eluate. The next 40 ml of acetone: DCM (3:7) was collected for analysis.



	1	2	3	4	5	6	7	8
Elution 1	0	2	2.4	2.3	66.7	5.1	0.8	0.1
Elution 2	0	0.6	0.9	7.1	83.7	3.1	3.3	1.1

Figure 4-2: Efficacy of two elution methods utilizing different eluent mixtures for preparatory chromatography showing percent elution of 17β-estradiol in extracts from 20 ml aliquots of municipal mixed sludge (n = 3)

Throughout the two experiments, the position of target compounds on the chromatogram was highly reproducible (see Figure 4-2).

4.1.5.7 FRACTIONATION OF ELUENT MIXTURES

Fine fractionation improves the reliability and sensitivity of the recombinant yeast assays (Nakada et al., 2004) and improves clean up for GC-MS analysis. Recovery efficacy of elution mixture 2 for E1, EE2, E3 and TT in addition to E2 was examined by comparing recoveries in fractionated preparatory chromatography elutes from spiked and unspiked mixed and digested sludge samples. Percent recoveries were estimated by subtracting concentrations found in unspiked controls from concentrations recovered from spiked sludge samples for three eluate aliquots (Table 4-13).

No target compounds were detected in the first elution mixture of 60 ml DCM for either mixed or digested sludges. The second eluate of 60 ml 3:7 acetone: DCM contained 86–106% of target compounds spiked into mixed sludge and 57–96% in digested sludge, with the exception of E3 (50% and 8%, respectively). The third eluate of 40 ml 7:3 acetone: DCM contained only 2% of estrone and no other target compounds spiked into mixed sludge samples. However, this third eluate recovered substantial quantities of E2 (38%) and E3 (22%) spiked into digested sludge, but only nominal amounts of estrone (3%) and testosterone (6%).

Table 4-13: Percent recoveries in three eluate solvent mixtures of acetone and dichloromethane (DCM) during preparatory chromatography elution of estrone (E1), 17β-estradiol (E2), 17α-ethinyl estradiol (EE2), estriol (E3) and testosterone (TT) in mixed (MS) and digested (DS) sludge and intersample percent relative standard deviations (% RSD)

Target com-pounds (n = 3)	Percent recoveries in preparatory chromatography eluates								% Relative standard deviations	
	60 ml DCM		60 ml 3:7 acetone:DCM		40 ml 7:3 acetone:DCM		Total recoveries			
	MS	DS	MS	DS	MS	DS	MS	DS	MS	DS
E1	ND	ND	92	96	2	3	94	98	5	5
E2	ND	ND	97	57	ND	38	97	95	2	8
EE2	ND	ND	86	82	ND	ND	86	82	1	8
E3	ND	ND	50	8	ND	22	50	30	69	14
TT	ND	ND	106	85	ND	6	106	91	11	4

ND = non-detectable concentrations

These findings are in agreement with similar fractionation studies (Nakada et al., 2004). This preparatory chromatography elution demonstrated good recoveries from both mixed and digested sludges for E1, E2, EE2, and TT, ranging from 82–106%, but poor recoveries for E3 at less than 50%. Therefore, it was the elution method of choice for wastewater and sludge treatment processes, using E1 and E2 as the model estrogens.

4.1.5.8 ADDITIONAL ELUTION STEP

To determine if the above preparatory chromatography elution is capturing the target compounds, an additional 40 ml elution was applied to mixed and digested sludge samples. An additional elution step with 40 ml 70% acetone in DCM recovered less than 3% of 17β-estradiol in both mixed and digested sludge. Similar recoveries of estrone, 17α-ethinyl estradiol, and testosterone were found in this final eluate. However, recoveries were substantially larger for estriol with averages of 60% for mixed sludge and 49% for digested sludge (Table 4-14).

Table 4-14: Percent recovery of estrogens and testosterone in extracts from 20 ml aliquots of mixed and digested sludge with an additional elution of 40 ml acetone:DCM (7:3) during preparative chromatography

Target compound in sludge samples (n = 3)	Percent recovery of target compounds in mixed sludge	Percent recovery of target compounds in digested sludge
Estrone (E1)	2.6	1.5
17β-estradiol (E2)	2.0	2.6
[$^2H_2$] 17β-estradiol (E2dd)	2.0	2.0
Ethylnylestradiol (EE2)	3.0	2.2
Estriol (E3)	60.0	48.6
Testosterone (TT)	0.0	1.1

Therefore, elution methods for recovery of estriol required more of the most polar eluents used for eluting 17β-estradiol from the preparatory chromatography column. If the target compounds in this study were to include estriol, then an additional elution of 40 ml 7:3 acetone:DCM would be added to the preparatory chromatography step.

4.1.6 Derivatization

Detecting trace amounts of 17β-estradiol with gas chromatography, followed by mass spectrometry (GC-MS), can be difficult due to the relatively low volatility of natural estrogens. Volatility can be increased by derivatization of the functional group with silylation agents

creating a trimethylsilyl (TMS) derivative. These derivatization techniques involved replacement of the acidic hydrogen in the alcohol groups of 17β-estradiol (-OH) with an alkylsilyl group (–OTMS).

Since some of the target compounds (i.e. estrone, testosterone) also contain a carbonyl group, another derivatizing reagent may improve chromatographic peak shape. Methoxyamine will react with the carbonyl group (C=O) forming an oxime derivative (CH_3ON). Oxime derivatives can only improve chromatographic performance, but also alter GC separations (Sellers, 2010).

4.1.6.1 SILYLATION AGENT RATIO

Six derivatization agent mixture ratios were tested for detection of 17β-estradiol by GC-MS (Table 3-6). Two of these derivatization agent mixture ratios, 70:30 and 50:50 mixtures of BSTFA:pyridine, resulted in greater peak abundance (GC-MS chromatogram) for E2 in spiked and unspiked mixed sludge matrix extracts (Figure 4-3). Results were similar for digested sludge matrix extractions. Due to the variability of E2 present in mixed and digested municipal wastewater sludges, the exact percentage of E2 derivatized for each derivatization agent mixture could not be determined in the sludge matrices.

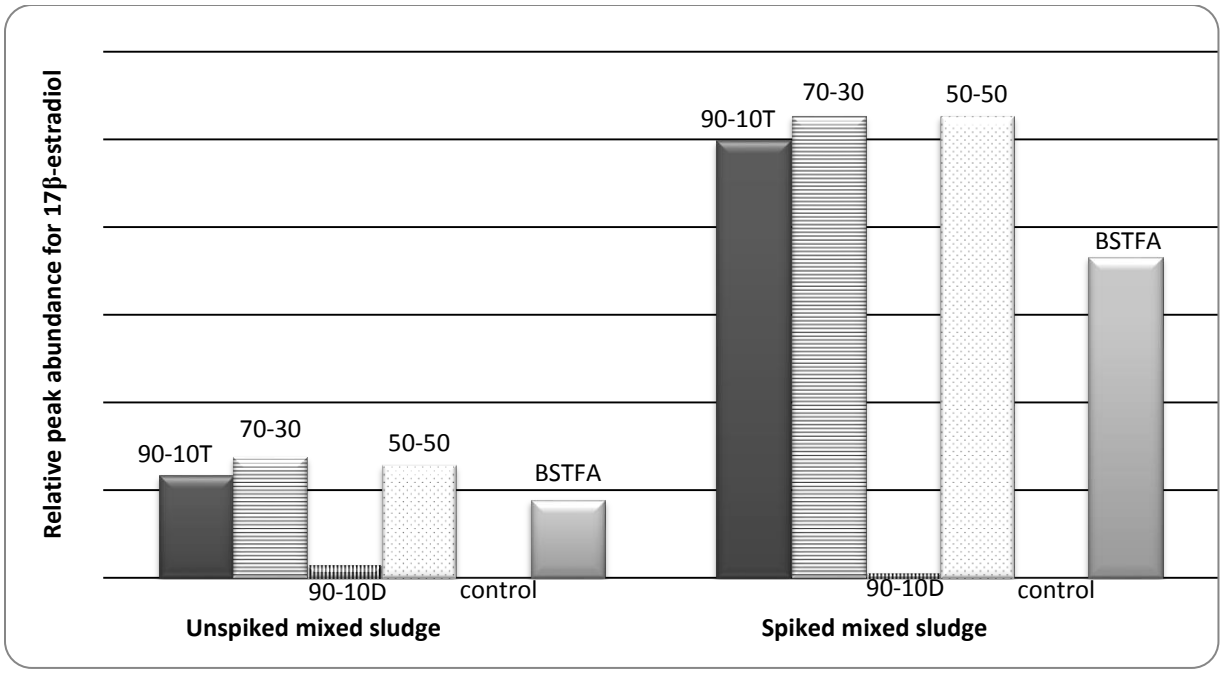


Figure 4-3: Comparison of six N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) based derivatization agent mixtures (Table 3-6) and GC-MS peak abundance for 17β-estradiol spiked in mixed domestic wastewater sludge after preparatory chromatography

Derivatization of cleaned mixed sludge extracts with 70:30 and 50:50 mixtures of BSTFA: pyridine, resulted in similar recoveries for E2 from the spiked samples. Silylation of E2 with either 70:30 or 50:50 BSTFA:pyridine were considered interchangeable within the laboratory protocol for optimization experiments. However, to provide uniform application of the laboratory protocol evaluation of wastewater and sludge treatment processes used a 50:50 mixture of BSTFA:pyridine for silylation steps when derivatizing E2 in municipal wastewaters and mixed and digested sludge extracts

4.1.6.2 SILYLATION TIME-TEMPERATURE

Sigma-Aldrich (1997) protocols for silylation with BSTFA + TMCS state derivatization times vary widely, depending upon the specific compound(s) being derivatized, while ThermoScientific (2008) recommend time-temperature derivatization at 60 °C for 15 minutes.

Many compounds are completely derivatized as soon as they dissolve in the reagent, others may require heating. Nakada et al. (2004) derivatized wastewater effluent extracts with BSTFA for 1 hour at 80 °C. However, derivatization temperatures in this study were kept at or below 70 °C, as ThermoScientific (2008) states decomposition of BSTFA can be significant at temperatures above 75 °C.

When derivatizing at 70 °C, increasing the time from 15 minutes to 7 hours resulted in 10% decreased peak abundance of E2 spiked in mixed sludge (n=6) and 43% decreased peak abundance of E2 spiked in digested sludge (n=6) (see Figure 4-4). This decrease in peak abundance on the GC-MS chromatogram may be due to degradation of E2 during the extended derivatization time at 70 °C. In addition, decreasing derivatization temperature from 70 °C to 60 °C, and increasing time from 15 minutes to one hour, also resulted in decreased peak abundance of E2, albeit a smaller decrease, of 3% for E2 in mixed sludge (n = 6) and 1.5% decrease for E2 in digested sludge (n=6) (see Figure 4-4).

Therefore, the optimized protocol for silylation of E2 in wastewater and mixed and digested sludge samples was carried out by derivatizing at 70 °C for 15 minutes, with a 50:50 mixture of BSTFA:pyridine.

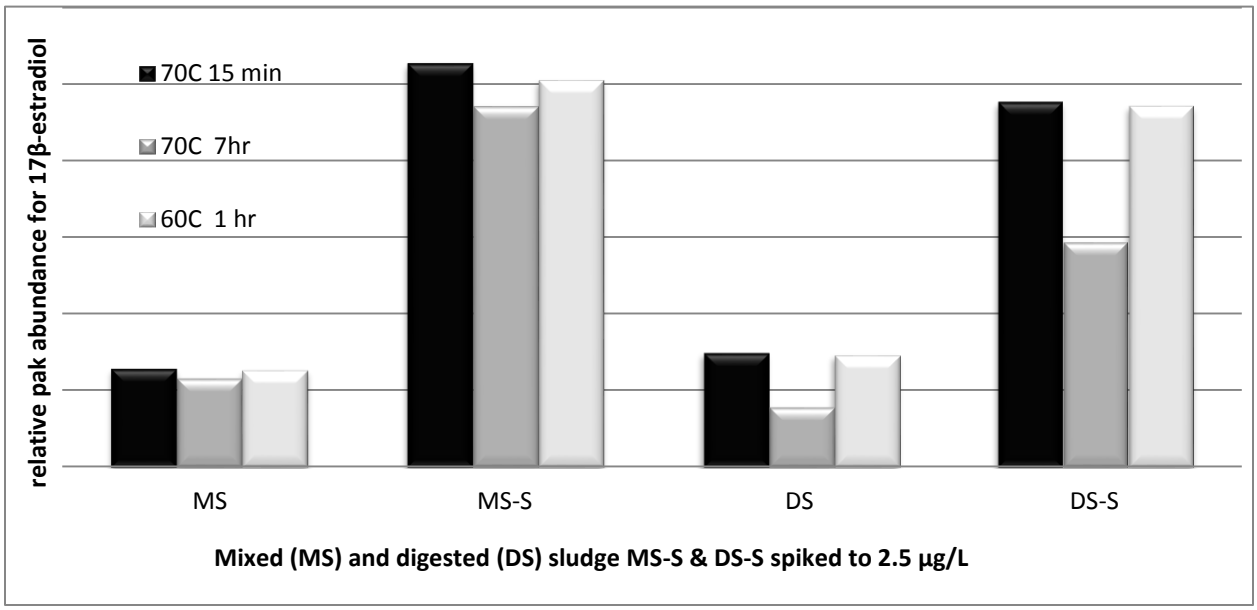


Figure 4-4: Effects of time and temperature on derivatization by silylation with BSTFA:pyridine (50:50) of 17β-estradiol in unspiked and spiked (-S) domestic mixed (MS) and digested (DS) sludge extracts

4.1.6.3 OXIMATION-SILYLATION AND PRE-SILYLATION

To determine if a two-step oximation-silylation derivatization procedure was appropriate for other sex hormones, four other steroids, estrone, 17α-ethinyl estradiol, estriol, and testosterone were targeted in addition to 17β-estradiol. In addition to the silylation reagent BSTFA with 1% TMCS, another derivatization agent, methoxyamine, was added in an attempt to form an oxime derivative (CH_3ON) with the carbonyl group in estrone and testosterone and improve chromatographic performance.

Due to GC-MS operation and maintenance issues, many samples derivatized by silylation only, had been stored frozen at -20 °C. To determine if previously silylated samples could be subject to oximation-silylation, without compromising peak strength or performance, 500 ng

standard mixtures of estrone, 17β-estradiol, 17α-ethinyl estradiol, estriol, and testosterone were derivatized at 60 °C. One-step (silylation), two-step (oximation-silylation), and three-step (silylation-oximation-silylation), were compared using seven sample sets and five methods that varied dosage and time (Table 4-16).

Silylation, oximation -silylation, and silylation- oximation -silylation derivatization methods of the target compounds are compared in Table 4-15 and Figure 4-5, using relative peak abundance from the GC-MS chromatograms for the target compounds. Both the two-step (oximation-silylation) and three-step (silylation-oximation-silylation) resulted in stronger peaks abundances for 17β-estradiol, with lower percent relative standard deviations for the three-step, silylation-oximation-silylation method.

Table 4-15: GC-MS peak abundance for one step (silylation, Si), two step (oximation-silylation, MoSi), and three step (silylation-oximation-silylation, SiMoSi) derivatization treatments at 60 °C and percent relative standard deviation (% RSD) for each target compound per treatment

Target compound (n = 3)	Derivatization treatments at 60 °C						
	Si[1]	MoSi[2]	MoSi[3]	SiMoSi[4]	MoSi[5]	SiMoSi[6]	MoSi[7]
Estrone	7667	8418	8318	8332	8407	8590	7914
E1 %RSD	3.9	12.1	22.1	3.2	8.8	6.5	5.9
17B estradiol	11766	13013	13278	13324	11983	13066	12997
E2 %RSD	3.2	6.6	9.6	1.4	7.3	1.5	5.8
17α-ethinyl estradiol	4365	4913	4963	5231	4290	5038	5097
EE2 %RSD	12.1	15.5	7.1	4.8	8.4	8.3	5.6
Estriol	3981	4531	5014	5100	5061	4918	4813
E3 %RSD	5.8	10.1	10.0	6.2	6.4	4.8	4.7
Testosterone	637	570	619	926	760	909	824
TT %RSD	9.7	16.7	13.5	7.7	6.8	7.6	6.4

[1] silylation 15 min
[2] oximation with 2% methoxyamine 30 min; silylation 15 min
[3] duplicate (of set 2-MoSi) — oximation with 2% methoxyamine 30 min; silylation 15 min
[4] silylation 15 min; oximation with 2% methoxyamine 30 min; silylation 15 min
[5] oximation-silylation performed in one step (30 min)
[6] duplicate (of set 4-SiMoSi) — silylation 15 min; oximation with 2% methoxyamine 30 min; silylation 15 min
[7] oximation 45 min with 2% methoxyamine 30 min; silylation 15 min

The higher, percent relative standard deviations noted for samples treated with the two-step oximation-silylation made it difficult to determine if the method improved detection of the other target compounds. Stronger peak abundance for testosterone was noted with the three-step silylation-oximation-silylation, which also produced an isomer of the testosterone derivative at a different retention time, when derivatization was repeated using a temperature of 70 °C (see Section 4.1.6.5 for further discussion).

Figure 4-5 illustrates the relative performance of the seven treatment sets in Table 4-18 in terms of the average peak abundance (n = 3). The three-step method derivatized mixed sludge extracts, as well as the two step method, outperformed one-step silylation. No difference was noted between duplicate sample sets (n = 3) for two step (MoSi sample sets 2 and 3) and three step (SiMoSi sample sets 4 and 6) derivatization.

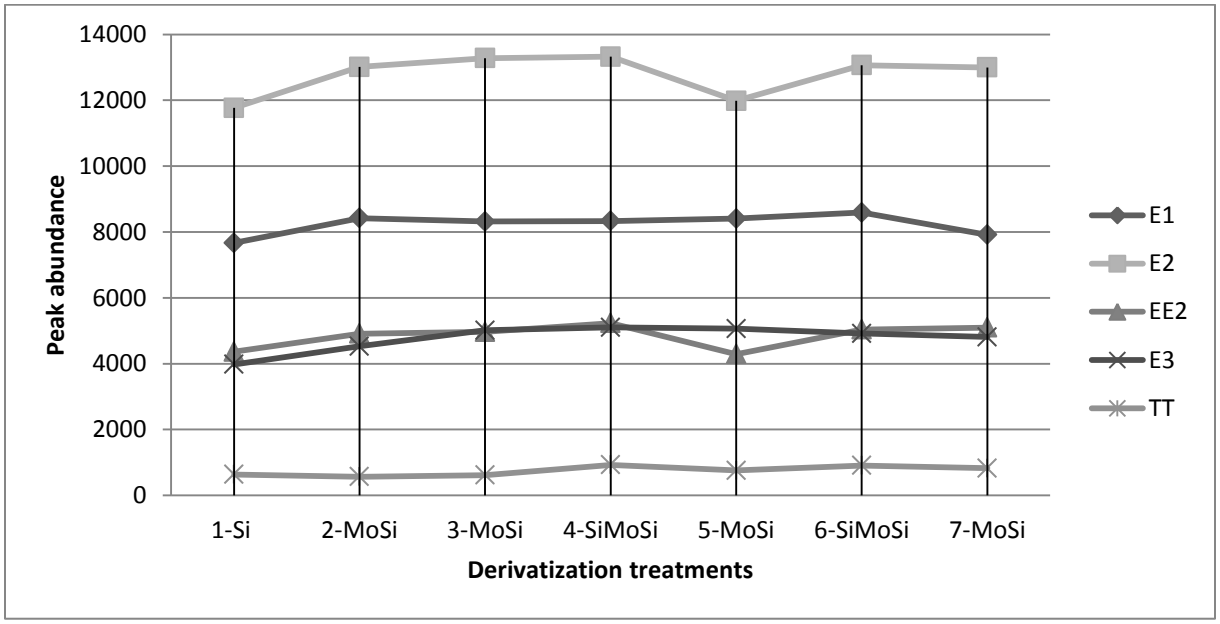


Figure 4-5: Average peak abundance (n = 3) for seven derivatization sample sets (listed in Table 4-15) at 60 °C for detecting five steroids; estrone (E1), 17β-estradiol (E2), 17α-ethinyl estradiol (EE2), estriol (E3), and testosterone (TT) by GC-MS analysis

Three-step derivatization performed as well, if not better than, the two-step oximation-silylation and both methods appeared to give higher peak abundances for the target compounds than silylation alone. Therefore, the two step oximation-silylation method was used to derivatize the previously silylated samples in storage at -27 °C. To ensure uniform application of the laboratory protocol, the three-step, silylation-oximation-silylation, derivatization was performed on wastewater and sludge samples used to evaluate sludge and wastewater treatment processes.

4.1.6.4 TIME-TEMPERATURE

Although many researchers are now freeze drying sludge, applying increasingly complex clean up methods and analyzing with LC-MS, a wide variation in derivatization time-temperatures for GC-MS analysis of estrogens in sludge and wastewater can be found in the literature. While some substances require no time or temperature application to achieve complete derivatization, the complex composition of municipal wastewater and sludges usually require more aggressive time-temperature regimes. Ternes et al. (2002) derivatized municipal sludge and wastewater using one-step silylation with MSTFA-TMSI for one hour at 60 °C, while Esperanza et al. (2007) derivatized pilot plant wastewater and sludge in a two-step oximation with 15% methoxyamine-HCl for 4 hours at 70 °C followed by silylation with 10% BSTFA-TMCS in pyridine for 15 hours at 70 °C. Use of a three step silylation-oximation-silylation could not be found in the published literature.

In an attempt to improve detection of the target compounds, twelve separate combinations of time-temperature were used to compare two step (oximation-silylation) and

three step (silylation-oximation-silylation) derivatization methods. The twelve treatments are detailed in the methodology Section 3.1.7.5, Table 3-8 and repeated as Table 4-16 in this section for convenient reference. Results for the twelve derivatization methods varying time and temperature during oximation and trimethylsilylation of estrone, 17β-estradiol, 17α-ethinyl estradiol, estriol, and testosterone are shown in Figure 4-6.

The results in Figure 4-6 and Figure 4-7 were expressed as measures of GC-MS peak abundance for detection of the target compounds: estrone (E1), 17β-estradiol (E2), 17α-ethinyl estradiol (EE2), estriol (E3), testosterone (TT). Increasing derivatizing time from 15 to 60 minutes during oximation in the two step method increased peak absorbance for E2.

Table 4-16: Twelve derivatization methods varying time and temperature during two step (oximation-silylation) and three step (silylation-oximation-silylation) derivatization of estrone, 17β-estradiol, 17α-ethinyl estradiol, estriol and testosterone

	70 µl BSTFA 30 µl pyridine			200 µl 10% methylamine + 700 µl pyridine			100 µl BSTFA			Toluene added as carrier solvent	Storage at -28°C for 2 weeks
Set #	Temp °C	Time (min)	N_2 dry	Temp °C	Time (min)	N_2 dry	Temp °C	Time (min)	N_2 dry		
1	—	—	—	70	15	—	70	15	—	—	—
2	—	—	—	70	60	—	60	15	—	—	—
3	—	—	—	70	60	—	70	60	—	—	—
4	—	—	—	60	15	—	60	15	—	—	—
5	—	—	—	60	60	—	70	15	—	—	—
6	—	—	—	60	60	—	60	15	—	—	—
7	—	—	—	70	15	yes	70	15	—	900 µl	—
8	—	—	—	70	15	yes	70	15	yes	1 ml	—
9	—	—	—	20	15	—	20	15	—	—	yes
10	70	15	yes	70	15	—	70	15	—	—	yes
11	70	15	yes	70	15	—	20	15	—	—	yes
12	—	—	—	70	60	—	70	60	—	—	yes

Pre-silylation, N_2 dry, methoximation, and silylation at 70 °C for 15 min each step (treatment 10) provided an excellent response and the strongest peak abundance for E1 and E2, while GC-MS peak abundances for EE2, E3, and TT were moderate and similar to the two step treatment sets 1 and 3. Relative peak abundance for TT and EE2 was greatest for the two step oximation-silylation treatment 2 when oximation time was increased from 15 to 60 minutes.

Treatment 2, a combination of methoximation followed by trimethylsilylation, provided the highest peak abundance for EE2, with good peak abundances for the other target compounds. Detection of EE2 appears to favour longer oximation time (60 minutes) with 2% methoxyamine and a lower silylation temperature (60 °C) and shorter silylation time (15 vs 30 minutes) with BSTFA in the two step method.

Treatment 3, oximation-silylation (similar to treatment 2 but with increased time-temperature) for silylation demonstrated adequate peak abundances but, compared to treatment 2, had a weaker response for all target compounds.

The outstanding performance of treatment set #10 (pre-silylation, N_2 dry, methoximation, and silylation at 70 °C for 15 min each step) providing strong peak abundance for estrone (E1) and 17β-estradiol (E2) (Figure 4-6), made it the preferred derivatization treatment for all sludge and wastewater samples processed in this study.

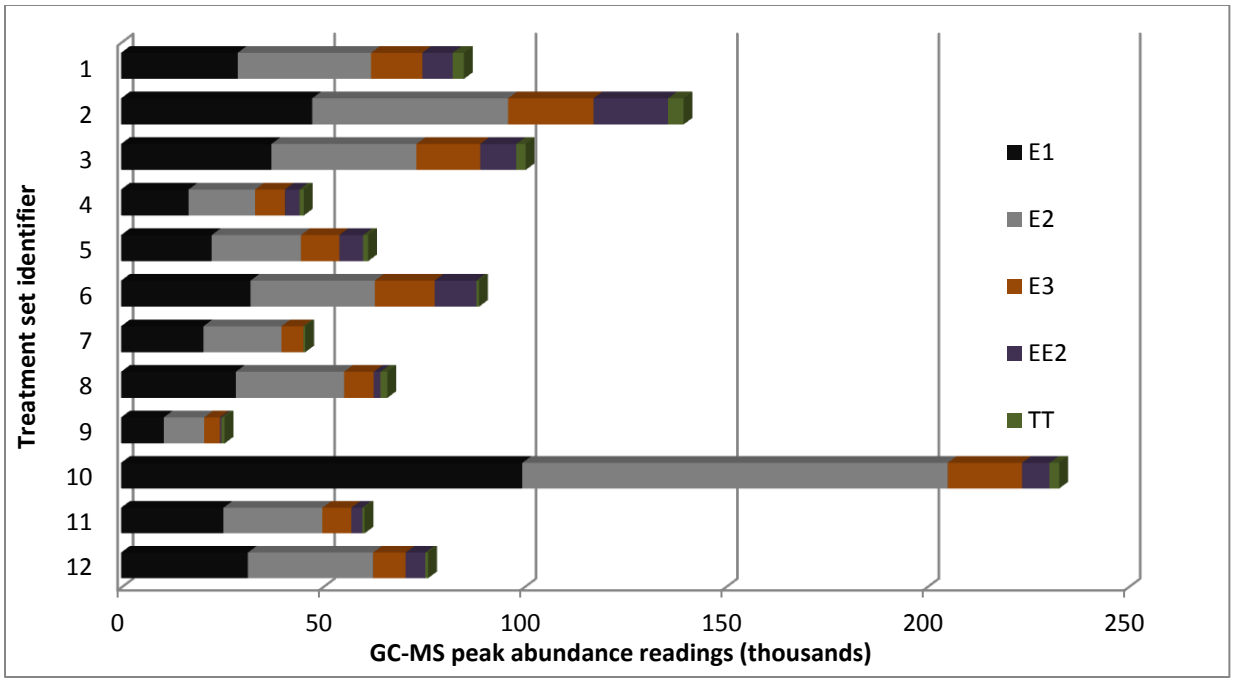


Figure 4-6: Comparison of twelve treatments (described in Table 4-16) varying time and temperature for two and three step derivatization methods for GC-MS detection of estrone (E1), 17β-estradiol (E2), estriol (E3), 17α-ethinyl estradiol (EE2), and testosterone (TT)

To optimize time-temperature for the three-step derivatization method, five treatment sets of three replicates, compared one-step (silylation), two-step (methoximation-silylation), and three time-temperature variations for a three-step (pre-silylation-methoximation-silylation), as detailed in Table 3-9 and repeated in Table 4-17 for the convenience of the reader. Results are shown in Figure 4-7 and expressed as peak abundance of the target compounds (E1, E2, E2dd, EE2, and TT) from GC-MS chromatograms.

Detection of EE2 was minimal for the five treatment sets, less than 1/100th of the relative peak abundance for E1, E2, and E3 in the five treatment sets. Although relative peak abundance for EE2 has been moderate to low compared with E1 and E2 in other experiments, the minimal response noted in the five treatment sets was unexpected. A laboratory error may have occurred in EE2 spiking or quality of the prepared EE2 analytical standard.

Table 4-17: One-step trimethylsilylation (Si) and two-step oximation-trimethylsilylation (MoSi) derivatization methods are compared to three time-temperature variations for a three-step pre-trimethylsilylation-methoximation-trimethylsilylation (SiMoSi) derivatization method

Set identifier (n=3)	70 µl BSTFA 30 µl pyridine			200 µl 10% methylamine + 700 µl anhydrous pyridine		100 µl BSTFA		GC carrier solvent added to make up a one millilitre volume
	Temp °C	Time (min)	N_2 dry	Temp °C	Time (min)	Temp °C	Time (min)	
1-Si	70	15						+ 900 µl pyridine
2-Mo-Si				70	30	70	15	
3-SiMoSi	70	15	yes	70	30	70	15	
4-SiMoSi	70	15	yes	70	60	70	15	
5-SiMoSi	70	15	yes	70	30	70	30	

Once again the three-step derivatization method outperformed both one and two step derivatization methods (Figure 4-7), with treatment #4 SMS giving the best response when oximation was increased from 30 to 60 minutes. The relative peak height for the three step method was also increased when final silylation step time was increased from 15 to 30 minutes but this may be a function of the increased time for oximation during the final silylation step.

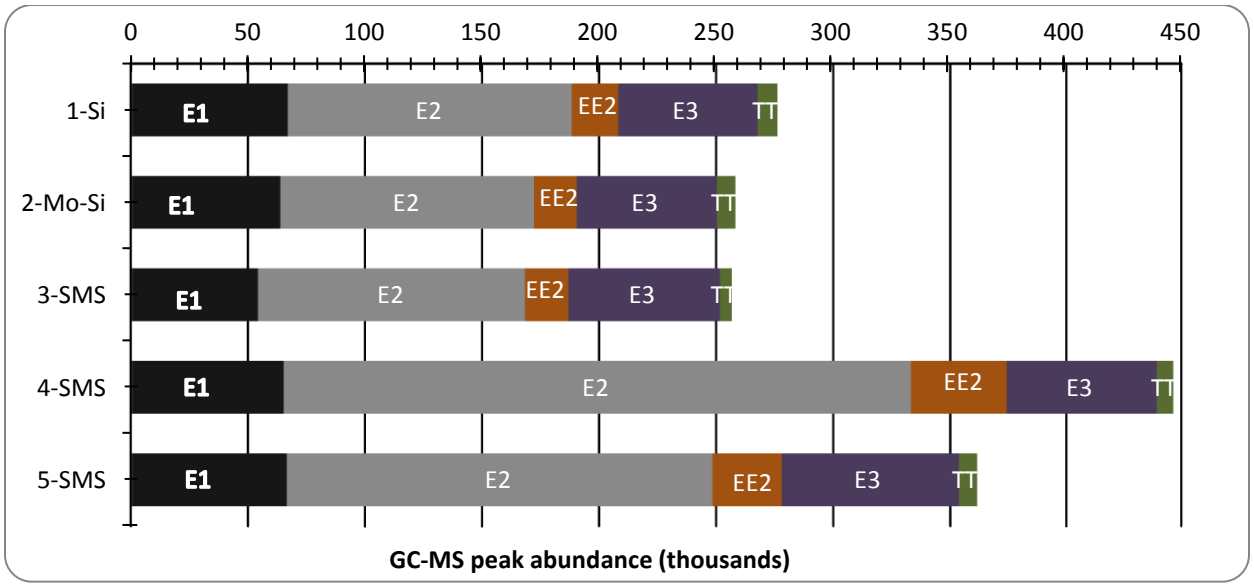


Figure 4-7: Five derivatization methods compare one-step (Si) and two-step (Mo-Si) derivatization methods with three time-temperature variations for a three step (SMS) derivatization method, as detailed in Table 4-17. Peak abundance on GC-MS chromatographs for estrone (E1); 17β-estradiol (E2); 17α-ethinyl estradiol (EE2); estriol (E3); and testosterone (TT) are compared for the five derivatization methods.

Oximation at 70 °C for 60 minutes, in the two and three step derivatization methods produced the highest relative peak abundance for E1 and E2, although peak abundance did not increase for the other compounds. Therefore the optimized time-temperature protocol for the oximation step in two and three step derivatization methods was 60 minutes at 70 °C.

4.1.6.5 PRE-SILYLATION IN SLUDGE MEDIA

To determine if the two and three step methods of derivatization are suitable for analysis of E1, E2, EE2, E3, and TT in sludge media by GC-MS, relative peak abundance of the target compounds were compared (Table 4-18).

When a pre-silylation step at 70 °C was added to oximation-silylation derivatization at 70 °C, the peak strength was increased by 200% for estrone and 17β-estradiol (see Table 4-18).

While chromatographic performance remained similar for 17α-ethinyl estradiol, testosterone and estriol, two retention times were noted for testosterone peaks. The two testosterone peaks were likely due to an isomer of the testosterone derivative (van de Kerkhof, 2001; Xu et al., 2010).

Table 4-18: Peak strength (abundance) using two step (oximation-silylation) and three step (silylation-methoximation-silylation) derivatization methods at 70 °C to detect estrone, 17β-estradiol, 17α-ethinyl estradiol, estriol, and testosterone in mixed sludge extracts by GC-MS

Target Compound (n = 3)	Two step derivatization		Three step derivatization		Percent difference
	Average peak abundance	% RSD[1]	Average peak abundance	% RSD[1]	
Estrone	91327	4.8	304622	5.6	234
17β-estradiol	115556	3.5	361127	2.3	213
17α-ethinyl estradiol	201134	1.9	205181	1.6	2
Estriol	178194	1.5	180566	1.8	1
Testosterone	73294	4.7	66712	5	-9
Testosterone (peak at second retention time)			20751	1.9	

1. % RSD = percent relative standard deviation

The increase in relative peak abundance for E1 and E2 was noted in solvent standards and sludge matrix spiked extracts derivatized with the three step method when compared to the two step method or one-step silylation. Estrone and E2 have the least number of reaction sites with one ketone (=O) on estrone and two hydroxyl (–OH) groups on 17β-estradiol. Silylated E1 and E2 may be more readily available to oxime formation. The subsequent silylation increases the exposure of E1 and E2 to the BSTFA-TMCS silylation agent to form methyloxime-trimethylsilyl (MO-TMS) ethers. The three-step method had greater time-temperature exposures during the pre- silylation step at 70 °C for 15 minutes, cooling and additional N_2 drying step at 50 °C, that may contribute to greater derivatization over the two-step derivatization step.

No significant difference in recovery of E2 was noted (n = 6; % RSD = 5.1) when derivatized samples were N_2 dried at 50 °C (n = 3; % RSD = 5.6) or at 37 °C (n = 3; % RSD = 6.2) before reconstituting samples in one millilitre toluene. Therefore, all remaining N_2 drying steps were performed at 50 °C.

The optimized protocol for derivatization of wastewater and sludges was the three-step method detailed in Section 3.1.7.1 and can be summarized as: trimethylsilylation (15 minutes); N_2 drying at 50 °C; oximation (60 minutes); and trimethylsilylation (15 minutes) at 70 °C.

4.1.7 Detection Limits

Derivatization of compounds often results in more than one peak for a metabolite of interest, owing to either partial silylation or isomerization in the case of some methoxyamine-treated compounds (Lisec et al., 2006). In this protocol this author identified all peaks of one compound, calculates their response independently, and picked the strongest, most reliable peak ion for quantification of the compound of interest. However, other methods such as summation of the peaks of one compound could be used as an alternative strategy (Lisec et al., 2006).

An equal quantity of internal standard (200 or 500 ng), deuterated 17β-estradiol was added to all sludge samples prior to derivatization steps.

Detection limits for estrone, 17β-estradiol, 17α-ethinyl estradiol, estriol and testosterone were calculated using the optimized methodology for mixed and digested sludges. Method

detection limits and limits of quantification, when calculated in accordance with standard laboratory procedures (Ripp, 1996; American Public Health Association, 2005), demonstrated low MDLs and LOQs for detection of estrone (0.8 ng and 2.5 ng per sample) and 17β-estradiol (0.5 ng and 1.4 ng per sample). 17α-ethinyl estradiol, estriol, and testosterone had higher MDLs and LOQs (Table 4-19) and were more difficult to detect in sludge and wastewater samples than E1 and E2.

Table 4-19: Analytical limits for GC-MS method to detect estrone, 17β-estradiol, 17α-ethinyl estradiol, estriol, and testosterone in domestic sludge and wastewaters (ng/sample)

Analytical limits	Estrone	17β-estradiol	17α-ethinyl estradiol	Estriol	Testosterone
Method detection limit	0.8	0.5	3.8	3.9	13.4
Limit of quantification	2.5	1.4	11.9	12.3	42.7

The MDL and LOQ for E1 and E2 (Table 4-19) were within the ranges reported for GC-MS analysis of wastewater and sludge matrices in the published literature (Ternes et al., 2002; Braga et al., 2005; Esperanza et al., 2007; Nakada et al., 2004; Servos et al., 2005; Muller et al., 2005). The MDL for EE2 and E3 (Table 4-19) were within the ranges reported by Muller et al. (2005) but higher than those reported by Esperanza et al. (2007). The MDL for testosterone (Table 4-19) was almost 7X higher than the MDL (2 ng/L) for testosterone reported by Esperanza et al. (2007). The derivatization method for this laboratory protocol is not ideal for testosterone and environmentally relevant concentrations may not be detected using this method.

This method can be used with confidence for the detection of E1 and E2 in wastewaters, mixed, digested, and activated sludges. However, concentrations of EE2, E3, and TT may be underestimated, as this method is not as sensitive to these compounds and will likely report low

levels as non-detectable (ND). This method can be improved for the detection of E3 by adding a final elution (20 ml of 7:3 acetone:DCM) to the preparatory chromatography step, as described in the Chromatography Section 3.1.6.9 Additional elution step.

Mixed and digested sludges are very complex matrices and despite the optimized extraction and cleanup procedures used in the developed chemical analysis, matrix interferences can cause decreased sensitivities and increased noise with GC-MS. Techniques that may be employed with GC-MS to increase sensitivity and decrease noise include the use of additional analytical equipment, such as tandem mass spectrometry, and/or intensive multi-step extraction and clean up procedures.

4.1.8 Recovery of Target Analytes

Recoveries of estrone, 17β-estradiol, 17α-ethinyl estradiol, estriol and testosterone were determined using the optimized methodology. Method recoveries were assessed in terms of absolute recovery using a deuterated E2 surrogate (500 ng) in 20 ml sludge and 1 L wastewater samples and relative recovery estimated from subtracting the concentration of target compounds found in control sludge and wastewater samples from the concentration of target compounds found in spiked (500 ng) sludge and wastewater samples (Table 4-20; Table 4-21; and Table 4-22).

Table 4-20 shows relative recoveries of estrone, 17β-estradiol, 17α-ethinyl estradiol, estriol and testosterone in 1 L wastewater influent (n = 3) and effluent (n = 3), estimated by subtracting concentrations of the target compounds in unspiked controls from the concentrations

found in spiked controls. Absolute recoveries were calculated as the recovery of 500 ng deuterated E2 from one litre of wastewater and were 95% ± 6 for influent and 97% ± 2 for effluent (Table 4-20). Absolute and relative recoveries were in the ranges reported in the published literature for recovery of estrogens (Esperanza et al., 2007; Braga et al., 2005; Nakada et al., 2004; Joss et al., 2004; Muller et al., 2008) and testosterone (Esperanza et al., 2007) from municipal wastewaters.

Table 4-20: Percent absolute recovery from 1 L municipal wastewater of deuterated 17β-estradiol (E2dd) and estimated percent recoveries of estrone (E1), 17β-estradiol (E2), 17α-ethinyl estradiol (EE2), estriol (E3) and testosterone (TT) with percent relative standard deviation

Steroid (n = 3)	Influent (1 L)		Effluent (1 L)	
	Percent recoveries of spike (500 ng)	Percent relative standard deviation	Percent recoveries of spike (500 ng)	Percent relative standard deviation
E1	101	16	97	9
E2	98	13	100	7
E2dd	95	11	97	4
EE2	94	14	95	11
E3	23	22	27	19
TT	92	14	96	10

Absolute recoveries of 17β-estradiol in mixed (92%) and digested sludge (95%) were determined by spiking 20 ml samples with 500 ng of deuterated 17β-estradiol prior to extraction (Table 4-21 and Table 4-22).

Relative recoveries were also estimated by comparing 20 ml unspiked controls to mixed (MS) and digested (DS) sludges spiked prior to extraction procedures, with 500 ng of estrone (MS 98%; DS 87%), 17β-estradiol (MS 81%; DS 89%), 17α-ethinyl estradiol (MS 91%; DS 86%), estriol (MS 21%; DS 17%), and testosterone (MS 98%; DS 94%) (Table 4-21 and Table 4-22).

Table 4-21: Recovery of 500 ng deuterated 17β-estradiol (E2dd) and estimated recoveries of 500 ng estrone (E1), 17β-estradiol (E2), 17α-ethinyl estradiol (EE2), estriol (E3), and testosterone (TT) from 20 ml mixed sludge (MS) with percent relative standard deviation

Steroid	Number of 20 ml MS samples	Average concentration		Contribution of spiked compound	% Relative standard deviation	Percent Recoveries of spike
		Spiked (500 ng)	MS controls			
E1	4	543	54	489	14	98
E2	5	448	43	405	16	81
E2dd	5	459	N/A	459	13	92
EE2	4	456	ND	456	15	91
E3	4	107	ND	107	27	21
TT	4	563	75	488	13	98

Recovery of estrone may be overestimated, and 17β-estradiol underestimated due to degradation of E2 into the by-product E1. To decrease bacterial degradation prior to and during the extraction process, the pH of sludge samples was reduced to 4.0, prior to adding target compounds. Gomes et al.(2004) spiked dried sludges and left them to air dry overnight and found recoveries of E1=238.5%, while recoveries of E2 = 0.9%. The odd recovery values were deemed to be biological degradation of E2 into E1. Subsequently, they autoclaved sludges prior to spiking and obtained recoveries of 75% for E1 and 96% for E2.

Recoveries for 17β-estradiol, 17α-ethinyl estradiol, estriol and testosterone in sludge matrices were within the ranges reported for recovery of estrogens in the published literature (Esperanza et al., 2007; Braga et al., 2005; Gomes et al., 2004; Nakada et al., 2004; Joss et al., 2004; Muller et al., 2008) and similar to recoveries of testosterone from sludge matrices reported by Esperanza et al. (2007).

Table 4-22: Absolute recovery of 500 ng 17β-estradiol 2D (E2dd) and relative recoveries of 500 ng estrone (E1), 17β-estradiol (E2), 17α-ethinyl estradiol (EE2), estriol (E3), and testosterone (TT) from 20 ml digested sludge (DS)

Steroid	Number of 20 ml DS samples	Average concentration		Contribution of spiked compound	% Relative standard deviation	Percent Recoveries
		Spiked (500 ng)	DS controls (ng)			
E1	5	525	90	435	19	87
E2	8	450	7	443	14	89
E2dd	8	476	N/A	476	15	95
EE2	5	483	55	483	11	86
E3	5	84	ND	84	12	17
TT	5	563	94	469	34	94

To evaluate where in the chemical analysis method losses of analytes were occurring, samples of four matrices; mixed sludge, digested sludge, influent, and effluent were spiked with 17β-estradiol, prior to preparatory chromatography or extraction procedures. Although inter-sample relative standard deviation varied from 2–10% for mixed and digested sludges, most of the analyte appears to be lost during preparatory chromatography (5% and 13%), as opposed to extraction (2% and 0.5%), respectively (Table 4-23). These findings agree with the analyte concentrations found in discarded portions of eluate during experiments to optimize preparatory chromatography as a clean up step (Table 4-13 and Table 4-14).

Table 4-23: Percent loss of 17β-estradiol (E2) in 20 ml mixed and digested sludges and 1 L wastewater influent and effluent during preparatory chromatography and extraction procedures

Sample matrix (n=3)	Mixed sludge		Digested sludge		Effluent		Influent	
Spiked with 1 ug E2 prior to:	% loss	% RSD	% loss	% RSD	% loss	% RSD	% loss	% RSD
Derivatization	N/A	7	N/A	2	N/A	6	N/A	6
Chromatography	5	2	13	3				
Extraction	2	10	0.5	6	1	4	-8	29
Note: percent loss of E2 assumes 100% recovery when spiked immediately prior to derivatization								

4.1.9 Quality Control

Throughout all of the experiments, no target compounds were found in the reagent blanks and method blanks.

To verify the method of using standards in solvents that are directly derivatized before GC-MS, to assess concentrations target compounds in wastewater sludge media, standard curves made up of target compounds in mixed and sludge extracts were compared to those in the derivatization agent. Standard curves in mixed and digested sludge were derived from adding five estrogens and testosterone (10 ng to 500 ng of E1, E2, EE2, E3, and TT) to cleaned sludge extracts, nitrogen dried, and immediately derivatized to produce standard curves (see Figure 4-8 and Figure 4-9).

Standard curves in mixed and digested sludges reflected the recoveries previously established for the target compounds and the concentrations of these compounds detected in the unspiked, sludge media controls. When all target compounds were spiked into mixed and digested sludge extracts, the effect of E1 increasing, as E2 decreased, was especially noticeable at higher concentrations (i.e. 250 ng and 500 ng), despite a pH of 4.0 prior to sample extractions.

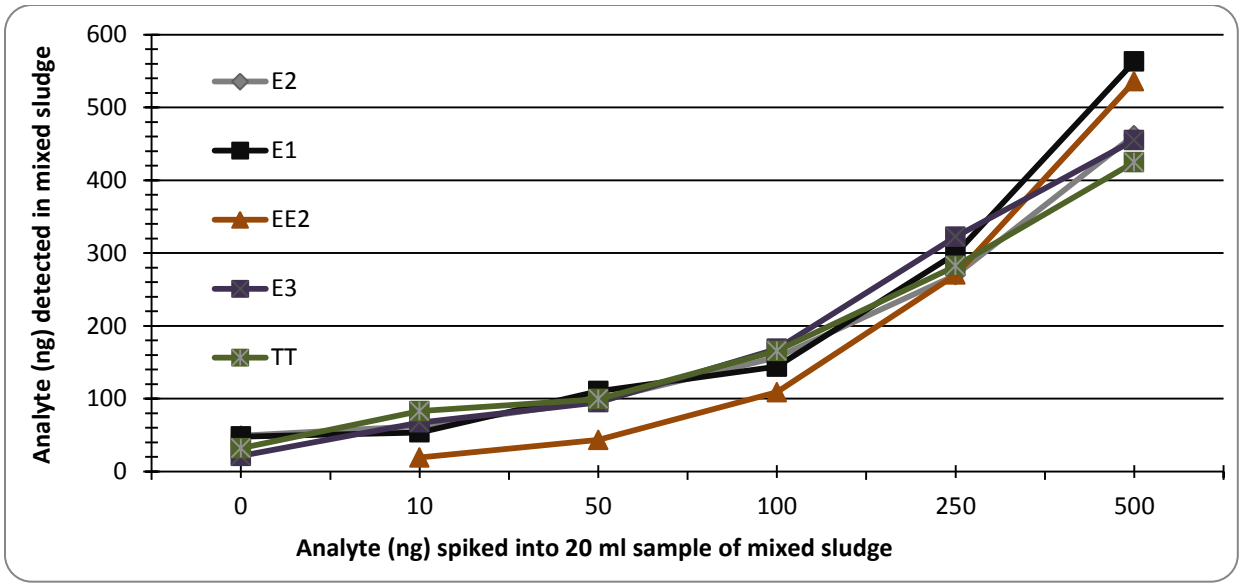


Figure 4-8: Standard curves of estrone (E1), 17β-estradiol (E2), 17α-ethinyl estradiol (EE2), estradiol (E3) and testosterone (TT) in 20 ml mixed sludge media

Sludge media standard curves have higher values at lower concentrations of the added standards, due to increased baselines from hormones already present in the mixed and digested sludges.

When using standard curves in mixed and digested sludge media to measure concentrations of the target compounds in sludge media, allowances for initial concentrations (estimated from controls) must be included in the calculations. Since standard curves of the target compounds in mixed (Figure 4-8) and digested (Figure 4-9) sludge were similar to the standard curves in GC-MS solvents (toluene and derivatization agent mixtures), it was concluded that concentrations of target compounds can be determined from standard curves in GC-MS solvents (Figure 4-10 and Figure 4-11).

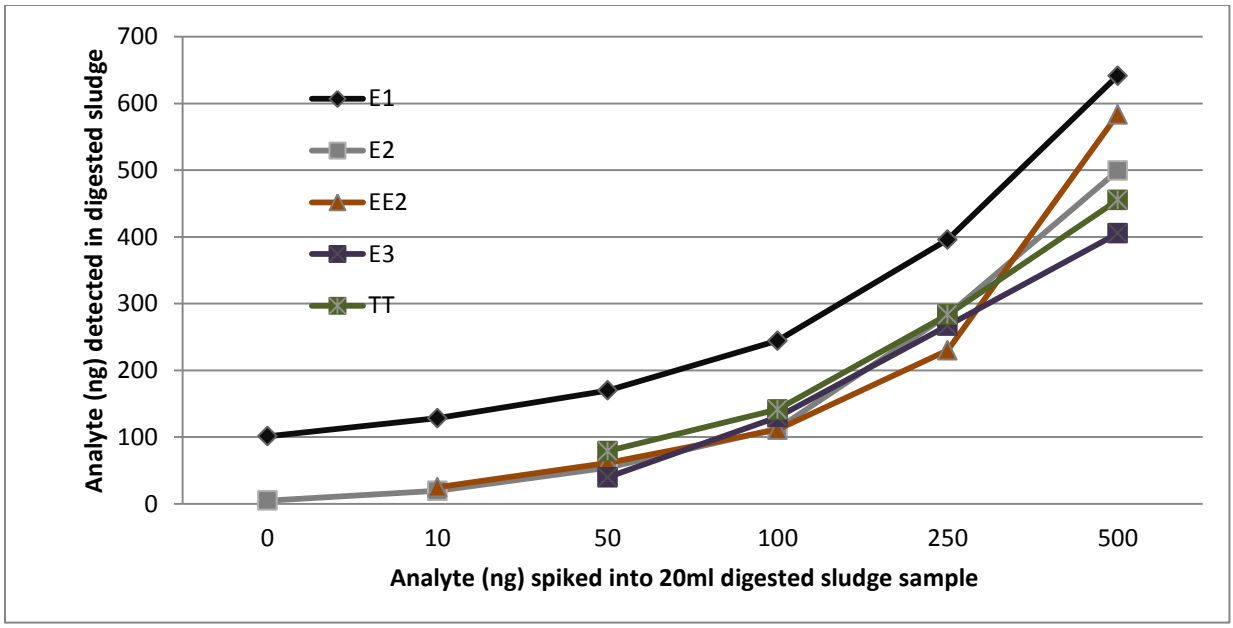


Figure 4-9: Standard curves of estrone (E1), 17β-estradiol (E2), 17α-ethinyl estradiol (EE2), estradiol (E3) and testosterone (TT) in 20 ml digested sludge media

Some variability in the GC-MS peak signal is to be expected between sludge wastewater samples, due to instrument performance and presence of target compounds in unspiked samples. An internal standard was added to samples prior to derivatization, standard sets run before and after every sample batch run, and a known standard in reagent run between sample sets; this evaluated instrument performance and allowed for corrections in peak abundance readings if necessary.

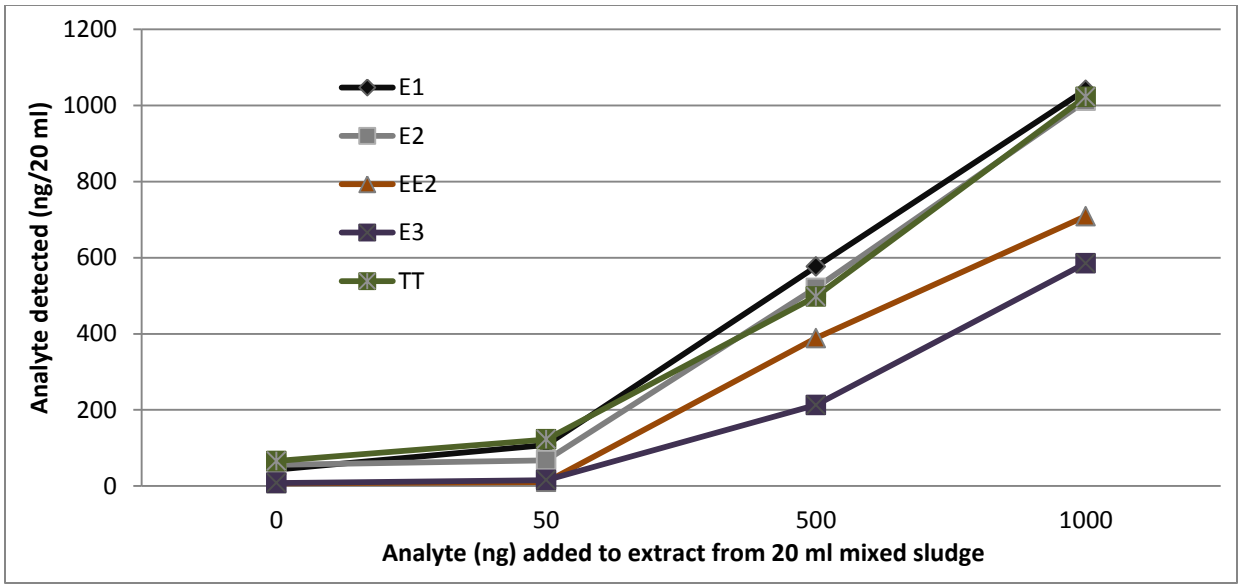


Figure 4-10: Analyte detected versus analyte added to extracts from 20 ml mixed sludge for estrone (E1), 17β-estradiol (E2), 17α-ethinyl estradiol (EE2), estriol (E3), and testosterone (TT)

In accordance with standard quality control measures for repeatability (Ripp, 1996), preparation of standard curves in mixed (Figure 4-10) and digested (Figure 4-11) sludge media and calculation of recoveries for various analyte concentrations was carried out six months apart. Standard curves in mixed and sludge media were, once again, comparable to standard curves prepared in reagent for all target analytes (Figure 4-11). Variability in analyte recoveries was a reflection of variability in unspiked samples, method recoveries, and analytical detection limits (see analyte recovery tables for standard curves in Appendix D-1).

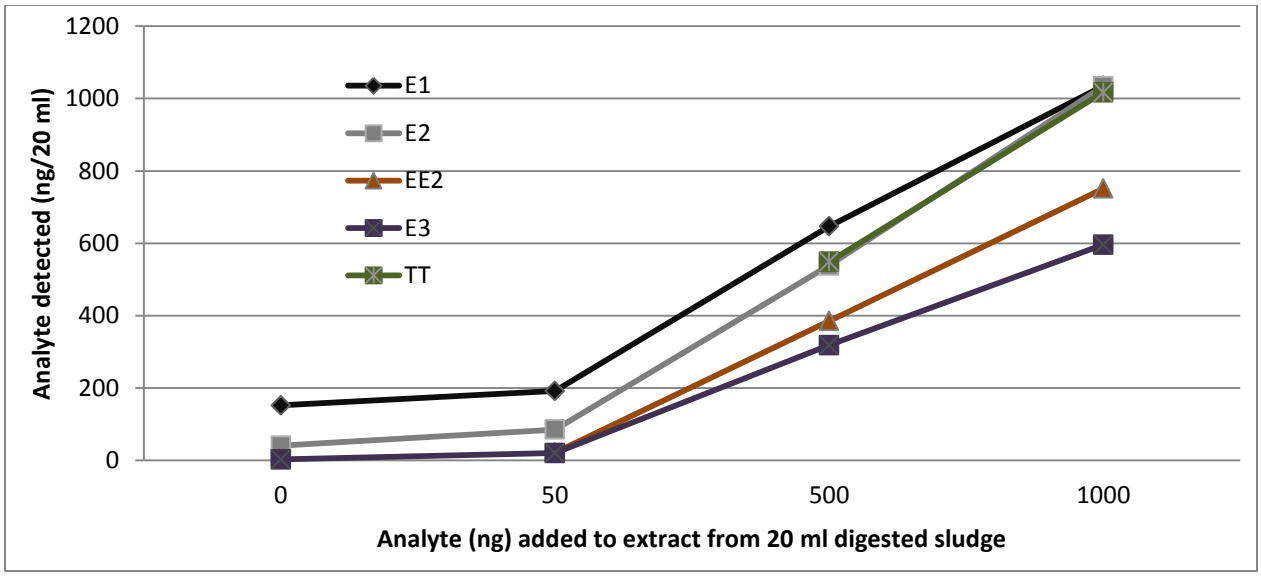


Figure 4-11: Analyte detected versus analyte added to extracts from 20 ml digested sludge for estrone (E1), 17β-estradiol (E2), 17α-ethinyl estradiol (EE2), estriol (E3), and testosterone (TT)

4.2 WASTEWATER TREATMENT PROCESSES

The average concentrations of target estrogens (**Error! Reference source not found.**) in he raw sewage were similar to that reported in other studies (Ifelebuegu, 2011; Sim et al., 2011). While the range of EE2 concentrations found in this domestic wastewater treatment plant fall within the range reported for domestic wastewaters (Debrow et al., 1998; Ternes et al., 1999; Servos et al., 2005; Ifelebuegu, 2011) and domestic wastewater treatment plant sludge (Ternes et al., 2002; Ifelebuegu, 2011; Drewes et al., 2005), due to the high method detection limit for EE2 associated with the analytical method used in this study, assessments for wastewater and sludge processes were based on E1 and E2 concentrations; and whole androgenic and estrogenic activities.

Greater than 80% removal of estrogens and estrogenic activity is commonly reported for wastewater treatment plants utilizing activated sludge technologies (Ternes et al., 1999; Servos et al., 2005; Drewes et al., 2005; Ifelebuegue, 2011; Fernandez et al., 2007; Leusch et al., 2006), which are considered to provide better removal of estrone (E1) and 17β-estradiol (E2) than tricking filter treatment of domestic wastewaters (Ternes et al., 1999; Servos et al., 2005). While Servos et al. (2005) found a trickling filter plant ineffective in removing estrogens, Ternes et al. (1999) reported E1 and E2 removal efficacies for domestic wastewater treatment plants utilizing trickling filter technology (67% and 92%) and activated sludge systems (83% and 99.9%) respectively.

In this study, the municipal wastewater treatment plant (WWTP) utilizes trickling filter technology. During each sampling event, three grab samples of wastewater were collected at each of six locations in the municipal wastewater treatment plant as described in Section 3.1.1 to evaluate treatment processes. Although the wastewater treatment plant reduced total E1+E2, as well as both E1 and E2 concentrations, some sampling days would show higher concentrations in the effluent than influent grab samples. It was important to repeat influent and effluent sampling at random days, to obtain a clear picture of whether the wastewater treatment process actually reduced or increased total E1+E2. It was even more important to obtain samples at each step in the wastewater treatment plant, before evaluating process performance in reducing estrogens and estrogenic activity.

Precipitation can saturate soils, carry contaminants and infiltrate sanitary transport pipes. It can increase contaminant loading through agricultural and urban runoff and, alternatively,

decrease contaminant loading through dilution of the wastewater stream. Precipitation can challenge wastewater treatment plant hydraulic load capacities and affect treatment processes. Flow into a sewage treatment plants can increase by up to 30% during rainfall events, due to infiltration (Tchobanoglous et al., 2003).

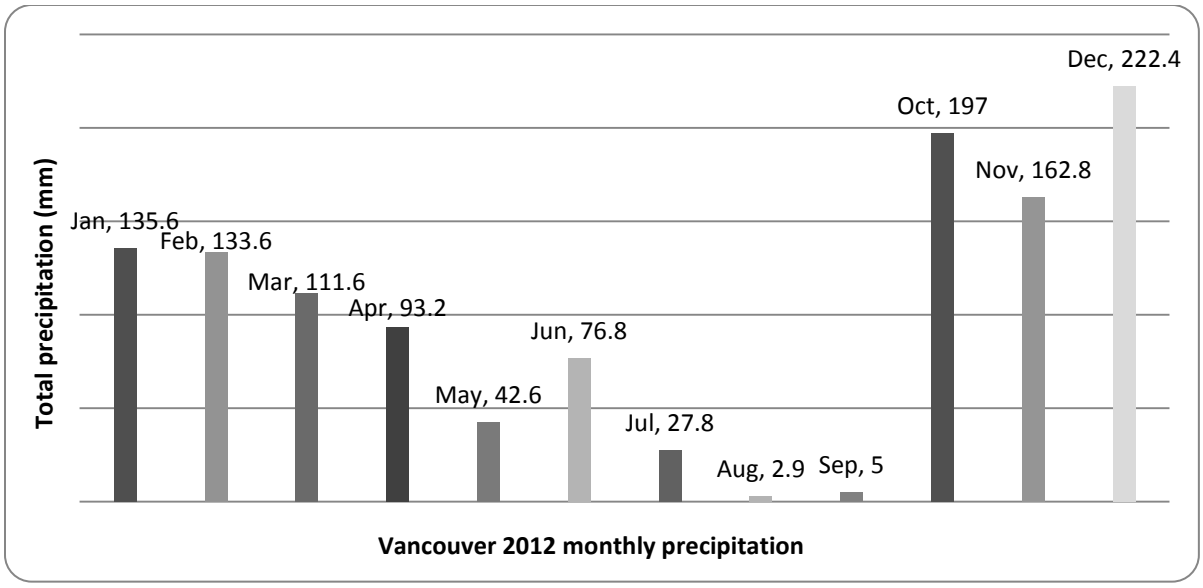


Figure 4-12: Total monthly precipitation (mm) at Vancouver International Airport in 2012. Data from Government of Canada (2013).

Concentrations of E1 or E2 (Figure 4-13) and ratio of E1 to E2 in the wastewater plant influent did not appear to be related to monthly precipitation (Figure 4-12 shows total monthly local precipitation). While the pattern of total E1+ E2 first appeared to directly correlate with monthly precipitation, the highest total E1+E2 concentration occurred in September, one of the driest months in 2012. Perhaps influent concentrations E1 and E2 would be better correlated to activities within the community population (e.g. summer holidays, large sporting events, etc.).

The influent (n = 6) concentrations of estrone (2–13 ng/L) and 17β-estradiol (4–19) to this wastewater treatment plant were within the range reported for municipal treatment plant inflows (Ifelebuegu , 2011; Sim et al., 2011). Not enough samples were processed to assess mass balance of E1 and E2 to determine diurnal or seasonal effects of runoff, temperature, precipitation, plant capacity, etc. In addition, the influent and effluent grab samples cannot provide a snapshot of plant efficacy, since influent E1 and E2 concentrations will vary greatly with time (Figure 4-13 and Figure 4-14); and the treatment time within the WWTP ensures that the initial contaminant content of the effluent prior to treatment cannot be represented by an individual influent grab sample. However, several samples together will give a clearer picture of plant efficacy in removal of E1 and E2.

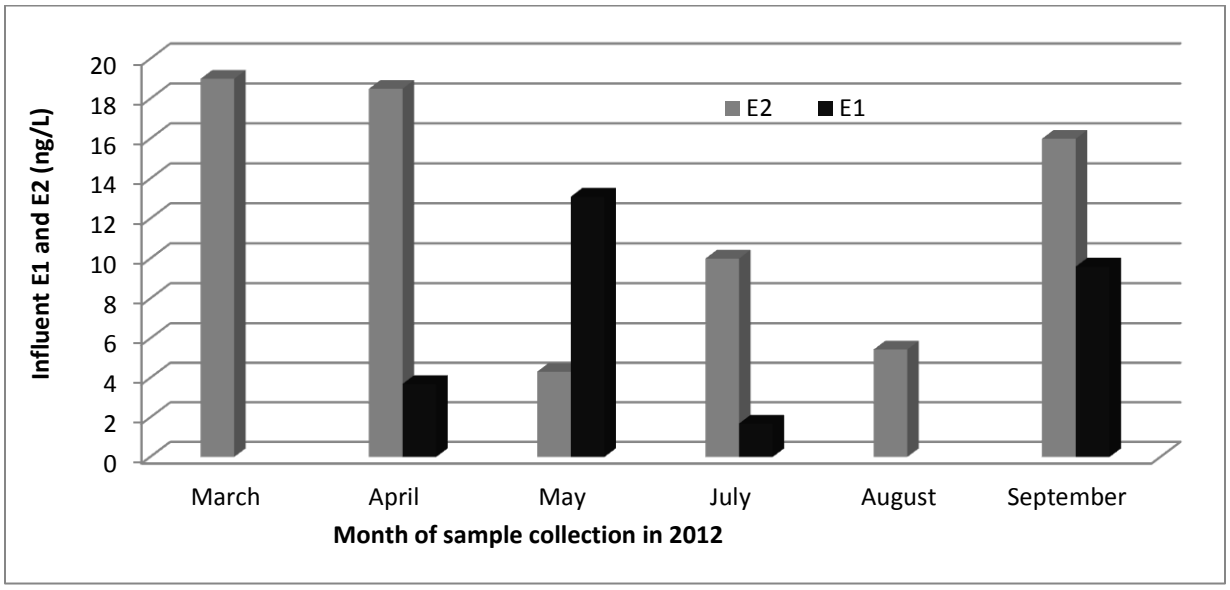


Figure 4-13: Influent (n = 3 grab samples collected in one day) estrone and 17β-estradiol per month of collection from a municipal wastewater treatment plant (samples were not analysed for estrone in March or August)

Effluent concentrations of E1 and E2 determined in this study are within the range of concentrations reported in recently published studies (Ifelebuegu, 2011; Drewes et al., 2005; Sim et al., 2011). All samples were collected between March 1st and October 1st, so all sampling events included pre-chlorinated effluent collected prior to the chlorine contact tank and post chlorinated effluent from the chlorine contact tank. While post-chlorinated demonstrated higher concentrations of total E1+E2 (Table 4-26), this effluent is treated to de-chlorinate, prior to release into the environment.

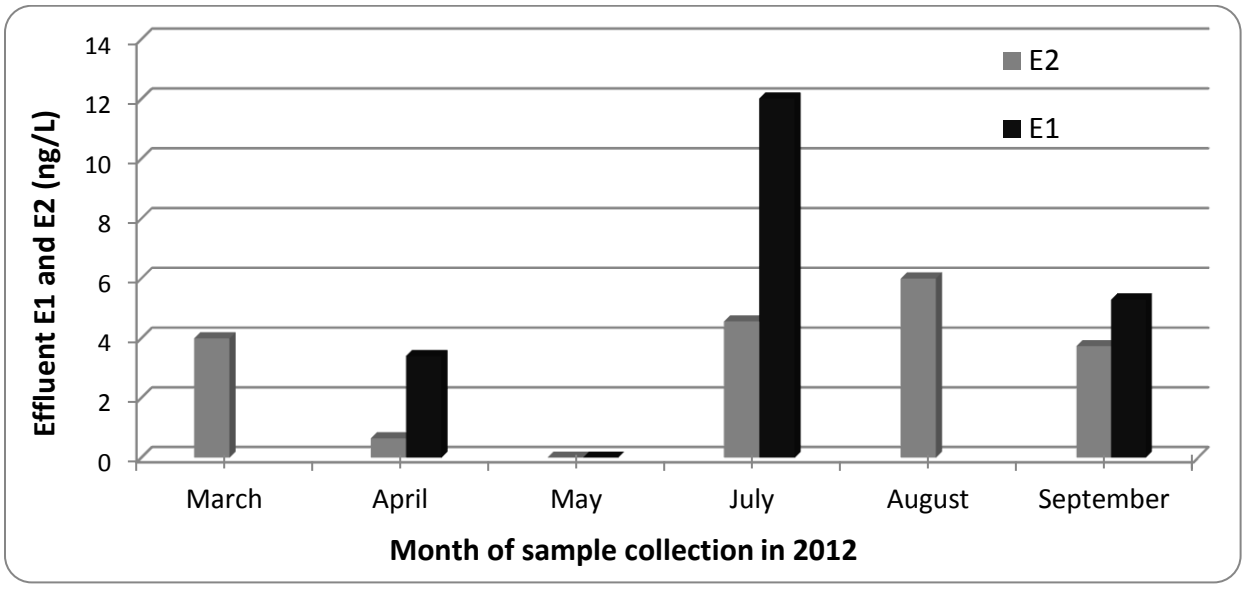


Figure 4-14: Effluent (pre-chlorination), (n = 3 grab samples collected in one day) estrone and 17β-estradiol concentrations per month of collection from a municipal wastewater treatment plant (samples were not analysed for estrone in March or August)

Although caution must be exercised when assessing results from a limited number of grab samples, there appears to be an inverse relationship between average monthly temperature and removal of E2 from the wastewater stream (Table 4-24).

Table 4-24: Average monthly temperature[1] and removal of 17β-estradiol in a municipal wastewater treatment plant using tricking filter technology

E2 (ng/L)	Influent	Effluent	% reduction	Average temperature (°C)
March	19.0	3.7	80.5	8.7
April	18.5	0.6	96.5	13.1
May	4.3	0.0	100.0	16.1
July	10.0	4.6	54.4	21.7
August	5.4	6.0	-10.4	23.5
September	16.0	3.7	76.6	19.6

[1] Source: Environment Canada (2014)

The percent reduction of E2 within the wastewater treatment plant decreases with increasing temperatures. The primary mechanism of E2 removal within a tricking filter treatment plant is thought to be associated with adsorption to solids and subsequent settling of solids. Ifelebuegu et al. (2010) determined partitioning coefficients (Kd) for E2 in mixed sludge at environmentally relevant temperatures. The partitioning coefficients were determined for a blended sludge mixture of primary and secondary sludges from a municipal wastewater treatment plant using conventional activated sludge treatment with ferric dosing for phosphorus removal. The mixed sludge, with a solids content of 4.3% and a pH of 6.8, and temperatures used to calculate the partitioning coefficients for E2 are particularly applicable to this research project (Table 4-24).

Table 4-25: Partition coefficients (Kd) and Gibbs free energy for adsorption of 17β-estradiol to a blended sludge mixture of primary and secondary sludges.[1]

Temperature (°C)	Partitioning coefficient (Kd) (L/Kg)	Gibbs free energy (-ΔG) (KJ/mol)
15	604	-15.42
20	487	-14.89
25	312	-14.37
30	245	-13.84

[1] Source: Ifelebuegu et al. (2010)

The Gibbs free energy values and partitioning coefficients determined by Ifelebuegu et al. (2010) demonstrated that the sorption of E2 to the mixed sludge biomass was spontaneous (exothermic) and inversely related to temperature. Given less adsorption to solids with increasing temperature, we assume percent removal of E2 with settling solids as mixed sludge. Therefore, more E2 will remain in the wastewater and the influent and effluent E2 concentrations would indicate less percentage removal by the wastewater treatment plant. These findings offer an explanation for the inverse relationship noted between average monthly temperatures and percent removal of E2 by the wastewater treatment plant. It is also interesting to note, the values of the enthalpy changes determined by Ifelebuegu et al. (2010) also suggest that the mechanism of sorption is predominantly physisorption and some elements of chemisorption.

Toxic substances, present in the complex matrices of wastewaters and sludges, can inhibit yeast estrogenic and androgenic assays. The auto-bioluminescent yeast strain, BLYR, can be used to detect toxic substances and quantify the degree of response inhibition. Through the concurrent use of BLYR, the inhibition of the response signal to estrogens by BLYES and androgenic activity by BLYAS, by a particular sample can by assessed and the inhibition corrected for by introducing a dilution factor to the sample. The greater the toxicity, the more diluted a sample must be before contact with the bioluminescent yeast strains (Table 4-26).

The IC_{20} and IC_{50} were defined in terms of sample size and dilutions required to produce (or, more accurately, avoid) 20% inhibition and 50% inhibition of the BLYR luminescent signal. The more a sample must be diluted to avoid inhibiting the yeast, the more toxic the undiluted substance is to the yeast. To ensure luminescence inhibition did not affect estrogenic activity

determinations in the wastewater and sludge, wastewater sample size was reduced and sludge sample dried extracts reconstituted in 2 ml ethanol, were further diluted to below IC_{20} concentrations (Table 4-26).

Table 4-26: Sample size adjustment and dilution factors to below luminescence inhibition concentrations IC_{50} and IC_{20} for bioluminescent yeast reporter assay (BLYR)

Collected after treatment process	Sample size(ml)	Maximum sample size (ml)		Dilution factor for sample reconstituted in 2 ml methanol	
		IC_{50}	IC_{20}	Dilution for IC_{50}	Dilution for IC_{20}
Influent	100	1250	500	1.0	1:2
Primary sedimentation	100	1250	500	1.0	1:2
Trickling filter	100	1250	500	1.0	1:2
Solids contact tank	100	1100	400	1.0	2:5
Effluent	1000	6000	2500	1.0	1.0
Chlorinated effluent	100	1500	750	1.0	1:4
Mixed sludge	20	10	4	1:2	1:5
Digested sludge	20	20	12	1.0	3:5

Table 4-26 lists the toxicity to the bioluminescent yeast strains in terms of the dilutions necessary to prevent percent inhibition of luminescence. If smaller samples (greater dilution) are required for the androgen or estrogen yeast screening assays, the relative toxicity to the yeast is greater. Influent, and primary sedimentation, trickling filter, and solids contact tank effluents exhibit similar toxicities to the yeast. Bioluminescent yeast toxicities were associated with high solids content wastewaters (e.g. trickling filter and solids contact effluents). While chlorinated effluent was less toxic, non-chlorinated effluent was the least toxic to the bioluminescent yeast assay. Non-chlorinated effluent exhibited very little toxicity to the yeast strains and represented an overall reduction in toxicity of 80% throughout the wastewater treatment plant.

Table 4-27: Estrogenic activity (EEQ) and androgenic activity (TEQ) at wastewater sampling locations throughout a municipal wastewater treatment plant (Figure 3-2)

Collected after process (n = 12)[3]	EEq[1] (ng/L)	EEq[1] Range (ng/L)	EEq[1] % RSD	TEq[2] (ng/L)	TEq[2] Range (ng/L)	TEq[2] % RSD
Influent	48	30–76	21	12	1.9–40	18
Primary sedimentation	48	22–91	13	8	1.9–24	33
Trickling filter	47	23–82	25	5	1.7–11	24
Solids contact tank	44	26–66	27	5	1.8–12	47
Effluent	35	18–53	12	4	1.3–10	5
Chlorinated effluent	43	21–67	40	6.5	1.7–17	31

[1] Estradiol equivalent (equivalency to 17β-estradiol)
[2] Androgenic equivalent (equivalency to testosterone)
[3] Four sampling events, three replicate samples/day

Estrogenic activity (EEQ) and androgenic activity (TEQ) (Figure 4-16) was determined for each sampling location (described in Table 3-1) throughout the wastewater treatment plant (process diagram shown in Figure 3-2). Estrogenic and androgenic activity was determined by auto-bioluminescent yeast assays using yeast strains BLYES and BLYAS, respectively.

Total E1+E2 was associated with higher solids content in the wastewater, with increased concentrations found in the influent, tricking filter effluent and solids contact tank (Figure 4-15). The influent had an average biochemical oxygen demand (BOD) of 263 mg/L and 200 mg/L suspended solids (SS). This particular wastewater treatment plant was efficient at removing both constituents, with an average of <5 mg/L BOD and 5 mg/L SS in the final effluent. Mixed liquor suspended solids in the solids contact tank averaged 1,480 mg/L, with an average mean cell residence time (MCRT) of 1.7 days.

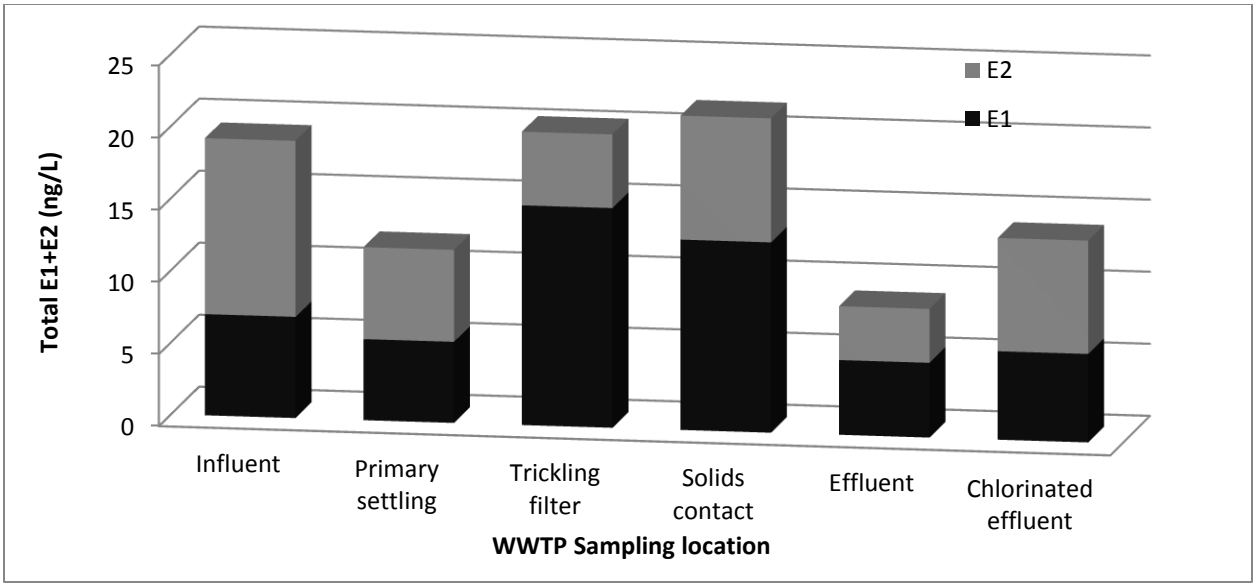


Figure 4-15: Average (n = 4) estrone (E1), 17β-estradiol (E2), and total E1+E2 concentrations at each wastewater sampling location within a municipal wastewater treatment plant

Table 4-27 and Table 4-26 and Figure 4-15 show some interesting trends, summarized in the following sections, relative to specific location in this wastewater treatment plant, where samples were extracted.

4.2.1 Primary Sedimentation

The average total suspended solids (TSS) in the raw influent was 190 mg/L but could vary by ± 20–24%. As expected, the highest degree of variation in concentrations of total E1+E2 occurred in raw influent samples, prior to wastewater treatment, (W-1) (see Figure 4-15) collected after the bar screen and before the sedimentation tank. The primary sedimentation tank removed 73% TSS, 20% E1; 48% E2; 38% total E1+E2, and increased the ratio of E1/E2, indicating biological degradation of E2 to E1 was a factor in estrogen removal in addition to solids adsorption. Primary sedimentation had no effect on estrogenic activity but lowered androgenic activity by 31% (Table 4-27).

4.2.2 Trickling Filter

The trickling filter influent from the primary sedimentation tank had an average of 50 mg/L TSS which increased during the biological treatment to an average of 86 mg/L TSS in the trickling filter effluent. The tricking filter is a biological solids contact process likely enriched with phylogenetically diverse community of organisms capable of degrading/converting estrogens – especially the more estrogenically potent E2 into the less potent E1. The ratio of E1/E2 increased from 0.9 (primary sedimentation) to 3.0 (tricking filter effluent), indicating that E2 was biologically converted to the less estrogenically active E1 in the tricking filter. However, estrogens present in the recirculating and sloughing solids could not be estimated by analyzing only the total phase of the influent and effluent. Removal rates across the tricking filter could not be calculated based only on the concentrations in the estrogen concentrations in the trickling filter influent and effluent total phase analysis. Since the hydraulic retention time (HRT) varies from the solids retention time (SRT) in the trickling filter, calculation of reduction of estrogens across the tricking filter would have required a separate sampling regime to account for the estrogens present in the solids inventory.

4.2.3 Solids Contact

Concentrations of E1 and E2 in the recycle line, to mix the solids (from trickling filter, secondary clarifier and solids contact tank) with trickling filter effluent before entering the solids contact tank were found to be similar to that of the solids contact tank effluent (Table 4-28). The solids contact tank mixed liquor suspended solids (MLSS) content was approximately 1500 mg/L and the effluent from the solids contact tank was approximately 1400 TSS. Activated

sludge processes, with both hydraulic retention times (HRT) and solids retention times (SRT) of several days, have been reported to reduce E2 (Ternes et al. 1999; Hamid and Eskicioglu, 2013). However, this solids contact tank, with a HRT of only 0.9-1.4 hours and a MCRT of about 1.7 days, functioned as a polishing unit to reduce BOD and SS. While the E1/E2 ratio of 1.5 from the solids contact tank was an increase from the influent (0.6) and primary sedimentation (0.9), it was lower than the trickling filter effluent (3.0) feeding into the solids contact tank. As with the trickling filter, removal of estrogens and a mass balance across the solids contact tank could not be calculated based only on the total phase analysis (aqueous and solid phases) of the influent and effluent of the solids contact tank since the SRT does not equal the HRT.

Table 4-28: Summary of individual treatment process efficacies in removing estrone (E1) and 17β-estradiol (E2) from the wastewater stream in a municipal wastewater treatment plant

Municipal wastewater treatment process / sampling location in MWWTP	Concentration (ng/L)		Range (ng/L)		Percent reduction (%) for each process			E1/E2 ratio
	E1	E2	E1	E2	E1	E2	Total E1+E2	
Influent (n = 6)	7	12	2–13	4–19				0.6
Primary settling (n = 4)	6	6	1–10	3–13	20	48	38	0.9
Trickling filter (n = 4)	15	5	5–28	1–12	—	—	—	3.0
Solids contact (n = 4)	13	9	8–20	2–24	—	—	—	1.5
Effluent (n = 6)	5	4	ND–12	ND–6	61	56	59	1.4
Chlorinated effluent (n = 4)	6	8	1–11	1–11	-18	-110	-57	0.8

If the wastewater treatment system is to be redesigned and the reduction of estrogens, estrogenic activity, and androgenic activity is to be considered, the effects of retention time in the solids contact tank must be examined, and adjusted accordingly. This also implies that a significant operational change may be needed, in the overall process.

4.2.4 Secondary Clarifier

Treatment in the secondary clarifier reduced E1 by 61%, E2 by 56% and total E1+E2 by 59% (Table 4-28). Since there was little to no change in the ratio of E1/E2 (1.4) between the influent to, and the effluent from, the secondary clarifier, removal was likely due to adsorption to, and settling with the solids. Similarly, the removal of 21% of estrogenic activity and 16% of androgenic activity in the secondary clarifier was associated with solids removal (Table 4-27). Influent to the secondary clarifier from the solids contact tank had an approximate TSS content of 1400 mg/L while TSS in the secondary clarifier effluent was, on average, 6 mg/L (range 4–7 mg/L). Since adsorption to, and settling of, solids in the secondary clarifier was effective at removing most of E1 and E2, as well as some of the estrogenic and androgenic activity from the wastewater stream, post-digestion sludge treatment processes should be examined for estrogen removal, where desired, for beneficial post-digestion sludge use or disposal.

4.2.5 Pre-and Post-Chlorinated Final Effluents

Overall, the wastewater treatment plant reduced total E1 and E2 by 54%; 27% of estrogenic activity; and 38% of androgenic activity in pre-chlorinated effluent and 24% total E1+E2; 10% estrogenic activity; and 15% of androgenic activity in post chlorinated effluent (see Figure 4-15and Figure 4-16). The most potent estrogen, E2, was reduced by 69% (n = 6; % RSD = 32) in pre-chlorinated effluent and E1 was reduced by 26% (n = 4; % RSD = 33). More importantly, the ratio of E1 to E2 was increased from 0.6 (influent) to 1.4 (pre-chlorinated effluent), indicating that E2 was biologically degraded to the less estrogenic E1 during the wastewater treatment process (Table 4-28).

Total E1+E2 concentrations were associated with higher solids content in all wastewater treatment processes, except the chlorine contact tank with the final effluent. Concentrations of total E1+E2 increased with chlorination were likely due to the release of bound E1+E2 from suspended solids and associated degradation/conversion of other compounds, such as E3 and EE2. It has been suggested that oxidative processes, such as chlorine and ozone, can increase estrogenic activity through chemical degradation/conversion of other compounds in the wastewater (Nakrst et al., 2011). Since the chlorinated wastewater is de-chlorinated prior to release to the environment, the total E1+E2 should be examined in the de-chlorinated effluent.

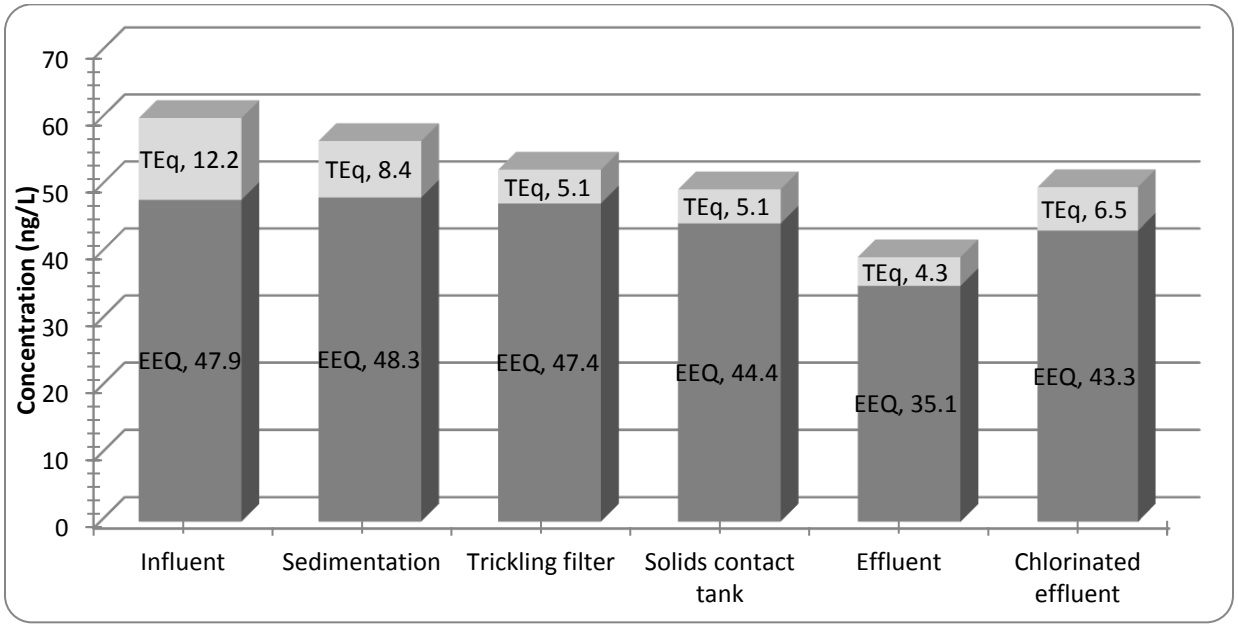


Figure 4-16: Estrogenic (EEQ) and androgenic (TEQ) activity for treatment processes in a trickling filter-solids contact municipal wastewater treatment plant

Nakrst et al. (2011) reported complete degradation of E2 and EE2 almost immediately after applying ozonation to an aqueous solution, confirmed by GC-MS measurements. However, even after one hour of ozonation, only 41% of estrogenic activity for E2 and 48% of estrogenic

activity for EE2, determined by YES assay, was removed. The authors suggested remaining estrogenic activity could be a consequence of formation of by-products during the ozonation process by oxidation via OH radical. They concluded that their results showed that the removal efficiency of estrogens from waters should be assessed by a combination of chemical analyses and bioassay.

The de-chlorinated effluent was not included in this sampling program. It is strongly recommended that this effluent be evaluated prior to assessing risk to the receiving environment.

4.2.6 Wastewater Treatment Performance

Servos et al. (2005) examined E1 and E2 concentrations and whole estrogenic activity (YES response) in influent and effluent from domestic wastewater treatment plants utilizing a variety of treatments and operating parameters. Concentrations of E1 and E2 varied considerably as did removal efficiencies. The trickling filter plant examined in by Servos et al.(2005) had higher levels of E1, E2, and whole estrogenic activity (YES response) in the effluent compared to the influent indicating negative percent removal of E1 (-18.5%) and E2 (-62.4%) and EEQ (-62%). However, Ternes et al. (1999), in a more extensive examination (n = 6 daily composite (4 hour interval) samples) of a trickling filter domestic wastewater treatment plant in Brazil, reported percent removal of E1, E2, and EE2 of 67%, 92% and 64%, respectively. Although these are higher removal efficiencies than reported here, the MWWTP examined in this study was capable of reducing estrogens E1 and E2 and whole estrogenic activity (Figure 4-17).

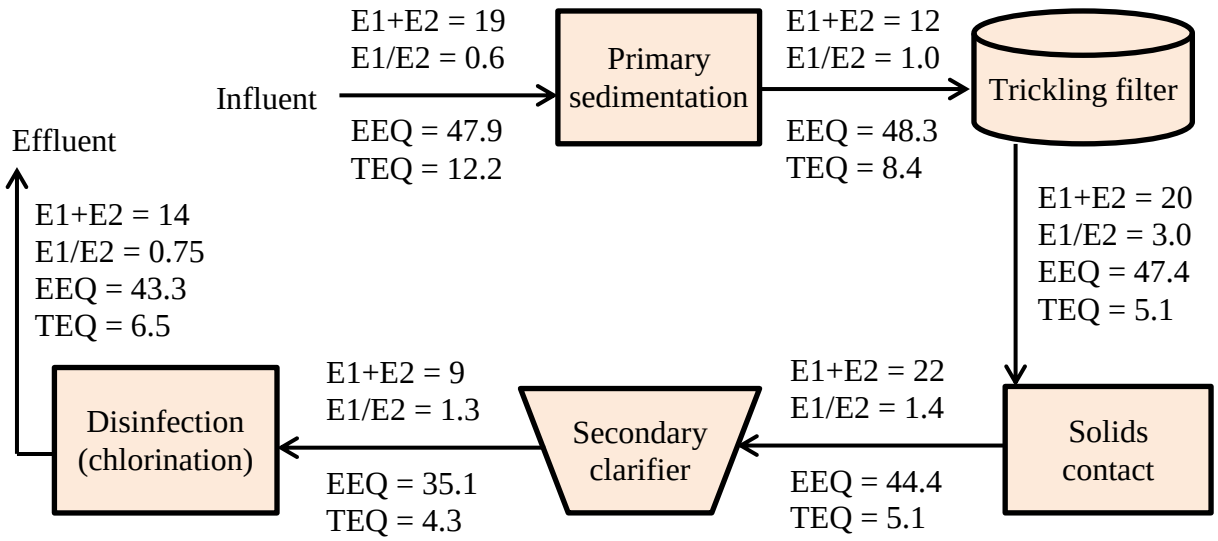


Figure 4-17: Trickling filter wastewater treatment plant sampling locations showing average (four sampling events (each with 3 replicates)) concentrations (ng/L) of: total 17-β estradiol and estrone (E1+E2); ratio of estrone (E1) to 17-β estradiol (E2); estradiol equivalent concentration (EEQ); and testosterone equivalent (TEQ)

Overall, the wastewater treatment plant reduced 54% of target estrogens (total E1 and E2); 27% of estrogenic activity; and 38% of androgenic activity. Significant reduction occurred in the secondary clarifier, leading to the conclusion that E1, E2, estrogenic activity and androgenic activity were adsorbed to solids and removal was through solids settling. Wastewater processes that reduced total suspended solids in the wastewater stream were associated with reduced total E1+E2.

The primary removal mechanism in this wastewater treatment plant was settling, with 38% E1+E2 removed in the primary setting tank. This indicates that primary treatment systems would reduce estrogen concentrations, but not to the levels typically reported for secondary treatment systems such as activated sludge systems. These findings are in agreement to those

reported by Drewes et al. (2005), in a comparison of primary and secondary treatment effluents for E1 and E2 (Table 1-3).

The ratio of E1/E2 was significantly increased in the tricking filter. Biological processes in the tricking filter degraded some of the more estrogenically potent E2 into the less estrogenic by-product, E1. An increase in total E1+E2 was noted during biological treatment in the trickling filter and solids contact processes, despite some biological degradation of more potent estrogen E2 to E1. The increase was likely due to the release from particulate matter and suspension in the high solids content, tricking filter and solids contact effluents. In an activated sludge system, Braga et al. (2005) found a significantly lower proportion of E2 in the mixed liquor suspended solids, compared to E1 and suggested that E2 is being oxidized to E1 during the biological oxidation process.

Subsequent settling in the secondary clarifier removed 59% of E1+E2 from the tricking filter/solids contact and was the most effective process for removing the target estrogens from the wastewater stream. The findings in this study, with respect to association of the target compounds with the solid phase and removal from the wastewater stream with the sludge component, are in agreement to other published literature (Esperanza et al., 2007; Drewes et al., 2005; Braga et al., 2005; des Mes et al., 2008). Although the primary removal mechanism of E1+E2 was settling of solids from the wastewater stream, secondary biological treatment improved effluent quality by increasing the ratio of E1/E2; and subsequent settling in the secondary clarifier reduced the overall E1+E2 concentration. The wastewater treatment plant

removed 69% of E2, the most potent estrogen, by solids removal (primary mechanism) and biodegradation.

Chlorination of final effluent increased E1+E2 concentrations, likely by releasing them from the solid phase and oxidative degradation/conversion of non-target compounds (e.g. conjugates, plant and animal sterols) present in the complex wastewater matrix. Braga et al. (2005) expected a low removal of E1 and E2, based on estrogen partitioning to effluents, given the low solids concentration, but was surprised to find high concentrations of E1 (24%) and E2 (43%) remained associated with the solids fraction in primary and secondary wastewater effluent. Effluent chlorination also increased estrogenic and androgenic activity in the chlorinated effluent, likely also due to the oxidative release of compounds from suspended solids and chemical degradation/conversion of organic matter in the wastewater matrix. The effects of dechlorination, on E1, E2, estrogenic and androgenic activity, prior to discharge into the environment were not examined in this study.

Estrogenic activity had a significant correlation with concentrations of the more potent estrogen, E2, in wastewater samples (Figure 4-18). With a Spearman's rank correlation coefficient (ρ) of 0.63 and 22 degrees of freedom, the significance level was $P < 1\%$, where P is the probability the null hypothesis was true and the observed correlation was strictly coincidental. Since E2 is considered to be an important contributor to estrogenic activity in domestic wastewater (Nakada et al., 2004; Sun et al., 2013), a correlation between concentrations of E2 and estrogenic activity in wastewater was expected. Although significant, it is not a perfect correlation between E2 and estrogenic activity, since the BLYES is a whole estrogenic analysis

and levels of synthetic estrogens (e.g. EE2) and estrogen mimicking substances (e.g. pesticides, plant sterols, etc.) in the wastewater will contribute significantly to the estrogenic activity concentration as EEQ (Nakada et al., 2004).

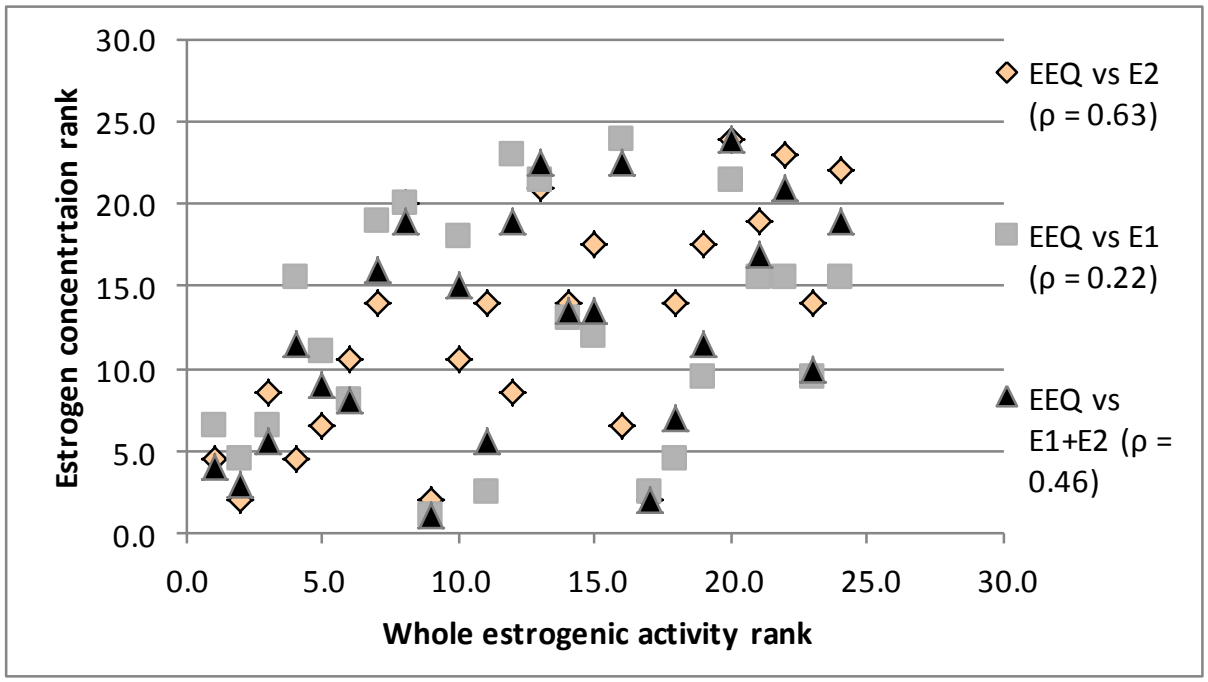


Figure 4-18: Spearman's rank correlation of ranked concentrations of whole estrogenic activity, measured as estradiol equivalents (EEQ), with: estrone (E1); 17-β estradiol (E2); and total E1+E2 ranked concentrations. Twenty-four data points represent four sampling events at six sampling locations (n=3 replicates) within a municipal wastewater treatment plant.

Estrone has approximately one-fifth to one-tenth the estrogenic activity of E2 (Matsui et al., 2000; Sun et al., 2013) (see Appendix B). Hence, as expected, there was no significant correlation between the much less estrogenically potent estrogen, E1, and estrogenic activity (ρ = 0.22) and, understandably, no significant correlation of estrogenic activity to total E1+E2 concentrations (ρ = 0.46) (Figure 4-18).

Toxicity to the bioluminescent yeast assay in Table 4-26 was expressed as the maximum sample size of wastewater or sludge that can be used in the analysis protocol without inhibiting bioluminescence in the yeast assay by 20% (IC_{20}) and 50% (IC_{50}). Toxicity was similar for influent, primary sedimentation effluent, and trickling filter effluent; all with an IC_{20} = 500 ml, and increased by approximately 20% for the solids contact tank effluent (IC_{20} = 400 ml). However, pre-chlorinated final effluent, collected after the secondary clarifier, had very little toxicity to BLYR (IC_{20} = 2500 ml). Chlorinated effluent showed increased toxicity to BLYR (IC_{20} = 750 ml), but this was not surprising since the chlorine was added to the effluent as a biocide, then dechlorinated before the effluent was released to the environment.

Removal of estrogens from the wastewater stream was associated with solids settling and subsequent removal from the wastewater stream. However, it was interesting to note that solids removal was not associated with a decrease in estrogenic or androgenic activity. No reduction in estrogenic activity was observed during primary sedimentation, indicating that secondary treatment may be required for removal of estrogenic activity in municipal wastewater treatment plants. Although the reduction of estrogenic activity could not be assessed for trickling filter and solids contact processes, there was some evidence of biological degradation of estrogens (e.g. ratio of E1/E2). While androgenic activity steadily declined throughout the wastewater treatment processes, most of the estrogenic activity was removed during settling in the secondary clarifier. The wastewater treatment plant removed 27% estrogenic activity and 38% androgenic activity, overall.

4.3 SLUDGE DIGESTION

Mixed sludge consisted of approximately 65% primary sludge and 35% secondary sludge, with a 4% total and 88% volatile solids. Mesophilic digestion (35–40 °C), with an average hydraulic retention time of 29 days, reduced these volatile solids by 60% and suspended solids by 63%, in the process examined at this plant. Sludge is fed into and exits the digester in liquid form (Figure 4-19).

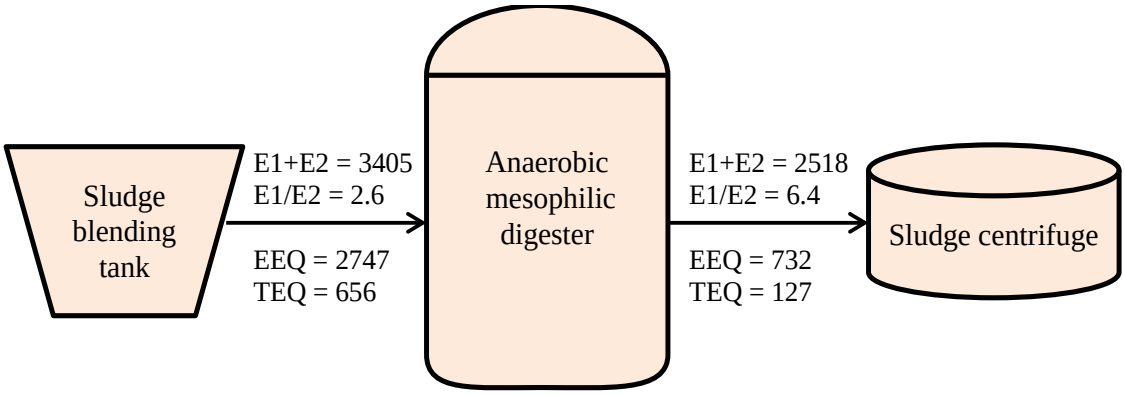


Figure 4-19: Anaerobic mesophilic sludge digestion with pre- and post- digestion average concentrations (ng/L) of: total estrone and 17-β estradiol (E1+E2); ratio of estrone to 17-β estradiol (E1/E2); estradiol equivalents (EEQ); and testosterone equivalents (TEQ)

Estrone and 17β-estradiol are associated with the solid portion of mixed and digested sludge with only 5% of E2 found in the liquid portion of centrifuged mixed sludge and 7.7% of E2 in the liquid potion of centrifuged digested sludge (Table 4-3). Since this mesophilic digestion system was so efficient at reducing total solids, when percent reduction of the target compounds are calculated as ng/g solids in digested sludge, it gives misleading results (Table 4-29) by showing an increase in total E1+E2 during the digestion process. Mixed and digested sludges were analysed for estrone and 17β-estradiol with the laboratory method using liquid-

liquid extractions. Therefore, a true mass balance of E1 and E2 for the mesophilic sludge digestion process was easily obtained by calculating ng/L sludge (Figure 4-19).

Table 4-29: Percent reduction of estrone (E1) and 17β-estradiol (E2) in mixed and digested sludge during sludge digestion process calculated as ng/g solids and ng/L sludge

Estrogen (n = 10)	Mixed sludge			Digested sludge			% reduction	
	ng/g	ng/L	range (ng/g)	ng/g	ng/L	range (ng/g)	ng/g	ng/L
E1	60	2469	14–118	145	2176	66–367	-142	12
E2	23	936	3–83	23	342	3–50	0	63
E1+E2	83	3405		168	2518		-102	26
Ratio E1/E2	2.6	2.6		6.3	6.4			

Mesophilic anaerobic sludge digestion reduced E1 by 12% and E2 by 63% and total E1+E2 by 26%. The mixed sludge had a ratio of E1/E2 of 2.6, while the digested sludge had a ratio of E1/E2 of 6.4, indicating that a significant degradation of E2 to the less estrogenically potent E1 was occurring during mesophilic digestion. In addition to the degradation of E2 to E1, the reduction of E1 indicated that E1 was also being degraded and the digester likely contains an enriched, phylogenetically diverse culture of E2 and E1 degrading bacteria (Table 4-30).

Table 4-30: Estrogenic activity (EEQ) and androgenic activity (TEQ) in mixed and digested sludge from a municipal wastewater treatment plant (Figure 3-2)

Sludge (n = 12)	EEq[1] ng/g	EEq[1] Range (ng/g)	EEq[1] (ng/L)	TEq[2] ng/g	TEq[2] Range (ng/L)	TEq[2] (ng/L)	EEq[1] %RSD	TEq[2] %RSD
Mixed	67	28–158	2747	16	2.4–54	656	15	30
Digested	49	26–87	732	8	1.7–26	127	4.8	3.9

[1] EEQ = Estradiol equivalent (equivalency to 17β-estradiol)
[2] TEQ = Testosterone equivalent (equivalency to testosterone)

Mesophilic anaerobic sludge digestion was effective at reducing estrogenic activity by 73%, while androgenic activity was reduced by 81%. Holbrook et al. (2002) measured estrogenic activity with the YES assay before and after anaerobic and aerobic digestion of sludge. Estrogenic activity increased drastically in the aqueous and solid phases following digestion. The authors suggest an increase in extractability for the digested sludge. However, Sarkar (2013) determined that, while there was no change in total E1+E2, estrogenicity of sludge measured by YES bioassay increased during anaerobic digestion due to reduction of E1 to more estrogenic E2.

The anaerobic mesophilic digestion process reduced toxicity to the bioluminescent yeast strain, with digested sludge IC_{20} threefold of the IC_{20} for mixed sludge. Although digested sludge had consistent (within 10%) toxicity values, mixed sludge toxicity (inhibition of luminescence) could vary as much as 2% to 84% in one grab sample. Mixed sludge had an overall average toxicity of 82% for a 20 ml sample of mixed sludge. In other words, an undiluted extract from a 20 ml sample of mixed sludge would, on average, inhibit 84% of the luminescence signal and render the yeast assay useless. Therefore, it was important to dilute all mixed sludge extracts prior to microplating with the bioluminescent yeast assay. Digested sludge, on the other hand, demonstrated one third the toxicity of mixed sludge and required only a 3:5 dilution to obtain reliable results (less than 20% luminescence inhibition) when using the bioluminescent yeast assay.

The effects of sludge treatments, for further sludge utilization or disposal of biosolids were not examined in terms of the fate of estrogens, androgenic activity and /or estrogenic

activity. However, due to the high affinity of E1 and E2 to solids, it is likely dewatering or drying processes alone will not eliminate estrogens or estrogenic activity.

4.4 SLUDGE TREATMENTS

Several sludge treatment processes were applied to mixed and digested sludges to examine the effects of estrone (E1) and estradiol (E2) concentrations. These treatment processes have been under active research investigation at the University of British Columbia, over the last 10 years.

1. Thermal with microwave irradiation
2. Oxidation (H_2O_2 additions)
3. Combinations of microwave irradiation and oxidation with H_2O_2
4. Conventional heat

Control samples were treated at laboratory room temperature (20 °C) and were not heated by microwave irradiation or conventional heat (water bath). Since samples were not preserved or prepared for solvent extraction (e.g. pH adjusted), they were biologically active prior to (perhaps during and after) all sludge treatments.

Estrone can be produced as a by-product of E2 degradation in wastewater and sludge treatments and it can also be converted to E2, during anaerobic digestion (de Mes et al., 2008; Sarkar, 2013). The organisms responsible for the biological degradation /conversion of E1 and E2 are phylogenetically diverse and populations are thought to be enhanced in engineered biological wastewater and sludge processing systems. Therefore, it's important to examine the mass balance of total E1 + E2 concentrations, as well as the individual values.

4.4.1 Microwave Irradiation

The predominant mechanism in which non-ionizing electromagnetic radiation from the microwave produces changes in sludge is the application of rapid, uniform heating with precise temperature control. The alternating electric field in microwave irradiation can cause athermal effects by inducing a rapid alignment and realignment of molecular dipoles within a polar solvent. It has been suggested microwaves athermally induce different biological effects by changing microbial structures (differentially partitioning ions; altering the rate and/or direction of biochemical reactions) (Banik et al., 2003; Samarketu et al., 1996; Porcelli et al., 1997).

Pre-treatment of sludge by microwave processing has shown to improve anaerobic digestion. Eskicioglu et al. (2007a) found waste activated sludge microwaved to 96 ^{0}C, produced 15–20% more biogas and 3.2–3.6 fold increase in soluble to total chemical oxygen demand (SCOD/COD). They also noted dewaterability of the microwaved sludge was enhanced after anaerobic digestion. Other researchers similarly reported an increase in biogas (methane) production and an increase in soluble COD (disintegration of sludge particles) accompanied by COD removal (Park et al., 2004; Wheeler et al., 2006; Wong et al., 2007).

Mixed and digested sludges in this research, responded differently to microwave treatments with respect to concentrations of the target compounds. Therefore microwave treatment sections are divided into separate subsections for mixed and digested sludges.

4.4.1.1 MIXED SLUDGE

While EE2, E3, and TT were present in the control (20 °C) mixed sludge, no detectable concentrations were found in mixed sludge after microwave treatments at 60 °C, 80 °C, and 100 °C (Figure 4-20). Microwave irradiation at 60 °C and 80 °C reduced concentrations of E1 (100% reduction) but had little effect on concentrations of E2. In a bench scale study, Hamid and Eskicioglu (2013) demonstrated an increase in E2 and a decrease in E1 in the influent sludge feed mixture to a mesophilic anaerobic sludge digester at a pretreatment microwave irradiation temperature of 80 °C. In fact, E2 was the only hormone in this study that showed consistent increase in concentration with increasing microwave pretreatment temperature.

In contrast to the decrease in E1 concentrations with microwave irradiation at 60 °C and 80 °C, microwave treatment at 100 °C did not reduce concentrations of either E1 or E2 in mixed sludge. In fact an increase in E2 concentration occurred, similar to that observed by Hamid and Eskicioglu (2013) at microwave pretreatment temperatures at and above 80 °C. Total (E1 + E2) concentration remained the same when mixed sludge was irradiated at 100 °C. However, the ratio of E1 to E2 changed dramatically, from 2.0 in the control samples to 0.75, with the 100 °C treatment (Figure 4-20 and Table 4-31). This increase in E2 and decrease in E1 concentrations agree with the findings of Hamid and Eskicioglu (2013). A combination of factors could have contributed to these findings including:

1. biological conversion of E2 into E1 during the 20 °C control treatment
2. organisms responsible for the conversion/degradation of E2 into E1 were inhibited during microwave irradiation at 100 °C. Although it would be expected that organisms acclimatized to mixed sludge temperatures (approximately 10 – 20 °C) would likely also be inhibited at microwave temperatures of 60 °C and 80 °C.
3. release/desorption of E2 from the solid phase at 60 °C, 80 °C and 100 °C, as well as a release/desorption of E1 from the solid phase at 100 °C.
4. transformation (e.g. autoxidation, denaturation) of non-target compounds such as plant sterols

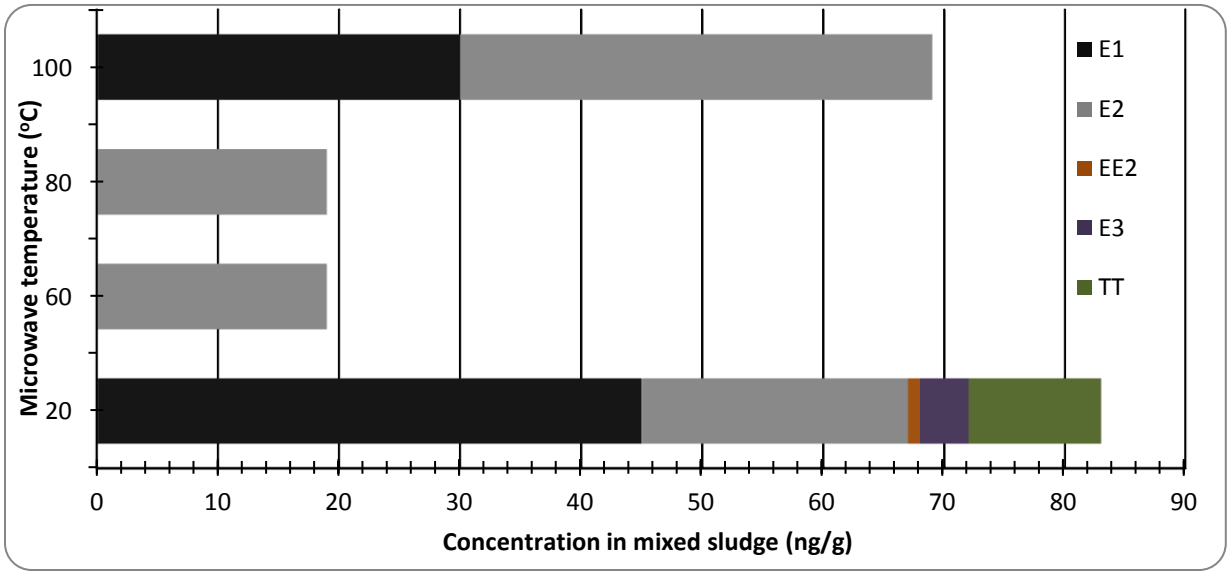


Figure 4-20: Average concentrations (n = 3) of estrone (E1), 17β-estradiol (E2), 17α-ethinyl estradiol (EE2), estriol (E3), and testosterone (TT) in 20 ml mixed sludge after microwave heat treatments of 60 °C, 80 °C or 100 °C

Table 4-31: Estrone (E1), 17β-estradiol (E2), 17α-ethinyl estradiol (EE2), Estriol (E3), testosterone (TT) concentrations in 20 ml mixed sludge microwave irradiation at 60 °C, 80 °C and 100 °C with percent relative standard deviations (% RSD) of treatment replicates (n = 3)

Mixed sludge (n = 3)	Room temp 20 °C (control sample set)			Microwave irradiation at 60 °C			Microwave irradiation at 80 °C			Microwave irradiation at 100 °C		
	ng/g	ng/L	% RSD	ng/g	ng/L	% RSD	ng/g	ng/L	% RSD	ng/g	ng/L	% RSD
E1	45	1862	18	ND			ND			30	1244	96
E2	22	897	16	19	799	45**	19	765	22	39	1594	4
EE2	1	49	20	ND			ND			ND		
E3	4	149	42	ND			ND			ND		
TT	11	448	26	ND			ND			ND		
*Analyte was detected in one of three replicates **Analyte was detected in two of three replicates												

Microwave treatments of mixed sludge at 60 °C and 80 °C appeared effective in reducing detectable concentrations of E1, EE2, E3 and TT, but were ineffective in reducing the more estrogenic E2 concentrations in mixed sludge. Microwave treatments at 100 °C reduced E1, but produced a higher concentration of the more estrogenically potent E2. Microwave irradiation at all experimental temperatures was ineffective for reducing E2 concentrations. This may be partially due to anaerobic conditions and may be improved when combined with oxidative treatments.

Table 4-32: Estrogenic and androgenic activity in municipal mixed sludge, after microwave irradiation at 60, 80, and 100 °C

Control and microwave irradiation treatment temperature (°C)	Estrogenic activity (EEQ)[1]		Androgenic activity (TEQ)[2]	
	ng/g	ng/L	ng/g	ng/L
20 control	25.7	1056	8.9	366
60	15.1	618	7.7	316
80	21.2	871	10.7	439
100	15.1	617	5.4	223

[1] EEQ = Estradiol equivalent (equivalency to 17β-estradiol)
[2] TEQ = Testosterone equivalent (equivalency to testosterone)

While a reduction in estrogenic activity was observed at 60 °C (40%); 80 °C (20%); and 100 °C (40%), no change in androgenic activity was noted for microwave irradiation temperatures of 60 °C and 80 °C. A reduction of androgenic activity was expected to correlate with non-detectable levels of testosterone at 60 °C and 80 °C. However, testosterone was the only androgen examined in this study and degradation by-products of testosterone (see the testosterone degradation pathway diagram in Appendix C) and non-targeted compounds (e.g. human and animal hormones) and their degradation by-products can exhibit androgenic activity by binding with the human androgen receptor in the YAS assay (Eldridge et al., 2007). Therefore, degradation by-products of testosterone and non-targeted compounds are likely responsible for the observed androgenic activity in the absence of testosterone at microwave irradiation temperatures of 60 °C and 80 °C. Androgenic activity was, however, reduced by 50% during microwave irradiation at 100 °C (Table 4-32), indicating androgens were being degraded at this temperature.

Overall, microwave irradiation of mixed sludge was ineffective in reducing concentrations of the most potent estrogen, E2. Microwave irradiation treatment did, however, reduce concentrations of other estrogens and estrogenic activity even at low temperatures (60 °C) and androgenic activity at 100 °C. It may be effective as a pre-treatment to digestion and/or in combination with other sludge treatments in reducing or eliminating estrogens, including E2, completely. Further research investigating microwave irradiation technology and the reduction of estrogens and estrogenic activity is needed.

4.4.1.2 DIGESTED SLUDGE

Estrone (E1) was undetectable in microwave treatments of digested sludge at 80 °C and 100 °C; however, irradiation at 60 °C appeared to have no effect on E1 concentrations, but reduced E2 by 50% (Figure 4-21and Table 4-33). Microwave irradiation at 80 °C reduced concentrations of E2 by 78% in digested sludges. While microwave treatment at 100 °C demonstrated an E2 reduction of only 32% in digested sludge, E1 was undetectable. Estriol (E3) was detected in only one of three replicates at 20 °C and 100 °C (Table 4-33). Due to the infrequent detection of E3 in digested sludge, the effects of microwave irradiation at 60 °C, 80 °C, and 100 °C could not be determined.

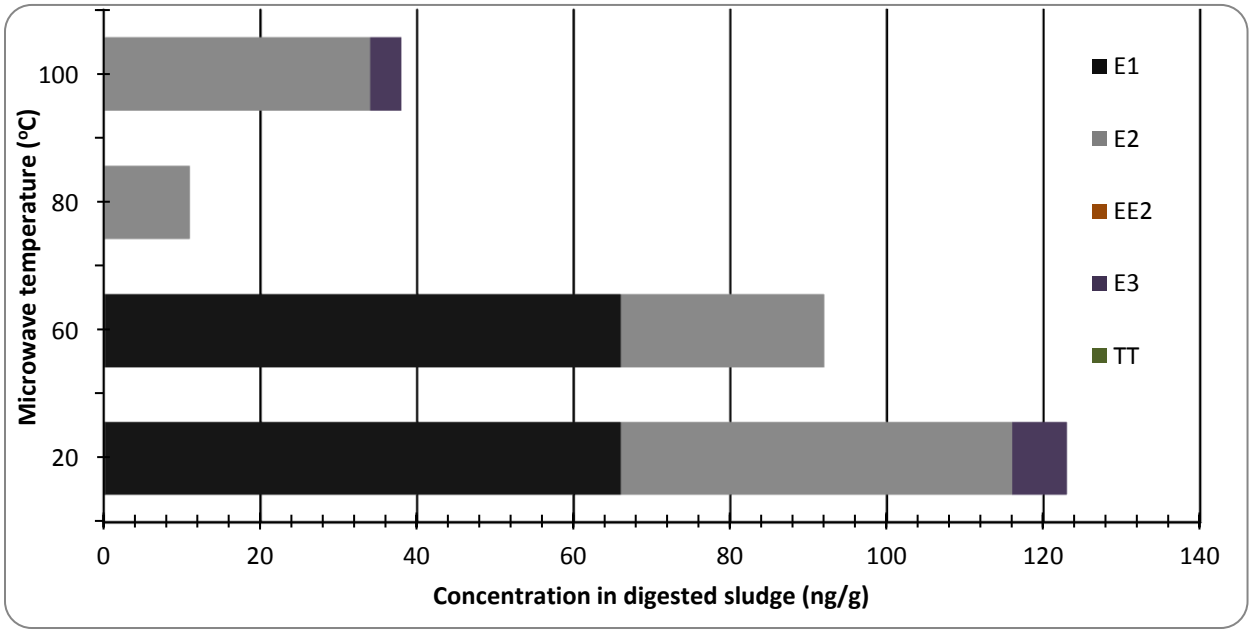


Figure 4-21: Average concentration of estrone (E1), 17β-estradiol (E2), 17α-ethinyl estradiol (EE2), estriol (E3), and testosterone (TT) in digested sludge (n = 3) after microwave heat treatments of 60, 80 or 100 °C compared to controls at 20 °C

Table 4-33: Estrone (E1), 17β-estradiol (E2), 17α-ethinyl estradiol (EE2), Estriol (E3), testosterone (TT) concentrations in 20 ml digested sludge microwave irradiation at 60 °C, 80 °C and 100 °C with percent relative standard deviations of treatment replicates (n = 3)

Digested sludge (n = 3)	Room temp 20 °C (control sample set)			Microwave irradiation at 60 °C			Microwave irradiation at 80 °C			Microwave irradiation at 100 °C		
	ng/g	ng/L	% RSD	ng/g	ng/L	% RSD	ng/g	ng/L	% RSD	ng/g	ng/L	% RSD
E1	66	992	*	66	989	4	ND			ND		
E2	50	756	16	26	385	22**	11	165	*	34	506	36
EE2	ND			ND			ND			ND		
E3	7	107	*	ND			ND			4	54	*
TT	ND			ND			ND			ND		

*Analyte was detected in one of three replicates
**Analyte was detected in two of three replicates

At all microwave irradiation temperatures, 60 °C, 80 °C, and 100 °C, the total (E1 + E2) concentration in digested sludge was reduced. The most effective microwave treatment was at 80 °C, where only one of the replicate samples had detectable levels of E2 (Table 4-33). The balance between E1 and E2 concentrations suggest E2 was converted to E1 and E1, in turn, was degraded with increasing microwave temperature applications to the digested sludge media.

While some degradation of estrogenic activity (~30%) was observed at 60 °C and 100 °C, an irradiation temperature of 80 °C had little effect (Table 4-34). Androgenic activity was actually increased at 60 °C, 80 °C, and 100 °C, with a three-fold increase during microwave irradiation at 80 °C (Table 4-34). It is interesting to note that the most effective irradiation temperature for the removal of estrogens was the least effective treatment for removal of estrogenic activity and actually increased androgenic activity.

The decreased estrogenic activity in digested sludge during microwave irradiation at 60 °C was likely due to estrogen degradation and coincided with decreased concentrations of

targeted estrogens. The increase in estrogenic activity observed at 80 °C did not coincide with increased estrogen concentration but, instead, a decrease in targeted estrogens was noted at this irradiation temperature. It would be reasonable to expect estrogenic activity to correlate to concentrations of the most potent estrogens, E2 and EE2. However, lingering estrogenic activity has been reported after estrogens have been removed from an aqueous solution (Nakrst et al., 2011). In addition, the combined effect of less potent estrogenic compounds released from the solids phase and degradation of non-target compounds could explain the rise in estrogenic activity during microwave irradiation at 80 °C. Microwave irradiation at 100 °C appeared to be successful at reducing estrogenic activity (25%) and E2 (33%) by a similar amount.

Table 4-34: Estrogenic and androgenic activity in municipal digested sludge, after microwave irradiation at 60 °C, 80 °C, and 100 °C

Control and microwave irradiation treatment Temperature (°C)	Estrogenic activity (EEQ)[1]		Androgenic activity (TEQ)[2]	
	ng/g	ng/L	ng/g	ng/L
20 Control	67.3	1010	30.1	452
60	46.0	690	38.7	581
80	65.2	979	102.8	1542
100	50.5	758	43.0	645

[1] EEQ = Estradiol equivalent (equivalency to 17β-estradiol)
[2] TEQ = Testosterone equivalent (equivalency to testosterone)

Androgenic activity in digested sludge increased with microwave irradiation at 60 °C and 80 °C and was not associated with testosterone concentrations since all samples, including controls, had non-detectable levels of testosterone. However, this rise in androgenic activity was likely due to release of non-targeted androgens from the solids phase and/or subsequent degradation into androgenic by-products. While increasing androgenic activity compared to control samples, an irradiation temperature of 100 °C appeared to be effective at reducing most androgenic activity that may have been created or released from the solid phase during

microwave temperature ramping to 100 °C. Overall, microwave irradiation at temperatures of 60 °C, 80 °C, and 100 °C were not effective at reducing androgenic activity.

Populations of organisms responsible for biological degradation of E1 and E2 are thought to be enhanced in certain engineered systems. This appears to be the case with the mesophilic digested sludge, where biological degradation was evident from the increase in the ratio of E1/E2 from 2.6 to 6.4. However, microwave irradiation encouraged the degradation of total E1 and E2 at all treatment temperatures. While it is possible more thermotolerant organisms present in the digested sludge may be biodegrading estrogens at microwave treatment of 60 °C and 80 °C, it is more likely organisms acclimatized at mesophilic temperatures would suffer lethal and sub-lethal effects during these irradiation temperatures. Microwave irradiation at 100 °C reduced E1 in digested sludge to non-detectable levels, but showed no reduction of E1 in mixed sludge; this may indicate the primary mechanism responsible for reduction of estrogens may be mechanical or chemical during thermal irradiation.

While microwave irradiation was effective at removing estrogens from digested sludge, it was only moderately effective at removing estrogenic activity and may have increased androgenic activity. The lack of correlation between estrogen concentrations and estrogenic activity may be partially explained by Nakrst et al. (2011), who reported persistent estrogenic activity after estrogens had been eliminated from an aqueous solution in a laboratory study examining ozone as an advanced oxidation process, for the removal of E1 and E2. They postulated estrogenic degradation by-products may be responsible for the lingering estrogenic activity.

4.4.2 Oxidation with Hydrogen Peroxide

Oxidation treatments were applied to mixed and digested sludges by hydrogen peroxide additions of 0.5%, 1%, 1.5% by weight (g/g) at 20 °C. Mixed and digested sludges differed in the response to this oxidative treatment.

4.4.2.1 MIXED SLUDGE

Concentrations of testosterone (TT) in mixed sludge were reduced to non-detectable levels when H_2O_2 was 1% and 1.5% (Figure 4-22). While 17α-ethinyl estradiol was below detectible levels at H_2O_2 addition of 1.5%, the relatively sporadic detection and low concentrations of EE2 casts some doubt as to whether H_2O_2 treatment at 1.5% will reduce EE2 concentrations in mixed sludge.

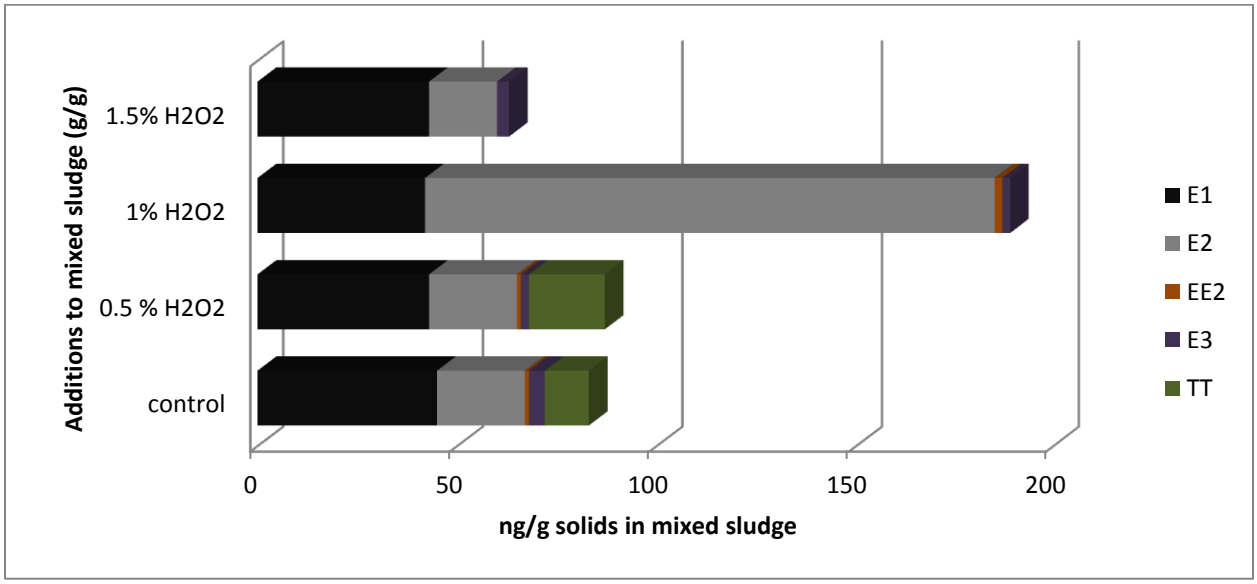


Figure 4-22: Effects of hydrogen peroxide (H2O2) additions to estrone, 17β-estradiol, 17α-ethinyl estradiol, estriol, and testosterone concentrations in mixed sludge at 20 °C

Hydrogen peroxide additions of 0.5%, 1%, 1.5% at 20 °C did not reduce concentrations of E1and E3 in mixed sludge. However, a 6.5 fold increase in E2 concentrations was noted when 1.0% H_2O_2 was applied at 20 °C (Table 4-35). Since concentrations of E1 remained unchanged, conversion of E1 to E2 did not contribute to this phenomenon. An increase in estrogenic activity was also observed where EEQ was increased by 46% when 1.0% H_2O_2 was applied at 20 °C in mixed sludge (Table 4-36).

Table 4-35: Estrone (E1), 17β-estradiol (E2), 17α-ethinyl estradiol (EE2), Estriol (E3), testosterone (TT) concentrations in 20 ml mixed sludge after additions of 0.5%, 1%, and 1.5% H_2O_2 (wt/wt solids) with percent relative standard deviations of treatment replicates (n = 3)

20 °C	Control			0.5% H2O2			1% H2O2			1.5% H2O2		
Mixed sludge	ng/g	ng/L	% RSD	ng/g	ng/L	% RSD	ng/g	ng/L	% RSD	ng/g	ng/L	% RSD
E1	45	1862	12	43	1757	53	42	1710	38	43	1772	11
E2	22	897	16	22	908	20	143	5883	16	17	717	36
EE2	1	49	20**	1	53	18**	2	88	61**			
E3	4	149	42	2	82	56	2	91	*	3	118	7**
TT	11	448	26	19	768	*						

*Analyte was detected in one of three replicates
**Analyte was detected in two of three replicates

Table 4-36: Estrogenic and androgenic activity in mixed sludge after hydrogen peroxide additions (wt/wt) to 20 ml municipal mixed sludge at 20 °C

Hydrogen peroxide (H_2O_2) additions to mixed sludge	Temperature (°C)	Estrogenic activity		Androgenic activity	
		EEQ (ng/g)	EEQ (ng/L)	TEQ (ng/g)	TEQ (ng/L)
Control	20	25.7	1056	8.9	366
0.5% H_2O_2	20	22.6	926	8.2	338
1.0% H_2O_2	20	37.7	1544	8.1	334
1.5% H_2O_2	20	20.0	822	8.1	334

[1] EEQ = Estradiol equivalent (equivalency to 17β-estradiol)
[2] TEQ = Testosterone equivalent (equivalency to testosterone)

It is possible that substances such, as other plant and animal sterols, not targeted by this chemical analysis; and EE2 and E3, present at concentrations less than this method is capable of

detecting; were converted to E2 in the presence of 1% H_2O_2. Hydrogen peroxide concentrations of 0.5% may not have been enough to push the reaction in the direction of E2 production and 1.5% may have chemically degraded E2 and /or inhibited any biological conversion/degradation processes to create E2 from other compounds present in the mixed sludge.

4.4.2.2 DIGESTED SLUDGE

Hydrogen peroxide additions of 0.5%, and 1.5% at 20 °C increased concentrations of E2 by 2.5 to 3 fold in digested sludge, while reducing E1 concentrations by 50% (Figure 4-23 and Table 4-37). However, concentrations of E2 were increased by 28.5 fold over the control samples of digested sludge when H_2O_2 was applied at a concentration of 1.0%. An increase in E2 concentration was also noted in mixed sludge with addition of 1% H_2O_2 at 20 °C (Figure 4-22). This increase in E2 concentration was accompanied by a reduction in E1 to non-detectable concentrations. Estriol concentrations in digested sludge were also reduced (70%) when 1.0% H_2O_2 was added; indicating other substances present in digested sludge, such as plant and animal sterols not targeted in the chemical analysis, may have been converted to E2. There was no change in the androgenic activity in mixed sludge with the H_2O_2 treatments.

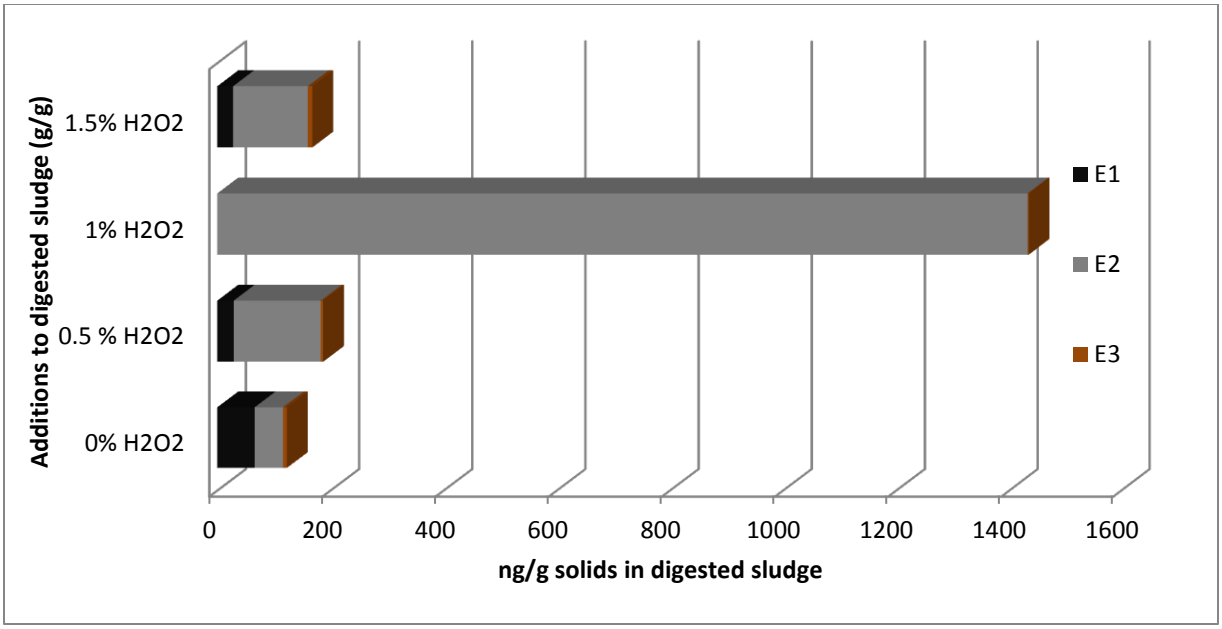


Figure 4-23: Effect of hydrogen peroxide (H_2O_2) additions to estrone (E1), 17β-estradiol (E2), and estriol (E3), in digested sludge (n = 3) at 20 °C

Table 4-37: Estrone (E1), 17β-estradiol (E2), 17α-ethinyl estradiol (EE2), Estriol (E3), testosterone (TT) concentrations in 20 ml digested sludge after additions of 0.5%, 1%, and 1.5% H_2O_2 (wt/wt solids) with percent relative standard deviations of treatment replicates (n = 3)

20 °C	Control			0.5% H2O2			1% H2O2			1.5% H2O2		
Digested sludge (n = 3)	ng/g	ng/L	% RSD	ng/g	ng/L	% RSD	ng/g	ng/L	% RSD	ng/g	ng/L	% RSD
E1	66	992	*	29	435	*				28	419	100**
E2	50	756	16**	154	2305	21**	1433	21501	6	132	1982	56
EE2	ND			3	39	*	ND			ND		
E3	7	107	*	4	55	*	2	27	*	8	114	*
TT	ND			ND			ND			ND		

*Analyte was detected in one of three replicates

**Analyte was detected in two of three replicates

The change in E1 to E2 ratio was much more pronounced in the digested sludge, indicating an enriched biological culture capable of producing E2 from E1, E3 and similar substances (plant and animal sterols) may play a role in this reaction. However, the expected

corresponding increase in estrogenic activity did not occur with the addition of 1% H_2O_2 at 20 °C (Table 4-38). Although a 40% increase in androgenic activity was noted, the estrogenic activity remained unchanged from digested sludge at 20 °C with no H_2O_2 additions.

Table 4-38: Estrogenic and androgenic activity in mixed sludge after hydrogen peroxide additions (wt/wt) to 20 ml municipal digested sludge at 20 °C

Hydrogen peroxide (H_2O_2) additions to digested sludge	Temperature (°C)	Estrogenic activity (EEQ)[1]		Androgenic activity (TEQ)[2]	
		ng/g	ng/L	ng/g	ng/L
Control	20	67.3	1010	30.1	452
0.5% H_2O_2	20	61.0	915	29.6	444
1.0% H_2O_2	20	66.2	994	50.0	750
1.5% H_2O_2	20	68.1	1022	35.6	533

[1] EEQ = Estradiol equivalent (equivalency to 17β-estradiol)
[2] TEQ = Testosterone equivalent (equivalency to testosterone)

Although E2 is the most potent natural estrogen and considered the most significant contributor to estrogenic activity in municipal sludges (Nakada et al., 2004), estrogenic activity may not always correlate with the presence or absence of E2. Nakrst et al. (2011) applied treatments of Fenton's reagent and H_2O_2 to water spiked with E2 and EE2 and observed an increase in estrogenic activity using the YES assay. Removal of E2 and EE2 was almost immediate. However, no significant decrease in E2 estrogenic activity was observed and only 57% of EE2 estrogenic activity was removed after 60 minutes of ozonation (Nakrst et al., 2011).

4.4.3 Combined Oxidation + Irradiation

All samples subjected to a combination of oxidation-microwave irradiation had non-detectable levels of EE2, E3, and TT, with the exception of one of three 100 °C replicates of mixed and digested sludge with low levels of E3. Therefore, oxidation-microwave irradiation

treatment efficacies for removal of total E1+E2 are compared for both mixed and digested sludge (Figure 4-24 and Figure 4-25).

4.4.3.1 MIXED SLUDGE

At temperatures of 100 °C, total E1+E2 concentrations were reduced with increasing H_2O_2 additions of 0.5% (26 % reduction), 1.0% (55% reduction), and 1.5% (72% reduction) (Table 4-41).

The most effective combination oxidative-irradiation treatments for reducing total E1+E2 in mixed sludge were 1.5% additions of H_2O_2 at microwave temperatures 60 °C (E1+E2 = 16 ng/g) and 80 °C (E1+E2 = 16 ng/g). However, without oxidative treatment, irradiation temperatures of 60 °C (Table 4-39) and 80 °C (Table 4-40) performed almost as well, with total E1+E2 concentrations reduced to 19 ng/g in mixed sludge (see Figure 4-24).

None of the oxidative-irradiation treatments reduced E2 in mixed sludge to non-detectible concentrations (Table 4-39, Table 4-40, Table 4-41). However, all control and oxidative-irradiation treatments at 60 °C and 80 °C reduced E1 to non-detectible concentrations (Table 4-39, Table 4-40).

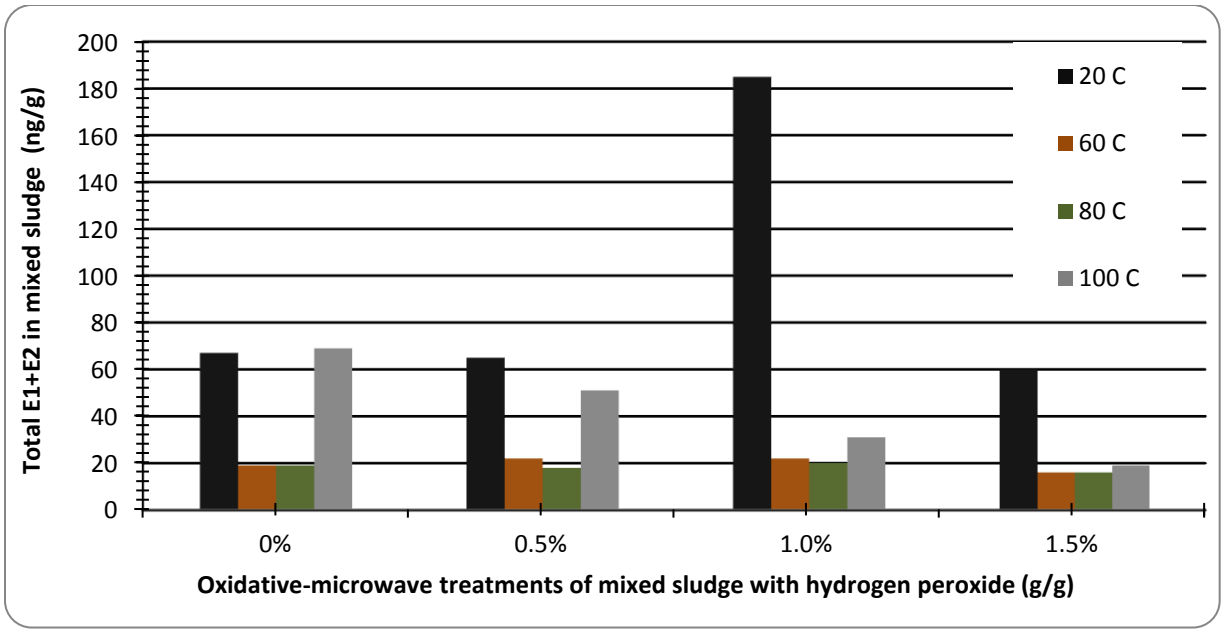


Figure 4-24: Combination of oxidative-microwave irradiation at 60 °C, 80 °C, and 100 °C, with 0.5%, 1%, and 1.5% H_2O_2 (wt/wt solids) additions to determine reduction of total estrone and 17β-estradiol (E1+E2) in mixed sludge

Table 4-39: Estrone (E1), 17β-estradiol (E2), 17α-ethinyl estradiol (EE2), Estriol (E3), testosterone (TT) concentrations in mixed sludge after treatment by microwave irradiation at 60 °C and hydrogen peroxide (H_2O_2) additions of 0.5%, 1.0%, 1.5% (g/g) with percent relative standard deviation (% RSD)

Target Steroid (n = 3)	Mixed sludge microwave irradiated at 60 °C											
	Control			0.5% H_2O_2 (g/g)			1% H_2O_2 (g/g)			1.5% H_2O_2 (g/g)		
	ng/g	ng/L	% RSD	ng/g	ng/L	% RSD	ng/g	ng/L	% RSD	ng/g	ng/L	% RSD
E1	ND			ND			ND			ND		
E2	19	799	45**	22	888	12	22	897	17	16	653	5**
EE2	ND			ND			ND			ND		
E3	ND			ND			ND			ND		
TT	ND			ND			ND			ND		
*Analyte was detected in one of three replicates **Analyte was detected in two of three replicates												

Table 4-40: Estrone (E1), 17β-estradiol (E2), 17α-ethinyl estradiol (EE2), Estriol (E3), testosterone (TT) concentrations in mixed sludge after treatment by microwave irradiation at 80 °C and hydrogen peroxide (H_2O_2) additions of 0.5%, 1.0%, 1.5% (g/g) with percent relative standard deviation (% RSD)

Target Steroid	Mixed sludge microwave irradiated at 80 °C											
	Control			0.5% H_2O_2 (g/g)			1% H_2O_2 (g/g)			1.5% H_2O_2 (g/g)		
(n = 3)	ng/g	ng/L	% RSD	ng/g	ng/L	% RSD	ng/g	ng/L	% RSD	ng/g	ng/L	% RSD
E1	ND			ND			ND			ND		
E2	19	765	22	18	755	7	20	827	20	16	657	11
EE2	ND			ND			ND			ND		
E3	ND			ND			ND			ND		
TT	ND			ND			ND			ND		
*Analyte was detected in one of three replicates **Analyte was detected in two of three replicates												

Table 4-41: Estrone (E1), 17β-estradiol (E2), 17α-ethinyl estradiol (EE2), Estriol (E3), testosterone (TT) concentrations in mixed sludge after microwave irradiation at 100 °C and hydrogen peroxide (H_2O_2) additions of 0.5%, 1.0%, 1.5% (g/g) with relative standard deviation (% RSD)

Target Steroid	Mixed sludge microwave irradiated at 100 °C											
	Control			0.5% H2O2			1% H2O2			1.5% H2O2		
(n = 3)	ng/g	ng/L	% RSD	ng/g	ng/L	% RSD	ng/g	ng/L	% RSD	ng/g	ng/L	% RSD
E1	30	1244	57	22	882	*	18	735	*			
E2	39	1594	4	29	1189	37	13	529	30	19	765	14
EE2	ND			ND			ND			ND		
E3	ND			0.5	19	*	ND			ND		
TT	ND			ND			ND			ND		
*Analyte was detected in one of three replicates **Analyte was detected in two of three replicates												

Oxidative-irradiation of mixed sludge did not reduce total E1+E2 (Figure 4-24) more effectively than thermal treatments alone by microwave irradiation (Figure 4-20). In addition, estrogenic and androgenic activity was not affected by H_2O_2 additions to mixed sludge at any experimental temperature (60 °C, 80 °C and 100 °C), other than an increase in estrogenic activity

observed with 1% H_2O_2 addition at 20 °C (Table 4-42). Reductions in estrogenic activity were associated with microwave irradiation temperatures only.

Table 4-42: Estrogenic and androgenic activity in municipal mixed sludge after hydrogen peroxide additions (wt/wt solids) and microwave irradiation at 60, 80, and 100 °C

Hydrogen peroxide (H_2O_2) additions (g/g) to mixed sludge	Control and microwave irradiation treatment Temperature (°C)	Estrogenic activity (EEQ)[1]		Androgenic activity (TEQ)[2]	
		ng/g	ng/L	ng/g	ng/L
Control	20 control	25.7	1056	8.9	366
0.5% H_2O_2	20 control	22.6	926	8.2	338
1.0% H_2O_2	20 control	37.7	1544	8.1	334
1.5% H_2O_2	20 control	20.0	822	8.1	334
Control	60	15.1	618	7.7	316
0.5% H_2O_2	60	14.3	588	8.4	345
1.0% H_2O_2	60	14.3	587	7.2	294
1.5% H_2O_2	60	13.7	561	7.6	313
Control	80	21.2	871	10.7	439
0.5% H_2O_2	80	18.2	745	8.3	339
1.0% H_2O_2	80	19.2	789	10.4	428
1.5% H_2O_2	80	16.9	693	9.5	391
Control	100	15.1	617	5.4	223
0.5% H_2O_2	100	14.9	612	5.4	223
1.0% H_2O_2	100	15.4	633	5.8	238
1.5% H_2O_2	100	15.7	646	5.8	239

[1] EEQ = Estradiol equivalent (equivalency to 17β-estradiol)
[2] TEQ = Testosterone equivalent (equivalency to testosterone)

4.4.3.2 DIGESTED SLUDGE

The most effective oxidative-irradiation treatments for reducing total E1+E2 in digested sludge were with 1.5% H_2O_2 at temperatures of 80 °C (89% reduction) and 100 °C (88% reduction) (Figure 4-25; and Table 4-44 and Table 4-45).

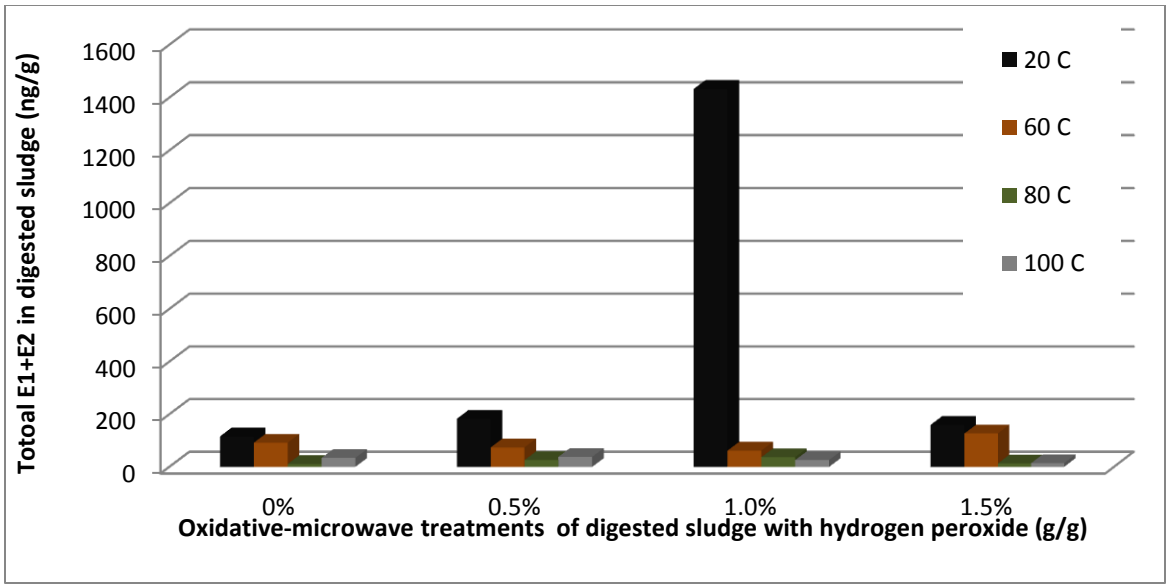


Figure 4-25: Combination of oxidative-microwave irradiation at 60 °C, 80 °C, and 100 °C, with 0.5%, 1%, and 1.5% H_2O_2 to determine reduction of total 17β-estradiol (E2) and estrone (E1) in digested sludge

Table 4-43: Estrone (E1), 17β-estradiol (E2), 17α-ethinyl estradiol (EE2), Estriol (E3), testosterone (TT) concentrations in digested sludge after microwave irradiation at 60 °C and hydrogen peroxide (H_2O_2) additions of 0.5%, 1.0%, 1.5% (g/g) with relative standard deviation (% RSD)

Target Steroids (n = 3)	Digested sludge microwave irradiated at 60 °C											
	Control			0.5% H_2O_2 (g/g)			1% H_2O_2 (g/g)			1.5% H_2O_2 (g/g)		
	ng/g	ng/L	% RSD	ng/g	ng/L	% RSD	ng/g	ng/L	% RSD	ng/g	ng/L	% RSD
E1	66	989	4	62	923	0.4	52	775	4	60	896	13
E2	26	385	22**	12	180	*	10	155	*	67	1003	5
EE2	ND			ND			ND			ND		
E3	ND			ND			ND			ND		
TT	ND			ND			ND			ND		

*Analyte was detected in one of three replicates
**Analyte was detected in two of three replicates

Table 4-44: Estrone (E1), 17β-estradiol (E2), 17α-ethinyl estradiol (EE2), Estriol (E3), testosterone (TT) concentrations in digested sludge after microwave irradiation at 80 °C and hydrogen peroxide (H_2O_2) additions of 0.5%, 1.0%, 1.5% (g/g) with relative standard deviation (% RSD)

Target steroids	Digested sludge microwave irradiated at 80 °C											
	Control			0.5% H_2O_2 (g/g)			1% H_2O_2 (g/g)			1.5% H_2O_2 (g/g)		
(n = 3)	ng/g	ng/L	% RSD	ng/g	ng/L	% RSD	ng/g	ng/L	% RSD	ng/g	ng/L	% RSD
E1	ND			ND			ND			ND		
E2	11	165	*	26	384	20	37	553	19	13	193	*
EE2	ND			ND			ND			ND		
E3	ND			ND			ND			ND		
TT	ND			ND			ND			ND		
*Analyte was detected in one of three replicates **Analyte was detected in two of three replicates												

Table 4-45: Estrone (E1), 17β-estradiol (E2), 17α-ethinyl estradiol (EE2), Estriol (E3), testosterone (TT) concentrations in digested sludge after microwave irradiation at 100 °C and hydrogen peroxide (H_2O_2) additions of 0.5%, 1.0%, 1.5% (g/g) with relative standard deviation (% RSD)

Target steroids	Digested sludge microwave irradiated at 100 °C											
	Control			0.5% H_2O_2 (g/g)			1% H_2O_2 (g/g)			1.5% H_2O_2 (g/g)		
(n = 3)	ng/g	ng/L	% RSD	ng/g	ng/L	% RSD	ng/g	ng/L	% RSD	ng/g	ng/L	% RSD
E1	ND			ND			ND			ND		
E2	34	506	36	38	565	19	26	383	21	14	207	26**
EE2	ND			ND			ND			ND		
E3	4	54	*	ND			ND			ND		
TT	ND			ND			ND			ND		
*Analyte was detected in one of three replicates **Analyte was detected in two of three replicates												

However, the combination oxidative-irradiation with 1.5% H_2O_2 at either 80 °C or 100 °C (Table 4-44, Table 4-45) was just as effective as thermal treatment alone by microwave irradiation at 80 °C (91% reduction), (Table 4-33). Estrogenic and androgenic activity was not affected by H_2O_2 additions to digested sludge at any experimental temperature (60 °C, 80 °C and

100 °C), other than the androgenic increase observed with 1% H_2O_2 addition at 20 °C (Table 4-46). Reductions and increases of estrogenic and androgenic activity were associated with microwave irradiation temperatures only.

Table 4-46: Estrogenic and androgenic activity in municipal digested sludge after hydrogen peroxide additions (wt/wt solids) and microwave irradiation at 60 °C, 80 °C, and 100 °C

Hydrogen peroxide (H_2O_2) additions (g/g) to digested sludge	Control and microwave irradiation treatment Temperature (°C)	Estrogenic activity (EEQ)[1]		Androgenic activity (TEQ)[2]	
		ng/g	ng/L	ng/g	ng/L
Control	20 Control	67.3	1010	30.1	452
0.5% H_2O_2	20 Control	61.0	915	29.6	444
1.0% H_2O_2	20 Control	66.2	994	50.0	750
1.5% H_2O_2	20 Control	68.1	1022	35.6	533
Control	60	46.0	690	38.7	581
0.5% H_2O_2	60	47.6	715	42.2	632
1.0% H_2O_2	60	45.3	679	39.0	586
1.5% H_2O_2	60	55.8	836	40.8	612
Control	80	65.2	979	102.8	1542
0.5% H_2O_2	80	66.5	998	118.0	1771
1.0% H_2O_2	80	67.6	1014	118.5	1778
1.5% H_2O_2	80	58.6	880	113.6	1705
Control	100	50.5	758	43.0	645
0.5% H_2O_2	100	47.1	707	43.6	654
1.0% H_2O_2	100	50.2	753	48.1	722
1.5% H_2O_2	100	49.0	736	43.8	656

[1] EEQ = Estradiol equivalent (equivalency to 17β-estradiol)
[2] TEQ = Testosterone equivalent (equivalency to testosterone)

Combination of oxidative-microwave treatment of digested sludge was not effective in reducing total E1+E2 concentrations in digested sludge. Microwave irradiation at 80 °C, without H_2O_2 addition, was preferred for reducing E1, E2 and total E1+E2 concentrations in digested sludge.

4.4.3.3 SUMMARY OF OXIDATION + MICROWAVE IRRADIATION

Although oxidative irradiation of mixed and digested sludges was not effective for reducing estrogens (total E1+E2) and estrogenic / androgenic activity, it has proven an effective pre-treatment for solubilizing ammonia and phosphorus, increasing biogas, reducing sludge mass, and increasing sludge dewatering (Laio et al, 2005; Wong et al., 2006a; Wong et al., 2006b; Yin et al., 2008). Laio et al. (2005) applied an oxidation process, a combination of hydrogen peroxide and microwave heating, to secondary municipal sludge and, thereby, increased solubilization of phosphate in an enhanced biological phosphorus removal process.

However, the oxidation mechanism in microwave irradiation has not been fully understood. Why estrogenic activity increased in mixed and digested sludges with 1% H_2O_2 at 20 °C, and not at microwave irradiation temperatures (60 °C, 80 °C and 100 °C), remains unexplained. It may indicate microwave irradiation counteracts the increase in estrogenic activity that accompanies the application of this oxidative treatment to the temperature control samples. Further study on the oxidation mechanism of H_2O_2/microwave is needed and is currently being investigated through graduate research projects at the University of British Columbia Environmental Engineering programs.

4.4.4 Conventional Heating

Conventional heating by a water bath and irradiation by a batch-process laboratory microwave were used to evaluate the effect of conventional heating and the athermal effects of MW irradiation on 17β-estradiol, estrone and whole estrogenic activity in mixed and digested

sludges. Mixed and digested sludge responded differently to conventional heating to 60 °C, 80 °C, or 100 °C by water bath, in terms of reducing total E1+E2 concentrations.

4.4.4.1 MIXED SLUDGE

Conventional heating of mixed sludge did not appear to reduce concentrations of EE2, E3, or TT at temperatures of 60 °C, 80 °C, or 100 °C (Table 4-47). Conventional heating at 60 °C produced similar results for reduction of E1 and E2. However, total E1+E2 was reduced by 60% at temperatures of 80 °C and 100 °C, with E1 being reduced by 67% and E2 by 31% (Table 4-47). These findings are in contrast to Carballa et al. (2006) that found no reduction of E1, E2 or EE2 associated with thermal pre-treatment (autoclave at 130 °C) for anaerobic mesophilic and thermophilic digestion.

Table 4-47: Estrone (E1), 17β-estradiol (E2), 17α-ethinyl estradiol (EE2), Estriol (E3), and testosterone (TT) concentrations in mixed sludge after conventional heating in a water bath at 60 °C, 80 °C and 100 °C with percent relative standard deviation (% RSD)

Target steroids (n = 3)	Mixed sludge Controls 20 °C			Water bath temperature								
				60 °C			80 °C			100 °C		
	ng/g	ng/L	%RSD	ng/g	ng/L	%RSD	ng/g	ng/L	%RSD	ng/g	ng/L	%RSD
E1	50	2033	14	60	2459	114	18	720	24	18	736	40
E2	15	611	18	14	578	63	9	376	33	11	449	38
E1+E2	65	2644		74	3037		27	1096		29	1175	
EE2	1	50	28	1	50	*	ND			0.6	25	30
E3	4	178	58	6	250	*	4	144	61	ND		
TT	7	300	44	2	100	22	7	300	13	1	50	*
*Analyte was detected in one of three replicates **Analyte was detected in two of three replicates												

In contrast, microwave irradiation at 60 °C and 80 °C reduced concentrations of E1, EE2, E3, and TT in mixed sludge to below detectable concentrations. While irradiation at 60 °C and

80 °C did not reduce levels of E2, total E1+E2 concentrations were reduced by 72% (Table 4-31). Microwave irradiation at 100 °C, while still reducing EE2, E3, and TT to non-detectable levels, did not reduce total E1+E2 concentrations, but it did reduce both androgenic and estrogenic activities by 40% (Table 4-32).

Conventional heating of mixed sludge at temperatures of 60 °C, 80 °C and 100 °C had little effect on estrogenic or androgenic activity (Table 4-48). However, microwave irradiation reduced estrogenic activity by 40% at 60 °C and 18% at 80 °C (Table 4-32).

Table 4-48: Estrogenic and androgenic activity in municipal mixed sludge, after application of conventional heat at 60 °C, 80 °C, and 100 °C

Mixed sludge control and conventional heat (°C)	Estrogenic activity[1]		Androgenic activity[2]	
	EEQ (ng/g)	EEQ (ng/L)	TEQ (ng/g)	TEQ (ng/L
20	44.3	1816	8.1	332
60	41.6	1704	8.9	364
80	41.9	1718	7.6	311
100	38.5	1580	9.2	376

[1] EEQ = Estradiol equivalent (equivalency to 17β-estradiol)
[2] TEQ = Testosterone equivalent (equivalency to testosterone)

Overall, microwave irradiation at 60 °C and 80 °C reduced EE2, E3, and TT to non-detectable levels and was the most effective method of reducing total E1+E2 in mixed sludge. Microwave irradiation at 60 °C was more effective than conventional heating at reducing estrogenic activity and concentrations of total E1+E2, EE2, E3, and TT in mixed sludge at all temperatures tested (60 °C, 80 °C, and 100 °C). Microwave irradiation of mixed sludge at 60 °C would be the most cost effective in terms of time and energy for reduction of estrogenic activity, and total E1+E2, EE2, E3, and TT in mixed sludge.

4.4.4.2 DIGESTED SLUDGE

Conventional heating of digested sludge at 60 °C reduced total E1+E2 by 31% (Table 4-49). However, increasing conventional heating to 80 °C and 100 °C did not demonstrate a further reduction in total E1+E2 in digested sludge. Conventional heating at 60 °C, 80 °C, and 100 °C did not reduce concentrations of EE2 and TT in digested sludge. Estriol was not detected in the conventional heat treatment or control digested sludge samples (Table 4-49).

Table 4-49: Estrone (E1), 17β-estradiol (E2), 17α-ethinyl estradiol (EE2), Estriol (E3), and testosterone (TT) concentrations in digested sludge after conventional heating in a water bath at 60 °C, 80 °C and 100 °C with percent relative standard deviation (% RSD)

Target steroids (n = 3)	Digested sludge Controls 20 °C			Water bath temperature								
				60 °C			80 °C			100 °C		
	ng/g	ng/L	%RSD	ng/g	ng/L	%RSD	ng/g	ng/L	%RSD	ng/g	ng/L	%RSD
E1	117	1762	33	75	1118	25	88	1320	31	75	1122	21
E2	20	300	18	20	300	17	17	250	15	10	150	19
E1 + E2	137	2062		95	1418		105	1600		85	1272	
EE2	0	5	48	2	25	7	1	20	77	1	10	87
E3	ND			ND			ND			ND		
TT	23	350	*	10	150	44	5	75	32	3	50	57

*Analyte was detected in one of three replicates
**Analyte was detected in two of three replicates

In comparison, microwave irradiation reduced total E1+E2 in digested sludge at temperatures of 60 °C (21%), 80 °C (91%) and 100 °C (31%) (Table 4-33). Estrone was below detectable concentrations in digested sludge irradiated at 80 °C and 100 °C. Detection of EE2, E3 and TT were infrequently detected or not detected at all in the digested sludge microwave heat treatment and control sets. Concentrations of E2 were indirectly related to temperature with concentrations reduced by 50%, 78%, and 32% at temperatures of 60 °C, 80 °C, and 100 °C, respectively (Table 4-33).

Table 4-50: Estrogenic and androgenic activity in municipal digested sludge, after application of conventional heat at 60 °C, 80 °C, and 100 °C

Control and microwave irradiation treatment temperature (^{o}C)	Estrogenic activity		Androgenic activity	
	EEQ (ng/g)	EEQ (ng/L)	TEQ (ng/g)	TEQ (ng/L)
20	49.0	736	11.1	166
60	42.2	633	10.6	159
80	52.0	780	13.1	197
100	54.4	815	13.5	203

Conventional heating slightly decreased estrogenic and androgenic activity at 60 °C, and increased estrogenic and androgenic activity at 80 °C and 100 °C (Table 4-50). This may be due to biological degradation of non-target compounds in digested sludge by thermotolerant organisms. In comparison, microwave irradiation reduced estrogenic activity by 30% at 60 °C and 25% at 100 °C (Table 4-34). However, microwave irradiation increased androgenic activity even more than conventional heating at 60 °C (29%); 80 °C (240%); and 100 °C (43%) (Table 4-34).

Overall, the most effective heat treatment for reducing E1 and E2 in digested sludge was microwave irradiation at 80 °C, although no change in estrogenic activity (and an increase in androgenic activity) was noted. While conventional heating to 60 °C by water bath reduced total E1+E2 by 31%, and was comparable to the 21% reduction with microwave irradiation at 60 °C, it was not nearly as efficient as the 91% reduction demonstrated by microwave irradiation at 80 °C. The combination of pressure and heat of microwave irradiation at 80 °C and 100 °C outperformed conventional heat and was the preferred method for reducing E1 and E2 in digested sludge.

5 SUMMARY AND CONCLUSIONS

A chemical analysis method protocol, using gas chromatography with mass spectrometry (GC-MS), was developed for detection of testosterone (TT), estrone (E1), 17β-estradiol (E2) and 17α-ethinyl estradiol (EE2) in mixed and digested sludges. In order to directly compare E1, E2 and testosterone concentrations with whole estrogenic analysis, the developed protocol was compatible for use with three auto-bioluminescent yeast estrogen screen assays. This chemical analysis targets the recovery of estrogens E1 and E2 in municipal sludge and wastewaters and utilizes: liquid-liquid mechanical shaker and ultrasonic extractions; combination Florisil and silica chromatography columns for clean up; and an oximation with pre- and post-silylation derivatization steps. This chemical analysis performed well for mixed and digested sludges, yielding average recoveries of estrone (98%, 89%), 17β-estradiol (81%, 89%), 17α-ethinyl estradiol (91%, 86%) and testosterone (98%, 94%), respectively. These protocols are elucidated and summarised for consideration and use by other researchers and the user community.

Based on the data collected in this research project, the following specifics are offered, with commentary, as it pertains to wastewater and several different sludge treatment technologies being investigated at the University of British Columbia.

5.1 WASTEWATER TREATMENT

Although removals of targeted estrogens, E1 and E2 were not as high for this trickling filter WWTP as reported by Ternes et al. (1999) for a trickling filter WWTP in Brazil, it did reduce total E1+E2 by 54%; the most potent estrogen, E2, by 69%; estrogenic activity by 27%; androgenic activity by 38%; and toxicity to BLYR by 90% from influent to pre-chlorinated

effluent. In addition, the mesophilic (35–40 °C) sludge digestion removed total E1+E2 by 26%; estrogenic activity by 73%; androgenic activity by 81%; and the most potent estrogen, E2, by 63%.

Overall, these data, taken collectively, indicate that the waste treatment configuration reduces estrogens and estrogenic activity in the wastewater stream by: solids settling in the primary sedimentation and secondary clarifier processes; and biological degradation in the trickling filter and solids contact processes. Although estrogens, estrogenic activity, and androgenic activity are reduced, they are not eliminated. These removal mechanisms need to be examined with respect to the operator's objectives for removal of these concentrations.

5.2 MICROWAVE IRRADIATION OF SLUDGE

Microwave irradiation at 60 °C and 80 °C produced similar results and was the most effective method of reducing total E1+E2, in mixed sludge. Microwave irradiation at 60 °C was more effective at reducing estrogenic activity and concentrations of total E1+E2, EE2, E3, and TT in mixed sludge, than conventional heating at all temperatures tested (60 °C, 80 °C, and 100 °C). Microwave irradiation of mixed sludge at 60 °C would be the most cost effective at full scale, in terms of time and energy for reduction of estrogenic activity and total E1+E2, E1, EE2, E3, and TT (but not E2) in mixed sludge.

The most effective heat treatment for reducing E1 and E2 in digested sludge was microwave irradiation at 80 °C, although no change in estrogenic activity (and an increase in androgenic activity) was noted. Although conventional heating to 60 °C by the water bath

reduced total E1+E2 in digested sludge by 31%, and was comparable to the 21% reduction with microwave irradiation at 60 °C, it was not nearly as efficient as the 91% reduction demonstrated by microwave irradiation at 80 °C. The combination of pressure and heat of microwave irradiation at 80 °C and 100 °C outperformed conventional heat and was the preferred method for reducing E1 and E2 in digested sludge.

Table 5-1: Potential for most effective domestic sludge treatments (thermal irradiation) used in combination to reduce concentrations of estrone (E1), 17β-estradiol (E2), 17α-ethinyl estradiol (EE2), estriol (E3), testosterone (TT), estrogenic activity (EEQ), and androgenic activity (TEQ).

Parameters	Mixed sludge (ng/L)	Microwave (60 °C)	Anaerobic mesophilic digestion	Microwave (80 °C)	Projected outcome (% removal)
E1	1862	100% (ND)	12%	100% (ND)	ND
E2	897	11%	63%	78%	94%
E1+E2	2759	71%	26%	91%	83%
EE2	49	100% (ND)	*	*	ND
E3	149	100% (ND)	*	100% (ND)	ND
EEQ	1056	41%	73%	0%	84%
TT	448	100% (ND)	*	*	ND
TEQ	366	14%	81%	-341%	44%

* Target compound was not consistently detected in digested sludge to calculate percent reduction during treatment

Table 5-1 projects the outcome of using a combination of the most effective sludge treatments (thermal irradiation) on mixed and digested sludge to reduce concentrations estrone (E1),), 17β-estradiol (E2), 17α-ethinyl estradiol (EE2), estriol (E3), testosterone (TT), estrogenic activity (EEQ), and androgenic activity (TEQ). Microwave treatment of mixed and digested domestic sludge at different temperatures was effective for reducing concentrations of the target compounds. Although, treatments demonstrating a reduction in target compounds were deemed effective, a process train utilizing treatments that increase concentrations by releasing them from the solid phase may be preferred prior to reducing concentrations with one of the preferred

methods in Table 5-1. Further research is required to assess the dynamics of estrogen and androgen release, breakdown, and conversion during microwave treatment of domestic sludge.

5.3 OXIDATIVE SLUDGE TREATMENTS

The addition of hydrogen peroxide, without the application of heat, did not reduce E1, E2, estrogenic or androgenic activity. However, an increase in the most potent estrogen E2 was noted, as well as an increase in androgenic activity. Hence the addition of hydrogen peroxide, without heat, is not recommended for the reduction of estrogens or estrogenic activity.

In addition, hydrogen peroxide additions did not improve the efficacy of microwave irradiation at 60 °C, 80 °C and 100 °C for reduction of total E1+E2, estrogenic activity and/or androgenic activity. Similarly, oxidative-irradiation of mixed sludge did not reduce total E1+E2 more effectively than thermal treatments alone by microwave irradiation.

Although oxidative irradiation of mixed and digested sludges was not effective for reducing estrogens (total E1+E2) and estrogenic / androgenic activity, it has proven an effective pre-treatment for solubilizing ammonia and phosphorus, increasing biogas, reducing sludge mass, and increasing sludge dewatering (Laio et al, 2005; Wong et al., 2006a; Wong et al., 2006b; Yin et al., 2008). Laio et al. (2005) applied an oxidation process, a combination of hydrogen peroxide and microwave heating, to secondary municipal sludge and, thereby, increased solubilization of phosphate in an enhanced biological phosphorus removal process.

5.4 IMPLICATIONS FOR FUTURE RESEARCH

The current study produced many exiting research questions that could not be addressed during the tenure of a single Ph.D. student. Recommendations for future research, in conjunction with potential full-scale application, include:

1. Estrogen partitioning between solid and water phases in municipal wastewaters and sludges should be examined in spiked and unspiked samples with high solids content. Spiking methods in wet and freeze-dried sludges should be examined to improve the reporting of method recoveries for laboratory protocols for quantification of estrogens.

2. Examine E1 and E2 concentrations during the process of wastewater chlorination. The sampling program in the current study assumed little difference in concentrations of E1, E2, estrogenic and androgenic activity between pre- and post-chlorinated effluents. However, all these concentrations increased in chlorinated effluents. Prior to assessing risk the of endocrine disrupting chemicals to the receiving environment, non-chlorinated, chlorinated and dechlorinated effluent should be evaluated, using concentrations of E1, E2, estrogenic and androgenic activity.

3. WWTP optimization for reduction of estrogenic and androgenic activity. Although a substantial decrease in estrogens and estrogenic and androgenic activity was noted, other wastewater treatment systems, such as extended-aeration type activated sludge systems, are thought to provide better removal than a trickling filter-solids contact process. However, the effects of retention time within the solids contact tank or other operational parameters have

not been examined. Treatment processes within the existing system may also need to be optimized and/or plant upgraded, to improve the removal of estrogens and estrogenic and androgenic activity.

4. Post-digestion sludge treatment optimization. Estrogens, estrogenic activity and androgenic activity were associated with adsorption to and settling of solids throughout the wastewater treatment system. Mesophilic anaerobic digestion (35–40 °C) effectively reduced estrogenic and androgenic activities. However, a substantial concentration of estrogens and estrogenic activity remained in digested sludge and these may be further reduced when mesophilic anaerobic digestion is combined with oxidative treatments. Due to the high affinity of E1 and E2 to solids, it is likely dewatering or drying processes alone will not eliminate estrogens or estrogenic activity prior to composting or land application. Therefore, the post digestion treatment processes should be examined and evaluated using E1, E2, estrogenic and androgenic activity in terms of biosolids use and conditions of the receiving environment. Similarly, it behooves the academic community to investigate the potential of thermophilic digestion (55–62 °C) to reduce or eliminate endocrine disruptors.

5. Identification of species capable of degrading endocrine disrupting compounds is required in order to develop enrichment cultures for seeding wastewater and sludge treatment systems. This research could be complemented with the examination of operating conditions enabling the enrichment of biological processes with cultures capable of reducing steroidal compounds in wastewaters and sludges. Determination of estrogen degradation pathways to

improve biological and chemical processes responsible for the degradation of endocrine disrupting compounds could also improve treatment system design and operating conditions.

6. Further research investigating microwave irradiation technology as pre- and post- sludge digestion for the reduction of estrogens and estrogenic activity is needed. Microwave irradiation of mixed sludge was ineffective for reducing concentrations of the most potent estrogen, E2. Microwave irradiation did, however, reduce concentrations of other estrogens and may be effective as a pre-treatment to digestion and/or in combination with other sludge treatments in reducing or eliminating estrogens completely, including E2. Microwave irradiation appeared promising as a sludge treatment for reducing estrogenic activity in mixed and digested sludges even at low irradiation temperatures (60 °C). However, higher temperatures were required to reduce androgenic activity, with 50% reduction observed at microwave irradiation at 100 °C.

7. Risk assessment should be conducted for biosolids and dechlorinated effluent to the receiving environment, utilizing recommendations #1 and #3 above. When assessing risk to the receiving environment, total E1+E2 and estrogenic activity in de-chlorinated effluent, distribution patterns, discharge rates, degradation factors for estrogens and estrogenic activity, etc. should be studied. Since aquatic life can be sensitive to low levels of estrogenic activity, it is important that treatment systems be engineered to reduce estrogens and estrogenic activity whenever possible, using more advanced treatment technologies now being put into practice.

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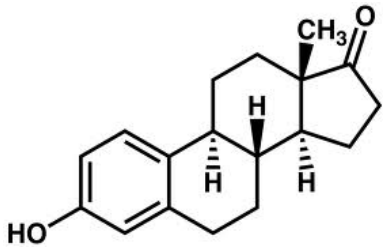
APPENDIX A: CHEMICAL STRUCTURES



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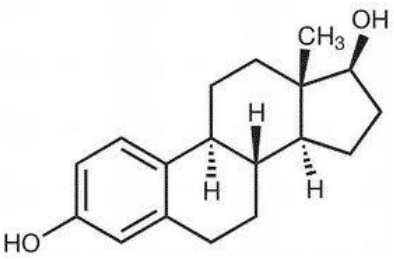
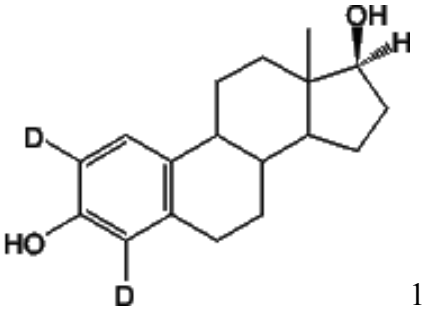
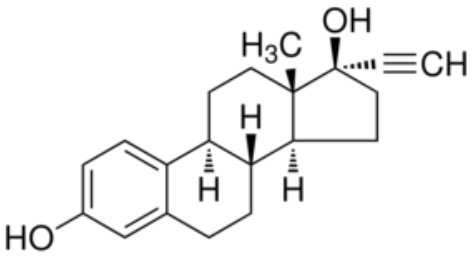


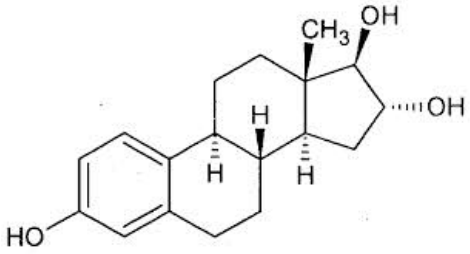
17β-estradiol



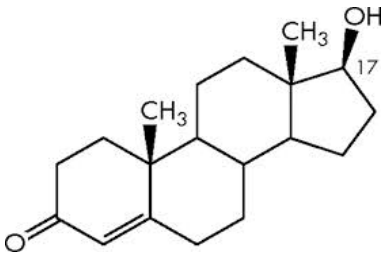
17β-estradiol-2,4-d_2



17α-ethinyl estradiol



Estriol



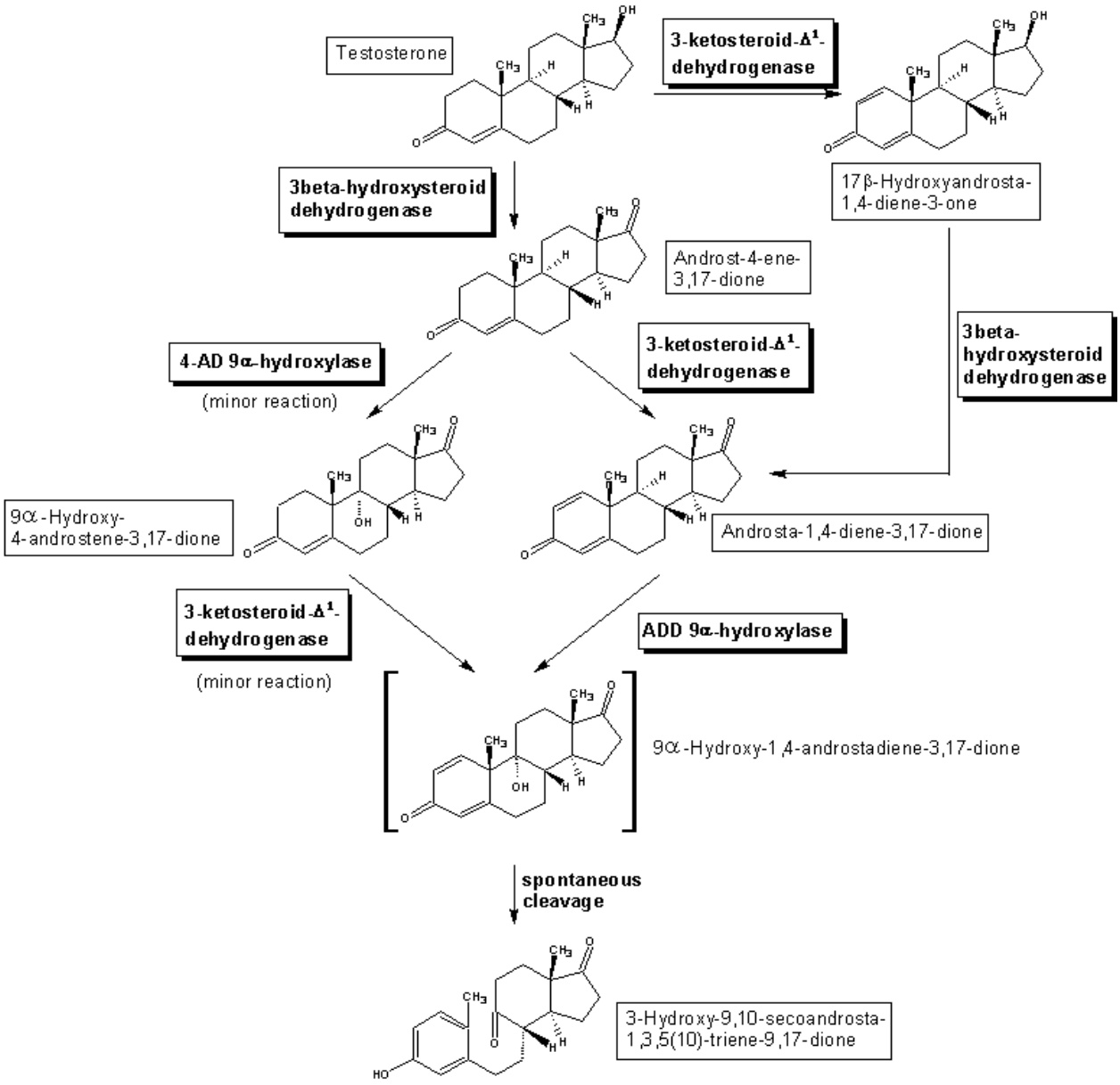
Testosterone

APPENDIX B: RELATIVE ESTROGENIC POTENCIES

Estrogen	Estrogenic potency (EEQ[1] as EEF[2])	Reference
17β-estradiol (E2)	1.0 (used as base value)	Matsui et al. (2000) Sun et al. (2013)
Estrone (E1)	0.21	Matsui et al. (2000)
Estrone	0.1	Sun et al. (2013)
Estriol (E3)	0.08	Sun et al. (2013)
Estriol	1.3×10^{-3}	Matsui et al. (2000)
17α-ethinyl estradiol	1.2	Sun et al. (2013)
17α-estradiol	0.01	Matsui et al. (2000)
17β-estradiol 3-sulphate	5.3×10^{-5}	Matsui et al. (2000)
β-estradiol 17-(β-D-glucuronide)	5.9×10^{-7}	Matsui et al. (2000)
β-estradiol 3-(β-D-glucuronide)	3.1×10^{-5}	Matsui et al. (2000)
17β-estradiol 3-sulphate 17-glucuronide	Not detectable	Matsui et al. (2000)
2-hydroxyestradiol	6.1×10^{-3}	Matsui et al. (2000)

[1] EEQ = 17β-estradiol equivalents
[2] EEF = Estradiol equivalency factor (where 17β-estradiol = 1.0)

APPENDIX C: TESTOSTERONE DEGRADATION PATHWAY



Testosterone Graphical Pathway Map 1
Source and permission to publish in this report: Gao et al. (2010)
Contributors: Andy Rabins and Carla Essenberg; University of Minnesota
December 05, 2012; http://umbbd.ethz.ch/tes/tes_image_map1.html

APPENDIX D: SUPPLEMENTARY DATA TABLES

D-1: CHEMICAL ANALYSIS DEVELOPMENT

Table D 1: Standard curves for target hormones in mixed and digested sludge matrices (September 2012)

Target compound added to 20 ml sludge (ng)	0	10	50	100	250	500
Estrone in mixed sludge (n=3)	48	54	110	144	299	563
Estrone in digested sludge (n=3)	101	128	170	244	396	641
17β-estradiol in mixed sludge (n=3)	49	62	97	157	270	462
17β-estradiol in digested sludge (n=3)	5	20	54	113	283	499
17α-ethinyl estradiol in mixed sludge (n=3)		19	43	109	270	536
17α-ethinyl estradiol in digested sludge (n=3)		25	62	111	230	583
Estriol in mixed sludge (n=3)	21	67	95	168	322	455
Estriol in digested sludge (n=3)			40	130	267	405
Testosterone in mixed sludge (n=3)	32	83	99	165	282	425
Testosterone in digested sludge (n=3)			79	141	284	455

Table D 2: Analyte recovery with standard additions of estrone (E1), 17B-estradiol (E2), 17a-ethinyl estradiol (EE2), estriol (E3), and testosterone (TT) to mixed and digested sludges

Analyte added (ng)	10	50	100	250	500
E1 mixed sludge	93	112	97	100	103
E1 digested sludge	115	112	121	113	107
E2 mixed sludge	106	97	108	88	83
E2 digested sludge	130	98	108	111	99
EE2 mixed sludge	193	87	109	108	107
EE2 digested sludge	249	123	111	92	117
E3 mixed sludge	214	133	139	119	87
E3 digested sludge	0	79	130	107	81
TT mixed sludge	198	121	125	100	80
TT digested sludge	0	158	141	113	91

Table D 3: Standard curves with percent recoveries of estrone (E1), 17β-estradiol (E2), 17α-ethinyl estradiol (EE2), estriol (E3), and testosterone (TT) in 20 ml samples of mixed and digested sludges (January 2013)

ng/sample	E1	E2	EE2	E3	TT
MS	43	55	7	8	66
MS + 50 ng	108	68	11	16	122
% recovery	116	64	19	27	106
MS + 500 ng	576	520	389	213	498
% recovery	106	94	77	42	88
MS + 1 ug	1041	1012	709	585	1022
% recovery	100	96	70	58	96
DS	152	41		2	
DS + 50 ng	192	85	22	20	
% recovery	95	94	44	39	0
DS + 500 ng	647	538	385	318	549
% recovery	99	100	77	63	110
DS + 1ug	1035	1034	751	596	1017
% recovery	90	99	75	59	102

Table D 4: Dichloromethane versus chloroform as an extraction solvent for recovery of estrone and 17β-estradiol in mixed and digested sludges

Target compounds	Dichloromethane (n=3)				Chloroform (n=3)					
	Mixed sludge		Digested sludge		Mixed sludge			Digested sludge		
	Average (ng)	% RSD	Average (ng)	% RSD	Average (ng)	% RSD	Loss %	Average (ng)	% RSD	Loss %
Estrone	66	14	90	5	48	61	27	87	12	3
17β-estradiol	52	11	7	43	38	60	27	5	20	25

Table D 5: Percent loss of 17β-estradiol during extraction and chromatography steps in the optimized chemical analysis protocol for recovery of estrogens from mixed and digested sludges

Samples spiked with 1 ug E2	Mixed Sludge		Digested Sludge		Effluent		Influent	
(% loss assumes 100% recovery for derivatization)	% loss	% RSD n = 3	% loss	% RSD n = 3	% loss	% RSD n = 3	% loss	% RSD n = 3
Derivatization	N/A	7	N/A	2	N/A	6	N/A	6
Chromatography	5	2	13	3				
Extraction	2	10	0	6	1	4	-8	29

Table D 6: Percent recovery of 17β-estradiol during extraction and clean up of mixed and digested sludges in the optimized chemical analysis protocol

20 ml sludge	17β-estradiol (E2) spiked prior to	% Recovery	% RSD
DS+1µg E2	extraction	104	11
DS+1µg E2	chromatography	103	5
MS+1µg E2	extraction	91	12
MS+1µg E2	chromatography	97	9
MS+1µg E2	derivatization	97	17

Table D 7: Calculation of analytical detection limits for 17β-estradiol (E2), estrone (E1), 17α-ethinyl estradiol (EE2), estriol (E3) and testosterone (TT) in one millilitre derivatization agent

	Peak abundan	0.5 ng E2	Peak abur	1.0 ng E2	Peak abur	E1 5 ng	Peak abur	EE2 5 ng	Peak abur	E3 10 ng	Peak abur	TT 10 ng
	23	0.7	44	1.3	102	5.4	22	6.1	13	9.3	7	8.8
	25	0.7	42	1.2	93	4.9	14	3.9	17	12.1	15	18.8
	15	0.4	38	1.1	88	4.6	20	5.6	15	10.7	5	6.3
	25	0.7	33	1.0	95	5.0	13	3.6	14	10.0	8	10.0
	18	0.5	42	1.2	91	4.8	21	5.8	16	11.4	9	11.3
	13	0.4	34	1.0	97	5.1	23	6.4	12	8.6	5	6.3
	16	0.5	28	0.8	98	5.2	14	3.9	14	10.0	7	8.8
Std dev	5.0	0.1	5.9	0.2	4.7	0.2	4.3	1.2	1.7	1.2	3.4	4.3
Average	19.3	0.6	37.3	1.1	94.9	5.0	18.1	5.0	14.4	10.3	8.0	10.0
MDL		0.5		0.5		0.8		3.8		3.9		13.4
LOQ		1.4		1.7		2.5		11.9		12.3		42.7
High spike	0.5<10*MDL?	4.6	1.0<10*MI	5.3	5.0<10*MI	7.7	5.0<10*MI	11.9	10.0<10*M	12.2	10.0<10*M	42.7
Low spike	0.5>MDL?	0.5	1.0>MDL?	0.5	5.0>MDL?	0.77	5.0>MDL?	1.2	10.0>MDL	1.2	10.0>MDL	4.3
signal/noise		3.9		6.4		20.3		4.2		8.4		2.3

Table D 8: Comparison of twelve methods varying time-temperature relationships for two- (methoxamine-silylation) and three- (silylation-methoximine-silylation) step derivatization protocols as discussed in sections 3.1.7.5 (methodology) and 4.1.6.4 (results)

Sample set	**E1**	**E2**	**E2dd**	**E3**	**EE2**	**TT**
1	28968.02	33079.39	7044.441	12759.55	7510.338	2844.209
2	47439.05	48652.99	5465.635	21196.05	18513.15	3889.275
3	37355.78	35991.68	5016.331	15822.77	8954.217	2347.658
4	16771.93	16524.01	2212.277	7404.325	3574.263	1086.716
5	22517.93	22144.39	3162.569	9521.929	5891.636	1290.525
6	32130.67	30864.25	4212.153	14850.56	10408.34	614.4525
7	20482.26	19361.07	2554.409	5394.706	0	488.212
8	28488.41	26912.28	24210.13	7341.692	1646.44	1751.202
9	10619.6	10038.14	1392.627	3918.894	367.3157	790.6594
10	99636.5	105611.1	66575.13	18427.99	6836.065	2418.479
11	25428.13	24550.84	19144.31	7209.941	2718.446	569.625
12	31495.76	31073.88	19088.56	8079.598	4910.433	682.6422

Table D 9: Comparison of time-temperature relationship variations for one-step (Si), two-step (MoSi) and three-step (SMS) derivatization methods as discussed in sections 3.1.7.5 (methodology) and 4.1.6.4 (results)

% difference in peak areas per treatment set average				
Si set 1	MoSi set 2	SMS set 3	SMS set 4	SMS set 5
116	104	85	97	99
83	71	70	163	112
83	73	72	158	114
93	138	66	88	116
98	95	98	100	109
125	110	76	97	92

Table D 10: Recovery of 17β-estradiol at pH 5.5 and 4.0 in digested sludge (DS) with 10% methanol added prior to extraction with dichloromethane and chromatography column clean up

Effect of lowering pH on recovery of E2 from mixed and digested sludges		
DS pH = 8.0 (20 ml)	10% MeOH + pH = 5.5	10% MeOH + pH = 4.0
E2 (ng)	8.1	9.6
% recovery	84	100
% RSD	5.8	11.0

Table D 11: Recovery of 17β-estradiol at pH 4.0 in mixed sludge (MS) with and without 10% methanol added prior to extraction with dichloromethane and preparatory chromatography

MS 20 ml	Control pH = 6.0	pH = 4.0	10% MeOH + pH = 4.0
E2 (ng)	21.4	32.1	33.1
% recovery	65	97	100
% RSD	4.1	11.1	7.3

Table D 12: Recovery of 17β-estradiol at pH 5.5 and 8.0 (control) in digested sludge (DS) with and without 10% methanol added prior to extraction

Effect of pH and MeOH on extraction efficiencies for E2 in digested sludge samples				
20 ml DS	Control	10% MeOH	10% MeOH + pH = 5.5	pH = 5.5
E2 (ng)	1.2	1.1	4.7	2.3
% Recovery	25	23	100	49
% RSD	2.2	33.1	19.7	7.3

Table D 13: Effect of pH 6, 4 and 2 on recoveries of E2 in 20 ml mixed sludge (MS) spiked (1 μg) with 17β-estradiol (E2) prior to dichloromethane extraction by combination wrist shaker/ultrasonic bath method

pH of 20 ml MS+1ug E2	Recovery (%)	% RSD
pH 6.0 (control)	69	12
pH 4.0	95	19
pH 2.0	85	11

Table D 14: Recovery of 17β-estradiol (E2) in unspiked mixed sludge (20 ml) during Soxhlet, wristshaker and shaker/ultrasonic extraction methods using dichloromethane (DCM) and comparison of three extraction solvent mixtures (acetone, DCM and 3:7 acetone:DCM) using the wrist shaker extraction method

Extraction (n = 3)	MS solids	weight (g)	Solvent	E2 (ng)	Range (ng)	E2 (ng/g solids)	Range E2 (ng/g solids)	% RSD
Soxhlet	freeze dried	0.766	DCM	37	20–41	49	26–54	43
Wrist shaker	freeze dried	0.74504	DCM	14	11–17	19	15–23	26
Wrist shaker	freeze dried	0.69894	30% acetone in DCM	13	ND–22	18	ND–31	89
Wrist shaker	freeze dried	0.7126	Acetone	15	13–17	21	18–24	15
Shaker / ultrasonic	wet solids	0.9024	DCM	37	35–44	41	39–49	17

Table D 15: Recovery of spiked (100 ng) 17β-estradiol (E2) in 20 ml mixed (MS) and digested (DS) sludges while varying preparatory chromatography packing materials—activated or deactivated, with acid (HCl) or water (H2O), for silica/Florisil or silica columns

Samples	Sludge	Deactivated	Silica/Florisil	Silica	% RSD	E2 (ng)	% recovery
1–3	MS+100 ng	H2O	X		3	94	72
4–6	DS+100 ng	H2O	X		17	107	99
7–9	MS+100 ng	HCl	X			ND	ND
10–12	DS+100 ng	HCl	X			ND	ND
13–15	MS+100 ng	HCl		X		ND	ND
16–18	DS+100 ng	HCl		X		ND	ND
19–21	MS+100 ng	H2O		X		ND	ND
22–24	DS+100 ng	H2O		X	87	65	59
25–26	MS	H2O	X		12	13	N/A
27–28	DS	H2O	X		28	4	N/A

Clarity of samples

Samples	Analyzed sample	Pre-sample elutions	Notes
1–3	very clean — light amber tint		
4–6	very clean — light amber tint		
7–9	very dirty, dark brown/green/black in colour		couldn't N2 dry after derivatization
10–12	very dirty, dark brown/green/black in colour		
13–15	very dirty, dark brown/green/black in colour	pre-elutions quite clear	
16–18	very dirty, dark brown/green/black in colour		couldn't N2 dry before derivatization. Lots of dark gel-like matter after derivatization couldn't transfer to GC-MS vial
19–21	clean with green tint with gel-like residue	light yellow	
22–24	clean with green tint with gel-like residue	dark yellow	
25–26	very clean — light amber tint		
27–28	very clean — light amber tint		

D-2: WASTEWATER AND SLUDGE PROCESSES

Table D 16: 17β-estradiol in mixed and digested sludges and influent and effluent from a municipal wastewater treatment plant utilizing tricking filter/solids contact technology (September 2012)

Unspiked samples (n = 3)	E2 ng/ sample	E2 ng/g solids	E2 ng/L	% RSD
Mixed sludge (20 ml)	20	25	1000	3
Digested sludge (20 ml)	14	46	700	20
Effluent (1 L)	14		14	10
Influent (1 L)	19		19	6

Table D 17: 17β-estradiol (E2) in mixed and digested sludges and influent and effluent from a municipal wastewater treatment plant utilizing tricking filter/solids contact technology (April 2012)

Unspiked samples (n = 3)	E2 (ng)	E2 (ng/L)	E2 (ng/g)	% RSD
Influent (1 L)	18.5	18.5		31
Effluent (1 L)	4	4		24
Mixed sludge (20 ml)	5	250	6	52
Digested sludge (20 ml)	2	100	7	18

Table D 18: Targeted hormones in wastewater samples collected at influent, after primary sedimentation (PS), tricking filter (TF), solids contact (SC), effluent and chlorinated effluent from a municipal wastewater treatment plant utilizing tricking filter/solids contact technology (July 2012)

July 12 2012 (n = 3)	Influent	PS	TF	SC	Eff	Cl Eff
Estrone	1.7	1.2	18.2	7.6	12.0	10.7
17β-estradiol	5.4	4.6	12.1	6.3	4.6	4.4
17α-ethinyl estradiol	1.5	1.8	3.6	2.0	ND	ND
Estriol	ND	ND	ND	ND	ND	ND
Testosterone	ND	7.6	ND	ND	ND	ND

Table D 19: Estrone (E1), 17β-estradiol (E2), 17α-ethinyl estradiol (EE2), estriol (E3) and testosterone (TT) in mixed (MS) and digested sludges (DS) from a municipal wastewater treatment plant utilizing mesophilic (35 °C) anaerobic digestion

November 2012	MS (ng/g solids)	DS (ng/g solids)
E1	20	100
E2	13	13
EE2	6.0	9.0
E3	ND	ND
TT	ND	6.2
TT was detected in only one sludge sample		
August 2012	MS (ng/g solids)	DS (ng/g solids)
E1	100	367
E2	83	33
EE2	ND	ND
E3	ND	ND
TT	154	ND
TT was detected in only one sludge sample		
April 2012	MS (ng/g solids)	DS (ng/g solids)
E1	27	180
E2	17	20
EE2	ND	ND
E3	57	30
TT	ND	71

September 2012	MS (ng/g solids)	MS % RSD	DS (ng/g solids)	DS %RSD
E1	118	12	213	1
E2	23	7	23	2
EE2	8	9	ND	N/A
E3	ND	N/A	ND	N/A
TT	280	*N/A	343	27
*TT was detected in only one sludge sample				
July 2012	MS (ng/g solids)	MS % RSD	DS (ng/g solids)	DS %RSD
E1	107	13	87	7
E2	21	4	13	8
EE2	ND	N/A	ND	N/A
E3	ND	N/A	ND	N/A
TT	311	3	393	26

Table D 20: Estrone (E1), 17β-estradiol (E2), 17α-ethinyl estradiol (EE2), estriol (E3) and testosterone (TT) in a municipal wastewater treatment plant (April 2012)

Hormones ng/L (n – 3)	Influent	Primary sedimentation	Trickling filter	Solids contact	Effluent	Chlorinated effluent
E1	3.7	2.8	9.6	7.2	3.4	2.2
E2	4.3	2.6	1.3	1.7	0.7	ND
EE2	2.4	2.1	8.3	4.6	1.5	ND
E3	14.5	12.3	ND	ND	ND	ND
TT	ND	ND	ND	2.8	4.4	ND
Notes:						
TT was only detected in one of three samples at both sampling locations 4 and 5						
Since only 10% of E3 was detected with chromatography elutions, results were expressed as 10X peak abundance						

Table D 21: Estrone (E1), 17β-estradiol (E2), 17α-ethinyl estradiol (EE2), estriol (E3) and testosterone (TT) in wastewater samples from a municipal wastewater treatment plant collected at influent, after primary sedimentation (PS), tricking filter (TF), solids contact (SC), effluent (eff) and chlorinated effluent (Cl eff); and mixed (MS) and mesophilic anaerobic digested sludges (DS) (May 2012)

Hormones ng/L (n = 3)	Inf	PS	TF	SC	Eff	Cl eff	MS (ng/g solids)	DS (ng/g solids)
E1	13.1	8.6	28.3	19.9	ND	1.3	14.8	30.5
E2	10.0	5.3	2.1	2.5	ND	ND	3.2	2.9
EE2	6.1	ND	4.7	1.0	ND	2.0	9.9	8.9
E3	38.4	25.2	ND	ND	ND	ND	ND	ND
TT	ND	27.8	38.3	16.4	ND	ND	40.8	15.7

Table D 22: Estrone (E1), 17β-estradiol (E2), 17α-ethinyl estradiol (EE2), estriol (E3) and testosterone (TT) in wastewater samples from a municipal wastewater treatment plant collected at influent, after primary sedimentation (PS), tricking filter (TF), waste activated sludge (WAS) solids contact (SC), effluent (eff) and chlorinated effluent (Cl eff) (September 2012)

Hormones (n = 3)	Inf (ng/L)	PS(ng/L)	TF (ng/L)	TF WAS (ng/L)	SC (ng/L)	Eff (ng/L)	Cl Eff (ng/L)
E1	9.6	9.9	4.9	17.7	18.1	5.3	10.4
E2	16	13	5	25	24	6	9
EE2	ND	ND	ND	ND	ND	ND	ND
E3	105	98	11	ND	ND	ND	4
TT	ND	ND	ND	ND	ND	ND	ND
Hormones (n = 3)	Inf %RSD	PS %RSD	TF %RSD	W3B % RSD	W4 %RSD	W5 %RSD	W6 %RSD
E1	6	6	4	9	14	19	10
E2	8	30	33	6	13	50	6
EE2	N/A	N/A	N/A	N/A	N/A	N/A	N/A
E3	17	10	21	N/A	N/A	11	N/A
TT	N/A	N/A	N/A	N/A	N/A	N/A	N/A

APPENDIX E: MATERIALS AND EQUIPMENT

E-1: CHEMICALS

α-17α-ethinyl estradiol: Sigma Aldrich

17β-estradiol: Sigma Aldrich

2-Propanol (used as disinfectant): Fisher Scientific, HPLC grade

Acetone, 4L: Fisher Scientific, HPLC grade

Adenine sulphate: Sigma Aldrich

Ammonium sulphate, $(NH_4)_2SO_4$: Sigma Aldrich

BSTFA (N,O-bis(trimethylsilyl) trifluoroacetamide) with 1% TMCS (trimethylchlorosilane): Thermo Scientific; derivatization grade

Calcium pantothenate: Sigma Aldrich

Chloroform, 4 L: Fisher Scientific, certified ACS

Copper sulphate, $CuSO_4 \cdot 5\ H_2O$: Sigma Aldrich

d-Biotin: Sigma Aldrich

Deuterated 17β-estradiol: Sigma Aldrich; β-estradiol-d_2 E4260-5 mg, (~$400)

Dichloromethane, 4 L: Sigma Aldrich, certified ACS (0.75% ethanol as preservative)

Estriol: Sigma Aldrich

Estrone: Sigma Aldrich

Ethanol 100%, 1 L: pure un-denatured grade, 200 proof, UBC Chemistry Store

Glucose 40%, 4 L: Fisher Scientific

Glycerin, 4 L: Fisher Scientific

Hexanes, 4L: Fisher Scientific, HPLC grade

Hydrochloric acid, HCl, 0.1 N: Fisher Scientific

Hydrochloric acid, HCl, 6 N: Fisher Scientific

Hydrogen peroxide 30%, 4 L: Fisher Scientific H325-4, certified ACS

Iron(II) Sulfate Heptahydrate, $FeSO_4 \cdot 7\ H_2O$: Sigma Aldrich

L-arginine-HCl: Sigma Aldrich

L-aspartic acid: Sigma Aldrich

L-glutamic acid: Sigma Aldrich

L-histidine: Sigma Aldrich

L-isoleucine: Sigma Aldrich

L-lycine-HCl: Sigma Aldrich

L-methionine: Sigma Aldrich

L-phenylalanine: Sigma Aldrich

L-serine: Sigma Aldrich

L-threonine: Sigma Aldrich

L-tyrosine: Sigma Aldrich

L-valine: Sigma Aldrich

Magnesium sulphate, $MgSO_4 \cdot 7\ H_2O$: Sigma Aldrich

Methoxyamine-HCl: Fisher Scientific; 98%, derivatization grade

Myo-inositol: Sigma Aldrich

pH buffers (pH meter calibration); 4.0, 7.0, 10.0: Fisher Scientific

Potassium hydroxide, KOH: Sigma Aldrich

Potassium phosphate monobasic, KH_2PO_4: Sigma Aldrich

Pyridine, anhydrous: Sigma Aldrich; extra dry, 99.8%

Sodium hydroxide, NaOH: Fisher Scientific

Testosterone: Sigma Aldrich

Thiamine-HCl: Sigma Aldrich

Toluene, 4L: Fisher Scientific, HPLC grade

E-2: MATERIALS

Cryogenic vials, 2 ml, sterile, Fisherbrand

Chromatography column, Agilent J&W DB-5ms, phenyl arylene polymer capillary column, 30 m length, 0.25 µm thickness, 0.25 inside diameter, temperature limits -60 to 325/350 °C

Extraction thimbles (Soxhlet), cellulose, single thickness, 43 mm X 123 mm, Whatman

Florisil adsorbent 100-200 mesh, Fisher Scientific

Florisil adsorbent 60-100 mesh, Fisher Scientific

Innoculation loops, Simport Ino-loop, sterile

Microplates, 300 µl 96 well assay plates, Costar

Nitrile gloves, purple: Kimberly Clark

Silica gel, 230-400 mesh, Fisher Scientific

Thermal adhesive sealing film, Fisherbrand

Transfer pipettes, Fisherbrand

E-3: EQUIPMENT

Autoclave (sterilizer): Market Forge SteriLMatic

Block heater: HACH, model: DRB 200

Centrifuge: Beckman CS-6

COD reactor: Bioscience Inc, Analytical products, model: COD-80150

Desiccator: Lab Com Co.

Forced air ovens: Isotemp oven; VWR Scientific 1350FM

Freeze Dryer: Ilshin Lab Co. Ltd., model: TFD5505

Incubator: New Brunswick Scientific; model: Innova 4230 refrigerated incubator shaker

Large magnetic stirrer: Corning

Magnetic stirrer: Fisher Scientific, Thermal magnetic stirrer, Fisher versamix

Microwave, 1000 watts: Ethos Touch Control, Advanced microwave labstation

Muffle furnaces: Lindberg GS; Thermolyne 30400 Furnace

Repeater pipette: Eppendorf, model: Repeater Plus

Rotary evaporator: Heidolph; Heidolph2 condenser unit; model: Laborota 4000

Tissue grinder/homogenizer: Brinkman, Brinkman Homogenizer; model: Polytron

Ultrasonic bath: Fisher Scientific,FS220H

Vortex: Fisher Scientific, model: Genie 2

Weigh balance: Mettler AC100

Wrist shaker: Burrell, model: E23 wristaction shaker

E-4: INSTRUMENTS

Gas chromatography unit: Hewlett Packard, model: HP6890

Mass spectrometer: Hewlett Packard, model: 5973 mass selective

pH meter: Beckman ϕ44 pH meter

APPENDIX F: YEAST GROWTH MEDIA

F-1: YEAST MINIMAL MEDIA (BASE MEDIUM)

55 g KH_2PO_4

8 g $(NH_4)_2SO_4$

17 g KOH pellets

0.8 g $MgSO_4$

Add above ingredients to 1 L distilled water in a 2 L Erlenmeyer flask and mix with a magnetic stirrer until dissolved. Add liquid ingredients as follows:

4 ml FeSO4 solution	(0.8 mg in 50 ml water)
20 ml L-histidine	(1 g in 100 ml water)
56 ml adenine sulfate	(0.4 g in 100 ml water)
8 ml L-arginine-HCl	(1 g in 100 ml water)
8 ml L-methionine	(1 g in 100 ml water)
12 ml L-tyrosine	(1 g in 100 ml water)
12 ml L-isoleucine	(1 g in 100 ml water)
8 ml L-lycine-HCl	(1 g in 100 ml water)
20 ml L-phenylalanine	(1 g in 100 ml water)
8 ml L-glutamic acid	(10 g in 100 ml water)
40 ml L-valine	(3 g in 100 ml water)
40 ml L-serine	(7.5 g in 100 ml water)

Combine above ingredients in a 4 L Erlenmeyer flasks and bring to 2 L with distilled water. Mix with magnetic stirrer. Divide in four 500 ml portions and autoclave for 20 minutes. Store at room temperature. Makes 2 L of 2X yeast minimal media, the base medium.

Vitamin solution

In 100 ml 0.05N HCl, add the following:

4 mg thiamine-HCl

4 mg Calcium pantothenate

4 mg Pyridoxine

25 mg Inositol

0.4 mg d-Biotin (2 ml @ 2 mg in 10 ml water)

Mix with magnetic stirrer until dissolved in solution. Sterilize by filtering through 0.2 µm filter into a sterile bottle. Store at 4 °C.

D-(+)-Glucose solution

Add 100 g glucose to 250 ml water and autoclave for 20 minutes. Store the 40% w/v glucose solution at room temperature.

L-Aspartic acid solution

Add 1 g L-aspartic acid to 250 ml 0.1N NaOH (0.56 g/100 ml) and autoclave for 20 minutes. Store at room temperature.

L-Threonine stock solution

Add 2.4 g L-threonine to 100 ml water and autoclave for 20 minutes. Store at room temperature.

Copper (II) sulphate stock solution

Add 0.75 g copper sulphate ($CuSO_4 \cdot 5H_2O$) to 100 ml water and filter sterilize. Store at room temperature.

Iron sulfate stock solution

Add 0.16 g iron(II) sulfate heptahydrate ($FeSO_4 \cdot 7\ H_2O$) to 100 ml water and acidify with 2 drops of H_2SO_4. Store solution in a dark area at room temperature.

F-2: FINAL GROWTH MEDIA YMM$_{(LEU-,\ URA-)}$

To 1 L of 2X base medium add the following stock solutions:

50 ml L-Aspartic acid

16 ml L-threonine

5 ml copper (II) sulphate

100 ml glucose

20 ml vitamin solution

Mix in a 2 L Erlenmeyer flask using a magnetic stirrer and add 50 ml 0.1N HCl to neutralize alkalinity of L-aspartic acid. Filter sterilize the final growth media into sterilized containers and store at 4 °C. Makes 1 L 2X YMM$_{(leu-,\ ura-)}$. If 1X YMM$_{(leu-,\ ura-)}$ is desired, dilute 1:1 with autoclaved distilled water at room temperature.