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Analysis of Endocrine Disrupting Compounds in Wastewater Treatment Plants: A Perspective of Trans-Boundary Waterborne Pollution

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ANALYSIS OF ENDOCRINE DISRUPTING COMPOUNDS IN WASTEWATER TREATMENT PLANTS: A PERSPECTIVE OF TRANS-BOUNDARY WATERBORNE POLLUTION

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DEDICATION

To the most important people in my life, my family.

ANALYSIS OF ENDOCRINE DISRUPTING COMPOUNDS IN WASTEWATER TREATMENT PLANTS: A PERSPECTIVE OF TRANS-BOUNDARY WATERBORNE POLLUTION

by

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DISSERTATION

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ABSTRACT

Recent decades have brought an increasing concern of potential adverse human and ecological health effects resulting from Endocrine Disruptor Compounds (EDCs). In particular from new emerging compounds such as natural estrogens (e.g., 17ß-estradiol, estrone), synthetic estrogens (e.g., 17α-ethynylestradiol), bisphenol A (BPA), nonylphenol ethoxylates (NPEOS), and nonylphenol (NP). These chemicals which are also known as organic wastewater contaminants are released directly to the environment after passing through wastewater treatment plants (WWTPs), which often are not designed to remove them from the effluent. The occurrence of the aforementioned compounds in surface water is becoming of increasing concern worldwide, and has led to a growing awareness that animals, and perhaps human health and function in ecosystems might become negatively impacted by continued release of these compounds into the environment at low levels (ng L^{-1}).

To determine the concentrations EDCs and the possible impact of WWTPs discharge, two different strategies were used. For the first approach, EDCs concentrations in wastewater were analyzed by Stir Bar Sorptive Extraction-Thermal Desorption-Mass Spectrometry. Simultaneously, the estrogenic activity was quantified by a chemiluminescent yeast assay which was developed to test water directly without concentration. EDCs concentration and estrogenic activity in the influent were lower in WWTPs in El Paso compared to the plants located in Mexico. Concentrations in effluent were 200% to 8000% higher in Mexico for the majority of the EDCs in study compared to the plants in El Paso. NP and NPEOS were the compounds with the higher concentration detected in influent ranging from non-detected to 8,144 ng L^{-1}. BPA levels in effluent water were below 581.6 ng L^{-1} and for estrogen the levels were below 65.2 ng L^{-1}. The removals of EDCs in WWTPs in El Paso were higher than 60% for the majority of the

plants, and in Mexico lower than 60%. Estrogenic activity was removed 31% to 98% in WWTPs in El Paso. Insignificant removal of estrogenic activity was determined in plants from Mexico ranging from no removal to 86%. Based on our observation, the WWTPs with at least secondary treatment process were able to remove EDCs more effectively with an average of 85% for the EDCs analyzed in this study.

In the second approach, a mass balance analysis was performed to determine the capability of two different treatment plants to remove EDCs from wastewater. Both plants were capable to eliminate up to 89 % of NP, NPEOS and estrogens. Denitrification treatment appeared to be inefficient for the removal of NP and ethoxylates from wastewater. Aerobic environment such as activated sludge treatment were responsible of degrading the majority of the compounds up to 90%. Advance tertiary treatment was more consistently efficient to remove EDCs from wastewater. The WWTPs in Mexico lacking activated sludge treatment performed the lowest removal efficiency of EDCs. It is essential that WWTPs in Mexico expand their facilities and upgrade their system to ensure that 100% of the wastewater can be treated by secondary treatment (activated sludge). The results from this study will undoubtedly serve as the initial framework upon which to expand and add more information in relation to EDCs or other contaminants in water resources along the border.

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CHAPTER 1: INTRODUCTION

The unremitting exponential growth in human population has created a corresponding increase in the demand for the Earth's limited supply of freshwater. Hence, protecting the integrity of our water resources is one of the most essential environmental issues, especially in semiarid-arid areas. Recent decades have brought an increasing concern for potential adverse human and ecological health effects resulting from Endocrine Disruptor Compounds (EDCs), especially new emerging compounds such as household chemicals, pharmaceuticals, and other consumables as well as estrogens [1]. These chemicals which are known as organic wastewater contaminants (OWCs) are released directly to the environment after passing through wastewater treatment processes, which are often not designed nor required to remove them from their effluent [1]. A nationwide study by the U.S Geological Survey revealed that various EDCs exist in 80% of stream samples [1]. Some of the most frequently detected EDCs include natural and synthetic estrogens such as estrone (E1), 17β-estradiol (E2), the synthetic contraceptive additive 17 α-ethynylestradiol (EE2), bisphenol A (BPA), and nonylphenols. The occurrence of the aforementioned compounds in surface water are becoming of increasing concern worldwide, and has led to a growing awareness that animal, and perhaps human health and function in ecosystems might become negatively impacted by continued release of these compounds into the environment even at low levels (e.g. ng L^{-1}). Research has shown reproductive effect in aquatic organisms exposed to low levels of these contaminants. Male fish expose to low levels (4 ng L^{-1}) of these contaminants have exhibited estrogenic responses, such as induction of vitellogenin, a protein normally produce by female fish, causing feminization [2, 3]. The consequences are remarkable because chronic exposure to these compounds can lead to a decrease in reproduction success and sustainability of fish population. Furthermore, the reuse of water and wastewater is

becoming a necessity for desert regions such as the Paso del Norte area. As an effort of the reuse of reclaimed water, El Paso Water Utilities (EPWU) has implemented extensive use of recycled water. For those reasons, the occurrence of trace EDCs in wastewaters and their behavior during wastewater treatment are very important. Unfortunately, such issues are not addressed because they are not on the regulatory lists as other environmental pollutants, such as heavy metals. Despite the known occurrence of EDCs in US water resources [4], no studies have been undertaken in El Paso, Texas and border city of Juarez, Mexico. Most current wastewater treatment plants (WWTPs) in El Paso and Mexico are not designed to treat all EDCs; thus, those emerging compounds and their metabolites can escape elimination in WWTPs and later enter the aquifer and surface water via recharge or sewage effluents. Furthermore, WWTPs in Ciudad Juarez, Mexico in comparison with WWTPs in El Paso, do not involve any activated sludge treatment which is the treatment that eliminate the majority of the pollutants in wastewater. Consequently, there are untreated discharges and flows that potentially reach surface water, such as the Rio Grande River, and/or groundwater bodies. This situation represents high health risks to human due to the potential contact with wastewater and vectors of waterborne diseases (e.g. giardiasis, helmitiasis), as well as environmental contamination risks

1.2 Objectives and Rationale

This research is the first initiative to identify the presence of EDCs in El Paso/Mexico water resources. *The key objective of this project was to understand the occurrence and fate of EDCs in wastewater treatment plants, and to study the possible impact to human health and aquatic ecosystem in El Paso/Ciudad Juarez border.* EDCs can survive water treatment and are known to induce estrogenic activity. E1, E2, and EE2 are known to elicit measurable ecological

change at concentrations below 1 nM [5, 6]. Nonylphenol and BPA are typically presented in treated water at nM to uM concentrations. As aforementioned these compounds can be responsible for the estrogenic effect on aquatic wildlife. Although advanced treatment technologies, such as ozonation, membrane filtration, and activated carbon adsorption, showed improved removal of EDCs in WWTPs, using these technologies would increase the operation costs of treating wastewater [7]. Therefore, a need remains to understand the removal mechanisms and fate of EDCs during current wastewater treatment processes to have better management of conventional practice in WWTPs. These problems has been recognized by scientists and engineers around the world and attention has been given to wastewater treatment as a means of mitigating current and future environmental damage from EDCs. The propagation of our studies to the Mexico area was critical because of the propinquity of the two countries, direct contact, and allotment of resources indispensable in United States- Mexico border cities.

Specifically, the following questions were addressed:

- *What is the levels and occurrence of selected EDCs within wastewater treatment plants?*
- *What is the performance of each treatment process in the different plants for removing EDCs?*
- *Are some plants are more efficient in removing EDCs than the others?*
- *Does EDCs concentration and estrogenic activity vary at different seasons in WWTPs influent and effluent?*
- *What is the fate of selected EDCs within wastewater treatment plants?*

It is well known that WWTPs with a secondary treatment remove pollutants more efficiently from wastewater. Therefore, it is hypothesized that WWTP located in Mexico will be the less effective in eliminate EDCs from wastewater. Elimination of pollutants in wastewater is dependant of several variables such as flow condition, temperature, and degradation rates. Thus, the concentration of EDCs and estrogenic activity in water could significantly varies with the seasons. To date, it is known that municipal WWTPs reduce EDCs to some extent, although frequently not to levels lower than the known effective concentrations for aquatics wildlife [2, 5, 8]. It was therefore hypothesized that the wastewater treatment plants in El Paso and Ciudad Juarez Mexico may not totally eliminate EDCs; thus a significant amount of EDCs will be detected in wastewater effluent.

1.3 Endocrine Disruptors Compounds

1.3.1 Nonylphenol , 4 -tert Octyphenol and ethoxylates

Nonylphenols (NP) are arguably the most ubiquitous anthropogenic compounds in the United States (Figure 1). It is the most commercially prevalent of the alkylphenol family, representing approximately 85% of the alkylphenol market. NP is produced from cyclic intermediates in the refinement of petroleum and coal-tar crudes [9]. The annual production of nonylphenol reached 154,200 tons in the USA, 73,500 tons in Europe 16,500 tons in Japan and 16,000 tons in China [10]. It is manufactured by alkylating phenol with mixed isomeric nonenes in the presence of an acid catalyst. The resulting product is a mixture of various isomers of nonylphenol, predominantly *para*-substituted nonylphenol with small amounts of *ortho*-substituted phenol and trace amounts of 2,4-dinonylphenol. Additional isomers, which represent the numerous branched structures that occur within the nonyl (nine carbons) group, add to the

complexity of the compound. Octylphenols (OPs) refer to a large number of isomeric compounds of the general formula $C_6H_4(OH)C_8H_{17}$. There are two main paths used in the production of OP, both of which involve the reaction of phenol and *tert*-octene (di-isobutene) in the presence of an ion-exchange resin or boron trifluoride complex in a batch reactor; or a fixed bed ion-exchange resin in a continuous process.

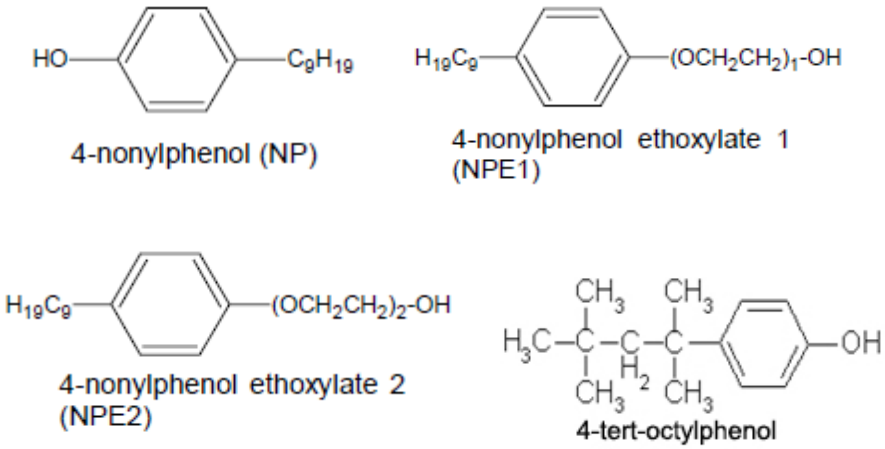


Figure 1: Structures of Nonylphenol and Alkylphenol Ethoxylates

There is little direct use for nonyphenol and 4-tert octylphenols. Rather it is further reacted to produce Alkylphenol ethoxylates (Figure 1). Alkylphenol ethoxylates (APEOs) are nonionic surfactants widely in industrial, institutional, commercial and household applications such as detergents, emulsifiers, wetting and dispersing agents, antistatic agents, demulsifiers and solubilisers [11]. Nonylphenol ethoxylates (NPEOs) and octylphenol ethoxylates (OPEOs) account for approximately 80% and 20% of the total APEO production. After use, the APEOs reach wastewater treatment plants (WWTP) or water bodies, where they undergo rapid transformation to short chains APEOs (mono and diethoxylates) and the parent compounds (nonyphenol and 4-tert octylphenol) [12,13]. The fate of nonylphenol in different environmental compartments (surface water, sediment, groundwater, soil and air) is controlled predominantly

by its physical–chemical properties and these in turn influence its degradation. Nonylphenol is a hydrophobic compound with a log K_{ow} value of 4.48 and low solubility in water, therefore it partitions favorably to organic matter and has low mobility, limiting its capacity for spreading in the aqueous phase of soil and sediments. In the surface layer of natural waters the concentration of nonylphenol can decrease due to photolysis induced by sunlight, but in sediments it has an estimated half-life of at least 60 year [10].

APEOs reach the aquatic system via wastewater treatment plants effluents, primarily attributed to incomplete removal during wastewater treatment processes. Because of it widely use, APEOS generated concerts in the scientist community after Giger and coworkers in 1984 established that nonylphenol ethoxylates and products were more toxic to aquatic life than their predecessors [14]. Its toxicity was reaffirmed when by accident, PVC tubing containing NP was used during experiments with human breast cancer cell line MCF-7 [15]. NP increased the cell proliferation of this breast cancer cell lines, and recently has been shown to cause uterine proliferation by acting on the estrogen receptor. These findings led to numerous studies related with the occurrence of APEOS in WWTP and in the aquatic ecosystem.

Nonylphenol is not completely eliminated from water by WWTPs, regardless the technology used at this time. In a study performed on three different WWTPs located in the mid-Atlantic of USA, concentration of alkylphenol in wastewater influent and effluent surpassed the part per billion levels, which is a common compare with the rest of the world [12]. Concentrations in influent for long chain NPEOs ranged from 894 to 425 µg L^{-1} and for short NPEOs from 262 to 47.6 µg L^{-1}. Amounts found in effluent ranged from 8.42 to 0.04 µg L^{-1} for long chain NPEOs and from 32.3 to 1.58 µg L^{-1} for short chain NPEOs. Octylphenol ethoxylates were detected in influent and effluent at concentrations of 3.1 and 0.5 µg L^{-1} respectively [13].

APEOs removals from wastewater were higher than 93.5 %. An investigations at three Northeast Kansas WWTPs found NPEOs and NP in influent wastewater at levels from nondetectable to more than 200 µg/L. Low levels (up to 23 µg/L) of NPEOs and NP were detected in the WWTP effluents that are discharged into the Kansas River [16]. A large portion of NPnEOs and NP appeared to adsorb to the biosolids. As much as 898 mg kg^{-1} NP was measured in biosolids from one WWTP [17]. High concentrations of alkylphenol have also been detected in wastewater effluent from Australia. The analysis performed in four different WWTPs with different technology revealed concentrations ranging from 0.084 to 2.4 µg L^{-1} for NPEO2, 0.047 to 3.9 µg L^{-1} for NPEO1, and 0.83 to 2.8 9 µg L^{-1} for NP. 4-t-OP concentrations were detected at level lower than 0.07 µg L^{-1}.

Naylor et al. [18] performed a study in thirty rivers in the continental U.S. in 1989 and 1990 to determine the frequency and concentrations of nonylphenol and its ethoxylates in water and sediments. Nonylphenol was found in approximately 30 percent of the water samples with concentrations ranging from about 0.20 to 0.64 µg L^{-1}. Approximately 71 percent of the sampling sites had measurable concentrations of nonylphenol in the sediments at concentrations ranging from about 10 to 2,960 µg L^{-1}. Ethoxylates of nonylphenol were found in 59 to 76 percent of the water samples, with amounts varying by extent of ethoxylation. Several years later a study conducted from 1990 to 2000 revealed the occurrences of 95 organic wastewater contaminants in 139 U.S. streams [9]. Nonylphenol was one of the most commonly occurring contaminants at higher concentrations than most of the other contaminants [9]. In this study the median concentration of nonylphenol ethoxylates in waters samples ranged from 0.1 to 1 µg L^{-1} with an occurrence ranging from 23 to 45 percent.

Aquatic biota appears to be highly sensitive to NP and OP exposure. Studies in oysters, showed developmental defects and increased death rates in embryos and larvae [17], resulting from by a single NP exposure of 0.1 μg L^{-1}. Similar concentrations also caused 17% of oyster larvae to grow both male and female sex organs [17]. After NP exposure, male trout hepatocytes showed increased levels of vitellogenin, an egg yolk protein normally produced only in females [19]. Studies on Japanese medaka (*Oryzias latipes*) exposed to NP revealed a decreases in eggs production and fertility [20]. Exposure of tadpoles to OP at relevant lose dose (10^{-1} M) disrupted the sexually dimorphin expression of SF-1 that occur during sexual differentiation [21]. Due to the toxicity to aquatic biota, NP and its ethoxylates were banned in Canada and Europe and were classified as priority hazardous substances (PHS) in the Water Framework Directive [10]. USA has not taken any action to prohibit the use of NP. However, after the abundant data demonstrating the effect of NP in the aquatic ecosystem the Environmental protection Agency (EPA) prepared a guideline for ambient water quality that recommends nonylphenol concentrations in freshwater be below 6.6 μg $L^{-1}$and below 1.7 μg L^{-1} in saltwater [9].

1.3.2 Bisphenol A

Bisphenol A (BPA) is a chemical produced in large volume and primarily used in the production of polycarbonate plastics and epoxy resins (Figure 2). It is estimated that 7 billion pounds of BPA is produced annually worldwide and 2.5 billion is produced in USA [22]. BPA is released into the environment through WWTPs effluents [23], landfill leachate (via hydrolysis of BPA from plastics [24], or natural degradation of polycarbonate plastics due to moderate water solubility and low vapor pressure. BPA is also used as a reactive agent in the production of

temperature-sensitive paper with color developing layers. Therefore, paper mill effluents and recycling paper products, such as toilet paper must also be considered as a major source for BPA [25]. BPA was also found in groundwaters from agricultural and industrial wells due to leaching of this compound. Other sources of BPA come from resins, lacquers, surfactants, and paints from pipes, gaskets, migration from packaging and bottling material, envelopes, and printer ink.

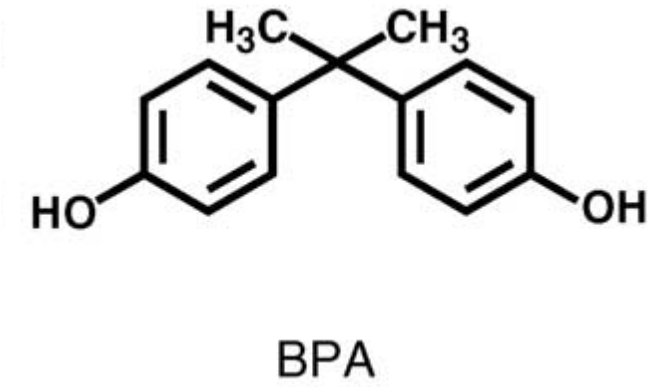


Figure 2: Structure of Bisphenol A

Limited information is available about BPA concentration in WWTPs and aquatic ecosystem. BPA has been detected in Canadian and US municipal waste treatment products and a high level has been measured in some industrial wastewater, most notably those associated with paper and allied products (maximum 149.2 µg L^{-1}; median 8.7 µg L^{-1}), chemicals and chemical products (maximum 91.3 µg L^{-1}; median 1.5 µg L^{-1}) and commercial laundries (maximum 43.6 µg L^{-1}; median 6.6 µg L^{-1}) [26] . In 2001 and 2002, BPA was not detected (< 0.001 µg L^{-1}) in effluent from a wastewater treatment plant in Louisiana, and concentrations were not quantifiable in samples collected from surface waters in Louisiana and in drinking water at various stages of treatment at plants in Louisiana [27]. Environmental monitoring of

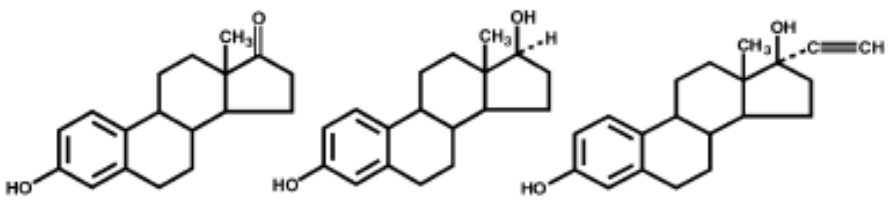
EDCs detected BPA at a median concentration of 0.14 µg $L^{-1}$and a maximum concentration of 12 µg L-in 41.2% of 85 samples collected from U.S. streams in 1999 and 2000 [1].

Human exposure to BPA is considerate very high because of it ubiquity. The heat, hydrolysis, sterilizing, microwave heating, warming prior to serving, and washing of containers result is increased leaching of the BPA into products that are consumed. Studies have shown the prevalence of human exposure to BPA. Trace levels of BPA in urine samples has been detected in various studies [28-30] and a report found that 92.6% of participants (n=2,517) in USA had urinary concentration in the range of 0.4 to 149 µg L^{-1} [31]. These findings have brought concern since several researches are showing harmful effects on animals and humans at very low doses. Very low doses of BPA (1 nM) were reported to cause proliferation of human prostate cancer cells, cardiovascular diseases, type 2 diabetes, and liver-enzyme abnormalities in sample of the adult US population [32]. BPA has also been found to have the paradoxical effect to block the beneficial effects of estradiol on neuronal synapse formation in rats at doses of 40 ug/kg, which is below EPA reference daily limit for human exposure [33]. However, for many years there has been disagreement between researchers and federal agencies in regard to what is considerate "low doses" effect of BPA. According to EPA and other agencies, low dose effect refer to effect being reported for chemicals at doses lower than used in traditional toxicological studies conducted for risk assessment [22]. Many researchers have classified BPA as weakly estrogenic and the levels in humans and environment are below the levels to cause adverse effects [22]. Moreover, abundant evidence indicates that BPA induces feminization during gonadal ontogeny of fishes, reptiles, and birds, but in all cases the amount of BPA necessary to cause such ontogenetic disruption exceeds concentrations in the environment [34]. Currently, the

use of BPA still allowed in USA, Europe and others countries. Canada is the only country that declared BPA as a health hazard and banned the use of it in products [22, 35].

1.3.3 Natural and synthetic estrogens

Natural estrogens (also known as the C18 steroidal group) such as estradiol and estrone share the same tetracyclic molecular framework which is composed of the four rings: a phenol, two cyclohexanes, and a cyclopentane (Figure 3) [36]. The difference in the compounds within the C18 group lies in the configuration of the D-ring at positions C16 and C17. E2 is used for the synthesis of ethinylestradiol (EE2), the commonly used active ingredient for oral contraceptive pills. Free estrogens, also known as unconjugated estrogens, are moderately hydrophobic and poorly soluble in water.



E1 **E2** **EE2**

Figure 3: Structures of Estrogens.

Sources of natural estrogen in the environment are contributed predominantly by humans and livestock through feces and urine. It is estimated that men excrete 1.6 and 3.9 µg day^{-1} of E1 and E2 respectively [37]. Women excretion of E2 can reach as high as 5000 µg day^{-1} and 259 µg day^{-1} of E1 in the case of pregnant women. During menstruation women excrete 3.5 and 8 µg day^{-1} of E1 and E2 respectively. Dairy cow excrete approximately 600 to 1200 µg day^{-1} of E1 and E2 and a pregnant cow could excrete up to 10800 µg day^{-1} of E2 [36]. EE2 primarily enters the

water treatment system as domestic sewage via excretion by women prescribed oral contraceptives [38]. Natural and synthetic are mostly excreted in feces and urines as the conjugated form. Conjugated estrogens do not possess biological activity and are formed by esterification of free estrogen by glucorinide and/or sulfate groups [36, 37]. In wastewater treatment plant or environment, conjugated estrogens are deconjugated back to their original form.

Concentrations of natural and synthetic estrogens in wastewater treatment plant effluents are very low, usually in the part per trillion (ng L^{-1}) levels. In effluents from wastewater treatment plants from Canada concentration of E2, E1 and EE2 were 1.8, 17.0 and 9.0 ng L^{-1} respectively [39, 40]. Concentration of E1 and E2 in WWTPS from California and New York followed same patterns with levels ranging between 0.77 to 18.0 ng L^{-1} [41]. Monitoring studies of surface water in USA found E1, E2 and EE2 in 7 to 15 % of the samples at median concentrations of 27.0, 9.0 and 73.0 0 ng L^{-1} respectively. This is an indication that wastewater treatment plant does not completely remove estrogens from wastewater and trace amount of the compounds are reaching our surface water resources.

A study in an artificial lake in Canada found that EE2 (4 ng L^{-1}) have led to feminization of males through the production of vitellogenin mRNA and protein, impacts on gonadal development as evidenced by intersex in males and altered oogenesis in females[3]. After 3 year of study the population of the fish from the lake was near to extinction. Compound such as E2 can reduce the reproductive fitness of adult male fish by suppressing their reproductive behaviors, including their ability to compete for nests and females[42].

1.4 Wastewater Treatments Plants

In this study 6 WWTPs were analyzed. Four plants are located in El Paso, Texas and two in Ciudad Juarez, Mexico. Locations of the WTTPs are shows in Figure 4.

1.4.1 Overview of wastewater treatment plants processes

Mechanical bar Screen: designed for the removal of coarse debris in the wastewater flow. These solids can clog and damage grit tank equipment, plant piping, or impede the hydraulic flow in open channels and pipes. Screening is the first step in treating water containing large solids.

Grit removal unit: Grit removal is a pre-aeration system designed to remove heavy non decomposable matter, such as sand and small rocks, from the sewage prior to primary sedimentation. Grit includes gravel, sand, and heavy particular matter such as bone chips and coffee grounds. Aeration mixing improves grit separation while freshening the water.

Primary clarifier: provide the removal of settleable solids and floating materials from the influent flow to provide an efficient, cost effective means of solids removal and to lessen the BOD loading on the activated sludge system.

Anoxic basins: The anoxic basins are a component that enhances the activated sludge. The main purpose of the anoxic basins is to allow for denitrification, or the conversion of nitrate and nitrite to nitrogen gas. Denitrification occurs when facultative microorganism use the oxygen molecules from NO3 which leave nitrogen gas N2. Oxygen chemically bound in nitrate and nitrite is a

readily available form of oxygen for many facultative bacteria. Bacteria continue to utilize food in anoxic zone but use nitrogen and not dissolve oxygen for their oxygen supply. This process reduces the organic load before the wastewater reaches the aeration basin and saves energy.

Activated Sludge: The activated sludge process aerates wastewater to allow microorganisms to consume BOD (biological oxygen demand) and reproduce in the aeration basin. The purpose of the activated sludge process is to reduce the concentration of dissolved, particulate and colloidal organic pollutants in the wastewater.

Aeration Basin: The wastewater is aerated to provide mixing and dissolve oxygen for the microorganisms feeding on the organic material contained in the wastewater.

Secondary clarifier: The mixed liquor is separated from the treated wastewater by gravity. Mixed liquor enters in the center of the clarifier which is called the flocculation well. The flocculated mixed liquor flows out of the bottom of the flocculation well. As the mixed liquor moves toward the effluent launder trough, the mixed liquor solid settle to the bottom of the clarifier. The settled solids are removed from the tank for return to the aeration tank to support microorganism population in the activated process.

Sand filter: Sand filters are beds of granular material, or sand, drained from underneath so that pretreated wastewater can be treated. Sand filter is use to physically stained particles incoming wastewater, chemical sorption, and assimilation, in which aerobic microbes eat the nutrients in the wastewater.

Powdered Activated Carbon (PAC): is a fine powder applied to an anaerobic or aerobic treatment system. The carbon in the biological treatment process acts as a "buffer" against the

effects of toxic organics in the wastewater. In such a system, biological treatment and carbon adsorption are combined into a single, synergistic treatment step.

Granulated Activated Carbon (GAC): is a system generally composed of carbon fixed-bed contactors used to absorb the relatively small quantities of soluble organic and inorganic compounds such as nitrogen, sulfides, and heavy metals remaining in the wastewater.

Ozone: is a very reactive gas that can oxidize bacteria, moulds, organic material and other pollutants found in water. Ozone Kills bacteria effectively and oxidizes substances such as iron and sulphur so that they can be filtered out of the solution.

Lime treatment: Lime inhibits pathogens by controlling the environment required for bacterial growth. Calcium hydroxide (hydrated lime) is an alkaline compound that can create pH levels as high as 12.4. At pH levels greater than 12, the cell membranes of harmful pathogens are destroyed.

Ultraviolet treatment (UV): UV rays inactivate microorganism by penetrating the cell walls, altering molecular compounds essential to cell function.

Clarifier/Thickener (densadeg): is a high-rate solids contact clarifier which combines optimized flocculation, internal and external sludge recirculation, and plate settling in two conjoined vessels to maximize hydraulic loading and treatment efficiencies.

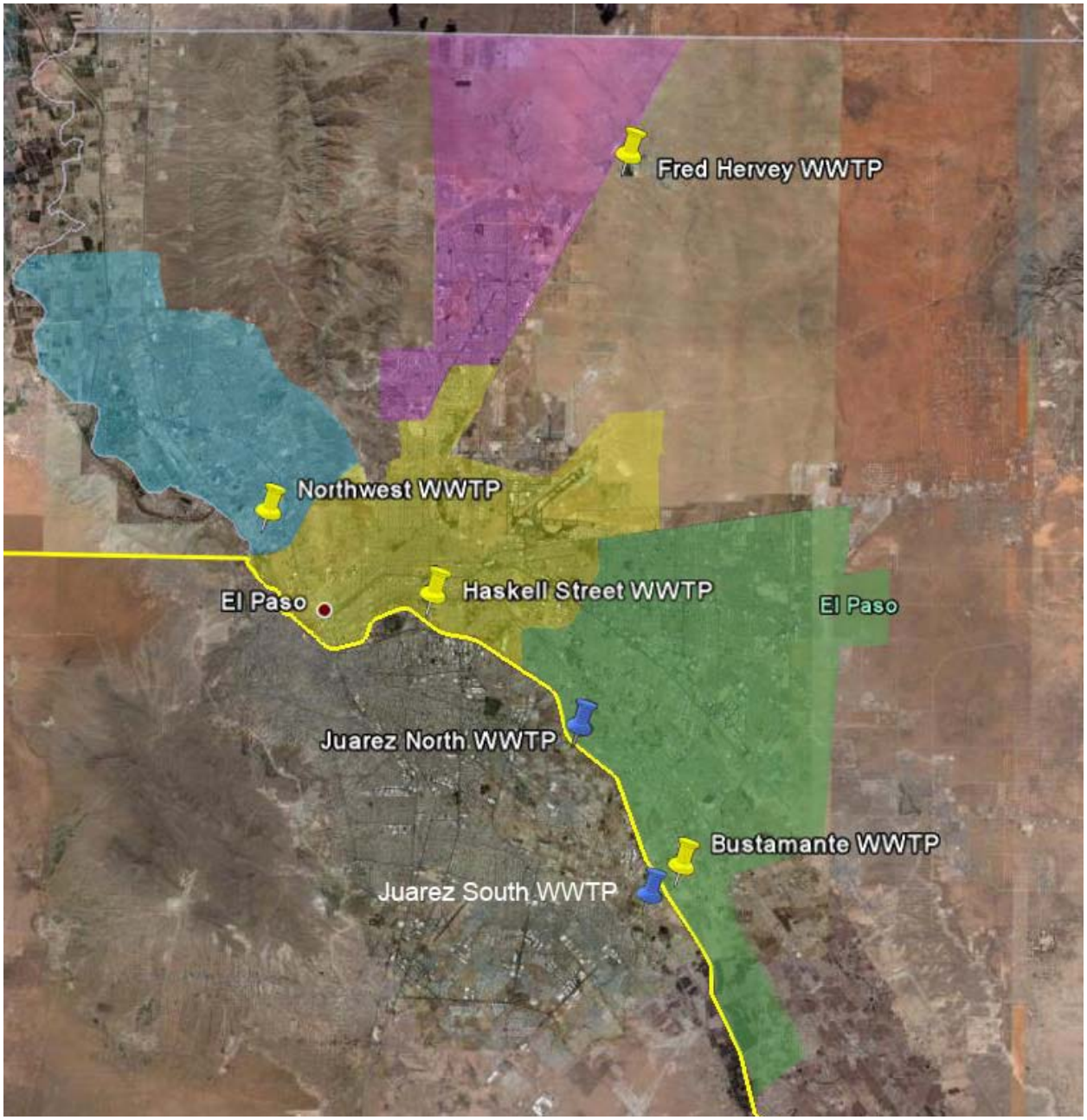


Figure 4: Locations of WWTPs.

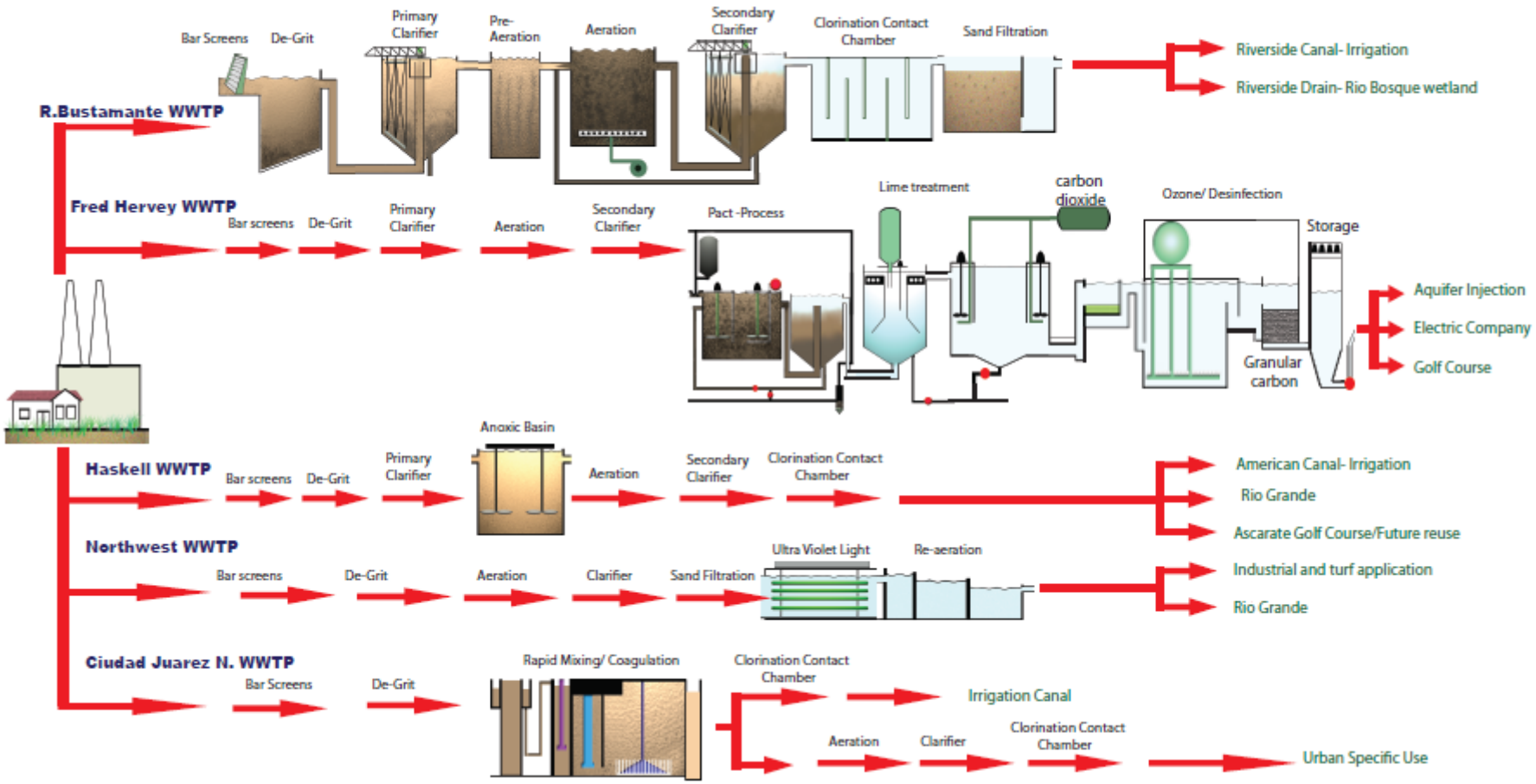


Figure 5: Schematics of Wastewater treatment Plants studied.

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CHAPTER 2: METHODOLOGY

2.1 Chemical and Biological Analysis

The compounds covered in the study afford analytical challenges when presented in complex matrices at low levels. Even with a complete chemical characterization, it is not possible to anticipate net estrogenic effect due to simultaneous presence of many other trace organic residuals in treated water. Moreover, EDCs are not defined by their chemical nature, but by their biological effect. Consequently, bioassays are necessary to measure net estrogenic activity in the whole water sample or corresponding concentrates. In other hand, since bioassays respond to all substances with receptor-mediated estrogenic activity regardless of the chemical structure, chemical analysis is needed to identify the individual estrogenic compound in environmental samples. ***Therefore, this study integrated the application chemical analysis and specific bioassay for the assessment of endocrine disrupting activity in complex environmental mixtures.*** Several studies combining chemical and biological analysis has demonstrated a relationship between concentration of EDCs in water samples and the estrogenic activity [1, 2]. High concentrations of EDCs in water correspond to a similar response in estrogenic activity. Although much effort has been made to identify the estrogenic substances in treated wastewater and surface water responsible for the reproductive disturbances observed in male fish, the results do not provide a uniform picture [3]. Bioassay-directed fractionation of WWTP effluents revealed the natural and synthetic estrogens as being the compounds mainly responsible for the estrogenic activity measured in a yeast reporter gene assay [4]. EC50 dose response is much lower for E2, E1, and EE2 compare with the rest of EDCs. On the other hand, high

concentrations of NP and nonylphenol monoethoxylate have been found in rivers and WWTP discharges that exceed the threshold levels for induction of vitellogenesis in adult male fish [5].

Chemical analysis of EDCs is typically performed using Gas Chromatography or Liquid Chromatography coupled with Mass or Tandem mass spectroscopy. Different bioassays have been used to determine estrogenic activity in samples. Some of the most commonly used are MCF-7 cell (breast cancer) proliferation assay, treating fish with vitellogenin as a biomarker, and yeast bioassay [6-8]. ***In this study Gas chromatography/Mass Spectroscopy (GC/MS) and an optimized recombination yeast assay were used for chemical and biological analysis, respectively.***

2.2 Stir Bar Sorptive Extraction –Thermal Desorption-Gas Chromatrography-Mass spectrometry (SBSE-TD-GCMS)

Stir Bar Sorptive Extraction (SBSE) was developed in 1999 using a stir bar coated with a 50-300 μL of PDMS (Figure 6). SBSE has been used to extract organic compounds from aqueous samples by putting the stir bar into the solution and stirring for a pre-determined time [9]. The stir bar is removed from the aqueous sample and the adsorbed compounds are thermally desorbed in a thermal desorption unit (TDU) and analyzed by GC-MS. Sorptive extraction is an equilibrium technique where the solutes from an aqueous phase into the extraction medium (PDMS) is controlled by the partition coefficient between the PDMS phase and the aqueous phases (KPMDS/w) [10]. The correlation between KPMDS/w and octanol-water coefficient (Ko/w) have been presented in several literatures. KPMDS/w values increased with increasing Ko/w, therefore, more nonpolar compounds are indeed partitioned more into the PDMS phase.

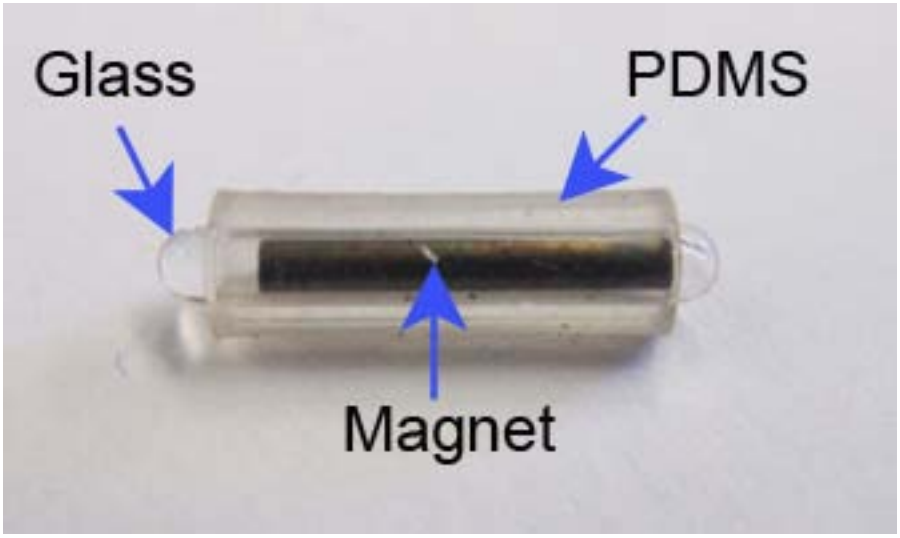

Figure 6: Stir bar Sorptive Extraction

Since PDMS phase is a non-polar liquid phase, it is preferable that the polarity of the analyte is low (e.g pesticides). Relatively high polarity compounds, such as estrogens and BPA, are not well recovered. Affinity of the estrogens for the polymer layer can be enhanced by in-situ derivatization of phenolics hydroxyl groups using acetic acid anhydride [11]. Furthermore, derivatization of EDCs produced a new compound that has properties more amenable for GC-MS analysis. Compounds containing hydroxyl group produce poor peak shape, separation and sensitivity. During the reaction with acetic anhydride, the polar phenolic hydroxyl groups are replaced with less polar acetate groups (Figure 7). The extraction is carried out by the simple addition of 200 mg sodium carbonate as a pH adjustment agent, and 200 uL of acetic acid anhydride into a 20 ml headspace vial with 20 mL of the water sample.

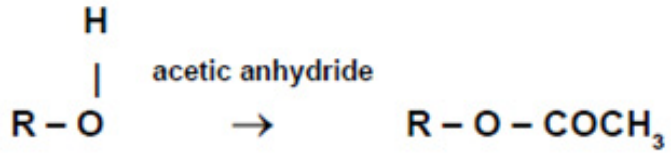

Figure 7: Derivatization by acetic anhydride

The stir bar is placed in each sample and stirred for 4 hours at 1000 rpm. After the SBSE process, the stir bar is removed from the sample solution, dried with lint-free paper, and then placed into a thermal desorption tube. The stir bar is heated in a thermal desorption unit (TDU) system to release the derivatized EDCs as gaseous phase from PDMS. EDCs recovery was dependent on sample matrix. Recovery ranged from 59% to >100% for alkylphenols, 49% to >100 % for Bisphenol A, 39% to >100% for estrogens. Limit of detection for were 1.6 ng L^{-1} for BPA, 1.5 ng L^{-1} for E2, 4.6 ng L^{-1} for E1, 7.9 ng L^{-1} for EE2. For alkylphenos the limit of detections was 2.5 ng L^{-1} for OP, 5.0 and 2.5 ng L^{-1} for NP and NPEO1, and 10 ng L^{-1} for NPEO1. Limit of detection will varied depending of sample matrix and instrumentation performance.

2.3 Yeast Assay Development: A four-hour yeast bioassay for the direct measure of estrogenic activity in wastewater without sample extraction, concentration, or sterilization

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Marc B. Cox : Participated in manuscript revision.

Wen-Yee lee: Participated in manuscript revision

During the last two decades, numerous studies have reported the presence of endocrine disruptor compounds (EDCs) in wastewater samples. However, EDCs are not defined by their chemical nature, but by their biological effect. For that reason, different kinds of assays have been employed to determine the overall estrogenic response of water samples. In-vivo assays that measure elevated levels of egg yolk protein, vitellogenin, in male fish is one the most sensitive and widely accepted test to determine estrogenic contamination in the aquatic ecosystem [12]. Vitellogenin is produced in the liver of female oviparous vertebrate species. Males produce very little vitellogenin [13]. This feminization has been linked to the presence of estrogenic compounds in water resources [14]. This type of assay has been performed in different manners. Fish in study can be collected from an expected contaminated area, and blood is taken to carry out vitellogenin analysis in the lab [13, 14]. The deployment of caged fish in wastewater effluent has been also used to detect in-vivo estrogenic activity [7, 15]. In this case, old rainbow trouts

were placed in cages close to wastewater effluent discharge for a specific time, and at the end of the period, blood samples were analyzed for vitellogenin using a homologous radioimmunology assay. Traditionally, estrogenicity by EDCs is tested in the lab where fish are exposed to known concentrations of the contaminants or to wastewater sample extract for weeks before analysis [16, 17].

In-vivo experiments are often time-consuming and expensive, and thus sophisticated analytical techniques for the measurement and assessment of EDCs are highly valued. For those reasons, especially in-vitro yeast (*Saccharomyces cerevisiae*) estrogen screen assays have successfully been used to assess estrogenic activity in environmental samples. Several studies have been performed in the past decade that have utilized recombinant yeast strains (rYES) capable of identifying compounds with the ability to interact with the human estrogen receptor (hERα). The rYES was first described in detail by Routledge and Sumpter [18] and has been employed in numerous subsequent studies for the assessment of estrogenic activity in a variety of sample types, including commercial chemical preparations as well as environmental samples, such as wastewater. In this assay, the hER is expressed in a form capable of binding to estrogen-responsive sequences (ERE) which is localized within a strong promoter sequence on the expression plasmid [19]. Since the ERE is linked to the β-galactosidase reporter gene, a colorimetric change is observed when an ER ligand binds the receptor and is translocated to the ERE [17]. This change in color occur when β-galactosidase is secreted into the medium, where it metabolizes the chromogenic substrate, chlorophenol red-b-D-galactopyranoside (CPRG), which is normally yellow, into a red product that can be measured by absorbance after 3 days incubation. However, the use of CPRG has demonstrated that the degradation products of chlorophenol red act as an estrogenic compound itself, which has led to the modification of the

rYES assay [20]. The subsequent modifications improved upon the original assay, but still resulted in time- and labor-intensive assay systems, some of which require 24-hour incubations [20, 21].

There are additional bioluminescent bio-reporter technologies based on activation of gene fusions using the firefly (*luc*) or bacterial (*lux*) luciferase [15-16]. These reporter assays showed similar detection levels to the rYES assay system based on certain estrogenic chemicals, but still requires a six hour incubation period to reach maximum bioluminescence, with measurement being taken every 60 minutes for 12 hours. Estrogenic activity has been also screened by the use of MELN cells [22-24]. These cells are estrogenic-sensitive breast cancer cells (MCF-7) steadily transfected with an estrogen-responsive gene. A significant draw-back to this and other mammalian cell-based reporter systems is the relatively high cost, long cell growth periods, and the need to sterilize or treat samples prior to applying them to the cells.

The yeast assay used in this research takes advantage of a commercially available chemiluminescent substrate for the detection of estrogen induced β-galactosidase expression. Using this assay an estrogen induced signal can be detected within 30 min and the total assay time from start to finish is no more than 4 h. More importantly, due to the short assay time, wastewater samples can be assayed without the need for sample extraction, concentration and sterilization. Therefore, wastewater samples can be tested immediately after sampling. This assay protocol represents a quicker and simpler alternative to the yeast based assays currently in use.

2.2.1: Yeast assay for non-concentrated samples.

The four-hour yeast bioassay illustrated in Figure 8 a modified version of a receptor-mediated β-galactosidase reporter assay that was previously described for use in the functional analysis of receptor regulatory proteins[25, 26]. W303α (MATα leu2-112 ura3-1 trp1-1 his3-11,15 ade2-1 can1-100 GAL SUC2) with a deleted pleiotrophic drug resistance gene (PDR5) was the parental yeast strain for all assays described. The deletion of PDR5 in this strain was performed using methods previously described [27]. The parent strain was cotransformed with a TRP1-marked constitutive human ERα expression plasmid (pG/ER) and a URA3-marked estrogen-inducible β-galactosidase reporter plasmid (pUCΔSS-ERE, both plasmids kindly provided by Didier Picard, University of Geneva) and maintained in synthetic complete media lacking uracil and tryptophan (SC-UW) to select for plasmid retention.

Using a sterile toothpick or swab pick three separate colonies from each transformation plate and inoculate each into 5 ml medium solution, SC-LUW, in a 50 ml conical tube and incubate overnight at 30C with shaking. The next morning the optical density at 600 nm (O.D.600 is determined for each culture and dilute each back to an O.D.600 of 0.08 in fresh SC-LUW in a new 50 ml conical tube. Place the diluted cultures in the water bath at 30C and incubate while shaking. The O.D. 600 of the cultures can be monitored during this time. Typically after 1 hour the cultures have exited lag phase and begun to grow. Once the cultures reach an O.D.600 of approximately 0.1, the culture was aliquoted into 15 ml centrifuge tubes. The cells were harvested by centrifugation at 2000 rpm for 2 minutes and supernatant was discarded. A mix of 2.25 mL of wastewater with 750µL of 4X concentrated SC-UW was added to each assay to be performed. The cultures were then incubated at 30 °C with shaking for 2

hours. One hundred µL from each of the cultures were then transferred into an opaque 96 wells plate and 100 µL of tropic gal screen (chemiluminescent substrate) was added. Cover the plate with tape or film, and incubate for two hours at room temperature. After the 2 hour incubation read the plate in the microplate luminometer using a gain of 1.0 and a voltage between 750 and 1100. The voltage used depends upon the strength of the signal. Reading at 900 volts is often taken at first which is sufficient in most cases. If the signal is weak the voltage can then be increased to improve the sensitivity.

2.2.2: Yeast assay for concentrated samples

Once the cultures reach an O.D 600 of approximately 0.1, 100 µL of the culture was transfered into an opaque 96 wells plate. Treat the cells with the standards or unknown samples and mix the solution by pipetting up and down. Once all samples have been delivered, cover the wells with tape or film to prevent evaporation and incubate the plate at 30 ˚C for 2 hours. Yeast can tolerate up to 1% ethanol or dimethylsulfoxide (DMSO) without any toxic effects. Thus, if know concentration is used always set up the compounds stock concentrations so that solvent is added at a final volume of no more than 1%. Prepare the Tropix Gal Screen reagent according to the instruction and place on ice. After the two hours incubation, add 100µl of the Tropix Gal Screen reagent to each well.

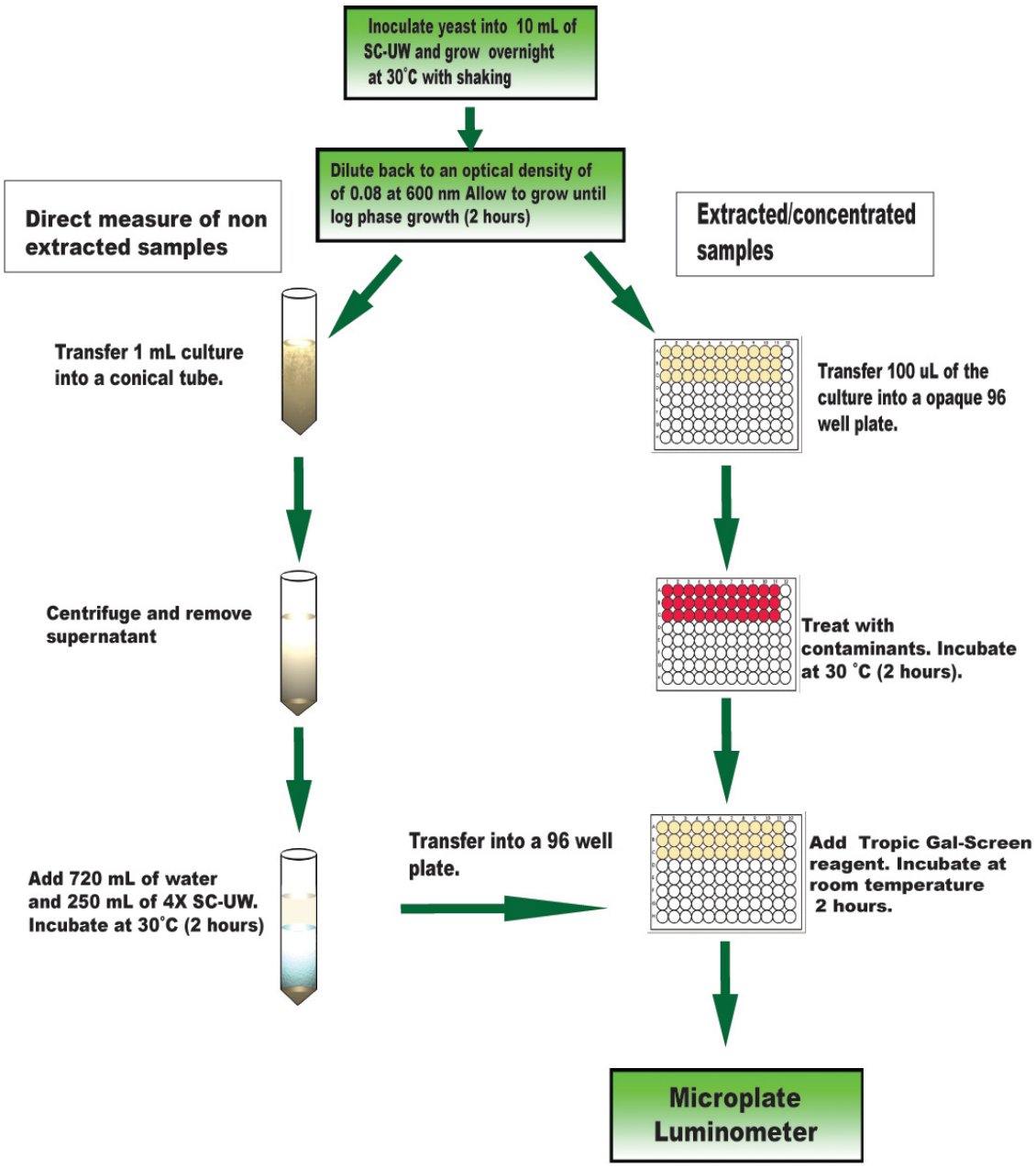

Figure 8: An illustration of the four-hour yeast bioassay protocol. A saturated overnight culture is diluted back to an O.D.600 of 0.08 and allowed to reach log phase growth before the addition of sample. Wastewater samples that typically have higher levels of contaminants can be assayed directly by mixing with concentrated yeast medium and placed on the cells for two hours prior to substrate addition (left panel). The samples can be extracted and concentrated as with traditional yeast assays when ligand levels are below values needed for direct detection. However, in this assay, the cells only need to be treated with concentrated sample for two hours prior to substrate addition and, thus, the concentrated samples can still be assayed in a four-hours time frame (right panel).

2.2.3: Results and Discussion

The two aforementioned procedures were tested and compared. An objective of this study was to develop a method in which the steps of extraction and concentration of a sample could be avoided. Measuring wastewater samples directly without extraction or sterilization significantly reduces the time between sample collection and data acquisition. Wastewater samples are usually extracted before analysis by solid phase extraction (SPE) and the eluted extract is concentrated, cleaned, and then followed by the assay. This process could take prolonged hours of work before being analyzed. The sensitivity of the chemiluminescent β-galactosidase substrate has allowed for the steroid hormone receptor-mediated reporter assays to be conducted within a more physiological time frame compared to the alternative assays in use.

Similar E2 dose-response curves (Figure 9) were observed for each of the assay protocols, demonstrating that there is no difference in the outcome of the assay regardless of whether water samples were tested directly or concentrated. No significant difference in EC50 was found between the two protocols. The EC50 of E2 obtained by essaying concentrated E2 and water diluted E2 (17β-estradiol was diluted into distilled, deionized water and treated in the same manner as the wastewater samples) was 1.45×10^{-10} M±0.1 and be 1.51×10^{-10} M±0.1, respectively. This demonstrates the relevance of the assay method in the evaluation of estrogenic compounds within wastewater samples without the need to concentrate samples prior to analysis.

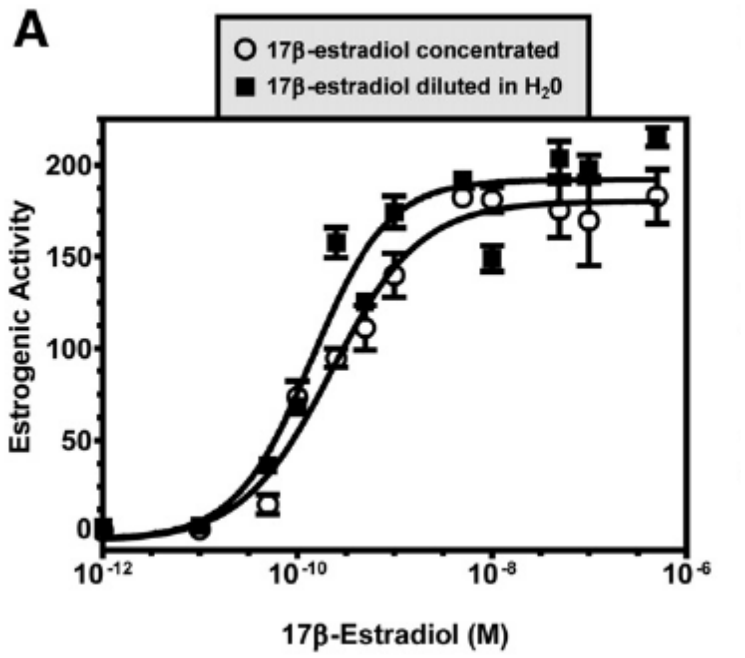

Figure 9: Four-hour yeast bioassay method comparisons. A) Representative 17β-estradiol dose-response curves in which the ligand solubilized in ethanol vehicle was added directly to the wells (open circles; Fig. 1 right panel) or was diluted in water and treated the same as wastewater samples (closed squares, Fig. 1 left panel) are shown. The resulting EC50 values averaged from three independent assays ± standard deviation were 1.45×10−10 M±0.1 for concentrated ligand and 1.51×10−10 M±0.1 for ligand diluted in water.

The Dose–response curves of estrogenic ligands in the study are shown (Figure 10). The EC50 values of the ligands range from the most sensitive, 17β-estradiol, with an EC50 of 1.45×10^{-10} M to the least sensitive, bisphenol A, with an EC50 of 3.43×10^{-6} M. The EC50 results using our yeast bioassay are very similar to the results obtained from other YES assay systems, with the exception of nonylphenol [28]. The EC50 of nonylphenol reported by Sanseverino et al [28] was 1.7×10^{-5} M using the BLYES assay system, which is 10^3 less sensitive than the EC50 value we determined using our yeast bioassay (2.48×10^{-8} M). EC50 for nonylphenol was similar to those obtained by E-screen assay [22]. In, addition this assay is 1 to 3 order of magnitude more sensitive than those assays that use vitallogenin as biomarker [29, 30].

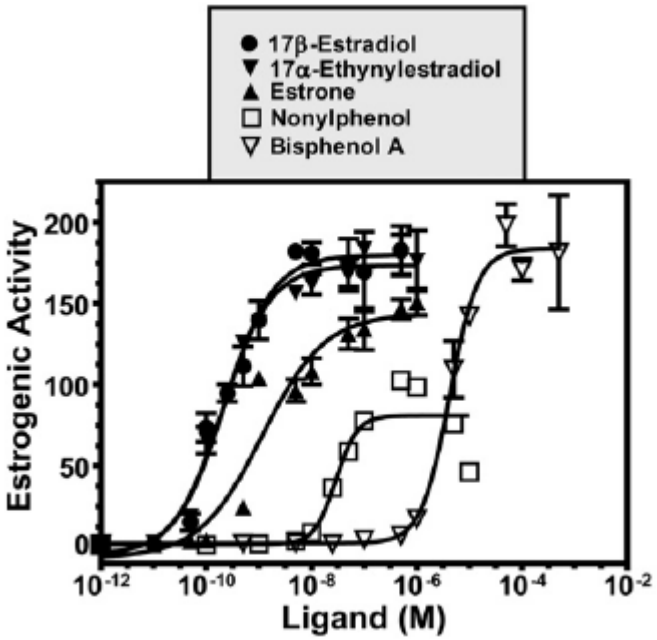

Figure 10: Dose–response curves for typical estrogenic wastewater contaminants using the four-hour yeast bioassay. Representative dose–response curves for the indicated ligands using the four-hour yeast bioassay are shown. The ligands used include 17β-estradiol (closed circle), 17α-ethynylestradiol (closed upside down triangle), estrone (closed upright triangle), nonylphenol (open square), and bisphenol A (open upsidedown triangle). All data points are averages of three independent replicates with error bars representing standard.

Table 1: EC50 values of estrogens receptor-Ligand interaction

EC^{50} Values of Estrogen Receptor-Ligand Interactions	
Compound	EC^{50} (M)[1,2]
β-estradiol	$1.5 \times 10^{-10} \pm 0.1$
17α-Ethynylestradiol	*$1.93 \times 10^{-10} \pm 0.0$*
Estrone	$1.28 \times 10^{-9} \pm 0.1$
Nonylphenol	$2.48 \times 10^{-8} \pm 3.9$
Bisphenol A	$3.43 \times 10^{-6} \pm 0.8$

[1] EC^{50} values (average ± SD)

[2] For all samples, n=3

For an exemplary comparison, the corresponding data for the samples taken in two plants in the winter of 2008 and the summer of 2009 are listed in (**Table 2**). The measured estrogenicities by bioassay (EEQyeast) for the wastewater samples from different wastewater treatment plants (WWTPs) were comparedwith the results (EEQGC–MS) calculated by EEF and concentrations determined by chemical analysis of the same samples. The (EEQyeast) measured in the samples ranged from no activity (NA) to 28.4 ng L−1 while the calculated estrogenic activity (EEQGC–MS) ranged from 2.1 to 155.9 ng L−1.

Table 2. Measured estrogenicities by bioassay compared to chemical analysis

		Measured EEQ_{yeast}		Calculated EEQ_{GCMS}	
Winter 2009		**EEQ**	**RSD**	**EEQ**	**RSD**
Plant A	Influent	20.26	2.78	102.59	4.45
	Effluent	6.75	6.30	6.13	2.66
Plant B	Influent	22.09	3.45	120.73	6.77
	Effluent	NA	NA	14.78	10.16
Summer 2009					
Plant A	Influent	18.30	2.58	75.34	4.45
	Effluent	7.40	0.99	1.36	0.16
Plant B	Influent	18.11	3.99	155.96	10.62
	Effluent	7.42	1.14	11.39	24.23

A 2nd order polynomial regression analysis using the measured and calculated EEQ values shows that there is a positive correlation between EEQyeast and EEQGC–MS (Figure 11). This implies that the higher the concentration of EDCs in the water, the higher the estrogenic activity reflected in the yeast assay. The authors observed that EEQGC–MS values were approximately 3 times greater than EEQyeast. A similar observation was reported in other studies [31, 32], in which chemical analysis predicted higher estrogenic activity than what was measured in the bioassay. Though the differences between measured and calculated EEQs could have various reasons, low EEQyeast values could suggest that potential interfering (antagonistic) compounds were present in the water samples [33]. It should also be noted that the chemical analysis-derived EEQ were found lower than the bioassay derived EEQ in the same sample in some studies [34]. The mixed results once again showed that the environmental sample matrices are complicated. Chemical analysis is only focused on the determination of target substances in wastewater. The result is limited in providing a complete account of all EDCs existing in

wastewater. The biological response of the yeast assay is complex and includes all estrogen-like compounds capable of binding to the receptor. This could lead to synergism, potentiation, and/or inhibition of the response, depending upon the quantities and combination of compounds present [35]. Despite the discrepancy, the estrogenic activities measured in wastewater influent samples were consistently higher than what were in the effluent. The results from this study demonstrate that this assay is a good sample screening tool for total estrogenic activity in wastewater samples.

In addition, our bioassay can function as a screening tool to determine whether further chemical analysis is necessary. Based on the positive correlation between bioassay and chemical analysis of screened samples, it is possible to use our modified yeast assay system as the first line of screening to select the samples which would require additional chemical analysis. This would allow the conservation of resources by eliminating unnecessary chemical analysis of samples which lack initial estrogenic activity in the yeast bioassay.

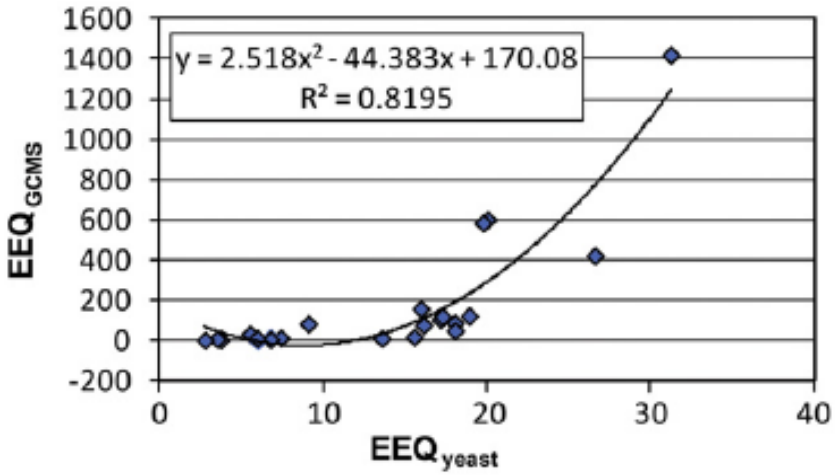

Figure 11: A correlation between EEQGCMS and EEQyeast. A second order polynomial regression analysis reveals a positive correlation between the estrogenic activity determined by direct biological measurement (EEQyeast) and by chemical analysis (EEQGCMS). EEQGCMS is the calculated overall estrogenicity based on the concentration of individual compounds detected and their relative potencies. EEQyeast, is the measured estrogenicity determined by the yeast bioassay.

3.2 Solid Phase Extraction

Solid-phase extraction (**SPE**) is a separation technique in which compounds that are dissolved or suspended in a liquid mixture are separated by passing the samples through a cartridge packed with a stationary phase. The result is that the target analytes in the sample are retained on the stationary phase. The portion retained can then be removed from the phase by eluting them with the appropriated solvents. Extraction of steroid, bisphenol A, and alkylphenol by SPE have been widely used for the determination of their concentration in wastewater samples [3, 36, 37]. In this study LC-18 reverse phase (octadecyl bonded-endcapped silica) was used for the extraction of the analytes in study, since is capable for separating moderately non-polar to nonpolar compounds from samples matrix.

Wastewater samples were filtered using 0.7 µm glass fiber membranes to eliminate the solid and avoid SPE clogging. SPE cartridges are first conditioned with methanol, ethylacetate and ultrapure water. After filtration 100 mL of the samples were spiked with the ideal isotopes and the water passed through the SPE cartridge by using a Visipred large volume sample manifold. The water passed through the cartridges slowly (5 mL per minute) by adjusting the pressure to -7.5 psi. Cartridge are dried by a gentle flow of nitrogen and the analytes are eluted with 10 mL of methanol, 5 mL of ethylacetate, and 5 mL of dimethylchlorine, which are solvents that covered wide range of polarity. Eluted extracts were concentrated to 0.5 mL in a nitrogen vaporator with a water bath at 60 °C. The concentrated samples were cleaned by 1 gram of silica gel deactivated with 5% water (w/w). Compounds from the silica gel were eluted by using 20% methanol in ethylacetate and dimethylchlorine. The extracts were concentrated again to dryness and reconstituted with 100 µL of dimethylformamide.

Similar to the SBSE procedure, the extract needed to be derivatized to obtain a higher sensitivity. Extract were derivatized by adding 100 µL of N-O Bis (trimethylsylil) trifluoroacetamide with 1% trimethylchlorosilane to the extracts, which were transferred to 2 mL vials. The vials were closed and heated in an oil bath at 70 °C for 30 minutes. In this reaction (silylation) an active hydrogen is replaced by an alkylsilyl group forming trialkylsilyl derivatives (Figure) After derivatization samples, were concentrated again to 100 µL. The reason to use dimethylformamide as the final solvent was to avoid transformation of EE2 to E1and E2 during the reaction. A study performed by Shareef et al. [38] revealed the formation of E1 after derivatization of EE2 using different solvents. These results were confirmed in our study (Figure 12) by derivatizating known concentration of EE2 in acetonitrile.

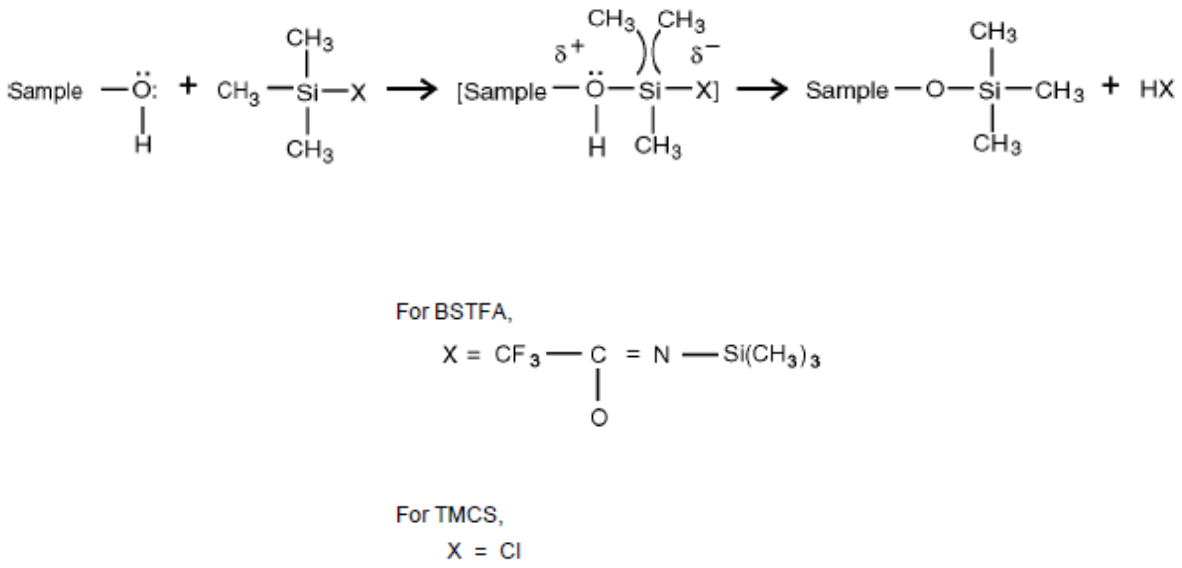

Figure 12: Derivatization by silylation

EDCs recovery was dependent on sample matrix. Recoveries were 85% for NPEO2, 77% for NPEO2 , 73% for Bisphenol A, 39% to >100% for NP, 90% for E1 for 88% for E2. These recoveries are similar to those obtained by Avila et al. using the same type of SPE cartridges[36]. Limit of detection were 1.6 ng L^{-1} for BPA, 4.2 ng L^{-1} for E2, 5.6 ng L^{-1} for E1, and 7.9 ng L^{-1} for EE2. For alkylphenols the limits of detection were 2.5 ng L^{-1} for OP, and 5 ng L^{-1} for NP and NPEOs. Limit of detection will varied depending of sample matrix and instrumentation performance.

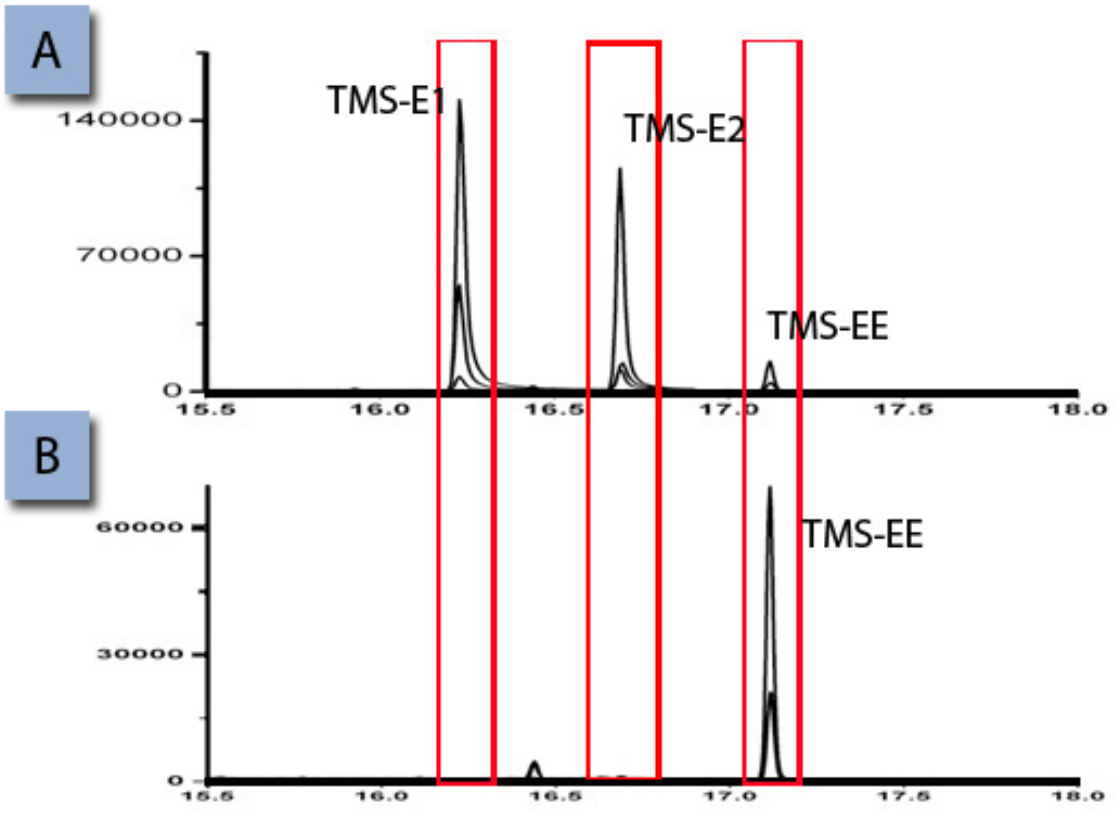

Figure 13: GC–MS Chromatograms of trimethylsilyl (TMS) ethinylestradiol (EE2) obtained using BSTFA+ 1%TMCS in various solvents: (A) acetonitrile,(B) dimethylformamide. Red rectangles indicate the location of the peaks.

4.2 Thermal Desortion-Gas Chromatography- Mass Spectrometry

The Stir Bars were thermally desorbed in a thermal desorption unit (TDU) (Gerstel, US) under splitless mode. The desorption process was programmed as follows: initial temperature at 40 °C with a ramp of 60 °C min^{-1} to 300 °C (held for 7 min). The transfer line temperature was set at 300 °C. The desorbed EDCs were then cryo-focused in a baffle liner in a cryo-injection system (CIS4) at −40 °C under liquid nitrogen prior to injection. The CIS 4 instrument was

programmed as follows: initial temperature at −40 ℃, ramp at 12 °C s^{-1} to 300 ℃ and held for 10 min. For SPE, 2 µL of samples were injected to the GC-MS. The transfer line temperature was set to 100 ℃, and after injection the temperature was increased to 300 ℃.

The separations were performed on a Zebron ZB-5MS capillary column (0.25 mm×30 m×0.25 µm, Phenomenex, CA). The oven was programmed as follows: initial temperature set at 60 °C with 15 ℃ min^{-1} ramp to 300 ℃ and held for 5 min. The carrier gas, (ultra-pure helium), was set at a constant flow of 1.2 mL min^{-1}. The mass spectrometer was operated in the selected-ion monitoring mode with electron-impact ionization (ionization voltage, 70 eV).

4.2.1 Single monitoring ion for compounds derivatizated by acylation.

Target compounds were measured based on the following quantification ions on selected ion monitoring mode: BPA: m/z=213, 228; E1 : m/z=270; E2: m/z=272; EE2: m/z=213, 296; NP: m/z=107, 135, 149; OCT: m/z= 135; NPEO1; m/z = 107, 179, 193; NPEO2; m/z = 223, 237, 251; BPA $^{13}C_{12}$: m/z=225, 240; p-n-NP $^{13}C_6$: m/z=113. Ten-point calibration curves were conducted ranging from 0.005 to 20 ng L^{-1}. The linear response of the curves produced correlation coefficients (R^2) higher than 0.99 for all EDCs. Figure 14 shows an example of peaks obtained by SBSE –GCMS.

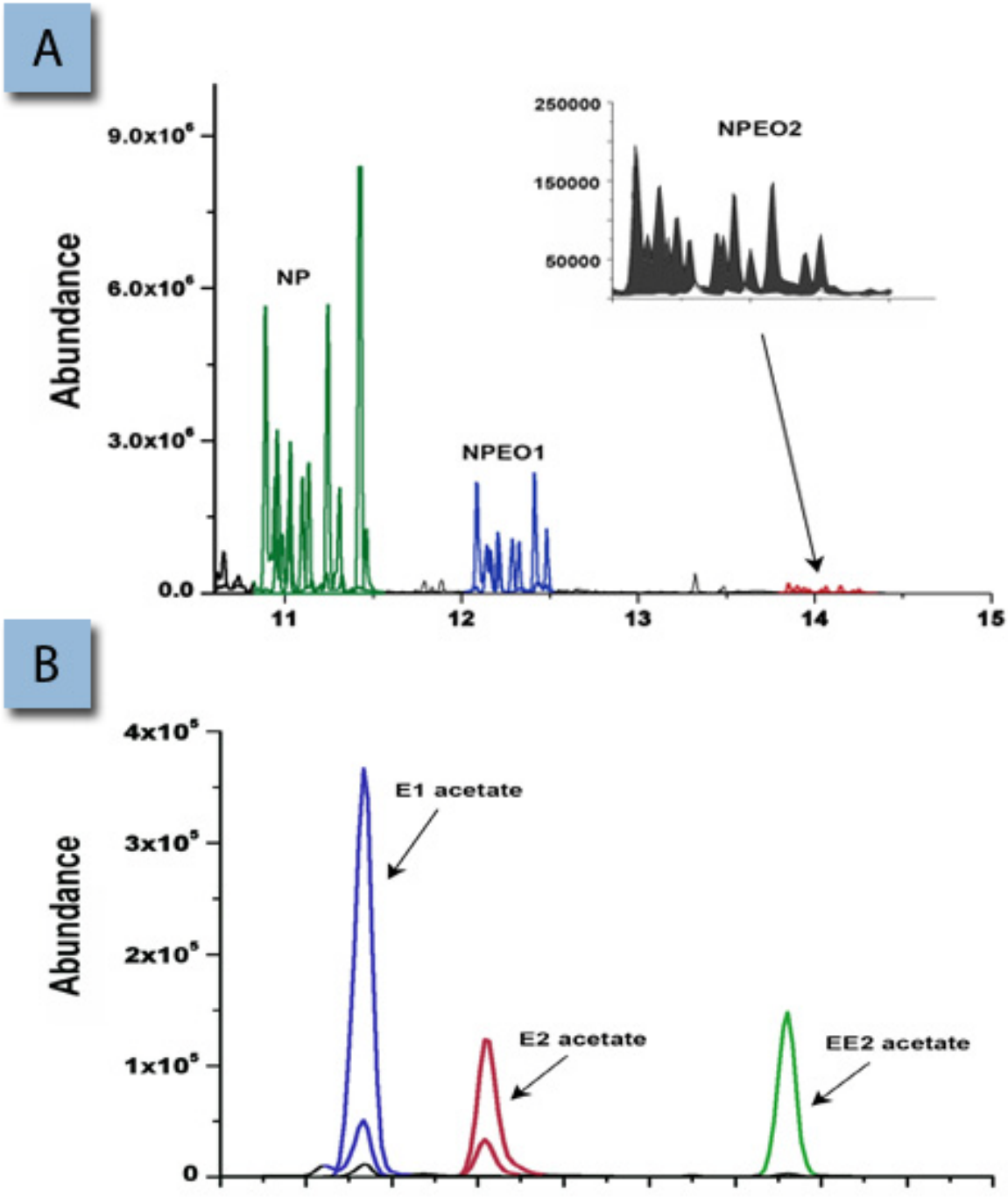

Figure 14: Chromatograms of Analytes by SBSE-TDU-GCMS. (A) NP and NPEOs; (B) Estrogens.

4.2.2 Single monitoring ion for compounds derivatizated by silylation

Target compounds were measured based on the following quantification ions on selected ion monitoring mode: BPA (Bisphenol A): m/z=357,372; E1 (Estrone): m/z=342, 257; E2 (17β-estradiol): m/z=416, 285; EE2 (17α-ethynylestradiol): m/z=425, 440; NP (Nonylphenol): m/z= 207, 221, 179, 249, 235; OCT: m/z= 207; NPEO1; m/z = 251, 265, 279, 293, 307; NPEO2; m/z = 323, 337, 295, 309, 351; E2 13C2: m/z= 418, 287; BPA 13C12 (Bisphenol, Ring 13C12): m/z=369, 384; p-n-NP 13C6 (para-n-Nonylphenol, Ring 13C6): m/z=185, 298. Ten-point calibration curves were conducted ranging from 0.005 to 20 ng L^{-1}. The linear response of the curves produced correlation coefficients (R2) higher than 0.99 for all EDCs. Figure 15 shows an example of chromatograms obtained by the GCMS.

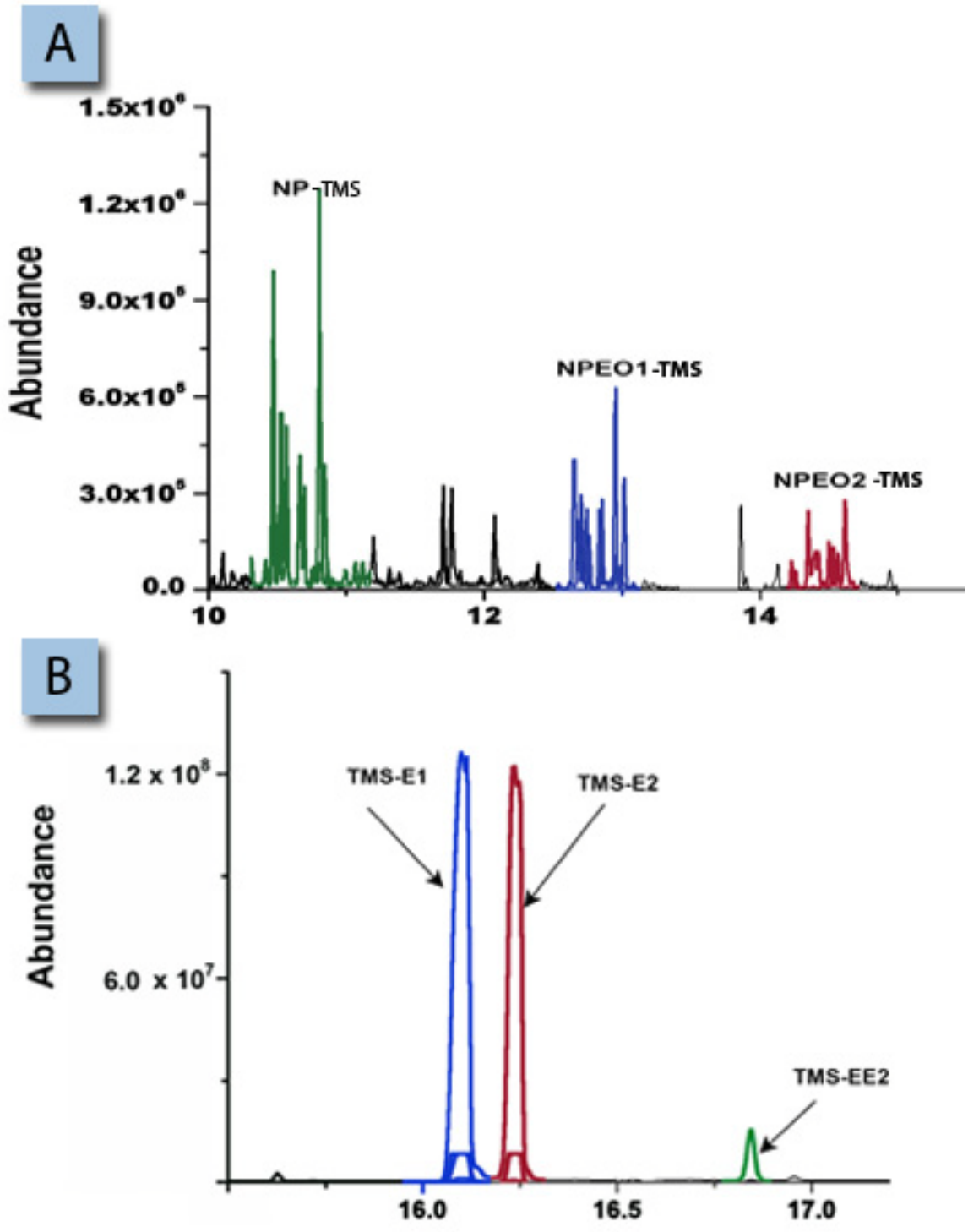

Figure 15: Chromatograms of analytes after SPE.

4.3 Quality control

4.3.1 Sampling:

All water samples will be collected using glass amber containers. Each bottle will be rinsed with high purity water and acetone, and baked for two hours at 400 o C prior to sampling. Upon collection, samples are preserved in ice, transported back to the laboratory, and stored at -80 o C prior to extraction, which is usually within 24 h. In case of storage, water samples should be analyzed within one week to avoid decomposition of organic compounds, especially estrogens.

4.3.2 Chemical analysis

Organic solvents used are HPLC or higher grade. Stir Bar will be soaked in 80:20 acetonitrile/methanol and then conditioned at 300 °C for 2 hours prior to use. Glassware for chemical analysis will be cleaned (water -solvent) and baked at 400 °C for at least 2 hours to prevent cross contamination. Positive identification of EDCs will be based on retention time and mass ion abundance ratios on GC/MS. For analyte identification and quantification, retention times for the analytes have to match retention times of reference compounds within 0.1 min. Calibration was performed with linear, nine-point curves from 0.005 to 100 μg L^{-1}. The limit of quantification was based on the lowest calibration point of the calibration curve. The method limit of detection is approximately one-third of the limit of quantification. Additionally, to be considered for quantification, the signal-to-noise ratios for the analytes needed to exceed six. Peak areas will be normalized to the surrogate standards area count to correct for variations in derivitization efficiency, analyte recovery in SBSE, SPE and GC/MS performance. The working solutions containing all the EDCs (target compounds and isotopes) at accurate defined above

mentioned concentrations were derivatized as described previously. Analysis will be run as triplicate for each sample. A blank was run every 5 samples for the elimination and verification of memory effect that could have affect the results.

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CHAPTER 3: CHEMICAL AND BIOLOGICAL ANALYSIS OF ENDOCRINE DISRUPTING COMPOUNDS IN WASTEWATER TREATMENT PLANTS FROM EL PASO, TX AND CIUDAD JUAREZ, MX

2.1 Introduction

A wide range of natural and man-made chemicals enter the environment through wastewater treatment plants (WWTPs). Some of these compounds can interfere with the endocrine system of humans and wildlife, altering reproduction and development functions [1]. The presence of such endocrine-disrupting compounds (EDCs) in natural ecosystems has been linked to adverse effects observed in wildlife, including feminization in male fish and development of oocytes in testes [2]. For instance, alkylphenols ethoxylates (APEOs) and their associated nonylphenol metabolites, as well as natural and synthetic hormones, have been identified as the major contributors to the estrogenic activity of wastewater [3, 4]. The release of alkylphenols ethoxylates into the environment has been reduced by restricting its marketing and use in manufactured products [5]. Such a strategy, however, cannot be applied to estrogens excreted by humans and animals. Consequently, the study of wastewater treatment processes efficiency to remove these chemicals before discharge into the environment is of particular concern.

Such issue is even more alarming when two cities share the same resources. El Paso, Texas and Ciudad Juarez, Mexico are twins cities located in the Chihuahuan desert with an estimated population of over 2 million. Under a program called Border 2012, USA and Mexico reached an agreement to protect the environment and promote public health in the border region. One of the goals of this program is to improve sewage treatment. Currently the wastewater treatment plants in Ciudad Juarez lack treatment capacity; consequently, there are untreated

discharges and flows that potentially reach surface water such as the Rio Grande and/or groundwater. This situation represents high health risks to human due to the potential contact with wastewater and vectors of waterborne diseases, as well as environmental contamination. Furthermore, the city of Ciudad Juarez lacks wastewater collectors that impede untreated sewage discharge to agricultural drain, which eventually will reach the river. Nevertheless, emerging compounds in wastewater effluent are no taken into account since they are not regulated by any agencies.

In this research, four WWTPs in El Paso, Texas and Two in Mexico were chosen to investigate the concentration of EDCs and estrogenic activity in influent and effluent samples. Stir bar sorptive extraction (SBSE) and Gas Chromatography/Mass Spectrometry (GCMS) and Chemiluminiscence yeast assay were used to determine the EDC concentrations and the estrogenicity, respectively. The aim of the present study was to investigate the seasonal variations of estrogenic compounds in the WWTPs, and the capability of each plant to remove EDCs from the wastewater. To our knowledge, this is the first study concerning EDCs in wastewater treatment plants from U.S.A-Mexico Border.

2.2 Materials and Methods

2.2.1: Materials

Estrone (E1), 17β-estradiol (E2), 17α-ethynylestradiol (EE2), Bisphenol A (BPA), nonylphenol technical mixtures (NP), 4-tert-octylphenol (OCT), nonylphenol monoethoxylate (NPEO1) and nonylphenol diethoxylate (NPEO2) were purchased from Sigma-Aldrich (MO, USA). Nonylphenol standard solution in methanol was supplied by AccuStandard (CT, USA). E2 (3,4-

$^{13}C_2$), BPA (ring $^{13}C_{12}$) and p-n-Nonylphenol (ring $^{13}C_6$) internal standards were from Cambridge Isotope Laboratories, Inc (MA, USA). HPLC grade methanol, acetic acid anhydride and sodium carbonate were purchased from VWR (USA). Stir bars (Twister®, 10 mm×1 mm; coated with polydimethylsiloxane (PDMS) were purchased from Gerstel Inc. (MD, USA). Stock solutions of the individual EDC and a combined working solution for GC/MS were prepared in methanol. For yeast assays, all EDCswere prepared as 10 mM stock solutions in ethanol. All solutions were stored at −4 °C until used.

2.2.2: Sample sites

Four WWTPs from El Paso, Texas, and two plants from Ciudad Juarez, Mexico were chosen for this study (Table 3). Each plant from El Paso employed typical primary treatment with the exception of plant one which lacks a primary clarifier, and wastewater influent pass through a bar-grit/de-grit process followed by the secondary treatment (activated sludge). Plant 2 is comprised of two-stage powdered activated carbon treatment activated sludge (PACT). It combines conventional biological treatment and the addition of powdered activated carbon to the wastewater during the process. After the PACT treatment the water passes through a nitrification /denitrification treatment where methanol is added in the second stage as carbon for denitrifiers). This plant is also the most advanced WWTP in the region involving a tertiary treatment consisting of high lime treatment, recarbonation, ozone disinfection, and granular activated carbon filtration (GAC). All WWTPs in El Paso used sand filtration. Plant 5 and 6 are located in Ciudad Juarez, Mexico and employed an advance primary treatment. This treatment consists of an aerated settling-degreasing process ($FeCl_3$ is added as coagulant) and a clarifier/thickener process which combines flocculation (a polymer is added as flocculant), internal and external

sludge recirculation, and plate setting in two conjoining vessels. Plant 5 has two effluents where 96% of the wastewater is treated by an above-mentioned primary treatment process and 2% passes through an activated sludge treatment. All plants use chlorine for disinfection as final treatment.

Table 3 Table: Description of wastewater treatment plants (WWTPs)

		Primary Treatment			Advance Primary Treatment		Secondary Treatment				Tertiary Treatment							
Plants	FLOW MGD	Bar-Grit	De-grit	PC	ASD	C-T	AS	PACTR	ANOX	SC	HLT	RECARB	O3	GAC	SF	UV	CL_2	Effluent Discharge
1	17.5	x	x				x			x					x	x	x	•Rio Grande River •Reservoir
2	10	x	x	x				x		x	x	x	x	x	x		x	•Golf course irrigation •Industrial cooling water •Ground water recharge
3	27.7	x	x	x			x		x	x					x		x	•Rio Grande River •Irrigation canal •Reclaimed water tower
4	39	x	x	x			x			x					x		x	•Rio Bosque Wetland •Irrigation canal •Reclaimed water tower
5	57	x	x		x	x	x			x							x	•Irrigation canal •Urban use
6	22	x	x		x	x											x	Irrigation canal

MGD= Million of gallons per day; PC = primary clarifier; ASD = aerated settling and degreasing; C-T = clarifier/thickener; AS = activated sludge, air diffused; PACTR = powdered carbon activated sludge; ANOX = anoxic basin; SC = secondary clarifier; HLT = high lime treatment; RECARB = recarbonation Treatment (CO_2); O_3 = ozone disinfection; GAC = granular activated carbon filtration; SF = sand Filtration; UV = ultraviolet light; Cl_2 = chlorine disinfectant

2.2.3: Sample collection

Twenty-four hour composite wastewater samples (influent and effluent) were collected from winter 2009 to summer 2010. Samples were labeled as "winter" if water temperature was at or below 21 °C, "spring" is temperature was between 22 to 25 °C and "summer" if it was at or above 26°C. Samplings were conducted upon availability and accessibility to the plant. All liquid samples were collected in previously baked amber glass containers, and transported in ice

to the laboratory. Samples were immediately filtered through Whatman GF-F glassfiber filters (pore size 0.7 μM) and extracted within 24 hours.

2.2.4: Sample extraction

Wastewater samples were extracted for EDCs using Stir Bar Sorptive Extraction with in-situ derivatization according to methods described previously [6-8]. Briefly, twenty milliliters of the water sample were transferred to a 20 mL screw cap vial. Into the vial, 200 mg of sodium carbonate and 200 μL of acetic acid anhydride were added as the pH adjustment agent (pH 11.5) and 200 μL of acetic acid anhydride were added as the derivatization reagent respectively. A pre-conditioned Stir Bar (three hours at 300 °C in a flow of nitrogen) was placed in each vial and the samples were stirred at 1000 rpm for four hours. After the extraction, the Stir Bar (Gerstel, Inc) was removed with forceps, rinsed with purified water, and dried with lint-free tissue paper. The Stir Bar was thermally desorbed in a Thermal Desorption (TDU) system that allowed EDCs to be released from the Stir Bar and subsequently analyzed by a GC–MS system (Agilent, Inc.).

2.2.5: Estrogenic Activity

Estrogenic activity of wastewater samples were determined using a yeast bioassay modified to measure wastewater samples directly without extraction, concentration, and sterilization as describe previously [9]. The yeast parent strain was co-transformed with a TRP1-marked constitutive human ERα expression plasmid (pG/ER) and a URA3-marked estrogen-inducible β-galactosidase reporter plasmid (pUCΔSS-ERE) and maintained in synthetic complete media lacking uracil and tryptophan (SC-UW) to select for plasmid retention. Briefly, yeast reporter strain was cultured overnight in SC-UW at 30 °C in a shaking water bath. After overnight culture the cells were diluted back to an optical density of 0.08 at 600 nm (O.D.600)

and incubated in a shaking water bath at 30 °C until the culture reached and O.D.600 of 0.1. The yeast culture in log phase growth was aliquoted into 15 ml centrifuge tubes at 1 ml per tube. The cells were then harvested by centrifugation and suspended in 1 ml of SC-UW prepared by mixing 750 µL of wastewater with 250 µL of 4× concentrated SC -UW for each assay to be performed. The cultures were then incubated at 30 °C with shaking for two hours. One hundred µL from each culture was then transferred to an opaque 96-well plate and 100 µl of Tropix Gal-Screen in Buffer B (Applied Biosystems, Foster City, CA) was added to each well. The plate was incubated for an additional two hours at room temperature. For all assays a 17β-estradiol standard curve was performed by diluting it into distilled, deionized water and treating it in the same manner as the wastewater samples. The hormone-induced chemiluminescent signal was then measured on a Luminoskan Ascent microplate luminometer (Thermo Fisher Scientific Inc., Waltham, MA).

2.2.6: Instrumental analysis — TDU–GC–MS

The Stir Bars were thermally desorbed in a thermal desorption unit (TDU) (Gerstel, US) under splitless mode. The desorption process was programmed as follows: initial temperature at 40 °C with a ramp of 60 °C min^{-1} to 300 °C (held for 7 min). The transfer line temperature was set at 300 °C. The desorbed EDCs were then cryo-focused in a baffle liner in a cryo-injection system (CIS4) at −40 °C under liquid nitrogen prior to injection. The CIS 4 instrument was programmed as follows: initial temperature at −40 °C, ramp at 12 °C s^{-1} to 300 °C and held for 10 min. The separations were performed on a Zebron ZB-5MS capillary column (0.25 mm×30 m×0.25 µm, Phenomenex, CA). The oven was programmed as follows: initial temperature set at 60 °C with 15 °C min^{-1} ramp to 300 °C and held for 5 min. The carrier gas, (ultra-pure helium), was set at a constant flow of 1.2 mL min^{-1}. The mass spectrometer was operated in the selected-

ion monitoring mode with electron-impact ionization (ionization voltage, 70 eV). Target compounds were measured based on the following quantification ions on selected ion monitoring mode: BPA: m/z=213, 228; E1 : m/z=270; E2: m/z=272; EE2: m/z=213, 296; NP: m/z=107, 135, 149; OCT: m/z= 135; NPEO1; m/z = 107, 179, 193; NPEO2; m/z = 223, 237, 251; BPA $^{13}C_{12}$: m/z=225, 240; p-n-NP $^{13}C_6$: m/z=113. Ten-point calibration curves were conducted ranging from 0.005 to 20 ng L^{-1}. The linear response of the curves produced correlation coefficients (R^2) higher than 0.99 for all EDCs.

2.2.7 Calculation of E2 Equivalents

The total estrogenic activity of a water sample was determined by the yeast assay. Based on the response, the activity of the unknown samples was interpolated from a dose–response curve of the standard compound E2 in mol L^{-1}, was converted into ng L^{-1} E2 equivalent (EEQ).

In addition, EEQ values could be determined by chemical analysis (expressed as $EEQ_{GC\text{-}MS}$). In addition, the estradiol equivalent factor (EEF) for each EDC(*i*), based on its half maximal effective concentration (EC_{50}) value obtained from yeast assay data, was calculated using the following equation:

$$EEF(i) = \frac{EC_{50}(E2)}{EC_{50}(i)} \quad (1)$$

Using EEF and the concentration of each EDC obtained by the GC-MS analysis, the EEQ $EEQ_{GC\text{-}MS}$ for each wastewater sample was calculated using the following equation:

$$\sum EEQ_{GC-MS} = \sum EEF(i) \times c(i) \quad (2)$$

Where, $EEQ_{GC\text{-}MS}$ is estradiol equivalents determined by GC-MS and c(i) is the concentration of EDC(i) determine by GC-MS. The sum of the calculated E2 equivalent values for the individual compounds represents the calculated overall estrogenecity of the sample.

The total estrogenic activity of a sample was determined by the yeast bioassay. A standard dose-response curve for E2 was conducted based on the four-parameter logistic equation (GraphPad prism version 5.00 Windows, Graphpad Software, San Diego California USA). Estradiol equivalents of water samples were calculated based on the following equation:

$$\log EEQ = \log EC_{50} - \frac{\log\left[\frac{(Top - Bottom)}{(y - Bottom)} - 1\right]}{HillSlope} \qquad (3)$$

Where Top and Bottom are the maximal and the basal responses respectively, EC_{50} of the agonist in the concentration that provokes a response half way between the basal (Bottom) response and the maximal (Top) response, and y is the activity response of the sample.

The EEQ obtained from the equation above has units of mol L^{-1}. To be consistent with the value obtained by chemical analysis, the value was then converted into ng L^{-1} by multiplying the molecular mass (in ng mol^{-1}) of the compound.

2.2.8 Statistical Analyses

All data treatments were checked for normality and equal variance. Data was log-transformed when necessary to follow normal distribution. Data was fixed by Kenward-Roger method and further analyzed by Tukey-Kramer Test. All differences were considered significant at $p < 0.05$. All statistical analyses were performed with the STATISTICA 8 package.

2.2.9 Calculation of removal rates

Since high analytical uncertainty can occur in the analysis of trace organic compounds the following system was applied to deal with low concentrations (< 10 ng L^{-1}). When concentrations were quantified below 10 ng L^{-1} in influent and effluent samples, removal rates were not calculated. When molecules were quantified in influent with concentrations higher than 10 ng L^{-1}, but not quantified in effluent, removal rates were calculated using the LOQ value for effluent. If a specific compound was not quantified in both influent and effluent, removal rate was also not calculated.

2.3 Results and Discussion

2.3.1: EDCs concentration in influent and effluent

Results from the analysis of EDCs in wastewater samples taken from the six WWTPs in El Paso, Texas and Ciudad Juarez, Mexico in different seasons are summarized in Table 4. Monitoring data for the years of 2009 and 2010 showed that NP, NPEO1, and NPEO2 had the highest concentrations in influent and effluents among the estrogenic compounds analyzed in this study. The concentrations of NP in influents of the four WWTPs in El Paso ranged from 727.0 to 64,457.6 ng L^{-1} and from non-detected (ND) to 14,985.3 ng L^{-1} in effluent water. Nonylphenol ethoxylates were also found at very high concentrations. NPEO1 and NPEO2 concentration in influent ranged from 801.3 to 51,175.2 ng L^{-1} and from 481.2 to 34,370.4 ng L^{-1} respectively. The high concentrations found in influent water are similar to those detected in other plants (Table 6) [10].The concentrations of these ethoxylates in effluent water ranged from ND to 8,144 ng L^{-1}. Levels of 4-t-Oct were lower than NP and ethoxylates with a maximum concentration of 6,836.9 ng L^{-1} in influents. NP concentrations in influents of WWTPs from Mexico were lower

than El Paso, ranging from 2,004.3 to 7,436.4 ng L^{-1} with a mean concentration of 4,482.9 ng L^{-1} (median of 2,964.6 ng L^{-1}). NPEO1 and NPEO2 also were found at higher concentration in influent from El Paso WWTPs as compared to the two plants in Mexico. The low concentration of NP and its ethoxylates in wastewater influent could be a reflection of low usage of domestic products, such as household cleaners and detergents, in developing countries like Mexico as compared to industrialized countries [1, 11, 12]. NP, NPEO1, and NPEO2 concentrations in effluent from WWTPs of Ciudad Juarez ranged from 899.2 to 3,169.1 ng L^{-1}, 1,896.0 to 3,169 ng L^{-1}, and from 865.0 to 6,462.0 respectively ng L^{-1}. The concentration of APEOS in the secondary effluent from Plant five ranged from below quantification levels to 4732.0 ng L^{-1}.

Table 4: Seasonal concentration of EDCs for each WWTP. Mean ± Standard deviation

	Seasons	N		BPA			Estrone			Estradiol			4-ort			NP			NPEO1			NPEO2		
Plant 1	Winter	4	Influent	305.3	±	63.0	64.9	±	24.7	106.5	±	119.2	966.0	±	1018.8	5946.2	±	3687.0	3888.2	±	5177.6	3858.9	±	2627.6
			Effluent	34.3	±	24.4	27.6	±	26.1	15.1	±	10.1	98.8	±	132.2	1051.4	±	1753.7	287.4	±	61.6	1190.9	±	1684.2
	Spring	3	Influent	319.6	±	132.2	50.2	±	11.2	20.6	±	15.8	1385.2	±	1384.6	5698.5	±	3689.1	10533.0	±	8549.2	6188.3	±	6691.9
			Effluent	20.7	±	18.7	5.8	±	4.0	6.8	±	1.7	75.8	±	2112.3	556.1	±	161.6	473.8	±	123.8	2373.7	±	2573.6
	Summer	5	Influent	175.2	±	126.0	33.1	±	9.9	8.1	±	5.2	2078.8	±	1336.4	3573.9	±	1501.2	4264.1	±	1914.2	2513.0	±	1425.8
			Effluent	11.1	±	9.2	11.3	±	8.8	5.7	±	6.6	20.8	±	12.0	388.5	±	475.5	331.3	±	322.0	568.5	±	1064.4
	Total		Influent	254.7	±	122.4	48.0	±	20.8	47.3	±	81.3	1534.5	±	1240.7	4895.8	±	2893.5	5871.3	±	5516.9	3882.4	±	3699.1
			Effluent	20.8	±	19.5	15.4	±	17.5	9.4	±	8.2	355.3	±	1054.3	651.4	±	1008.4	358.2	±	226.2	1050.4	±	1496.6
Plant 2	Winter	4	Influent	575.6	±	228.0	82.7	±	22.8	53.5	±	45.2	819.4	±	748.5	3557.5	±	2478.4	3991.1	±	3601.4	3190.6	±	1814.7
			Effluent	16.4	±	16.7	6.3	±	1.5	42.2	±	71.4	7.2	±	5.9	88.5	±	170.4	BQL	±		BQL	±	
	Spring	3	Influent	965.9	±	1094.2	85.3	±	61.6	7.1	±	3.6	3693.7	±	2610.2	9531.9	±	6393.5	18653.5	±	19593.6	14802.2	±	15851.0
			Effluent	214.4	±	288.2	5.9	±	6.4	4.1	±	3.7	15.7	±	12.0	458.3	±	368.7	430.3	±	745.3	1109.8	±	1922.1
	Summer	4	Influent	437.3	±	156.4	58.8	±	20.5	13.9	±	18.2	2706.1	±	1258.2	5309.3	±	949.3	7220.6	±	3312.3	2252.3	±	1462.9
			Effluent	29.0	±	47.3	6.9	±	4.0	2.5	±	2.9	18.8	±	29.9	71.6	±	107.6	157.9	±	83.5	754.0	±	1282.0
	Total		Influent	631.8	±	558.8	74.7	±	34.6	26.5	±	34.4	2289.4	±	1879.1	5823.9	±	4071.1	9681.7	±	11496.5	6298.8	±	9585.4
			Effluent	75.0	±	159.4	6.4	±	3.7	17.4	±	43.8	13.8	±	18.3	183.2	±	265.8	193.8	±	396.1	621.8	±	1254.8
Plant 3	Winter	3	Influent	331.7	±	53.0	63.8	±	14.8	41.9	±	26.6	1112.1	±	410.1	11218.6	±	9658.0	6710.6	±	3934.1	5224.8	±	2943.1
			Effluent	19.0	±	12.1	10.7	±	11.2	36.6	±	50.9	37.0	±	1.3	267.4	±	104.7	466.5	±	90.0	718.4	±	1016.0
	Spring	1	Influent	755.4			71.3			25.0			1030.3			66457.7			51175.2			11599.5		
			Effluent	84.1			13.8			11.7			21.4			743.6			1972.6			1038.9		
	Summer	2	Influent	414.6	±	54.1	41.4	±	18.8	ND	±	ND	3692.8	±	957.2	19181.1	±	6591.8	18084.1	±	13264.3	6329.8	±	4887.0
			Effluent	32.6	±	39.0	3.1	±	4.4	ND	±	ND	11.3	±	16.0	118.8	±	168.0	219.7	±	310.7	780.2	±	1103.4
	Total		Influent	429.9	±	169.6	57.6	±	18.0	25.1	±	26.5	1958.7	±	1433.8	23079.3	±	22645.6	20152.9	±	19517.6	6941.7	±	3901.4
			Effluent	34.4	±	31.7	8.7	±	8.6	20.2	±	37.1	23.6	±	15.2	303.2	±	275.6	669.0	±	756.6	807.2	±	761.7
Plant 4	Winter	1	Influent	380.0			92.5			14.4			515.7			2756.9			3112.3			2504.5		
			Effluent	203.5			76.1			6.6			257.5			973.5			738.3			2046.6		
	Spring	2	Influent	632.3	±	570.1	125.8	±	131.1	37.8	±	37.9	2958.4	±	2253.0	28175.0	±	14656.4	38287.6	±	698.0	21282.8	±	17235.3
			Effluent	25.5	±	25.8	53.6	±	36.9	12.2	±	17.2	301.8	±	110.6	7899.5	±	10020.8	3685.5	±	4757.8	7516.0	±	889.0
	Summer	1	Influent	229.4			66.3			27.5			2694.1			7981.5			3041.6			4961.6		
			Effluent	19.5			25.9			18.3			252.1			1046.4			1621.2			1699.1		
	Total		Influent	468.5	±	384.6	102.6	±	81.0	29.4	±	24.5	2281.7	±	1758.9	16772.1	±	15796.2	20682.2	±	20332.9	12507.9	±	14236.9
			Effluent	68.5	±	91.3	52.3	±	29.6	12.3	±	11.0	278.3	±	69.4	4454.7	±	7021.1	2432.6	±	3125.4	4694.4	±	3301.3

Mexico																							
Plant 6	**Spring**	2	Influent	594.1	±	16.8	124.9	±	16.8	15.7	±	22.2	259.3	±	81.1	2412.8	±	106.2	2000.6	±	64.3	2639.3	±	3238.2
			Effluent	570.3	±	39.2	89.4	±	39.2	12.1	±	17.2	383.2	±	34.6	2445.8	±	201.6	2105.6	±	282.0	2867.1	±	2830.5
	Summer	1	Influent	1476.8			179.6			33.9			690.1			9590.4			9257.5			7791.7		
			Effluent	685.6			65.2			16.2			416.1			4654.9			3904.8			3046.8		
	Total		Influent	888.3	±	513.7	143.1	±	33.8	21.7	±	18.9	402.9	±	255.2	4805.3	±	4144.6	4419.6	±	4190.0	4356.7	±	3754.0
			Effluent	608.7	±	82.8	81.4	±	31.1	13.5	±	12.4	394.2	±	30.9	3182.1	±	1283.4	2705.3	±	1057.7	2927.0	±	2004.2
Plant 5	**Spring**	3	Influent	729.4	±	53.8	76.3	±	53.8	25.5	±	0.3	383.5	±	161.8	4557.4	±	2730.8	4038.1	±	703.8	4142.6	±	2738.5
			Effluent	569.7	±	46.8	72.9	±	46.8	17.1	±	19.7	176.4	±	45.0	2219.0	±	1179.5	2228.3	±	373.3	4317.2	±	2565.7
				88.1	±	5.6	6.3	±	5.6	6.3	±	9.9	28.1	±	35.6	458.0	±	543.0	1114.1	±	589.0	3975.7	±	561.8
	Summer	1	Influent	645.5			124.4			ND			490.8			2965.0			2172.7			369.0		
			Effluent	535.7			76.8			ND			498.6			2626.5			2160.6			634.0		
			Effluent2	41.0			10.7			ND			131.1			656.0			1515.9			1473.2		
	Total		Influent	708.4	±	254.6	88.4	±	50.1	17.0	±	14.8	410.3	±	142.6	4159.3	±	2367.6	3571.7	±	1095.5	3199.2	±	2925.7
			Effluent	561.2	±	185.9	73.9	±	38.3	12.8	±	18.2	257.0	±	165.3	2320.9	±	984.4	2211.4	±	306.7	3396.4	±	2789.2
			Effluent2	76.3	±	82.3	7.4	±	5.1	4.7	±	8.7	53.8	±	59.2	507.5	±	454.3	1214.5	±	521.2	3141.5	±	1498.5

The concentrations of APEOs and its parent found in effluents from Plant four were two to 800 times higher compared to the other plants studied in El Paso. These finding should be of concern for El Paso community since millions of gallons of effluent are discharged to Rio Bosque Wetlands Park via a channel into a series of large shallow wetland cells. These cells are dry during most of the year, but during winter time they are refilled with effluent water and serve as a refuge for thousands of migratory birds and several species of amphibians during that season. EDCs in this water could affect the wildlife in this area. However, studies need to be performed in the wetland to determine the concentration of EDCs in water and sediments, and the possible impact to the ecosystem. Overall, the concentrations of APEOS in effluents were statistically higher in WWTPs from Mexico than in El Paso. The levels of APEOs detected in secondary effluents from Plant 6 were statically higher than El Paso with the exception of NP. However, there is a decrease in NP concentration (53%) in effluents from the secondary treatment employed in plant 5 in comparison to the effluents from the advance primary treatment. Plant 4 and WWTPs in Ciudad Juarez, Mexico have similar concentration of NP and ethoxylates to those found in China and Santa Maria Nativitas, Mexico [1, 12].

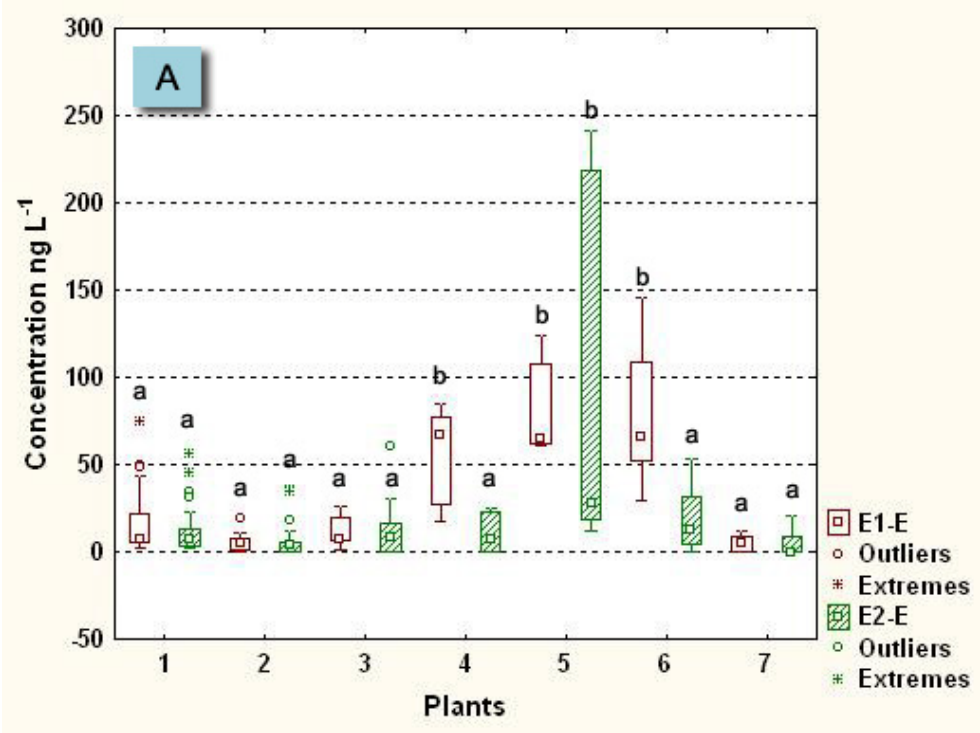
Table 5: Concentration of EDCs in WWTPs from different studies. Concentration in ng L^{-1}

	NP		NPEO1		NPEO2		OP		BPA		E1		E2	
	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent	Influent	Effluent
USA														
Massachussetts	25000–3300	160	15000–21000	5000	6400–8000	800	200-740		94-150	20-55				
California												< 12.0		< 4.5
Kansas	20000	8000			13500	6500								
Mid Atlantic	34780-37000	380-1148				921-1040	45.8-263							
Mexico	3200–13020	450–4890	ND–2630	1200–1700	18910–40310	810–4090								
Europe														
Spain	57634	650	42700	960	39690	380								
Belgium		0.18										< 0.6	23	< 0.2
France		0.99										4	54.6	3.3
Netherlands		0.52										< 1.8	71	3.4
Germany												< 0.9	48	2.7
Greece	230	180	5760	890	3990	1840	730	150						
Australia 1	3070	335					229	23.5	140	86.7	13.1	41.9	16	1.6
Australia 2							19-66				54	4.1	22	0.95
Australia 3		938-2114		47-583		84-2373		11.5-48		12-148		13.3-37.6		1-4.2
	24,791.60													
China		4,292.60							421.5	39.8	38.6	12.6	21.4	4.4

the concentration on NP in those plants ranged from 860 to 4,296 ng L^{-1} for NP, 47.0 to 3980 ng L^{-1} for NPOE1, and 84 to 4,009 ng L^{-1} for NPEO2. The concentrations of APEOs in effluent from WWTPs in Texas were comparable with quantities detected in Europe, Australia, Greece, and United states (mid-Atlantic and mid-west), where concentration ranged between 200 to 1,200 ng L^{-1} [11, 13-15].

BPA concentration in influent from WWTPs in El Paso and Mexico were very similar with the exception of plant 1 where concentrations were lower with concentrations as low as 88 ng L^{-1}. This low concentration of BPA in influent could be related to the sources of Plant 1 which are primarily composed of residential waste in comparison with the others plants where substantial amount of industrial waste is treated. The concentration of BPA in effluent from El Paso was 48.9 ng L^{-1} (median of 19.5 ng L^{-1}) and in Mexico 581.6 ng L^{-1} (median of of 550.5 ng L^{-1}). The level of BPA in the secondary effluent from plantfive was 82.3 ng L^{-1} (median of 37.6 ng L^{-1}). BPA concentrations in primary effluent from Plant five and effluent from plant six were one to 500 times higher than any amount found in other studies, where concentrations ranged between 10 to 190 ng L^{-1}[12, 15-17]. In comparison to plants in El Paso, concentrations of BPA in effluent from WWTPs of Mexico were statically different from about 300 to 3000% higher. The concentrations of BPA in effluent from WWTPs in Texas were comparable with quantities detected in Europe, Australia, Greece, and United states (mid-Atlantic and mid-west), where < 112 ng L^{-1} [11, 13-15]. The concentrations of BPA in all the WWTPs in study are considerate low to cause an adverse effect to aquatic environment according to several studies [18, 19].

As for the estrogens, the synthetic estrogen EE2 was not detected in influent and effluent of all WWTPs in this study. Similar results have been seen in others studies where EE2 was either not detected or the levels were below quantification limits [16, 20-22]. Low variation of natural estrogen levels was observed in influent samples between WWTPs, especially E2. Mean values of E2 in WWTPs influent was 35.8 ng L^{-1} in the US and 19.8 ng L^{-1} in Mexico. E2 concentrations in effluent from El Paso were 15.6 ng L^{-1} and 13.1 ng L^{-1} in effluents from WWTPs in Mexico. On the other hand, E1 average concentrations in influent and effluent from El Paso WWTPs were 65.25 ng L^{-1} and 15.6 ng L^{-1} respectively. Influent and effluent samples of Ciudad Juarez WWTPs revealed a mean concentration of 111.8 ng L^{-1} and 77.1 ng L^{-1} respectively. Estrogen values in effluents were consistent with those reported in the literature (Table 5) where concentration varied between BQL to 39.0 ng L^{-1} for E1 and between BQL to 8.0 ng L^{-1} for E2 [12, 14, 17, 20, 23]. E2 concentrations in effluents Plant 5 (in Ciudad Juarez) were 50 % to 200% higher than El Paso WWTPs. Estrone in Mexico plants was 7 to 13 times higher than plants one,two and three and higher than reported E2 values in others countries and states from the USA [12, 14, 20, 24]. No differences in E2 concentration were found between WWTPs in Mexico and Plant 4. The concentration of E2 in effluent from plant four and Mexico plant are within the levels to cause potential effect to the aquatic biota according to studies performed by Panter et al. However, further analysis is needed in the surface water in the studied area since when water is discharged into the river compound concentration is diluted to lower levels.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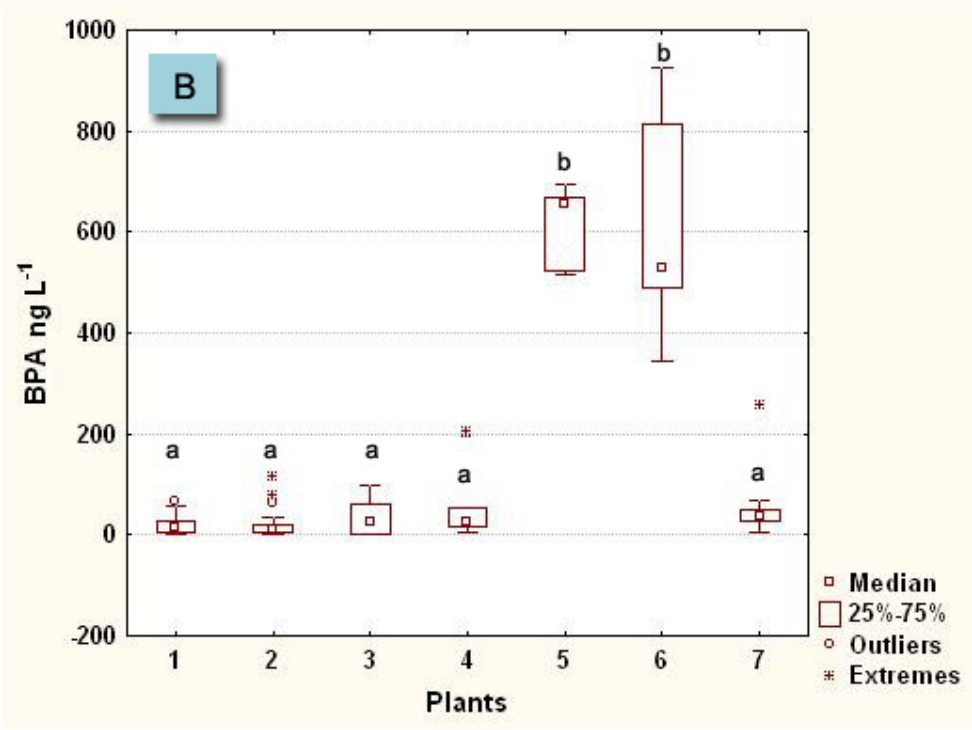
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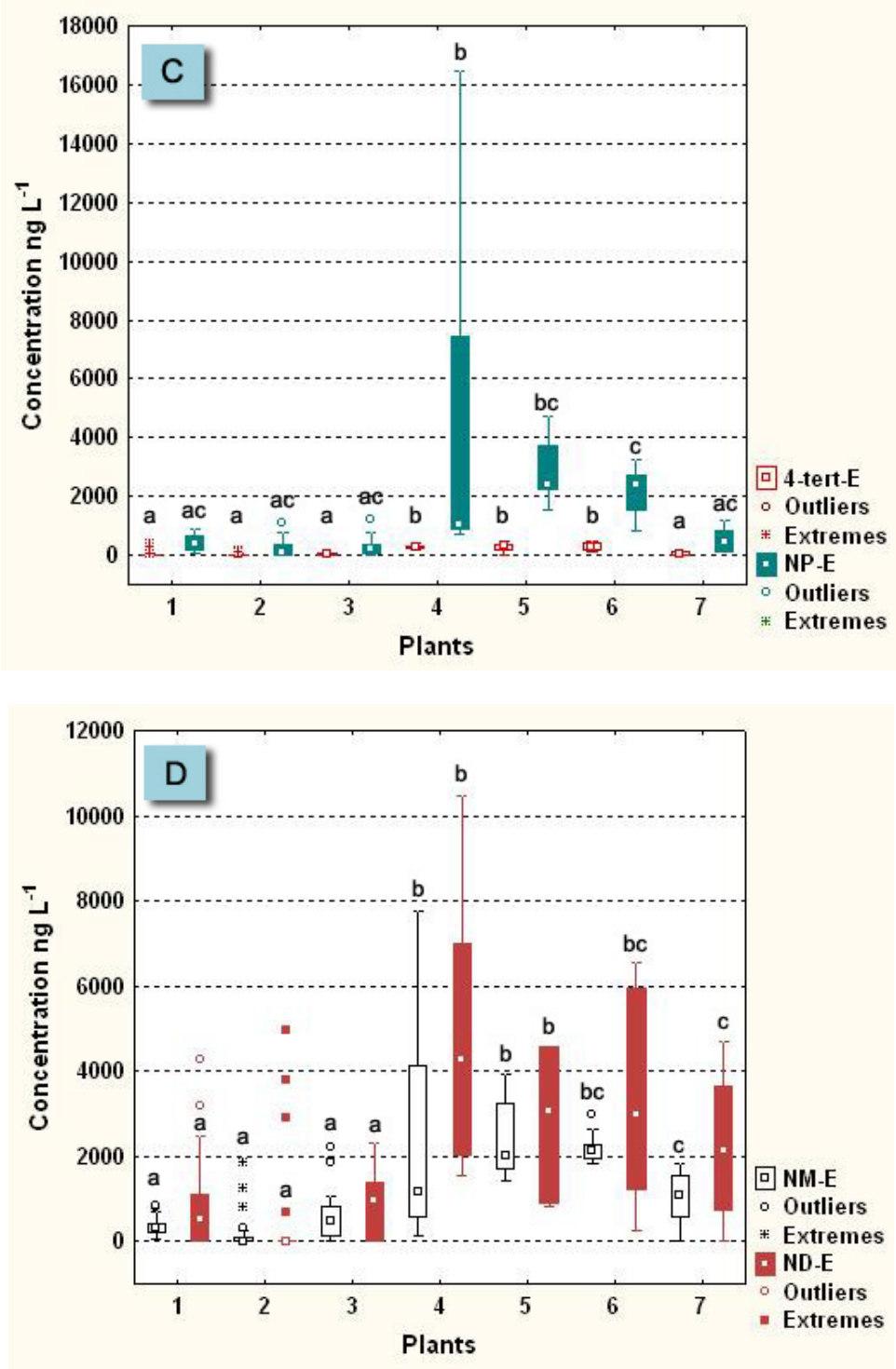
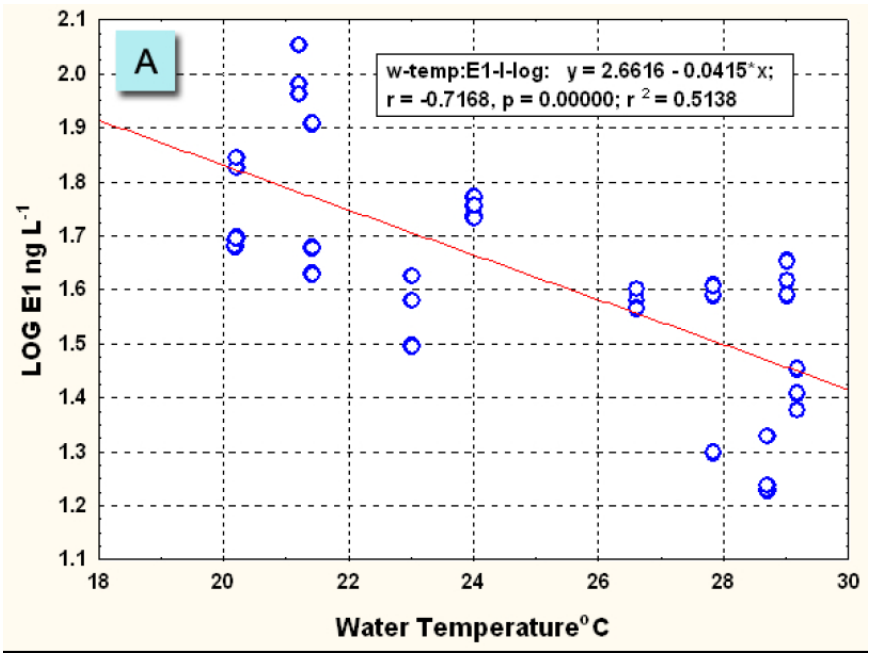


Figure 1: Box plot showing EDCs concentrations in effluents from WWTPs in El Paso, TX, and Ciudad Juarez, MX. Plant 7 = secondary effluent from Plant 5 (activated sludge treatment). Different letters denote significant differences between groups (p, < 0.05, Tukey-Kramer test). (A) E1 and E2, (B) Bisphenol A, (C) 4-tert-Oct and NP, (D) NPEO1(NM) and NPEO2 (ND).

2.3.2: Seasonal Variation of EDCs concentration in Influent and Effluent

To understand seasonal distribution levels of estrogenic compounds in WWTPs, sewage samples from January 2009 to June 2010 were analyzed. The results are shown in Table 4. As mentioned in the methodology the samples were labeled as "winter" if water temperature was at or below 21 °C, "spring" is temperature was between 22 to 25 °C and "summer" if it was at or above 26°C. El Paso-Ciudad Juarez ambient temperatures during winter and spring seasons can fluctuate vastly during weeks from very cold temperature (~2°C) to warmer temperature (~26.6 °C). Since the concentration of EDCs in wastewater samples has been related to temperature, the labeling of seasons was therefore determined in such manner to address the oscillation of temperature due to the desert climate of the area in study.

As seen in Table 4 , high variation was observed in EDCs concentrations in each season, regardless of the month or year of sample collection. EDCs concentrations in influent and effluent were not significantly different (P 0.05) among seasons. However, concentrations of EDCs in water with temperatures higher than 25°C were significantly different from the respective concentrations in water at temperatures below 25 °C. Concentrations were correlated to temperature and some patterns were observed for some of the plants. Significant correlation (Pearson correlation, $P < 0.05$) was found between temperature and ECDs levels, nevertheless, the correlations coefficients were low (Figure 16). Levels of natural estrogens in influent of Plant one to Plant four decreased at high temperatures (r^2= 0.22-0.57). It is therefore hypothesized that some degradation of E2 and E1 has occurred during wastewater treatment process. Temperature is one of the main factors in controlling microbial activity in wastewater.



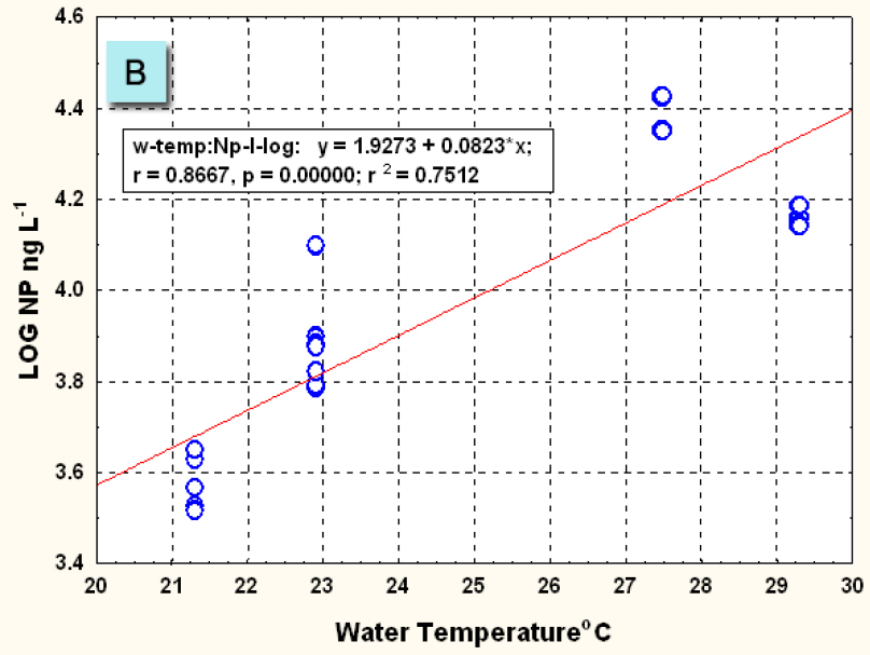


Figure 16: Concentration of EDCs from two WWTPs as a function of water temperature. (A) Plant 1 correlation for estrone, (B) Plant 4 correlation for nonylphenol. Significant correlation (Pearson Correlation P< 0.05)

Warmer temperature can accelerate the degradation and transformation rate of organic compound in the water, and low temperature can inhibit or slowdown this process. A similar trend was observed in a wastewater treatment plant from China [3]. On the other hand, no significant correlation was found in this study for effluents in comparison with temperature with the exception of Plant one where the concentration of E2 was lower at higher temperature.

In the case of NPs and APEOs, the concentrations of OCT and NP increased at higher temperature in several of the plants analyzed. APEOs rapidly degraded into NP and shortened alkylphenol ethoxylates at high temperature, whereas the degradation rate became very slow at low temperatures [12]. Significant correlations were found in influent for all the plants (except Plant five), specially for OCT (r^2 =0.31 -0.62). However, only plant 6 shows some positive correlation in the secondary effluent. These results are comparable with others studies which reported that APEOs degraded into shortened alkyphenols ethoxylates at higher temperature [11, 12].

Bisphenol A concentration varied among seasons and plants. Plant 5 revealed high concentration in warmer temperature in the influent as well as in the effluent. In plant 3 and 4 the concentration of BPA decreased as the temperature increase. Little variation in BPA was observed in plant 1 and 2, regarding the change in temperature. Those small variations in BPA concentration in plant 1 and 2 are consistent with the results found by Yang el al. in a municipal treatment plant in China. Further studies are needed to understand the behavior of BPA in relation to change in temperature.

These small variations of EDCs concentrations during different seasons and weak correlation with temperature might be related to the climate of the study area. WWTPs in this study sustained warmer water temperatures in comparison to others studies where temperatures were recorded as low as 9 ºC. Water temperature in most of the plants studied ranged from 20 to 29 ºC. Plant 2, which is located north El Paso, was the only one to record temperature as low as 14 ºC for the effluent water, but influent temperatures were higher than 20 ºC. The consistency of warmer water temperature in the WWTPs could have maintained similar microbial activity during the research period. To our knowledge, however, no researches have been performed in similar semiarid areas and it was not possible to compare these results to determine any resemblances.

2.3.3: Estrogenic Activity in WWTPs Influent and Effluent

Estrogenic activities measured by the yeast assay in influent and effluent water from wastewater treatment plants are shown in Table 6. Measured estrogenic activities in influent samples were remarkably similar for every plant with minimum and maximum levels of 10.7 ng L^{-1} EEQs and 58.2 ng L^{-1} EEQs respectively. The lower estrogenic activities were found in plant 1 and the higher activities in plant 5. Estrogenic activity in effluent water also revealed the same trends as the influent samples having very similar values among the WWTPs ranging from ND to 16.0 ng L^{-1} EEQs (median=6.60 ng L^{-1} EEQs), with the exception of Plant 4, Plant 5, and Plant 6 (primary effluent) where higher estrogenicities were measured (6.8 to 48.0 ng L^{-1} EEQs). Estrogenic activities in effluent samples were in the same ranges as the values obtained in other studies. Authors reported level ranging from 0.7 to ng L^{-1} EEQs 7.8 (E-Screen) in effluents of 16 WWTPs in Germany [4], 0.6 to 5.2 ng L^{-1} EEQs in Japan (yeast assay) [25], 0.1 to 5.5 ng L^{-1} EEQs (Yeast estrogen screen) in two WWTPs from France [26], and from $\leq$1 to 15 ng L^{-1} EEQs

(Yeast estrogen screen) in effluents from New York and Texas treatment facilities [27]. E2 equivalents in Plant 4 to Plant 6 were similar to levels detected in China (29.0 to 41.1 ng L^{-1} EEQs).

To evaluate the contribution of individual EDCs in the study to overall estrogenic activity of wastewater, the measured estrogenicity by the yeast assay was compared to the estrogenic activities calculated based on chemical analysis. Concentrations obtained for each particular EDC in the chemical analysis was multiplied by its EEF (Equation 1), resulting in an EEQ for the particular substance. Despite their relatively high concentrations in wastewater, the contribution of BPA, OCT, NPEO1 and NPEO2 to the total estrogenicity in influent and effluent were minor (< 2%). Same results have been reported in other studies. As expected the contribution of natural estrogen to the total estrogenicity of the samples are high, contributing for 18.9 to 21.6 % of the total estrogenic activity. Estradiol, E2, was the most active estrogen accounting in some samples for 99% of the total estrogenic activity. However, unlike other studies, NP was the compound contributing for most of the estrogenicity in wastewater samples analyzed. Typically, NP contribute for less than 5 % of total activity even when concentrations detected are higher, because of it low estrogenic potency compared to natural estrogens [4, 12, 28]. NP accounted for an average of 45.9 to 63.8 % of the activity in the six plants studied. The reason for this dissimilarity compared to other studies is related, in fact, to the low EC_{50} (2.48×10^{-8} M) of NP obtained in our bioassay. EC_{50} reported in literature are 4 orders of magnitude higher in comparison to the EC_{50} of E2 [12, 29, 30]. The bioassay used in this study is more sensitive concerning NP when compared to other published data. That sensitivity might be related to the deletion of PDR5 “drug pump” gene from the W303α genetic background to avoid

possible transport of estrogen-like ligands from the yeast cells. Ligand retention within the yeast cells allows for a more sensitive bioassay[31].

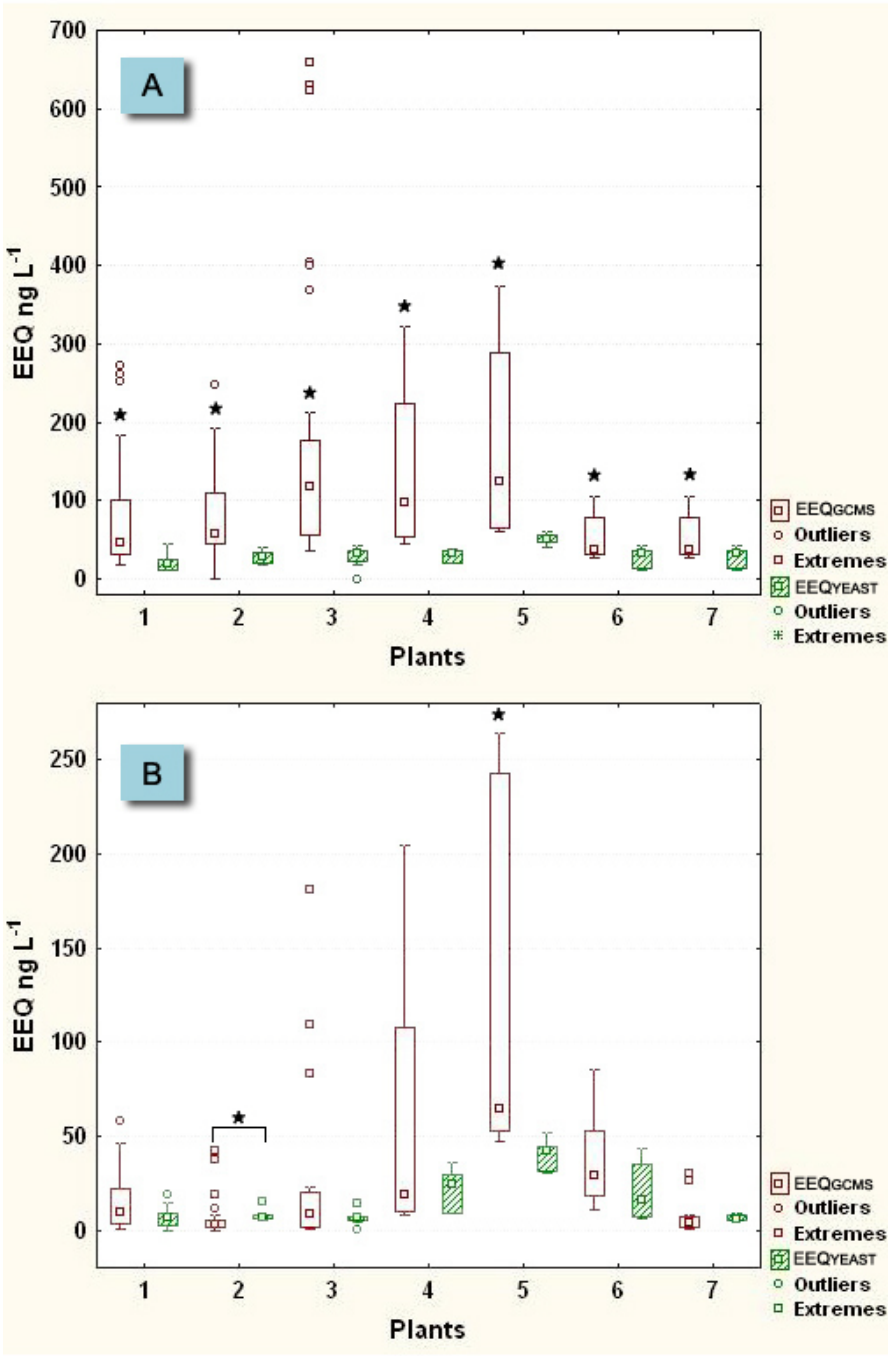


Figure 17: Estrogenic Activity obtained by Chemical Analysis and measured analysis. (A) Influent (B) Effluent. *Significant differences in EEQ between EEQ_{yeast} and $EEQ_{GC\text{-}MS}$, p , 0.05 (Tukey-Kramer Test).

The measured estrogenicities by bioassay (EEQ_{yeast}) for the wastewater samples from different WWTPs were compared with the results ($EEQ_{GC–MS}$) calculated by EEF and concentrations determined by chemical analysis of the same samples (Equation 2). The EEQ_{yeast} measured in the samples ranged from no activity (NA) to 58.6 ng L^{-1} while the calculated estrogenic activity ranged from 0.6 to 317.3 ng L^{-1}. In general, the calculated EEQ concentrations were higher than those measured in the assay, by between 1–fold and 10-fold in 61 % of the influents samples tested (n=40), and by between 1- and 7-fold in 30% of the effluents. Significant difference was mainly found in the influent samples (Figure 3). Aerni et al. [4] found in five WWTPs in Europe that the calculated EEQ concentrations tended to overestimate the EEQ concentrations in the recombinant yeast assay. Similarly, Thorpe et al. [32] found deviations in the measured responses in rYES versus the calculated estrogenic potency, with the calculated EEQ concentrations been higher by 2 to 24-fold in 43 effluents samples. Differences were also reported in other studies in which chemical analysis predicted higher estrogenic activity than what was measured in the bioassay [15, 19]. Although, the calculated EEQ concentrations were generally higher than those measured in the yeast assay, the opposite was observed in effluent samples. Though the differences in EEQ between the two analyses were not significant for effluent samples, in 57% of the samples the EEQ levels to some extent were higher than those obtained in the chemical analysis. Tanaka et al. [33] found in their investigation that in a number of the WWTPs studies, the estrogenic activity was higher in the rYES than predicted based on the measured chemical concentrations. Pawlowski et al. [34] also reported a lower calculated estrogenic activity, compared with the measured estrogenicity for two WWTPs effluents in Germany. The mixed results once again showed that the environmental sample matrices are complicated. Problems in making this approach by comparing EEQ_{yeast}

versus $EEQ_{GC–MS}$ arises as a consequence of an incomplete knowledge of the chemical composition of the WWTPS influent and effluent and constraints caused by uncertainties in the accuracy of the data. Chemical analysis is only focused on the determination of target substances in wastewater. The result is limited in providing a complete account of all EDCs existing in wastewater. The biological response of the yeast assay is complex and includes all estrogen-like compounds capable of binding to the receptor. This could lead to synergism, potentiation, and/or inhibition of the response, depending upon the quantities and combination of compounds present. It has been reported that antagonist in wastewater samples can inhibit the estrogen response leading to a reduction and underestimation of the estrogenic activity. This effect was proved by indirectly measured antiestrogenic compounds by an effluent-volume-dependent suppression of the β-galactosidase activity induced by E2 [35]. The results revealed that antiestrogens were responsible for a 50 % reduction in estrogen-induced activity in WWTP effluents. Similar results were obtained by Otakuye et al. [36] when a depression of the EE2- dependent curve occurred after treating the cells with a mixture of EE2 standards and concentrated from effluent samples. In this study, however, no significant differences were found in several of the effluent samples which is could be an indication of a substantial removal of antagonist compounds during water treatment. Otakuye et al. [36] also found that when EE2 gene controls were supplemented with some effluent extracts the response was amplified, indicating vast removal of antiestrogen for those specific samples.

2.3.4: Seasonal Variation of Estrogenic Activity in Influent and Effluent

Estrogenic activity variations during seasons were studied statistically in the same manner as EDCs concentration in water samples. Table 6 illustrates the average EEQ in influent and effluent for each plant at different seasons. Weak but significant correlations were observed by comparing EEQ_{yeast} versus water temperature. However, the correlation results were WWTP specific and some of the plants did not show any relationship to temperature ($r^2 < 0.33$). Plant 1 has a negative significant correlation for both EEQ_{GCMS} ($r^2 = 0.30 - 0.33$) and EEQ_{yeast} ($r^2 = 0.48$-0.68) with the highest correlation obtained for estrogenic activity in influent water (Figure 4).

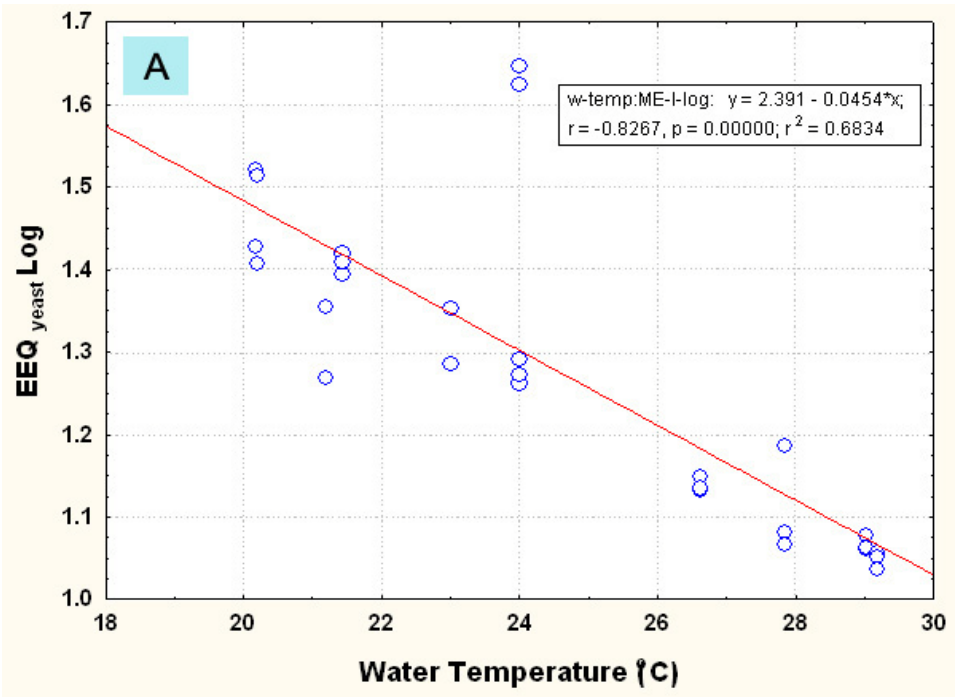
Table 6: EDCs concentrations from WWTPs for every season. The data is presented as average ±standard deviation.

Measured EEQ ng L^{-1} (yeast)																			
		Plant 1			**Plant 2**			**Plant 3**			**Plant 4**			**Plant 5**			**Plant 6**		
Winter	Influent	26.6	±	5.7	31.2	±	9.4	27.5	±	12.1	35.9			N/A			N/A		
	Effluent	8.7	±	4.1	7.9	±	4.7	6.7	±	10.1	29.4			N/A			N/A		
	S.effluent	N/A			N/A			N/A			N/A			N/A			N/A		
Spring	Influent	30.1	±	11.4	32.2	±	4.8	36.1	±		33.8	±	0.9	42.7	±		25.2	±	13.7
	Effluent	11.9	±	3.9	6.8	±	0.8	5.9	±		27.7	±	0.9	48.9	±		23.6	±	14.1
	S.effluent	N/A			N/A			N/A			N/A			N/A			4.5	±	2.5
summer	Influent	12.2	±	1.1	25.4	±	4.5	19.3	±	4.7	21.3			40.7	±	7.7	30.0		
	Effluent	3.6	±	4.4	5.8	±	0.6	6.0	±	0.1	10.4			33.8	±	20.1	24.7		
	S.effluent	N/A			N/A			N/A			N/A			N/A			6.4		
Calculated EEQ ng L^{-1} (GC-MS)																			
		Plant 1			**Plant 2**			**Plant 3**			**Plant 4**			**Plant 5**			**Plant 6**		
Winter	Influent	147.3	±	97.2	82.7	±	56.5	178.8	±	16.8	44.9			N/A			N/A		
	Effluent	27.0	±	17.8	11.5	±	11.5	18.0	±	28.9	22.4			N/A			N/A		
	S.effluent	N/A			N/A			N/A			N/A			N/A			N/A		
Spring	Influent	73.7	±	35.5	106.5	±	85.4	513.6	±		227.9	±	119.1	63.4			60.1	±	36.1
	Effluent	10.6	±	6.2	1.7	±	2.2	9.9	±		103.4	±	133.5	55.2			42.5	±	30.6
	S.effluent	N/A			N/A			N/A			N/A			N/A			2.7	±	2.8
summer	Influent	38.1	±	16.7	66.8	±	42.5	148.0	±	57.5	63.8			199.9	±	166.0	46.0		
	Effluent	4.8	±	4.0	2.6	±	0.6	1.7	±	1.1	11.4			237.0	±	149.5	31.7		
	S.effluent	N/A			N/A			N/A			N/A			N/A			13.7		

S.Effluent : Secondary effluent from plant 5

Similar results were observed for in the rest of the plants, where the highest correlation was obtained for measured estrogenicity in influent water. However, the correlations were weaker ranging from 0.33 to 0.50. Plant 1, 2, and 4 were the only facilities to express a significant correlation for estrogenic activity in effluents (r^2 = 0.33 -0.48). Correlation of calculated estrogenicity versus water temperature was extremely inconsistent, following similar patterns obtained in the chemical analysis, which is varied amongst the WWTPs studied.

A decrease in estrogenic activity denotes that an increment in temperature resulted in faster chemical degradation or an enhancement in microbial activity. However, it is noteworthy to emphasize that the yeast assay measured the overall activity in a sample and the reduction in estrogenecity does not only account for the compounds in study, but to any ligand capable to bind the estrogen receptor. Our results are comparable with the study performed by Hemming at al. where plasma vitellogenin in male fish increased during the months of March, and December and decreased during August, and June. Nonetheless, the opposite results have been observed in effluent waters [37]. Fernandez et al. [37] found that measured EEQs by recombinant strain (RYA) were higher during summer, as consequence of more free estrogens forming by deconjugation of estrogens-glucuronide or sulfate groups at high temperature. Once again, small variation in estrogenic activity as well as EDCs concentration in different seasons might be due to the constantly warmer water temperature in El Paso-Mexico arid area.



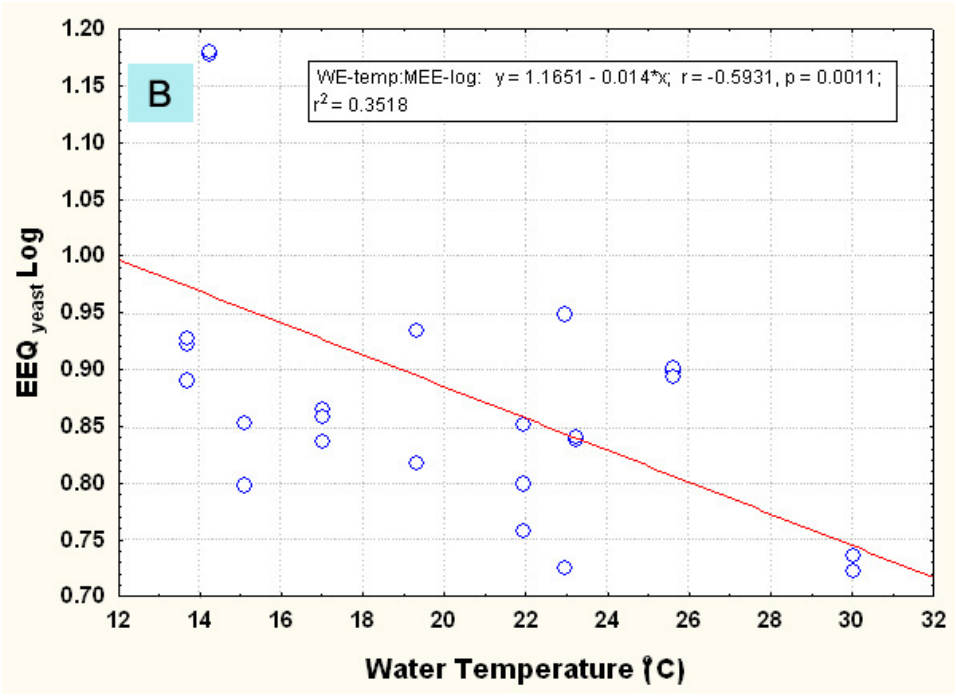


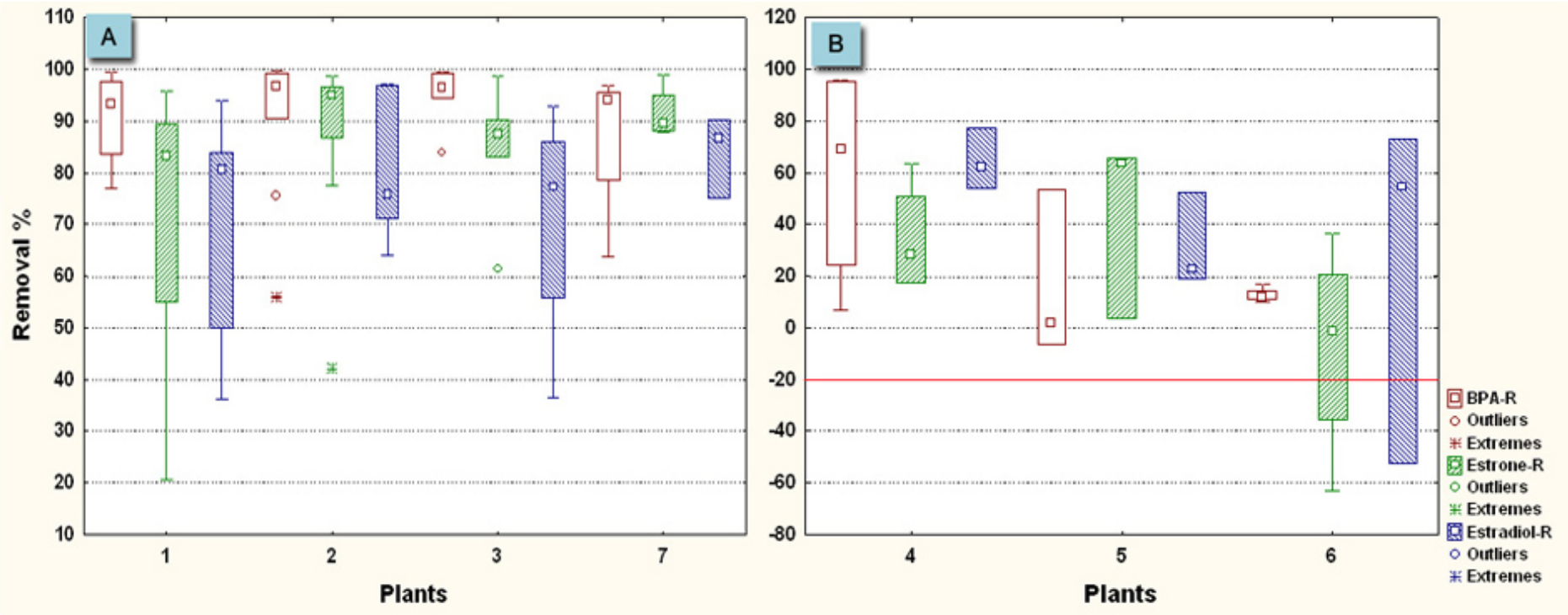
Figure 18: Estrogenic activity from two WWTPs as a function of water temperature. A: Plant 1, B: Plant 2. Significant correlation (Pearson Correlation P<0.05)

2.3.5: EDCs and Estrogenic Activity Removal

It was observed that the removal of EDCs from wastewater were higher in the WWTPs located in El Paso, Texas in comparison to the plants in Mexico (Figure 20). Removal of EDCs in WWTPs from El Paso ranged between 31 to 98 % and in Mexico from "no removal" to 86%. Plant 2 which is the facility comprising a tertiary treatment, was the plant with the highest removal (> 82%) for the overall sampling period, followed by plant 3 (secondary treatment). Plant 1 also provided satisfactory removals, however, at a lower rate than plant 2 and 3. The lower removal rate might be related to the elimination of the primary treatment in this particular WWTP where raw water is directly transferred to the aeration tank without removing sludge from the incoming water. Primary treatment (PT) can remove high amount of organic solid from the water in which EDCs are eliminated since they can be solid-bound into the sludge. Without primary treatment the elimination of EDCs in the water might depend vastly on the sludge retention time in the activated sludge system to allow bacteria to degrade organic compounds. However, PT account for only 10 to 30 % of EDCs elimination and in some cases an increase in the concentration of a compound like E2 has been observed [5]. Plant 4, surprisingly, had very low removal (< 77%) even when the wastewater treatment system in that facility is similar to plant 2. This is of great concern since 30 to 70% of the compounds are still reaching the aquatic ecosystem, and as aforementioned plant 4 discharge water directly to a wetland that create artificial ponds that many animals adopt as home during winter season. High concentration of E2 (27.4 -79.6 ng L^{-1}), NP (813.7 -14985.3 ng L^{-1}), and 4-tert (223 -379 ng L-^{1}) was detected in effluent samples from this plant. Concentrations of E2, NP, and 4-tert were high enough to potentially cause adverse effect to reproduction of fish and amphibians, although, NP concentrations were below the EPA quality criteria standard for freshwater species [38].

However, further analysis is needed in this plant since efficient removals (> 90%) were obtained for BPA, OCT, and NP for some sampling dates, and the quantity of samples collected might not represent the overall capability of the plant.

The removal of EDCs from WWTPs is Mexico was insignificant with a highest percentage removal of 44% (estrone) during the sample period. The highest removal for a specific sampling was 72% (estradiol) obtained in plant 6 (primary effluent). For both plants in Mexico negative removals were obtained for BPA, NPEO2, OCT, E1 and E2. These increase of EDCs in final effluent can be linked to analytical uncertainty in trace analysis, or explained by desorption of molecules from sludge and suspended particulate matter [5]. Also, molecules such as E1 can be produced during the treatment process by the degradation (oxidation) of E2 and the opposite can occur by the reduction of E1 to E2 [4]. This inefficient removal by Ciudad Juarez WWTPs is the result of the physical-chemical treatment as the only the procedure applied to the wastewater which focuses in removing sludge, grease, and solid. The removal of EDCs will depend of the attachment of the molecules to the colloids. Concentration of EDCs in effluent water from WWTPs in Mexico reaches the aquatic ecosystem at the $\mu g\ L^{-1}$ (ppb) levels. Even though effluent are discharged into the irrigation canal the water can reach the Rio Grande River via agricultural runoff, and return flows conducted to the river, about 150 km downstream near FortQuitman,Texas.


A
B
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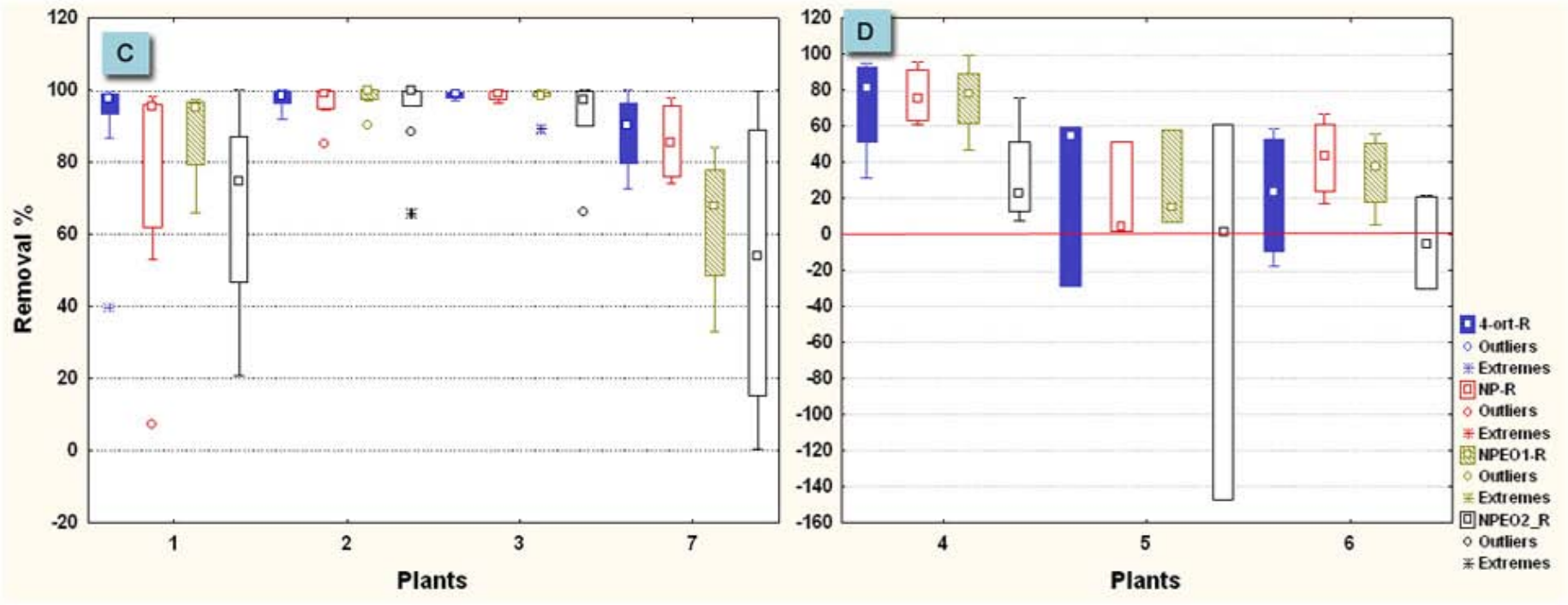

Figure 19: Removal rate (%) of EDCs for 6 WWTPs studied. (A-B) : Removal of BPA, E2 and E1. (C-D): Removal of 4-tert-oct, NP, NPEO1 and NPEO2. Plant 7: Secondary effluents from plant 5.

In contrast, the removals obtained in secondary effluent from plant 6 are similar to WWTPS in El Paso, with the exception of NPEO1 and NPEO2 that were eliminated in lower proportions. Once again, this is a reaffirmation of how important is the application of activated sludge treatment is for the elimination of organic compounds in wastewater.

The removal of estrogenic activity in the water is lower in comparison to the chemical analysis (Figure 20). The higher removal rate was 74% of the overall estrogenicity in the water. However, the results are in agreement with the chemical analysis. WWTP 1, 2, and 3 represent the facilities with the higher efficiency in El Paso. Plant 4 removed only 30% of the total estrogenic activity in wastewater. Secondary treatment from plant 6 (Mexico) presented a mean removal of 74% of estrogenic activity in wastewater. Primary treatment of plant 6 and plant 5 eliminated less than 25% of the overall estrogenicity in wastewater. These results from the wastewater treatment in Mexico are alarming, because the estrogenic activity not only represent the compounds in study, but other compounds capable of cause adverse effect to the endocrine system are potentially reaching the water source as well. However, the low removal of estrogenic activity in comparison to the chemical analysis could be a possible underestimation of the real efficiency of each WWTP. The inhibition of estrogenic activity by antagonist in the influent samples might be occurring; therefore, the low removal is a result of the underestimation of estrogenicity in the input water.

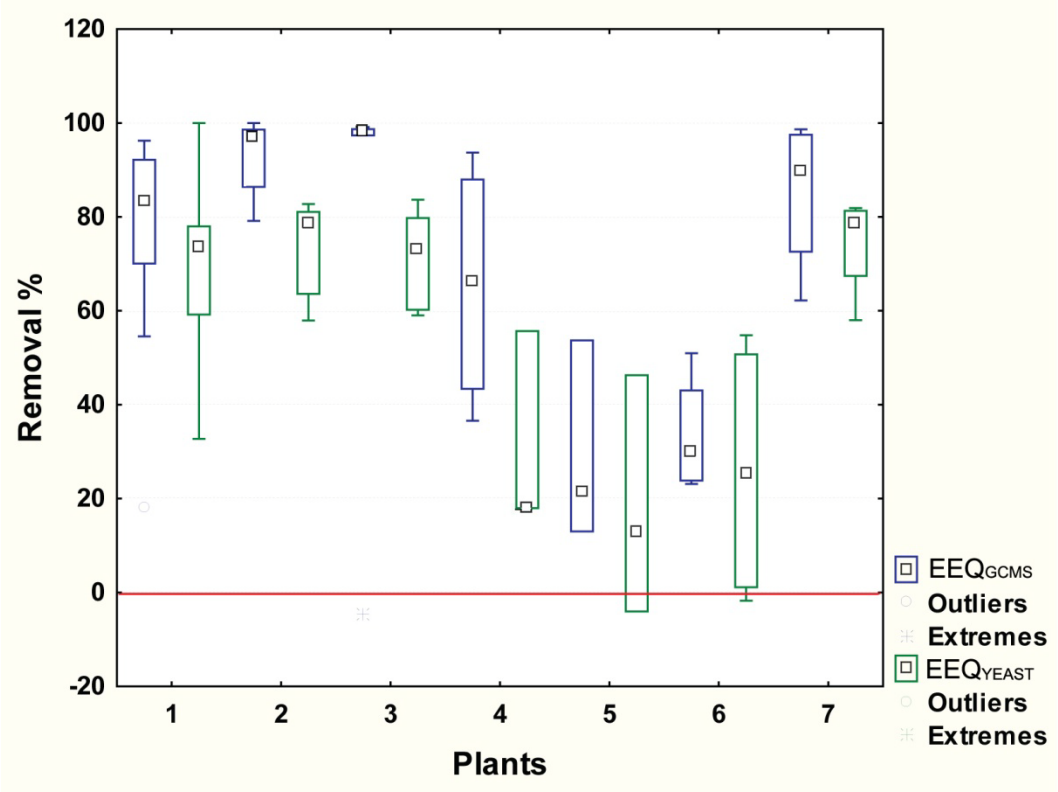

Figure 20. Removal rate (%) of estrogenic activity for 6 WWTPs studied. Plant 7: Secondary effluents from plant 5.

2.4. Conclusion

In the present study, we have assessed the occurrence and removal of EDCs and estrogenic activity in 4 WWTPs in El Paso, and 2 plants in Mexico. The seasonal variations of EDCs concentration and estrogenicity in the influent and effluent were assessed by chemical analysis and chemiluminiscense yeast assay. Little variation was found between seasons and weak correlations were obtained when comparing concentration, and estrogenic activity versus water temperature. Probably, as a result of the warmer temperature in the area of study, the bacterial population has maintained similar activity during the investigation. The concentrations

of EDCs and estrogenic activity in effluent were higher in WWTPs from Ciudad Juarez, Mexico. Moreover, the removal of EDCs and estrogenicity from wastewater treated was ineffective in comparison to the process implemented in the WWTPs in El Paso. Primarily, because WWTPs plants in Mexico do not apply any secondary treatment (e.g. activated sludge) to the wastewater, and most of the process is centered to eliminate solid from wastewater Plant 6 encompassed a biological treatment, but only 4 % of 57MGD is treated by this system. It is of great urgency that WWTPs in Mexico expand their facilities and upgrade their system to ensure that 100% of the wastewater can be treated by secondary treatment. From this study we can concluded that the current effluents from WWTPs in Mexico are not adequate for river discharge. Low removal and high concentration of EDCs were detected in effluents from Plant 4 located in El Paso, Texas. The reasons are unknown since the plant includes same systems of some of the others plants studied. Further research is needed to determine the impact of the effluent released to the wetland ponds.

Because of the high estrogenic potency and of these estrogenic compounds in the environment, further studies are needed to determine if dilution of the effluent in the receiving environment could affect the biota. In the USA-MEXICO border the effluent contributes a large volume of the flow, especially during the summers, when the flow in the rivers is primarily the result of effluent discharge; it is possible that aquatic organisms may be exposed to estrogenic chemicals at levels sufficient to produce biological responses. This needs to be investigated by conducting further assessments of the receiving aquatic environment.

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CHAPTER 4: FATE OF ENDOCRINE DISRUPTING COMPOUNDS IN TRADITIONAL AND ADVANCE WASTEWATER TREATMENT PLANTS

1. Introduction

Natural estrogens (estrone and estradiol), and degradation products of alkylphenol ethoxylates (nonyphenol ethoxylates, and nonylphenol) have been found to cause adverse effects to the aquatic biota at low concentrations [1-3]. High concentrations of these compounds were found in surface water in a nationwide study of emerging contaminants in rivers from the United States[4]. These findings have induced an intense focus on the study of sources and environmental fate of these compounds.

Natural estrogens are mainly excreted by humans and livestock [5]. Estradiol (E2) is the primary metabolite with the highest potency (EC50 = 1.45 E^{-10} M), whereas estrone (E1) is the secondary metabolite with reduced potency (EC50=1.28 E^{-9} M). Alkylphenol compounds also possess strong capabilities to mimic natural hormones by interacting with the estrogen receptors [6]. Most of these compounds have been detected in both influent and effluent from wastewater treatment plants (WWTPs) at concentrations high enough to produce adverse effects in the environment. Nonylphenol is the most ubiquitous in wastewater discharge with concentrations in effluent ranging from < 0.05 to 262 μg L^{-1} [7]. Since WWTPs are one of the primary sources of EDCs, it is important to understand the efficiency of the treatments to remove EDCs from wastewater.

In this study two wastewater treatments plants (WWTPs) were chosen to determine their efficiency to remove micropollutants from wastewater by performing mass balance analysis. Each WWTP selected has different technologies with one plant comprising of a secondary

treatment process and one involving tertiary treatment. Effluents from these plants are discharger into the river or injected to the aquifer.

2. Material and methods

2.1: Materials

Estrone (E1), 17β-estradiol (E2), 17α-ethynylestradiol (EE2), Bisphenol A (BPA), nonylphenol technical mixtures (NP), 4-tert-octylphenol (OCT), nonylphenol monoethoxylate (NPEO1), and nonylphenol diethoxylate (NPEO2) were purchased from Sigma-Aldrich (MO, USA). Nonylphenol standard solution in methanol was supplied by AccuStandard (CT, USA). E2 (3,4-13C2), BPA (ring 13C12), and p-n-Nonylphenol (ring 13C6) internal standards were from Cambridge Isotope Laboratories, Inc (MA, USA). HPLC grade methanol, dimethyl chlorine, and ethyl acetate were from VWR (USA). Silica gel (70-230 mesh ASTM) was purchased from Sigma- Aldrich.

2.2 Sample sites

The schematic of WWTPs in El Paso is shown in (Figure 21). The Plant 1 treatment consists of a screen and de–grit basin, followed by an aeration tank (activated sludge treatment) for biological decomposition of pollutants. Before the aeration system, ammonia is removed from the water in a denitrification basin (not shown in diagram). In the second stage, effluent from the aeration tanks is directed to clarifiers to remove the sludge. A portion of the sludge is returned to the aeration basins to treat more wastewater and the excess sludge is removed from the process, dewatered and stabilized with lime. Water from the clarifier is directed to sand filtration to reduce effluent turbidity and then treated by ultraviolet light as a disinfection

process. Effluent is discharged to the Rio Grande River. Plant 2 comprises an advance tertiary treatment. As plant 1, raw water passes through a bar screen and a de-grit basin, but the water is then directed to a primary clarifier where the sludge is collected and then pumped into anaerobic digesters (36°C). The sludge from the digesters is dried and composted for community use. The primary effluent is directed to the activated sludge system (aerated) and powdered activated carbon (PACT) is added in the water input to remove organic compounds by absorption. A secondary clarifier is used to remove the sludge and carbon from the water and is directed to a centrifuge system for dewatering and lime is used for stabilization. Effluent from the secondary clarifier is treated in a denitrification tank where bacteria convert ammonia to nitrate, and nitrate to harmless nitrogen gas, which is vented to the atmosphere. Methanol (210GPD) is added as a carbon source for bacteria. Subsequently, a tertiary clarifier is used to remove the rest of the sludge and carbon. A portion of the sludge from the second and third clarifier is returned to the aeration tank to maintain bacterial population. Water is then treated by raising the pH (11) with lime to kill pathogens, and remove hardness and heavy metals. Carbon dioxide is added to lower the pH. Sand filters are used to reduce turbidity, followed by ozone disinfection for sterilization. Finally, effluent is passed over granulated activated carbon (GAC) to adsorb any remaining organic contaminants. The water is then injected into the aquifer.

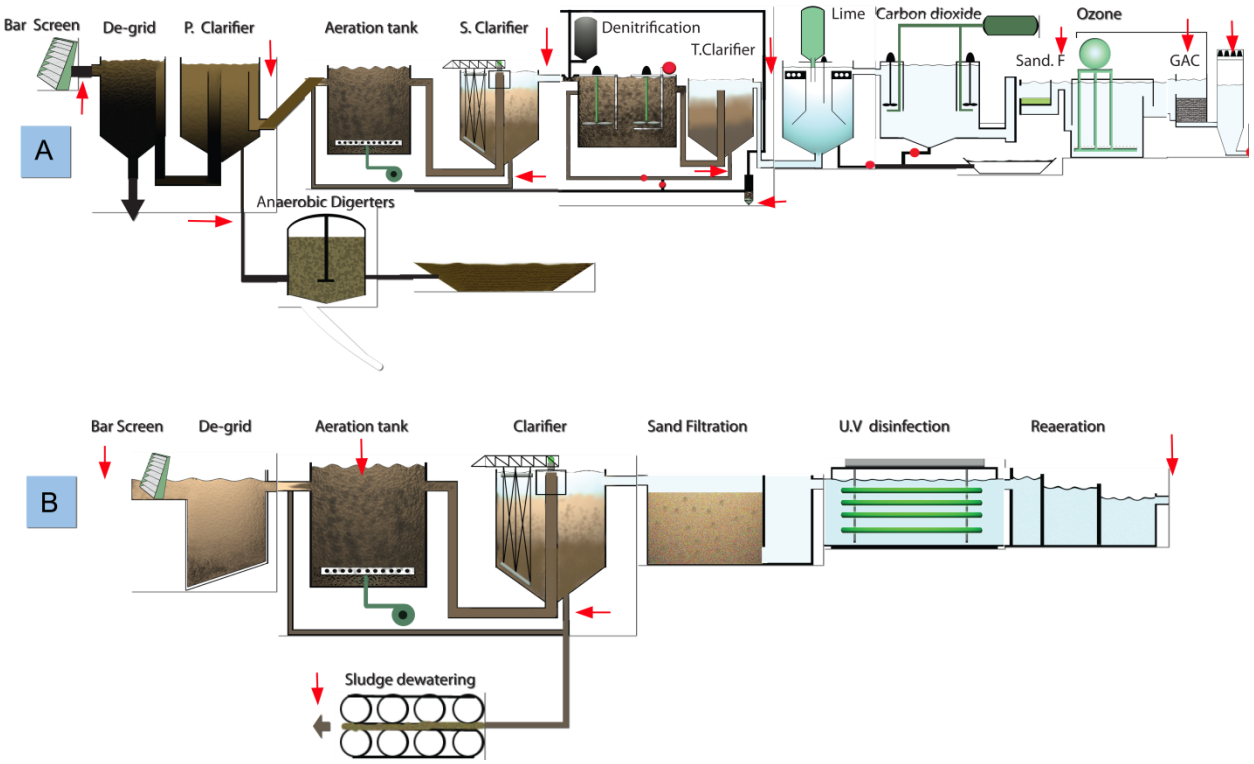

Figure 21: Flow scheme for two wastewater treatment plants in El Paso, TX with sampling location (Red arrows). (A) Plant with advance tertiary treatment; (B) Plant with secondary treatment. P.Clarifier: Primary Clarifier; S.Clarifier: Secondary clarifier; T.clarifier : Tertiary clarifier. Sand F: Sand filtration; GAC: Granulate activated carbon. Red arrows indicate sample site.

2.3 Sample collection

Twenty-four hour composite wastewater samples were collected from different treatments in winter 2010 and summer 2010. All liquid samples were collected in previously baked amber glass containers, and transported in ice to the laboratory. Samples were immediately filtered through Whatman GF-F glassfiber filters (pore size 0.7 µM) and extracted within 24 hours.

2.4 Sample Extraction

For determination of dissolved NPEs and estrogens' concentrations, samples were filtered through a 0.7-μm Whatman GF/F filter (Whatman, USA). The filtered aqueous phase (1000 mL) was then extracted through solid phase extraction (SPE) 1 g discovery DSC-18 cartridges (Supelco, PA, USA). SPE cartridges were pre-conditioned before extraction with 5 mL of methanol, 5 mL of ethylacetate, and 5 mL of ultrapure water. Once extraction was completed, the cartridges were dried by a gentle flow of nitrogen and the analytes were eluted using 10 mL of methanol, 5 mL of ethylacetate, and 10 mL of a 50/50 dichloromethane/hexane mixture. A nitrogen evaporator (Organomation, USA) was employed to concentrate extracts to 0.5 mL. The extracts were passed through 2 g of silica gel for cleanup and eluted with 20% methanol in ethylacetate. The extracts were concentrated again to dryness and reconstituted with 100 µL of dimethylformamide.

2.5 Sludge and suspended solid extraction

Sludge and suspended solids collected in the filters were freeze dried and extracted by sonication. Five hundred grams of sludge and the dry filter papers were extracted twice with 10 mL of methanol, 10 mL of ethyl acetate, and 10 mL of dimethylchlorine for 30 minutes. Each extract was combined and concentrated to 0.5 mL with a nitrogen evaporator, cleaned with silica gel, and concentrated for a second time as aforementioned.

2.6 Concentrated derivatization

Derivatizations were performed by adding 100 µL of N-O Bis (trimethylsylil) trifluoroacetamide with 1% trimethylchlorosilane to the extracts, which were transferred to 2 mL vials. The vials were closed and heated in an oil bath at 70 °C for 30 minutes. After derivatization, samples were concentrated again to 100 µL.

2.7 Gas Chromatography-Mass Spectrometry

Two µL of samples were injected into the GC-MS. The transfer line temperature was set at 100 °C and after injection, the temperature increased to 300 °C. The separations were performed on an HP-MS capillary column (0.25 mm×30 m×0.25µm, Phenomenex, CA). The oven was programmed as follows: initial temperature set at 60 °C with 15 °C min^{-1} ramp to 300 °C and held for 5 min. The carrier gas used was ultra-pure helium at a constant flow of 1.2 mL min^{-1}. The mass spectrometer was operated in the selected-ion monitoring mode with electron-impact ionization (ionization voltage, 70 eV). Target compounds were measured based on the following quantification ions on selected ion monitoring mode: BPA (Bisphenol A): m/z=357, 372; E1 (Estrone): m/z=342, 257; E2 (17β-estradiol): m/z=416, 285; EE2 (17α-ethynylestradiol): m/z=425, 440; NP (Nonylphenol): m/z= 207, 221, 179, 249, 235; NPEO1; m/z = 251, 265, 279, 293, 307; NPEO2; m/z = 323, 337, 295, 309, 351; E2 13C2: m/z= 418, 287; m/z=369, 384; p-n-NP 13C6 (para-n-Nonylphenol, Ring 13C6): m/z=185, 298. Ten-point calibration curves were conducted ranging from 0.005 to 20 ng L^{-1}. The linear response of the curves produced correlation coefficients (R2) higher than 0.99 for all EDCs.

2.8 Mass balance analysis of Endocrine disruptor Compounds

To understand the fate and transport of endocrine disruptors in the WWTPs in study, mass balance analysis was performed on each compound by multiplying the concentration by the average daily flow rates:

$$W = Q(C_{aqueous} + (C_{SS} \times C_{TSS})) \quad (1)$$

where W is the total mass load of target analytes (g d^{-1}); Q is the water flow for the specific treatment; $C_{aqueous}$ is the concentration of the analyte in the wastewater and C_{SS} is the concentration of the analyte in suspended solid; C_{TTS} is the concentration of total suspended solid.

The return activated sludge (RAS) loading was calculated by the following equation:

$$W_{sludge} = C_{sludge} \times Q_{sludge} \times TSS_{sludge} \quad (2)$$

where W_{sludge} is the total mass load of target analytes (g d^{-1}); Q_{sludge} is the sludge flow of RAS; and TSS_{sludge} is the concentration of total suspended solid in the RAS. Sorbed effluent concentration entering into the centrifugation process was assumed to be equal to the sum of the sorbed effluent concentration from the secondary clarifier and tertiary clarifier. TSS concentration was not obtained for the primary sludge. Mass load (W_{PS}) of the primary can be calculated by equation 3:

$$W_{PS} = C_{PS} \times Q_{PS} \times SP \times \rho \quad (3)$$

Where C_{PS} is the concentration in the primary sludge; SP is the percent solid in the primary sludge effluent; and ρ is the density of the water which was used as volume to mass conversion. The mass in the dehydrated sludge (disposal) was calculated by multiplying the concentration in the sludge, the percent solid, and the amount of sludge (grams) per day disposed.

The mass removal percentage for each EDC in each the treatment process was calculated as:

$$W_{removal} = \frac{W_{\text{inf}\,luent} - W_{effluent}}{W_{\text{inf}\,luent}} \times 100 \quad (4)$$

where $W_{influent}$ and $W_{effluent}$ is the mass load of a specific EDC in the influent and effluent for each unit, respectively. When the concentration of a compound was below corresponding quantification limit, half of the limit of detection was used for calculation.

Since natural estrogens are subjected to transformations (oxidation and reduction) between them, the combined concentrations of E1 and E2 were calculated [8-10]. NP and NPEOs were also combined in the mass balance analysis as they represent the final metabolites during the transformation process of long chain to short chain ethoxylates. Since the sludge and the dissolved solid were freeze dried, the concentration for theses samples are a conglomeration of EDCs sorbed to the sludge and EDCs attached to the sludge from the aqueous phase.

As for the second plant in study, a complete mass balance analysis was not possible because of the lack of composite sampling collected by the WWTP's operators. Composite samples are only collected in the influent, mix liquor, and effluents. The absence of automatic sampling is one of the reasons for the small amount of sampling implemented in that plant. Concentrations of EDCs detected in the mix liquor were not taken into account for the mass balance analysis since the samples were obtained inside the aeration system and not after the process. A mass balance of the aeration system involved numerous variables which are beyond the scope of the steady state approach used in this study.

Table 7: Parameters for each WWTP

Plant 1		Influent	P.E	S.Clarifier	T.Clarifier	Sand	Gac	effluent	D. sludge
	Flow MGD	6.62	6.62	7.33	6.63	5.35	5.40	4.86	
	TSS (mg L^{-1})	252.31	83.85	22.00	18.00	<1	<1	<1	
	Sludge Flow(MGD)		0.03	8.96	3.43				
	TSS sludge (mg L^{-1})			7056.92	7429.23				
	Solid %		3.00						15.00
	Disposal (tons)								49.33
Plant 2		**Influent**		**Aeration**	**Clarifier**			**Effluent**	
	Flow MGD	7.31		7.31				6.52	
	TSS (mg L^{-1})	164.65		3296.45				1.81	
	Sludge Flow(MGD)				2.70				
	TSS sludge (mg L^{-1})				10872.00				
	Solid %								31.35
	Disposal (tons)								6.00

3. Results

3.1 Mass balance of Estrogen in WWTP with Tertiary Treatment

The E1 and E2 concentrations (Table 7) were reduced by 38% in the primary treatment tank owing mainly to degradation of estrogen by bacteria (Figure 22). Elimination of estrogens by sorption to the sludge was minimal, accounting for only less than 1% of the total removal. Comparably, poor removal of estrogen by primary treatment has been observed in other studies [8, 9, 11]. The sorbed concentrations of estrogens in a WWTP from Australia were found to be slightly higher or below detection limits, estimating that the sorbed load is less than 12% [9]. Approximately 50% of the solid is removed by primary treatment, but the reduction in the concentration of E1 and E2 is minimal. The low percentage removal is an indication that estrogens are not partitioning into the nonpolar material in the raw water mainly because of the low concentration of solids in the influent[8]. However, mass loading of estrogens from the primary treatment for sampling performed in the summer was higher than mass input. This might be attributed to the cleavage of conjugated E2 and E1 (glucuronides and sulfates) by sludge bacteria [9, 10, 12]. On the other hand, oxidation and reduction processes of these compounds could be occurring during this treatment [8]. However, both compounds showed an increase in concentration (

Table **8**). Thus, the increment in concentration is mainly related to the deconjugation to free estrogen, rather than transformation between them. Similarly, Carballa et al. [10] found an increased on mass flux of estrogens from 0.517 g d^{-1} in influent to 0.652 g d^{-1} in pretreated effluents.

In the activated sludge system, estrogens were removed by 92%, and in the following aerated reactor, the natural estrogens are further reduced by biological degradation so that a total of more than 99.7% is eliminated. Elimination of estrogens by sorption to the sludge was minimal in comparison to the estrogen degradation, accounting for 31% to34% of the total removal in the aeration treatment and < 1% in the denitrification treatment. The low concentration of estrogen after the tertiary clarifier suggests that microbial degradation is occurring in both aqueous (dissolved) and sludge (sorbed) phase. It has been estimated in batch experiments that 50% -70% of estrogen will be sorbed to sludge in activated sludge treatment [13]. Nevertheless, this study represents a lower sorption and estrogens are mainly eliminated by biodegradation (62%-99%). Our findings are comparable to the studies of Andersen et al. [9] and Braga et al. [8]. Andersen et al. found that 90% of the estrogens are reduced by biological degradation after the second denitrification tank. A mass balance performed by Braga et al. indicated that 25% of total estrogens accumulate in mixed liquor suspended solid (MLSS), whereas 6% of the total mass load is removed from the activated sludge process in the form of waste activated sludge (WAS). The high removal of estrogen in the denitrification treatment in water samples from winter where concentrations of estrogens are virtually eliminated is interesting. It has been found that denitrification is an important treatment step for the removal of estrogens since it has been shown that nitrifying bacteria possess superior estrogenic removing capability [5, 9, 14]. Contrariwise, some studies have demonstrated low elimination of estrogens during anaerobic condition and the majority of the degradation occurred in aerobic condition (activated sludge treatment) [12, 15, 16]. This circumstance was observed in samples obtained in the summer where denitrification treatment eliminated 14% of the incoming estrogen concentration. Further analyses are needed to verify the efficiency of anaerobic condition in the

removal of E2 and E1. It is noteworthy to mention that the mass loading in effluent from the denitrification treatment is similar for both sampling seasons. Since concentrations of estrogen were below the limit of quantification for both sampling periods, half the limit of detection was used in the mass balance.

Loss of estrogens by volatilization is likely limited due to the low vapor pressures [5]. Henry's law constants for E1 (3.0×10^{-8} kPa) and E2 (3.0×10^{-8} kPa) are very small, thus they are not easily volatilized under normal temperature and pressure [5].

After biological treatment, the concentrations of E1 and E2 are slightly above the limit of quantification or below the limit of quantification. For samples collected in the winter, a 5-fold increase in the mass loading occurred in the sand filtration. It is well known that conjugated estrogens are not removed completely from wastewater treatment plants, and can account for 40% of the total estrogen discharge from the plant via effluents [17, 18]. The vast increase of the mass loading in the sand filtration might be explained by deconjugation of trace amounts of conjugated estrogens into free estrogens. Estrogen concentrations after granulated activated carbon were reduced by 90%, which is an indication that E1 and E2 are getting sorbed by the GAC phase. However, ozone treatment has been proven to eliminate estrogens at a great extent, thus the removal of estrogen by the GAC treatment might be overestimated. Since water samples from the ozone treatment were not available for analysis, the actual reduction after the sand filtration is unknown. Excess sludge was not analyzed in this research. However, according to various studies, the sorption of estrogen to excess sludge is very low: less than 3% of the total concentration sorbed [9, 13]. Overall, high efficiency to remove estrogens (99%) was obtained by this plant for both sampling periods.

Table 8: Concentration and Mass rate of Estrogens in Advance Tertiary Treatment Plant

Concentration of estrogens (ng L^{-1})					Mass rate of estrogen (g d^{-1})				
Location	**Period**		**E1**	**E2**	**Location**	**Period**	**E1**	**E2**	**Σ Estrogens**
Influent	Winter	$C_{dissolved}$	345.2	65.2	**Influent**	Winter	8.8	2.1	10.9
		C_{sorbed}	23.9	70.5					
	Summer	$C_{dissolved}$	77.7	53.5		Summer	2.1	1.8	3.9
		C_{sorbed}	19.3	70.5					
P.E	Winter	$C_{dissolved}$	242.0	17.0	**P.E**	Winter	6.3	0.5	6.8
		C_{sorbed}	101.8	28.1					
	Summer	$C_{dissolved}$	100.1	57.3		Summer	2.6	1.4	4.0
		C_{sorbed}	24.3	28.1					
S.Clarifier	Winter	$C_{dissolved}$	16.0	2.1	**S.Clarifier**	Winter	0.5	0.1	0.5
		C_{sorbed}	28.4	4.0					
	Summer	$C_{dissolved}$	8.5	3.0		Summer	0.2	0.1	0.3
		C_{sorbed}	12.0	4.0					
T.Clarifier	Winter	$C_{dissolved}$	5.3	1.8	**T.Clarifier**	Winter	0.1	0.0	0.2
		C_{sorbed}	2.5	4.0					
	Summer	$C_{dissolved}$	8.2	3.0		Summer	0.2	0.1	0.3
		C_{sorbed}	3.0	4.0					
Sand F.	Winter	$C_{dissolved}$	8.9	5.8	**Sand F.**	Winter	0.2	0.1	0.3
	Summer	$C_{dissolved}$	9.3	0.0		Summer	0.2	0.0	0.2
GAC	Winter	$C_{dissolved}$	3.0	0.0	**GAC**	Winter	0.1	0.0	0.1
	Summer	$C_{dissolved}$	3.0	0.0		Summer	0.1	0.0	0.1
Product	Winter	$C_{dissolved}$	3.2	0.0	**Product**	Winter	0.1	0.0	0.1
	Summer	$C_{dissolved}$	3.0	0.0		Summer	0.1	0.0	0.1
Sludge					**Sludge**				
P.E sludge	Winter		24.0	52.2	**P.E sludge**	Winter	0.1	0.2	0.2
	Summer		49.2	52.2		Summer	0.2	0.2	0.3
RAS1	Winter		12.2	4.0	**RAS1**	Winter	1.5	0.5	2.0
	Summer		6.5	4.0		Summer	6.4	3.9	10.3
RAS2	Winter		2.5	4.0	**RAS2**	Winter	0.1	0.2	0.3
	Summer		3.0	4.0		Summer	0.2	0.2	0.4
CAKE	Winter		53.9	4.0	**CAKE**	Winter	0.4	0.0	0.4
	Summer		62.9	4.0		Summer	0.9	0.1	1.0

$C_{dissolve}$: concentration in the aqueous phase; C_{solved}: concentration in the sludge; PE: preliminary effluents; S. Clarifier: secondary clarifier; T.Clarifier: tertiary clarifier; Sand F: sand filtration; GAC: granulated activated carbon; RAS; return activated sludge; Cake: dewatered sludge by centrifugation.

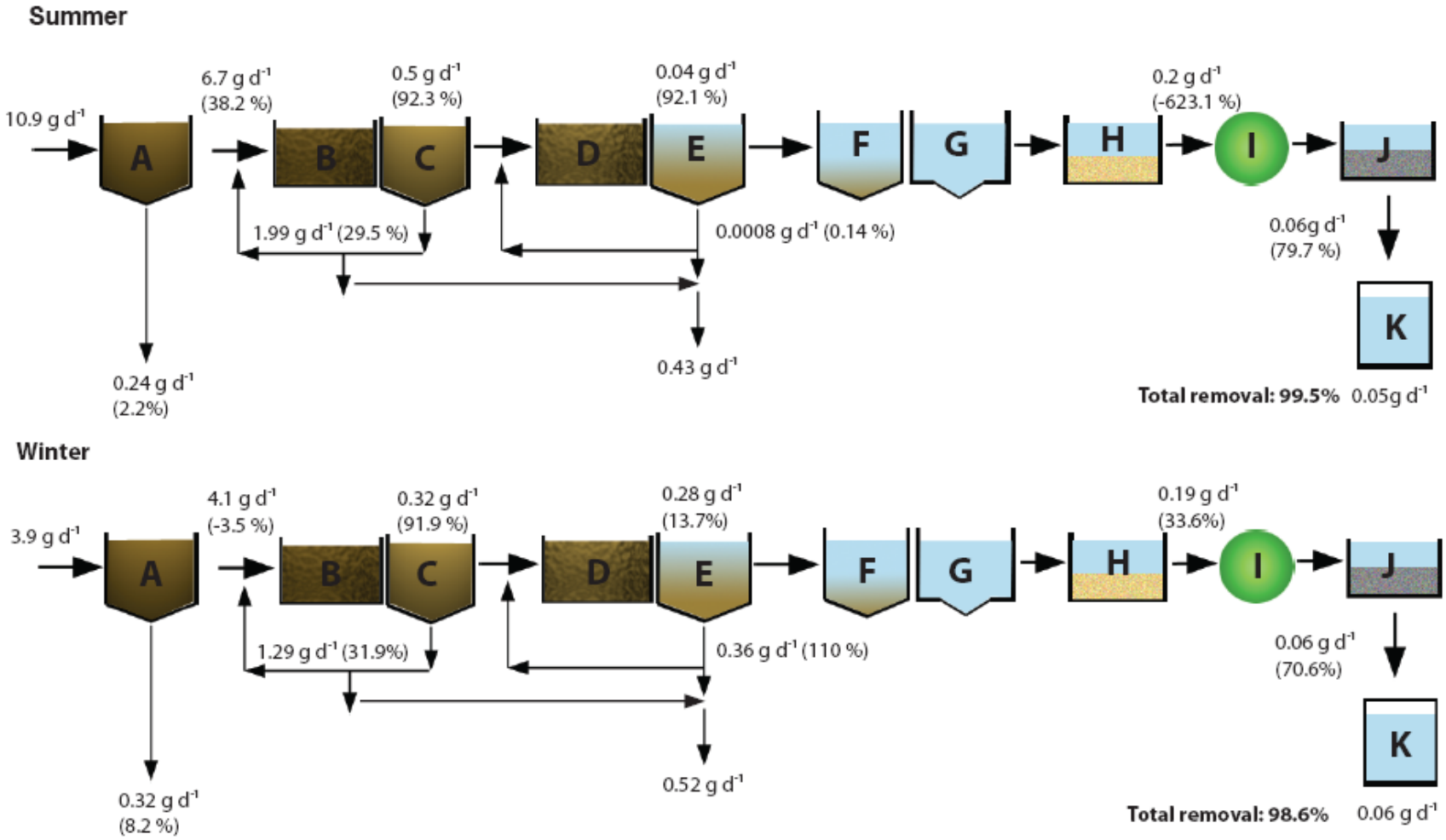

Figure 22: Mass Balance of estrogens in tertiary treatment plant. A: primary clarifier; B: Aeration; C: secondary clarifier; D: denitrification; E: tertiary clarifier; F: Lime treatment, G: carbon dioxide treatment; H: sand filtration; I: ozone disinfection; J: granulated activated sludge; K: product.

3.2 Mass Balance of Nonyphenol and Ethoxylates in WWTP with Tertiary Treatment

Based on the mass fluxes, elimination of ΣNPE in the primary treatment was 24% and 9.5% for winter and summer samples respectively (Figure 23). If we compare the mass loading of the sludge to the mass loading input to the primary treatment, 20% of the ΣNPE were removed by the sludge. However, if we compare the mass loading of the sludge with the total mass lost during the treatment, 82% and 77% of NPE were removed via sorption to sludge. Partition of short chain NPEOs to organic matter is very high because of their relative hydrophobicity (log Kow ˜ 4) [19, 20]. It is expected that a great portion of NPEOs entering to the primary treatment process are removed by sludge sorption since microbial activity is very low at this stage. Though hydrolysis could take action in the primary clarifier, sedimentation is the predominant mechanism in this unit [21]. A similar pattern was observed in plants of Mid-Atlantic where 60% of NP, NPEO1 and NPEO2 were removed in primary treatment [19]. However, even when by some degree the Σ NPE are removed in the primary treatment, a slight increase (22 -34.6%) in concentration was detected for NP and NPEO1 in each sampling period (Table 9). This augmentation might be related to the transformation of long chain ethoxylates to short chain ethoxylates (NP, NPEO1 and NPEO2) [19, 22]. According to McAdam et al [22], the composition of ethoxylates in the settled sewage was 78% to 90% of long chain ethoxylates and 8% to 16% of nonylphenol. The high concentration of long chain ethoxylates in the influent can be transformed by bacteria in the wastewater leading to an increase in short ethoxylates in the primary treatment. A decrease of NPEO2 was observed in primary effluent, which might be related to the transformation of it to NPEO1.

Similar results were observed when the molar concentrations expressed as NP (ΣM_{NP}) for both treatments were compared (Table 10). Higher concentrations were found after the primary

treatment for this group of alkylphenols. This conversion was implemented to take into account the transformation occurring within this group of alkylphenols and expressed as NP, which is the parent compound and final product.

After the aeration treatment, the ΣNPE were removed from the incoming water in winter and summer by 78% to 88%, for a total elimination of 83% to 89%. From the total removal of ethoxylates and nonylphenol, 52% and 43% (66% and 47% of the total mass lost) were removed by sorption into the sludge and the rest by biodegradation. These data are consistent with other studies in WWTPs and batch experiments, where short chain ethoxylates and NP were efficiently removed by degradation and sorption to the large quantity of biomass [23, 24]. Aerobic conditions appear to be the main environment for the partial degradation of ethoxylates and NP [25]. Literatures have reported high removal levels of NPEOs in aeration/nitrification processes by 43% [22, 23]. Nevertheless, some reports have shown a different observation where concentrations of NPEO1 and NPEO2 increase by 100 to 1100%, and NP by 52% in carbonated activated sludge process [22]. In batch experiments under aerobic conditions, NPEO2 was the predominant metabolite after degradation of long chain ethoxylates.

Table 9: Concentration and Mass rate of NPE in Advance Tertiary Treatment Plant

Concentration of NPEs (ng L^{-1}) in Wastewater Treatments						Mass rate (g d^{-1}) of NPEs in Wastewater Treatments					
Treatment	**Period**		**NP**	**NPEO1**	**NPEO2**	**Treatment**	**Period**	**NP**	**NPEO1**	**NPEO2**	**ΣNPES**
Influent	Winter	$C_{dissolved}$	12217.7	4800.5	1306.4	**Influent**	Winter	380.5	167.1	51.2	598.8
		C_{sorbed}	10524.1	6629.2	2612.8						
	Summer	$C_{dissolved}$	6792.1	8148.1	3954.3		Summer	217.6	274.8	132.0	624.4
		C_{sorbed}	5788.2	8700.8	4049.8						
P.E	Winter	$C_{dissolved}$	7806.1	5136.7	777.5	**P.E**	Winter	298.6	134.4	20.7	453.7
		C_{sorbed}	47253.7	2613.0	543.6						
	Summer	$C_{dissolved}$	8175.8	9661.0	1791.5		Summer	233.3	251.1	48.7	533.0
		C_{sorbed}	11615.7	2743.7	1444.2						
S.Clarifier	Winter	$C_{dissolved}$	2863.5	55.3	400.1	**S.Clarifier**	Winter	82.5	2.5	13.1	98.2
		C_{sorbed}	5059.1	1546.0	3246.3						
	Summer	$C_{dissolved}$	826.1	282.3	1211.7		Summer	24.0	8.4	34.4	66.8
		C_{sorbed}	1517.7	956.3	789.0						
T.Clarifier	Winter	$C_{dissolved}$	5370.9	128.4	253.9	**T.Clarifier**	Winter	139.2	3.4	6.6	149.1
		C_{sorbed}	9978.6	312.3	436.4						
	Summer	$C_{dissolved}$	587.3	281.6	1062.5		Summer	16.0	7.7	28.7	52.3
		C_{sorbed}	475.5	353.9	435.3						
Sand F.	Winter	$C_{dissolved}$	2277.6	150.3	346.3	**Sand F.**	Winter	46.1	3.0	7.0	56.2
	Summer	$C_{dissolved}$	111.1	189.4	1058.5		Summer	2.9	5.0	28.0	36.0
GAC	Winter	$C_{dissolved}$	1545.0	98.8	244.0	**GAC**	Winter	31.3	2.0	4.9	38.2
	Summer	$C_{dissolved}$	120.0	191.7	679.8		Summer	3.2	5.1	18.0	26.3
Product	Winter	$C_{dissolved}$	932.1	23.5	276.8	**Product**	Winter	17.1	0.4	5.1	22.7
	Summer	$C_{dissolved}$	344.0	147.3	200.0		Summer	6.6	2.8	3.9	13.3
Sludge						**Sludge**					
P.E sludge	Winter		31628.5	3900.7	2386.2	**P.E sludge**	Winter	100.4	12.4	7.6	120.4
	Summer		11615.7	2026.7	744.2		Summer	36.9	6.4	2.4	45.7
RAS1	Winter		1182.3	312.3	436.4	**RAS1**	Winter	145.8	38.5	53.8	238.2
	Summer		800.0	295.3	477.4		Summer	101.5	37.5	60.5	199.5
RAS2	Winter		569.1	516.7	215.8	**RAS2**	Winter	29.4	26.7	11.1	67.2
	Summer		569.3	258.4	567.9		Summer	11.8	5.4	11.8	29.0
CAKE	Winter		2403.7	2942.1	4133.5	**CAKE**	Winter	17.8	21.8	30.6	70.1
	Summer		841.2	1732.0	538.8		Summer	34.6	71.3	22.2	128.0

$C_{dissolve}$: concentration in the aqueous phase; C_{solved}: concentration in the sludge; PE: preliminary effluents; S. Clarifier: secondary clarifier; T.Clarifier: tertiary clarifier; Sand F: sand filtration; GAC: granulated activated carbon; RAS; return activated sludge; Cake: dewatered sludge by centrifugation

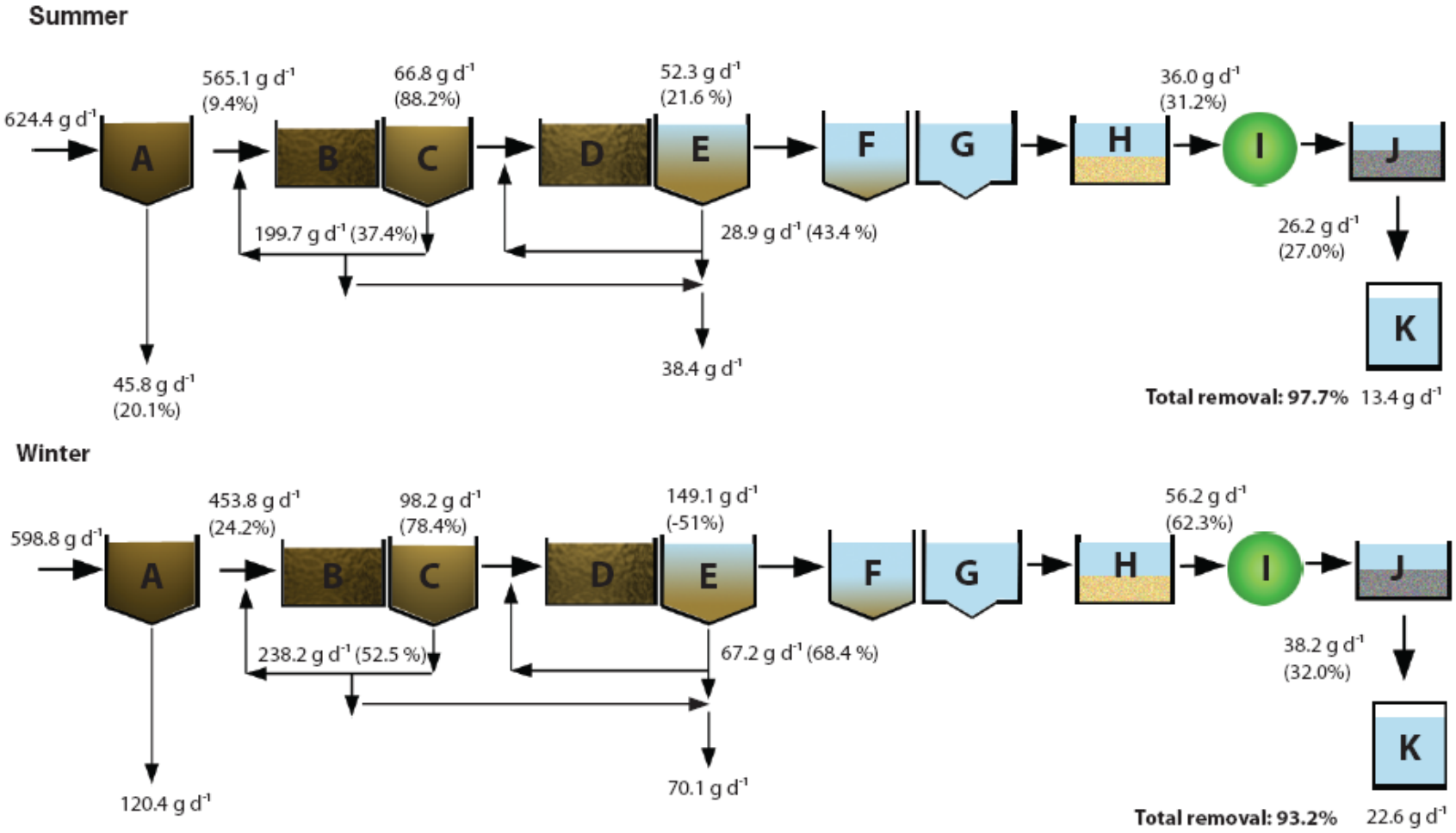

Figure 23: Mass Balance of ΣNPE in advance tertiary treatment plant. A: primary clarifier; B: Aeration; C: secondary clarifier; D: denitrification; E: tertiary clarifier; F: Lime treatment, G: carbon dioxide treatment; H: sand filtration; I: ozone disinfection; J: granulated activated sludge; K: product.

Short chain NPEOs have been insignificantly removed by anaerobic processes. Mass loading of ΣNPE for winter sampling increased from 98 g d^{-1} to 149 g d^{-1} (removal: -25%) in the nitrification/denitrification treatment due to an increase in concentrations of NP and NPEO1. Poor biodegradations are consistently obtained by systems comprising of both nitrification and denitrification treatments and anaerobic conditions [7, 22]. An increase of NPE has been observed in this process up to 2150% for NPEOs and 51 % for NP [22], and wide variations in ΣNPE were observed in 3 plants in the USA with removals ranging from 12 % to 59% . Moreover, analysis of NPE in anaerobic digestion systems has shown limited effect on nonylphenol mass [26, 27]. This trend illustrates short chain carboxylate species formation during the process followed by the progressive shortening of the long chain ethoxylates to NPE. No significant difference was found between the ΣM_{NP} after the nitrification/denitrification treatment and the concentration of the secondary clarifier influent-an indication of zero removal of NPE during that treatment. However, both the mass of ΣNPE as well as ΣM_{NP} after the nitrification/denitrification process in samples collected in summer decreased by 21%. The decrease of NPE during the summer season could be attributed to the high temperature of the water (28 °C), which allows bacteria to degrade NPE at a higher rate [28]. Nevertheless, this might be considered a poor removal, which resembles the results obtained by Loyo et al. [7]. These results demonstrated the complexity of NPE fate in wastewater treatment plants as this encompasses both adsorption and biodegradation mechanisms. Moreover, the specific biodegradation route of these compounds remains unknown [22, 29].

Mass loading of ΣNPE decreased after sand filtration for both sampling periods. However, it is uncertain if the removal is related to the process of filtering by itself or if other mechanisms of elimination occurred through the lime treatment. Samples were not collected in that process and there is not information in the literature about removal of NPEOS and NP by lime treatment. Granulated activated carbon appeared to remove 26% to 66% of ΣNPE. Lab experiment testing the absorption of EDCs to GAC found effective removal of NP from water samples [30]. Ozone treatment studies in lab scale found that compounds like NP can be degraded by over 50% at pH of 7 and the contribution increased substantially with pH>7. Therefore, the total removal of ΣNPE cannot be attributed solely to GAC. NPEOs and NP were removed from wastewater by 93% and 98% in the winter and summer, respectively.

Table 10: Concentration of NP and Ethoxylates (Mol L^{-1})

Location	Period	NP	NPEO1	NPEO2	ΣM_{NP} [(a)]
Influent					
	Winter	1.03E-07	4.32E-08	1.27E-08	3.50E-05
	Summer	5.71E-08	6.37E-08	2.60E-08	3.23E-05
P.E					
	Winter	2.50E-07	2.93E-08	4.29E-09	6.24E-05
	Summer	8.98E-08	4.69E-08	1.05E-08	3.24E-05
S.Clarifier					
	Winter	3.60E-08	6.06E-09	1.18E-08	1.18E-05
	Summer	1.06E-08	4.68E-09	6.50E-09	4.80E-06
T.Clarifier					
	Winter	6.97E-08	1.67E-09	2.24E-09	1.62E-05
	Summer	4.82E-09	2.40E-09	4.86E-09	2.66E-06
Sand F.					
	Winter	1.03E-08	5.69E-10	1.31E-09	2.69E-06
	Summer	5.04E-10	7.16E-10	3.44E-09	1.02E-06
GAC					
	Winter	7.01E-09	3.74E-10	9.23E-10	1.83E-06
	Summer	5.45E-10	7.25E-10	2.21E-09	7.65E-07
Product					
	Winter	4.23E-09	8.88E-11	1.05E-09	1.18E-06
	Summer	1.56E-09	5.57E-10	6.49E-10	6.09E-07

(a) Molar concentration expressed as NP

3.3 Mass Balance of Nonylphenol and Ethoxylates in WWTP with Secondary Treatment

For comparison purposes, the advance tertiary treatment plant was compared with a plant comprising of a secondary treatment. NP and NPEOs were selected since they are the contaminants with the highest levels detected in effluent. Furthermore, this WWTP is the only one discharging water into the Rio Grande daily. It is noteworthy to emphasize that mix liquor samples were not taken into account for the mass balance analysis. Nonetheless, the concentrations detected in those samples are shown in (Table 11). Based on the mass balance analysis, ΣNPE were removed from wastewater by 89% and 92% in summer and winter, respectively (Figure 24).

Table 11: Concentration and mass rate of NP and NPEOS in secondary treatment plant.

Concentration of NPEs (ng L^{-1}) in Wastewater Treatments						Mass rate (g d^{-1}) of NPEs in Wastewater Treatments					
Treatment	**Period**		**NP**	**NPEO1**	**NPEO2**	**Treatment**	**Period**	**NP**	**NPEO1**	**NPEO2**	**ΣNPES**
Influent	Winter	$C_{dissolved}$	15093.0	3487.0	6496.2	**Influent**	Winter	409.9	90.0	162.9	662.8
		C_{sorbed}	12949.4	1743.2	1946.3						
	Summer	$C_{dissolved}$	4705.5	2943.9	4422.4		Summer	121.9	75.9	112.5	310.3
		C_{sorbed}	2456.0	1432.0	1756.0						
Mix Liquour	Winter	$C_{dissolved}$	3550.9	976.3	209.4	**Mix Liquour**	Winter	N/A	N/A	N/A	N/A
		C_{sorbed}	2053.6	933.7	570.2						
	Summer	$C_{dissolved}$	184.6	417.4	745.6		Summer	N/A	N/A	N/A	N/A
		C_{sorbed}	140.2	432.6	565.3						
Effluent	Winter	$C_{dissolved}$	344.0	849.3	1721.0	**Effluent**	Winter	7.9	19.5	39.5	66.8
	Summer	$C_{dissolved}$	201.6	145.2	653.3		Summer	4.6	3.3	15.0	22.9
Sludge						**Sludge**					
RAS	Winter		954.0	976.3	209.4	**RAS**	Winter	89.4	91.5	19.6	200.5
	Summer		836.8731	275.59	357.8		Summer	78.4	25.8	33.5	137.8
Belt press	Winter		1050.5	1037.0	324.1	**WAS**	Winter	3.8	3.9	0.8	8.5
	Summer		2031.6	3114.9	697.0		Summer	3.3	1.1	1.4	5.9
						Belt press	Winter	2.2	2.2	0.7	5.1
							Summer	4.3	6.5	1.5	12.3

Belt press: dewatering sludge after belt press; RAS: return activated sludge; WAS: waste activated sludge.

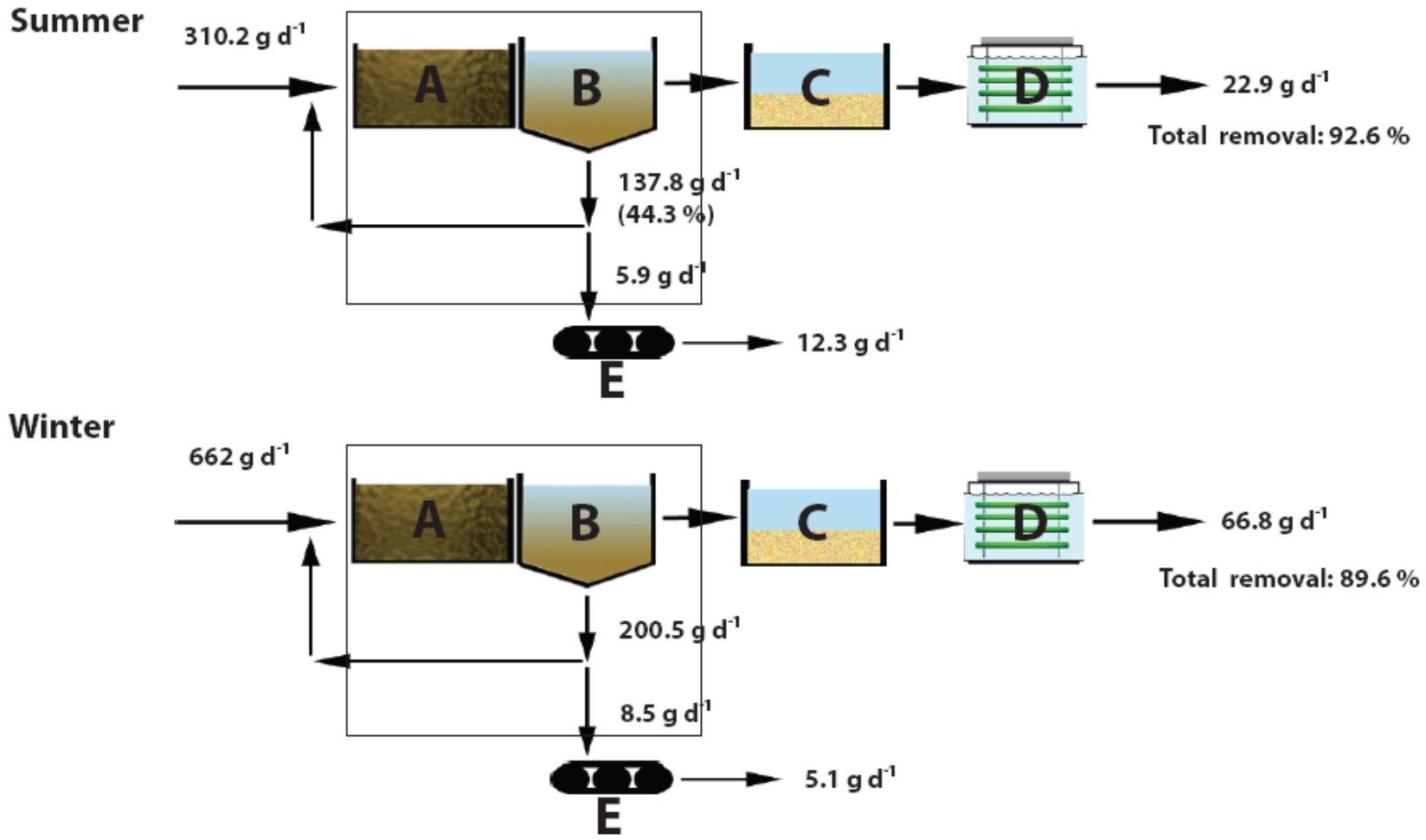

Figure 24: Mass balance in secondary treatment plant. A: aeration; B: Clarifier; C: Sand filtration; D; UV disinfection; E: belt press (dewatering).

The total amounts of ΣNPE removed by the sludge were 30% (winter) and 44% (summer). However, it is difficult to identify the real elimination of ΣNPE by the system due to the lack of information obtained regarding the aeration system and the clarifier effluent. On the other hand, if the ΣM_{NP} are taken into consideration there is a decrease in the ΣNPE during the whole treatment. Concentrations of NP were higher in the sludge as compared to the NPEOs due to the high K_{ow}. Similar results were found in wastewater treatment plants from Kansas [23]. Mass loading in the wastes' activated sludge can be estimated by using the concentrations detected in the RAS. WWTPs distribute the sludge output from the clarifier by returning a portion to the aeration system and the remaining portion is directed to the dewatering process for sludge disposal. The mass flux in the waste activated sludge (WAS) was 8.5 g d^{-1} for winter and 12.5 g d^{-1} for summer samples. Mass in the dewatered cake (belt press) was similar to the WAS in winter samples, and increased in the summer. It is evident that ΣNPE are not totally removed after the dewatering process. The same results were found by Zhang J. [27] where the mass flux in the dewatered cake is comparable to the input coming from the anaerobic digester. In this study, the mass flux for the digester was 520.9 kg d^{-1} and 520.5 9 kg d^{-1} in the dewatered cake. The total removal for each period was 89.9% (winter) and 92.6% (summer). However, the elimination of ΣNPE in this plant cannot just be attributed to sorption to sludge or biodegradation. This plant comprises of a UV treatment after the clarifier, which, according to Ike et al. [31], is efficient to degrade NPE. In that study, NPE were degraded by exposing them to UV/TiO2 (ultraviolet in presence of titanium dioxide). Nonetheless, further analyses are needed regarding the degradation of NP and NPEOs by UV treatment.

Table 12: Concentration of NP and Ethoxylates (Mol L^{-1})

Location	Period	NP	NPEO1	NPEO2	ΣM_{NP} [(a)]
Influent					
	Winter	1.27E-07	2E-08	3E-08	8E-10
	Summer	3.25E-08	1.7E-08	2E-08	3E-10
Mix Liquor					
	Winter	2.54E-08	7.2E-09	3E-09	2E-10
	Summer	1.47E-09	3.2E-09	4E-09	4E-11
Effluent					
	Winter	1.56E-09	3.2E-09	6E-09	5E-11
	Summer	9.15E-10	5.5E-10	2E-09	2E-11
Sludge					
RAS	Winter	4.33E-09	3.7E-09	7E-10	4E-11
	Summer	9.15E-10	5.5E-10	2E-09	2E-11
Belt press					
	Winter	4.77E-09	4.2E-09	5E-10	4E-11
	Summer	2.16E-20	1.6E-20	2E-21	2E-22

a)Molar concentration expressed as NP

4. Conclusion

The efficiency in removing ΣNPE loading in secondary treatment and tertiary treatment has been assessed. From this study, it is suggested that advance tertiary treatment processes comprising biodegradation and filtration can provide an effective NP and NPEOS removal of up to 98%. Secondary treatment delivered a removal of 90%. Since a complete mass balance could not be performed in the secondary treatment WWTP, it is difficult to arrive at some conclusion about capability of the process within the aeration system. As aligned with the results of previous studies, the denitrification process appears to produce the poorest degradation of the ΣNPE, which induce an increase in NP due to de-ethoxylation of long chains ethoxylates. However a different result was obtained in the summer sampling where the concentration of ΣNPE

decreases after denitrification treatment. These results demonstrated the complexity of NP and NPEOs degradation in WWTPs which is still not understood [22]. To determine how NP and NPEOs increase during the treatment process, more analyses concerning long chains ethoxylates are needed. The elimination of estrogen mainly occurs from the biodegradation process (<60%). Interesting GAC treatment seems to remove estrogen from the water even with their low k_{ow} in comparison with NPE. However, more analyses are needed in the lime treatment, ozone treatment, and UV treatment to corroborate the potential degradation of target compounds by this process. The use of tertiary treatment allows for a better removal of trace compounds. Nevertheless, the use of advanced tertiary treatment technologies (e.g., ozone) for the removal of micropollutants in wastewater presents both financial and environmental constraints, particularly in reference to energy and carbon footprint [32].

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CHAPTER 5: CONCLUDING REMARKS

In this study we have analyzed endocrine disruptor compounds by integrating chemical and biological analysis. This approach allows us to determine not only the concentration of the analytes in the wastewater, but also the estrogenic activity of the samples. Determining the in the water gave us a perspective of how significant are the levels found in wastewater effluent, and the capability of the plant to eliminate them. The developing of a bioassay to test wastewater by direct assayed without concentration of the samples represent a quicker and simple alternative in comparison to the yeast based assay currently in use. However, this type of assay is exceptional in the screening of wastewater where high concentrations of contaminants are expected. In case of surface water or underground water the concentration of the sample might be the best procedure.

By using chemical and biological analyses, we have assessed the occurrence and removal of EDCs and estrogenic activity in 4 WWTPs in El Paso, and 2 plants in Mexico. The concentrations of EDCs and estrogenic activity in effluent were higher in WWTPs from Ciudad Juarez, Mexico. Moreover, the removal of EDCs and estrogenicity from wastewater treated was ineffective in comparison to the process implemented in the WWTPs in El Paso. Primarily, because WWTPs plants in Mexico do not apply any secondary treatment (e.g. activated sludge) to the wastewater, and most of the process is centered to eliminate solid. Low removal and high concentration of EDCs were detected in effluents from one plant located in El Paso, Texas. The reasons are unknown since the plant includes same systems of some of the others plants studied. This plant is in charge of release effluent to Rio Bosque to generate an artificial wetland. The pods formed are the habitat for many migration birds, usually in winter seasons. Further research is needed to determine the impact of the effluent released to the wetland ponds.

Analysis of effluent, surface water and sediment could be the first approach for determining possible impact to the wildlife. Little variation was found between seasons and weak correlations were obtained when comparing concentration, and estrogenic activity versus water temperature. Probably, as a result of the warmer temperature in the area of study, the bacterial population has maintained similar activity during the investigation. Additional research is needed to determined antiestrogenic compounds in the type of assay used in this study. In this moment, cleaning procedures are used to evade interference during the assay, but several approaches have failed to accomplish the purposes. Those who have prevailed did not provide specific indication of the kind of compound blocking estrogen receptors. Although technically challenging, it may also be possible in the future to develop procedures that will allow us to determine estrogenic activity without having underestimation of the real receptor response.

Mass balance analyses were performed in two different plants in El Paso. These plants are responsible for water discharge to the river and to the groundwater. From this study, it is suggested that advance tertiary treatment processes comprising biodegradation and filtration can provide an effective NP and NPEOS removal of up to 98%. Secondary treatment delivered a removal of 90%. As aligned with the results of previous studies, the denitrification process appears to produce the poorest degradation of the ΣNPE, which induce an increase in NP due to de-ethoxylation of long chains ethoxylates. However a different result was obtained in the summer sampling where the concentration of ΣNPE decreases after denitrification treatment. In other hands, denitrification process cannot be eliminated from WWTPs process since is essential for the removal of nutrients which can cause adversed effect to the aquatic ecosystem if high concentration is discharge. To determine how NP and NPEOs increase during the treatment

process, more analyses concerning long chains ethoxylates are needed. The elimination of estrogen mainly occurs from the biodegradation process (<60%) and they were remove in at the end of the treatment by 99.5 % . Interesting GAC treatment seems to remove estrogen from the water even with their low k_{ow} in comparison with NPE. However, more analyses are needed in the lime treatment, ozone treatment, and UV treatment to corroborate the potential degradation of target compounds by this process. The use of tertiary treatment allows for a better removal of trace compounds. Nevertheless, the use of advanced tertiary treatment technologies (e.g., ozone) for the removal of EDCs in wastewater presents both financial and environmental constraints, particularly in reference to energy and carbon footprint.

Taking in account the information obtained from the chemical, biological and mass balance analyses the use of at least secondary treatment is enough to removed micropollutants such as EDCs in the wastewater from 60% to 99%. However, the plant with tertiary treatment removed EDCs from wastewater at a high rate constantly whereas in the others plants high variability in removal was observed during the study. I estimated that at least 6325 g of alkylphenols are discharge directly to the Rio Grande per year and 1450 g per year is injected to the aquifer. BPA and estrogen are discharge from about 248 g per year to the river and 140.0 g per year is injected.

Because of the high estrogenic potency and of these estrogenic compounds in the environment, further studies are needed to determine if dilution of the effluent in the receiving environment could affect the biota. In the USA-MEXICO border the effluent contributes a large volume of the flow, especially during the summers, when the flow in the rivers is primarily the

result of effluent discharge; it is possible that aquatic organisms may be exposed to estrogenic chemicals at levels sufficient to produce biological responses. This needs to be investigated by conducting additional assessments of the receiving aquatic environment.

As shown by the experiments presented herein, I believe that it is of great urgency that WWTPs in Mexico expand their facilities and upgrade their system to ensure that 100% of the wastewater can be treated by secondary treatment. A major technical issue in this study was the difficulty to obtain more samples from the wastewater treatment plants in Mexico. More studies concerning the fate of EDCs in WWTPs from Mexico will help to find answers that facilitate the upgrading of the treatments for future expansions of the facilities. The results presented in the previous experimental chapters will undoubtedly serve as the initial framework upon which to expand and add more information in relation to EDCs or other contaminants in water resources along the border.

CURRICULUM VITAE

Roberto De La Torre Roche was born in July 9, 1978 in San Juan, Puerto Rico. He graduated from La Escuela Central de Artes Visuales, a high school specialized in visual art. He entered to the University of Puerto Rico at Arecibo and earned a Bachelor of Science degree in Chemical Industrial Processes in December 2001. After graduation, he worked for one semester in the Food and Drug Administration at Los Angeles California, as a chemist, before enrolling in the graduate program in Environmental Science in the University of Texas at El Paso. He received his Master of Science degree in 2004. In 2006, he joined the doctoral program of Environmental Science and Engineering.

Roberto de La Torre was recipient of the National Science Foundation Scholarship and received numerous travel awards that allowed him to present his research to the science community. He has presented his research at international and national conferences, such as The International Chemical Congress of Pacific Basins Society, Society of Environmental Toxicology and Chemistry, American Chemical Society, and Society of Advancing Hispanic/Chicanos and Native Americans in Science.

While pursuing his degree, Roberto worked as full-time staff research assistant for the Department of Chemistry at The University of Texas at El Paso, and has published two science articles. In the future, he is interested in preforming research on the effect of pollution on wildlife and human health. Furthermore, he is interested in the adverse effects of human population growth, and habitat fragmentation.

Roberto De La Torre's dissertation entitled, "Analysis of Endocrine Disrupting Compounds in Wastewater treatment Plants: A Perspective of Trans-Boundary Waterborne pollution" was supervised by Dr. Wen-Yee lee.

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