The Fate of Net Estrogenicity and Anti-Estrogenicity During Conventional and Advanced Biosolids Treatment Processes

by

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ABSTRACT

THE FATE OF NET ESTROGENICITY AND ANTI-ESTROGENICITY DURING CONVENTIONAL AND ADVANCED BIOSOLIDS TREATMENT PROCESSES

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Biosolids are the nutrient-rich organic residual materials resulting from the treatment of domestic sewage at a wastewater treatment facility, and are increasingly land-applied for agricultural and land-reclamation purposes as part of the wastewater management process. While the presence and fate of estrogenic endocrine-disruptors (eEDCs) in wastewater has been extensively studied, much less focus has been given to examining the presence and fate of eEDCs during biosolids treatment. In particular, little work has been done to measure the net estrogenic potency of biosolids using in vitro bioassays, such as the Yeast Estrogen Screen (YES) assay. This is despite the fact that widespread land-application of biosolids provides for the direct introduction of eEDCs into terrestrial and aquatic environments. The relative scarcity of bioassay-based net estrogenicity data for sludges and biosolids is in large part due to the analytical challenges involved in working with such a complex sample matrix.

Comprehensive sampling at wastewater treatment plants in Guelph and London, ON, demonstrated that the estrogenicity of anaerobically-treated biosolids is considerably lower (12.0-19.7 ng/g estradiol-equivalents) than that reported in earlier published studies. The results of the present study were made possible due to the development of a
sample preparation methodology that overcame the toxic effects that sludge and biosolid samples typically exert on yeast cells in the YES assay. An anti-estrogenicity assay was also applied for the first time to sludges/biosolids to measure the extent to which antagonistic compounds ‘block’ the response of the YES assay. The results of these tests suggest that although the net estrogenicity of anaerobically treated solids is indeed low, up to twice the amount of estrogenicity measured by the YES assay may be masked in biosolids by the presence of antagonistic compounds.

While aerobic treatment conditions reduced net estrogenicity to at-or-below detectable levels, net estrogenicity remained relatively constant throughout the unit processes of the anaerobic treatment train. Biosolid ageing during storage led to an overall decrease in net estrogenicity of both conventionally-treated “restricted use” and advanced-treated “unrestricted use” anaerobic biosolids. However, levels of net estrogenicity were observed to spike during the early stages of storage, particularly under freeze/thaw conditions.
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1. INTRODUCTION

1.1. STATEMENT OF THE PROBLEM AND SIGNIFICANCE

The Canadian Council of Ministers of the Environment (CCME) defines biosolids to be the “organic product obtained from the physico-chemical and/or biological treatment of wastewater… biosolids can be derived from the treatment of either municipal wastewater or industrial wastewater” (CCME, 2005; pg. 3). In comparison, the United States Environmental Protection Agency (US EPA) defines biosolids as “… the nutrient-rich organic materials resulting from the treatment of sewage sludge,” where ‘sewage sludge’ refers to the solids present in domestic wastewater (2010). While the differences between the two are subtle, the American definition captures two important practical realities: biosolids are increasingly valued as a form of nutrient-rich soil amendment, and such beneficially-reused organic matter originates almost exclusively from municipal wastewater as opposed to on-site industrial treatment systems. Given that solids management represents approximately 50% of the total operating cost of municipal wastewater treatment (CCME, 2011), the production of useable, value-added biosolids as an end-product can make good economic sense. From an environmental perspective land-application of biosolids, whether for agricultural, silvicultural or land-reclamation purposes, replenishes nitrogen, phosphorus, and trace elements, increases the organic content of soil and improves soil structure, and reduces the need for landfill or incinerator capacity (US EPA, 1999).

Nonetheless, beneficial-use applications of biosolids remain a contentious issue (Reilly, 2001; Beecher et al., 2005; Goodman and Goodman, 2006). Historically, public aversion to land-application centred on concerns regarding the potential detrimental
effects of pathogens and heavy metals in biosolids, as well as odour issues (Reilly, 2001). Enhanced biosolids stabilization processes, pre-treatment regulations for industrial wastewater dischargers and direct-injection/direct-tillage (as opposed to spraying or spreading directly over land) can all aid in mitigating pathogen, metal and odour concerns, respectively (O’Connor et al., 2005). However, since roughly the early-1990’s, academic and public scrutiny has arisen over another potentially detrimental side-effect of biosolids reuse – the transfer of organic micropollutants (OMs) originally present in wastewater to terrestrial and aquatic environments via land-applied biosolids. The term organic micropollutants (which may also be referred to by other congener terms, such as ‘microconstituents’, ‘trace organic compounds (TOC)’, and ‘emerging contaminants’) encompasses a chemically-diverse array of compounds (Wells et al., 2008). The list includes, but is not limited to, natural and synthetic hormones, human and veterinary pharmaceuticals and their metabolites, fire retardants, surfactants, plasticizers, plant-derived phytoestrogens and anti-microbial compounds (Clarke and Smith, 2011).

Synthetic organic compounds are ubiquitous in daily life - 143,000 organic chemicals are registered in the European Union for industrial use (Clarke and Smith, 2011) – and hormones are produced by all multicellular organisms. Therefore, it is inevitable that OMs will be present to some extent in the domestic, institutional, commercial and industrial waste streams that end up in municipal wastewater treatment plants (WWTPs). During the wastewater treatment process, some OMs may be effectively removed via mineralization or chemical/microbial degradation; others, as shown in Figure 1, will be discharged from the WWTP in the effluent stream either in soluble form or adsorbed to colloidal or particulate matter, while still others will end up
adsorbed to sludge that is subsequently treated or disposed. While removal rates vary depending on WWTP configuration, process conditions and the OM under consideration, the presence of a wide variety of OMs in sludges and biosolids in multi-site studies is well-established (Kinney et al., 2006; US EPA, 2009; Water Environment Research Foundation, 2010).

Figure 1: Removal mechanisms for organic micropollutants in an activated sludge WWTP (after Meakins et al., 1994); activated sludge aeration basin at left, secondary clarifier at right

Given that OMs are so chemically diverse, their proposed deleterious effects on the environment following land application of biosolids are equally varied. In the case of antimicrobial residues, there is concern that their introduction to the soil via biosolids may reduce the soil’s microbial diversity (Sullivan et al. 2006), although recent research appears to dispute this (Ying et al., 2007; Zerzghi et al., 2010). Concerns over the emergence of antibiotic-resistant bacteria resulting from persistent exposure to pharmaceutical residues in biosolids are met with mixed results in the literature. Studies
have found no increase in bacterial resistance to antibiotics in biosolid-amended soil (Brooks et al., 2007), increases in some soils but not in others after biosolids application (Munir and Xagorakaki, 2011), and consistent bacterial resistance to the antibiotic oxytetracycline, albeit only when biosolids were applied above the standard agronomic rate (Stromberger, 2006). However, of all the various types OMs that have elicited scientific, regulatory and public interest, in wastewater and by extension in sludges and biosolids, it is endocrine disrupting compounds (EDCs) that have taken centre stage. Indeed, from the emergence of the first publications on EDCs in the environment in the early 1990’s, annual peer-reviewed publications grew exponentially through the beginning of the 21st century (Matthiessen, 2003).

The term ‘endocrine disruptor’ encompasses a broad range of compounds that share a common ability to interfere with the normal chemical-messaging function of the endocrine system found in all animals. The endocrine system consists of glands (such as the adrenal gland, pineal glands, thyroid and ovarian follicles, amongst others) that excrete chemical substances called hormones, which travel through the bloodstream and ultimately bind to specific cellular receptors. The binding of a hormone molecule to its corresponding receptor can either promote or inhibit a specific response within the target cell (i.e. the binding of parathyroid hormone to the parathyroid hormone receptor can both promote the release of calcium into the bloodstream, and reduce the concentration of phosphate in the bloodstream).

In 1999 the Canadian Environmental Protection Act (CEPA) defined a hormone disrupting chemical as “a substance having the ability to disrupt the synthesis, secretion, transport, binding, action or elimination of hormones in an organism, or its progeny, that
is responsible for the maintenance of homeostasis, reproduction, development, and behaviour of an organism” (Department of Justice Canada, 1999). As the CEPA definition of “EDC” implicitly states, the endocrine system is key to regulating the homeostasis, reproduction, development, and behaviour of an organism. Disruption of normal endocrine function can be effected by the binding of structurally similar molecules to hormone receptors, which may lead to (i) agonism (the binding molecule promotes a response similar to what would be elicited by the actual hormone) or (ii) antagonism (the binding molecule suppresses the response that would be elicited by the binding of the proper hormone molecule). Finally, EDCs can interfere with the way natural hormones and receptors are synthesized, metabolized, or regulated (Filali-Meknassi et al., 2004). It has been known since the 1920’s and 1930’s that exposure to natural and synthetic substances could induce enhanced or abnormal endocrine responses in mammals (Matthiessen, 2003). Complicating the issue as to what precisely constitutes an EDC is the fact that complex mixtures and effluents where the active chemical(s) are not precisely known have also been observed to elicit hormone-like responses (Hewitt and Servos, 2001).

The ability to detect and quantify a wide range of EDCs in environmental matrices through chemical analyses is continuously being refined, with nanogram-per-litre detection limits now being commonplace, and picogram-per-litre detection limits emerging (Richardson, 2008). In comparison to chemical analyses, in vitro bioassays do not provide insight into the concentrations of discrete compounds present in a sample. Rather, in vitro bioassays provide a measurement of the net endocrine-disrupting potential of all endocrine-active substances that are present in a sample (Leusch et al.,
The concept of ‘net’ activity with regards to the measurement of estrogenic activity in environmental samples is particularly important. Environmental samples often contain complex mixtures of compounds, some of which may act synergistically (i.e. provoke a response greater in magnitude when mixed than what would be predicted based on their individual concentrations), others of which may act antagonistically (i.e. reduce the magnitude of the expected response). It is for these reasons that the correlation of calculated estrogenicity (based on chemical analysis of target EDCs) and measured estrogenicity (based on bioassays) is often poor, since the calculated estrogenic activities include the contribution of only a limited number of target compounds and do not account for interaction between compounds or the presence of anti-estrogenic substances (Petrovic et al., 2004). Thus, the response of an in vitro bioassay to an environmental sample provides a more meaningful picture of the potential organism-level effects than chemical analyses alone (Global Water Research Coalition, 2006).

Four classes of in vitro bioassays are commonly used for evaluating the estrogenic activity of environmental samples (Petrovic et al., 2004; Global Water Research Coalition, 2006).

- **Receptor-binding assays**, which measure the ability of chemicals in environmental sample extracts to compete with E2 in binding to estrogen receptor sites.
- **Cell-proliferation assays**, in which cell lines that require estrogen for growth (often human breast cancer cells) are incubated in the presence of environmental sample extracts. Cell proliferation is used as a measurable endpoint.
- **Gene expression assays**, in which cells (such as fish primary hepatocytes) that express protein(s) under estrogenic stimulation are incubated in the presence of
environmental sample extracts. Protein production (in the case of fish hepatocytes, vitellogenin) is the measured endpoint.

- Reporter gene assays, in which the binding of estrogenic compounds to the estrogen receptor within a cell activates a cascade of molecular events that ultimately leads to a particular gene being expressed. In the case of the commonly-used Yeast Estrogen Screen (YES), the cells are yeast (*Saccharomyces cerevisiae*) which have been genetically modified to include the human estrogen receptor (hER). The binding of estrogenic compounds to the hER causes the activation of a gene that produces the enzyme β-galactosidase, which then causes a colour change in the yeast incubation medium.

The latter assay (YES) is not without its shortcomings – most notably that yeast cells have a cell wall which can impede the active and passive transport of test substances to the intracellular space (Global Water Research Coalition, 2006). However, the YES assay has become the most widely-used tool for the evaluation of net estrogenicity in environmental samples, as it can be easily used for high-throughput screening and provides reproducible results (Leusch et al., 2010). Accordingly the YES assay has been widely applied in analyses of net estrogenicity in wastewater streams and wastewater-impacted surface waters worldwide (Murk et al., 2002; Beck et al., 2006; Nelson et al., 2007; Li et al., 2010; Mnif et al., 2010). Recently, a modification of the YES assay was developed (Buckley, 2010) which allows for the parallel determination of the anti-estrogenic potency of samples. Using the two versions of the YES assay together, both the net estrogenic content and the extent to which anti-estrogens “mask” the presence of additional estrogenic potency can be determined.
Studies focussing on the presence of natural and synthetic hormones in WWTPs first appeared upwards of forty years ago (Stumm-Zollinger and Fair, 1965; Tabak and Bunch, 1970; Tabak et al., 1981). This early research was instrumental in providing a justification for further investigation into the occurrence and fate of EDCs in wastewater streams; however, limitations in the abilities and accuracy of early trace analytical methods meant that the results of these older studies now appear unreliable (e.g. the study of Tabak et al. (1981) reported wastewater concentrations of 17α-ethynylestradiol one- to two-orders of magnitude higher than contemporary studies). Advances in sample preparation methods and analytical instrumentation have allowed for the reliable quantification of EDCs in complex wastewater matrices. Appendix 1 presents the measured influent and, when available, corresponding effluent concentrations of estrone (E1), 17β-estradiol (E2), estriol (E3), and 17α-ethynylestradiol (EE2) from a number of studies conducted at various WWTPs. The first three of these compounds are naturally-excreted human hormones, while EE2 is the synthetic estrogen analogue used in birth control and hormone replacement therapy formulations. Multiple WWTPs investigated within a study are presented separately in this table, along with descriptions of the wastewater treatment process, where such information was provided. An emphasis has been placed on Canadian studies, as the results of studies confirm that operational parameters, including temperature, affect the extent of EDC removal during the wastewater treatment process (Holbrook et al., 2002; Braga et al., 2005; Lishman et al., 2006). Of note is that the first two entries in Appendix 1 provide early EDC data for the City of Guelph.
Broadly speaking, the pattern that emerges is that the estrogenic steroid hormones show high degrees of removal in wastewater treatment plants that employ secondary or tertiary treatment, and considerably less removal when only primary treatment is used. Within this generalisation, the work of Lee and Peart (1998) showed that the concentrations of E1, E2 and E3 in Montreal primary effluent were essentially unchanged from the influent values. Similarly, Braga et al. (2005) found that while up to 85% of E1 and 96% of E2 were removed in a process involving secondary biological treatment and tertiary microfiltration/reverse osmosis, a primary-treatment WWTP removed only 14% and 5% of E1 and E2, respectively. Johnson et al. (2005), in a survey of E1, E2, EE2 and nonylphenol removal at 17 WWTPs across Europe also concluded that secondary treatment was consistently more effective than primary treatment, with a weakly significant correlation between E1 removal and solids retention time (SRT) and hydraulic retention time (HRT) being noted. EDC removal at WWTPs has also been evaluated in terms of net estrogenic potency using in vitro bioassays.

In comparison, very little work has been conducted to determine the extent to which anti-estrogens may mask the presence of estrogenic compounds. Net estrogenic potency may provide a more relevant picture of the overall reduction of EDCs during wastewater treatment than individual identification of specific compounds. Table 1 shows the reduction in estrogenic effect measured at a number of full- and pilot-scale WWTPs. Amongst these studies, the work of Leusch et al. (2006) used GC-MS analysis in tandem with two separate bioassays to determine the percentage of total estrogenic effect in WWTP influent that was attributable to steroid estrogens. Based on 12 samples, E1 and E2 were found to account for 62% of estrogenic activity, while industrially-derived
compounds (nonylphenol, octylphenol and bisphenol-A) accounted for an average of 1.6% of the estrogenic load. Considering that the wastewater in this study was largely domestically-sourced, the residual estrogenic activity was ascribed to natural (E3) and synthetic (EE2) steroid estrogens. These data corroborate the earlier work of Desbrow et al. (1998) who determined that over 80% of the estrogenicity in WWTP effluent was due to residual E1, E2 and EE2.

Table 1: Estrogenic potency reductions during conventional wastewater treatment

<table>
<thead>
<tr>
<th>Year</th>
<th>Study</th>
<th>Influent ng/L E2-Eq</th>
<th>Effluent ng/L E2-Eq</th>
<th>Bioassay(s) Used</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>2000</td>
<td>W. Körner et al.</td>
<td>58-70</td>
<td>6</td>
<td>ER screen using MCF-7 breast cancer cells</td>
<td>WWTP used conventional activated sludge (CAS)/biological nutrient removal (BNR) process</td>
</tr>
<tr>
<td>2002</td>
<td>A.J. Murk et al.</td>
<td>1-120</td>
<td>&lt;1-16</td>
<td>1)YES (2) ER-mediated chemically activated luciferase gene expression (ER-CALUX), (3) ER competitive ligand binding assay</td>
<td></td>
</tr>
<tr>
<td>2002</td>
<td>L.A. Kirk et al.</td>
<td>20-80</td>
<td>&lt;3-13</td>
<td>YES</td>
<td></td>
</tr>
<tr>
<td>2002</td>
<td>K. Onda et al.</td>
<td>35-72 (n=12 samples)</td>
<td>4-35 (n=12 samples)</td>
<td>YES</td>
<td>Earlier portion of study used enzyme immunoassay (EIA) to measure E2 concentrations but suggested that this test yielded false positives in effluents</td>
</tr>
<tr>
<td>2003</td>
<td>A. Svenson et al.</td>
<td>1-30 (n=204 samples)</td>
<td>0.1-15 (n=204 samples)</td>
<td>YES</td>
<td></td>
</tr>
<tr>
<td>2006</td>
<td>F.D.L. Leusch et al.</td>
<td>&lt;4-185 (n=15 samples)</td>
<td>&lt;1-4.2 (n=15 samples)</td>
<td>Sheep estrogen receptor/trout androgen receptor binding assays</td>
<td></td>
</tr>
</tbody>
</table>

Limited data exists on the distribution of EDCs amongst suspended solids/colloidal material during the wastewater treatment process at the full-scale. Cicek et al. (2007) determined that the percentage of E1, E2 and EE2 associated with wastewater
solids >0.7µm in diameter was 33%, 25% and 52%, respectively, at the WWTP in Brandon, Manitoba. Stasinakis et al. (2008) analyzed the partitioning of five weak endocrine disruptors – 4-n-nonylphenol, nonylphenol monoethoxylate, nonylphenol diethoxylate, triclosan and bisphenol-A – at six wastewater treatment plants in Greece employing either secondary or tertiary treatment (chlorination). Between 45 and 50% of compounds tested were sorbed to suspended solids (particle size >1.5µm) in the influent, while in treated wastewater this fraction ranged from 18-35%, though these percentages were highly variable. Heidler and Halden (2007) performed a similar analysis of the fate of triclosan (an antimicrobial compound used in soaps and detergents) during tertiary wastewater treatment. Their results indicated that 80% ± 22% of triclosan in the influent was associated with particulate matter. Both studies found that while the apparent removal efficacy of triclosan was high when comparing influent and effluent concentrations (98% ± 1% for Heidler and Halden, 2007; 91% ± 6% for Stasinakis et al., 2008), a large portion of the compound was not actually transformed or lost, but rather, remained in active form in the activated sludge (50% ± 19% for Heidler and Halden, 2007; 45% ± 27% for Stasinakis et al., 2008).

At the bench scale, Schäfer et al. (2002) demonstrated that adsorption of estrone at concentrations ranging from 5-500ng/L to activated sludge particles was linear at pH’s ranging from 3-12 and at suspended solids concentrations of 2-8g/L. As shown in Table 2, the natural and synthetic steroid estrogens are all moderately hydrophobic and remain un-ionized at the pH ranges that are typical of municipal wastewater. As such, they would be expected to sorb to the hydrophobic organic carbon flocs and colloids created by microbes during aerobic wastewater treatment processes. This tendency was confirmed.
by Holbrook et al. (2003), who found that wastewater treatment processes that operated at longer solids retention times (aerobic digestion > membrane bioreactor > conventional activated sludge process) produced colloidal matter with greater affinity for E2 and EE2. Thus, three interlinked questions arise for environmental engineers and scientists, which are: (i) to what extent do current municipal wastewater treatment practices truly remove estrogenic EDCs versus transfer them to the solid phase, (ii) to what extent do anti-estrogenic compounds in wastewater/sludge mask the full load of eEDCs that are present, and (iii) if estrogenic EDCs are being partitioned to sludge, to what extent are they then removed during solids digestion/treatment processes? An in-depth discussion of these issues is offered in Chapter 2 of this thesis, which also highlights the reasons why net-estrogenicity measurements of sludges and biosolids using in vitro bioassays are exceedingly rare compared to wastewater data.

Table 2: Log $K_{ow}$ and pKa of E1, E2, E3 and EE2 (Westerhoff et al., 2005)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Log$K_{ow}$</th>
<th>pKa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrone (E1)</td>
<td>3.13</td>
<td>10.3</td>
</tr>
<tr>
<td>17β-estradiol (E2)</td>
<td>4.01</td>
<td>10.4</td>
</tr>
<tr>
<td>Estriol (E3)</td>
<td>2.45</td>
<td>&gt;10.4 &amp; &gt;15*</td>
</tr>
<tr>
<td>17α-ethynylestradiol (EE2)</td>
<td>3.67</td>
<td>~10.5</td>
</tr>
</tbody>
</table>

*For deprotonation of first and second hydroxyl hydrogen, respectively

The presence of estrogenic EDCs in wastewater is an inescapable reality. Even if industrial estrogenic inputs were reduced via product reformulation, usage restrictions or bans, or other source-control measures, the most potent estrogenic EDCs on a unit basis remain those that are excreted by the human body, either naturally or via the therapeutic use of hormone mimics. For the specific case of Guelph, Ontario, an estimate of the magnitude of estrogenic loading that might be expected in the Guelph WWTP influent is
provided in the following set of calculations. Looking only at inputs from the three most estrogenic substances, EE2, E2 and E1, the following demographic data were used:

- Guelph population data for 2006, as shown in Table 3 (Statistics Canada). Note that this data includes only permanent residents of the city, and does not account for the seasonal student population at the University of Guelph
- The Ontario birth rate in 2006
- The average age of Canadian females at menarche (12 years; Moisan et al., 1990)*
- The average age of Canadian females at menopause (51.3 years; McKinlay et al., 1992)*
- The percentage of menstruating Canadian females using oral birth control (~18%; Wilkins et al., 2000)*
- The percentage of Canadian post-menopausal females using hormone replacement therapy (~15%; Csizmadi et al., 2002)*
- The range of per capita E1/E2/EE2 excretion rates summarised by Johnson and Williams (2004)
- The E2-equivalency factor for E1 and EE2 provided by Johnson and Sumpter (2001)
- Average daily Guelph WWTP influent flow for 2006 (55,900m$^3$/day)

*Female demographic data are summarized in Table 4.

The results of this analysis, shown in Table 5, provide an estimated influent estrogenic load of 21.8-35.3ng/L E2-equivalents (E2-Eq), with a median concentration of 28.5ng/L E2-Eq. The numbers derived from these calculations include only the
estrogenic input of three compounds, yet do fit well within the lower range of estrogenic potency observed in wastewater influent elsewhere (see Table 1) and would presumably provide a similar baseline across Canada. Questions concerning the fate of eEDCs during sludge/biosolids treatment processes - and particularly the magnitude of estrogenic activity in biosolids destined for land application - remain a pressing concern for engineers, scientists and policy makers in Ontario and beyond (US EPA, 2009; Water Environment Association of Ontario, 2010; Water Environment Research Foundation, 2010; Langdon et al., 2011).

Table 3: Guelph, Ontario demographic data for 2006

<table>
<thead>
<tr>
<th>POPULATION</th>
<th>YEAR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guelph</td>
<td>114,943</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Age Range</th>
<th># Men</th>
<th>Age Range</th>
<th># Women</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-4</td>
<td>3,485</td>
<td>0-4</td>
<td>3,390</td>
</tr>
<tr>
<td>5-9</td>
<td>3,525</td>
<td>5-9</td>
<td>3,435</td>
</tr>
<tr>
<td>10-14</td>
<td>3,820</td>
<td>10-14</td>
<td>3,515</td>
</tr>
<tr>
<td>15-19</td>
<td>3,835</td>
<td>15-19</td>
<td>3,730</td>
</tr>
<tr>
<td>20-24</td>
<td>4,460</td>
<td>20-24</td>
<td>4,730</td>
</tr>
<tr>
<td>25-29</td>
<td>4,305</td>
<td>25-29</td>
<td>4,255</td>
</tr>
<tr>
<td>30-34</td>
<td>4,115</td>
<td>30-34</td>
<td>4,350</td>
</tr>
<tr>
<td>40-44</td>
<td>4,775</td>
<td>40-44</td>
<td>4,775</td>
</tr>
<tr>
<td>45-49</td>
<td>4,350</td>
<td>45-49</td>
<td>4,605</td>
</tr>
<tr>
<td>50-54</td>
<td>3,665</td>
<td>50-54</td>
<td>3,895</td>
</tr>
<tr>
<td>55-59</td>
<td>3,105</td>
<td>55-59</td>
<td>3,330</td>
</tr>
<tr>
<td>60-64</td>
<td>2,185</td>
<td>60-64</td>
<td>2,430</td>
</tr>
<tr>
<td>65-69</td>
<td>1,635</td>
<td>65-69</td>
<td>1,940</td>
</tr>
<tr>
<td>70-74</td>
<td>1,470</td>
<td>70-74</td>
<td>1,855</td>
</tr>
<tr>
<td>75-79</td>
<td>1,340</td>
<td>75-79</td>
<td>1,805</td>
</tr>
<tr>
<td>80-84</td>
<td>925</td>
<td>80-84</td>
<td>1,470</td>
</tr>
<tr>
<td>Over 85</td>
<td>615</td>
<td>Over 85</td>
<td>1,210</td>
</tr>
<tr>
<td>TOTAL</td>
<td>55,940</td>
<td>TOTAL</td>
<td>59,015</td>
</tr>
<tr>
<td>PERCENT</td>
<td>48.7</td>
<td>PERCENT</td>
<td>51.3</td>
</tr>
</tbody>
</table>
Table 4: Data on female population composition of Guelph, Ontario in 2006

<table>
<thead>
<tr>
<th>Ovulating Women</th>
<th>Menopausal Women</th>
<th>Guelph Births</th>
<th>Oral Birth Control Use</th>
<th>H.R.T. Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>12-14</td>
<td>2109</td>
<td>51.3-54</td>
<td>2,882</td>
<td>15-19</td>
</tr>
<tr>
<td>15-19</td>
<td>3,730</td>
<td>55.5-59</td>
<td>3,330</td>
<td>20-24</td>
</tr>
<tr>
<td>20-24</td>
<td>4,730</td>
<td>60-64</td>
<td>2,430</td>
<td>25-29</td>
</tr>
<tr>
<td>25-29</td>
<td>4,255</td>
<td>65-69</td>
<td>1,940</td>
<td>30-34</td>
</tr>
<tr>
<td>30-34</td>
<td>4,350</td>
<td>70-74</td>
<td>1,855</td>
<td>35-39</td>
</tr>
<tr>
<td>35-39</td>
<td>4,295</td>
<td>75-79</td>
<td>1,805</td>
<td>40-44</td>
</tr>
<tr>
<td>40-44</td>
<td>4,775</td>
<td>80-84</td>
<td>1,470</td>
<td>45-49</td>
</tr>
<tr>
<td>45-49</td>
<td>4,605</td>
<td>Over 85</td>
<td>1,210</td>
<td>1,224</td>
</tr>
<tr>
<td>50-51.3</td>
<td>1,013</td>
<td>TOTAL</td>
<td>16,922</td>
<td>TOTAL</td>
</tr>
<tr>
<td>TOTAL</td>
<td>33,862</td>
<td>% All Women</td>
<td>29</td>
<td>% All Women 18</td>
</tr>
<tr>
<td>% All Women</td>
<td>57</td>
<td>% Guelph Pop. 15</td>
<td>% All Women 9</td>
<td>% Guelph Pop. 5</td>
</tr>
<tr>
<td>% Guelph Pop.</td>
<td>29</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Ontario Births, 2006: 134,514
Ontario Pop., 2006: 12,665,300
ON Birth Rate., 2006: 10.65/1000 persons
Ontario O.B.C.: 5,533
Ontario H.R.T.: 2,162
TOTAL ON O.B.C.: 14,040
Table 5: Calculated estrogenic loading (due to E1, E2 and EE2) in the Guelph WWTP using 2006 demographic/flow data

<table>
<thead>
<tr>
<th>Demographic Group</th>
<th># in Guelph</th>
<th>E1 (Mean)</th>
<th>E1 (Min)</th>
<th>E1 (Max)</th>
<th>E2 (Mean)</th>
<th>E2 (Min)</th>
<th>E2 (Max)</th>
<th>EE2 (Mean)</th>
<th>EE2 (Min)</th>
<th>EE2 (Max)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>55,940</td>
<td>2.6</td>
<td>1.4</td>
<td>2.9</td>
<td>1.8</td>
<td>1.3</td>
<td>2.4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Menstrual Females</td>
<td>33,862</td>
<td>11.7</td>
<td>7.5</td>
<td>15.4</td>
<td>3.2</td>
<td>1.7</td>
<td>4.6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Menopausal Females</td>
<td>14,760</td>
<td>1.8</td>
<td>0</td>
<td>5.7</td>
<td>1</td>
<td>0</td>
<td>3.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Menopausal Females on HRT</td>
<td>2,162</td>
<td>28.4</td>
<td>24</td>
<td>33</td>
<td>56</td>
<td>51.5</td>
<td>61.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pregnant Women</td>
<td>1,224</td>
<td>550</td>
<td>432</td>
<td>668</td>
<td>393</td>
<td>340</td>
<td>445</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Women on Oral Birth Control</td>
<td>5,533</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10.5</td>
<td>9.6</td>
<td>11.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Demographic Group</th>
<th># in Guelph</th>
<th>E1 (Mean)</th>
<th>E1 (Min)</th>
<th>E1 (Max)</th>
<th>E2 (Mean)</th>
<th>E2 (Min)</th>
<th>E2 (Max)</th>
<th>EE2 (Mean)</th>
<th>EE2 (Min)</th>
<th>EE2 (Max)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>55,940</td>
<td>145.4</td>
<td>78.3</td>
<td>162.2</td>
<td>100.7</td>
<td>72.7</td>
<td>134.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Menstrual Females</td>
<td>33,862</td>
<td>396.2</td>
<td>254.0</td>
<td>521.5</td>
<td>108.4</td>
<td>57.6</td>
<td>155.8</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Menopausal Females</td>
<td>14,760</td>
<td>26.6</td>
<td>0</td>
<td>84.1</td>
<td>14.8</td>
<td>0</td>
<td>51.7</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Menopausal Females on HRT</td>
<td>2,162</td>
<td>61.4</td>
<td>51.9</td>
<td>71.4</td>
<td>121.1</td>
<td>111.4</td>
<td>133.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pregnant Women</td>
<td>1,224</td>
<td>673.3</td>
<td>528.8</td>
<td>817.7</td>
<td>481.1</td>
<td>416.2</td>
<td>544.7</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Women on Oral Birth Control</td>
<td>5,533</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>58.1</td>
<td>53.1</td>
<td>62.5</td>
</tr>
</tbody>
</table>

**TOTAL** | **1302.9** | **913.0** | **1656.9** | **826.0** | **657.8** | **1019.4** | **58.1** | **53.1** | **62.5** |

**Estimated Excreted Output in µg/person/day (from Johnson and Williams, 2004)**

**UNITS/NOTES**
- **mg/day in Guelph**
- **ng/L in Influent**
- **E2 Equivalence Factor**
- **E2-EQ**

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Min</th>
<th>Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2-Eq (ng/L)</td>
<td>28.5</td>
<td>21.8</td>
<td>35.3</td>
</tr>
</tbody>
</table>

*Based on an average daily flow of 55,900 m³ (55,900,000 L/d)

*Based on equivalency factors from Johnson and Sumpter (2001)
1.2. **Scope and Objectives of the Work**

The overall scope of this study was to assess the fate and transport of net estrogenic and anti-estrogenic potency through sludge and biosolid unit treatment processes in full-scale wastewater treatment plants and during storage. The specific objectives of the research conducted over the course of this study were:

- To develop and validate a sample extraction and cleanup method that would allow the yeast estrogen screen (YES) assay to be consistently applied to sludge and biosolid samples,
- To determine changes in net estrogenicity during successive anaerobic and aerobic unit processes at full-scale WWTPs,
- To modify the YES assay to allow quantification of the anti-estrogenic (inhibitory) content of sludges and biosolids in parallel with net estrogenicity, and;
- To determine the effects of storage conditions on the net estrogenicity/anti-estrogenicity of ‘Class A’ and ‘Class B’ biosolids

1.3. **Research Significance**

This study is one of only three in which the fate of net estrogenicity has been examined in full-scale wastewater treatment plants throughout the unit processes that comprise anaerobic and aerobic sludge treatment. Unlike the previous two studies (Holbrook et al., 2002; WERF, 2010), the present research involved sample extraction and cleanup method development that overcame the toxic effects that commonly occur when YES assay yeast are incubated in the presence of sludge and biosolid samples.
Thus, it is the only one of the three studies in which YES assay results are not compromised by incomplete or unclear dose-response relationships. To the author’s best knowledge, no studies have currently been published in which the antiestrogenic potency of wastewater solids has been measured and no data presently exists on the fate of net estrogenicity/antiestrogenicity in biosolids during storage.
2. **LITERATURE REVIEW**

An abridged version of the following literature review was submitted to the journal *Environmental Science and Technology* on July 15, 2010; re-submitted with revisions on October 5, 2010; and accepted for publication October 6, 2010.


2.1. **INTRODUCTION**

The first study indicating that human hormones were present in wastewater effluent due to incomplete elimination during the wastewater treatment process emerged in the 1960’s (Stumm-Zollinger and Fair, 1965). However, the discovery of aquatic organisms with disrupted sexual development characteristics in the 1990s near the outfalls of municipal wastewater treatment plants (WWTPs) drew attention to the presence and potential risks of endocrine-disrupting compounds (EDCs) in wastewater streams from human excretion and industrial, pharmaceutical, veterinary and personal-care sources (Colborn et al., 1993; Purdom et al., 1994; Desbrow et al., 1998; Jobling et al., 1998). The work of Colin Purdom and colleagues (1994) in identifying elevated levels of vitellogenin, an egg-yolk protein precursor, in the blood of male fish exposed to WWTP effluent in the UK was seminal in bringing attention to the chronic effects of trace concentrations of EDCs. This was quickly followed by further research in the UK that identified the presence of intersex roach (fish with oocytes in the testes) downstream of numerous WWTPs (Jobling et al., 1998). Both
types of abnormalities involved feminising effects, and suggested that the fish had been exposed to estrogenic substances. In a controlled study by Kidd et al. (2007) in northern Ontario experimental lakes, fathead minnows were exposed to a concentration of ~6ng/L of 17α-ethinylestradiol (EE2), a synthetic birth-control hormone. The fish population collapsed in the second year of dosing because of a loss in population of young fish (Kidd et al., 2007), brought on by the chronic exposure to trace levels of an estrogen mimic. Thus, the term ‘EDC’ has often become synonymous for those chemicals that have the potential to bind to the estrogen receptor and either mimic or block the effects of the endogenous steroid hormone, 17β-estradiol (E2).

Brzozowski et al. (1997) elucidated the crystal structure of the ligand binding domain of the human estrogen receptor (ERα). The gap of the ligand binding domain is much larger than 17β-estradiol requires, and hence there is space for a variety of other molecules to interact with the receptor. While the liquid and solid streams in WWTPs are complex matrices that may contain a wide range of known and suspected estrogenic EDCs (Tan et al., 2007), it is E2 and its metabolites estrone (E1) and estriol (E3) and synthetic contraceptive analogue, 17α-ethynyl estradiol (EE2) that appear to account for the majority of estrogenic potency in these samples (Rutishauser et al., 2004; Johnson and Sumpter, 2001). Indeed, bioassay-based studies suggest that the potency of E1/E2/E3/EE2 in terms of estrogenic effect is significantly – often orders of magnitude – higher than other putative eEDCs. While the natural estrogenic steroid hormones are largely excreted from the human body as functionally inactive sulphate or glucuronide conjugates, as shown in Figure 2,
bacteria in wastewater readily re-convert the derivatized estrogens back to their original form (Andreolini et al., 1987; Guengerich, 1990; Baronti et al., 2000; Johnson et al., 2000). The estrogenic potencies of various compounds relative to E2, as determined in various *in vitro* tests, are shown in Table 6 to Table 10. These tables emphasize that while the exact estrogenic potencies of compounds vary according to the test employed, the overall distinction between the potency of naturally-occurring estrogenic steroid hormones (and their close synthetic analogue, EE2) and synthetic/industrial eEDCs is clear.

![Chemical structures of E1, E2, E3, EE2 and their sulphate and glucuronide conjugates](image)

**Figure 2:** Chemical structures of E1, E2, E3, EE2 and their sulphate and glucuronide conjugates
Table 6: Relative potencies of selected estrogens as determined by various *in vitro* biossays (Rutishauser et al., 2004)

<table>
<thead>
<tr>
<th>Compound</th>
<th><strong>BIOASSAY</strong></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yeast Estrogen Screen</td>
<td>Zona Radiata Proteins Production</td>
<td>Rainbow Trout Gonadal Cell Line RTG-2 Assay</td>
<td></td>
</tr>
<tr>
<td>17β-estradiol</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Estrone</td>
<td>0.38</td>
<td>Not determined</td>
<td>Not determined</td>
<td></td>
</tr>
<tr>
<td>Estriol</td>
<td>0.0024</td>
<td>Not determined</td>
<td>Not determined</td>
<td></td>
</tr>
<tr>
<td>17α-ethynylestradiol*</td>
<td>1.19</td>
<td>0.70</td>
<td>1.62</td>
<td></td>
</tr>
<tr>
<td>Bisphenol A*</td>
<td>1.1x10⁻⁴</td>
<td>3.6x10⁻⁵</td>
<td>2.5x10⁻³</td>
<td></td>
</tr>
<tr>
<td>4-nonylphenol*</td>
<td>2.5x10⁻⁵</td>
<td>7.5x10⁻⁵</td>
<td>1.1x10⁻³</td>
<td></td>
</tr>
<tr>
<td>4-tert-octylphenol*</td>
<td>7.8x10⁻⁶</td>
<td>3.3x10⁻⁵</td>
<td>1.3x10⁻³</td>
<td></td>
</tr>
</tbody>
</table>

*Synthetic compound; all others are naturally-occurring

Table 7: Relative potency of alkylphenol polyethoxylates (surfactants) and degradation products as determined using trout hepatocytes (Jobling and Sumpter, 1993)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Relative Potency</th>
</tr>
</thead>
<tbody>
<tr>
<td>17β-estradiol</td>
<td>1</td>
</tr>
<tr>
<td>NP9EO*</td>
<td>2x10⁻⁷</td>
</tr>
<tr>
<td>NP2EO*</td>
<td>6x10⁻⁶</td>
</tr>
<tr>
<td>NP1EO*</td>
<td>6.3x10⁻⁶</td>
</tr>
<tr>
<td>Nonylphenol*</td>
<td>9x10⁻⁶</td>
</tr>
<tr>
<td>Octylphenol*</td>
<td>3.7x10⁻⁵</td>
</tr>
<tr>
<td>p-tert-butylphenol*</td>
<td>1.6x10⁻⁴</td>
</tr>
</tbody>
</table>

*Synthetic compound; all others are naturally-occurring

Table 8: Potencies of EDCs as determined with the E-screen assay (Andersen et al., 1999)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Relative Potency</th>
</tr>
</thead>
<tbody>
<tr>
<td>17β-estadiol</td>
<td>1</td>
</tr>
<tr>
<td>17α-ethynylestradiol*</td>
<td>0.9</td>
</tr>
<tr>
<td>Diethylstilbestrol*</td>
<td>0.05</td>
</tr>
<tr>
<td>Testosterone</td>
<td>1x10⁻⁵</td>
</tr>
<tr>
<td>Bisphenol A*</td>
<td>1x10⁻³</td>
</tr>
<tr>
<td>Bisphenol A dimethacrylate*</td>
<td>2x10⁻⁵</td>
</tr>
<tr>
<td>4-n-octylphenol*</td>
<td>1x10⁻⁶</td>
</tr>
<tr>
<td>4-n-nonylphenol*</td>
<td>7x10⁻⁷</td>
</tr>
<tr>
<td>Benzylbutylphthalate*</td>
<td>1x10⁻⁶</td>
</tr>
<tr>
<td>Methoxychlor*</td>
<td>1x10⁻⁶</td>
</tr>
<tr>
<td>o,p-DDT*</td>
<td>1x10⁻³</td>
</tr>
<tr>
<td>p,p-DDE*</td>
<td>2x10⁻⁴</td>
</tr>
<tr>
<td>Chlormequat chloride*</td>
<td>4x10⁻⁶</td>
</tr>
</tbody>
</table>

*Synthetic compound; all others are naturally-occurring
Table 9: Relative potencies of estrogenic steroid hormones as determined with the Yeast Estrogen Screen (YES) assay (Johnson and Sumpter, 2001)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Relative Potency</th>
</tr>
</thead>
<tbody>
<tr>
<td>17β-estradiol</td>
<td>1</td>
</tr>
<tr>
<td>Estrone</td>
<td>0.5</td>
</tr>
<tr>
<td>Estriol</td>
<td>0.005-0.04</td>
</tr>
<tr>
<td>17α-ethynylestradiol*</td>
<td>1-2</td>
</tr>
</tbody>
</table>

*Synthetic compound; all others are naturally-occurring

Table 10: Relative potencies of estrogenic steroid hormones as determined with the Yeast Estrogen Screen (YES) assay (Svenson et al., 2003)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Relative Potency</th>
</tr>
</thead>
<tbody>
<tr>
<td>17β-estradiol</td>
<td>1</td>
</tr>
<tr>
<td>Estrone (n=10 tests)</td>
<td>0.19</td>
</tr>
<tr>
<td>Estriol (n= 15 tests)</td>
<td>0.0035</td>
</tr>
<tr>
<td>17α-ethynylestradiol (n=8 tests)*</td>
<td>2.2</td>
</tr>
</tbody>
</table>

*Synthetic compound; all others are naturally-occurring

In the years following the initial reports of EDC-induced environmental effects, considerable effort was placed on identifying and quantifying known and putative EDCs in WWTP influents and effluents worldwide, as reviewed by Teske and Arnold (2008) and Liu et al. (2009). Studies have revealed the tendency of many endocrine-active compounds to partition to the sludge flocs over the course of conventional wastewater treatment (Layton et al., 2000; Huang and Sedlak, 2001; Khanal et al., 2006; Carballa et al., 2008). However, relatively little work has been conducted using chemical analysis to systematically track the fate of EDCs during various common sludge stabilisation processes, such as anaerobic or aerobic digestion, alkaline treatment and composting. Far less research has focused on using *in vitro* bioassays to determine the total endocrine-active potency of biosolids, as opposed to pinpointing the concentrations of specific known and suspected EDCs. For the purpose of this paper, the operational definition of ‘biosolids’ common to most municipal jurisdictions is considered to encompass the treated residual matter resulting from the biological stabilization and/or digestion of

Many scientists, engineers and policy makers consider the beneficial reuse of biosolids for agricultural and reclaimed-land soil amendment as an increasingly acceptable and necessary endpoint in wastewater management (Goldstein, 2000; Epstein, 2003; Overcash et al., 2005; US EPA, 2006). Historically, public wariness and opposition to biosolids reuse has focused on concerns over human-health risks from exposure to microbial, viral, and toxic/irritant chemicals via aerosols and ground/surface water contamination (Magoarou, 2000; Reilly, 2001; Epstein, 2003). In the past decade, these worries have been compounded by concerns regarding the presence of endocrine-disrupting compounds in biosolids, and the impacts that EDCs may have on ecological and human-health when biosolids are land-applied (Overcash et al., 2005; O’Connor et al., 2005; Kinney et al., 2006; Banga et al., 2009). Current research has demonstrated the potential for emerging contaminants, including EDCs, to enter water bodies due to leaching or runoff from biosolids-augmented agricultural land (Lissemore et al., 2006; Lapen et al., 2008; Larsbo et al., 2009).

As outlined by Beecher et al. (2005), there are a number of “outrage factors” that are perceived by the public with regard to the land-application of biosolids. The presence of known or suspected endocrine-disrupting compounds in biosolids magnifies the unease related to several of these factors. These include fear of the: artificial/industrial, as many EDCs are anthropogenic substances; hard-to-understand (the causes and effects of endocrine disruption in an ecological context is complex); delayed effects/affecting future generations, as EDCs may be viewed as having unknowable future consequences on
environmental and/or human health (Beecher et al., 2005; Goodman and Goodman, 2006).

Determination of the presence and fate of EDCs in wastewater solids across the stabilization train is vital to evaluating the potential environmental loads of endocrine disruptors that might result from reuse applications. A better understanding of which unit processes promote the degradation of EDCs can allow for treatment facilities to be designed in which process parameters and emerging solids treatment technologies (US EPA, 2006) minimize EDC concentrations in the finished biosolids. Demonstrable removal during biosolids treatment may in turn help to allay public fears over EDC inputs to the environment and help to promote acceptance of biosolids usage. The goal of this review paper is to provide a comprehensive look at the current state of knowledge with regards the chemical and in vitro analytical techniques for detecting EDCs in sludges and biosolids, and to compile what is presently known about the fate of EDCs during common biosolids-treatment unit processes. Suggestions are also provided for future research directions.

2.2. MUNICIPAL BIOSOLIDS BACKGROUND

The nutrient content and soil-conditioning properties of biosolids have made their land application a common practice (US EPA, 1999; Magoarou, 2000; Apedaile, 2001). This is illustrated in Figure 3 where the end-uses and per-capita annual production of dry biosolids are shown for the USA, Canada, and 13 European Union nations. In all instances, the land application of biosolids accounted for roughly half or more of total usage. Hence, a significant proportion of the pollutants that become concentrated in processed biosolids will be exposed to subsequent weathering processes. The traditional
objectives of biosolids stabilization are to reduce the concentrations of pathogens and nuisance odours, and to facilitate the reduction in volume and/or mass of the sludge, generally through digestion and dewatering processes (US EPA, 1999). Major methods of stabilization include anaerobic digestion, aerobic digestion, alkali stabilization, composting and heat drying (US EPA, 1999). These processes may be applied singularly or as a series of steps, shown in Figure 4. Biosolids stabilization involves a complex interplay of physical, chemical, mechanical and biological changes which could affect the solids partitioning, degradation, adsorption, and to a much lesser extent, volatilization and photolysis pathways of EDCs in sludge matrices (Barnabé et al., 2009). As is discussed in Chapter 2.4, the occurrence of EDCs in biosolids has been well-established, although the efficacy of unit processes or process conditions in promoting their degradation/removal is not always clear.
Figure 3: Biosolids fate and annual per capita production in Canada, USA and Europe for the year 2000; *EU = 15 member states as of 2000, with the exception of IT and SE (From US EPA, 1999; Magoarou, 2000; Apedaile, 2001)

Figure 4: Schematic of advanced wastewater and biosolids treatment processes
2.3. **Analytical Methods**

Research into endocrine disruptors grew nearly exponentially during the 1990s (Matthiessen, 2003), much of which concerned the presence and ecological consequences of EDCs entering and being discharged from WWTPs. However, quantitative measurements of the EDC content of wastewater sludges and biosolids have been much slower to appear, due to the complex composition of sludge/biosolids and associated matrix interferences hindering analyses. A number of preparative and cleanup steps are typically required in order to reliably measure endocrine disruptors in biosolids. One of the first challenges to be overcome when analyzing wastewater solids is adequate sample stabilization. Because of the high microbial population in sludges/biosolids, near-total loss of trace endocrine disruptors can occur via biodegradation if bacterial growth is not arrested (Gomes et al., 2004).

Successful preservation methods for solid and wastewater samples used for EDC/trace organics analyses include lyophilization (Ternes et al., 2002; Das and Xia, 2008; Chiu et al., 2009; Mnif et al., 2010), autoclaving (Löffler and Ternes, 2003; Gomes et al., 2004; Gomes et al., 2009) and the addition of formaldehyde (Baronti et al., 2000; Simonich et al., 2000; Spring et al., 2007). It should be noted, however, that autoclaving wastewater solids may cause desorption of some EDCs from sludge flocs (Suzuki and Maruyama, 2006; Chen and Hu, 2010), which could lead to underestimations of solid-phase EDC concentrations. The use of formaldehyde as a preservative has been shown to introduce impurities into the sludge samples which interfere with subsequent chemical analyses (Simonich et al., 2000), while formaldehyde residue may be cytotoxic if *in vitro* bioassay-based analyses are applied (Tan et al., 2005).
Quantitative analysis of the EDCs present in biosolids requires that these trace target analytes be extracted as effectively and selectively as possible from the other matrix constituents. Figure 5 shows the results of EDC screening undertaken by the US EPA as part of the Targeted National Sewage Sludge Survey (TNSSS) of 84 biosolids samples collected from 74 WWTPs across the USA (US EPA, 2009). The results show a general tendency for moderately- to highly-hydrophobic compounds, as indicated by the increasing QSAR-calculated octanol-water partition coefficients, Log $K_{OW}$, (ChemDraw Ultra v. 11.0, Cambridgesoft, Cambridge, MA) to accumulate at higher concentrations in processed biosolids. While variations exist both among and between calculated and experimentally-derived Log $K_{OW}$ values, the TNSSS results corroborate that the tendency of many EDCs to sorb to biosolids can be predicted by the octanol-water partition coefficient (see Figure 6). Nonetheless, even at the higher range, the EDCs selected for analysis in this survey were present at parts-per-million (mg/kg) levels. Thus, extraction processes typically use non-polar or polar-aprotic organic solvents, e.g. acetone, ethyl acetate, toluene, hexane, to selectively solubilize EDCs from the biosolid matrix.

To facilitate contact between the extraction solvent(s) and biosolid particles, a variety of extraction methods have been used, including wrist/rotary shaking (Gomes et al., 2004; Lorenzen et al., 2004; Chiu et al., 2009), ultrasonication (Ternes et al., 2002; Chenxi et al., 2008; Stasinakis et al., 2008), Soxhlet extraction (Bagó et al., 2005; US EPA, 2009), and pressurized solvent extraction (La Guardia et al., 2001; Keller et al., 2003; Arnold et al., 2008; Minamiyama et al., 2008; Patureau et al., 2008). Even with extensive extraction processes, recoveries of estrogenic compounds from biosolids in the range of ~50-70% are common (Ternes et al., 2002; Gomes et al., 2004; Lorenzen et al.,
However, the sample mass used for extraction can have a significant effect on the overall method-recovery of EDCs. Chiu et al. (2009) compared the recovery of natural estrogens (estrone, E1; 17β-estradiol, E2; estriol, E3) and the oral birth-control analogue of E2, 17-α-ethinylestradiol (EE2) from 0.1, 0.2 and 0.5g samples of primary sludge, and found that only the 0.1g samples allowed for reliably quantitative (>95%) recovery. As the mass of sample increased, so too did the amount of co-extracted matrix compounds, which decreased the sensitivity of the detection method (liquid chromatography/tandem mass spectrometry). This is important to bear in mind when considering that the methods published in the literature for EDC analysis call for the extraction of anywhere from 20mg (Stasinakis et al., 2008) to 250mg (US EPA, 2007) to 500mg (Gomes et al., 2004).
Figure 5: Occurrence of selected EDCs in treated biosolids in the USA (after US EPA, 2009). Quantities in brackets after analyte name refer to number of detects above LOQ in n = 84 samples; ‘ND’ = ‘not detected’
Figure 6: Extracted dry-weight concentrations of endocrine disrupting compounds as a function of calculated Log $K_{OW}$ (after US EPA, 2009)

The presence of co-extracted compounds from the biosolids samples can interfere with the sensitivity and/or reproducibility of quantitative analytical steps. While some researchers have measured EDCs in biosolid solvent extracts without extensive post-extraction cleanup (Moeller and Reeh, 2003; Amir et al., 2005; Bagó et al., 2005; Minamiyama et al., 2008), extracts must typically be subjected to further clean-up steps before quantification. Chromatographic cleanup with silica, Florisil, alumina, or combinations of the three is widely used to retain unwanted highly polar and/or ionic species on the stationary phase while allowing low- to moderately-polar endocrine-active compounds to be collected in the solvent phase (Kinney et al., 2006; US EPA, 2007; Das
and Xia, 2008; Chiu et al., 2009; Fernandez et al., 2009). However, care must be taken when applying cleanup steps using silica or alumina adsorbents, as some endocrine disruptors (such as estriol) are polar enough to be strongly retained, and hence poorly recovered, from the stationary phase (Gomes et al., 2004; Liu et al., 2010). The use of metallic copper as a cleaning agent, either during the sample extraction step or as part of the subsequent cleanup process, is recommended to remove the high levels of sulphur that are typically present in biosolids (DeWil et al., 2009) and which can greatly interfere with analytical tools such as gas chromatography/mass spectrometry (GC/MS) (Kester et al., 2005; Osemwengie, 2006; US EPA, 2007). Gel permeation chromatography (GPC) separates analytes based on molecular size as opposed to polarity and has also been used as a cleanup step for the removal of coextracted organic compounds and sulphur from biosolids extracts (Ternes et al., 2002; Gomes et al., 2004; Osemwengie, 2006; Yang and Metcalfe, 2006). Kester et al. (2005) provide a case study in which the effect of sequential cleanup steps (Florisil/GPC/silica/alumina) of a biosolid solvent extract on removing extraneous peaks from GC/MS chromatograms are clearly illustrated.

One of the most commonly encountered approaches for the cleanup of sample extracts, either alone or in combination with some or all of the above steps, is reversed-phase solid phase extraction (SPE). EDCs present in the solvent extracts from biosolids are reconstituted in water, and concentrated on the SPE material under vacuum (Gomes et al., 2004; Carballa et al., 2006; Minamiyama et al., 2008; Patureau et al., 2008). The basic principles underlying SPE methodology specific to environmental matrices has been extensively reviewed by Comerton et al. (2009). Following desorption from the SPE matrix, the concentrations of specific endocrine disrupting species present in sample
extracts are typically determined using liquid- or gas-chromatography for analyte separation followed by quantitation with mass spectrometry (LC or GC/MS). Many variations on this approach exist, and are reviewed in the context of environmental analyses in great depth by Richardson (2008). While these methods can detect EDC concentrations down to the ng/g level in biosolid samples if suitable sample preparation steps have been followed, the potency of EDCs varies over orders of magnitude.

Measurements of EDC concentrations alone do not necessarily give a full picture of the endocrine-disrupting activity of a sample, as the suite of chemicals chosen for determination is chosen a priori and (a) likely will not account the entire range of EDCs present in a sample, (b) does not account for differences in endocrine-active potency amongst different chemical species, nor possible antagonism, synergism or potentiation of chemical mixtures. In vitro bioassays have been applied in wastewater research to determine whole-sample endocrine-disrupting effects, usually estrogenic (Leusch et al., 2006; Fernandez et al., 2007; Pauwels et al., 2008a; Fernandez et al., 2009) but also androgenic (Leusch et al., 2006; Conroy et al., 2007), either in place of or as a complement to chemical analyses. The relative simplicity in terms of equipment and expertise needed to conduct in vitro estrogenicity bioassays in comparison to GC- or LC-MS analyses may allow for more routine monitoring of estrogenicity in WWTP settings (Water Environment Research Foundation, 2007). In comparison to wastewater, very little work has been conducted that extends in vitro EDC testing to biosolids samples.

Attempts to apply the yeast estrogen screen (YES) of Routledge and Sumpter (1996) or its variant (De Boever et al., 2001) to biosolid samples have been hampered by the sample extracts causing cell death (WERF/GWRC 2004; Teske et al., 2007;
McNamara et al., 2009). The Water Environment Research Federation/Global Water Research Coalition (WERF/GWRC) report (2004) also found that the type of extraction solvent can interfere with the YES assay results, as yeast-cell toxicity was encountered with acetone/dichloromethane biosolids extracts but not with methanol extracts from the same samples. This is consistent with recent method development work by Frische et al. (2009), who found that the degree of estrogenicity measured by a YES assay could be drastically underestimated by the presence of confounding toxic substances. It is likely that the choice of extraction solvent will vary the efficiency with which cytotoxic compounds present in biosolids will be co-extracted along with EDCs. It is also worth noting that the choice of solvent that optimizes desorption of EDCs from the SPE cartridges/discs for subsequent YES assay testing has not been well defined for biosolids samples.

In a variety of papers in which C$_{18}$ SPE cartridges or discs were used to clean and concentrate wastewater extracts, the optimal desorption solvent for eliciting a maximal estrogenic response in the Routledge and Sumpter YES assay ranges from 60% v/v methanol/water (Conroy et al., 2005), 80% v/v methanol/water (Desbrow et al., 1998), and 100% methanol (Conroy et al., 2007; Fernandez et al., 2007). Fernandez et al. (2009), using a recombinant yeast strain to analyze WWTP influent and effluent, found that the 80% v/v methanol/water SPE eluate elicited minimal estrogenic response in the assay, whereas diethyl ether extracts were consistently estrogenic. The establishment of standardized extraction protocols and quality assurance and control measures when working with biosolids, such as US EPA Method 1698 (US EPA, 2007), may facilitate further study towards standardizing approaches when applying bioassays to biosolids.
2.4. BIOSOLIDS UNIT PROCESSES

2.4.1. ANAEROBIC AND AEROBIC DIGESTION

Anaerobic and aerobic digestion processes are designed to optimise the microbial degradation of organic matter, and as such, the potential exists for endogenous and exogenous organic EDCs to be removed during biosolids treatment. Laboratory-scale studies have helped to identify specific classes of heterotrophic bacteria present in wastewater matrices that are capable of degrading EDCs (Haiyan et al., 2007; Ren et al., 2007; Gaulke et al., 2008). Conversely, a wide variety of microorganisms of different taxa, e.g., bacteria, fungi, yeasts, algae and protozoa, can convert C\textsubscript{17}-ketosteroids excreted by humans, such as androstenedione and androsterone (non-estrogenic) and E\textsubscript{1} (moderately-estrogenic) into highly-estrogenic 17β-estradiol, as well as converting plant-based sterols to testosterone (Donova et al., 2005). Winter et al. (1984) were also able to identify five strains of bacteria originating from human faeces that could reduce non-estrogenic C\textsubscript{17}-ketosteroids to 17β-estradiol under anaerobic conditions. Thus, it is difficult to make a priori assumptions regarding the degree to which a given compound will be biologically degraded during biosolids processing.

Holbrook et al. (2002) examined the evolution of total estrogenicity in biosolids during two separate treatment processes – two-stage mesophilic anaerobic digestion and combined three-stage thermophilic/two-stage mesophilic aerobic digestion process. The total estrogenicity of the biosolids increased during the anaerobic process, and increased through the three thermophilic stages and first mesophilic stage of the aerobic digestion, before falling in the second mesophilic aerobic digestion step. The general trend of increasing estrogenicity through the anaerobic digestion processes is attributed by
Holbrook et al. to the possible generation of estrogenic metabolites during the biosolids treatment process. This theory is borne out in studies that have monitored the fate of nonylphenol polyethoxylate surfactants (NPnEOs, \(n = 1-16\)) and their degradation product, nonylphenol (NP), in biosolids. As the number of ethoxy units in the NPnEO chain decreases, the resulting molecules exhibit successively greater estrogenic potency, albeit still markedly less than steroid hormones such as E2.

Keller et al. (2003), compared the concentrations of NPnEOs and NP in the biosolids from three WWTPs. Two WWTPs used aerobic digestion, and the third employed a two-stage anaerobic digestion process. The two sets of aerobically-digested biosolids samples contained roughly similar concentrations of NP (24 and 13 mg/kg dry weight, respectively) and similar profiles and concentrations of ethoxylated nonylphenol (from \(n = 1\) to 10 ethoxylate units, at concentrations of up to 10 mg/kg(dw)). In contrast, the anaerobically digested biosolids contained NP concentrations of 40 to 60 times that of aerobic biosolids, along with elevated NP1EO (nonylphenol monoethoxylate) concentrations of up to 61.5 mg/kg(dw). Longer-chain NPnEO concentrations in the anaerobically treated biosolids were, however, markedly lower than in the aerobically treated biosolids.

This apparent tendency for the anaerobic digestion process to promote the transformation of NPnEOs to estrogenic NP was confirmed by Minamiyama et al. (2006). Using bench-scale mesophilic anaerobic digesters fed with thickened sludge from a full-scale WWTP and operated at a 28-day solids retention time (SRT), the authors monitored the transformation of NP1EO and two other NP precursors, nonylphenol mono- and dicarboxylate (NP1EC and NP2EC), that were spiked into the sludge. Throughout the
study, 40% of all added NP1EO and 100% of all NP1EC were converted to NP, while NP2EC was converted to NP only after 20 days of sludge acclimation. The high degree of conversion of NP1EO and NP1EC to NP is significant because the estrogenic potency of NP is approximately 4 and 33 times higher than that of NP1EO and NP1EC, respectively (Dussault et al., 2005).

Contrary to the studies cited above, Hernandez-Raquet et al. found that anaerobic digestion of secondary sludge at an SRT of 20 days and temperature of 30 °C did not yield any reduction in spiked NP, or conversion of NP1EO or NP2EO to NP (2007). A bioassay (human breast cancer cells transfected with an estrogen-responsive reporter-gene coding for luciferase) was used to determine the total estrogenicity of the anaerobically digested sludge. The recalcitrance of NP/NP1EO/NP2EO to degradation, combined with a decrease of total suspended solids during digestion, meant that the net estrogenicity of the treated sludge on a dry-weight basis actually increased during the anaerobic digestion process. No other EDCs were chemically analyzed, so the amount of estrogenicity attributable to compounds other than NPnEOs cannot be determined from this study. However, aerobic digestion of secondary sludge at an SRT of 20 days and temperature of 30 °C resulted in the removal of all three targeted analytes to below the detection limit (0.1µmol/kg dry-weight). Near-quantitative removal of NP/NP1EO/NP2EO was also reported when anaerobically-treated biosolids were subsequently subjected to aerobic digestion. Both aerobic treatment conditions removed total estrogenicity by approximately 90%, to below 20µg E2-equivalents (EEQ) per kilogram of dry-weight solids. Given the orders-of-magnitude difference in estrogenic potency between NP/NP1EO/NP2EO and steroid hormones such as E2 and E1, the 90%
reduction in total estrogenticity suggests that the treatment conditions successfully removed EDCs beyond the three analyzed.

Patureau et al. (2008) found that mesophilic anaerobic digestion (20 day SRT, 35 °C) of an unspiked primary/secondary (1:1 v/v) sludge led to the complete removal of pre-occurring NP2EO and 80% removal of NP1EO. NP production also occurred, although the amount produced was stoichiometrically less than the moles of NP1EO and NP2EO lost, suggesting that NP itself can be degraded under anaerobic conditions. Increasing the temperature of the anaerobic digester to 55 °C did not significantly improve total NPnEO removal relative to mesophilic conditions. While Hernandez-Raquet et al. found near-total NPnEO removal during mesophilic aerobic digestion, Patureau et al. reported only 39 ± 7% removal of NPnEOs during thermophilic (65 °C) aerobic digestion at a 20-day SRT. By comparison, an earlier batch study by Banat et al. (2000) reported 66% removal of NP during aerobic digestion of sewage sludge at 60 °C and 10-day SRT.

To the extent that the fate of EDCs during (an)aerobic sludge digestion has been studied, it is clear that a considerable amount of the work has focused on NPnEOs alone. This is likely due to the fact that nonylphenol polyethoxylates are found at much higher concentrations (µg- and mg/kg) in wastewater sludges than most other EDCs, and as such can be more easily quantified. Few studies exist in the literature that investigate the fate of other EDCs during aerobic or anaerobic processing, despite the fact that E1, E2 E3 and EE2 have been found to exert the majority of estrogenic activity in wastewater (Nelson et al., 2007; Salste et al., 2007). As part of a comprehensive study on the fate of estrogens throughout a full-scale WWTP, Andersen et al. (2003) measured E1, E2 and EE2
concentrations in certain WWTP processes and found a notable increase in the
centrations of E1 and E2 during sludge stabilization. The waste activated sludge
(WAS) contained 7 ng/g(dw) E1, 1.7 ng/g(dw) E2, and 3 ng/g(dw) EE2 while the
anaerobically digested biosolids (33 °C, 20-day SRT) contained 25.2 ng/g(dw) E1, 5.1
ng/g(dw) E2 and no detectable EE2 (detection limit of 1.5 ng/g). The reported
concentrations of E1 and E2 in the digested biosolids are different than those reported in
earlier related work by Ternes et al. (2002), who found E1 concentrations of 2-
16ng/g(dw) and E2 concentrations of 9-49ng/g(dw). Based on the results of two grab
samples, Fernandez et al. (2009) reported far lower E1 and E2 concentrations of
0.056ng/g(dw) and 0.155ng/g(dw), respectively, in anaerobically-digested biosolids. The
details of the biosolids treatment processes given in the three studies are not enough to
offer an explanation as to whether the orders-of-magnitude difference in E1 and E2
concentrations in the 2002 and 2003 papers versus the 2009 results are related to
wastewater source composition, process variables, or improvements in the analytical
method.

Carballa et al. (2006) determined that process variables of the anaerobic digestion
process had little or no effect on E1, E2 and EE2 removal at the bench-scale, using 70:30
v/v primary and biological sludge from a full-scale WWTP as a feed source. Neither
alkaline pre-treatment with lime nor thermal pre-treatment via autoclaving conferred any
improvement in EDC removal following mesophilic (37 °C) or thermophilic (55 °C)
aerobic digestion. SRT was also found to have no effect on the extent of EDC removal.
Mesophilic treatment slightly enhanced the degree and consistency of EE2 removal (85 ±
5% removal versus 75 ± 15% under thermophilic conditions), though for E1 and E2
temperature appeared to play no role in net removal (85% in either case). Carballa et al. (2007) noted that EDC removal increased with sludge adaptation during anaerobic digestion, although no clear definition was provided as to what constituted ‘adaptation’ or how this was quantified. Subsequent work by the same research group (Carballa et al., 2008) modelled EDC partitioning based on the solid-water distribution coefficient ($K_d$) and organic carbon distribution coefficient ($K_{OC}$). Modelling confirmed that the sorptive properties of sludges with respect to natural estrogens and musks are not greatly affected by variations in anaerobic digester operating conditions, which helps to account for the similar removal efficiencies observed at the bench scale despite various pre-treatments and SRT’s. Conversely, sludge acclimation was not observed in the long-term study conducted by Czajka and Londry (2006), in which EE2 was spiked at a high concentration of 5 mg/L into sludge from a municipal WWTP anaerobic digester and monitored over a 271-day period. Their results suggested that even though reduction of electron acceptors (nitrate, sulphate, iron) and methanogenesis occurred, EE2 degradation was minimal, and not statistically different from a negative control.

Polybrominated diphenyl ether (PBDE) flame retardants have been identified as EDCs (Legler and Brouwer, 2003), and nearly 90% of PBDEs entering WWTPs end up sorbed to sludge (North, 2004; Song et al., 2006). Sludge acclimation appeared not to affect the removal of PBDE fire retardants in the work done by Arnold et al. (2008). The concentrations of various PBDE congeners decreased by 6-24% during mesophilic anaerobic digestion, but no improvements in their removal efficiency were observed over a 30-day period. Bisphenol-A (BPA) is commonly encountered in municipal wastewater sludge and also appears to be resistant to mesophilic anaerobic degradation, as confirmed
through both full-scale and bench-scale testing with 2:1 v/v primary sludge:WAS (Weltin et al., 2002). The mean concentrations of BPA in primary sludge and WAS from 12 German WWTPs was 950 and 565 µg/kg(dw), respectively, while the mean concentration in the digested biosolids was 1,260 µg/kg (dw). The BPA that desorbed from the sludge during anaerobic digestion appeared to re-sorb during the dewatering process, as the mean concentration in the dewatered cake increased to 5,130 µg/kg(dw).

An earlier study by Lee and Peart (2000) of BPA concentrations in the biosolids from 31 Canadian municipal WWTPs similarly found that BPA was released during anaerobic digestion, although the BPA concentrations encountered in the digested biosolids were considerably lower than those reported by Weltin et al. (2002), with a maximum reported concentration of 36.7 µg/kg(dw).

2.4.2. COMPOSTING

Composting can be applied as either a stabilization treatment for dewatered sludge, or as an enhanced treatment strategy for biosolids that have already been stabilized by other means (US EPA, 1999). Due to its aerobic nature and high microbial activity, the composting process has been suggested as a beneficial method of reducing the presence of EDCs in biosolids (Minamiyama et al., 2006). However, as with aerobic/anaerobic sludge stabilization, much of the work presented in the literature with respect to biosolids composting focuses only on the occurrence and fate of NP/NPnEOs. An early study by Jones and Westmoreland (1998) of high-strength sludge resulting from the cleaning of raw wool found that composting was able to reduce total NP/NPnEO from 14 g/kg(dw) to 1.2 g/kg(dw) after 98 days. However, in order to achieve final concentrations on the order of mg/kg(dw), the authors suggested a further 98 days of
composting. In comparison, the USEPA’s Biosolids Guidance document suggests 3-4 weeks of composting time for municipal biosolids to meet 40 CFR Part 503 “Class B” reuse regulations (US EPA, 1999). Samples analyzed at intervals during the composting process showed that longer-chain polyethoxylate oligomers were quantitatively converted to NP, NP1EO and NP2EO after 98 days. In this respect, sludge composting appears to yield a similar NP\(n\)EO degradation pathway as anaerobic digestion, with higher-estrogenicity NP, NP1EO and NP2EO being formed at the expense of less-estrogenic longer-chain oligomers. This trend may not be conclusive, however, with Minamiyama et al. (2008) finding roughly equal concentrations of NP and NP\((n=1-4)\)EO in composted biosolids at ~50 µg/kg(dw), and total NP\((n\geq5)\)EO concentrations at ~25 µg/kg(dw). However, the results presented by Minamiyama et al. (2008) provide no details as to the composting conditions; hence the presence of longer chain polyethoxylates in the compost may be an artefact of different process conditions.

Studies conducted at full-scale municipal WWTP have validated that composting can indeed be effective in removing NP\(n\)EOs. Keller et al. (2003) found that the composting of anaerobically digested WWTP biosolids in windrows for two months at 40-50 °C yielded a finished product with NP\(n\)EO \((n>2)\) levels comparable to that of the starting biosolids, but with NP/NP1EO/NP2EO levels reduced by 85.5, 59.5 and 84.5%, respectively. The consequence of this finding is that the composting process appears to preferentially remove moderately estrogenic short-chain nonylphenol derivatives, while leaving the longer-chain (non-estrogenic) oligomers unchanged, under the conditions used in the study. Total NP\(n\)EO levels measured at 13 full-scale WWTPs in the USA were 65-100% lower in composted biosolids vis-a-vis the fresh biosolids (Xia et al.,
A limited number of studies have investigated the factors that influence removal efficiency of NPnEOs during biosolids composting. Xia et al. (2005) tested the effects of different composting temperatures (25, 45 and 65 °C) and initial proportions of biosolids-to-wood shavings (43:57, 65:35 and 84:16 by dry weight) on NP removal at the bench scale. Removal during the early stages (i.e. up to eight days) of composting was strongly and positively associated with temperature. However after 15 days both the 45 and 65 °C conditions yielded approximately 80% NP removal, while the 25 °C condition yielded approximately 50-60% NP removal. While higher temperatures promoted faster NP removal, the authors’ data showed that similar degrees of removal can be achieved using lower temperatures and longer treatment times. Under all temperature conditions, lower biosolids-to-wood shavings ratios favoured higher rates of NP removal.

In subsequent work, Das and Xia (2008) determined that NP degradation at 43:57 biosolids-to-wood shavings ratio (C:N ratio of 65:1) followed second-order kinetics, while at ratios of 65:35 and 84:16 (C:N of 30:1 and 20:1, respectively) NP degradation followed first-order kinetics. Moeller and Reeh (2003) collected WWTP sludge blend that had been mixed 1:1:1 (v/v) with straw and park waste and allowed to compost for one week in windrows prior to being fed into bench-scale composters. At temperatures of 35, 50 and 55 °C Moeller and Reeh noted a significant reduction in NP after approximately 30 days of treatment and no associated detection of NP1EO and NP2EO. However, at a process temperature of 65 °C, both NP1EO and NP2EO were detected within the first several days of composting, and their concentrations, along with that of NP, increased during the first four days of treatment. While NP1EO and NP2EO eventually disappeared, the concentration of NP – the most estrogenic of the degradation
by-products — steadily increased from during days 12 to 25 of composting. Pakou et al. (2009) conducted bench-scale compost studies using blended, undigested primary and waste active sludges. Unlike the previously cited investigations, Pakou et al. (2009) did not maintain isothermal conditions in the compost, but rather allowed the temperature to vary up to a maximum of approximately 64 °C according to microbial activity. Over the course of 62 days, Pakou et al. observed a progressive decline in NP1EO and NP2EO concentrations. However, NP concentrations increased and reached maxima during the first 11 days of composting, before declining thereafter. Thus, choosing higher composting temperatures and shorter treatment times may lead to increased concentrations of estrogenic NP in the compost relative to the fresh biosolids.

The fate of EDCs other than NP/NPnEOs in biosolids composting remains poorly studied, and this subject is prioritized as an area of waste management in need of further research (Farrell and Jones, 2009). Yamashita and Ozaki (2005) reported that E1, E2, E3 and EE2 were not detected in the biosolids compost from 17 different Japanese WWTPs, with only an E2 metabolite (17β-estradiol-3,17-disulphate) being measured (0-2.8 mg/kg(dw)). Full-scale windrow composting of chicken manure amended with hay, straw, leaves and seed manure showed a considerable reduction in E2 levels (Hakk et al., 2005). E2 degradation followed first-order kinetics, with water-soluble concentrations dropping from 83 µg/kg(dw) to 13 µg/kg(dw) after 139 days. However, it is likely that the concentrations of E2 encountered in the work of Hakk et al. (2005) may be higher than those present in municipal wastes, given the high levels of E2 that are excreted by chickens. Based on the analysis of samples from four different municipal WWTPs in the USA, Kinney et al. (2006) concluded that biosolids composting was relatively ineffective...
in removing a suite of micropollutants, some of which were estrogenic surfactants and phytoestrogens. EDC concentrations varied considerably between the four sites for some compounds, i.e. NP 2.18-491 mg/kg, NP1EO 3.96-17.2 mg/kg, NP2EO 2.85-7.01 mg/kg, BPA 4.69-9.03 mg/kg, 3β-coprostanol 8.1-72.8 mg/kg, cholesterol 19.1-157 mg/kg, β-sitosterol 50.8-200 mg/kg and stigmastanol 2.76-17.4 mg/kg (note that all concentrations are given per unit mass of organic carbon). Pauwels et al. (2008b) were able to isolate six strains of bacteria from compost that were able to co-metabolise EE2 along with E2 at ng/L levels, whereas no bacteria strains present in fresh faeces or activated sludge were capable of using EE2 as a metabolic substrate. While these results are helpful in elucidating potential EDC removal mechanisms, it is not clear to what extent bacteria would preferentially metabolise trace-level EDCs in the presence of the wide range of other organic substrates present in a full-scale composting facility.

The plasticizer diethylhexyl phthalate (DEHP) is ubiquitous in biosolids (Marttinen et al., 2004; Bagó et al., 2005; Kinney et al., 2006), and is an antiandrogenic endocrine disruptor (Parks et al., 2000). The DEHP content of raw and anaerobically digested sludges (57 and 77 mg/kg(dw)), mixed 2:1 (v/v) with a commercial chipped bark/peat amendment, decreased by 58% and 34%, respectively, during 85 days of composting (Marttinen et al., 2004). Bagó et al. (2005) report a higher level of DEHP removal when anaerobically digested sludge was composted, versus aerobically digested sludge (85% removal versus 76%). While the extent of DEHP removal from anaerobically digested sludge determined by Bagó et al. (2005) is considerably higher than that of Marttinen et al. (2004), no details are provided with respect to composting conditions (i.e. temperature, oxygen content, composting duration). Thus, the source of
this discrepancy is unclear. DEHP removal from both activated sludge and anaerobic lagoon sludge followed first-order degradation kinetics during a composting study by Amir et al. (2005). However, the half-life of DEHP in activated sludge composting was considerably shorter (28.9 days) than that of anaerobic sludge (45.4 days).

2.4.3. Alkaline Stabilization

Very little work exists in the literature with respect to the effects of alkaline stabilization on the fate of EDCs present in sludges, despite the fact that this remains a common, well-established method of processing biosolids (US EPA, 2006). In a lab-scale study, Ivashechkin et al. (2004) monitored the fate of BPA during sludge conditioning with ferric chloride, followed by stabilization with calcium hydroxide (Ca(OH)$_2$). While ferric chloride addition had no effect on the high affinity of BPA for the sludge, addition of Ca(OH)$_2$ to a final pH of 12.4 led to the near-quantitative desorption of BPA. The degree of desorption was particularly pronounced above a pH of 10.3, the $pK_a$ of BPA. Ivashechkin et al. (2004) similarly found that EDCs such as E1, E2 and NP, which all possess similar $pK_a$ values as that of BPA also desorbed from sludge during lime stabilization. The implication of this research is that the extreme pH values encountered during alkaline processing will cause deprotonation of EDC molecules and biosolids, both of which are weakly acidic, leading to desorption of EDCs from floc particles and solubilization. Soil-application tests conducted by Koulombos et al. (2008) confirmed that treatment of anaerobically-digested biosolids to a pH of 11 with Ca(OH)$_2$ led to higher leaching rates of radiolabelled NP than soils amended with biosolids that had been conditioned either by freeze-thawing, polymer thickening, or ferric chloride addition. Increased NP leaching potential occurred as a result of desorption, in accordance with
Ivashechkin et al. (2004). The application of alkaline-stabilized biosolids also changed the NP degradation pathway in soil, with microbial mineralization becoming negligible and sequestration to organic acids being favoured by the elevated pH. Further research into the fate of other EDCs during alkali addition would be particularly valuable, considering that there are numerous emerging biosolids treatment technologies that continue to incorporate alkaline stabilization as a process step (US EPA, 2006).

2.5. Bioassay Measurements

Method development work conducted by WERF and GWRC (2004) found that both mesophilic-anaerobically digested (MAD) biosolids and heat-dried biosolids had measurable estrogenic activity. Two in vitro bioassays were used - the Routledge and Sumpter (1996) YES assay and the estrogen-receptor chemically activated luciferase gene expression (ER-CALUX) assay. The freeze-dried biosolids samples were extracted using an accelerated solvent extraction system, and the extracts were not further cleaned prior to analysis. The extraction solvent had to be changed from a dichloromethane/acetone mixture to methanol during method development, due to the toxicity of the initial extracts on the yeast assay. The two tests provided discrepant results, with MAD biosolids registering at 15µg/kg(dw) E2-equivalents (EEQ), according to the YES assay, and 73µg/kg(dw) EEQ according to the ER-CALUX assay. Whereas the YES assay indicated an increase in total estrogenic potency after MAD biosolids were heat dried (15 → 27µg/kg(dw) EEQ) the ER-CALUX indicated a decrease from 73 to 25µg/kg(dw) EEQ.

In comparison, Lorenzen et al. (2004) analyzed 13 anaerobically-digested biosolids samples and four aerobically-digested samples with the ER-CALUX assay
and a similar YES screen to that used in the WERF/GWRC research. The YES results were consistently higher than the corresponding estrogen-equivalent values derived from the ER-CALUX assay, by an average factor of 25. Thus, while anaerobically-digested biosolids ranged from approximately 500-2000µg/kg(dw) EEQ according to the YES results, the corresponding range for ER-CALUX results was approximately 20-80µg/kg(dw). While Lorenzen et al. did not provide ER-CALUX results for aerobically-digested biosolids, the YES screen results indicated a substantial reduction in estrogenicity relative to anaerobic digestion (mean values of 11.3µg/kg(dw) EEQ versus 1233µg/kg(dw) EEQ). Aerobic digestion was also more effective than anaerobic digestion at reducing total androgenicity, with anaerobically digested biosolids having an average of 543µg/kg(dw) testosterone-equivalents versus non-detectable levels of testosterone-equivalent compounds in the aerobically treated biosolids.

The orders-of-magnitude differences in YES-estrogenicity reported by Lorenzen et al. (2004) and WERF/GWRC (2004) also appear in the results presented by Teske et al. (2007). Teske et al. applied a modified version (per De Boever et al., 2001) of the Routledge and Sumpter YES assay to determine the estrogenicity of membrane-bioreactor (MBR) WAS and centrifuge-thickened anaerobically digested sludge from two full-scale WWTPs. The YES results were expressed as 17α-ethinylestradiol (EE2) equivalents, rather than the more common E2-equivalents; however, as shown in Table 1, EE2 is roughly similar in estrogenic potency to E2. The MBR waste sludge had a total estrogenic potency of 12.2µg/kg(dw) EE2-equivalents, which is in line with the values for aerobically-digested sludge determined by Lorenzen et al. The estrogenicity of anaerobically-digested biosolids was reported as 299,364µg/kg(dw) EE2-equivalents,
which is considerably higher than the estrogenicity reported elsewhere in the literature for wastewater, solids, or sediments. The authors note that digested biosolids extracts tended to be more toxic to the yeast screen than samples derived from wastewater, but no further details are provided as to how this toxicity affected the interpretation of the YES screen results. McNamara et al. (2009) attempted to quantify the extent of total estrogenicity reduction associated with three biosolids-treatment processes: (1) mesophilic anaerobic digestion (MAD), (2) MAD followed by post-aerobic digestion, and (3) MAD preceded by a thermal sludge pre-conditioning step (heating to 170°C for 30 minutes). While the aerobic post-treatment reduced estrogenicity relative to the anaerobically-digested biosolids, overall reduction comparisons could not be made because both the raw starting sludge and thermally pre-treated samples were toxic to the yeast used in the Routledge and Sumpter YES assay.

2.6. Future Outlook

The development of in vitro assays to detect endocrine-disrupting substances in wastewater has led to a shift from quantifying the presence of individual chemicals alone to augmenting these results with measurements of total estrogenic or androgenic potency. However, such an approach has had limited success when applied to biosolids, due to cytotoxicity. Recent efforts at standardizing the processes associated with extraction and concentration of EDCs for quantitative chemical analysis, such as US EPA Method 1698, has paved the way for similar work to be conducted with regards to bioassay preparation. Future method development is warranted to determine what extraction and cleanup steps can be undertaken to prevent cytotoxicity from occurring when applying in vitro bioassays to biosolids extracts. Method development work is also needed to clarify which
extraction and cleanup steps extract the widest consortium of endocrine-active compounds while minimizing false-negative results due to the co-extraction of compounds that act as antagonists or toxicants in the assays.

Such work is merited, because the fate of overall endocrine-disrupting potency during sludge treatment unit processes has not been clearly established. While the presence and behaviour of certain analytes, particularly surfactants and plasticisers, have been better studied in biosolids treatment processes, these chemicals are part of a long list of known or suspected EDCs, many of which are significantly more potent endocrine disruptors. While total-estrogenicity or total-androgenicity bioassay measurements do not pinpoint the specific compounds that are present in a sample, they do offer insight into the hormonal potency of complex mixtures. Being able to compare total-estrogenicity or –androgenicity equivalents in biosolids between unit processes may also help in disseminating the fate of EDCs during biosolids-treatment to the public. Public perception of the safety of biosolids, particularly for land-application, may be weakened by reports of the presence of a myriad of compounds, even if the chemicals in question have vanishingly slight or unclear endocrine-disrupting potential. Work aimed at establishing correlations between bioassay measurements and total chemical concentrations using concentration-addition models may also aid in validating the use of in vitro bioassays as screening tools prior to more in-depth chemical analyses.

Beyond overcoming analytical challenges, one of the most important research needs with respect to delineating the fate of EDCs in biosolids lies in taking a holistic approach to EDC measurement throughout the entire chain of conventional WWTP solids-handling unit processes. To date, certain processes (e.g. anaerobic digestion,
composting) have received considerable attention, whilst others (e.g. alkaline stabilisation, thermal treatment) have received little, with side processes, such as dewatering or polymer addition, having received almost none. Bench-scale research is valuable for establishing a baseline grasp of removal efficiencies and fate mechanism of EDCs during unit treatment operations. However, rigorous sampling from full-scale WWTPs is invaluable for assessing the interplay of treatment processes on EDC fate, and can be used to better understand the impact of operating variables, such as solids- and hydraulic-retention time on EDC removal efficiency. Full-scale sampling also offers opportunities for research into the nature of the bacterial communities and strains and gene complexes found within bacterial communities, which may then be related to the efficacy of EDC degradation during biosolids digestion. When considering full-scale treatment operations, there is also a need for research that examines the fate of EDCs during prolonged biosolids storage conditions. Particularly in northern climates, seasonal agricultural practices and regulatory requirements may dictate periods of non-application of municipal biosolids, during which the potential exists for further biodegradation of EDCs beyond that provided during unit processes.
3. MATERIALS AND METHODS

While specific details of the analytical methodology are dealt with in the individual chapters of this thesis, the following section provides an overview of the general methodology and materials that were used for key tests.

3.1. PREPARATION AND SILANIZATION OF GLASSWARE

In order to minimize cross-contamination, all glassware was scrupulously cleaned, following the protocol below. In addition, the silanization of glassware is recommended when working with trace-level eEDCs to prevent their adsorption to the surface of glassware (Huang and Sedlak, 2001; Gomes et al., 2004). The silanization method described below was adapted from the work of Deyhimi and Coles (1982), and was applied to all glass sampling bottles, vials used for sludge/biosolid extraction, and to test tubes containing sludge/biosolid solvent extracts.

- Wash all glassware in hot tap water with DeCon detergent
- Rinse glassware x5 with hot tap water
- Rinse glassware x5 with distilled water
- Rinse glassware x3 with ethanol
- Cover mouth of glassware with tinfoil
  - For analytical glassware: place glassware in the drying oven (105 °C) overnight
  - For sample bottles, sludge extraction vials: bake in muffle oven (550 °C) for two hours
• To silanize glassware:
  o When glassware is cool, add several drops of 12.5% v/v trimethylchlorosilane (CAS# 75-77-4) in hexane
  o Cover glassware tightly with tinfoil
  o Place in drying oven (105 °C) for a minimum of two hours
  o When cool, rinse glassware x3 with ethanol, cover tightly with new tinfoil, and use green lab tape to indicate that the piece has been silanized

3.2. **YEAST ESTROGEN SCREEN (YES) ASSAY**

The initial sample of concentrated human estrogen-receptor (hER) transfected recombinant yeast stock was kindly provided via Prof. C. Metcalfe, Trent University (Peterborough, ON) from original stock from Prof. J. Sumpter, Brunel University (Uxbridge, UK); the methodology shown below, except where noted otherwise, is from Routledge and Sumpter (1996) and SOPs provided by Prof. C. Metcalfe.

3.2.1. **PREPARATION OF STOCK SOLUTIONS**

3.2.1.1. **Estradiol (E2) Standard**

- Accurately weigh 55.6mg of 98% pure 17-β estradiol (CAS# 50-28-2, Sigma-Aldrich)
- Transfer E2 to a 100mL volumetric flask
- Use Pasteur pipette to wash down any remaining E2 from the weighing boat into the volumetric flask using absolute ethanol
- Add ethanol until the volumetric flask is filled; stopper the flask, seal with Teflon tape, and hand-shake for ~30 seconds until the E2 is dissolved
- Charge a disposable 1mL glass pipette x3 with E2 stock solution; transfer 1mL to a second 100mL volumetric flask; reseal the stock solution with stopper and Teflon tape, and place in freezer for storage
- Fill second 100mL volumetric flask with absolute ethanol, stopper, seal with Teflon tape, and hand-shake for ~30 seconds
- Charge a disposable 1mL glass pipette x3 with intermediate E2 solution; transfer 1mL to a third 100mL volumetric flask; dispose of the intermediate solution after use
- Fill third 100mL volumetric flask with absolute ethanol, stopper, seal with Teflon tape, and hand-shake for ~30 seconds, store in the -20 °C freezer, but return to room temperature before use. This is the working E2 standard for the assay, and has a concentration of 54.48µg/L (2 x 10^-7 M) E2

3.2.1.2. Minimal Medium

- Add the following to 1000mL of Milli-Q water and stir with magnetic stirrer on a hot plate set to 75 °C until all constituents appear dissolved:
  - 13.61g potassium phosphate monobasic (KH$_2$PO$_4$, CAS# 7778-77-0)
  - 1.98g ammonium sulphate ((NH$_4$)$_2$SO$_4$, CAS# 7783-20-2)
  - 4.2g potassium hydroxide (KOH, CAS# 71769-53-4)
  - 0.2g magnesium sulphate (MgSO$_4$, CAS# 7487-88-9)
  - 1mL of 40mg/50mL ferric sulphate (Fe$_2$(SO$_4$)$_3$, CAS# 10028-22-5)
  - 50mg L-leucine (CAS# 61-90-5)
  - 50mg L-histidine (CAS# 71-00-1)
  - 50mg adenine (CAS# 73-24-5)
- 30mg L-tyrosine (CAS# 60-18-4)
- 30mg L-isoleucine (CAS# 73-32-5)
- 30mg L-lysine hydrochloride (CAS# 7274-88-6)
- 25mg L-phenylalanine (CAS# 63-91-2)
- 20mg L-arginine hydrochloride (CAS# 74-79-3)
- 20mg L-methionine (CAS# 63-68-3)
- 100mg L-glutamic acid (CAS# 56-86-0)
- 150mg L-valine (CAS# 72-18-4)
- 375mg L-serine (CAS# 56-45-1)

- Pipette 45mL aliquots into 100mL Kimax bottles
- Autoclave at 121 °C for 10 minutes
- Store sealed containers at room temperature

### 3.2.1.3. Glucose Solution

- Weigh out 20g of (+)-D-Glucose, anhydrous (CAS# 50-99-7), add to 250mL Kimax bottle
- Measure 100mL of Milli-Q water into the Kimax bottle
- Autoclave at 121 °C for 10 minutes
- Store sealed container at room temperature

### 3.2.1.4. Vitamin Solution

- Add the following to 180mL of Milli-Q water in a sterile beaker:
  - 8mg of pyridoxine (CAS# 65-23-6)
  - 8mg of thiamine hydrochloride (CAS# 67-03-8)
  - 8mg (+)-pantothenic acid (CAS# 137-08-6 → D-calcium pantothenate)
- 40mg inositol (CAS# 87-89-8)
- 20mL of 2mg/100mL biotin (CAS# 58-85-5) in Milli-Q water
  - Sterilize the solution by syringe-filtering (using a 0.2µm filter tip) into a 250mL sterilized Kimax bottle
  - Store at 4 °C

3.2.1.5. **L-Aspartic Acid Solution**
- Add 100mg of L-aspartic acid (CAS# 56-84-8) to 25mL of Milli-Q water
- Sterilize the solution by autoclaving at 121 °C for 10 minutes
- Store at room temperature

3.2.1.6. **L-Threonine Solution**
- Add 600mg of L-threonine (CAS# 72-19-5) to 25mL of Milli-Q water
- Sterilize the solution by autoclaving at 121 °C for 10 minutes
- Store at 4°C

3.2.1.7. **Cupric Sulphate Solution**
- Add 320mg of anhydrous cupric sulphate (CAS# 7758-98-7) to 100mL of Milli-Q water in a sterile beaker
- Sterilize the solution by syringe-filtering (using a 0.2µm filter tip) into a 100mL sterilized Kimax bottle
- Store at room temperature

3.2.1.8. **Chlorophenol Red-β-Galactopyranoside (CPRG) Solution**
- Add 100mg of chlorophenol red-β-D-galactopyranoside (CAS# 99792-79-7) to 10mL of absolute ethanol in an amber glass vial
• Store at 4°C

3.2.2. PREPARATION OF HER YEAST CULTURE

3.2.2.1. Day One

• Retrieve 10x concentrated yeast stock from the -20°C freezer; place in a beaker of ice to thaw

• In a laminar flow hood, prepare growth medium as follows, by pipetting solutions into a 45mL sterile aliquot of minimal medium:
  o 5mL glucose solution
  o 1.25mL aspartic acid solution
  o 0.5mL vitamin solution
  o 0.4mL threonine solution
  o 0.125mL cupric sulphate solution

• Vortex mix thawed 10x concentrated yeast stock to suspend cells

• Add 250µL of 10x concentrated yeast stock to the growth medium, and place in incubator/shaker set to 28 °C and 250 rpm

3.2.2.2. Day Two (~ 24 hours after initiating incubation)

• Mix a second batch of growth medium, as per Day 1 instructions

• Using a sterile pipette, transfer 2mL growth medium to a disposable cuvette, and use this as a blank to measure the optical density of the yeast solution; use a second sterile pipette to transfer 2mL of the cultured yeast solution to a disposable cuvette
• Measure the blank-corrected optical density of the yeast stock at 630nm (Genesys 10 UV-Vis Scanning Spectrophotometer or equivalent)

• If \( O_{630nm} \sim 1.0 \), yeast stock is ready to be used (after a 24-hour incubation period, the OD_{630nm} has typically been 0.90 – 1.25)

• To remaining growth solution, add cultured yeast in 0.5mL increments, and check the OD_{630nm} after each addition until an absorbance of \( \sim0.1 \) is achieved (this typically requires approximately 2.5mL \pm 0.5mL of yeast culture) – *note that Routledge and Sumpter (1996) added a fixed volume of yeast culture for each assay; in the modified approach, the volume is adjusted during each run of the assay to account for slight variations in yeast growth in the initial starting culture, as per De Boever et al., 2001*

• Prepare enough bottles of growth solution to fill the amount of microplates that will be used, and add sufficient yeast culture to each to achieve OD_{630nm} of \( \sim0.1 \), and then add 0.5mL of CPRG solution to each bottle; this mixture can now be used to fill the microplates

3.2.3. **Preparation of the Microplates**

• Use 300\( \mu \)L, clear, flat-bottomed microplates (Whatman)

• Prepare a “dummy row” (sufficient to create a quadruplicate set of standard curves) as follows:
  - Autopipette 100\( \mu \)L of absolute ethanol into wells C2-C12 of a plate
  - Autopipette 200\( \mu \)L of E2 working standard into well C1; then withdraw 100\( \mu \)L from this well and add to well C2, fill and discharge
the pipette x5 to mix the contents (this will yield a 2x dilution in well C2)

- Withdraw 100µL of solution from well C2, and add to well C3, charging the pipette x5 to mix; repeat this process across the remaining wells, and discard the final excess 100µL from well C12
- Cover the “dummy row” with lab or autoclave tape, and then – working from the lowest concentration well, C12 – pipette four 10µL aliquots of diluted E2 standard into wells E/F/G/H 12; repeat this process, successively uncovering wells C11 \( \rightarrow \) C1 and adding the solution to the corresponding E/F/G/H wells

- Pipette 100µL of absolute ethanol into each of wells A1 \( \rightarrow \) A12 to create a negative blank
- Allow ethanol/E2 solution to evaporate by placing the microplate in the laminar flow hood
- After addition of yeast culture and incubation, the standard curve/negative control plate should resemble Figure 7.
Figure 7: Example of an ethanol negative control row (top row) and E2 standard curve (bottom four rows)

3.2.3.2. Environmental Extracts

- To create an environmental-sample curve, autopipette 80µL of absolute ethanol across wells 2 → 12 of a row
- Add 80µL of cleaned solvent extract from an environmental sample to well 1 of the row; add another 80µL of extract to well 2, and as with the E2 curve procedure, serially-dilute the contents of well 2 across the remaining wells; repeat as desired to obtain replicate curves
- Allow solvent to evaporate by placing the microplate in the laminar flow hood
3.2.3.3. Incubation of Microplates

- When all solvent has evaporated from individual wells, add 200µL of the combined growth medium/CPRG/yeast mixture to each well in the sample assay microplate.
- Seal the plate(s) with autoclave tape.
- Place plates in the incubator/shaker and tape securely to the shaker plate; incubate at 32 °C with the shaker tray set to 150 rpm, for 72 hours.

3.2.4. Reading the Microplates

- Use a microplate reader (Molecular Devices SpectraMax 190 plate reader with SoftMax Pro 4.3.1 software, or equivalent) to determine the absorbance of the samples at both 630nm (to evaluate sample turbidity) and 575nm (to evaluate colour development) – note that Routledge and Sumpter (1996) evaluated colour development at 540nm, however chlorophenol-red absorbance is higher at 575nm than at 540nm (De Boever et al., 2001).
- Use the absorbance data to calculate the corrected absorbance values as follows:
  - Corrected Absorbance = Test\textsubscript{575 nm} – (Test\textsubscript{630 nm} – Control\textsubscript{630 nm, average})

3.2.5. Preparation and Storage of 10x Concentrated Yeast Stock

3.2.5.1. Day One

- Make growth medium (minimal medium + vitamin solution/etc.) and add 125µL of concentrated yeast stock (stored in the -80 °C freezer) thawed in a beaker of ice in the laminar flow hood.
• Place in incubator at 28 °C with the orbital shaker set to 250 rpm for approximately 24 hours

3.2.5.2. Day Two

• Make more growth medium and add 1mL of yeast from “Day 1” culture per ~50mL (several can be made at one time)
• Incubate at 28°C with the orbital shaker set to 250rpm for approximately 24 hours

3.2.5.3. Day Three

• Transfer each culture made on Day Two to a sterile 50mL centrifuge tube (with closure)
• Centrifuge at 4 °C for 10 minutes at 2000g
• In laminar flow hood, decant supernatant and re-suspend centrifuged yeast pellet in 5mL of a minimal medium/glycerol mixture (45mL minimal medium + 5.5mL glycerol, previously autoclaved at 121 °C/10 minutes)
• Transfer 0.5mL aliquots via autopipette to sterile GC vials (previously autoclaved at 121 °C/10 minutes), cap and crimp, and store at -20 °C
• Yeast stocks stored in this manner are good for 4 months

3.3. Measurement of Carbohydrates and Proteins

3.3.1. Extraction of Sludge/Biosolid Samples

Total protein and carbohydrate content of sludge and biosolid samples was measured after heat-extraction of the solids, a method that has been widely applied to wastewater solids (Kiff and Thompson, 1979; Chang and Lee, 1998; Fan et al., 2006).
• Add ~50mL of liquid sludge sample to a disposable centrifuge tube and centrifuge for 30 min at 2000g (Note: high high-solids-content samples may not require centrifuging; proceed to the next step)

• Discard supernatant and resuspend 0.5g of centrifuged sludge pellets in 10mL of 0.9% saline solution in a Hach vial

• Shake sample ~5 seconds to disperse sludge, and then vortex mix for ~5 seconds per sample

• Place rack of samples in sonication bath (Cole-Parmer, 100W) and sonicate 15 minutes at maximum power

• Place vials in a beaker of boiling water and heat for 1 h at 100°C.

• Plunge samples into an ice bath for 5 minutes to cool off

• Vortex mix each sample ~5 seconds

• Centrifuge for 30 min at 2000g.

• Collect supernatant for carbohydrate/protein analysis; saline extracts can be stored in the -20 °C freezer if not being analyzed the same day

### 3.3.2. **Analysis of Carbohydrates**

The methodology for carbohydrate testing follows that of Raunkjær et al. (1994)

• Dissolve 0.1 g anthrone in 50 mL of 96% sulphuric acid; sulphuric acid solution must be made daily 4 hours before use and mixed with a magnetic mixer in an ice water bath

• Add 2 mL of saline sample extract to 10 mL glass Hach vials; a blank vial containing only 0.9% saline solution is also prepared
• Add 5 mL of anthrone reagent to each vial
• Cap tubes and mix with a vortex mixer for 5 seconds
• Place tubes in a boiling water bath for 14 minutes
• After boiling, place vials in an ice water bath for 5 minutes
• Measure the absorbance of each sample at 625 nm (Genesys 10 UV-Vis Scanning Spectrophotometer or equivalent) using a 1 cm cuvette, using the saline-only sample as a blank
• Calculate the total carbohydrate concentration from a standard curve based on 4 glucose standard solutions (12.5, 25, 50 and 100 mg/L)

3.3.3. **ANALYSIS OF PROTEINS**

The methodology for carbohydrate testing follows that of Raunkjær et al. (1994).

3.3.3.1. **Preparation of Reagents**

• **Reagent A** (143mM NaOH (40g/mol), 270mM Na$_2$CO$_3$, 106g/mol) → to make a volume of 1000mL, measure 5.72g NaOH and 28.62g Na$_2$CO$_3$ and bring to a final volume of 1000mL with Milli-Q water → in order to fully dissolve the solutes, may need to place the mixture on a warm hotplate

• **Reagent B** (57mM CuSO$_4$, MW 159.6g/mol) → to make a volume of 250mL, measure 2.2743g CuSO$_4$ and bring to a final volume of 250mL with Milli-Q water

• **Reagent C** (124mM of sodium tartrate dihydrate, MW 230.1g/mol) → to make a volume of 250mL, measure 6.0171g of Na$_2$C$_4$H$_4$O$_6$ and bring to a final volume of 250mL with Milli-Q water
- Bovine Serum Albumin (BSA) Standard  
  Bring 250mg to a final volume of 500mL with fresh Milli-Q in a volumetric flask, yielding a stock concentration of 500mg/L BSA (note that the BSA doesn’t readily dissolve, and foams when mixed). **Solution must be made fresh weekly.**

- Prepare “reagent D” by mixing reagents A → C as follows: 100mL “A” with 1mL “B” and 1mL “C”

3.3.3.2. **Sample Analysis**

- Add 1.0 mL of sludge/biosolid saline extract and 1.5mL of saline solution to 10mL Hach vials; for the blank, use 2.5mL of 0.9% saline solution only

- Dilute stock 2 N Folin-Ciocalteu Phenol reagent 1:1v/v with distilled water.

- Add 3.5 mL of reagent D to the vials and mix for ~5 seconds with a vortex mixer; let mixture sit at room temperature for 10 minutes

- Add 0.5 mL of diluted Folin-Ciocalteu Phenol reagent to each of the vials, mix immediately with vortex mixer and keep for 45 min at room temperature to develop colour.

- Measure absorbance at 735 nm (Genesys 10 UV-Vis Scanning Spectrophotometer or equivalent) using 1 cm cuvettes, using the saline-only sample as a blank

- Calculate protein concentrations from the standard curves (20, 40, 60 and 100mg/L BSA)

3.4. **Statistical Analyses**

All measures of statistical significance were conducted at the 95% confidence level. The regressions used to construct YES assay response curves for various samples were compared to the response curves of estradiol (E2) standard using the parametric \( F \)-test.
Comparisons between data sets were made using the parametric Student’s $t$-test for data sets that were normally-distributed, and using the non-parametric Mann-Whitney $U$-test for data sets that were not normally-distributed. Outliers in data sets were detected using Grubb’s test.
4. OVERCOMING THE TOXICITY AND ANTAGONISTIC EFFECTS OF MUNICIPAL WASTEWATER SLUDGE AND BIOSOLID EXTRACTS IN THE YEAST ESTROGEN SCREEN (YES) ASSAY

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4.1. INTRODUCTION

Biosolids are the organic, nutrient-rich solids that result from the stabilization of sludge during the engineered wastewater treatment process, and are increasingly accepted as a beneficial-reuse product for agricultural, silvicultural and land-reclamation purposes (Goldstein, 2000; Overcash et al., 2005). However, the potential for endocrine disrupting compounds (EDCs), pharmaceuticals, and other personal-care products to leach from land-applied biosolids into surrounding soil and water bodies has emerged as an issue of concern (Larsbo et al., 2009; Langdon et al., 2010). Estrogenic EDCs in particular are likely to partition to the solid phase during wastewater treatment, due to their moderate-to-high hydrophobicity and organic-carbon partition coefficients (Yamamoto et al., 2003). A considerable amount of
research has been devoted to determining the presence of known and suspected EDCs in wastewater sludges and biosolids via chemical analyses (Citulski and Farahbakhsh, 2010). The quantification of individual endocrine-active analytes does not, however, necessarily give insight into the overall estrogenic activity of a complex sample. The relative estrogenic potency of various EDCs spans several orders of magnitude, and synergistic and/or antagonistic interactions between EDCs and other matrix components can yield net agonistic responses that differ from levels predicted based on concentration-addition models (Rajapakse et al., 2002). Accordingly, various *in vitro* bioassays have been developed to measure the net estrogenic potency of environmental samples. Of these tests, the yeast estrogen screen (YES, Routledge and Sumpter, 1996) is amongst the most robust and widely used in environmental analyses (Leusch et al., 2010).

While the YES assay has been widely used for the study of WWTP influent and effluent streams, its use for the testing of wastewater solids has been less successful. Prior to applying the YES assay, solid samples must undergo an extraction step. This process releases both EDCs and cytotoxic and antagonistic substances from the sample matrix, and the sample extract can lead to yeast cell death when the assay is conducted (WERF/GWRC, 2004; Lorenzen et al., 2006; Teske et al., 2007; McNamara et al., 2009; WERF, 2010). Extensive cleanup procedures are typically required when preparing biosolids extracts for chemical analyses (Kester et al., 2005).

Of the limited studies that appear in the literature in which the YES assay has been applied to biosolids, Teske et al. (2007) and WERF (2010) employ a solid-phase extraction (SPE) step to clean up biosolid extracts and Lorenzen et al. (2006)
use high-pressure liquid chromatography (HPLC), while the remaining studies do not report any cleanup process (Lorenzen et al., 2004; WERF/GWRC, 2004; McNamara et al., 2009). The only study which does not mention extract cytotoxicity is that of Lorenzen et al. (2004), despite the lack of an extract cleanup process. Overall, toxicity confounds the use of the YES assay by making it difficult or impossible to establish dose-response curves, and hence EC$_{50}$ values, as more highly-concentrated points on the curve must be excluded due to cell death. A statistical approach to dealing with incomplete dose-response curves is suggested by WERF (2010), in which estrogenic activity is determined from the lowest sample dilution exhibiting an estrogenic response statistically greater than the baseline. However, the WERF (2010) authors acknowledge that such an approach may increase the risk of false-positive estrogenicity measurements.

To the authors’ knowledge no work has been presented that develops and validates a cleanup process to overcome biosolid toxicity and antagonistic effects in the YES assay, and thereby evaluate estrogenic potency based on full dose-response relationships. Consequently, the aim of this research was to develop a relatively simple yet robust cleanup methodology that would allow for the reliable measurement of net estrogenicity of municipal wastewater sludges and biosolids using the YES assay.

4.2. MATERIALS AND METHODS

4.2.1. SAMPLE COLLECTION

All sampling was conducted at the City of Guelph (ON, Canada) WWTP, which has a maximum treatment capacity of 64,000 m$^3$/d and serves a population of
approximately 125,000 persons. Influent and effluent samples were collected as 24-hour equal-volume composites in refrigerated (4 °C) autosamplers, while sludges/biosolids were collected via grab sampling. Solid and liquid samples were initially collected in 1 L polypropylene Nalgene bottles that had been thoroughly cleaned with DeCon detergent and autoclaved prior to use. Samples were stored at 4 °C for a maximum of two hours after collection prior to transport to the University of Guelph. All samples were then transferred to 1 L amber glass bottles that were silanized prior to use with 1:7 v/v trimethylchlorosilane/ hexane (Sigma-Aldrich, Oakville ON) and acetone-rinsed (HPLC-grade, Fisher Scientific, Oakville, ON). Wastewater samples were solid-phase extracted within 24 hours of arrival at the lab, and solids samples were transferred to a drying oven within three hours of arrival at the lab.

4.2.2. Sample Processing – Liquids

Liquid samples (500 mL) were sequentially filtered through pre-ashed Whatman 934-AH glass fibre filters (1.5µm pore size) and Millipore nitrocellulose membranes (0.45µm pore size), using 5 g of infusorial earth (Fisher Scientific, Oakville, ON), previously baked at 550 °C for two hours, as a filtration aid in each case. 2.5 mL of ethanol was used to aid in desorbing any retained analytes from the filters, for a total volume of 5 mL ethanol per 500 mL sample (1% v/v). Filtered samples were transferred back to silanized amber glass bottles and 1 cm squares of copper foil (Fisher Scientific, Oakville, ON) activated as per US EPA Method 1698 (2007) were added to the filtrate for 30 minutes to aid in sulphur removal. Filtrates were then refrigerated pending solid-phase extraction later the same day.
4.2.3. **Sample Processing – Solids**

Solid samples (100g) were transferred to acetone-rinsed aluminum weighing boats and dried at 105 °C for 16 hours. After drying, solids were homogenized in a glass mortar and pestle with an equal mass of sodium sulphate (Sigma-Aldrich, Oakville, ON), previously baked at 550 °C for two hours. Pulverized samples were transferred to ashed glass sample vials with Teflon-lined caps for storage at -20 °C until extraction.

Accurately-weighed portions of approximately 0.2 g solids-and-sodium sulphate were transferred to 15 mL silanized glass centrifuge tubes. Samples were sequentially extracted with 2x 5 mL ethanol and 2x 5mL ethyl acetate (HPLC grade, Fisher Scientific, Oakville, ON). Extraction was conducted by ultrasonicating the samples at 100 W and 65 °C (Cole Parmer Model 8851, Montréal, PQ) for 15 minutes each cycle, followed by centrifuging the extracts at 2000 g for 5 minutes. The solvent supernatants were transferred to silanized amber glass bottles, and after all four fractions had been collected, 1 cm squares of activated copper foil were added to the extract for 30 minutes. After removing the copper foil, the solvent extracts were concentrated to approximately 2 mL in a hot-water bath set to 80 °C. The extracts were then reconstituted with 250 mL of MilliQ-filtered water (Millipore, Bedford, MA), and refrigerated pending solid-phase extraction later the same day.

4.2.4. **Sample Cleanup**

Filtered wastewater samples and sludge/biosolid extracts were cleaned by solid-phase extraction (SPE). HyperSep C_{18} cartridges with 500 mg bed weight/3 mL volume (Fisher Scientific, Oakville, ON) were sequentially conditioned under gravity flow with 3 mL each of toluene, ethyl acetate, ethanol and MilliQ water. Samples were passed
through the cartridges under vacuum at a flow rate of less than 5 mL/minute using a Supelco Visiprep vacuum manifold (Sigma Aldrich, Oakville, ON). After filtration, the cartridges were dried under vacuum (-2 kPa) for 5 minutes. The cartridges were rinsed by passing 3 mL of 10% v/v methanol/MilliQ water by gravity flow and then further dried at -15° Hg for 30 minutes and were foil-wrapped and stored at -18 °C until extraction.

Analytes were desorbed by passing 3x 3 mL portions of ethanol by gravity through the dried cartridges. The eluates were collected in silanized borosilicate glass test tubes, and were concentrated to near-dryness in a block heater (Techne, Burlington, NJ) set to 95°C under a gentle stream of compressed air filtered through a 0.45 µm pore-size PTFE filter.

Near-dry SPE extracts were solvent-exchanged by adding 2 mL of a 95:5 v/v mixture of hexane/toluene (Optima grade, Fisher Scientific, Oakville, ON), and heating again to near-dryness under a stream of filtered air. This process was repeated a second time, and the final extracts brought up to 1 mL in 95:5 v/v hexane/toluene. Cleanup columns were prepared by dry-packing 1 g of sodium sulphate, 2.5 g of 5%-activated basic alumina, 2.5 g of 5%-activated silica gel (Fisher Scientific, Oakville, ON) and a further 1 g of sodium sulphate into 10 mL disposable syringe barrels. Ten mL of methanol, followed by 10 mL of 95:5 v/v hexane/toluene was passed through the columns. Sample extracts exchanged into hexane/toluene were layered onto the columns and were rinsed with a further 10 mL of 95:5 v/v hexane/toluene. Estrogenic compounds were desorbed from the columns using 20 mL of methanol (Optima grade, Fisher Scientific, Oakville, ON), and the eluate was collected in silanized glass test tubes. The eluate was dried to near-dryness as described above, and finally reconstituted in 2 mL of
ethanol. The cleaned extracts were transferred to amber glass, Teflon-capped GC vials and stored at 4 °C until use.

4.2.5. YES ASSAY

The YES assay was conducted as described by Routledge and Sumpter (1996), using hER-transfected recombinant yeast kindly provided by Prof. Chris Metcalfe (Trent University, Peterborough, ON). All chemicals used for the assay media were 98% or greater purity. Yeast growth was measured as optical density at 630 nm (OD$_{630}$), while colour development was measured as optical density at 575 nm (OD$_{575}$). The following slight modifications were made to the original assay methodology:

- After reaching an OD$_{630}$ of approximately 1.0, yeast culture was added to the assay medium to achieve an OD$_{630}$ of approximately 0.1. The exact volume added varied slightly from run-to-run, depending on the yeast turbidity at the end of the incubation period
- The titre plates were shaken at 150 rpm throughout the 3-day incubation period to promote suspension of the yeast cells and constant mixing with the assay medium

For each test, a negative-control row consisting of 12 wells of 100 µL ethanol was prepared. Standard curves for each test were prepared from positive-control rows of 17-β-estradiol (E2) in ethanol, diluted from 10 nM to 5x10$^{-3}$ nM nominal concentration.

Environmental samples were tested by initially placing 80 µL of sample extract into the first well of a row, and sequentially diluting two-fold with ethanol across the 12 wells in each titre plate row to a final dilution of 1/2048 (i.e. 2$^{11}$ times dilution). Titre plates were scanned after 20 seconds of mechanical shaking using a Molecular Devices SpectraMax
190 plate reader with SoftMax Pro 4.3.1 software. Optical density measurements for E2 standards and environmental samples were transformed as follows:

Corrected OD = \( \text{OD}_{575\text{nm}}(\text{Sample}) - [\text{OD}_{630\text{nm}}(\text{Sample}) - \text{OD}_{630\text{nm}}(\text{Ethanol control, average})] \)

All dose-response curves for standards and environmental samples were calculated in SigmaPlot v. 11 (Systat Software Inc., San Jose, CA) using a four-parameter sigmoidal Hill equation. To obtain estrogenicity values for environmental samples, the logarithm of extract dilution that yield a 50% response was computed from the fitted curve. This value was converted to an E2-Eq per gram of solid as follows:

\[
\text{E}2 = \text{Eq/g} = \left( \frac{\text{E}2 \text{ EC}50 (\text{ng/L})}{\text{EC}50 \text{ dilution factor } \times 0.4} \right) \div \text{mass of biosolid extracted (g)}
\]

The E2 EC50 was calculated from the estradiol standards run with each set of environmental extracts. The factor of 0.4 accounts for the fact that while 80 µL of extract or dilution thereof was placed in each well of the titre plate, the extract was dried out and then reconstituted with 200 µL of yeast growth medium in each well. The method detection limit for this assay, based on E2 standards, was \(4 \times 10^{-3}\) nM E2 (1.1 ng/L as E2).

4.3. RESULTS AND DISCUSSION

In order to accurately determine the estrogenicity of wastewater-derived sample matrices, biodegradation of EDCs must be arrested as effectively as possible. Sludges are particularly susceptible to analyte loss (Gomes et al., 2004; Smyth et al., 2007). Four stabilization treatments were considered in this study – heat drying at 105 °C for 16
hours, lyophilisation at -80 °C, and autoclaving (121 °C/98 kPa) and formaldehyde addition followed by drying at 60 °C for 16 hours. Bacterial inactivation was assessed by observing general bacterial re-growth in samples of mixed primary/thickened waste activated sludge (TWAS) pre- and post-anaerobic digestion, and dewatered anaerobically digested biosolids pre- and post-Lystek treatment (heat + alkaline Pasteurisation), using the method of Lotrario et al. (1995). As shown in Appendix 2, all four treatments inhibited visible bacterial growth at 28 °C over at least a 24 hour period. However, the impact of each pre-treatment on yeast viability in the YES assay was highly different. Yeast growth, measured as cell turbidity at 630 nm is shown in Figure 8 for extracts of mixed primary/thickened waste activated sludge. Following stabilization, samples were extracted as described in Section 4.2.2. and 4.2.3. and cleaned by SPE alone, and the extracts were serially-diluted for YES assay testing. The average yeast turbidity in the ethanol blank (OD_{630nm}) was 0.785 ± 0.098 (n = 12). The formaldehyde-treated sample was toxic to the yeast across nearly the entire dilution range, while the freeze-dried sample extract also had significantly reduced growth in all except the most dilute wells. The autoclaved sample extract was less toxic to the yeast, but yeast growth was nonetheless greatly reduced relative to the heat-stabilized sample. Only the sample dried at 105 °C exhibited yeast growth that was consistently within range of the negative control values. Note that the average yeast turbidities in the more highly-concentrated (right-hand side of the graph) heat-treated and autoclaved samples are higher than the negative control. This stimulatory effect on yeast growth (hormesis) has been described previously in YES assay yeast cultures inoculated in the presence of environmental extracts (Frische et al., 2009). The potential for preservation methods to release cytotoxic
compounds from the sludge matrix or to alter the composition of estrogenic compounds in waste solids has been observed (Aerni et al., 2004; Combalbert et al., 2010). The present work demonstrates that toxicity effects observed when applying the YES assay to wastewater solids arise in part due to the sample stabilization process itself. Based on these results, the decision was made to preserve all samples in this study by drying at 105°C to avoid the introduction of toxic artefacts in the YES assay.

Figure 8: Yeast growth as OD$_{630nm}$ for extracts of mixed primary sludge/TWAS after various stabilization methods, extracts plated in triplicate, error bars are ± 1 S.D. (Dashed black lines = average OD$_{630}$ of 12 ethanol control wells ± 3S.D.)

Preliminary work confirmed that SPE cleanup alone was insufficient to yield reliable estrogenic responses from sludge and biosolids extracts in the YES assay (Teske et al., 2007; WERF, 2010). In addition to cytotoxicity, this lack of apparent estrogenicity
in samples from WWTPs can be due to the masking effects of co-extracted compounds that act as estrogen receptor antagonists (Conroy et al., 2007; Frische et al., 2009). Elemental sulphur is prevalent at high concentrations in municipal WWTP sludges and biosolids (DeWil et al., 2009), and inhibits yeast growth (Okamura et al., 1996). Accordingly, a sulphur removal step using copper foil was added to the extract cleaning process, as was the silica/alumina cleanup step for further removal of toxic and interfering coextracted compounds. This sequence of cleanup steps in advance of bioassay applications was chosen due to the efficacy of similar steps in preparing sludge/biosolid samples for quantitative chemical analyses (Kester et al., 2005).

To test the effects of the extraction and cleanup processes on the recovery of E2, 1 g portions of infusorial earth (pre-baked at 550 °C for two hours) were mixed with 10 mL of the 54.5 µg/L E2 standard used for positive control curves. The slurry was allowed to dry overnight under a stream of nitrogen, and samples of approximately 0.2 g were accurately weighed and solvent-extracted. The extracts were subjected to one of four treatment steps: (i) copper treatment alone, (ii) SPE alone, (iii) silica/alumina cleanup alone or (iv) all three treatments in sequence. The 2 mL portions of cleaned extract resulting from each process would, in theory, have the same E2 concentration as the starting stock solution. Figure 9 shows the YES assay results for each cleaned extract, along with an E2 standard curve.

All four cleanup conditions yielded similar EC50 values ranging from 12.6 to 19.5 ng/L E2, versus an EC50 of 19.2 ng/L for the E2 standard curve. However, in order to meaningfully compare sigmoidal dose-response curves to each other, the curves must be parallel in the central sloped portion of the curve (the Hill slope). To check whether the
response curves obtained for cleaned extracts were comparable to that of the untreated E2 standard, the curves were tested for statistically significant variations using a constrained curve-fit model. While SPE is the most common sample cleanup procedure applied to biosolids in literature studies, it was the only treatment to yield a response curve that was statistically different (F-test, $\alpha = 0.05$) from the E2 positive control. Thus, a combination of all three cleanup steps was judged to be appropriate for further sample analyses.

Figure 9: Response curves for extracts of E2-spiked matrix blank after cleanup steps (extracts plated in triplicate, error bars are ±1 S.D.)

The effect of the cleanup process on raw sludge samples is demonstrated in Figure 10. Raw sludge samples (mixed primary/TWAS) were heat-dried, extracted, and cleaned with either SPE alone or the full copper, SPE, silica/alumina workup. None of
the samples were toxic to the yeast – due to hormesis, yeast growth (as measured by the turbidity of the yeast incubated with sludge extracts) was statistically higher than the ethanol negative controls (Mann-Whitney rank-sum test, P<0.05). However, the samples cleaned by SPE alone elicited no estrogenic response from the YES assay, whereas the fully cleaned extracts exhibited clear dose-dependent estrogenicity. As shown in Figure 11, copper treatment in conjunction with SPE allowed for partial expression of estrogenic activity by the yeast relative to the lack of response that was commonly encountered with SPE alone; however more highly-concentrated extracts still yielded no estrogenic response in the absence of the chromatographic cleanup step.

Figure 10: Estrogenic response from heat-dried mixed primary sludge/TWAS samples – extracts plated in triplicate, error bars are ±1 S.D., grey curves are 95% confidence bands for the regression curves
### Figure 11: Comparison of YES assay results on biosolid extracts with and without chromatography cleanup.

<table>
<thead>
<tr>
<th>Ethanol Blank</th>
<th>SPE-COPPER-CHROMATOGRAPHY-CLEANED EXTRACTS</th>
<th>SPE-COPPER-CLEANED EXTRACTS (CHROMATOGRAPHY STEP OMITTED)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2 Curve</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thickened Waste Activated Sludge (TWAS)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary Anaerobic Digester (fed with 1º sludge &amp; TWAS)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary Anaerobic Digester (fed with 1º/TWAS/Lystek solids)</td>
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<td></td>
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<tr>
<td>Secondary Anaerobic Digester #4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Belt Press thickened solids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lystek digested solids</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Decreasing Concentration**

Ethanol blank and E2 curves are for reference purposes, and have been plated as-is without cleanup steps.
Assessing how completely EDCs are retained in cleanup steps leading up to the YES assay is complicated. Wastewater solids contain a myriad of estrogenic substances and are complex in composition, making it difficult to pick appropriate estrogenic surrogates. In order to determine whether the SPE/copper/chromatography cleanup process yielded representative results, composite wastewater influent and effluent samples were subjected to the same cleanup steps that were applied to solids, followed by the YES assay. Representative response curves for a set of SPE/copper/chromatography-cleaned liquid and solid samples, along with method blanks (sodium sulphate alone extracted in lieu of biosolids, MilliQ water in lieu of wastewater samples) are shown in Figure 12. The assay results for liquid samples showed a median estrogenicity of 49.3 ng/L E2-Eq for influent samples (n = 11 samples collected between August and December, 2010; max. = 65.5 ng/L E2-Eq, min. = 22.1 ng/L E2-Eq) and 2.7 and 1.6 ng/L E2-Eq for effluent samples (collected December 1st and 15th, 2010).

The calculated E2-Eq’s for the influent and effluent in this study are in very close agreement to previous studies. Kirk et al. (2002) report 11-75 ng/L E2-Eq in raw wastewater in the UK, whilst Onda et al. (2002) report 35-72 ng/L E2-Eq in Japanese wastewater, and Svenson et al. (2003) report E2-Eq’s of 1.4-1.8 ng/L in Swedish WWTP effluent, all using the YES assay. The YES assay E2-equivalents in this study also correspond well to the summed estrogenic potency of measured concentrations of EDCs (such as estradiol, estrone, and estriol) reported in Canadian WWTPs, including Guelph’s WWTP, in a study by Lee and Peart (1998). It appears that the cleanup process does not lead to noticeable losses of estrogenic substances for wastewater samples. Conversely, if the cleanup process did lead to the preferential removal of antiestrogenic compounds, the
measured values of net estrogenicity in the effluent and influent would be higher than the comparable reference values from the above-noted studies. Hence it is likely that such compounds in sludge/biosolid extracts are also carried through the cleanup steps.

![Graph showing corrected absorbance at 575 nm against log volume fraction for different samples including influent, effluent, TWAS, Primary AD, Secondary AD, BP AD Solids, Lystek solids, solids blank, and liquid blank. Error bars represent ± 1 S.D.]

Figure 12: YES assay curves for wastewater, sludge/biosolid samples and method blanks post-cleanup – extracts are plated in quadruplicate, error bars are ± 1 S.D. ‘TWAS’ = thickened waste activated sludge, ‘AD’ = anaerobic digester, ‘BP’ = belt-press thickened. All samples collected December 1st, 2010

Reducing the toxicity and inhibitory effects of compounds in sludges and biosolids (whether native to the solids or introduced as an artefact of sample processing) is a critical step in accurately evaluating the estrogenicity of these samples using the YES assay. The few net estrogenic potency values that are currently reported for biosolids are inconsistent. In applying the YES assay to anaerobically digested municipal biosolids, the WERF/GWRC (2004) study reported a potency of 15 ng/g E2-Eq, Lorenzen et al. (2004)
Research conducted by WERF (2010) is the most comprehensive to date in terms of YES measurements of biosolids, yet here too the results were frequently confounded by toxicity and/or inhibition, including samples from almost every sampling point at one of the four WWTPs under consideration. The present study has found that net estrogenicity measurements on raw and biosolid samples are readily obtainable using the YES assay if adequate cleanup steps are taken. Raw TWAS samples \((n = 9)\) analyzed with the YES assay after cleanup had a median estrogenicity of 9.8 ng/g E2-Eq (min. = 3.6 ng/g E2-Eq, max. = 19.0 ng/g E2-Eq). In comparison, anaerobically digested biosolids \((n = 9)\) exhibited statistically higher net estrogenicity, with a median of 17.0 ng/g E2-Eq (min. = 5.8 ng/g E2-Eq, max. = 21.1 ng/g E2-Eq). These concentrations are congruent with the earlier WERF/GWRC (2004) results, and suggest that later studies may have significantly overestimated the potential estrogenic load of municipal biosolids. The apparent increase in net estrogenicity on a unit-mass basis after anaerobic digestion in this study is also consistent with the results of both YES assay (Teske et al., 2007) and chemical analysis (Keller et al., 2003) of digested solids.

4.4. CONCLUSIONS

The purpose of this work was to develop and validate an effective method of sample processing and cleanup of municipal wastewater sludge and biosolid samples to allow for net estrogenicity to be measured using the YES assay. In particular, this method development was focused on overcoming the toxic and inhibitory effects that have been
reported elsewhere when the YES assay was applied to wastewater solids. The results of this study indicate that common methods of sample stabilization, such as autoclaving, lyophilisation and formaldehyde addition, may in fact add to the toxicity of sludge/biosolid extracts. In seeking a balance between retarding the microbial degradation of estrogenic compounds and reliably applying the YES assay, heat drying at 105 °C as soon as possible after sample collection is recommended as the best strategy. The widely used SPE cleanup process is not sufficient to remove toxic compounds and estrogen-receptor antagonists from sludge and biosolid samples. Additional cleanup using copper, silica and alumina did not cause the loss of E2 from spiked blank matrix blanks, and when applied to wastewater influent and effluent samples, the YES results remained very similar to those determined in other studies. Using this cleanup approach raw sludge and digested biosolid sample extracts were not toxic to yeast, and estrogenic dose-response relationships were reliably obtained over a full range of sample dilutions. Based on this approach, the net estrogenicity of treated biosolids was found to be on the low end of the wide range of current literature values. However, this work confirms that the anaerobic digestion process does increase the estrogenic potency relative to raw solids.
5. The Fate of Net Estrogenicity During Municipal Anaerobic and Aerobic Biosolids Treatment – ‘YES’, You Can Measure It

This chapter will be submitted in manuscript form to the journal Environmental Science and Technology, under the authorship of J. Citulski and K. Farahbakhsh

5.1. Introduction

Municipal wastewater treatment plants (WWTPs) produce an average of 115-150 grams of dry digested biosolids for every cubic metre of wastewater treated (FCM/NRC, 2004), with solids treatment and disposal expenses comprising upwards of 50% of the total cost of wastewater treatment (Wei et al, 2003). The beneficial reuse of biosolids via land application in agricultural, land-reclamation and silvicultural settings allows for the recycling of nutrients and organic matter while simultaneously reducing solids disposal costs (Overcash et al., 2005; Singh and Agrawal, 2008). However, acceptance of land-application amongst policy-makers and the public is held back in part due to concerns over the presence of estrogenic endocrine-disrupting compounds (eEDCs) in biosolids (Beecher et al., 2005; Goven and Langer, 2009). The presence of known and suspected eEDCs in wastewater sludges and biosolids has been well-established in multi-site studies (Kinney et al., 2006; US EPA, 2009; WERF, 2010; Langdon et al., 2011), as has the potential for certain EDCs and other micropollutants to leach from biosolids following land application (Lissemore et al., 2006; Lapen et al., 2008; Larsbo et al., 2009). The impact of solids treatment processes on the fate of specific eEDCs, particularly surfactants, has also been the subject of considerable research (Citulski and Farahbakhsh, 2010).
What is not clearly understood is what the total effective endocrine-active potency of biosolids is, nor the impact that individual WWTP unit processes may have in altering the overall endocrine-active potency of solids as they are treated. *In vitro* bioassays have been widely applied in measuring the total - usually estrogenic - potency of wastewater and surface water. Such assays provide a more meaningful picture of the estrogenicity of complex matrices than can be inferred from chemical measurements alone, particularly when the chemical composition of a sample and thus the interactions between compounds are not fully known (Leusch et al., 2010). One of the key *in vitro* bioassays used in the monitoring of estrogenic compounds in the environment is the Yeast Estrogen Screen (YES) assay. The YES assay has been applied to the analysis of biosolids on a far more limited scale than wastewater in large part because sludge and biosolid samples proved to be toxic to the yeast culture used in the assay (GWRC/WERF, 2004; Lorenzen et al., 2004; Teske et al., 2007; McNamara et al., 2009; WERF, 2010). These studies have presented net estrogenicity values for anaerobically digested municipal biosolids ranging from 15ng/g estradiol-equivalents (E2-Eq; GWRC/WERF, 2004) to over 300,000ng/g E2-Eq (Teske et al., 2007). This is but an indication of the difficulties inherent in processing and analysing samples of biosolids for net estrogenicity using the YES assay.

In the present study, a set of enhanced sample extraction and cleanup steps were used to mitigate the toxicity of sludge and biosolid samples and thus obtain consistent dose-response curves when using the YES assay. Net estrogenicity measurements were obtained for solids at each unit process in the treatment train of two full-scale WWTPs, one employing mesophilic anaerobic solids digestion, and the other employing an aerobic membrane-bioreactor (MBR) thickening system.
5.2. MATERIALS AND METHODS

5.2.1. SITE DESCRIPTION AND SAMPLE COLLECTION

Sampling was conducted at the City of Guelph (ON, Canada) WWTP and the City of London (ON, Canada) Oxford Water Pollution Control Plant. Details of the two plants are provided in Table 12 and process flow diagrams showing sampling points are provided in Figure 13. Sampling was conducted biweekly in Guelph, between August 25, 2010, and December 15, 2010. Samples were collected in London on three successive weeks between April 5th and 19th, 2011. At the time of this study, the Guelph WWTP was pilot-testing the Lystek process for enhanced biosolids treatment. Lystek treatment involves a sequencing batch reactor where heat and alkaline chemicals are used to convert dewatered anaerobically-digested solids into a pathogen-free liquid that meets US EPA ‘Class A’ standards (US EPA, 2006). Solids were sampled from two of the Guelph WWTP’s four primary anaerobic digesters, one of which (Digester A) processed a mixture of thickened primary and waste-activated sludge (WAS), the other of which (Digester B) processed a recycled stream of Lystek-treated solids along with thickened primary and WAS. Other key differences between Digester A and Digester B are summarized in Table 11.

Table 11: Overview of differences between Guelph WWTP’s mesophilic anaerobic digester A & B

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Digester A</th>
<th>Digester B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solids retention time (SRT); days</td>
<td>14</td>
<td>16.5</td>
</tr>
<tr>
<td>Average TS content of digestate; % ± S.D.</td>
<td>2.2 ± 0.2</td>
<td>3.7 ± 0.2</td>
</tr>
<tr>
<td>Average VS content of digestate solids; % ± S.D</td>
<td>56.1 ± 2.1</td>
<td>48.9 ± 1.2</td>
</tr>
</tbody>
</table>
Table 12: Overview of sample site operating conditions

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Guelph WWTP</th>
<th>Oxford WPCP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rated flow (m$^3$/day)</td>
<td>64,000</td>
<td>17,250</td>
</tr>
<tr>
<td>Average flow (m$^3$/day)</td>
<td>55,000</td>
<td>8,800</td>
</tr>
<tr>
<td>Influent source composition</td>
<td>60% residential, 40% industrial/commercial/institutional</td>
<td>100% residential</td>
</tr>
<tr>
<td>Average sludge production (m$^3$/day)</td>
<td>457</td>
<td>54</td>
</tr>
<tr>
<td>Average SRT during secondary aerobic treatment (days)</td>
<td>8</td>
<td>12.5</td>
</tr>
<tr>
<td>Solids treatment process description</td>
<td>Mixture of primary and thickened WAS fed to 4 mesophilic (35-37 °C) anaerobic digesters, SRT ~ 14 to 16.5 days; digestate transferred to secondary anaerobic digester, SRT ~ 7 days; solids dewatered via belt press, landfilled; Lystek advanced heat/alkaline treatment at the pilot scale</td>
<td>1° solids thickened in the primary clarifier; WAS thickened in a batch-fed aerobic membrane bioreactor, SRT ~ 16 hours; mixture of thickened 1° sludge/TWAS trucked off-site for fluid-bed incineration</td>
</tr>
</tbody>
</table>

Unless otherwise noted, influent and effluent samples were collected as 24-hour equal-volume composites in refrigerated (4 °C) autosamplers, while all other samples were collected via grab sampling. Samples were initially collected in 1 L polypropylene Nalgene bottles that had been thoroughly cleaned with DeCon detergent and autoclaved prior to use. Samples were stored at 4 °C for a maximum of two hours after collection prior to being transported in coolers on ice to the University of Guelph. All samples were then transferred to 1 L amber glass bottles that were silanized prior to use with 1:7 v/v trimethylchlorosilane/ hexane (Sigma-Aldrich, Oakville ON) and acetone-rinsed (HPLC-grade, Fisher Scientific, Oakville, ON). Liquid samples were solid-phase extracted within
24 hours of arrival at the lab, and solid samples were transferred to a drying oven within three hours of arrival at the lab.

5.2.2. **Analytical Methodologies**

5.2.2.1. **YES Assay**

Sample extraction and cleanup steps prior to the YES assay are described in Chapter 4. In brief, solid samples were heat dried at 105 °C, pulverized in a mortar and pestle with sodium sulphate, and solvent extracted using ethanol and ethyl acetate in an ultrasonicator at 65 °C and 100W; liquid samples were filtered through glass fibre filters with diatomaceous earth as a filter aid. Filtered liquid samples and sludge/biosolid solvent extracts were treated with activated copper to remove sulphur, and then cleaned using reverse-phase 3mL/500mg C<sub>18</sub> solid-phase extraction (SPE) cartridges sequentially preconditioned with 3mL each of toluene, ethyl acetate, ethanol and MilliQ water. Target analytes were desorbed from the SPE cartridges using ethanol, solvent-exchanged into 95:5% v/v hexane/toluene, and further cleaned using layered alumina/silica chromatography columns. Methanol was used to elute estrogenic compounds from the columns, and the eluate was evaporated to near-dryness under a stream of filtered air in a block heater. The near-dry extracts were reconstituted in ethanol, and the cleaned extracts were then analyzed for net estrogenicity using the YES assay. The YES assay procedure of Routledge and Sumpter (1996) was followed, and further details on test conditions and data analysis are provided in Chapter 4. Yeast growth was measured as optical density at 630 nm (OD<sub>630</sub>), while colour development (production of chlorophenol-red due to β-galactosidase activity) was measured as optical density at 575 nm (OD<sub>575</sub>). Each sample
extract was run in quadruplicate in the YES assay. Optical density measurements for E2 standards and environmental samples were transformed as follows:

\[
\text{Corrected OD} = \text{OD}_{575\text{nm}}(\text{Sample}) - [\text{OD}_{630\text{nm}}(\text{Sample}) - \text{OD}_{630\text{nm}}(\text{Ethanol control, avg.})]
\]

### 5.2.2.2. Total/Volatile Solids, Protein and Carbohydrate Analyses

The total and volatile solids contents of sludge and biosolid samples and total suspended/volatile solids of liquid samples were measured in accordance with Standard Method 2540 (Eaton et al., 1998). Total protein and carbohydrates were analyzed using the Lowry and anthrone methods, respectively, as per Raunkjær et al. (1994). Total proteins were quantified using bovine serum albumen (BSA) as the reference standard, and total carbohydrates were quantified against glucose as a reference standard. Solid samples were heat-extracted in 0.9% w/v saline solution (Chang and Lee, 1998) and the extract was used for protein and carbohydrate testing, while liquid samples were analyzed directly. Lystek samples could not be analyzed for protein or carbohydrate content due to the poor settleability of fine solids in the saline extract, even after extensive centrifugation.

### 5.2.2.3. Chemical Analysis of Selected Natural and Industrial eEDCs

Chemical analysis of natural human estrogens (17β-estradiol, E2; and estrone, E1) and a synthetic analogue, 17α-ethinylestradiol (EE2) and industrial eEDCs (4-octylphenol, 4-OP; 4-\(n\)-nonylphenol, 4-NP; and bisphenol-A, BPA) was conducted on select sample extracts at the Worsfold Water Quality Centre, Trent University (Peterborough, ON), by Dr. James Yuan. Extracts were analyzed by liquid chromatography/tandem mass spectrometry (LC-MS/MS) using an ABSciex QTrap 5500 mass spectrometer system in negative ion mode using multiple-reaction monitoring.
Instrument control, data acquisition and processing were performed with Analyst 1.5 software. Chromatographic separation was conducted on a Dionex Ultimate 3000 HPLC system with a Genesis C$_{18}$ column (150 × 2.1 mm i.d., 4µm) at a flow rate of 0.20-0.22 mL/min. The mobile phases A and B were MilliQ water and methanol, respectively. The applied gradient elution was as follows: mobile phase B is increased from 60% to 97% over 4 minutes, held for 6 minutes, and then decreased from 97% to 60% over 2 minutes and held for 3 minutes. The column was kept at room temperature and the injection volume was 20 µL.
Figure 13: WWTP Process flow diagrams and sampling points for the Oxford (above) and City of Guelph (below) WWTPs
5.3. **RESULTS AND DISCUSSION**

5.3.1. **WASTEWATER AND SLUDGE CHARACTERISTICS**

Properties of the solid and liquid samples collected in this study are presented in Table 13. Although the two WWTPs in this study receive wastewater of very different source composition (Table 12), the average total and volatile suspended solids levels as well as protein and carbohydrate concentrations in the two influent streams were statistically indistinguishable (Students’ t-test, \( \alpha = 0.05 \)). A further key similarity between the two sites was the net estrogenic potency of the influent streams entering the plants, as measured by the YES assay, which averaged 46.9 ± 18.1 ng/L E2-Eq at the Guelph WWTP and 59.4 ± 4.1 ng/L E2-Eq at the Oxford WPCP. These values are congruent with those obtained in other studies for wastewater influents using both the YES assay and other *in vitro* tests of net estrogenicity (Kirk et al., 2002; Murk et al., 2002; Svenson et al., 2003; Leusch et al., 2006).
Table 13: Wastewater and sludge/biosolids characteristics; values in parentheses are 1 S.D.; ‘n/d’ = not detected, ‘n/a’ = not analyzed

<table>
<thead>
<tr>
<th>SOLID SAMPLES</th>
<th>Guelph WWTP</th>
<th>London - Oxford WPCP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total Solids (%)</td>
<td>Volatile Solids (% of Total Solids)</td>
</tr>
<tr>
<td>Primary sludge, $n = 9$</td>
<td>3.5 (0.2)</td>
<td>73.9 (1.8)</td>
</tr>
<tr>
<td>Thickened WAS, $n = 8$</td>
<td>3.3 (1.0)</td>
<td>69.6 (3.5)</td>
</tr>
<tr>
<td>Primary anaerobic digester A, $n = 9$</td>
<td>2.2 (0.2)</td>
<td>56.1 (2.1)</td>
</tr>
<tr>
<td>Primary anaerobic digester B, $n = 9$</td>
<td>3.7 (0.2)</td>
<td>48.9 (1.2)</td>
</tr>
<tr>
<td>Secondary anaerobic digester, $n = 9$</td>
<td>2.6 (0.5)</td>
<td>53.6 (3.0)</td>
</tr>
<tr>
<td>Belt press solids, $n = 8$</td>
<td>23.4 (1.5)</td>
<td>57.3 (3.4)</td>
</tr>
<tr>
<td>Lystek solids, $n = 8$</td>
<td>15.2 (1.0)</td>
<td>51.6 (1.9)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>LIQUID SAMPLES</th>
<th>Guelph WWTP</th>
<th>London - Oxford WPCP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total Suspended Solids (mg/L)</td>
<td>Volatile Suspended Solids (mg/L)</td>
</tr>
<tr>
<td>Belt press filtrate (grab), $n = 9$</td>
<td>3249.6 (2198.1)</td>
<td>1563.6 (1138.6)</td>
</tr>
<tr>
<td>Influent (24 hr. composite), $n = 9$</td>
<td>259.3 (57.5)</td>
<td>225.6 (47.2)</td>
</tr>
<tr>
<td>Effluent (24 hr. composite), $n = 2$</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>London - Oxford WPCP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Influent (24 hr. composite), $n = 3$</td>
<td>291.7 (35.5)</td>
<td>265.0 (34.9)</td>
</tr>
<tr>
<td>MBR permeate (grab), $n = 3$</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>MBR thickener permeate (grab), $n = 2$</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td>Final effluent, post-UV, $n = 3$</td>
<td>n/d</td>
<td>n/d</td>
</tr>
</tbody>
</table>
5.3.2. YES ASSAY TECHNIQUES AND VALIDATION

Dose-response curves for YES assay data were fitted by a four-parameter nonlinear logistic model using the Marquardt-Levenberg algorithm (SigmaPlot 11.0, Systat Software Inc., Chicago, IL), with turbidity-corrected colour production at 575nm plotted on the ordinate and the logarithm of sample extract volume-fraction plotted on the abscissa. Sample estrogenicity was calculated based on EC50 values from E2 standard curves, which were run in quadruplicate alongside each set of samples. EC50 values and the $r^2$ value for each E2 standard curve are presented for each run in Table 14. Prior to fitting dose-response curves to the absorbance data for environmental samples, yeast growth (OD630nm) was checked to detect potential toxic effects resulting from the biosolids/wastewater extracts. Yeast growth was compared to the average turbidity (±3 standard deviations) of 12 ethanol negative-control wells that were plated in parallel with each set of samples. If the sample’s yeast growth was below the (average – 3S.D.) turbidity value for the negative control, the datum was excluded from the dose-response curve analysis.

Due to the sample cleanup process used in preparing sample extracts (Chapter 4), low yeast growth was observed in less than 0.5% of the sample wells, and an example of a typical sample microplate is shown in Figure 14. However, turbidity values greater than the (average + 3S.D.) value for the negative control were observed in concentrated sample-extract wells (typically x1, x2, x4 and occasionally x8 dilution) for some sludge/biosolid extracts. This stimulation of yeast growth was likely the result of hormesis (Stebbing, 1982), which has been described previously in YES assay yeast cultures inoculated in the presence of environmental extracts (Frische et al., 2009).
Sample wells in which hormesis was observed were included in the calculation of dose-response curves. Field blanks, consisting of one-litre samples of MilliQ water, and biosolid extraction blanks, consisting of Na$_2$SO$_4$ baked at 550 °C for two hours prior to extraction were also analyzed for estrogenicity. As shown in Figure 15, none of the blank samples elicited an estrogenic response from the YES assay.

Figure 14: Example of YES assay medium after 72-hour incubation with biosolid ethanol extracts; two different biosolid samples are plated in quadruplicate rows, numbers below each column refer to the times-dilution of the extract. Note the concentration-dependent fuchsia colour production, indicative of the presence of estrogenic compounds in the extracts.
Figure 15: YES assay results for field and extract blanks. Error bars are ± 1S.D. Each data point for field/extract blanks is the average of 4 samples. E2 standard curve and 95% confidence band (in grey) are based on the six sets of E2 standards run in quadruplicate in parallel with the field/extract blanks, therefore each data point is the average of 24 measurements.

5.3.3. YES ASSAY – SAMPLING RESULTS

Results of the YES assay for each sample collected in this study are presented in Table 14, and are summarized in Figure 16. What is immediately noteworthy in the results for the Guelph WWTP is that there is little apparent change in the net estrogenicity of solids between the unit processes of the anaerobic treatment train. The average net estrogenic potency on a unit basis was roughly double in the thickened WAS compared to the settled primary sludge. Given the hydrophobicity of most eEDCs (see Chapters 1 and 2), and the hydrophobic nature of microbial flocs and colloids produced...
during the activated-sludge process (Liao et al., 2001) this unit-mass increase was likely due to the partitioning of eEDCs from the liquid phase to the WAS. It is also possible that bacteria in the activated sludge converted natural hormones excreted by the human body as inactive derivatives back to their original active form (Baronti et al., 2000), or degraded weakly-estrogenic surfactant molecules into more estrogically-active metabolites (WERF, 2010).

Primary and thickened WAS are the sole feed source for primary anaerobic Digester A, and are augmented with Lystek-treated solids in anaerobic Digester B. Despite the differences in feed composition and SRT between the two digesters, the net estrogenicity of the two sets of digestate was not statistically different (Mann-Whitney ranked sum test, α = 0.05; P = 1.00), but was significantly higher in the digested solids on a unit basis compared to the primary sludge/WAS. As is discussed in further depth later in this chapter, there are a number of known and hypothesized mechanisms by which the anaerobic digestion process increases the estrogenticity of sludges. On average, there was no difference in the net estrogenicity of the solids from the primary and secondary anaerobic digesters. The secondary digestate was dewatered using belt-presses to yield the equivalent of US EPA ‘Class B’ biosolids, and on a unit basis this was similar in net estrogenic potency to the primary/secondary digestate. While the average unit net estrogenicity of Lystek-treated ‘Class A’ solids was slightly lower than that of the dewatered starting material (14.7 ± 5.5 ng/g versus 17.6 ± 7.3 ng/g E2-Eq), this difference was not statistically significant (Students’ t-test, α = 0.05; P = 0.377). Comparisons between sampling points at the London-Oxford plant are complicated by the fact that net estrogenicity values at all of the solid samples were considerably lower
than those at the Guelph plant, and frequently non-detectable. Interestingly, though net estrogenicity was measured in all three primary sludge samples collected at London-Oxford, these samples averaged less than half of the net estrogenicity of the Guelph primary sludge samples. This is despite the fact that the net estrogenicity of the London-Oxford influent was higher than that of the Guelph influent.
Table 14: YES Assay net estrogenicity measurements; “E2 EC\textsubscript{50}” refers to the EC\textsubscript{50} value for the E2 standard curve run with each set of samples, from which sample EC\textsubscript{50} values were calculated.

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<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Influent (composite)</td>
<td>38.8</td>
<td>94.0</td>
<td>35.6</td>
<td>53.7</td>
<td>43.3</td>
<td>34.5</td>
<td>59.2</td>
<td>41.9</td>
<td>n/a</td>
<td>50.7</td>
<td>46.9</td>
<td>18.1</td>
</tr>
<tr>
<td>Primary sludge</td>
<td>5.6</td>
<td>6.3</td>
<td>5.1</td>
<td>7.1</td>
<td>4.4</td>
<td>5.2</td>
<td>3.5</td>
<td>5.9</td>
<td>n/a</td>
<td>3.3</td>
<td>3.5</td>
<td>1.1</td>
</tr>
<tr>
<td>Thickened WAS</td>
<td>16.6</td>
<td>14.0</td>
<td>n/a</td>
<td>10.1</td>
<td>13.3</td>
<td>6.9</td>
<td>12.0</td>
<td>12.1</td>
<td>n/a</td>
<td>3.6</td>
<td>11.1</td>
<td>4.1</td>
</tr>
<tr>
<td>Primary anaerobic digester &quot;A&quot;*</td>
<td>12.5</td>
<td>8.4</td>
<td>5.8</td>
<td>21.3</td>
<td>16.4</td>
<td>14.7</td>
<td>18.7</td>
<td>40.0</td>
<td>n/a</td>
<td>17.0</td>
<td>17.2</td>
<td>9.9</td>
</tr>
<tr>
<td>Primary anaerobic digester &quot;B&quot;**</td>
<td>15.3</td>
<td>16.3</td>
<td>7.1</td>
<td>20.2</td>
<td>13.3</td>
<td>6.9</td>
<td>12.0</td>
<td>12.1</td>
<td>n/a</td>
<td>17.0</td>
<td>18.8</td>
<td>9.7</td>
</tr>
<tr>
<td>Secondary anaerobic digester</td>
<td>15.7</td>
<td>20.2</td>
<td>10.4</td>
<td>21.5</td>
<td>25.8</td>
<td>8.9</td>
<td>19.7</td>
<td>21.6</td>
<td>n/a</td>
<td>10.1</td>
<td>17.1</td>
<td>6.1</td>
</tr>
<tr>
<td>Belt press solids</td>
<td>10.7</td>
<td>23.1</td>
<td>9.1</td>
<td>25.6</td>
<td>n/a</td>
<td>13.4</td>
<td>19.8</td>
<td>29.1</td>
<td>17.7</td>
<td>10.2</td>
<td>17.6</td>
<td>7.3</td>
</tr>
<tr>
<td>Belt press filtrate</td>
<td>53.6</td>
<td>89.2</td>
<td>159.8</td>
<td>115.6</td>
<td>75.5</td>
<td>56.2</td>
<td>98.1</td>
<td>88.2</td>
<td>n/a</td>
<td>78.3</td>
<td>90.5</td>
<td>32.4</td>
</tr>
<tr>
<td>Lystek solids</td>
<td>12.0</td>
<td>12.7</td>
<td>n/a</td>
<td>19.5</td>
<td>24.4</td>
<td>8.8</td>
<td>17.1</td>
<td>n/a</td>
<td>15.1</td>
<td>8.5</td>
<td>14.7</td>
<td>5.5</td>
</tr>
<tr>
<td>Effluent (composite)</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>2.7</td>
<td>n/a</td>
<td>1.6</td>
<td>2.1</td>
<td>0.8</td>
</tr>
</tbody>
</table>

| E2 EC\textsubscript{50} (ng/L) | 12.0 | 19.8 | 12.9 | 30.8 | 11.2 | 9.7 | 15.5 | 26.2 | 26.2 | 12.0 | 17.6 | 7.6 |

<table>
<thead>
<tr>
<th>LONDON-OXFORD WPCP</th>
<th>Apr. 5</th>
<th>Apr. 12</th>
<th>Apr. 19</th>
<th>Avg.</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Influent (composite)</td>
<td>64.0</td>
<td>58.1</td>
<td>56.1</td>
<td>59.4</td>
<td>4.1</td>
</tr>
<tr>
<td>Primary sludge</td>
<td>2.8</td>
<td>2.4</td>
<td>1.4</td>
<td>2.2</td>
<td>0.7</td>
</tr>
<tr>
<td>Mixed liquor suspended solids</td>
<td>n/d</td>
<td>1.6</td>
<td>n/d</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Aeration tank WAS</td>
<td>n/d</td>
<td>1.2</td>
<td>1.4</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>MBR tank WAS</td>
<td>n/d</td>
<td>2.0</td>
<td>1.5</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>MBR permeate (grab)</td>
<td>0.2</td>
<td>0.3</td>
<td>0.2</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>MBR thickened WAS</td>
<td>n/d</td>
<td>n/a</td>
<td>2.2</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>MBR thickener permeate (grab)</td>
<td>0.2</td>
<td>n/a</td>
<td>0.2</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Post-UV effluent</td>
<td>0.3*</td>
<td>0.4*</td>
<td>0.2**</td>
<td>0.3</td>
<td>0.1</td>
</tr>
</tbody>
</table>

- E2-Eq values are in ng/L for liquid samples, ng/g (dry weight) for solid samples
- “n/d” → not detected
- “n/a” → not analyzed; sample could not be collected and therefore no analysis was conducted
- *Mixture of primary and thickened WAS
- **Mixture of primary and thickened WAS and Lystek-digested solids
- *Grab sample
- **24 hr. Composite sample

| E2 EC\textsubscript{50} (ng/L) | 8.5 | 8.0 | 7.2 | 7.9 | 0.7 |

| E2 Curve Regression r\textsuperscript{2} | 0.980 | 0.969 | 0.983 |
Figure 16: Summary of YES assay net estrogenicity measurements; ‘AD’ = anaerobic digester, error bars are ± 1S.D.
5.3.4. **Chemical Analysis of Selected EEDCs**

Estrone (E1) was detected in thickened WAS and dewatered digested biosolid samples at low (1-2 ng/g) levels. E2, EE2, 4-NP, 4-OP and BPA were not detected in the WAS or treated solids. In comparison, Ternes et al. (2002) measured E1 at 2-16 ng/g and E2 at 9-49 ng/g in anaerobically digested solids, and Andersen et al. (2003) measured E1, E2 and EE2 at 7 ng/g, 1.7 ng/g and 3 ng/g respectively in TWAS and 25.2 ng/g, 5.1 ng/g and non-detectable, respectively, in anaerobically digested solids. Matrix effects caused by interfering compounds that were co-extracted from the solid samples were observed in the chromatograms. These effects likely led to the inability to detect eEDCs, which is a particularly common problem when environmental samples are analyzed using LC-MS/MS (Zhao and Metcalfe, 2008; Comerton et al., 2009). In comparison to the anaerobically-treated solids from the Guelph WWTP, the solids from the London-Oxford WPCP exhibited considerably lower net estrogenic activity, with no detectable estrogenicity in five of the 11 aerobically-treated samples. Due to the matrix effects encountered during chemical analysis of the Guelph samples, the samples from London were not analyzed by LC-MS/MS.

5.3.5. **Comparison of Current Results to Previous Studies**

In previous studies where the YES assay was applied to sludge/biosolid samples, toxic interferences have led to inconclusive results (Teske et al., 2007; McNamara et al., 2009; WERF, 2010). In other instances toxicity has necessitated the calculation of E2-equivalents based on sample estrogenic responses that are less than the EC_{50} of the positive control (Lorenzen et al., 2004; WERF, 2010), which may increase the chance of false-positive results. In the earliest study of estrogenicity during biosolids treatment, E2-
Eq values were calculated based on a single concentration-factor of sample extract, rather than establishing full dose-response relationships across a range of dilutions (Holbrook et al., 2002). The current study expands upon and complements these earlier endeavours by providing YES assay data that are derived from EC\textsubscript{50} values based on full sample dose-response curves in the absence of toxic interferences. The intensity of sample collection, particularly at the Guelph WWTP, greatly exceeds that of any previous studies.

The volume of sampling conducted in this work helps to address a research gap articulated in the WERF (2010) report; namely that focused sampling at high frequency, with concomitant monitoring of plant operating parameters, such as flow, suspended solids and nutrient concentrations, was required. Such a strategy was deemed necessary to better assess whether the variations in estrogenic potencies observed in previous studies were due to variations in plant performance or reflective of difficulties in sample analysis. The relative consistency of the results obtained for each of the sampling points considered in this study contrasts with the orders-of-magnitude ranges reported in previous work.

The average net estrogenicity of mesophilic anaerobically-treated biosolids in this study – 17.2 ± 9.9 ng/g E2-Eq for digested primary sludge/WAS, 18.8 ± 9.7 ng/g E2-Eq for digested primary sludge/WAS/Lystek – is in good agreement with the results presented by GWRC/WERF (2004), who reported an average of 15 ± 9 ng/g E2-Eq in five samples treated by mesophilic anaerobic digestion. Holbrook et al. (2002) reported slightly higher levels of 23 ± 5.6 to 57 ±8.5 ng/g E2-Eq in samples from two mesophilic anaerobic digesters operating at a municipal WWTP. In contrast, Lorenzen et al. (2004) reported an average net estrogenic potency of 1,233 ng/g E2-Eq in 13 samples of
anaerobically-digested biosolids from Ontario WWTPs. Teske et al. (2007) reported 299,000 ng/g EE2-Eq in anaerobically-digested biosolids from a WWTP in Arizona, with EE2 exhibiting an estrogenic potency ranging from equal to double that of E2 in the YES assay (Johnson and Sumpter, 2001). Similarly, the WERF (2010) study reported estrogenic loads that varied over nearly three orders of magnitude between sampling dates within the same WWTPs, albeit the report acknowledged that toxicity effects frequently compromised the YES assay.

5.3.6. **COMPARISON OF ANAEROBICALLY- AND AEROBICALLY-TREATED SLUDGE**

Compared to anaerobic digestion, aerobic sludge treatment processes generally have been reported to be more effective in reducing the net estrogenicity of wastewater solids. In the present study, of the Oxford WPCP mixed liquor suspended solids, waste activated sludge and MBR recycled activated sludge, the highest net estrogenicity value measured was 2.0 ng/g E2-Eq. The thickened aerobic MBR solids were equally low in net estrogenicity. The low levels of net estrogenicity measured in this study with the MBR system are similar to the results of Coleman et al. (2009), who reported 1.63 ± 0.24 ng/g E2-Eq in MBR biomass. Coleman et al. (2009) attributed the enhanced removal of estrogenicity during MBR treatment to the longer SRT versus the conventional activated sludge (CAS) process. While the MBR system in the present study was operated in a 16-hour batch-feed mode, i.e. an SRT less than that of the CAS treatment train, the CAS process at London-Oxford did indeed operate at a higher SRT than at the Guelph WWTP, which averaged 12 and 8.5 days, respectively. The net estrogenicity values reported by Teske et al. for MBR solids (12.2 ng/g EE2-Eq) and by Lorenzen et al. for aerobically-
digested municipal biosolids (average of 11.3 ng/g E2-Eq for four samples) were considerably lower than the comparable results for anaerobically-treated solids in their studies. The net estrogenicity of biosolids from three parallel thermophilic aerobic digesters in the study of Holbrook et al. (2002) ranged from 4.2 ± 0.7 to 11 ± 1.5 ng/g E2-Eq.

In comparing levels of net estrogenicity in WWTP effluent to that of the influent, the overall removal of eEDCs across the wastewater treatment process is consistently higher than 80% (Körner et al., 2000; Murk et al., 2002; Leusch et al., 2006; Muller et al., 2008; Li et al., 2010). The results of this study show equally high overall removal rates, with an average of 95% removal of net estrogenicity at the Guelph WWTP and 99% removal at the London-Oxford WPCP, based on influent-to-effluent calculations. The higher removal rate for net estrogenicity at the London plant may be a consequence of the longer SRT in the CAS process (12 days at London-Oxford plant versus 8.5 days at Guelph), or a comparative lack of refractory industrial eEDCs. One of the main criticisms, though, of influent-to-effluent comparisons has been that they may not give a true picture of the net “removal” of eEDCs if such compounds are potentially just being partitioned to wastewater solids (WERF, 2010).

When the focus is shifted to examine the removal of eEDCs from sludge during unit treatment processes, the picture becomes less straightforward. Based on chemical analyses, Muller et al. (2010) report a five-fold increase in E1 concentrations in WAS compared to primary sludge. Concentrations of E1 and E2 increased by a factor of four and three, respectively, when WAS was anaerobically digested (Andersen et al., 2003), while long-chain alkylphenol polyethoxylate (APnEO) surfactant molecules were
converted to more estrogically-potent nonylphenol (NP) during anaerobic digestion 
(Keller et al., 2003; Minamiyama et al., 2006). However, aerobic digestion can 
completely remove APnEOs (Hernandez-Raquet et al., 2007). Aerobic post-treatment of 
aerobically-digested biosolids has also been shown to quantitatively remove NP and 
APnEOs (Hernandez-Raquet et al., 2007) and to lower net estrogenicity (McNamara et 
al., 2009). Muller et al. (2010) found that the concentration of E2 and EE2 increased 
during the dewatering of anaerobically-digested solids, and suggested that overall rate of 
removal of E1/E2/EE2/E3 across the entire full-scale sludge treatment process is <40%.

5.3.7. NET ESTROGENICITY BALANCE

Table 15 presents a ‘net estrogenicity balance’ for the Guelph WWTP, based on 
flowrate and sludge production data provided by WWTP staff, solids content data shown 
in Table 13, and the net estrogenicity data from Table 14. The median reduction of 
estrogenicity in the solid phase, calculated on the difference in estrogenic load in the 
aerobic digester feed (primary sludge + WAS) versus the dewatered digestate, was 
48% which is comparable to the reduction in E1/E2/EE2/E3 reported by Muller et al. 
(2010). However, the percent-reduction between the digester influent and effluent ranged 
from just under 20% to just over 60%. Holbrook et al. (2004) did find a marginal 
relationship between the protein and carbohydrate content of wastewater colloids and the 
extent to which E2 and EE2 sorbed to solids. In this study there was no discernable 
relationship between either the protein or the carbohydrate content of the solid or liquid 
streams and the corresponding estrogenicity of the sample.

This is illustrated in Figure 17 for influent samples and undigested solids (TWAS) 
and digested solids from Guelph’s secondary anaerobic digester. The protein and
carbohydrate contents of wastewater solids may serve as a partial indicator of the sorption affinity of specific compounds, but they do not predict net estrogenic content. It is important to bear in mind that the YES assay provides a measure of the net estrogenic potency of a sample, i.e. the potency of all agonistic compounds that are present, less the impact of any antagonistic or inhibitory compounds that may also be present. Though the sample preparation and cleanup steps used in this study have been shown to minimize inhibitory effects, the net estrogenicity of the samples is still likely to be affected by the presence of antagonistic substances that are present in wastewater (Conroy et al., 2007; Buckley, 2010). Nonetheless, the data in Table 15 give insight into the effective daily load of net estrogenicity entering the Guelph WWTP, and exiting in the biosolids and the finished effluent. Note that although sludges and biosolids were collected as grab samples in this study, the SRT’s through the activated sludge and anaerobic digestion processes inherently provide for a time-averaged composite sample (WERF, 2010). When taking into the account the net estrogenicity of effluent samples, as measured on 01/12/2010 and 15/12/2010, the net daily estrogenic load exiting the plant averages 7% of that entering the plant (189 mg E2-Eq versus 2828 mg E2-Eq), with 4.7% (130 mg E2-Eq) being discharged in the effluent and 2.2% (59 mg E2-Eq) being discharged in the biosolids. Assuming similar influent composition and WWTP operating conditions and performance, it appears that current common wastewater and anaerobic solids treatment practices readily provide >90% overall removal of net estrogenicity.
Figure 17: Correlations between protein/carbohydrate content and net estrogenicity
Table 15: Net estrogenicity balance for the Guelph WWTP

<table>
<thead>
<tr>
<th>Date (2010)</th>
<th>Total daily influent flow (x1000 m$^3$)</th>
<th>Total influent estrogenic load (mg E2-Eq/day)</th>
<th>Primary sludge production (mg E2-Eq/day)*</th>
<th>TWAS production (mg E2-Eq/day)*</th>
<th>Total TWAS estrogenic load (mg E2-Eq/day)*</th>
<th>Total estrogenic load into digesters (mg E2-Eq/day)*</th>
<th>Dewatered belt press solids, kg/day</th>
<th>Total belt press estrogenic load (mg E2-Eq/day)*</th>
<th>% E2-Eq reduction (influent to belt press solids)</th>
<th>% E2-Eq reduction (1°/TWAS to belt press solids)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aug. 25</td>
<td>59.4</td>
<td>2,301</td>
<td>324</td>
<td>64</td>
<td>61</td>
<td>51</td>
<td>115</td>
<td>20,812</td>
<td>59</td>
<td>97.4</td>
</tr>
<tr>
<td>Sept. 8</td>
<td>50.0</td>
<td>4,702</td>
<td>375</td>
<td>89</td>
<td>82</td>
<td>48</td>
<td>137</td>
<td>18,190</td>
<td>95</td>
<td>98.0</td>
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<tr>
<td>Sept. 22</td>
<td>52.1</td>
<td>1,855</td>
<td>363</td>
<td>69</td>
<td>85</td>
<td>n/a</td>
<td>n/a</td>
<td>14,585</td>
<td>31</td>
<td>98.3</td>
</tr>
<tr>
<td>Oct. 6</td>
<td>53.5</td>
<td>2,870</td>
<td>600</td>
<td>114</td>
<td>212</td>
<td>40</td>
<td>154</td>
<td>21,590</td>
<td>124</td>
<td>95.7</td>
</tr>
<tr>
<td>Oct. 20</td>
<td>63.3</td>
<td>2,738</td>
<td>172</td>
<td>45</td>
<td>146</td>
<td>69</td>
<td>114</td>
<td>7,111</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Nov. 3</td>
<td>57.6</td>
<td>1,985</td>
<td>697</td>
<td>106</td>
<td>71</td>
<td>15</td>
<td>121</td>
<td>18,235</td>
<td>59</td>
<td>97.0</td>
</tr>
<tr>
<td>Nov. 17</td>
<td>66.6</td>
<td>3,944</td>
<td>519</td>
<td>107</td>
<td>153</td>
<td>40</td>
<td>147</td>
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<tr>
<td>Dec. 1</td>
<td>61.3</td>
<td>2,572</td>
<td>494</td>
<td>104</td>
<td>74</td>
<td>27</td>
<td>131</td>
<td>14,171</td>
<td>92</td>
<td>96.4</td>
</tr>
<tr>
<td>Dec. 15</td>
<td>60.9</td>
<td>3,084</td>
<td>476</td>
<td>54</td>
<td>122</td>
<td>14</td>
<td>68</td>
<td>12,037</td>
<td>27</td>
<td>99.1</td>
</tr>
</tbody>
</table>

*Based on daily sludge/biosolids production volume, multiplied by % TS content (Table 13) and E2-Eq concentration (Table 14) to yield a total mass load of E2-Eq
A detailed net estrogenicity balance for the London-Oxford WPCP is not practical, given that the plant was sampled less intensively. Nonetheless, the present study confirms that aerobic solids treatment systems, such as MBR, do appear to provide better removal of eEDCs than anaerobic digestion. The higher reduction in net estrogenicity at the London-Oxford WPCP versus the Guelph WWTP should be kept in context of the fact that the London-Oxford plant receives 100% of its influent from residential sources. Research conducted by WERF (2010) found that industrial eEDCs, surfactants in particular, may contribute more strongly to the net estrogenicity of sludge/biosolid samples than natural estrogens, and are generally more refractory to degradation. Thus, given the lack of industrial wastewater input at London-Oxford, it is possible that the daily load of industrial estrogens is lower than at the Guelph WWTP, although detailed chemical analyses would be required to confirm this.

Given that surfactants may actually increase in estrogenicity as they are partially degraded (Dussault et al., 2005) and are not readily removed during anaerobic digestion, their presence may play a partial role in the Guelph biosolids being higher in estrogenicity than the solids from London-Oxford. The lack of refractory industrial eEDC inputs to the London-Oxford plant may also explain that while the influent to the London plant was higher in net estrogenicity than in Guelph, the primary and WAS were all considerably lower. While the long-held paradigm is that the majority of estrogenic activity in wastewater is attributable to the presence of natural estrogens (Desbrow et al., 1998), industrial estrogens may actually play a more prominent role in sludge/biosolid estrogenicity than is widely accepted. Further work, tying together detailed chemical analyses of industrial eEDCs and YES testing at plants receiving various degrees of
industrial input would be helpful in (dis)confirming the contribution that industrial estrogens may make to the estrogenic potency of biosolids. The WERF (2010) report also found that alkaline stabilization using lime was able to reduce the concentration of surfactants in dewatered sludge by 80%. In this study, alkaline (Lystek) treatment had a moderate effect, reducing the average estrogenicity from an average of $17.6 \pm 7.3$ ng/g E2-Eq to $14.7 \pm 5.5$ ng/g E2-Eq.

5.4. CONCLUSIONS

This study represents the first time that net estrogenicity was comprehensively surveyed through full-scale wastewater solids treatment processes using the YES assay without toxicity issues potentially obscuring the validity of the data. The range of net estrogenicity values reported for biosolids in a limited number of previous studies spans over three orders of magnitude. This study confirms that the net estrogenic potency of anaerobically treated biosolids as measured with the YES assay is indeed low, never having exceeded 30 ng/g E2-Eq in nine separate sampling events. The overall load of net estrogenic activity leaving the Guelph WWTP in the finished biosolids was <5% of estrogenic load entering the plant, and was approximately half the amount that was discharged in the final effluent. Alkaline treatment (Lystek process) appears to slightly reduce the net estrogenicity of anaerobically digested solids. However, in accordance with previous studies, aerobic treatment was confirmed as being more effective at reducing the estrogenicity of wastewater sludge than anaerobic digestion or alkaline treatment. While the post-anaerobic Lystek alkaline treatment process may be advantageous as a pathogen-reduction measure, it does not appear to confer any strong advantage with regards to reducing net estrogenicity. Future research into the effects of
aerobic post-treatment on the net estrogenicity of anaerobically-digested biosolids is warranted; one existing pilot-scale study (McNamara et al., 2009) provides mention that aerobic post-treatment effectively reduces net estrogenicity without providing quantitative data. In addition, as technologies for the pre-treatment of sludge prior to digestion such as ultrasonic, microwave and enzyme conditioning enter into the mainstream (US EPA, 2006), the opportunity to examine their impacts on net estrogenicity arises.
6. MEASURING ANTIESTROGENIC ACTIVITY IN MUNICIPAL WASTEWATER SLUDGES AND BIOSOLIDS

This chapter will be submitted in manuscript form to the journal Environmental Toxicology and Chemistry, under the authorship of J. Citulski and K. Farahbakhsh

6.1. INTRODUCTION

The Yeast Estrogen Screen assay (YES; Routledge and Sumpter, 1996) has become a widely-used screening tool in evaluating the total estrogenic activity of wastewater streams. It has been used in evaluating the effectiveness of engineered wastewater treatment processes in reducing overall estrogenicity, and the loading of estrogenic substances that occurs when treated wastewater is discharged to the environment (Murk et al., 2002; Huggett et al., 2003; Svenson et al., 2003; Nelson et al., 2007; Muller et al., 2008; Li et al., 2010). Municipal wastewater treatment plant (WWTP) effluent represents the largest discharge, by volume, of anthropogenic pollution into Canadian waters (Environment Canada, 2001, pg. 1), a situation which is similar in most developed countries (UN-HABITAT, 2008). However, municipal wastewater treatment also creates large quantities of solid residual matter (Table 16), typically labelled as ‘sludge’ in its untreated state and ‘biosolids’ following stabilization. As with WWTP effluent these solids contain estrogenic substances (Kinney et al., 2006; US EPA, 2009; WERF, 2010; Langdon et al., 2011) which may be directly introduced to the environment through land application of biosolids for agricultural, silvicultural or land-reclamation purposes. The potential toxicity of sludge/biosolid extracts to yeast has led to relatively
fewer studies in which the YES assay was applied to these samples versus wastewater. Nonetheless several researchers have been able to quantify the estrogenic potency of sludges and biosolids using the YES assay (Holbrook et al., 2002; Lorenzen et al., 2004; Teske et al., 2007; McNamara et al., 2009; WERF, 2010).

The human estrogen receptor (hER-α) that is integrated into the yeast genome in the YES assay is responsive to the binding of both estrogenic and antiestrogenic ligands. These stimulate and inhibit, respectively, the production of the enzyme β-galactosidase by the reporter gene lac-Z (Routledge and Sumpter, 1997). The measurable endpoint of the YES assay is colour development induced by the production of β-galactosidase. Thus, antiestrogenic compounds in a sample may compete with estrogenic substances in binding to the hER-α, and in so doing yield an apparent estrogenic response from the YES assay that is lower than would occur if only estrogenic compounds were present (Fernandez et al., 2007). Accordingly, it has been suggested that the estrogenic activity measured by the YES assay is more accurately described as the ‘comprehensive estrogenic activity’ (Yuan, 2003), as the assay yields a net response to the total pool of both agonistic and antagonistic compounds present in environmental samples.

Modifications to the original YES assay methodology have allowed for the antiestrogenic potency of environmental samples to be measured in parallel with total estrogenicity. Conroy et al. (2005, 2007) quantified the antiestrogenic potency of wastewater effluent relative to the dose-dependent inhibition of β-galactosidase production in the YES assay by the antagonist tamoxifen. A similar approach was used by Svenson et al. (2011) to measure the antiestrogenicity of landfill leachate, and by Jeffries et al. (2011) to measure the antiestrogenic activity of sediments impacted by
agricultural runoff. Recently, Buckley (2010) developed a method in which the antiestrogenicity of wastewater effluent was measured by co-incubating varying dilutions of effluent extract with a 17β-estradiol (E2) standard. The dose-dependent inhibition of E2 expression in the presence of effluent extract was then used to determine the sample IC$_{50}$, i.e. the concentration of effluent at which the response of the YES assays to E2 standard was inhibited by 50%. The advantage in calculating a sample’s EC$_{50}$ and IC$_{50}$ based on the YES assay’s response to E2 is that it provides a common basis for quantitative comparison of estrogenic and antiestrogenic effects. Using this approach, the present study is the first in which the antiestrogenic potency of wastewater sludges and biosolids at various stages of treatment has been systematically measured.

Table 16: Estimated annual sewage sludge production (UN-HABITAT, 2008)

<table>
<thead>
<tr>
<th>Country</th>
<th>Total annual sludge production (dry tonnes)</th>
<th>Annual per capita sludge production (dry kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brazil</td>
<td>372,000</td>
<td>2.0</td>
</tr>
<tr>
<td>China</td>
<td>2,966,000</td>
<td>2.3</td>
</tr>
<tr>
<td>Turkey</td>
<td>580,000</td>
<td>8.2</td>
</tr>
<tr>
<td>Slovakia</td>
<td>55,000</td>
<td>10.1</td>
</tr>
<tr>
<td>Hungary</td>
<td>120,000</td>
<td>12.0</td>
</tr>
<tr>
<td>Japan</td>
<td>2,000,000</td>
<td>15.7</td>
</tr>
<tr>
<td>Canada</td>
<td>550,000</td>
<td>16.6</td>
</tr>
<tr>
<td>Italy</td>
<td>1,000,000</td>
<td>17.2</td>
</tr>
<tr>
<td>Norway</td>
<td>86,500</td>
<td>18.8</td>
</tr>
<tr>
<td>Czech Republic</td>
<td>200,000</td>
<td>19.5</td>
</tr>
<tr>
<td>USA</td>
<td>6,514,000</td>
<td>21.8</td>
</tr>
<tr>
<td>Portugal</td>
<td>236,700</td>
<td>22.3</td>
</tr>
<tr>
<td>Germany</td>
<td>2,000,000</td>
<td>24.3</td>
</tr>
<tr>
<td>United Kingdom</td>
<td>1,500,000</td>
<td>24.7</td>
</tr>
<tr>
<td>Slovenia</td>
<td>57,000</td>
<td>28.4</td>
</tr>
<tr>
<td>Finland</td>
<td>150,000</td>
<td>28.7</td>
</tr>
<tr>
<td>Netherlands</td>
<td>1,500,000</td>
<td>91.0</td>
</tr>
</tbody>
</table>
6.2. MATERIALS AND METHODS

6.2.1. SITE DESCRIPTION AND SAMPLE COLLECTION

Detailed descriptions of the two full-scale wastewater treatments plants (WWTP) sampled in this study (Guelph and London-Oxford, Ontario) are given in Chapter 5, and the preparative steps applied to solid and liquid samples prior to application of the YES assay are provided in Chapter 4. Briefly, one-litre grab samples of wastewater solids and filtrate from the biosolids dewatering belt press, and 24-hour equal-volume composite samples of raw influent and treated effluent were collected as explained in Table 17 between August 25th and December 15th, 2010 at the Guelph WWTP and on April 5th, 12th, and 19th, 2011, at the London-Oxford WPCP. Following filtration (liquid samples) and ultrasonication-assisted solvent extraction (solid samples), the samples were sequentially cleaned using activated copper, solid-phase extraction on C18 cartridges, and layered silica-alumina chromatography columns. Cleaned sample extracts were reconstituted in ethanol, and these extracts were analyzed by the YES assay as described in Chapter 4 for total estrogenticity and by a parallel YES assay-based antiestrogenicity test as described below.
### Table 17: Description of sampling points

<table>
<thead>
<tr>
<th>Sampling Point</th>
<th>Sample Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Guelph WWTP</strong></td>
<td></td>
</tr>
<tr>
<td>1 Raw influent</td>
<td>Municipal wastewater (~60% residential, 40% industrial/commercial/institutional, by volume) after passing through bar screen</td>
</tr>
<tr>
<td>2 Primary clarifier</td>
<td>Gravity-thickened primary sludge; ferric sulphate added for phosphorus removal</td>
</tr>
<tr>
<td>3 Thickened waste activated sludge (TWAS) line</td>
<td>Activated sludge from the aeration tank (~8.5 day sludge retention time); thickened via rotary drum thickeners</td>
</tr>
<tr>
<td>4 Primary digester A</td>
<td>Anaerobic digester, fed with solids from (1) and (2); operated at 35-37°C, ~14 day sludge retention time</td>
</tr>
<tr>
<td>5 Primary digester B</td>
<td>Anaerobic digester, fed with solids from (1), (2) and (9); operated at 35-37°C, ~16.5 day sludge retention time</td>
</tr>
<tr>
<td>6 Secondary digester</td>
<td>Anaerobic holding tank, fed with solids from (4) and (5); operated at 35-37°C, ~7 day sludge retention time</td>
</tr>
<tr>
<td>7 Belt press</td>
<td>Polymer-conditioned solids from (6), dewatered using a belt filter</td>
</tr>
<tr>
<td>8 Filtrate collection line</td>
<td>Liquid extracted from the solids in (7) as they are dewatered</td>
</tr>
<tr>
<td>9 Lystek reactor</td>
<td>Heat- and alkaline-stabilized dewatered solids from (7); run on a pilot-scale basis; a portion of these solids are fed into (5)</td>
</tr>
<tr>
<td>10 Effluent discharge pipe</td>
<td>Treated wastewater; post-activated sludge process, polished using rotating biological contactors, rapid sand filtration, chlorination/dechlorination</td>
</tr>
<tr>
<td><strong>London-Oxford WPCP</strong></td>
<td></td>
</tr>
<tr>
<td>1 Raw influent</td>
<td>Municipal wastewater (100% residential) after passing through 2mm screen</td>
</tr>
<tr>
<td>2 Primary clarifier</td>
<td>Gravity-thickened primary sludge</td>
</tr>
<tr>
<td>3 Aeration tank</td>
<td>Mixed liquor suspended solids</td>
</tr>
<tr>
<td>4 Return activated sludge line</td>
<td>Biological solids from the membrane bioreactor (MBR) tank, prior to return to the aeration tank</td>
</tr>
<tr>
<td>5 MBR waste activated sludge line</td>
<td>Biological solids from the membrane bioreactor (MBR) tank prior to introduction to the MBR thickener</td>
</tr>
<tr>
<td>6 MBR thickener</td>
<td>Thickened WAS from the batch-fed MBR thickener</td>
</tr>
<tr>
<td>7 MBR Permeate</td>
<td>Permeate stream from the MBR tank</td>
</tr>
<tr>
<td>8 MBR Thickener permeate</td>
<td>Permeate stream from the MBR thickener</td>
</tr>
<tr>
<td>9 Post-UV treatment effluent</td>
<td>Combined MBR/MBR thickener permeate after UV disinfection</td>
</tr>
</tbody>
</table>

#### 6.2.2. Antiestrogen Assay

Antiestrogenicity was measured using the YES assay modification developed by Buckley (2010). Based on the tests conducted on liquid/sludge/biosolid extracts, as discussed in Chapter 5, the estrogenic EC$_{50}$ – i.e. the dilution at which each extract elicited a half-maximal response from the YES assay – was known for each sample. For
the antiestrogen assay, sample extracts were diluted in ethanol in silanized glass test tubes to their respective EC$_{50}$ concentration, at a total volume of 4 mL. For the YES assay testing, an initial 80µL aliquot of each sample extract was serially diluted across the 12 wells of a microplate row. Thus, for the antiestrogen assay, extracts that had been diluted to their EC$_{50}$ concentration were added in 160µL, 80µL, 40µL and 20µL volumes across individual microplate rows and evaporated under a stream of air in a laminar flow hood. This yielded a fixed concentration of 2x EC$_{50}$, 1x EC$_{50}$, ½x EC$_{50}$ and ¼x EC$_{50}$ for each extract in each well of a given row. 10µL aliquots of serially-diluted E2 standard solution with a nominal concentration of 200nM were then added to the first 9 wells of each row and evaporated under a stream of air in a laminar flow hood. When diluted with yeast medium to a final volume of 200µL/well the final concentration of E2 spiked into each well ranged from 10nM (well #1) to 4x10$^{-2}$ nM (well #9).

The remaining three sample wells in each row were left unspiked, giving triplicate measurements of β-galactosidase activity induced by the sample extract alone. Along with each set of antiestrogen assay plates, a plate was prepared with a row of negative-control wells (100µL ethanol per well) and four positive-control rows of serially-diluted E2 (final concentrations of 10nM to 5x10$^{-3}$nM), and the ethanol/E2 standard solutions were evaporated under a stream of air in a laminar flow hood. Then 200µL of yeast assay medium containing the chromogenic substrate chlorophenol red-β-D-galactopyranoside (CPRG) prepared as per Routledge and Sumpter (1996) was added to each negative-control/positive-control/test well. The microplates were sealed with autoclave tape and incubated in an incubator/shaker at 32°C/150rpm for 72 hours. At the end of this period, yeast turbidity and β-galactosidase-induced colour production were measured using a
microplate reader, and from these optical density (OD) measurements turbidity-adjusted net colour production was calculated as described in Chapter 4. Post-incubation microplates showing negative controls, positive controls and sample extract/E2-spiked wells are presented in Figure 18.
Figure 18: Example of negative control wells and quadruplicate E2 standard curve (top) with E2 nanomolarity values shown below wells; example of antiestrogen assay for two separate samples, showing E2-spiked sample wells at left (spiked E2 nanomolarity values above wells) and unspiked sample wells in triplicate with corresponding EC$_{50}$ dilution at right.
6.2.3. Antiestrogenicity Calculations

Based on previous results (Chapter 5), it was determined that the vast majority of the sludge, biosolid and liquid samples considered in this study elicited an estrogenic response in the YES assay, which confirmed the presence of estrogen receptor agonists in these samples. If the sample extracts contained only agonistic compounds, then the dosing of fixed concentrations of extract on top of E2 standard dilutions should lead to increased colour production in the YES assay, and shift the standard curves upwards in a dose-dependent manner. As an illustrative example, Figure 19 shows that the presence of a biosolid extract did slightly shift the three least-concentrated points of the E2 curve upwards. Conroy et al. (2005) and Buckley (2010) observed a similar response when incubating wastewater effluent extracts with E2 and its synthetic analogue, 17α-ethinylestradiol (EE2). At low E2 concentrations the presence of agonists in the environmental sample was manifested in an upward shift in the E2 curve.

The impact of antiestrogenic compounds in the sample extracts on the E2 standard curves was revealed by subtracting the average β-galactosidase activity of the 2x EC_{50}, 1x EC_{50}, ½x EC_{50} and ¼x EC_{50} extract-only wells from the β-galactosidase activity of the corresponding 7x, 8x and 9x-dilution (extract + E2) wells. These wells were chosen because they consistently bracketed the upward inflection point in the E2 dose-response curve. This process is shown as a sample calculation in Table 18, for the same set of optical density measurements depicted in Figure 19. The β-galactosidase activity stimulated by E2 alone (bottom 4 rows of the table) clearly decreases in tandem with increasing sample extract concentrations, as shown in Figure 20, due to the presence of antiestrogenic compounds in the sample extracts. The dose-dependent decrease in β-
Figure 19: Comparison of the YES assay response to a serially-diluted E2 standard alone, and the response to a mixture of serially-diluted E2 and fixed concentrations of biosolid extract from a secondary anaerobic digester (sampled December 1, 2010). Error bars on E2 standard curve are ±1S.D., lines bracketing the E2 dose-response curve show the 95% confidence interval for the regression.

galactosidase production induced by E2 alone due to increasing amounts of co-incubated sample extract was used to calculate the IC$_{50}$. The IC$_{50}$ statistic represents the concentration of extract sufficient to inhibit the activity of the E2 standard-induced β-galactosidase activity by 50%. To calculate the antiestrogen IC$_{50}$, the residual β-galactosidase activity from (extract + E2) wells 7, 8 and 9 after extract-only activity had been subtracted was expressed as a percentage of the corresponding E2-only activity. The residual percent-response relative to the standard was then averaged for each extract concentration (1/4x, 1/2x, 1x, 2x EC$_{50}$), and plots were created of percent-E2 activity.
versus the volume-fraction of sample extract in each row. In the present example, the December 1st, 2010 secondary anaerobic digester sample’s YES assay EC$_{50}$ occurred when the extract was diluted 16-fold. So, for the antiestrogenicity assay the extract was plated at a volume-fraction of 1/8$^{th}$ (2x EC$_{50}$), 1/16$^{th}$ (1x EC$_{50}$), 1/32$^{nd}$ (½x EC$_{50}$) and 1/64$^{th}$ (¼x EC$_{50}$). From the regression, shown in Figure 21, the IC$_{50}$ (volume fraction of sample extract causing a 50% decrease in colour production relative to the maximum E2 response) was calculated.
Table 18: Turbidity-adjusted absorbance values (OD_{575nm}) indicating β-galactosidase activity in E2 standard curves, sample extracts + E2, and sample extracts alone

<table>
<thead>
<tr>
<th>Well # →</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
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<tr>
<td>E2 Standard Curve</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>E2 conc. (nM)</td>
<td>10</td>
<td>5</td>
<td>2.5</td>
<td>1.25</td>
<td>0.63</td>
<td>0.31</td>
<td>0.16</td>
<td>0.08</td>
<td>0.04</td>
<td>0.02</td>
<td>0.01</td>
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<tr>
<td>E2 Curve #1</td>
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<td>3.48</td>
<td>3.49</td>
<td>3.45</td>
<td>3.46</td>
<td>3.52</td>
<td>3.43</td>
<td>3.33</td>
<td>2.62</td>
<td>1.58</td>
<td>1.31</td>
<td>1.14</td>
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<td>E2 Curve #2</td>
<td>3.73</td>
<td>3.70</td>
<td>3.70</td>
<td>3.72</td>
<td>3.72</td>
<td>3.73</td>
<td>3.67</td>
<td>3.43</td>
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<td>2.60</td>
<td>1.69</td>
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<td>Sample Extract + E2 Spike</td>
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<tr>
<td>Spiked E2 conc. (nM)</td>
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<td>2.5</td>
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<td>0.63</td>
<td>0.31</td>
<td>0.16</td>
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<tr>
<td>2x EC_{50}</td>
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<td>3.56</td>
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<td>3.42</td>
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<td>3.67</td>
<td>3.64</td>
<td>3.62</td>
<td>3.65</td>
<td>3.62</td>
<td>3.60</td>
<td>3.59</td>
<td>3.42</td>
<td>2.10</td>
<td>2.23</td>
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<tr>
<td>1/2x EC_{50}</td>
<td>3.71</td>
<td>3.70</td>
<td>3.66</td>
<td>3.69</td>
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<td>3.08</td>
<td>1.52</td>
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<tr>
<td>1/4x EC_{50}</td>
<td>3.67</td>
<td>3.68</td>
<td>3.68</td>
<td>3.70</td>
<td>3.68</td>
<td>3.66</td>
<td>3.64</td>
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<tr>
<td>(Sample Extract + E2) Absorbance – Average Sample Extract-Only Absorbance</td>
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<tr>
<td>2x EC_{50}</td>
<td>0.78</td>
<td>0.67</td>
<td>0.62</td>
<td>0.78</td>
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<td>22.0</td>
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<td>36.4</td>
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<tr>
<td>1/2x EC_{50}</td>
<td>2.11</td>
<td>2.07</td>
<td>1.52</td>
<td>2.11</td>
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<td>2.07</td>
<td>1.52</td>
<td>59.9</td>
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<td>58.3</td>
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<tr>
<td>1/4x EC_{50}</td>
<td>2.50</td>
<td>2.45</td>
<td>1.92</td>
<td>2.50</td>
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<tr>
<td>% of E2 Abs</td>
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<td>Avg.</td>
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</table>
Figure 20: Plots from Figure 18 with sample extract-only average β-galactosidase activity subtracted from each point in the corresponding (extract + E2) spiked curves. The 7x, 8x and 9x-dilution E2 and E2+extract wells (shown inside the boxes) were used in the IC\textsubscript{50} regression analysis.

Figure 21: Regression line from which the IC\textsubscript{50} value was determined. Data points are the average ± S.D. (n=3) for each of the four sample dilutions in Figure 20, normalized as % response of the corresponding point on the E2 standard curve.
6.3. **RESULTS AND DISCUSSION**

6.3.1. **ANTIESTROGENICITY ASSAY RESULTS**

The regression analysis described above and illustrated for one sample was used to determine the antiestrogen IC\(_{50}\) values for a number of solid and liquid samples from the Guelph WWTP. The antiestrogenicity test is based upon comparing the response of the YES assay to E2 in the presence of various concentrations of a sample’s estrogenic EC\(_{50}\) value. In the case of the samples collected at the London-Oxford WPCP – which, as described in the previous chapter, frequently had non-detectable estrogenicity – running the corresponding antiestrogen assay was often not possible. Even in the case where samples did exhibit a slight estrogenic response in the YES assay, the volume of sludge/biosolid/permeate/effluent solvent extract required to run the antiestrogen assay surpassed the amount of extract available. This is because of the inverse relationship between EC\(_{50}\) and sample volume; the lower a sample’s estrogenic potency, the more of it is required to elicit a measurable response in the YES assay, and hence in the antiestrogen assay. Thus, while data are also presented in this chapter for a select few samples from London, the emphasis is on samples from Guelph.

Since the antiestrogen assay uses the same yeast cell medium as the YES assay, it too can be susceptible to the toxicity effects that are caused by wastewater samples in general and sludge/biosolid samples in particular. Inhibition of cell growth would lead to a false-positive result in the antiestrogen assay, as this inhibition would lead to reduced production of β-galactosidase. The resulting reduction in colour production would then appear to be the consequence of antiestrogens binding to the yeast’s hER-α (Buckley, 2010). However, the sample extracts used in this antiestrogenicity study were the same
as those used for the YES assays described in the preceding chapter. As previously mentioned the post-extraction cleanup steps successfully minimized yeast toxicity, even when yeast were incubated with full-strength solvent extracts. For the antiestrogen screen all extracts were first diluted to their YES assay EC\textsubscript{50} concentration, and then plated at a maximum concentration of 2x EC\textsubscript{50}. While the extent of the dilution depended on the sample being analyzed, extracts were never plated at any less than 4x dilution, and hence none of the samples showed inhibited yeast growth.

Table 19 presents the IC\textsubscript{50} values that were determined for each of the Guelph WWTP samples that were analyzed using the antiestrogenicity assay. In this table, the IC\textsubscript{50} values are presented as the times-dilution of the initial sample extract that caused a 50% reduction in β-galactosidase activity when co-incubated with E2. For solid samples, accurately-weighed portions of approximately 0.1g of dried sludge/biosolid were extracted, as per the details in Chapter 4. To account for variations in the precise amount of solid that was extracted for each sample, IC\textsubscript{50} values have all been normalized to 0.1g. A similar approach was used to normalize the times-dilution of each sample that elicited half-maximal estrogenic response (EC\textsubscript{50}) in the YES assay, based on the analyses discussed in Chapter 5. Both sets of values – the IC\textsubscript{50} dilution for the antiestrogenicity assay, and EC\textsubscript{50} dilution for the YES assay – are presented in Table 19, along with the \( r^2 \) value for the linear regression used to determine each sample’s IC\textsubscript{50}. Note that some sample extracts were submitted for chemical analysis and others were used for duplicate testing in the YES assay for quality control. Therefore, not enough extract was available to run an antiestrogen assay for each sample that was analyzed with the YES assay.
Table 19: Sample-mass normalized antiestrogenic IC\textsubscript{50} and estrogenic EC\textsubscript{50} values determined by the YES assay for Guelph WWTP samples; “Prim.” = primary sludge, “1\textsuperscript{o} AD” = primary anaerobic digester, “2\textsuperscript{o} AD” = secondary anaerobic digester, “BP” = belt press solids, “LYS” = Lystek treated solids, “INF” = raw influent, “BP FILT” = filtrate from belt press, “EFF” = effluent

<table>
<thead>
<tr>
<th>DATE</th>
<th>SOLID SAMPLES</th>
<th></th>
<th>LIQUID SAMPLES</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Value</td>
<td>Prim.</td>
<td>TWAS</td>
<td>1\textsuperscript{o} AD ‘A’</td>
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<td>16.9</td>
<td>27.6</td>
<td>20.9</td>
</tr>
<tr>
<td></td>
<td>IC\textsubscript{50} \textsuperscript{r2}</td>
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<td>0.959</td>
<td>0.778</td>
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<td>24.0</td>
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<td></td>
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<td>15.3</td>
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<td>9.0</td>
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<td></td>
<td>IC\textsubscript{50} \textsuperscript{r2}</td>
<td>0.941</td>
<td>0.874</td>
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Table 18 (Continued): Antiestrogenic IC$_{50}$ and estrogenic EC$_{50}$ values determined by the YES assay for Guelph WWTP samples; “Prim.” = primary sludge, “1o AD” = primary anaerobic digester, “2o AD” = secondary anaerobic digester, “BP” = belt press solids, “LYS” = Lystek treated solids, “INF” = raw influent, “BP FILT” = filtrate from belt press, “EFF” = effluent

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"IC$_{50}$" and "EC$_{50}$" are the inhibitory and effective concentrations, respectively, required to achieve a 50% effect. "IC$_{50}$ $r^2$" values indicate the goodness of fit for the regression analysis.
The regression analyses used to determine the antiestrogen IC\textsubscript{50} values displayed in Table 19 yielded strong correlation coefficients. Of the 78 tests conducted, 64 had $r^2$ values $>0.9$, nine had $r^2$ values between 0.8 and 0.9, and five had $r^2$ values between 0.7 and 0.8. The significance of the results in Table 19 is that in the majority of samples, the median antiestrogenic concentration of the extracts was lower (i.e. occurred at a greater times-dilution) than that of the corresponding estrogenic EC\textsubscript{50}. In other words, the YES assay was sensitive to the antiestrogenic compounds present in the sludge/biosolid/wastewater extracts at extract dilutions higher than (and thus, extract concentrations lower than) those required to elicit an estrogenic response. A visual means of interpreting the data in Table 19 is provided in Figure 22, where the ratio of (EC\textsubscript{50} times dilution/IC\textsubscript{50} times dilution)*100\% is shown for each sample. The significance of this ratio is that if it equals 100\%, i.e. the IC\textsubscript{50} and EC\textsubscript{50} values are equal, half of the estrogenic potency of a sample is effectively “masked” by the presence of antiestrogens. At values less than 100\%, proportionately more of the substances capable of inducing estrogen-receptor expression in the YES assay are masked by the presence of antiestrogens. Conversely, at values greater than 100\%, proportionately less of the estrogen-receptor agonists in a sample are masked by competing antiestrogens. A similar presentation is given in Figure 23 for data from the London-Oxford treatment plant.
Figure 22: Ratio of EC_{50} to IC_{50} values for Guelph solid (top) and liquid (bottom) samples; “A.D.” = anaerobic digester
Figure 23: Ratio of EC$_{50}$ to IC$_{50}$ values for London samples. All samples are from April 12, 2011, unless otherwise indicated. “MLSS” = mixed liquor suspended solids, “CAS RAS” = aeration tank return activated sludge, “MBR RAS” = membrane bioreactor return sludge.

In the original development of the YES-based antiestrogen assay, Buckley (2010) tested three separate composite samples (two in duplicate) of chlorinated effluent from a conventional activate sludge (CAS) WWTP. The (EC$_{50}$/IC$_{50}$) quotient for these samples ranged from 94% to 137%, with a mean value of 116%. Therefore, amongst this limited set of samples, estrogenic potency slightly dominated over antiestrogenic potency. The overall pattern that emerges in the present study is the opposite; antiestrogenic potency predominated over estrogenic potency in both solid and liquid samples at the Guelph...
WWTP. The median (EC\textsubscript{50} times dilution/IC\textsubscript{50} times dilution) quotient for solid samples from Guelph was 69.2%, while that for liquid samples from Guelph was 70.5%.

However, as shown in Figure 22 there was considerable variation in these values among certain sampling points (particularly primary anaerobic digester A, the secondary anaerobic digester, and the belt press filtrate) between sampling events.

The implication of having antiestrogenic compounds in a sample exert an effect at lower concentrations than are required to yield an estrogenic response is that over half of the estrogenic potency will be masked when running the YES assay. True to the concept of the YES assay being a reporter of “comprehensive” estrogenic activity, the present results indicate that the estrogenic potency measured in sludge and biosolid samples is a net value, i.e. estrogenicity in excess of that being masked by the presence of antiestrogens. While antiestrogenic responses have been described previously in \textit{in vitro} testing of wastewater samples (Gagné and Blaise, 1998; Conroy et al., 2005; Conroy et al., 2007; Fernandez et al., 2007; Buckley, 2010), no work has currently been published measuring this effect in wastewater solids. In the context of sludge treatment, the presence of antagonistic compounds means that apparent changes in estrogenicity (as measured by the YES assay) may be reflective not just of the loss or production of estrogenic substances, but also the loss or production of antiestrogens.

\textbf{6.3.2. \textit{Comparison of Treatment Processes}}

Figure 22 shows that despite variations in the magnitude of the (EC\textsubscript{50}/IC\textsubscript{50}) ratio between sampling events, antiestrogenic potency decreased relative to estrogenic potency as solids progressed through the anaerobic treatment process. This is illustrated in Figure 24, in which the average (EC\textsubscript{50} times dilution/IC\textsubscript{50} times dilution) values for each
sampling point at the Guelph WWTP across all sampling events are displayed. The primary sludge samples and the waste activated sludge samples had similar ratios of estrogenic to antiestrogenic potency. However, by examining the actual EC\textsubscript{50} and IC\textsubscript{50} values for primary and thickened WAS samples (Table 19), both estrogenic and antiestrogenic potency tended to increase in the TWAS relative to the primary sludge. It appears that both estrogenic and antiestrogenic compounds were formed during activated sludge treatment, albeit in similar relative proportions. The average EC\textsubscript{50}/IC\textsubscript{50} quotient for the solid samples increased during anaerobic digestion relative to the primary/TWAS, yet generally again so too did the individual EC\textsubscript{50} and IC\textsubscript{50} values (Table 19). Thus, the increase in the average EC\textsubscript{50}/IC\textsubscript{50} quotient was due to a more profound increase in the estrogenic potency of the digested solids than antiestrogenic potency.

As discussed in the preceding chapter, the average estrogenic potency of the primary and waste-activated sludge was 5.3 and 11.1 ng/g E2-equivalents, respectively, rising to an average of over 17 ng/g E2-Eq in the biosolids from the primary and secondary anaerobic digesters and the dewatered finished biosolids. There is a well-established body of research documenting the production of estrogen-receptor agonists during anaerobic digestion (Weltin et al., 2002; Andersen et al., 2003; Keller et al., 2003; Minamiyama et al., 2006). It appears in this study that estrogen-receptor antagonists are also produced during the anaerobic digestion process, but that the total production of antiestrogens is less than that of estrogenic compounds. Similarly, though the liquid filtrate released during the dewatering of digested solids showed a reduction in the average EC\textsubscript{50}/IC\textsubscript{50} quotient relative to the WWTP influent, the data in Table 19 indicate that both the estrogenic EC\textsubscript{50} and antiestrogenic IC\textsubscript{50} increased in the filtrate relative to
the influent. This further supports the hypothesis that antiestrogenic compounds were produced during the anaerobic digestion process, but to a lesser extent than estrogenic substances. On average, there was an increase in the relative antiestrogenic potency between the influent and effluent at the Guelph WWTP. The Guelph treatment plant uses chlorination for the disinfection of its effluent. Wu et al. (2009) found that chlorination increased the antiestrogenic potency of biologically-treated wastewater, as measured by a modified yeast-based assay. Chemical analyses suggested that this increase was due to the formation of chlorinated aromatic amino acids and humic/fulvic acids. In comparison, London-Oxford disinfects its effluent with ultraviolet treatment, and the antiestrogenic potency of the effluent extracts was considerably less than the estrogenic potency (Figure 23).
Figure 24: Changes in antiestrogenic potency relative to estrogenic potency at Guelph WWTP sampling points; “A.D.” = anaerobic digester; note that the Lystek system is a pilot process, not full-scale. Arrows indicate transfer of solids between treatment processes.

The limited number of antiestrogenicity results from the London-Oxford WPCP makes direct comparisons between the anaerobic and aerobic sludge treatment process difficult. However, the samples from London all exhibited lower antiestrogenic potency than those collected in Guelph. As shown in Figure 23, amongst the samples from London that were analyzed, the estrogenic potency was either roughly equal to, or significantly greater than the corresponding antiestrogenic activity, including in the influent samples. A fundamental difference between the Guelph and London-Oxford WWTPs is that while the former receives influent with approximately 40% industrial input by volume, the influent reaching the London-Oxford plant is 100% residential in
composition. The lower antiestrogenic potency observed in the London samples relative to those from Guelph may be linked to this lack of industrial wastewater input. Indeed, irrespective of the differences in solids treatment processes between the two plants, the antiestrogenicity of the Guelph raw influent was higher than that of the London-Oxford influent.

A wide range of industrial chemicals exert antiestrogenic effects (Navas and Segner, 1998). Gagné and Blaise (1998) found that amongst 10 wastewater treatment plants in Québec, antiestrogenic effects in the effluent (as measured by a trout hepatocyte bioassay) were correlated to the percentage of industrial wastewater content. Svenson et al. (2011) measured the estrogenic and antiestrogenic potency of Swedish municipal landfill leachates using yeast-based bioassays. Those landfills receiving only domestic waste appeared to have both low estrogenic and low antiestrogenic activity, while the highest antiestrogenic activity was found in the leachate from the landfill receiving only industrial waste. Similarly, Jeffries et al. (2011) found both in vivo and in vitro evidence of antiestrogenic compounds present in sediments collected from water bodies downstream of intensively-farmed agricultural areas and industrial-scale cattle raising facilities. Antiestrogenic effects were not observed in tests conducted on sediments from non-impacted reference sediments. The results of the present study suggest that the antiestrogenicity of municipally-treated sludges and biosolids is also linked to the presence of compounds originating from industrial rather than municipal sources.

6.4. CONCLUSIONS

This study is the first to present measurements of antiestrogenicity in municipal wastewater sludges and biosolids. Antiestrogenic compounds were indirectly detected by
the YES assay as a dose-dependent decrease in E2-induced β-galactosidase activity. Antiestrogens were then quantified by an IC\textsubscript{50} statistic expressed in term of sample extract volume sufficient to inhibit the E2 standard-induced β-galactosidase activity by 50%. The detection of antiestrogenic potency in sludges and biosolids by the modified YES assay confirms that, when the classic YES assay is applied to wastewater solids, the estrogenicity values that are measured are ‘net’ values, i.e. estrogenic potency in excess of that masked by antiestrogens. Overall, both solid and liquid samples from the London-Oxford plant were lower in antiestrogenic potency than those from Guelph. Based on these results, and other bioassay studies of environmental media conducted by other researchers, it appears that this difference can be explained by the presence of estrogen receptor antagonists in the industrial wastewater inputs to the Guelph WWTP. The antiestrogenic potency of solid samples from Guelph increased as solids treatment progressed from primary settling through the activated sludge process, and then increased again during the anaerobic digestion stage. However, this increase in the antiestrogenic IC\textsubscript{50} was usually accompanied by an even greater increase in the estrogenic EC\textsubscript{50}. Thus, the potency of the estrogenic substances in the solids increased relative to that of the antiestrogenic compounds during stabilization. The tandem increases in both estrogenic and antiestrogenic potency suggest that both types of compounds are formed during anaerobic treatment. Although more work is needed to confirm the observation, it appears that chlorination increases the potency of antiestrogens relative to estrogenic compounds in treated wastewater, while UV disinfection does not.
7. Measuring the Effects of Storage Conditions on the Estrogenic and Antiestrogenic Potency of Municipal Biosolids as Measured by the YES Assay

This chapter will be submitted in manuscript form to the journal Environmental Science and Technology, under the authorship of J. Citulski and K. Farahbakhsh

7.1. Introduction

Beneficial reuse of municipally-treated biosolids on agricultural land is typically subject to restrictions on the timing and frequency of such applications by national or provincial/state-level regulations. These restrictions often necessitate the prolonged storage of stabilized biosolids, particularly in temperate zones where biosolids application must be matched to seasonal growing periods (UN-HABITAT, 2008).

Microbial activity remains high in biosolids during storage (Ahmed and Sorensen, 1995) and bacterial re-growth can readily occur during storage even in previously sterilized solids (Zaleski et al., 2005). Thus, storage periods may allow for biodegradation of organic constituents in biosolids above and beyond the level of treatment provided by engineered stabilization processes such as aerobic/anaerobic digestion or alkaline treatment. Indeed, in some jurisdictions the prolonged storage of digested biosolids is considered an enhanced treatment step in its own right (UN-HABITAT, 2008).

The fate of organic microconstituents and endocrine-disrupting compounds (EDCs) under biosolids storage conditions has not been well studied. Chenxi et al. (2008) found that of eight pharmaceuticals analyzed over a 77-day period in samples of aerobically-digested biosolids stored at ambient conditions (summer/fall, Ohio, USA),
three showed no discernible removal, two had intermediate degradation half-lives of 37-77 days, and three had half-lives of less than 17 days. Half-lives were roughly twice as long under anaerobic storage conditions as compared to aerobic, but the presence of sunlight had no impact on the removal of the selected compounds. By comparison, the lowest half-life reported by Walters et al. (2010) for a suite of pharmaceuticals and personal care products in a 1:2 mixture of anaerobically-digested biosolids/soil stored at ambient conditions (Maryland, USA) over a three-year period was 187 days. As with Chenxi et al. (2008), Kouloumbos et al. (2008) found that the type of stabilization method impacted the subsequent persistence of organic pollutants during biosolid ageing. Kouloumbos et al. (2008) reported that the EDC nonylphenol (NP) was more persistent over an 87-day period in biosolid-amended soil when anaerobically-digested solids were subjected to a freeze-thaw cycle, compared to the application of unfrozen biosolids. There was also a slight reduction in NP mineralization potential in alkaline-treated anaerobically-digested solids, as compared to biosolids that had not undergone the additional treatment step.

There is a growing body of evidence that estrogenic EDCs are present in biosolids based on both chemical analyses (Kinney et al., 2006; US EPA, 2009; Langdon et al., 2011) and bioassay-based measurements of net estrogenic potency (GWRC/WERF, 2004; Lorenzen et al., 2004; Teske et al., 2007; McNamara et al., 2009; WERF, 2010). There have not, however, been any studies that have examined the fate of net estrogenic and antiestrogenic potency during biosolids storage. The present study seeks to address this knowledge gap by determining the changes in net estrogenic and antiestrogenic potency in Class A (unrestricted use) and Class B (restricted use) biosolids under three
storage conditions (ambient freeze-thaw, 4 °C, 25 °C) over a four month period (December – April). During this period, sewage-derived biosolids may not be applied to agricultural land in the province of Ontario, as per the Nutrient Management Act (Ontario MOE, 2011). Both estrogenic and antiestrogenic potency were measured using the yeast estrogen screen (YES) assay.

7.2. MATERIALS AND METHODS

7.2.1. SAMPLE COLLECTION

Biosolids were collected from the City of Guelph wastewater treatment plant (WWTP). The plant serves a population of approximately 125,000, with 60% of the plant influent coming from residential sources and 40% from industrial, institutional and commercial wastewater streams. Ferric sulphate is added to the grit tank and primary clarifier for phosphorus removal. Gravity-thickened primary sludge is combined with rotary drum-thickened waste activated sludge from the secondary treatment process and distributed to four mesophilic anaerobic digesters (35-37 °C, solids retention time of 14 – 16.5 days). Digested solids are polymer-conditioned and dewatered using a belt press, yielding a final product that is approximately 23% solids and meet US EPA ‘Class B’ (restricted use) criteria. A pilot system at the Guelph WWTP provides further treatment for a portion of the dewatered belt press biosolids using the Lystek process. Dewatered solids are transferred to a batch reactor where heat and potassium hydroxide are used to produce a pathogen-free liquid with approximately 16% total solids content that meets US EPA ‘Class A’ standards (US EPA, 2006). Ten-litre grab samples of belt press and Lystek solids were collected on December 3rd, 2010, and split into portions for each treatment condition, described below.
7.2.2. **Biosolids Storage Test Set-Up**

Accurately-weighed portions of approximately 250g of biosolids were transferred to 1L glass jars that were silanized prior to use with a 1:7 v/v trimethylchlorosilane/hexane solution to prevent adsorption of EDCs to the glass surface. The jars were capped and lengths of 1/8” vinyl tubing with a plug of glass wool were inserted into the caps to facilitate moisture exchange and prevent gas build-up and ingress of any foreign matter. The jars were wrapped in aluminum foil to exclude light, as shown in Figure 25.

Triplicate samples of Lystek and belt press solids were prepared for storage at each of three conditions: ambient (freeze-thaw), 4 °C and 25 °C. Ambient samples were kept outside in an unshaded west-facing locked wire-mesh cage, while samples at 4 °C and 25 °C were kept in a refrigerator and incubator, respectively. The maximum and minimum daily ambient temperatures, as recorded for the City of Guelph by Environment Canada, are shown in Figure 26. The 4 °C and 25 °C samples were thoroughly mixed on a weekly basis, and the ambient samples were mixed when the temperature allowed (i.e. when the samples were not frozen solid). Sub-samples were removed at weeks 2, 6, 11, 15 and 18 for analysis of total and volatile solids, and for analysis of net estrogenic and anti-estrogenic potency.
Figure 25: Biosolids storage jars

Figure 26: Sub-sampling dates and temperature profile for ambient samples (temperature data from Environment Canada)
7.2.3. **Sample Analysis**

After initial collection on December 3rd, 2010, a further five portions were removed from each sample on the dates shown in Figure 26, corresponding to 2, 6, 11, 15 and 18 weeks of storage. Prior to sub-sampling, ambient samples were allowed to reach room temperature in the lab on occasions where the biosolids were completely frozen. Total solids (TS) content was determined as the difference in mass after 16 hours of drying at 105 °C, and volatile solids (VS) were determined as the change in mass of the dried solids following combustion at 550 °C for two hours, in accordance with Standard Method 2540 (APHA/AWWA/WEF, 2005). Equal portions of solids from each of the triplicate samples within each treatment condition were mixed together in acetone-rinsed aluminum boats and dried at 105 °C for 16 hours for subsequent analysis of estrogenicity and anti-estrogenicity. Dried solids were solvent-extracted and cleaned according to the procedure in Chapter 4. Cleaned extracts were then tested for net estrogenicity according to the procedure in Chapter 5, and tested for anti-estrogenicity according to the procedure in Chapter 6.

7.3. **Results**

As shown in Figure 26, the ambient samples underwent several freeze-thaw cycles over the course of 18 weeks of storage. Due to the need to thaw these samples in order to obtain portions for TS/VS/(anti-)estrogenicity testing, each sub-sampling date corresponds to a thaw event as well. Figure 27 shows replicates of each sample at the termination of the study (April 4th, 2011). Both the belt press and Lystek samples became visibly thinner in composition and the belt press solids finer in texture after repeated freeze-thaw cycles. This is consistent with the physical breakdown of organic floc.
particles due to ice-crystal intrusion (Diak et al., 2011). However, the data presented in Figure 28 indicate that there were only slight, not statistically significant (Students’ t-test, \( \alpha = 0.05 \)) reductions in TS and VS content between the initial belt press and Lystek samples and the ambient condition sub-samples collected throughout the study. In comparison, both TS and VS declined steadily and linearly throughout the storage period in the belt press and Lystek biosolids stored at 25 °C (Students’ t-test, \( \alpha = 0.05 \)). A similar linear decline in TS occurred in the belt press and Lystek samples stored at 4 °C, albeit at approximately half the rate of the comparable 25 °C samples. While VS also declined linearly in the belt press samples stored at 4 °C at roughly half the rate of the 25 °C samples, the pattern of VS destruction in the Lystek solids stored at 4 °C was inconsistent. Overall, VS decreased in the 4 °C Lystek samples, but the decline was not statistically significant (Students’ t-test, \( \alpha = 0.05 \)).

Figure 27: Lystek and belt press thickened solids (shown in quadruplicate) at the end of four months of storage
Figure 28: Total solids (top) and volatile solids (bottom) content of samples throughout the study period; error bars are ± 1S.D., n = 3
For the stored solids, portions from each of three triplicate samples were mixed to create a composite prior to extraction and analysis of (anti)estrogenic activity. The net estrogenic potencies of the samples, expressed in terms of estradiol-equivalents (E2-Eq), are shown in Figure 29. None of the subsamples analyzed during the study were toxic to the yeast cells in the YES assay. Hence the estrogenicity values calculated for each sampling event are based on the EC$_{50}$, i.e. the sample-extract concentration that elicited a half-maximal response in the YES assay relative to an E2 standard curve. As discussed in the preceding chapter, the antiestrogenic potency of samples was evaluated based on the IC$_{50}$ - the concentration of sample extract (expressed in terms of times-dilution) that caused a 50% reduction in the response of the YES assay when co-incubated with known concentrations of E2. Table 20 presents the EC$_{50}$ and IC$_{50}$ values for each sample as the times-dilution of the solvent extract that yielded a half-maximal effect in the respective assay. Table 20 also shows the coefficient of determination ($r^2$ value) for the linear regressions used to determine each antiestrogenic IC$_{50}$. The magnitude of a sample’s estrogenic potency relative to its antiestrogenic potency was then determined by examining the ratio of (EC$_{50}$/IC$_{50}$)*100%. The significance of this ratio is that if it equals 100%, i.e. the IC$_{50}$ and EC$_{50}$ values are equal, half of the estrogenic potency of a sample is effectively “masked” by the presence of antiestrogens. At values less than 100%, proportionately more of the substances capable of inducing estrogen-receptor expression in the YES assay are masked by the presence of antiestrogens; at values greater than 100%, proportionately less of the estrogen-receptor agonists in a sample are masked by competing antiestrogens. Ratios of estrogenic EC$_{50}$ to antiestrogenic IC$_{50}$ for the samples analyzed in this study are shown in Figure 30.
Figure 29: Net estrogenic potency of initial and stored biosolids samples as measured by the YES assay; note the logarithmic scale for the abscissa. The percent change in net estrogenicity between initial collection and after four months of storage is shown above each sample.

Table 20: Antiestrogenic IC\textsubscript{50} and estrogenic EC\textsubscript{50} values determined by the YES assay for stored biosolid samples; “BP” = belt press solids, “LYS” = Lystek solids, “Amb.” = ambient temperature conditions.

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Figure 30: Ratios of estrogenic EC$_{50}$ (as ‘times-dilution’ of the sample extract) to antiestrogenic IC$_{50}$ (as ‘times-dilution’ of the sample extract) for belt press solid samples (top) and Lystek samples (bottom)
7.4. DISCUSSION

The YES assay is responsive to the binding of both estrogen-receptor agonists and antagonists, which provoke and inhibit, respectively, the assay’s measured colour-production endpoint. Previous studies have shown that estrogen-receptor agonists can be both created and degraded during sludge treatment processes, particularly under anaerobic conditions (Andersen et al., 2003; Keller et al., 2003; Minamiyama et al., 2006), though little is known of the fate of specific antiestrogenic compounds during sludge stabilization processes. Studies on the fate of estrogenic compounds during the anaerobic storage of pig manure (Burnison et al., 2003) and cow manure (Lorenzen et al., 2004), as well as aged manure-based mushroom compost (Andaluri et al., 2011) all indicate that estrogenic substances persist and in some instances increase in concentration during storage, although the length of storage was not clearly specified in the studies.

In the present study, the evolution of net estrogenicity during biosolids storage was time-dependent in both the belt press and Lystek solids, as shown in Figure 29. After four months of storage, net estrogenicity was reduced by 18-77% in five of the six treatment conditions, with a slight (9%) increase in net estrogenicity relative to the initial sample in the Lystek solids stored at 25 °C. A common feature of both sets of solids under all three temperature conditions was that net estrogenicity showed a steep decline after the first two weeks of storage. This decline continued into the sixth week of storage for the belt press and Lystek solids stored at 4 °C. Conversely, samples stored at 25 °C and under ambient conditions exhibited sharp increases in net estrogenicity when sampled at week six. This phenomenon was particularly striking in the ambient samples, with net estrogenicity rising from 17.7 ng/g E2-Eq to 72.9 ng/g E2-Eq in the belt press
solids and from 15.1 ng/g E2-Eq to 335.4 ng/g E2-Eq in the Lystek solids. Broadly speaking, the overall pattern of net estrogenicity during storage was one of initial decline, followed by increases during the middle of the storage period, followed by a second phase of decline. Three scenarios may potentially account for these patterns:

- Degradation (either via microbial processes or physicochemical mineralization) of the estrogenic compounds originally present in each sample, followed by the formation of estrogenic substances via microbial deconjugation of initially inactivated hormones (USEPA, 2010) or degradation of surfactants into more estrogenically-potent byproducts (Keller et al., 2003; Minamiyama et al., 2006), which are then subsequently degraded;

- Contrarily, decreases in net estrogenicity as measured by the YES assay could be the result of the initial formation of antagonistic compounds (again, either via microbial processes or physicochemical mineralization) which mask the presence of estrogen-receptor agonists; a cycle of antagonist formation/degradation/secondary formation would thus lead to an apparent cycle of estrogenicity reduction/increase/reduction;

- The observed changes in net estrogenicity over time may also reflect the interplay of both estrogen-receptor agonist formation/destruction, in tandem with antagonist formation/destruction.

Enumeration and/or classification of the microbial composition of the biosolids used in this study was not conducted. However, both belt press and Lystek samples tested positive for general microbial growth even after 16 hours of drying at 105 °C in an earlier phase of this research (see Appendix 2). Given that the Lystek solids undergo thermal
and alkaline treatment which removes a number of pathogen indicator bacteria (Singh et al., 2007), it is likely that the microbial consortia in the stored Lystek solids would be different in composition from that of the dewatered solids. Indirect evidence of the microbial activity in the stored samples is provided by the time-dependent decrease in TS and VS in the belt press and Lystek solids stored at 4 °C and 25 °C (Figure 28). The TS and VS content of the ambient belt press and Lystek samples remained unchanged throughout the study period, likely as a consequence of minimal microbial activity due to the predominantly freezing temperatures that the samples were exposed to (Figure 26). Thus, the ambient samples provide an opportunity to compare the evolution of net estrogenic and antiestrogenic potency over time under conditions of reduced microbial metabolism, relative to the 4 °C/25 °C samples.

As presented in Figure 30, the antiestrogenic potency of the stored solids varied over time relative to the net estrogenic potency. In freshly-treated biosolids the YES assay was generally sensitive to the presence of antiestrogens at lower sample extract concentrations than were required to elicit a half-maximal estrogenic response, as discussed in Chapter 6. Conversely, the EC_{50}/IC_{50} ratios shown in Figure 30 indicate that during storage the relative potency of antiestrogens was frequently less than that of the estrogenic compounds present in the samples. This suggests that antiestrogens present in the initial biosolid samples were either partially removed, or converted to less active compounds. Changes in antiestrogenic potency over time did not exhibit any clearly discernible patterns, either within samples of the same type (i.e. belt press versus Lystek solids) or between samples stored at the same temperature. However, with the exception of the belt press solids stored at 25 °C, the remaining five samples all shared a relative
increase in antiestrogenicity when tested at the end of four months of storage. For the Lystek samples stored at 4 °C and both the Lystek and belt press solids stored under ambient conditions, the final antiestrogenic potency relative to estrogenic potency was actually greater than that of the starting samples.

It appears that changes in the estrogenic potency of the stored solids cannot be explained only in terms of ‘masking’ or ‘unmasking’ by the formation and destruction of antiestrogens over time. Data plots of samples’ net estrogenicity versus antiestrogenic IC\textsubscript{50} (not shown) revealed no strong correlations between the two variables. Both belt press and Lystek solids stored at 4 °C showed successive declines in net estrogenicity at weeks two and six of sub-sampling, despite IC\textsubscript{50} values that remained stable relative to the EC\textsubscript{50}. This indicates that reductions in net estrogenicity were ‘true’ losses, as opposed to masking by antiestrogens, as the antiestrogenic potency remained stable while net estrogenic potency decreased. Belt press solids stored at 25 °C also did not demonstrate direct links in changes of net estrogenic and antiestrogenic potency.

Between weeks two and six of storage, the relative antiestrogenic potency of these solids increased, which would theoretically further mask estrogenic compounds; however, the net estrogenicity increased three-fold. While there was a decrease in antiestrogenic potency between weeks six and 11, which would potentially unmask the presence of estrogenic compounds, net estrogenicity decreased by a third. Thus, it appears that the concentrations of estrogenic and antiestrogenic compounds in biosolids are dynamically transformed over the course of prolonged storage periods. It is important to consider that antiestrogenic compounds vary in their ability to act as antagonists in the YES assay. Some compounds act as full antagonists, while other weak antagonists may
even act as estrogen-receptor agonists depending on their concentration and the presence of other compounds (Fang et al., 2000). The results of the present study confirm the presence and persistence of estrogenic and antiestrogenic compounds during biosolids storage. The lack of clear correlation between changes in concentrations may be reflective of complex underlying fate mechanisms and chemical transformations for the two classes of compounds, which cannot be discerned by the ‘net’ measurements provided by the YES assay.

To account for the masking of estrogenic compounds by estrogen-receptor antagonists, E2-Eq values were adjusted for each sample by adding back the calculated amount of inhibited estrogenicity. This calculation was carried out as follows: since the (antiestrogenic) IC$_{50}$ and (estrogenic) EC$_{50}$ were known for each sample, the percent-inhibition of estrogenicity was calculated as $(IC_{50}/(EC_{50} + IC_{50})) \times 100\%$. As an example using the data in Table 20, the belt press sample stored at 4 °C had an IC$_{50}$ of 15.5 times-dilution and an EC$_{50}$ of 17.5 times-dilution on December 16$^{th}$, so the percentage of masked estrogenicity was calculated as $(15.5/(17.5 + 15.5)) \times 100\% = 47.0\%$. Thus, the measured net estrogenicity value of 3.9 ng/g E2-Eq was recalculated as being 47.0% higher, or 5.7 ng/g E2-Eq. This adjustment was based on the fact that both estrogenicity and antiestrogenicity values were calculated using the YES assay, an approach that provides a common basis for quantitative comparison of estrogenic and antiestrogenic effects (Buckley, 2010). Figure 31 provides an overview of the changes in net and calculated total estrogenicity of the belt press and Lystek solids throughout the storage period, based on the net and calculated total estrogenic content of the initial samples.
Figure 31: Percent-changes in net estrogenicity (‘unadjusted’ curves) and total estrogenicity as calculated by adding masked estrogenic potency (‘adjusted’ curves) over time, relative to initial samples

Whether based on net estrogenicity values as determined by the YES assay alone or total calculated estrogenicity values that take into account antiestrogenic masking, the percent-changes in estrogenic potency during storage show similar profiles (Figure 31).
Thus, even if an antiestrogenicity assay cannot be run in parallel to the YES assay, the latter still provides a useful insight into the pattern of estrogenicity changes with respect to storage time. The patterns shown in Figure 31 appear to indicate that changes in estrogenic potency during storage depend on the type of pretreatment the biosolids have undergone. The Lystek heat- and alkaline-treatment process creates a finished product that is lower in VS (see Figure 28) and significantly lower in pathogenic bacteria content than belt press solids, while decreasing the particle size and increasing the surface area of the biosolid flocs. The Lystek process also changes the chemical composition of the belt press solids, most notably decreasing the ammonia content and increasing potassium through the addition of potassium hydroxide (Singh et al., 2007). The belt press and Lystek solids initially shared comparable levels of net estrogenicity (17.7 vs. 15.1 ng/g E2-Eq, respectively). The YES assay responded to both initial sample extracts at similar EC\textsubscript{50} concentrations (15.4 and 14.6 times-dilution, respectively) and the YES antiestrogen screen responded at similar IC\textsubscript{50} concentrations (18.5 and 15.5 times-dilution, respectively). While the evolution of estrogenicity over time showed a similar profile for both types of solids under ambient conditions, storage at 4 °C and 25 °C led to very different patterns of increases and decreases in estrogenicity.

Reductions in net estrogenicity between 47% and 78% occurred in belt press and Lystek samples under all three temperature conditions when sampled after two weeks of storage. As previously discussed, this drop in net estrogenicity cannot be explained by a corresponding increase in antiestrogenic masking, suggesting that both belt press and Lystek solids contain a pool of estrogenic compounds that are readably-degradable. Previous studies (Andersen et al., 2003; Keller et al., 2003; Minamiyama et al., 2006)
have noted that estrogenic loads can also increase with time under anaerobic conditions due to the formation of secondary estrogenic compounds via microbial degradation. This is consistent with the estrogenicity patterns observed in the belt press and Lystek samples stored at 4 °C and 25 °C (Figure 31), in which initial decreases in estrogenicity were followed by intermediate periods of relative increase. Indirect evidence that the increases in estrogenicity observed partway through the storage periods were microbially-mediated comes from the fact that such increases were observed sooner (week six) in the samples stored at 25 °C, and later (week 11) in the samples stored at 4 °C. This lag in the secondary formation of estrogenic compounds at 4 °C versus 25 °C may reflect reduced microbial growth rates at the lower temperature.

Samples stored at 25 °C suggest that multiple phases of estrogen-receptor agonist formation and destruction may also occur, as two peaks in estrogenicity – slight in the belt press solids, prominent in the Lystek samples – occurred. While the belt press and Lystek samples show similar profiles in estrogenicity changes at 4 °C and 25 °C, the magnitude of these changes varied. The minimum net estrogenicity reduction in the belt press samples stored at 4 °C relative to the fresh biosolids was 46%, whereas for the Lystek samples stored at 4 °C the minimum reduction was only 12%. Belt press samples stored at 25 °C exhibited net estrogenicity 25% higher than the fresh biosolids at week six of sub-sampling, and Lystek solids had a net estrogenicity 75% higher at week 15 than when initially collected. Further work is necessary to evaluate whether these differences are related to factors such as the Lystek solids having a different consortium of microbes than the dewatered solids, differences in the chemistry of the two matrices, increases in
bioavailability of sorbed estrogenic compounds due to the increased surface area of
Lystek solids, or combinations of the above.

An initial decline in estrogenicity occurred after two weeks of storage even under
the frequently below-freezing conditions that the ambient samples were exposed to (58% reduction in belt press solids and 56% reduction in Lystek solids). This was in spite of the fact that TS and VS measurements over time in these samples remained minimally changed, indicating low levels of microbial activity. The near-identical estrogenicity profiles of the two ambient samples (Figure 31) contrast with the differences between the belt press and Lystek samples at 4 °C and 25 °C. Both ambient profiles show the sharp spike in estrogenicity that was registered during sampling at week six, which followed a period of several days’ thaw, with daytime maximum temperatures reaching 10.8 °C.

This spike in estrogenicity was far in excess of any seen in the samples stored at 4 °C or 25 °C, and was accompanied by equally steep increases in antiestrogenic activity (refer to the IC$_{50}$ values in Table 20).

Given the overall differences in the microbial and physical composition of the belt press versus Lystek solids, and given the differences in their estrogenicity profiles at 4 °C and 25 °C, the similarity in these profiles under ambient conditions may indicate that changes in estrogenicity were governed by physical freeze-thaw processes. Freeze-thaw cycles have been shown to increase the concentrations of organic compounds in anaerobically digested biosolids (Kumar et al., 2006). Indeed, a USEPA study (2010) on the stability of micropollutants in biosolids during frozen storage found that concentrations of the estrogenic compounds 17α-estradiol and estrone, along with certain pharmaceuticals, increased during the first two weeks of storage. The same study found
that after four weeks of storage the concentrations of these compounds had returned to levels that were not statistically elevated above the initial levels. More research would be required to delineate the role of physical conditions on altering the estrogenic potency of biosolids. However, the present research suggests that freeze-thaw cycles may lead to changes in the estrogenic and antiestrogenic potency of biosolids that are distinct from the fate profiles encountered under temperate conditions. Overall, more frequent sampling of biosolids during storage, along with a wider suite of testing to more fully characterize physicochemical changes in the biosolids (ammonia and oxygen concentrations, ionic strength, TS/VS, particle-size distribution) would allow for principal components analysis (PCA) to better delineate underlying causes of net (anti)estrogenicity changes.

The persistence – and depending on storage time, increased concentrations – of both estrogenic and antiestrogenic compounds during biosolids storage means that transfer of these substances to the environment is inevitable when biosolids are land-applied. On the one hand, it must be emphasized that in vitro detection of (anti)estrogenicity is not a guarantee that deleterious endocrine-disrupting effects will occur in individual organisms exposed to the test matrix, let alone amongst an entire population of organisms (Lister and Van Der Kraak, 2001). Also, certain compounds, such as benzotriazole, elicit an in vitro endocrine-disruptive response, but are not disruptive in vivo (Harris et al., 2007). On the other hand, in vitro testing may actually underestimate the in vivo potency of endocrine disruptors (Huggett et al., 2003).

While comparatively less research has focused on the presence of antiestrogenic contaminants in the environment relative to estrogenic compounds (reviewed by Bolong
et al., 2009), evidence suggests that antiestrogens are widespread (Hilscherova et al., 2002; Lorenzen et al., 2003). Antiestrogens can bioaccumulate to concentrations orders-of-magnitude higher than the trace concentrations that organisms may encounter in the environment (Schreurs et al., 2004). This in turn may exacerbate the impairments to normal growth and reproductive fitness that have been observed when organisms are exposed to low doses of antiestrogens (Mackenzie et al., 2003). In the context of biosolids, Lind et al. (2010) found that pregnant ewes and their fetuses exhibited statistically significant evidence of antiestrogenic effects in the form of decreased bone density and mineral content when grazed on biosolid-amended land versus non-amended control plots.

7.5. CONCLUSIONS

The results of this study show that both estrogenic and antiestrogenic potency persists in Class A (Lystek) and Class B (belt-press dewatered) anaerobically treated biosolids during storage under three temperature conditions: 4 °C, 25 °C and ambient freeze-thaw cycles. While storage to a period of four months did incur a reduction in net estrogenicity as measured by the YES assay in five of six samples, Lystek solids stored at 25 °C showed a slight increase in net estrogenicity. Moreover, sub-samples collected within the four-month storage period showed wide variations in both estrogenic and antiestrogenic potency. This was particularly so in samples exposed to ambient conditions, in which both estrogenic potency spiked to levels over four-fold higher in belt press solids and 22-fold higher in Lystek solids relative to initial levels following the first freeze-thaw cycle. Thus, while extended storage may provide for enhanced removal of endocrine disruptors above-and-beyond the level provided by engineered treatment
processes, shorter storage periods may actually increase the load of EDCs introduced to
the environment via land application. Future research into the effects of enhanced storage
or post-stabilization treatment practices, such as aerated storage, composting and heat
drying/pelletization may help to identify best-management practices to further reduce
(anti)estrogenicity in land-applied biosolids. Furthermore, storage durations beyond the
four-month mandated period should be considered to fully evaluate the effects of long-
term ageing.
8. SUMMARY, CONCLUSIONS, CONTRIBUTIONS AND RECOMMENDATIONS

8.1. SUMMARY

Although considerable focus has been placed on measuring the chemical profiles of estrogenic endocrine disruptors (eEDCs) through various stages of wastewater treatment, little attention has been paid to the fate of eEDCs during sludge treatment processes. Even less work has examined the net estrogenic and antiestrogenic potency of sludges and biosolids using in vitro bioassay-based tests. In the present study, two in vitro bioassays were used to quantify the net estrogenic and antiestrogenic potency of wastewater solids as they proceeded through the unit treatment processes of two full-scale wastewater treatment plants: the City of Guelph and City of London (ON) - Oxford WWTP. While the two WWTPs had similar levels of net estrogenicity in the influent, they differed in both the source composition and the type of treatment provided to the solids (anaerobic versus aerobic). In addition, a Lystek pilot system at one of the WWTPs allowed for the determination of the effects of advanced biosolids treatment on changing the net estrogenic and antiestrogenic potency of stabilized biosolids. Ageing tests were conducted on both Class A and Class B biosolids to simulate the effects of seasonal storage conditions on (anti)estrogenic stability and fate. The testing of estrogenic and antiestrogenic potency by the Yeast Estrogen Screen (YES) assay was made possible due to method-development work which identified sample stabilization, extraction and cleanup conditions that effectively overcame toxic interferences that routinely plague the YES assay when testing sludges and biosolids.
8.2. CONCLUSIONS

The sludge/biosolid sample preparation steps developed in this research were found to reliably yield sample extracts that could be applied to the YES assay without the decreases in yeast growth frequently encountered in previous studies. Based on the YES assay results for the Guelph WWTP, net estrogenicity increased in the first two phases of sludge stabilization (primary → aeration tank, aeration tank → mesophilic anaerobic digester). The two stages of net estrogenicity increase corroborates the results of previous researchers who found that estrogenicity increases on a unit-mass basis during initial aerobic treatment (due to the deconjugation of inactive hormonal metabolites), and during anaerobic digestion (due to the breakdown of long-chain surfactant molecules into more active degradation by-products). Net estrogenicity was similar in samples from both of the anaerobic digesters that were sampled, despite one of the digesters running at a longer SRT (16.5 versus 14 days) and a higher total solids content (3.7% versus 2.2%).

Net estrogenicity values were also similar when comparing samples from the primary anaerobic digesters, secondary anaerobic digester, and dewatered solids, indicating that the estrogenic substances present in the biosolids were recalcitrant under the given treatment conditions. Lystek thermal/alkaline treatment did yield a slight (16.5%, on average) reduction in net estrogenic potency relative to the dewatered digested solids fed into the system. The antiestrogenicity assay confirmed the presence of antiestrogenic potency in sludge/biosolid samples from throughout the treatment train. In fact much like estrogenic potency, antiestrogenic potency tended to increase as the solids underwent successive treatment steps. However, overall percent-increases in antiestrogenic potency between treatment processes were typically less than the
corresponding percent-increases in net estrogenic potency. It is important to simultaneously consider the evolution of both estrogenicity and antiestrogenicity, as apparent increases in a sample’s estrogenicity can be the result of a reduction in antiestrogenic compounds, which ‘mask’ the presence of estrogen receptor agonists in the YES assay. Thus in this study, the observed increases in net estrogenic potency throughout the stabilization process were indeed true increases, i.e. the measured increases in estrogenicity occurred despite parallel increases in the presence of antiestrogens.

While the London-Oxford WWTP had slightly higher average net estrogenicity in the influent compared to the Guelph WWTP, the levels measured in the sludge were considerably lower than those in the Guelph samples, and the MBR permeate and post-UV effluent samples were always below 0.5 ng/L E2-Eq, compared with an average of 2.1 ng/L E2-Eq in Guelph WWTP final effluent. Other research has suggested that aerobic treatment is more effective at removing eEDCs than anaerobic digestion, and that industrial estrogens are less effectively removed than natural estrogens. These factors likely influence the disparity in the results between Guelph and London, as: (i) London-Oxford’s influent is purely residential in composition, whereas 40% of Guelph’s influent comes from industrial/commercial/institutional discharges, (ii) the SRT of the London-Oxford plant’s CAS system is longer than Guelph’s (12 days versus 8.5), allowing for greater contact time between eEDCs and microbes, and (iii) sludge at the London-Oxford plant is treated solely by aerobic processes (CAS/MBR/MBR thickener) rather than the anaerobic systems at Guelph. Furthermore, London-Oxford’s final effluent is disinfected with UV treatment, while Guelph’s final effluent is chlorinated/de-chlorinated. This may
account for the higher antiestrogenic potency observed in Guelph’s effluent vis-a-vis London-Oxford’s as chlorination has been demonstrated to produce antiestrogenic by-products (Wu et al., 2009).

Due to sampling limitations, it was not possible to calculate net estrogenic loads between treatment processes at London-Oxford. However, the intensive sampling at the Guelph WWTP did allow for the development of a clearer picture of net estrogenicity changes throughout the treatment process. In total, approximately 2.5% of the net estrogenic load entering the Guelph WWTP remained in the treated biosolids, with approximately double the amount being discharged in the final effluent. In spite of the increase in net estrogenicity on a unit-mass basis when the mixed primary/thickened waste-activated sludge feed underwent anaerobic digestion, the overall estrogenic load in the digested solids still decreased by 41% on average, due to the overall solids destruction. This finding helps to resolve uncertainties that have arisen in past research concerning the fate of eEDCs during wastewater treatment, in which the focus was largely on quantifying eEDC loads in the treatment plant influent and effluent. While the percent-removal of most eEDCs appeared high on the basis of influent-to-effluent comparisons, such studies did not fully resolve whether eEDCs were truly ‘removed’ from wastewater, or rather partitioned (partially or fully) to the solid phase. The results of the biosolids storage studies showed that the estrogenic potency of land-applied biosolids may in fact be even lower than what is measured immediately post-treatment. Biosolids storage, whether for logistical reasons of supply-and-demand or mandated due to seasonal application restrictions, allow for ongoing microbial activity or physicochemical processes to alter the (anti)estrogenic compounds present in the solids.
In this study, dewatered Class B biosolids underwent a 77% and 75% reduction in net estrogenicity after four months of storage at 4 °C and 25 °C, respectively, and an 18% decrease after four months of storage under ambient freeze-thaw conditions. However, Lystek (Class A) biosolids showed much less consistency in net estrogenicity changes; while it decreased by 29% after four months storage at 4 °C and 50% under ambient conditions, it increased by 9% at 25 °C. Moreover, all samples showed spikes in estrogenicity within the four-month period. Thus while ultimate removals were observed in five of the six study conditions after four months, shorter (or potentially, longer) storage periods could potentially lead to increased estrogenic loads relative to fresh biosolids. This study also found that under temperate conditions, estrogenicity profiles over time appeared to depend on the source composition of the biosolids (and presumably on the physical, microbial and chemical properties of the solids). In comparison, both belt press and Lystek samples showed similar changes in estrogenicity profiles under freeze-thaw conditions, indicating that under these conditions physicochemical processes may be more important than microbial degradation pathways. Antiestrogenic compounds also persisted during storage, and while overall potency (in terms of IC$_{50}$ values) either decreased or remained the same by the end of four months’ storage, spikes occurred within this period in five of the six samples.

8.3. CONTRIBUTIONS

The main engineering, scientific, and policy/guideline-related contributions of this research are summarized as follows.

- From an engineering perspective, this research is the first in which the effects of solids stabilization unit processes in altering the net estrogenic and antiestrogenic
potency of municipal wastewater sludges has been studied over the long-term at the full-scale. With regards to process conditions, this research makes the following contributions to the body of wastewater engineering knowledge and practice:

- Aerobic stabilization conditions, particularly at longer solids retention time (SRT) reduces the net estrogenicity of municipal wastewater sludges;
- Conversely, anaerobic stabilization increases both the net estrogenicity and antiestrogenicity of biosolids, although the overall increase in estrogenicity is greater than the corresponding increase in antiestrogenic potency;
- The Lystek provided minimal reduction of both net estrogenic and antiestrogenic potency relative to anaerobically-digested feed solids, indicating that such compounds are recalcitrant to further degradation under combined alkaline/high-temperature processing;
- Despite initially similar levels of net estrogenicity in the raw wastewater, sludge/biosolid samples from the London-Oxford WPCP (which treats only domestically-sourced sewage) were consistently lower in net estrogenicity than samples from the Guelph WWTP, which treats sewage that averages 40% industrial/commercial/institutional input. Therefore, while the contribution of industrial estrogenic endocrine disruptors towards the overall estrogenic potency of wastewater (and by extension, sludge/biosolids) has been presumed in the past to be relatively minor compared to natural estrogens, anaerobic solids stabilization processes
appear to increase the potency of industrially-derived eEDCs

- This study is the first to present a comprehensive mass balance for eEDCs throughout the conventional activated sludge treatment process and associated solids-stabilization processes. Despite slight increases in net estrogenicity between certain stabilization steps, the overall results of the mass balance confirm that aerobic biological wastewater treatment provides a true reduction in the net estrogenic potency, rather than facilitating the transfer of estrogenic species from the liquid to the solid phase

- From a scientific perspective, this work is the first to systematically study and develop sample preparation processes which consistently mitigate the toxic effects that sludge/biosolid samples have on recombinant yeast-based (anti)estrogenicity bioassays. Accordingly, the steps developed in this research allow for the application of such assays on sample matrices for which data were frequently incomplete or unreliable, and allow for the establishment of an eEDC mass balance across the aerobic biological wastewater treatment process;

- Furthermore, this study is the first to the author’s knowledge in which the net antiestrogenicity of wastewater sludges and biosolids has been quantified. With research into the environmental effects of exposure to antiestrogens beginning to complement the ongoing focus on exposure to estrogenic trace contaminants, this research provides a starting point from which the potential environmental impacts of antiestrogens in land-applied biosolids can be determined;

- With respect to potentially informing future policies and/or guidelines
surrounding land-application of biosolids, the following recommendations can be made based on this research:

- Industrially-derived eEDCs appear to contribute a significant proportion of the net estrogenic potency in municipal biosolids, particularly following anaerobic digestion. As such, aerobic post-treatment whether in the form of windrows or active composting should be considered for anaerobically stabilized solids (based on the wastewater source composition for a given treatment plant) prior to land-application to reduce eEDC loading if such loads are determined to be ecologically deleterious;

- As an alternative to post-anaerobic digestion enhanced treatment steps, mandating storage periods prior to the land-application of biosolids could be used to reduce the loading of eEDCs to the environment. However, given that spikes in both net estrogenic and anti-estrogenic potency were observed during the four-month storage period in this study, a minimum overall ageing time would need to be determined through further study to ensure that the storage period allowed for sufficient eEDC degradation;

If minimization of the loading of estrogenic and anti-estrogenic substances to terrestrial environments becomes a primary objective within land-application practices, the use of biosolids from plants receiving only domestic wastewater inputs may be recommended. However, more research involving a wider number of municipal WWTPs is needed to confirm that industrially-sourced compounds do indeed increase the (anti)estrogenic load of biosolids relative to biosolids that result from purely domestic inputs. Alternative technologies such as sludge pyrolysis could then be considered for the sustainable reuse
of mixed-source biosolids to reduce the transfer of (anti)estrogenic compounds to the environment.

8.4. RECOMMENDATIONS

In this study, differences in wastewater source composition and treatment processes between the Guelph and London-Oxford WWTPs were confounded (mixed-source/anaerobic treatment versus residential-source/aerobic treatment). Ideally, net estrogenicity and antiestrogenicity measurements would be made on samples from four treatment plants: (i) mixed-source/anaerobic, (ii) mixed-source/aerobic, (iii) residential-source/anaerobic, (iv) residential-source/aerobic. This approach would better resolve to what extent each factor contributes to increases or decreases in the relative (anti)estrogenic potency of the final biosolids.

The effects of further biosolids treatment methods, such as aerated storage, composting, and heat drying/pelletization on the (anti)estrogenicity of biosolids would be useful in developing future best-management practices for land-applied biosolids. Currently, pelletization is carried out to a limited extent in Ontario (Toronto, Windsor, Smiths Falls), and biosolids composting is not currently conducted at the municipal scale, though both processes are more commonplace in the USA and Europe. However, declining landfill capacity and public pressure for biosolids treatment processes that further reduce odour and vector-attraction problems may increase the extent to which advanced biosolids treatment and subsequent land-application becomes the norm in Ontario.

From a risk-assessment perspective, ultimately more research is needed to determine what the actual *in vivo* effects are of estrogenic and antiestrogenic compounds
entering the terrestrial and aquatic environment via land-applied biosolids. In the meantime, the sample processing techniques developed in this study may allow for wider application of \textit{in vitro} bioassays to screen for potential exposure to estrogenic and antiestrogenic substances in land-applied biosolids and biosolid-impacted soils.
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http://water.epa.gov/polwaste/wastewater/treatment/biosolids/index.cfm Date accessed: 30.05.2011


**APPENDIX 1: PRESENCE OF E1, E2, E3, EE2 IN WASTEWATER TREATMENT PLANTS WORLDWIDE**

<table>
<thead>
<tr>
<th>Year</th>
<th>Country</th>
<th>Authors</th>
<th>WWTP Operating Details</th>
<th>E2 (ng/L)</th>
<th>E1 (ng/L)</th>
<th>EE2 (ng/L)</th>
<th>E3 (ng/L)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Influent</td>
<td>Effluent</td>
<td>Influent</td>
<td>Effluent</td>
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<tr>
<td>1998</td>
<td>Canada</td>
<td>H.-B. Lee and T.E. Peart</td>
<td>Preservedgrab samples from <strong>Guelph</strong>: influent and tertiary effluent</td>
<td>N/D (&lt;5)</td>
<td>N/D (&lt;5)</td>
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<td>1998</td>
<td>Canada</td>
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<td>Grab samples from <strong>Guelph</strong>: influent and tertiary effluent</td>
<td>9.0-21</td>
<td>N/D (&lt;5)</td>
<td>29-53</td>
<td>11-17</td>
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<tr>
<td>1998</td>
<td>Canada</td>
<td>“</td>
<td>Grab samples from Burlington; primary and tertiary effluent</td>
<td>5.2-8.8</td>
<td>N/D (&lt;5)</td>
<td>23-29</td>
<td>4.5-7.5</td>
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<td>Canada</td>
<td>“</td>
<td>Grab samples from Burlington; primary and tertiary effluent</td>
<td>8.0-20</td>
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<td>41-65</td>
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<td>Canada</td>
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<td>8.0-9.0</td>
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<td>1999</td>
<td>Canada</td>
<td>T.A. Ternes et al.</td>
<td>Relatively high EE2 effluent concentration; authors themselves question this</td>
<td>N/A</td>
<td>3.0</td>
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<td>1999</td>
<td>Germany</td>
<td>“</td>
<td>Influent sampled with flow-proportional autosampler; effluent came from WWTP’s using iron coagulants for phosphorus removal</td>
<td>15.0</td>
<td>N/D</td>
<td>27</td>
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<td>2000</td>
<td>Italy</td>
<td>C. Baroni et al.</td>
<td>Cobis WWTP; composite samples</td>
<td>8.1-25</td>
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<td>5.4-17</td>
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<td>Fregene WWTP; composite samples</td>
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205
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<th>EE2 (ng/L)</th>
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<td>Effluent</td>
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<td>C. Baronti et al.</td>
<td>Ostia WWTP; composite samples</td>
<td>5.9-22</td>
<td>0.72-3.5</td>
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<td>13-82.1</td>
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<td>Italy</td>
<td>&quot;</td>
<td>Roma Sud WWTP; composite samples</td>
<td>4.7-10</td>
<td>0.53-3.1</td>
<td>25-48</td>
<td>8.7-51</td>
</tr>
<tr>
<td>2000</td>
<td>Italy</td>
<td>&quot;</td>
<td>Roma Est WWTP; composite samples</td>
<td>6.3-11</td>
<td>0.62-0.82</td>
<td>34-68</td>
<td>3.7-10</td>
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<td>2000</td>
<td>Italy</td>
<td>&quot;</td>
<td>Roma Nord WWTP; composite samples</td>
<td>6.3-14</td>
<td>0.44-1.9</td>
<td>30-49</td>
<td>6.4-40</td>
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<td>2000</td>
<td>Italy</td>
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<td>Roma Sud WWTP; composite samples</td>
<td>4.7-10</td>
<td>0.53-3.1</td>
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<td>8.7-51</td>
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<td>2000</td>
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<td>Roma Est WWTP; composite samples</td>
<td>6.3-11</td>
<td>0.62-0.82</td>
<td>34-68</td>
<td>3.7-10</td>
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<td>2000</td>
<td>Italy</td>
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<td>Roma Nord WWTP; composite samples</td>
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<td>0.44-1.9</td>
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<td>30-49</td>
<td>6.4-40</td>
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<td>6.4-40</td>
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<td>2000</td>
<td>Italy</td>
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<td>2000</td>
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<td>0.62-0.82</td>
<td>34-68</td>
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<td>Italy</td>
<td>&quot;</td>
<td>Roma Nord WWTP; composite samples</td>
<td>6.3-14</td>
<td>0.44-1.9</td>
<td>30-49</td>
<td>6.4-40</td>
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<td>H.-B. Lee et al.</td>
<td>Ashbridges Bay WWTP; composite samples</td>
<td>9.6-15</td>
<td>&lt;1-4.4</td>
<td>35-43</td>
<td>16-56</td>
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<td>&quot;</td>
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<td>&quot;</td>
<td>Humber WWTP; composite samples</td>
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<td>2004</td>
<td>Canada</td>
<td>&quot;</td>
<td>North Toronto WWTP; composite samples</td>
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<td>&lt;1-1.7</td>
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<td>O. Braga et al.</td>
<td>CAS (SBR - anoxic/ aerobic) Tertiary treatment = continuous micro- filtration (CMF), RO and chlorination/dechlorination</td>
<td>2.2-72 (avg. = 22)</td>
<td>N/D (&lt;1)</td>
<td>29-93 (avg. = 55)</td>
<td>N/D (&lt;5)</td>
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<td>&quot;</td>
<td>Effluent from advanced primary treatment (ferric chloride addition)</td>
<td>6.3-29 (avg. = 12)</td>
<td>Avg. 11.5</td>
<td>36-81 (avg. = 53)</td>
<td>Avg. 45.8</td>
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<td>Canada</td>
<td>L. Lishman et al.</td>
<td>24-hour composite samples from Thames River area; no correction was made for incomplete recovery</td>
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<td>N/D (&lt;5)</td>
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<td>7.6-38</td>
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<td>Authors</td>
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<td>Effluent</td>
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<tr>
<td>2007</td>
<td>Australia</td>
<td>B.L.L. Tan et al.</td>
<td>Secondary effluent; CAS process</td>
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<td>N/D-13.6</td>
<td>3.5-22.7</td>
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<tr>
<td>2007</td>
<td>Australia</td>
<td>B.L.L. Tan et al.</td>
<td>Secondary effluent; revolving-plate/spiral to aerate bioreactor</td>
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<td>N/D-9.5</td>
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<td>Australia</td>
<td>B.L.L. Tan et al.</td>
<td>Secondary effluent; CAS process</td>
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<td>N/D</td>
<td>2.9-13.7</td>
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<tr>
<td>2007</td>
<td>Australia</td>
<td>B.L.L. Tan et al.</td>
<td>Secondary effluent; BNR process</td>
<td>N/D-709</td>
<td>N/D</td>
<td>N/D-9.5</td>
<td>N/A</td>
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<td>2007</td>
<td>Australia</td>
<td>B.L.L. Tan et al.</td>
<td>Secondary effluent; CAS process</td>
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<td>5.1-31.5</td>
<td>1.3-12.1</td>
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<td>Canada</td>
<td>A.J. Spring et al.</td>
<td>Primary treatment incl. Ferric chloride, secondary clarification UV disinfection</td>
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<td>N/D (&lt;0.5)</td>
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<td>N. Cicek et al.</td>
<td>Brandon MB WWTP; SBRs with UV disinfection</td>
<td>26.5</td>
<td>4.4</td>
<td>72.26</td>
<td>4.91</td>
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<td>2007</td>
<td>U.S.A.</td>
<td>M. Esperanza et al.</td>
<td>Pilot CAS plants with 3-stage secondary digesters and secondary clarification</td>
<td>38.9-50.3</td>
<td>N/D-2.2</td>
<td>44.7-54.9</td>
<td>6.9-11.1</td>
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<td>2008</td>
<td>Australia</td>
<td>B.L.L. Tan et al.</td>
<td>Grab samples from the influent of a 5000 p.e. BNR WWTP with tertiary treatment (sand filtration/activated carbon/UV)</td>
<td>12.2</td>
<td>N/A</td>
<td>37.5</td>
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APPENDIX 2: DETECTION OF GENERAL BACTERIAL GROWTH IN BIOSOLIDS

The general presence of bacterial growth was evaluated using the method of Lotrario et al. (1995) in the following samples:

- Mixed primary/thickened waste activated sludge (feedstock for the Guelph WWTP’s mesophilic anaerobic digesters)
- Anaerobically digested solids
- Belt-thickened anaerobically digested solids
- Lystek-treated thickened solids (i.e. heat- and alkaline-Pasteurised)

Portions of each sample were sterilized by four different methods:

- Heat drying at 105 °C for 16 hours
- Lyophilisation at -80 °C for 36 hours (VirTis, Toronto, ON)
- Autoclaving (121 °C/98 kPa) for 30 minutes followed by drying at 60 °C for 16 hours
- Addition of 37% w/v formaldehyde followed by drying at 60 °C for 16 hours

Tryptic soy broth (TSB; Fisher Scientific, Oakville, ON) was prepared by mixing 9 g of TSB powder into 300 mL of MilliQ water and autoclaving at 121 °C for 30 minutes. Borosilicate glass test tubes and HDPE test tube covers were autoclaved at 121 °C for 15 minutes, and 7 mL of sterilized TSB medium was aseptically pipetted into each test tube. After addition of TSB, each test tube lip was flame-sterilized before replacing the test tube cover. For anaerobic digester influent/effluent samples, 6 g of sludge was added per test tube. For belt-press thickened and Lystek solids, only 2 g of sample was
added to each test tube, due to higher solids content. After addition of sludge samples, each test tube was vortex-mixed using a whirly-mixer. The test tubes were placed in incubators at either 28 °C, 35 °C or 44.5 °C with their lids slightly ajar to allow for air/gas exchange. The test tubes were vortex mixed every 24 hours and incubated for a total of 5 days. Each sample was prepared in triplicate.

During the incubation period, an agar mixture (20 g agar + 30 g TSB powder in 1000 mL MilliQ water, autoclaved at 121 °C for 30 minutes) was prepared, and 10 mL of agar mixture pipetted into sterile 10 cm diameter Petri dishes. After incubation, 250 µL aliquots of incubated sample were aseptically pipetted onto agar-filled Petri dishes. For anaerobic digester influent/effluent samples, the 250 µL sample was taken from the supernatant layer (as the solids had settled to the bottom of the test tube). The solids in Lystek influent/effluent samples didn’t settle, and hence these samples were centrifuged at 1300g for 5 minutes and supernatant was used for plating. The aliquots of sample were spread evenly over the agar layer using the spread-plate method. Samples were then further incubated at 28/35/44.5 °C, and the visible growth of bacterial colonies on the agar layer was recorded over 96 hours. Control plates were prepared by spread-plating 250 µL portions of autoclaved MilliQ water on agar plates and incubating these alongside the test plates. The results of the testing for the four different pre-treatments are present in Tables A1-A4. No growth was observed in any of the MilliQ control plates.
Table A1: Visible bacterial regrowth in extracts of wastewater solids treated by heat drying at 105 °C; N = no visible colonies, Y = visible colonies present

<table>
<thead>
<tr>
<th>Heat Drying (105 °C)</th>
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<th>44.5°C</th>
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<tr>
<td>HOURS →</td>
<td>24</td>
<td>48</td>
<td>72</td>
</tr>
<tr>
<td>1º/TWAS A</td>
<td>N</td>
<td>N</td>
<td>Y</td>
</tr>
<tr>
<td>1º/TWAS B</td>
<td>N</td>
<td>N</td>
<td>Y</td>
</tr>
<tr>
<td>1º/TWAS C</td>
<td>N</td>
<td>N</td>
<td>Y</td>
</tr>
<tr>
<td>Digested solids A</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Digested solids B</td>
<td>N</td>
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<td>N</td>
</tr>
<tr>
<td>Digested solids C</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Belt-thickened solids A</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>Belt-thickened solids B</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>Belt-thickened solids C</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>Lystek-treated solids A</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Lystek-treated solids B</td>
<td>N</td>
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<td>N</td>
</tr>
<tr>
<td>Lystek-treated solids C</td>
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Table A2: Visible bacterial regrowth in extracts of wastewater solids treated by lyophilisation at -80 °C; N = no visible colonies, Y = visible colonies present

<table>
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<td>24</td>
<td>48</td>
<td>72</td>
</tr>
<tr>
<td>1º/TWAS A</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>1º/TWAS B</td>
<td>N</td>
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<td>N</td>
</tr>
<tr>
<td>1º/TWAS C</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Digested solids A</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Digested solids B</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Digested solids C</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
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<td>Belt-thickened solids A</td>
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<td>N</td>
<td>Y</td>
</tr>
<tr>
<td>Belt-thickened solids B</td>
<td>N</td>
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<td>Y</td>
</tr>
<tr>
<td>Belt-thickened solids C</td>
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<td>Y</td>
</tr>
<tr>
<td>Lystek-treated solids A</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Lystek-treated solids B</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Lystek-treated solids C</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
</tbody>
</table>
Table A3: Visible bacterial regrowth in extracts of wastewater solids treated autoclaving; N = no visible colonies, Y = visible colonies present.

<table>
<thead>
<tr>
<th>30 min. Autoclaving</th>
<th>28°C</th>
<th>35°C</th>
<th>44.5°C</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HOURS</strong></td>
<td>24</td>
<td>48</td>
<td>72</td>
</tr>
<tr>
<td>1º/TWAS A</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>1º/TWAS B</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>1º/TWAS C</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Digested solids A</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Digested solids B</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Digested solids C</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Belt-thickened solids A</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>Belt-thickened solids B</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>Belt-thickened solids C</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>Lystek-treated solids A</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Lystek-treated solids B</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Lystek-treated solids C</td>
<td>N</td>
<td>N</td>
<td>N</td>
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</tbody>
</table>

Table A4: Visible bacterial regrowth in extracts of wastewater solids treated by formaldehyde addition; N = no visible colonies, Y = visible colonies present.

<table>
<thead>
<tr>
<th>Formaldehyde</th>
<th>28°C</th>
<th>35°C</th>
<th>44.5°C</th>
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<tbody>
<tr>
<td><strong>HOURS</strong></td>
<td>24</td>
<td>48</td>
<td>72</td>
</tr>
<tr>
<td>1º/TWAS A</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>1º/TWAS B</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>1º/TWAS C</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Digested solids A</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Digested solids B</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Digested solids C</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Belt-thickened solids A</td>
<td>N</td>
<td>N</td>
<td>Y</td>
</tr>
<tr>
<td>Belt-thickened solids B</td>
<td>N</td>
<td>N</td>
<td>Y</td>
</tr>
<tr>
<td>Belt-thickened solids C</td>
<td>N</td>
<td>N</td>
<td>Y</td>
</tr>
<tr>
<td>Lystek-treated solids A</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Lystek-treated solids B</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Lystek-treated solids C</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
</tbody>
</table>