Characterization and Hazard Assessment of Endocrine-Disrupting Chemicals in Municipal Wastewater Effluents

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Author's declaration

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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ABSTRACT OF THESIS submitted by:
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There is growing concern about exposure to chemicals, especially those that have been shown to interfere with the endocrine system of vertebrates, hence called endocrine disrupters. Reported endpoints include effects on the reproductive system of humans and other animals, such as feminization of males, breast and testicular cancer, altered sex ratios in wildlife, and birth defects. The main point source of endocrine disrupters into the environment is wastewater treatment plant (WWWTP) effluents.
A preliminary screening for estrogenicity and (anti-)androgenicity was performed on samples from two WWTPs in Greece, Athens and Mytilene. Estrogenic potentials were found in both effluents, with higher activity found in Mytilene. The apparent higher efficiency of removal of estrogenic potential in the Athens WWTP could be due to an extra step of clarification, which removes inorganic solids. Neither androgenic nor anti-androgenic activity was found on the samples; therefore these potentials are not of concern. The expected positive correlation between endocrine potential and wastewater influx was not observed. On the other hand, the effect of a difference in wastewater treatment technology could not be fully clarified. While the addition of primary clarification in the Athens WWTP is the possible cause of a lower estrogen potential, such correlation could not be associated with a longer hydraulic retention time.
Although a complete assessment of risks require more studies, *in vitro* assays such as those applied in this thesis provide useful data for initial screening of hazards, and to design further assessments, particularly when integrated in approaches such as effect-directed analysis.

Keywords: endocrine disruptors, wastewater treatment plant effluents, effect-directed analysis

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"You are capable of more than you know. Choose a goal that seems right for you and strive to be the best, however hard the path. Aim high. Behave honorably. Prepare to be alone at times, and to endure failure. Persist! The world needs all you can give."

- Edward O. Wilson

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List of Abbreviations

ATCC	American Type Culture Collection
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichloro-diphenyl-tricloroethane
DHT	Dihydrotestosterone
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E2	Estradiol
EDA	Effec-directed analysis
EDC	Endocrine-disrupting chemical
FBS	Fetal bovine serum
fM	Femtomolar
g	Gram
h	Hour
HF	Hydroxyflutamide
L	Liter
max	Maximum
mL	Mililiter
mm	Milimeter
mRNA	Messenger ribonucleic acid
n	Sample size
nm	Nanometer
nM	Nanomolar
NR	Nuclear hormone receptor
PBS	Phosphate-buffered saline
PCBs	Polychlorinated biphenyls
pH	Potential of hydrogen
pM	Picomolar
PPCPs	Pharmaceuticals and personal care products
rpm	Revolutions per minute
SPE	Solid-phase extraction
TIE	Toxicity identification and evaluation
v/v %	Volume percent concentration
WWTP	Wastewater treatment plant
µL	Microliter

1. fIntroduction

Agricultural and industrial activities, wastewater effluents, and storm water runoff from urban areas result in the discharge of many chemicals into aquatic ecosystems, affecting their quality. Both public and scientific interest regarding exposure to chemicals has increased as more evidence on sources, pathways, and negative impacts on humans and wildlife is reported (Ritter, 2002). Particularly, recent concerns involve chemicals that have been shown to interfere with the endocrine system of vertebrates, hence called endocrine disrupters (Crisp *et al.*, 1998; Jobling *et al.*, 1998; Hecker and Hollert, 2011; Swart and Pool, 2013). Reports include effects on the reproductive system of humans and other animals, such as feminization of males, breast and testicular cancer, altered sex ratios in wildlife, and birth defects (Crisp *et al.*, 1998; Ternes *et al.*, 1999; Swart and Pool, 2013). These effects are further alarming because most endocrine disrupters bioaccumulate in some organisms, complicating scenarios of how environmental concentrations of toxicants translate into ecological effects (Tyler *et al.*, 1998).

Endocrine disrupters, or endocrine-disrupting chemicals (EDCs), are “exogenous agents that interfere with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development and/or behavior” of organisms (Crisp *et al.*, 1998). EDCs encompass a wide spectrum of chemicals defined by their biological activity rather than their chemical structure. Endocrine activity has been identified for dioxins, pesticides, pharmaceuticals and personal care products (PPCPs), phytoestrogens, plasticizers and many other substances (Ternes *et al.*, 1999; Rotchell and Ostrander, 2003; Swart and Pool, 2013). PPCPs are an important

subgroup among EDCs due to their ubiquity, and because they have been found in soils, sediments, and water. Although some PPCPs are readily biodegradable, an ever-increasing human consumption causes a “pseudopersistent” state in sewage and effluents (Garric, 2013). Even if passing through a sewage treatment plant, the efficiency of removal of PPCPs and other EDCs varies among systems (Garric, 2013). Incomplete elimination of EDCs could result in their dispersal in surface waters, which raises concern about potential effects in non-target species. Aquatic organisms might be exposed to contaminants during their entire lifecycle and many physiological consequences are still unknown. Although there is evidence that EDCs affect the development and function of the reproductive and nervous systems of animals under laboratory conditions (Kloas *et al.*, 2009), it is still unclear how concentrations found in the environment are posing a risk to wildlife or humans (Tyler *et al.*, 1998; Hecker *et al.*, 2002). The ecotoxicity of EDCs raises issues such as environmental and human health, wastewater treatment, and environmental risk assessment, relevant to both scientists and policy-makers (Garric, 2013).

Environmental risk assessment of EDCs requires precise measurements of such compounds, as well as a clear understanding of their mechanism of action and dispersal in the environment (Ternes *et al.*, 1999). However, characterization of environmental exposure to EDCs is not straightforward due to wide variety of chemical families that have been identified as such, along with the fact that many chemicals have not yet been identified as endocrine disruptors or there are currently no viable methods to analyze them. Furthermore, environmental samples usually contain complex mixtures of several chemical compounds, increasing the possibility of various interactions between the components therein that could result in agonistic,

antagonist or synergistic effects. Toxicity Identification and Evaluation (TIE), and Effect-Directed Analysis (EDA) are proposed approaches for the identification of EDCs that overcome these analytical complications (Ternes *et al.*, 1999), by assessing toxicity through the integration of chemical and biological analyses. These methods are being applied by an increasing number of research groups and environmental agencies (Hecker and Hollert, 2009). The United States Environmental Protection Agency has stated that a better understanding of causes and effects of endocrine disruption, as well as the identification of EDCS, will improve risk prevention capacity.

Even while not all EDCs have been recognized, and consequences of endocrine disruption are still uncertain, there is a need to regulate exposure to these chemicals and include them in standard risk assessments (Hecker and Hollert, 2011). Moreover, increased understanding of the dynamics of EDCs will allow for an improvement of current treatment technologies of wastewater treatment plants (WWTPs) with the goal to increase efficiencies in removing this type of chemicals. Public policies attending toxicity issues require as much evidence as possible, as decision makers must also respond to public concern and economical interests (Vindimian, 2013). Therefore, characterization of potential risks to humans and wildlife is essential in context with assessing impacts and translating them into regulatory guidelines and requirements.

In order to set a background for this thesis, the current knowledge on EDCs, such as evidence on their effects on biota and mechanism of action are described. EDA was explored as an approach to environmental analysis of EDCs in WWTP effluents. Then, WWTP technologies are evaluated, in relation to the removal of EDCs.

a. Endocrine disrupting chemicals

There is rising concern about chemicals in the environment that mimic or interfere with the action, synthesis, metabolism or transport of endogenous hormones, and thus, disrupting reproductive function (Crisp *et al.*, 1998; Tyler *et al.*, 1998). EDCs may act as agonists or antagonists at the nuclear hormone receptors, enhancing or inhibiting the action of hormones, or they can interfere with the production or elimination of these hormones, which can result in the disruption of endogenous hormone homeostasis (Crisp *et al.*, 1998).

As mentioned above, endocrine activity has been identified for diverse chemicals. One group of contaminants that has been widely studied is organochlorine pesticides. For example, the well-known case of the pesticide DDT (dichloro-diphenyl-trichloroethane) and its effect on eggshell thinning in birds has been linked to endocrine pathways (Crisp *et al.*, 1998). Although DDT and other organochlorine pesticides have been banned in western countries, they are still present in the environment due to their persistence. PCBs (polychlorinated biphenyls) present a similar problem: they are found at around the same quantities, they were heavily used, and they are persistent contaminants (Tyler *et al.*, 1998). Dioxins are also chlorinated compounds that have been found to interfere with the endocrine system of mammals (Tyler *et al.*, 1998)

Endocrine activity has also been identified for substances other than chlorinated compounds. Surfactants such as nonylphenol ethoxylates, plasticizers such as bisphenol-A and other compounds such as phthalates have been reported to act as estrogens in wildlife (Tyler *et al.*, 1998; Kloas *et al.*, 2009). The latter represent a major issue, since they are used as plasticizers, but are also present in cosmetics,

lubricants, and many other products. Due to their ubiquity, they have been found in rivers, wastewaters, marine systems, and aquatic organisms (Tyler *et al.*, 1998).

Effects may be reversible or latent, so they might not be identified promptly. Furthermore, EDCs have different mechanisms of disruption, and their effects also depend on dose, duration of exposure, type of organism they're affecting and its developmental stage. (Crisp *et al.*, 1998).

i. Mechanisms of endocrine disruption

EDCs, and in particular PPCPs, have a specific biological activity or therapeutic effect by design (Garric, 2013). However, it is because of this characteristic, or by their side effects, that they may cause endocrine disruption in non-target organisms such as aquatic wildlife (Tyler *et al.*, 1998). There is enough homology across the endocrine systems of vertebrates for EDCs to impact them in similar ways (Swart and Pool, 2013). While less conclusive, effects have also been identified in invertebrates (Tyler *et al.*, 1998).

As previously mentioned, endocrine disruption may occur by several mechanisms, depending on the type and dose of the compound, as well as the life stage of the impacted organism. A common disruption pathway is by agonistic or antagonistic interaction with hormone receptors. Some EDCs might mimic endogenous hormones due to structural resemblance. Such EDCs often bind more weakly to receptors than the natural ligands, but can interfere with natural endocrine action (Figure 1). This could occur through competition for binding sites against endogenous hormones resulting in changed cell function by the expression of proteins (Tyler *et al.*, 1998), or by the induction of an abnormal effect, such as estrogenic activity in males. Other

compounds can block binding sites so receptors are unable to respond to endogenous ligands.

EDCs can also affect the endocrine system by interfering with the synthesis, degradation, or transport of endogenous hormones, or altering the quantity or affinity of receptors (Tyler *et al.*, 1998). Other pathways include alteration of the hormone structure, and antagonism of the effect of endogenous hormones (Swart and Pool, 2013).

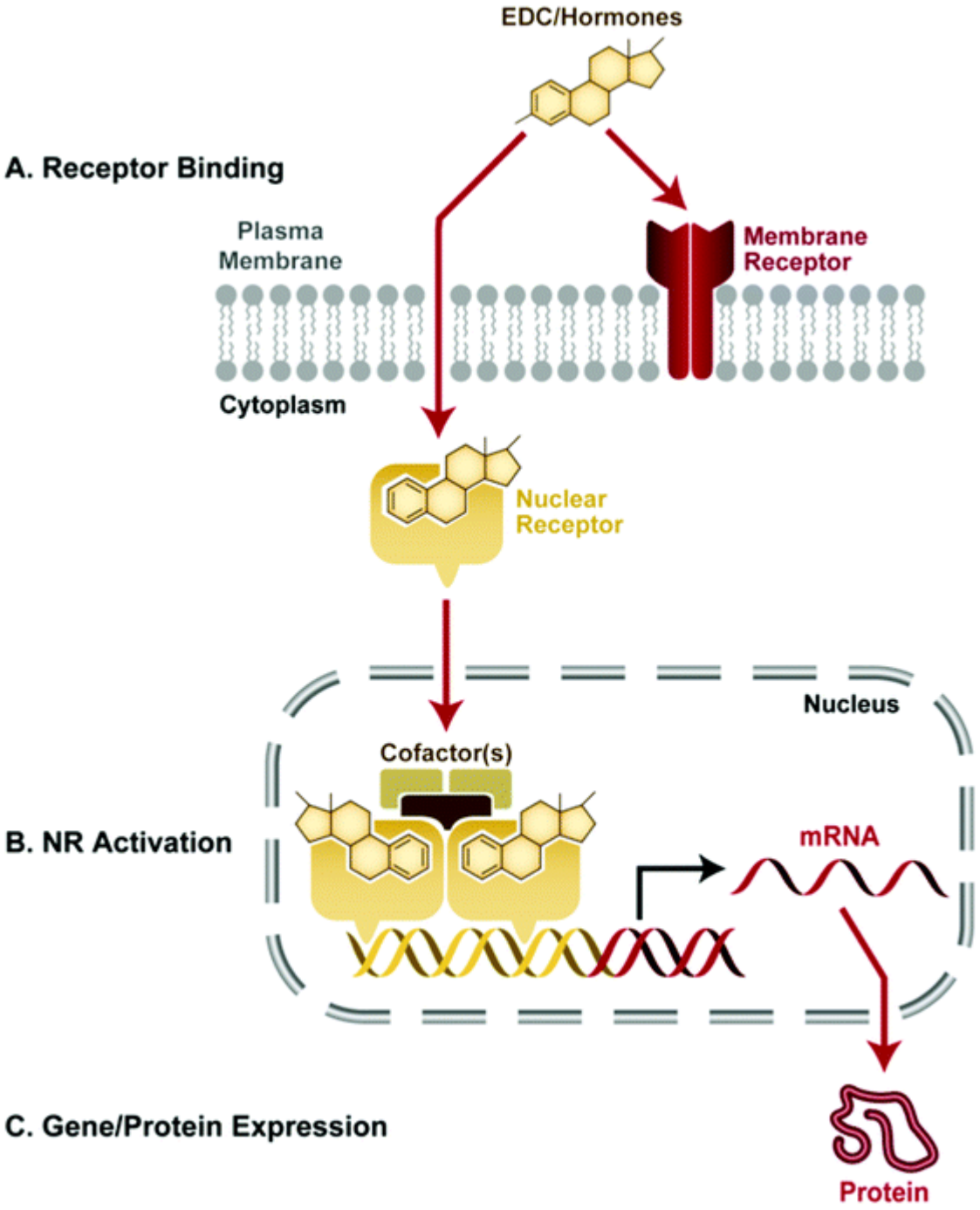

Figure 1. Schematic representation of disruption of receptor signaling by an EDC.

A: The EDC could be a small lipophilic molecule, which can pass through the plasma membrane and bind to a nuclear hormone receptor (NR). B: The NR is activated by EDC binding, and it translocates to the nucleus where the cell's transcriptional machinery, such as cofactors, are recruited to form a complex on the hormone response element of a hormone-responsive gene. C: The assembled complex promotes transcription of downstream DNA into mRNA and eventually translation into protein. Ultimately, gene and protein expression of hormone responsive genes may be influenced by EDC binding to nuclear hormone receptors (Schug *et al.*, 2013).

Laboratory studies have confirmed that EDCs can have effects on wildlife, especially on aquatic organisms (Tyler *et al.*, 1998). EDCs are readily bioavailable to fish and their impacts have been widely seen. One important effect is the “feminization” of males. This could occur through a number of pathways, and the expression of vitellogenin is one of the most studied cases. Vitellogenin is an egg yolk precursor active in females. Males also possess the vitellogenin gene, but do not express it under normal conditions. However, the presence of estrogenic compounds, such as ethinylestradiol, the active component of the contraceptive pill, as well as other natural and synthetic estrogens has been shown to promote the expression of vitellogenin in juvenile and male fish (Tyler *et al.*, 1998; Swart and Pool, 2013). Compounds that act as estrogen receptor agonists up regulate endogenous estrogen production, which leads to downstream manifestations such as the expression of vitellogenin. This phenomenon has led to vitellogenin becoming a popular biomarker for the exposure of male oviparous vertebrates to estrogenic chemicals (Rotchell and Ostrander, 2003). Extended exposure of wild populations of such vertebrates can lead to more biological relevant impacts such as intersexuality, the simultaneous presence of both male and female gonadal characteristics (Jobling *et al.*, 1998), reduced egg production, and altered gonadal development (Swart and Pool, 2013).

In addition to estrogens, androgens and antiandrogens are an important group of EDCs. These compounds bind to the androgen receptor, thus either stimulating or preventing the action of endogenous androgens, respectively. It has actually been suggested that the mechanisms by which estrogens disturb the male reproductive system is also through antiandrogenic activity, rather than, or in addition to, estrogenic pathways (Crisp *et al.*, 1998). However, differential effects of parent

compounds and their metabolites or other transformation products further complicate the interaction of certain chemicals with nuclear receptors. For example, DDT is know for its estrogenic activity, but its metabolite DDE (dichlorodiphenyldichloroethylene) has been proved to be an antiandrogen (Crisp *et al.*, 1998).

There are also several non-receptor-mediated mechanisms of endocrine disruption, such as the obstruction of synthesis, degradation, and transportation of endogenous hormones, resulting in altered hormone levels. Examples of these pathways are the decreased availability of cholesterol, a steroid precursor, by the phytoestrogen β-sitosterol, and the inhibition of androgen-to-estrogen conversion by tributyl tin (Tyler *et al.*, 1998).

Endocrine disruption through the above reviewed mechanisms has been hypothesized to translate into breast, testicular and prostate cancers, abnormal sexual development, reduced sperm count, and even neurobehavioral effects in humans (Crisp *et al.*, 1998). In wildlife, endpoints include feminization of males, masculinization of gastropods, decreased hatching success and offspring survival, and alteration of behavioral function (Tyler *et al.*, 1998)

Although our knowledge of the phenomenon of endocrine disruption is constantly increasing, studies are still limited to very few species and specific hotspots of EDCs discharges (Tyler *et al.*, 1998).

b. EDCs in the environment

EDCs can readily be dispersed in the environment. The main route of dispersion is through effluents of WWTPs, but also through agricultural runoff and industrial

effluents. Residues of personal care products and other EDCs enter the sewage system immediately following their application or improper disposal. Active compounds of pharmaceuticals or their metabolites are introduced into wastewater by excretion, hospital waste, or by improperly discarding expired drugs. Veterinary drugs are also released by excretion, but they typically enter surface water along with other EDCs directly through surface water runoff, infiltration into groundwater, or through the application of manure as fertilizer. A minor but potential source is also industrial production and distribution (Richardson and Bowron, 1985; Swart and Pool, 2013).

EDCs, particularly antibiotics and synthetic hormones, have been identified in biota, sediment, wastewater, surface water, and groundwater (Garric, 2013). However, the dynamics, effects and interactions of PPCPs and other EDCs, once released into the environment, are complicated and poorly understood. EDCs can undergo biomagnification, especially those that are of lipophilic nature. EDC concentrations have been assessed in water and primary consumers such as fish, and their concentration up the food chain has yet to be studied. As stated before, some EDCs can be bioaccumulated, and this usually occurs in specific tissues, which would concentrate the contaminants in potentially sensitive organs (Tyler *et al.*, 1998).

Endocrine disruption is relatively well understood in vertebrates, as EDCs will have similar effects on many animals compared to humans. However, their interaction with the endocrine system of invertebrates could be very different. Even if no endocrine-disrupting properties are found, EDCs could be metabolized by invertebrates into more hormonally active compounds (Tyler *et al.*, 1998).

PPCPs are released to the environment where they mix and potentially interact with other EDCs, and many other contaminants and substances. In addition, every water compartment has its own biodiversity, pH, salinity and other unique characteristics that could influence chemical behavior, speciation, affinity, and other properties that would make generalizations from laboratory experiments impossible. These factors also influence transformation processes such as biodegradation that would change the chemical nature of the parent compound (Casellas *et al.*, 2013). Specific interactions, including additive/synergistic/antagonist toxicity would need to be assessed (Tyler *et al.*, 1998).

In any case, additional knowledge on EDCs such as their behavior in the environment, bioaccumulation in organisms, effects on non-target organisms, and the efficiency of their elimination trough WWTPs is required to improve current risk assessment strategies of the exposure of humans and wildlife to this group of chemicals.

c. EDCs in municipal waste water effluents

A wastewater treatment plant (WWTP) is a facility designed to receive sewage from residential, commercial and/or industrial sources for its cleanup and subsequent discharge into aquatic systems (Kestemont and Depiereux, 2013). Although WWTPs often include physical, chemical, and biological processes, removal of many EDCs is incomplete, and these plants represent a major source of EDCs in the environment (Kasprzyk-Hordern *et al.*, 2009; Hughes *et al.*, 2013). There is a wide range of elimination efficiencies, which depends on initial discharge, retention time, technology, and performance (Garric, 2013). For example, PPCPs were first identified in WWTPs in the 1980s (Richardson and Bowron, 1985), and now all

classes of pharmaceuticals have been found in WWTP effluents and receiving aquatic ecosystems. This has been possible due to advances in analytical techniques which allow for lower detection levels (Garric, 2013). PPCPs can be detected at concentrations of up to parts per million in influent and effluent samples of certain WWTPs (Kestemont and Depiereux, 2013).

Wastewater treatment processes vary significantly among different plants. An advanced system, involving primary to quaternary treatment, is shown here (Figure 2). Most WWTPs consist of a pre-treatment that eliminates raw materials from the influent (physical removal of debris) and a primary treatment (sedimentation of particular matter and lighter phases such as oil are skimmed off). Secondary treatment encompasses degradation of the biological content in the sewage, and is part of most modern treatment systems. In cases where the effluent is discharged into sensitive zones, a tertiary treatment is added. For discharge into swimming zones or for drinking water, quaternary treatment is required as the final step for purification in some advanced treatments (Kestemont and Depiereux, 2013).

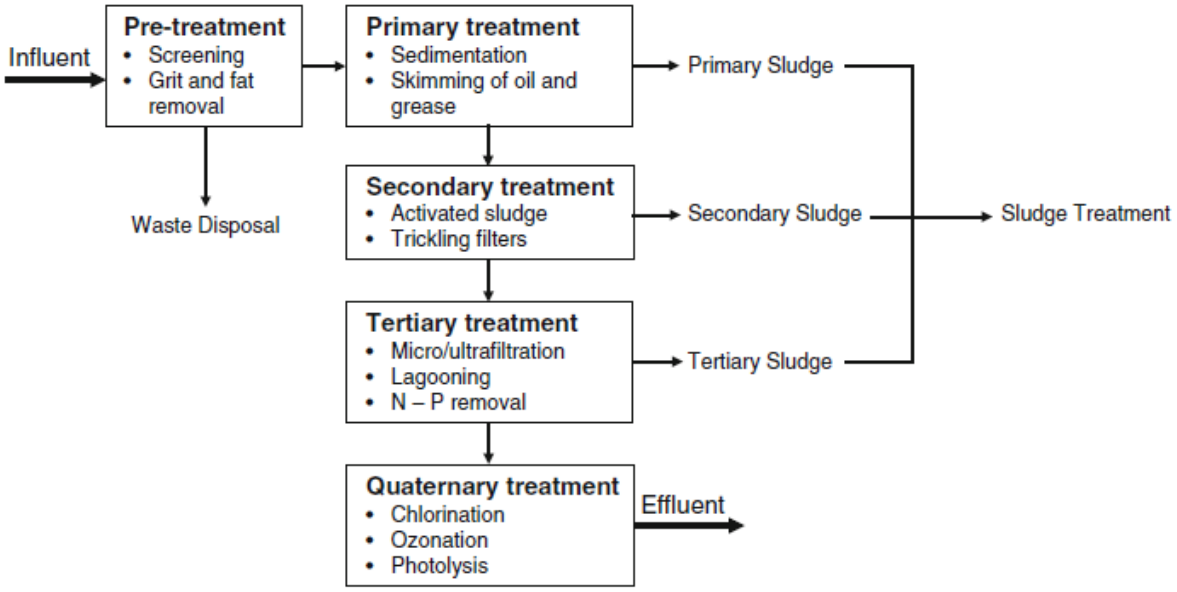

Figure 2. Process flow diagram of a WWTP (Kestemont and Depiereux, 2013).

Historically, the main objective of WWTPs was the removal of organic materials, so facilities have been engineered for that purpose. Elimination of EDCs by WWTPs occurs mainly via biodegradation or adsorption on suspended solids (Garric, 2013). Removal efficiencies for EDCs can be up to 99%. For example, the highest estrogen content has been found in effluents WWTPs with only primary treatment. On the other hand, activated sludge seems to remove more estrogenic compounds than trickling filters, although hydraulic retention time is also important (Kestemont and Depiereux, 2013). Tertiary and quaternary treatments further improve the efficiency of WWTPs. Chlorination has been reported to eliminate estrogenic activity due to the oxidation effect on the phenolic ring of some exogenous hormones; however, it might be the case that some of the metabolites formed during chlorination are more hormonally active than parent compounds (Kestemont and Depiereux, 2013). Another treatment type, ozonation, has been reported to further decrease estrogenic activity (Maletz *et al.*, 2013), and remove up to 90% of antibiotics, synthetic and

natural steroid compounds, and other EDCs (Kestemont and Depiereux, 2013). Nonetheless, ozonation has been reported to also result in the formation of active metabolites with endocrine properties (e.g. interaction with steroidogenesis; Maletz et al. 2013)

While individual EDCs are usually found at non-toxic concentrations in both influent and effluent waters, their combinatory effects remain unknown and toxicity analysis of their mixtures is essential for a proper risk assessment under realistic field situations (Garric, 2013).

d. Assessment of EDCs

Although EDCs have been identified in many environmental compartments, their presence alone does not represent a risk. Bioavailability, concentration in the environment and in the organism must also be assessed.

Since the early 1960s it has been recognized that many environmental samples include complex mixtures of numerous chemical compounds (Schuetzle and Lewtas, 1986). It also became apparent that classic analytical approaches to identify biologically active compounds were neither economically or technically feasible due to the lack of *a priori* knowledge of the sample components (Brack, 2003; Hecker and Hollert, 2009). By the 1980s, approaches combining bioassays and analytical techniques were taking place in the environmental toxicology field (Schuetzle and Lewtas, 1986). Brack (2003) described effect-directed analysis (EDA) as “a combination of biotesting, fractionation procedures and chemical analytical methods”.

Several bioassays have been developed for the analysis of endocrine activity. *In vivo* assays are an essential component of a comprehensive risk assessment of

endocrine disruption, since they provide information on organismal endpoints and have a direct application on the ecotoxicology of EDCs (Tyler *et al.*, 1998). However, initial screening with *in vivo* assays would be too costly and time-consuming, and they do not provide details on the mechanism of action of toxicants. On the other hand, *in vitro* tests are based on specific biological mechanisms and more definitive cause-response relations at the cellular level can be investigated using these systems (Zacharewski, 1997). Nevertheless, *in vitro* assays are not necessarily predictive of whole-organism and population effects (Tyler *et al.*, 1998).

As stated before, target chemical analysis of environmental samples is usually not possible or very time- and labor-consuming under complex exposure scenarios where many contaminants are unknown. On the other hand, non-target analysis is time-consuming and might not deliver appropriate results (Brack, 2003). Biotesting alone allows for detection of biological activity, but it does not provide conclusive information on the identity of the sample components (Brack, 2003). Moreover, *in vitro* effects might be different in mechanism or intensity than *in vivo* effects due to factors such as biodegradation, bioaccumulation, biomagnification, metabolism and synergy/antagonism with other compounds or the matrix (Tyler *et al.*, 1998). However, such screening tests give clues for the toxicological significance of EDCs (Crisp *et al.*, 1998), as they are based on specific steps of endocrine pathways (Wang *et al.*, 2013). EDA brings these approached together and allows for a more comprehensive analysis.

Under the EDA process (Figure 3), a sample is first analyzed for biological action and general toxicity using a battery of bioassays. If significant biological action is found in a sample, then it undergoes fractionation to separate individual or types of chemicals.

Fractions are further tested with bioassays and the process is repeated until biological activity is pinpointed to specific fractions. Finally, the fractions are chemically tested to identify individual compounds (Hecker and Hollert, 2009).

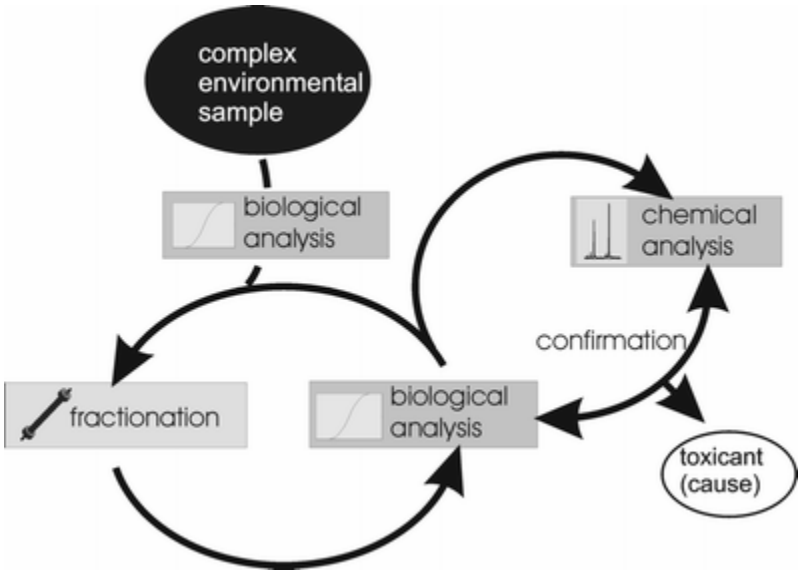

Figure 3. Principle of EDA identification of toxicants (Hecker and Hollert, 2009)

EDA of EDCs released through municipal effluents into surface waters focuses on *in vitro* characterization of specific endocrine disruptive potentials up- and downstream of target WWTPs as well as their effluents. Tests include *in vitro* assays for cytotoxicity, (anti)estrogenicity, (anti)androgenicity, and steroidogenesis disruption to identify specific endocrine potentials and to separate these from general toxicity; and measurements of selected water quality parameters. Subsequent chemical analysis of target compounds allows for the identification of specific EDCs and synergistic/antagonist mechanisms (Hecker and Hollert, 2009).

EDA is becoming a routine approach for environmental exposure assessments; however, many studies are often focused on specific endpoints. Moreover, different effects are found when using a different combination of bioassays, pointing to a need

for more holistic screening approaches (Hecker and Hollert, 2009). Emerging contaminants, including PPCPs, are of concern due to the fact that specific bioassays are non-existent or scarce in a way that they cannot be properly assessed. Furthermore, EDA techniques still need refinement and homologation so results can be compared among different studies and exposure scenarios.

e. Synthesis

It has been recognized that EDCs are widely present in the environment and can potentially have detrimental effects on non-target organisms. They exist as mixture of compounds for which interactions have not been fully studied. Classic chemical analytical techniques are insufficient for timely assessment of EDCs as they are limited such that only known chemicals can be identified for which analytical methods exist. Furthermore, bioassays enable characterization of specific toxicities of samples that may be indicative of organismal effects. On the other hand, biological assays alone do not provide sufficient information regarding the specific chemicals responsible for the observed effects, which is necessary for the identification of appropriate removal technologies for these contaminants. Therefore, complementary analyses are required for the identification of emerging contaminants and their hazard assessment.

EDA represents a comprehensive approach for the characterization of EDCs that can be translated to real environmental conditions. Although the technique has not been standardized yet, its application on case studies could allow for the development of a 'toolbox' method to be implemented as an environmental regulation. In this thesis, the first step of the EDA process, biological analysis, was performed on selected samples from WWTPs in Mytilene and Athens, Greece. Both estrogenic and (anti-

)androgenic activity were studied on the samples. The locations have similar climatic conditions; however, they differ in population size, which correlates with in input to treatment facilities, as well in the type of treatment itself. This analysis allows for a comparison between these factors with the purpose to assess the efficiency of each treatment and identify the appropriate technology for each case.

2. Objectives

a. Aim

The aim of this thesis was to assess how the efficiency of different types of wastewater treatment plants to remove endocrine-disrupting chemicals from wastewater varies among different treatment technologies using bio-analytical tools. In order to achieve this, the following specific aims were established.

i. Specific aims

- Analysis of endocrine disrupting activity in selected WWTP effluents using two *in vitro* biotests to characterize (anti-)estrogenic and (anti-)androgenic potentials.
- Assessment and comparison of EDC removal efficiency of the WWTP of a small city (Mytilene) and a large city (Athens) in a Mediterranean climate.
- Correlation of EDC removal efficiency with population size, and wastewater treatment technology.

ii. Null hypotheses

- No statistically significant (anti-)estrogenic or (anti-)androgenic potentials are present in WWTP effluents.
- If any, (anti-)estrogenic and (anti-)androgenic potentials found in both WTTPs are statistically different.

iii. Objectives

The overall objective of this thesis is to characterize the efficiency of removal of EDCs from WWTP effluents across different treatment technologies and population

sizes, using targeted *in vitro* bioassay-directed analysis. Specifically, the objectives of this study are to:

Objective 1: Learn to conduct two *in vitro* bioassays, namely the T47D-kbluc and the MDA-kb2 assays, to assess (anti-)estrogenic and (anti-)androgenic potentials, respectively, and optimize these assays for use with WWTP effluents.

Objective 2: Assessment of endocrine disrupting activity, namely (anti-)estrogenicity and (anti-)androgenicity, of effluents of two WWTPs in Greece using the two bioassays listed under Objective 1.

Objective 3: Compare removal efficiency of EDCs between the WWTPs of Mytilene and Athens, and correlate efficiencies with treatment technology and population size.

3. Methodology

a. Samples

Samples were collected from effluents of WWTPs located in Mytilene and Athens. Altogether, 14 samples each were available (Table 1).

Table 1. Descriptions of tested samples.

Code	Sample description
M Oct1	Sample from Mytilene WWTP effluent obtained on October 1, 2013
M Oct2	Sample from Mytilene WWTP effluent obtained on October 2, 2013
M Oct3	Sample from Mytilene WWTP effluent obtained on October 3, 2013
SBA	Sample blank A
SBB	Sample blank B
TBA	Travel blank A
TBB	Travel blank B
LBA	Lab blank A
LBB	Lab blank B
SBA	Solvent blank A
Blank	Solvent blank B
A M14	Sample from Athens WWTP effluent obtained on March 14, 2013
A M15	Sample from Athens WWTP effluent obtained on March 15, 2013
A M16	Sample from Athens WWTP effluent obtained on March 16, 2013

24-hour flow-proportional composite samples of WWTP effluents were taken in 3 consecutive days in each sampling campaign. Samples were collected in 1 L pre-cleaned amber glass bottles. All samples were transported to the laboratory in a cooler.

b. Extraction

Samples were filtered through a 47 mm GF-F Whatman filter, prebaked at 450 ºC. Solid-phase extraction (SPE) was done using an OASIS HLB. Each cartridge was pre-conditioned with 5mL of methanol (Sigma, Mississauga, ON, Canada) and then 5mL of Millipore water. Samples were loaded on at 1 drop/second. Elution of the cartridge is done using 5mL of methanol and then 10mL of dichloromethane/hexane (Sigma).

Samples were dried under nitrogen stream, and stored at -80 ºC.

c. Reconstitution and sample preparation

Solvent blank B was selected as a procedural blank, and was reconstituted in 1 mL ≥99.9% dimethyl sulfoxide (DMSO, Sigma), along with samples M Oct1, M Oct2, M Oct3, A M14, A M15, and A M16. 500 µL of each sample were taken for analysis, while the rest was dried out under a nitrogen stream to store again at -80 ºC.

d. Cytotoxicity test

No previous cell work had been done with the samples, and thus, general toxicity was unknown. Therefore, samples were first screened for cytotoxicity by the use of the cell proliferation reagent WST-1 (Roche, Laval, QC, Canada). WST-1 is a tetrazolium salt that is cleaved to formazan by cellular enzymes. An increase in the

number of viable cells results in an expansion of formazan amount, which can be measured in a spectrophotometer (Roche, 2006).

Following manufacturer's guidelines (Roche, 2006), cells were cultured normally (described in the following sections). Cells were dosed with DMSO and samples, diluted 1:1000 in the appropriate medium. After dosing the cells, 10 µL of the reagent were added to each well. Medium was used as control. Absorbance was read at 450 nm at 30 and 60 minutes after adding the reagent. 620 nm was the reference wavelength.

e. *In vitro* (anti-)estrogenicity test

The estrogenicity of the samples was tested using T47D-kbluc cells, based on methods described by Wilson *et al.* (2004) and He *et al.* (2011).

i. Cell culture

A stock of frozen T47D-kbluc cells (ATCC CRL-2865) maintained in liquid nitrogen was thawed to use in the assay. Frozen cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were thawed by gently rubbing the vial between the hands. Cells were poured into a vial containing 10 mL of filter-sterilized maintenance RPMI culture medium (Sigma) with a pH of 7.3, supplemented with 2.5 g/L dextrose, 1.5 g/L sodium bicarbonate, 1 mM sodium pyruvate, 10 mM HEPES, and 10% FBS. The vial was centrifuged at 1500 rpm for 5 minutes. Medium was removed from the vial and the cell pellet was re-suspended in 10 mL of fresh medium and transferred to a petri dish.

T47D-kbluc cells were cultured in maintenance RPMI medium at 37 ºC in a 5% CO_2 atmosphere. Maintenance medium was changed every 2-3 days. When confluent,

cells were detached from the culture dish with 1.5 mL of TrypLE™ Express (Life Technologies, Burlington, ON) and split 1:3 or 1:4. In preparation of exposure experiments, cells were maintained in 10 mL of withdrawal medium, prepared with 10% dextran-charcoal stripped FBS, rather than normal FBS. Withdrawal medium was changed in day 4 or 5 after splitting.

ii. Cell assay

On day 8 after splitting, cells were re-suspended in assay medium, prepared with 10 % dextran-charcoal treated FBS. Cells were seeded into 96-well luminometer plates on day 8 after splitting. Rows A and G, and column 1 were avoided to eliminate any light-contamination in the outer wells. 100 µL of cell solution in assay medium were added per well at a density of 40,000 cells/mL and left overnight. Cells were dosed the following day adding 100 µL of fresh assay medium containing 0.1% v/v sample.

An estradiol (E2) standard curve was also included on each plate. A stock standard solution of estradiol was prepared in DMSO, and serial dilutions were prepared in assay medium, with final concentrations ranging from 10 fM to 300 pM. The final concentration of DMSO in all exposures was 0.1%

After dosing, cells were incubated for 24 h, and luciferase activity was determined as a measure of estrogenicity by use of the steadylite plus luciferase assay system (Perkin-Elmer, Woodbridge, ON, Canada) according to manufacturer recommendations. Cells were washed once with 100 µL PBS with calcium and magnesium per well. 50 µL of PBS with calcium and magnesium were added to each well, along with 50 µL of steadylite plus reagent. Luminescence was read at 15, 30, 45, and 60 minutes after addition of reagent.

f. *In vitro* (anti-)androgenicity test

Samples were tested for androgenic and anti-androgenic activity in MDA-kb2 cells, based on methods described by Wilson *et al.* (2002) and He *et al.* (2011).

i. Cell culture

A stock of frozen MDA-kb2 cells (ATCC CRL-2713) maintained in liquid nitrogen was thawed to use in the assay. Frozen cells were obtained from the ATCC. Cells were thawed by gently rubbing the vial between the hands. Cells were poured into a vial containing 10 mL of filter-sterilized L-15 culture medium (Sigma) supplemented with 10% FBS. The vial was centrifuged at 1500 rpm for 5 minutes. Medium was removed from the vial and the cell pellet was re-suspended in 10 mL of medium and transferred to a petri dish.

MDA-kb2 cells were maintained at 37 ºC without CO_2. Medium was changed every 2-3 days. At about 60% confluence, cells stacked onto each other, then they were split 1:3 or 1:4 with 1.5 mL of trypsin to be plated.

ii. Cell assay

Cells were seeded into 96-well luminometer plates with 100 µL of cell solution in medium at a density of 200,000 cells/mL. Again, rows A and G, and column 1 were not used. Cells were dosed on day 3 after seeding, adding 100 µL of fresh medium containing 0.1% v/v sample.

A dihydrotestosterone (DHT) standard curve was included on each plate as positive control. A stock standard solution of DHT was prepared in DMSO, and serial dilutions were prepared in medium, with final concentrations ranging from 10 pM to 3 nM. The final concentration of DMSO in all exposures was 0.1%.

In subsequent anti-androgenicity assays hydroxyflutamide (HF) was used as antagonist control. Cells were dosed with either 100 pM or 300 pM DHT plus sample or 1mM HF in medium. After dosing, cells were incubated for 48 h, and luciferase activity was determined as a measure of (anti-) androgenicity by use of the steadylite plus luciferase assay system (Perkin-Elmer) according to manufacturer recommendations. Cells were washed once with 100 µL PBS with calcium and magnesium per well. 50 µL of PBS with calcium and magnesium were added to each well, along with 50 µL of steadylite plus reagent. Luminescence was read at 30, 45, and 60 minutes after addition of reagent.

g. Data analyses

Statistical analyses were performed in Microsoft Excel for Mac 2011 and SPSS version 20 for Mac. T47D-kbluc was tested for normality and homogeneity of variance by the use of the T-test. MDA-kb2 data was tested for normality and homogeneity of variance with Kruskal Wallis test followed by Mann Whitney U. Data are expressed as means and standard deviations or standard errors. F-tests were used to evaluate the equality of means. Differences with $p < 0.05$ were considered significant.

4. Results

a. Cytotoxicity

Samples were screened for cytotoxic effects on each cell line. Medium and medium containing solvent (DMSO) used on each assay were used as control. The T47D-kbluc cell viability in DMSO was statistically different from medium (Figure 4). Sample M Oct3 also showed a statistical difference with respect to medium. However, in this case cell viability was over 95%, and it was decided to continue with the experimentation. None of the other samples was cytotoxic at the greatest concentrations tests.

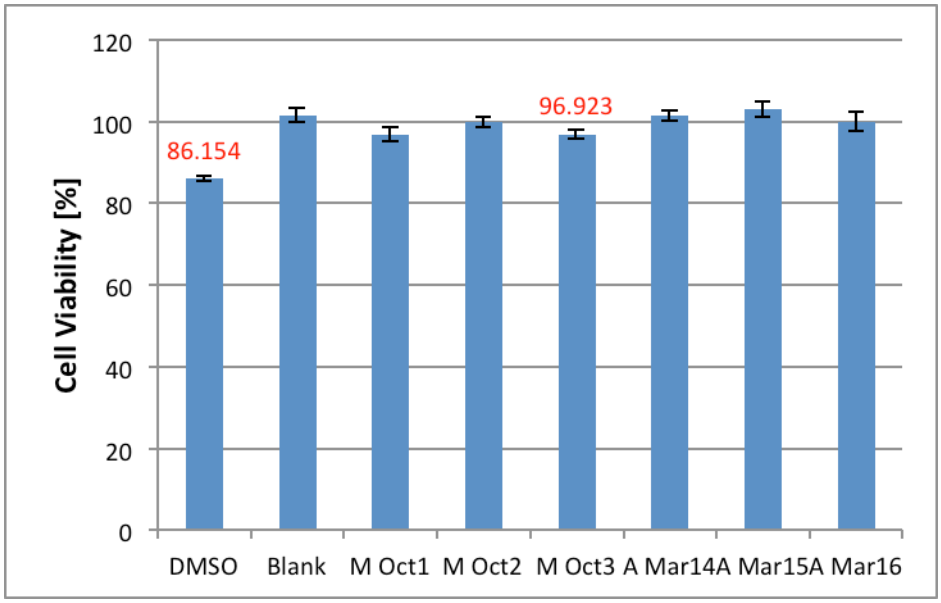

Figure 4. Determination of the cytotoxic activity of samples on T47D-kbluc cells, expressed as percentage of cell viability relative to medium.

Data is given as means ± standard error (n=6). Values are shown for samples statistically different from medium (t-test).

When samples were tested in the MDA-kb2 cell assay, two samples were significantly different from the media and solvent controls (Figure 5). In this case, DMSO was administered at 0.1% v/v, and therefore no statistically different cytotoxicity was observed. Cell viability of the procedural blank was statistically greater than medium. In contrast, M Oct1 had significantly lesser cell viability. However again the change was only marginal (-15%) in M Oct1 with respect to the medium), so it was appropriate to continue with the next phase.

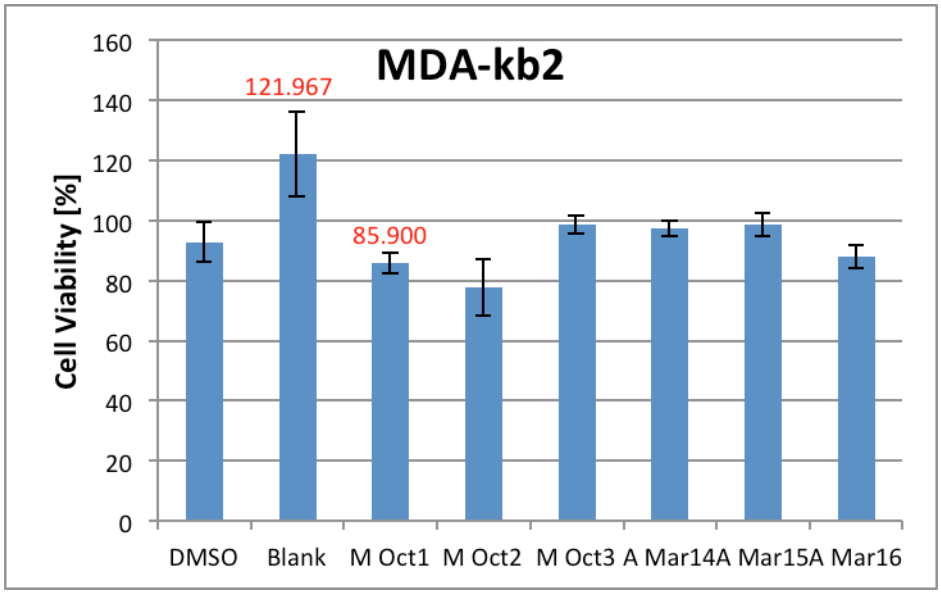

Figure 5. Determination of the cytotoxic activity of samples on MDA-kb2 cells, expressed as percentage of cell viability relative to medium.

Data is given as means ± standard error (n=4). Values are shown for samples statistically different from medium (t-test).

b. Estrogenicity

An 8-point calibration curve for estradiol was constructed. The following concentrations of estradiol were used: 10 fM, 30 fM, 100 fM, 300 fM, 1 pM, 3 pM, 10 pM, and 30 pM (Figure 6).

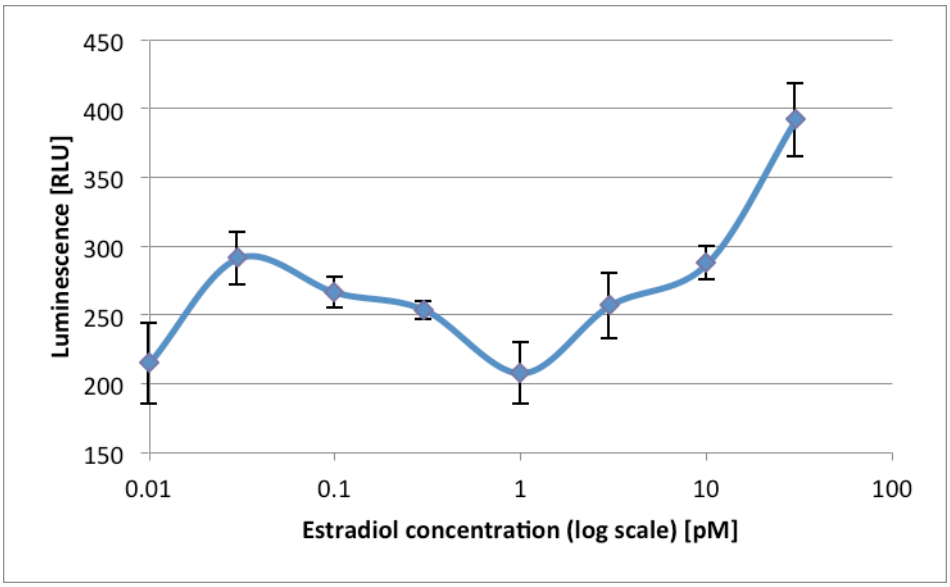

Figure 6. Estradiol (E2) calibration curve on T47D-kbluc cells.

Data is showed as means ± standard error (n=4).

Estrogenicity of the majority of effluent samples was significantly induced compared to the solvent controls (Figure 7). All means of samples fell within the 10-30 pM range, which are the 2 highest points of the estradiol calibration curve (Figure 6).

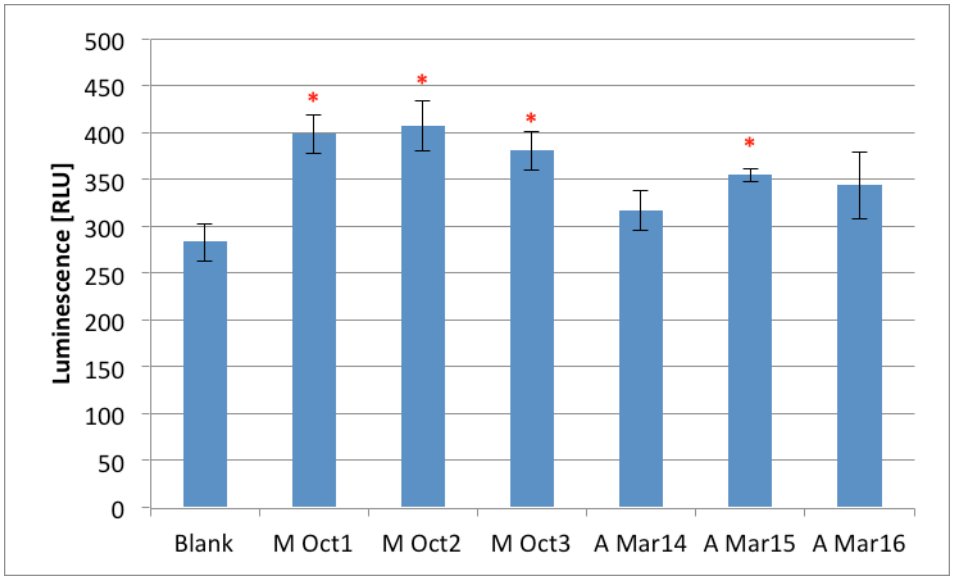

Figure 7. Estrogenic activity of samples expressed as luminescence [RLU].

Data is given in mean RLU ± standard error (n=4). * indicates sample is statistically different from solvent controls.

The percent of maximum effect caused by estradiol was calculated for each sample, taking the mean maximum response of the positive control, 30 nM estradiol, as 100% (Figure 8), as suggested by (Coors *et al.*, 2003). The results clearly showed that the samples from Mytilene had the greatest estrogenic potency with over 100% of the maximum induction caused by 30pM estradiol in two out of three cases. Greater estrogenic potentials were also observed in the samples collected from the Athens WWTP but only the March 15 sample was significantly different from the solvent controls, and maximum inductions did not exceed 80% of the maximum response observed after exposure to E2. Finally, a weak estrogenic potential was also observed for the procedural blank.

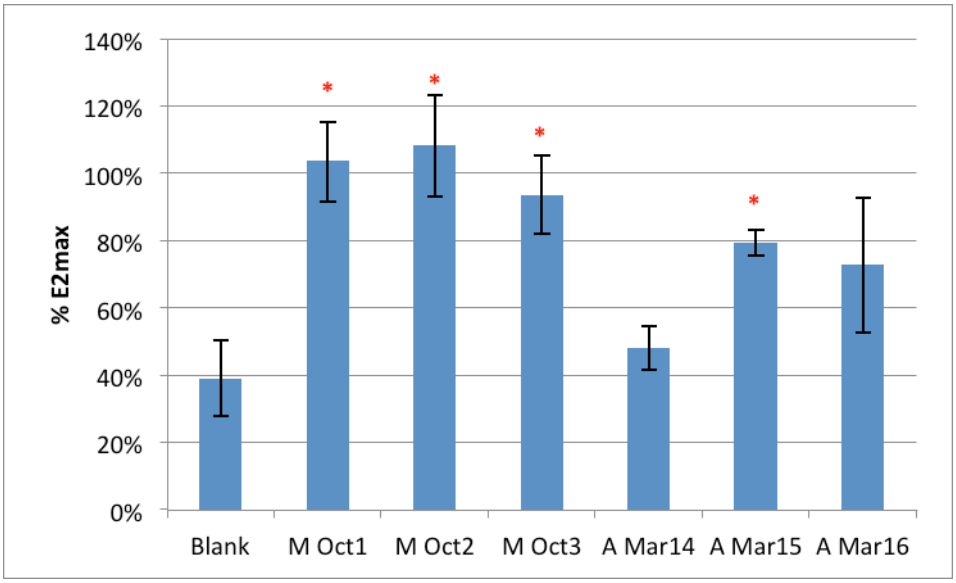

Figure 8. Estrogenic activity (luminescence to the maximum achievable response with estradiol, RLU in %E2max) in the samples, detected with the T47D-kbluc assay.

Presented as means ± standard errors (n=4). Mean medium RLU has been subtracted from each replicate. * indicates sample is statistically different from solvent controls.

c. (Anti-)Androgenicity

The MDA-kb2 androgenicity assay was performed twice (Figure 9). Replicate 1 was performed in quadruplicates, with an 8-point dihydrotestosterone (DHT) calibration curve. Calibration points included 10 pM, 30 pM, 100 pM, 300 pM, 1 nM, 3 nM, 10 nM, and 30 nM DHT. Replicate 2 was performed in triplicates, with a 6-point calibration curve, omitting the 2 highest points of Replicate 1.

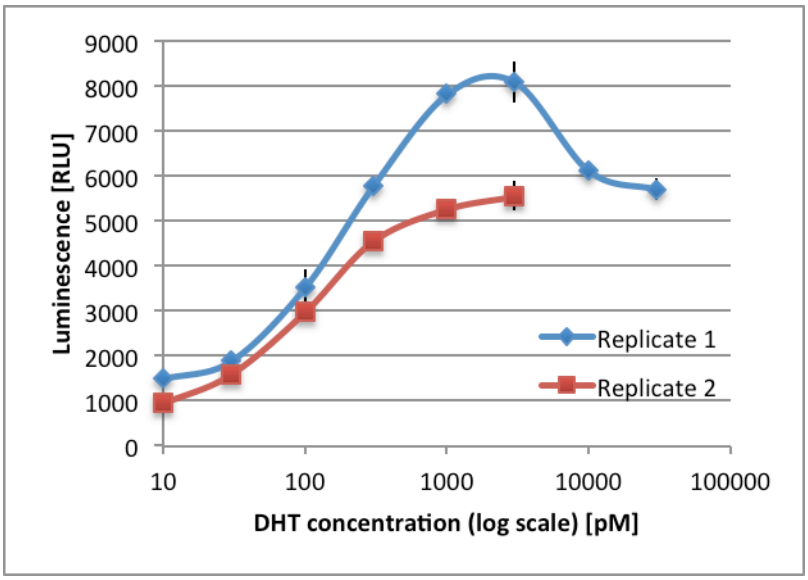

Figure 9. Dihydrotestosterone (DHT) calibration curve on MDA-kb2 cells.

Data is showed as means ± standard error (n=4 for replicate 1, n=3 for replicate 2).

In replicate 1, all samples but the blank were below the mean of 10 pM DHT at that experiment, which is the lowest point of the calibration curve. In the case of replicate 2, all Mytilene samples are also below the lowest point of the curve, while Athens samples fall between 10 and 30 pM DHT. However, samples M Oct2, M Oct3, A Mar14, A Mar15, and A Mar16, while being statistically different from the medium, they are not so from the blank. On the other hand, M Oct1 is not statistically different from the medium, but it is from the blank.

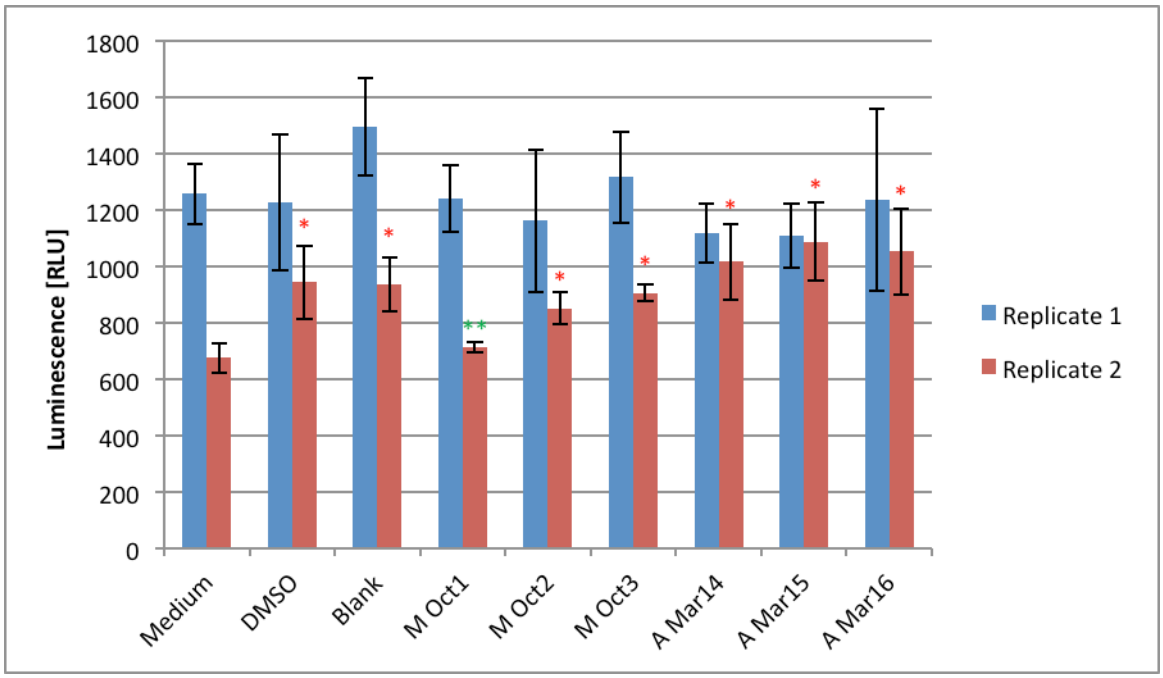

Figure 10. Androgenic activity of tested samples expressed as luminescence.

Data is given in mean RLU ± standard deviation (n=4 in replicate 1, n=3 in replicate 2). * indicates statistical difference relative to the medium in the corresponding replicate. ** indicates statistical difference relative to the procedural blank in the corresponding replicate. Statistical differences were evaluated with t-tests.

The percent of maximum effect caused by DHT was calculated for each sample, taking the mean maximum response of the positive control, 3 nM DHT, as 100% (Figure 11). Although samples show up to 20% of the maximum response, means were not statistically different from the controls; therefore, the observed effect is probably a matrix effect.

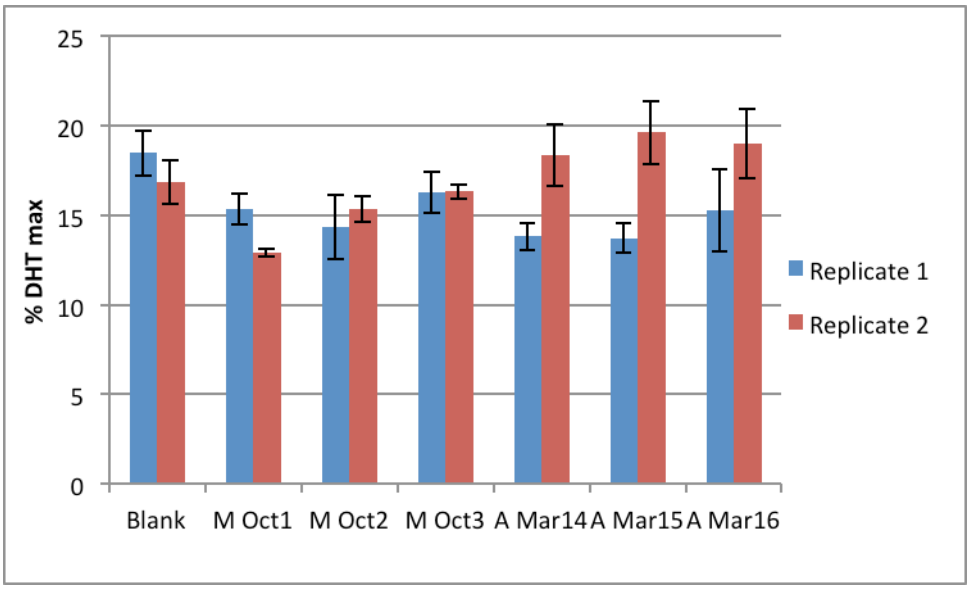

Figure 11. Androgenic activity (luminescence relative to the maximum achievable response with estradiol, RLU in percentage of DHTmax) in the samples, detected with the MDA-kb2 assay.

Data is showed as means ± standard error (n=4 for replicate 1, n=3 for replicate 2).

The MDA-kb2 anti-androgenicity assay was also performed twice with for 4 replicates for each sample on each experiment (Figure 12). Hydroxyflutamide significantly reduced the DHT-induced androgenic response, proving the assay worked as expected. There was an indication of anti-androgenic activity in sample A Mar15 in replicate 1; however, it was not observed in replicate 2.

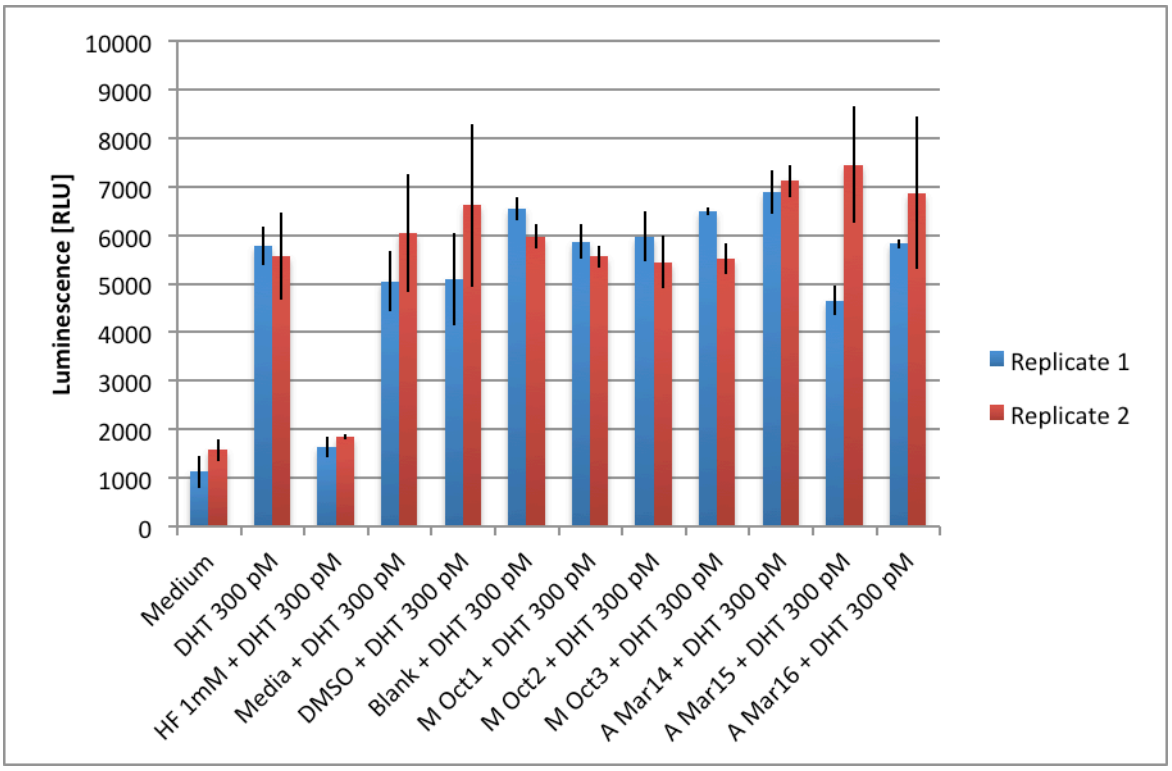

Figure 12. Anti-estrogenic activity of tested samples expressed as luminescence.

Data is given in mean RLU ± standard deviation (n=4 for all samples, except DHT 300 pM in which n=3).

A difference in mean luminescence was observed between replicates, but overall the values were still comparable. Furthermore, contrasted to the mean luminescence of 300 pM DHT and the reduction of the value with the addition of HF, it is evident that there is not significant anti-androgenic potential in any of the samples.

5. Discussion

a. General toxicity/cytotoxicity

The overall results of the cytotoxicity assay indicated that the majority of samples did not contain substances that were acutely toxic to T47D-kbluc cells. The only sample that was statistically different from the medium used as control was M Oct3 (Figure 4). It is unclear what the exact reason for this difference of M Oct3 is, but possible reasons could be the presence of cytotoxic constituents, or variations introduced during the extraction or assay procedure. However, the absolute difference was very small, with less than 4% decrease in cell viability, and therefore, the observed decline was not considered significant from a biological perspective. Consequently, estrogenic experiments were conducted for all samples up to the greatest concentrations of extracts tested. Efforts were made to perform the assay at lower sample concentrations (1:200 and 1:4000, as opposed to 1:1000); however, cells did not grow to confluence, and the assay could not be completed in the available timeframe. Interestingly, the solvent control showed a statistically significant decrease (by 14 %) in cell viability compared to the medium control. This could also be due to deviations originated in the procedure. The fact that the rest of the samples did not show statistical difference with respect to the medium, even though the same DMSO concentration was present in all of them, could confirm the variation theory. However, it is also possible that the samples contained elements that stimulated the growth of cell, thus suppressing the cytotoxicity of DMSO.

Similarly, only one sample, M Oct1, showed a significant decrease in cell viability when tested in the MDA (anti-)androgenicity assay (Figure 5). Again, however, the

decrease was not considered biologically relevant (15%), especially because the mean value for the sample was not statistically different from the value for DMSO. Interestingly, in the case of the cytotoxic effect on MDA-kb2 cells, the absorbance of the blank was statistically higher than the medium (Figure 5). This phenomenon could have been caused by a procedural error. The higher value of only the blank could also be caused by a stimulating effect from the blank on the growth of cells, which in turn could have been inhibited by the samples. However, considering that there was no difference between the solvent controls and any of the effluent samples, as well as the medium-only treatment group, it is unlikely that the samples caused actual cytotoxicity to the cells. Nonetheless, a replication of the assay is recommended.

b. Estrogenic potency of effluents

The initial experiment with the T47D cells presented here clearly showed that the effluents of both the Mytilene and Athens WWTPs contain contaminants with estrogenic properties (Figure 8). All responses obtained for the samples fell within the linear range of the E2 standard calibration curve of the assay (Figure 7). In fact, samples collected at Mytilene had potencies equal or greater to the maximum response elicited by the greatest concentration of E2 (30 pM), and thus, can be considered highly potent. Samples collected from the effluents of the WWTP at Athens were less potent than those from Mytilene but still reached potencies of up to 80% of that of the maximum effect induced by 30 pM E2. This clearly indicated that elimination of estrogenic contaminants was incomplete in both WWTPs tested in this study, with Athens are likely to be more efficient in removal of natural and synthetic estrogens than Mytilene.

The higher efficiency of Athens WWTP in the removal of estrogenic activity should come from primary clarification step that is not part of the Mytilene WWTP (Samaras *et al.*, 2013). Clarification is the process separation of inorganic solids. Although the Athens WWTP receives almost 150 times the input of Mytilene WWTP, samples from Athens presented less than half of the estrogenic activity than Mytilene samples in some cases (A Mar14 vs. M Oct2). Furthermore, while both WWTPs include secondary treatment with biological nitrogen and phosphorus removal, the hydraulic retention time in Mytilene WWTP is higher than in Athens (23 h vs. 9 h), which also points to the clarification step as the main source of difference in the efficiency of removal of estrogenic potential.

The finding if estrogenic potential in Athens and Mytilene WWTPs is in accordance with previous studies investigating the estrogenic activities of municipal effluents (Ternes *et al.*, 1999; Kasprzyk-Hordern *et al.*, 2009; Garric, 2013; Kestemont and Depiereux, 2013), confirming the incomplete removal of contaminants with estrogenic properties during conventional wastewater treatment processes. Johnson *et al.* (2005) found a positive correlation between hydraulic retention time and removal of estrogenic activity; however, this was not the case on this preliminary screening.

It should be acknowledged that the procedural blank also showed some estrogenic activity; however, this activity was relatively weak (less than 40% E2 max) compared to the actual sample extracts, indicating that the observed effects are truly due to the presence of estrogens in the investigated effluents. Nevertheless, considering that the here-presented data is only from one initial experiment, the assay needs to be repeated to confirm these results. Furthermore, it is recommended that in addition to effluent samples, matching influent samples be also analyzed to enable

characterization of the actual efficiency of the different WWTPs to remove estrogenic compounds from raw sewage.

c. (Anti-)Androgenic potency of effluents

Androgenic activity was not observed for any of the samples (Figure 10), and in both replicates most of the samples were below the values of the calibration curve. Athens samples, and M Oct2 and M Oct3 fell within the lower portion of the DHT standard curve during the second replicate experiment; however, they were not statistically different from the blank. This difference suggests an increase of variation between replicates, probably due to procedural issues such as the advanced generation of the cells. Further testing is recommended, including the addition of at least 2 lower points on the calibration curve.

In the anti-androgenicity experiment the addition of 1 mM hydroxyflutamide to DHT-stimulated cells decreased luminescence values down to basal levels as they were reported for medium-exposed cells only. Thus, the assay performed as expected and previously reported (Bittner *et al.*, 2012). There was indication of significant anti-androgenic activity of sample A Mar15 during the first replicate experiment. However, this was not confirmed on replicate 2 (Figure 12). The rest of the samples seemed to maintain 300 pM DHT levels, and did not show inhibiting activity comparable to hydroxyflutamide. Although the existence of antiandrogens has been reported or predicted in WWTP effluents, estrogens are more common (Jobling *et al.*, 2009; Rostkowski *et al.*, 2011)

Despite the lack of perceived anti-androgenic activity, differences between replicates are noticeable in some of the samples (DMSO, A Mar15); therefore further testing is recommended.

6. Conclusions

A preliminary screening for estrogenicity and (anti-)androgenicity was performed on samples from two WWTPs in Greece, namely Athens and Mytilene. Estrogenic potentials were found in both effluents, with higher activity found in Mytilene. The apparent higher efficiency of removal of estrogenic potential in the Athens WWTP could be due to an extra step of clarification, which removes inorganic solids. Neither androgenic nor anti-androgenic activity was found on the samples; therefore these potentials are not of concern. The expected positive correlation between endocrine potential and wastewater influx was not observed. On the other hand, the effect of a difference in wastewater treatment technology could not be fully clarified. While the addition of primary clarification in the Athens WWTP is the possible cause of a lower estrogen potential, such correlation could not be associated with a longer hydraulic retention time, as suggested by Johnson *et al.* (2005).

a. Recommendations

It must be noted that this thesis represents an initial screening of endocrine activity. Consequently, replication of the assays is highly recommended to obtain conclusive results. Furthermore, the addition of anti-estrogenic potential and steroidogenesis disruption assays would provide a more comprehensive assessment of risks. Moreover, if any endocrine disruptive potential were confirmed, the next steps would include the assignment of endocrine activity to specific compounds, and their quantification in the effluents, which would involve a series of chemical analyses. A broader sampling campaign is also suggested, to evaluate if and how endocrine potentials vary throughout the year, especially to contrast seasons of low and high tourist inflow. Finally, ultimate ecotoxicological consequences should be addressed,

in order to translate EDC concentration into human health endpoints and biological responses, at organismal, population, and ecosystem levels.

Although a complete assessment of risks require more studies, *in vitro* assays such as those applied in this thesis provide useful data for initial screening of hazards, and to design further assessments.

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