

APPLICATION OF WATER/WASTEWATER TREATMENT IN TRACE ORGANIC  
COMPOUNDS REMOVAL AND OTHER INDUSTRY SECTORS

by

Bingfeng Dong

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This dissertation is dedicated to my family,

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My parents, Yi Dong and Liping Shi;

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And my advisor

Prof. Robert G. Arnold

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## ABSTRACT

Wastewater reuse is fast becoming an imperative issue based on the developments in water/wastewater engineering coupled with increasing pressures on water resources. Trace organic compounds (TOrcs) that exist in water/wastewater, are a serious threat once they were released in the environment. During the past decade, there has been much progress toward understanding the occurrence, fate and toxicology of trace organic pollutants that enter the environment in treated wastewater. The objective of the first part of this research was to evaluate the combined effects of sequential anaerobic/aerobic digestion on residual TOrcs, concentrating on chemicals that are responsible for observed estrogenic/androgenic activities in biosolids. Full-scale digestion was simulated using bench-scale bioreactors in which the primary independent variables were retention time, temperature, and oxygen loading during aerobic digestion. Treatment-dependent changes in estrogenic/androgenic activity and concentrations of specific Endocrine disrupting compounds (EDCs) were measured. Results suggest that standard mesophilic anaerobic digestion increases the total estrogenic/androgenic activity of sludge while aerobic digestion was effective in the reduction of estrogenic/androgenic activity as a supplementary treatment stage.

The second part of the study was focused on the fate of TOrcs and estrogenic activity in water and sediment of the Santa Cruz River, which is effluent dependent except during infrequent periods of rainfall/runoff in Tucson area. Several sampling campaigns were carried out from 2011 to 2013. Results suggest that some organic

TOrCs, including those that contribute to estrogenic activity, were rapidly attenuated with distance and time of travel in the Santa Cruz River. Indirect photolysis of estrogenic compounds through the river might play an important role for the observation of estrogenic activity changes in the SCR. Hydrophobic TOrCs may accumulate in river sediments during dry weather periods. Riverbed sediment quality is periodically improved through storm-related scouring during periods of heavy rainfall and runoff.

Wastewater effluent can be applied to the algal biodiesel industry based on regional water stress across the world. In the third part of the research, reclaimed wastewater was explored for this purpose, simultaneously satisfying the needs for water, macronutrients such as nitrogen and phosphorus, and micronutrients necessary for growth of microalgae. At the same time, algal growth in conventionally treated wastewater will improve water quality through the same nutrient removal processes and perhaps by lowering residual levels of trace organics that are an impediment to potable reuse. Results showed that metals levels in most municipal wastewaters are unlikely to disrupt growth, at least by metals tolerant microalgae like *Nannochloropsis salina*. Cells can grow without inhibition on nutrients from treated municipal wastewater or a centrate stream derived from wastewater treatment. The results also suggest while wastewater provides a suitable nutrient source for algal growth, there is simply not enough municipal wastewater available to support a meaningful biofuels industry without water recycling and nutrient recovery/reuse from spent algae.

The last part of the dissertation was the application of water/wastewater treatment techniques, specifically advanced oxidation processes (AOPs) in other industrial sectors. In the integrated circuit production industry, chemical formulations used for megasonic

cleaning typically contain hydroxides, peroxides and carbonates, which can affect particle removal efficiency and feature damage. The role of carbonates and ammonia in modulating the oxidation power of megasonic irradiated alkaline solutions through the scavenging of hydroxyl radicals by varying levels of carbonates, bicarbonates, ammonia and solution temperatures on net generation of hydroxyl radicals for applications in semiconductor industry was investigated in this study. The simulation of actual megasonic cleaning process was carried out at acoustic frequency of  $\sim 1$  MHz and different power densities. Carbonate ions were better scavengers of hydroxyl radicals than bicarbonate ions. The effect of bulk solution temperature revealed that the rate of generation of hydroxyl radicals at a power density of  $8 \text{ W/cm}^2$  increased with temperature from  $10\text{-}30^\circ\text{C}$ , which suggests an increase of transient cavitation with temperature.

## CHAPTER 1 GENERAL INTRODUCTION

### 1.1 Fate of estrogens/androgens during sludge digestion

#### 1.1.1 Introduction to endocrine disrupting compounds

Trace organic compounds that exhibit endocrine disrupting properties have been discovered by environmental scientists in waters and wastewaters throughout the world (Filali-Meknassi et al., 2004; Kolpin et al., 2002). Endocrine Disrupting Compounds (EDCs) are defined as “exogenous substances that alter function(s) of the endocrine system and consequently cause adverse health effects in an intact organism, or its progeny, or (sub)-populations” (WHO/IPCS, 2002). Although these compounds are generally present in the environment at very low concentrations (ng/L to µg/L), their ecological effects on wildlife and human beings have been noticed (Colborn, 1995).

Estrogens and estrogen mimics are the most studied among the known EDCs. Since the early 1990s, there have been numerous observations of aquatic organisms with disrupted sexual development characteristics. Purdom et al. (1994) in the United Kingdom were the first to find out that effluents from municipal wastewater treatment plants were estrogenic. They observed that male fish located downstream of wastewater treatment plant outfalls sometimes produce vitellogenin, a precursor protein for egg yolk production. Jobling et al. (1998) found that wild fish downstream of wastewater treatment plants have greater intersex characteristics, which included histological alterations, abnormal ratios of sex steroids, and the simultaneous presence of both sex specific gonads. Another good example in this field was a seven-year research project done by Kidd et al. (2007). They observed the collapse of the fathead minnow population in a Canadian freshwater lake due to the periodic addition of 5-6 ng/L 17 $\alpha$ -ethinylestradiol

(EE<sub>2</sub>), a primary birth control pill ingredient, over a period of three years. Vitellogenin mRNA and the protein itself were produced in male fish, and impacts on gonadal development were also observed as evidences for intersex characteristics in male fish. Even after two years following the EE<sub>2</sub> dosage events, the population of fathead minnow was not restored in that lake.

The endocrine system is a chemical messaging system which regulates a wide range of physiological responses in animals and human beings. It integrates the functions of individual organs, the nervous and immune systems through the action of circulating hormones (Colborn et al., 1993). Hormones are secreted in very low concentrations, with blood level concentrations in the range of 10<sup>-10</sup> to 10<sup>-12</sup> M. Many of them are lipid soluble, and they all have highly specific receptor sites. A number of human tissues express estrogen receptors including the brain, immune system, cardiovascular system, lung, mammary glands, liver, kidneys, reproductive tract, adipose tissue, and bone (Mueller, 2004).

EDCs can interfere with the synthesis, storage, release, secretion, transport, elimination, binding, or action of endogenous hormones. Physiological studies have demonstrated that even very subtle effects on the endocrine system can result in changes of growth, development, reproduction or behavior that can affect the organism itself, or even the offspring of exposed organisms. The specific mechanisms by which substances disrupt endocrine systems are very complex, and not yet completely understood. A possible mechanism is shown in Figure 1.1. Effects on reproductive, neurological and immunological function and carcinogenesis are endpoints of greatest concern from the long-term exposure to EDCs.

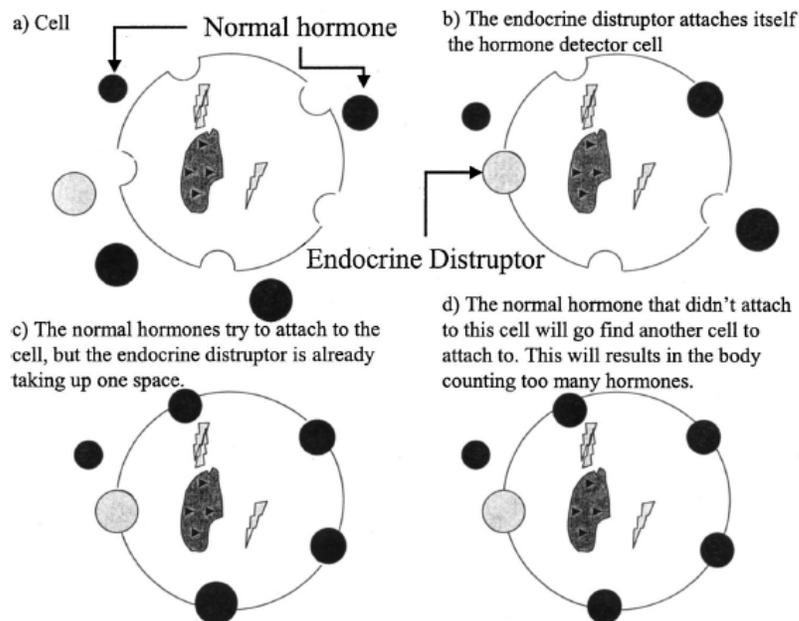


Figure 1.1 Endocrine disruptors mechanisms of action (Filali-Meknassi et al., 2004).

For example, at the cellular level, estrogens can cross cell membranes into the cytosol and then the nucleus, where they bind to estrogen receptors (Figure 1.2). The estrogen-receptor complex then binds to the estrogen response element (ERE) in the promoter region of DNA. If induced, estrogen-dependent RNA and protein synthesis begins.

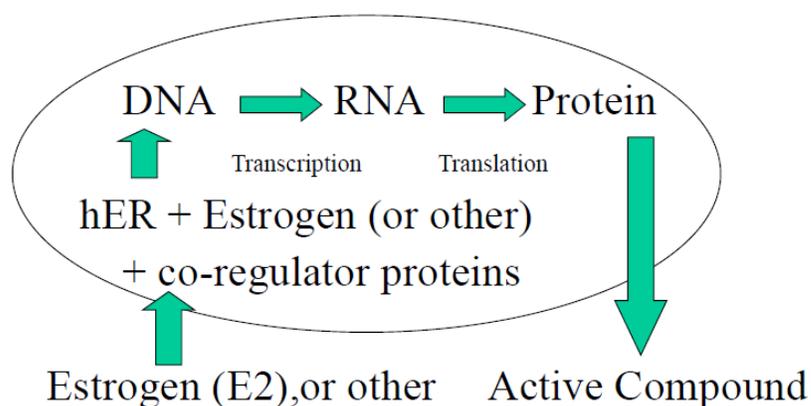


Figure 1.2 Mechanism of estrogen-dependent response at the cellular level.

There are two kinds of estrogen receptors in animal and humans, estrogen receptor-alpha (ER- $\alpha$ ) and estrogen receptor-beta (ER- $\beta$ ). They share many similarities, but the main distinction between the two receptors is their distributions among target organs (Nilsson et al., 2000). The function of ER- $\alpha$  is to regulate gene transcription among target organs such as the pituitary gland, vagina, and uterus (Dechering et al., 2000). It is a part of the nuclear receptor superfamily and is expressed in the MCF-7 breast cancer cell line. ER- $\beta$  is present in the ovaries and the prostate, and tends to be overproduced in ovarian cancers.

Estrogenic compounds can be classified as agonists, which mimic the body's natural estrogen; or as antagonists, which bind to estrogen receptors, although the complex so formed is unable to attach to the DNA, and thus the transcription process is suppressed (Dechering et al., 2000; Kuiper et al., 1997). Both types tend to interfere with the normal cellular response to natural estrogens. Estrogen mimics can exhibit an estrogenic response in the absence of normal estrogens or enlarge the regulated response to estrogens.

### **1.1.2 Endocrine disrupting compounds in wastewater treatment processes**

Conventional wastewater treatment processes were originally designed for the removal of suspended solids, biodegradable organic material and pathogens (Metcalf & Eddy, 2003), but not trace organic chemicals which are present in municipal wastewater. More recently, wastewater treatment processes have been amended to remove nutrients such as nitrogen and phosphorus in order to reduce the potential of eutrophication in receiving bodies (Metcalf & Eddy, 2003). EDCs are commonly not of much concern during wastewater treatment because they are difficult to measure at levels commonly

present and because there is no federal regulation governing their levels in waters released to the environment (Kolpin et al., 2002; Purdom et al., 1994). The levels of EDCs in biosolids used as soil amendments are also unregulated (National Research Council., 2002). This is of particular concern for the more hydrophobic EDCs, which are expected to partition on solids during wastewater treatment.

Wastewater effluent discharges are major sources of EDCs in the receiving water bodies (Purdom et al., 1994). Estradiol ( $E_2$ ) and estrone ( $E_1$ ), and the synthetic hormone  $EE_2$ , which are incompletely degraded in the human body, are probably the predominant estrogenic compounds in wastewater in terms of their respective contributions to overall estrogenic activity (Desbrow et al., 1998; Drewes et al., 2005). Other EDCs present in WWTP receiving waters include detergent metabolites, plasticizers, polychlorinated biphenyls, polyaromatic hydrocarbons, pesticides and insecticides (Kolpin et al., 2002).

In humans and other animals, estrogens such as  $E_2$  and  $E_1$  are conjugated with glucuronide or sulfate functional groups in the liver. This step increases their solubility and facilitates their excretion in urine (Khanal et al., 2006). Conjugated estrogens do not possess biological activity and dissolve in aqueous solution at much higher concentrations than their unconjugated counterparts (Khanal et al., 2006). During the collection of wastewater or at the headwork of WWTPs, the water-soluble conjugated forms are frequently hydrolyzed by fecal bacteria that express glucuronidase or sulfatase, so that the glucuronide and sulfate moieties will be cleaved, restoring their original bioactive forms. In this way, hormonal activity can increase during wastewater treatment processes (D'Ascenzo et al., 2003; Ternes et al., 1999b).

Alkylphenol ethoxylates (APnEOs) are another important group of EDCs that are of concern during wastewater treatment. They are used in the formulation of a large variety of detergents, paints, lubricants, resins, and pesticides (Sharma et al., 2009). The annual worldwide production of APnEOs is approximately 500,000 metric tons (Sharma et al., 2009). APnEOs consist of an alkylphenol connected to a poly-ethoxylate chain. Of the total production of APnEOs, greater than 80% are nonylphenol ethoxylates (NPnEOs), with most of the remainder being octylphenol ethoxylates (OPnEOs) (Brooke et al., 2005).

Alkylphenols (APs) are degradation products of APnEOs that are formed during secondary wastewater treatment. They are significantly more estrogenic than their precursors (Laws et al., 2000). Based on the yeast estrogen screening assay (YES) results, however, APs are at least  $10^3$  times less estrogenic on a molar basis than are  $E_2$  and  $EE_2$ , even though they are more potent estrogens than their parent compounds (Routledge and Sumpter, 1997). Ethoxylate chains of APnEOs are normally not shortened during the wastewater collection and transport, but the chains are shortened or eliminated altogether by bacterial cleavage during anaerobic digestion of sludge (Ahel et al., 1994a). Both aerobic and anaerobic degradation lead to AP1EO and AP2EO. Aerobic biodegradation can also lead to the formation of alkylphenol carboxylates (APEC). With the shortage of the ethoxylate chain of the APs during the sludge digestion process, their hydrophobicity increases, and tend to partition on the organic-rich solid phase. Anaerobic treatment forms APs as the final products, which are important contributors to the estrogenic activity forming in the wastewater treatment process based on their high concentrations,

usually about  $10^3$  times more concentrated than  $E_2$  in municipal wastewater effluent (Teske and Arnold, 2008).

#### **1.1.2.1 Preliminary treatment**

Preliminary treatment processes, such as screening and grit removal, remove only a small part of the dissolved organic material from the influent. Screening removes objects such as rags, paper, plastics, and metals to prevent damage and clogging of downstream equipment and piping system. Grit removal can only be effective for relatively large particles that are primarily inorganic and not the main sources of EDCs entering municipal WWTPs (Metcalf & Eddy, 2003). Thus, preliminary treatment processes are not a major factor in the removal of EDCs or total estrogenic/androgenic activity during the wastewater treatment process.

#### **1.1.2.2 Adsorption of EDCs to Suspended Solids**

Due to low solubility and the moderately hydrophobic character of EDCs in water, the octanol-water distribution coefficients ( $D_{OW}$ ) of the estrogens of greatest environmental concern indicate that they should be removed from the liquid phase by adsorption onto suspended solids and solids removal during conventional wastewater treatment.

This assumption was confirmed by the detection of high concentrations of estrogens in dewatered and digested sludge (49 ng/g of  $E_2$  and 37 ng/g of  $E_1$ ) (Matsui et al., 2000). In the same way,  $D_{OW}$  values suggest that these substances are hydrophobic in character and should associate preferentially with organic matter. Adsorption of  $E_2$  and  $EE_2$  to activated sludge from WWTPs was investigated (Clara et al., 2004), and results showed they had a high affinity for the sorbent, despite of the use of very high initial

concentrations to overcome experimental difficulties. During a contact period of 24 hours, no difference between the adsorption to activated and inactivated (no biological activity by adding mercury sulphate) sludge could be detected, which suggests that adsorption to the sludge is one important elimination pathway for this type of compounds.

The properties of APnEOs and APs have important consequences for their fate during wastewater treatment. In wastewater treatment, the degradation of APnEOs produces metabolites that differ in hydrophobicity from the parent compounds, so that changes in the distributions of APnEOs and their metabolites between liquid and solid phases are expected during wastewater treatment processes. Long-chain APnEOs are more hydrophilic and will tend to stay in the liquid phase. When these compounds are digested in either aerobic or anaerobic digestion processes, the ethoxylate chains are shortened by microbes, and the products are more hydrophobic, as well as more estrogenic (see above). The final digestion products, such as AP1EO, AP2EO and APs, are preferentially sorbed onto the biosolid fraction when the digestion process is finished. Similarly, the levels of these compounds increased in river sediments that are exposed to effluent from WWTPs (T. Isobe et al., 2001; Shang et al., 1999).

If contaminants are adsorbed on sludges, they are likely to accumulate in biosolids. In this case, the application of digested sludge as fertilizer or soil conditioner on agricultural fields may result in the contamination of soil and ground water (Ternes et al., 1999b). If contaminants are dissolved or associated with dissolved natural organics or even colloidal organics, then they may be transported with relative ease through wastewater treatment plant (Schäfer et al., 2002). In addition to sorption onto particles as

a removal mechanism, these compounds may partition into the non-polar fat and lipid material in raw sewage according to mass balance data for EDCs in wastewater treatment plants (Ternes et al., 1999b).

### **1.1.2.3 Biodegradation of EDCs**

#### **1.1.2.3.1 Aerobic digestion**

Biological treatment is frequently used worldwide to stabilize municipal wastewater prior to discharge to surface waters. The activated sludge process is the most commonly used biological treatment method. Johnson and Sumpter (2001) showed that not all estrogenic compounds were completely broken down or converted to biomass. Based on several field studies, it appears that E<sub>2</sub>, E<sub>3</sub>, and EE<sub>2</sub> can be removed during conventional wastewater treatment, although E<sub>1</sub> appears to be less efficiently treated and results are more variable (Johnson et al., 2000; Lee and Liu, 2002; Ternes et al., 1999b). In four out of thirty WWTPs sampled, the concentration of E<sub>1</sub> in effluent was higher than that of influent (Baronti et al., 2000). This could have been due to the biodegradation of E<sub>2</sub> by sewage bacteria, which initiate biochemical attack at the D ring of E<sub>2</sub>, leading to the formation of the major metabolite E<sub>1</sub> (Johnson and Sumpter, 2001; Lee and Liu, 2002).

Conversely, because estrogens are excreted in the urine of humans and other animals as glucuronide or sulfated conjugates (Andreolini et al., 1987; Layton et al., 2000), deconjugation during wastewater treatment could have accounted for the increase in E<sub>1</sub> concentrations. D'Ascenzo et al. (2003) confirmed through laboratory biodegradation tests that glucuronated estrogens are readily deconjugated in domestic

wastewater, presumably due to the large amount of the  $\beta$ -glucuronidase enzyme produced by fecal bacteria (e.g., *Escherichia coli*).

Shi et al. (2004) observed the biodegradation of natural and synthetic estrogens in mixed nitrifying activated sludge (NAS) and pure cultures of the ammonia-oxidizing bacterium *Nitrosomonas europaea*. Results confirmed that NAS significantly degrades both natural and synthetic estrogens. Among the estrogens, E<sub>2</sub> was the most rapidly degraded. Although ammonia-oxidizing bacteria, such as *N. europaea* contribute to estrogen degradation, NAS degrades estrogens and their degradation intermediates while *N. europaea* only degrades estrogens with no further transformation of their intermediates. The biological transformation of trace organic compounds also depends on the age of the activated sludge (Ternes et al., 2004). A trace pollutant's affinity to the bacterial enzymes in the activated sludge influences the pollutant's transformation or decomposition. Biological decomposition of various compounds increases with the age of the sludge. The bacterial population may become more diversified with increasing sludge age (longer residence time of microorganisms), possibly because slow-growing bacteria eventually reach relevant numbers (Carballa et al., 2004). The conjugated form of EE<sub>2</sub> makes it more soluble in wastewater than EE<sub>2</sub> itself. In experiments with activated sludge, earlier research shows that EE<sub>2</sub> will not degrade under non-nitrifying conditions, while nitrifying sludge oxidized EE<sub>2</sub> to more hydrophobic compounds (Vader et al., 2000).

Surfactants such as APnEOs are biodegraded aerobically by the abbreviating their polyethoxylate chains. However, mineralization of these compounds is more difficult due to the presence of the highly branched alkyl group on the phenol ring (Abd-Allah and

Srorr, 1998), and chain abbreviation can provide more estrogenic daughter compounds such as NP and OP (Ahel et al., 1994a). These products can be further degraded via ring cleavage, leading to compound mineralization during extended aerobic treatment, as in aerobic digestion (Ahel et al., 1994a).

#### **1.1.2.3.2 Anaerobic digestion**

NPnEOs are present at much higher concentrations ( $\mu\text{g}/\text{kg}$  to  $\text{mg}/\text{kg}$ ) in sludges than most other EDCs, and as such they can be more easily quantified and studied. In fact, there have been relatively few investigation to confirm the fates of other EDCs during anaerobic process, despite the fact that  $E_1$ ,  $E_2$ ,  $E_3$  and  $EE_2$  account for the majority of estrogenic activity in wastewater (Desbrow et al., 1998).

During anaerobic digestion process, the polyethoxylated chains of NPnEOs are shortened, and more hydrophobic metabolites are produced, such as NP1EO, NP2EO and NP (Brunner et al., 1988; Giger et al., 1984). Similar results for NPnEOs were obtained under controlled (lab) conditions as well (McNamara et al., 2012; Minamiyama et al., 2006). However, NP resists biodegradation under anaerobic conditions, leading to its accumulation during anaerobic digestion (Ejlertsson et al., 1998; Giger et al., 1984). As discussed previously, NP1EO, NP2EO and NP tend to absorb on biosolids, so that their accumulation in sludge is a potentially critical pathway to environmental exposure (Ahel et al., 1994a). The use of biosolids as soil amendments may eventually threaten the natural environment and even human or animal health through the consumption of agricultural products. The potential role of aerobic digestion in the destruction of short-chain APnEOs has been noted (McNamara et al., 2012).

Field-scale measurements around anaerobic reactors treating sludge concluded that hormones are poorly attenuated under methanogenic conditions (Joss et al., 2004; Khanal et al., 2006). These results agreed with laboratory experiments performed under simplified anaerobic conditions (Czajka and Londry, 2006). Under anaerobic conditions,  $E_2$  were transformed into  $E_1$ , which was also partially removed. In the same study,  $EE_2$  was not degraded. Similarly, De Mes et al. (2008) observed no reduction in the spiked concentration of  $EE_2$  during a bench-scale simulation of anaerobic digestion. On the other hand, Carballa et al. (2006) reported that the concentrations of  $E_1$ ,  $E_2$  and  $EE_2$  were reduced by almost an order of magnitude during the mesophilic (37°C) anaerobic digestion of sludge. Similar fractional reductions for these compounds were reported under thermophilic conditions (55°C).

In the first part of the study, sequential anaerobic-aerobic digestion was simulated in bench scale reactors. Independent variables for the aerobic digestion step included sludge retention time, temperature, and aeration rate. Dependent variables included estrogenic activity, androgenic activity and the concentrations of major endocrine disrupting compounds (such as NP and OP).

## **1.2 EDCs and estrogenic activity in waters and sediments of the Santa Cruz River in Tucson, AZ**

WWTPs are considered to be the major source of EDCs in the environment (Khanal et al., 2006). The presence and fates of EDCs and estrogenic activity in surface water and sediments of Santa Cruz River, an effluent-dependent stream in the vicinity of Tucson, AZ will be reported on, as will the concentrations of trace organics of

wastewater origin in monitoring wells proximate to the river downstream from two major wastewater outfalls.

### **1.2.1 Water shortage problem**

Tucson is located in the northern semi-arid reaches of the Sonoran Desert in eastern Pima County, Arizona. Few surface streams in Arizona contain perennial flow. Although at one point, the Santa Cruz River, which flow northwest through the Tucson area was among those, the river reach south of Tucson is now dry except for periods of exceptional rainfall, and in the reach extending northwest of Tucson the river is effluent dependent. Until the early 1990s, the Tucson community relied almost exclusively on groundwater to meet water demand (Pepper et al., 2004). However, due to rapid population growth and associated water demand, the groundwater system transitioned from an approximate state of equilibrium to one of accelerating depletion. Before the arrival of Colorado River water in the Tucson area via the Central Arizona Project (CAP) canal, groundwater overdraft was commonplace, and in some proximate areas, the local water table fell as much as 200 feet over the preceding 50 years.

Full utilization of the Tucson regional allotment of CAP water from Colorado River (~260,000 acre-feet per year (AFY) [ $3.2 \times 10^8 \text{ m}^3 \cdot \text{yr}^{-1}$ ]) will not indefinitely satisfy area water requirements without again resorting to ground water mining or initiating the widespread recovery and reuse of wastewater. It is almost inevitable that water reclamation/reuse will become an increasingly important part of regional water resources portfolio. The natural rate of ground water replenishment in the hydrologic unit surrounding Tucson (the Tucson Active Management Area or TAMA) has been estimated at 60,000 AFY ( $7.5 \times 10^7 \text{ m}^3 \cdot \text{yr}^{-1}$ ). Total water use in the TAMA is now about

400,000 AFY ( $4.9 \times 10^8 \text{ m}^3 \cdot \text{yr}^{-1}$ ) (UNESCO-IHP, 2009). Clearly, a sustainable water supply in the Tucson area depends on incorporation of imported water sources and broadening of reclaimed water use in the regional water resources portfolio (Figure 1.3).

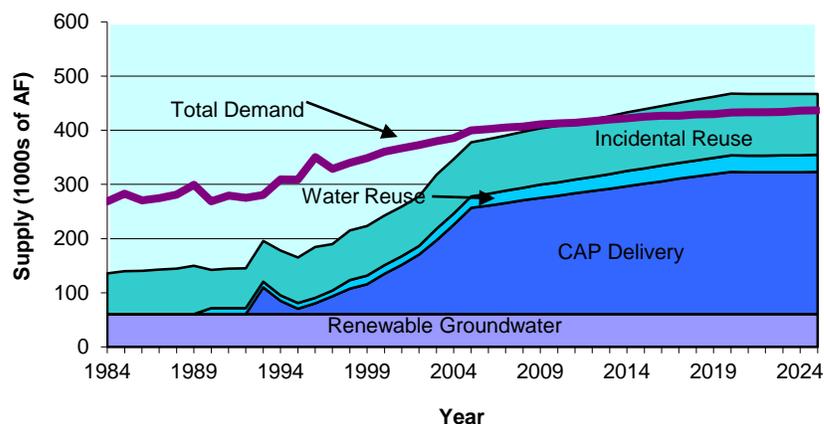


Figure 1.3 Annual water demand and supply projections for the TAMA. The uppermost (purple) line represents the total rate of water demand. Shaded regions correspond to the four major components of supply. The difference between the demand and total supply curves represents mining or storage. Data were derived from the ADWR 3<sup>rd</sup> Management Plan (Arizona Department of Water Resources, 1999).

Although the regional CAP allotment is projected to expand to  $3.2 \times 10^8 \text{ m}^3 \cdot \text{yr}^{-1}$ , competition among western states for the water source of Colorado River suggests that increases much beyond that figure are unlikely in the future. Only the quantity of reclaimed or reused wastewater seems to be a solution.

### 1.2.2 Santa Cruz River

The Santa Cruz River (SCR) is 184 miles (296 km) long and has its headwaters in the high intermontane grasslands of the San Rafael Valley to the southeast of Patagonia, Arizona (Figure 1.4). It flows southward through northern Sonora, Mexico before returning to southern Arizona. The river is effluent dependent in the Tucson area.



Figure 1.4 Map of the Santa Cruz River Watershed.

The wastewater effluent generated in the Tucson area, mainly from Agua Nueva Wastewater Reclamation Facility (WRF) and Tres Rios WRF, is either recharged at Sweetwater Recharge Facility (SRF), Tucson, AZ or discharged to the Santa Cruz River (Quanrud et al., 2004). Both the Santa Cruz River and the SRF obtain chlorinated/dechlorinated secondary effluent from Pima County's Agua Nueva WRF. About five miles (7.8 km) downstream from the Agua Nueva WRF outfall, chlorinated/dechlorinated secondary effluent from Pima County's Tres Rios WRF (activated sludge process) is discharged directly into the SCR. The Tres Rios WRF treats an average of 40,000 acre-feet ( $4.93 \times 10^7 \text{ m}^3$ ) per year. Except during large rainfall events, effluent is the only water source in the effluent-dominated SCR channel. Galyean (1996) estimated that 90% of the effluent discharged to the Santa Cruz infiltrated within the 25-mile (40 km) reach of the Santa Cruz River below the Agua Nueva WRF. Below that point, the river is again dry during perhaps 11 months of the year. Altogether, ~20%

of the water treated at the two regional plants is reused for landscape irrigation via a managed water reclamation and distribution system. The remainder is discharged to the SCR, where most of it infiltrates in the river basin and reenters the regional groundwater resource. From the perspective of water quality, this is an interesting situation since water from the regional aquifer is withdrawn from wells, chlorinated and distributed for potable use without further treatment. Travel time on the river (as estimated from data of USGS gauging stations) is about 9 hours during dry weather flow.

### **1.2.3 EDCs in Santa Cruz River**

According to the discussion in previous section, many chemicals used in domestic households are persistent and they can pass through conventional wastewater treatment process. Among these, trace organic chemicals, including EDCs, are of particular interest to environmental engineers and the public. In a 2002 nationwide survey (Kolpin et al., 2002), the USGS measured some of the highest in-stream concentrations of EDCs in the effluent-dependent lower SCR near Tucson. Nonylphenol in the Santa Cruz River was present at the highest concentration measured in the country (about 40 µg/L at Cortaro Road). Since flow in the Tucson area reaches of the Santa Cruz River is entirely wastewater effluent most of the year, it is expected to contain estrogens and estrogenic activity comparable to that of secondary effluent from the Agua Nueva WRF and Tres Rios WRF.

Because some of the important EDCs have moderate to high hydrophobicities (i.e., high *D<sub>ow</sub>* values) and tend to partition onto the solid phase, suspended solids and the sediments are potentially significant contributors to overall EDC exposure in the Santa Cruz River. Trace organics may accumulate in riverbed sediments due to

deposition of suspended solids as well as by sorption during effluent infiltration in the riverbed.

A possible mechanism for the degradation of trace organics in surface waters is photodegradation (Lin and Reinhard, 2005; Wols and Hofman-Caris, 2012). Photodegradation occurs via both direct and indirect photolysis. In direct photolysis, the absorption of light is by the chemical itself, eventually leading to compound transformation. Indirect photolysis occurs when light energy is absorbed by photosensitizers, the most important of which, in the SCR, are probably dissolved organic matter (DOM) of wastewater origin in the river. The contribution of this mechanism to EDC destruction is investigated in the research described here.

To better understand seasonal variations of the fates of EDCs and the changes of estrogenicity loadings to the effluent-dependent lower SCR, in the second part of the dissertation, a series of water and sediment samples were taken before and after periods of heavy rainfall in Tucson. The data are used to assess the fate of trace organic compounds and the importance of specific fate mechanisms in surface waters and sediments of the SCR. The study could provide substantial new information on transport and fate of selected emerging organic contaminants including estrogens in the lower SCR.

### **1.3 Cultivation of *Nannochloropsis salina* in wastewater for biomass and lipid production**

The purpose of this portion of the dissertation work was to evaluate the feasibility of using treated municipal wastewater and/or nutrient rich side streams derived from

municipal wastewater treatment as a source of water and nutrients for algal growth. The rationale for this line of investigation follows.

There is fairly good evidence that the water and nutrient demands of a viable algal biofuels industry would prove prohibitively large unless waste sources can be utilized to advantage. There is both water and nutrient value in conventionally treated secondary effluent that can be exploited. A representative value for total available nitrogen in secondary treated municipal wastewater effluent (absent denitrification) is around 30 mg/L as N (Metcalf & Eddy, 2003). Similarly, a representative total phosphate concentration was estimated at 3 mg/L. The nutrient value of effluent suggests that wastewater can be used to defray, although perhaps not eliminate, nutrient costs for large scale growth of algae.

Similarly, wastewater effluent can reduce the demand of an algae industry for more conventional potable water resources—those that can be prepared for potable use at much lower cost. The value of effluent used for algal growth is highly case specific, and any figure that is used for the purpose of economic analysis should be reexamined in a local or regional context. In an economic sense, use of reclaimed water displaces the most expensive water source necessary to satisfy potable demand. It follows that the economic value of such practice (reclaimed water for algal growth) is equal to the marginal cost of water resource development for potable needs. In southern Arizona, for example, that value is at least as high as the cost of purchasing Colorado River water from the Central Arizona Project, estimated here at \$140/acre-foot and probably much higher since water to western cities is heavily subsidized through federal water projects. The USBR (2011)

estimated the marginal cost of potable water resources development for the lower Colorado River basin at \$1000-\$4000 per acre foot.

Use of treated wastewater for algal growth can also greatly reduce the cost of wastewater treatment in cases such as Tucson's, where nutrient removal is a condition for effluent discharge to a surface water. Pima County has just retrofitted Tucson's major wastewater treatment facilities for nitrogen and phosphorus control. The capital cost is on the order of a billion dollars.

When water is discharged from algae-producing facilities, certain water quality characteristics may actually be improved. Nutrient removal is only the most obvious example. It is also possible that algae will take up metals and trace organics (such as EDCs) that survive conventional wastewater treatment. Water quality changes, however, may not be entirely favorable since salts will inevitably accumulate due to evapotranspiration during algal growth. Consequently, water quality changes that arise during algal growth will be established experimentally, but will not be taken as a potential economic benefit at this point.

The primary liability associated with use of reclaimed water for algal growth is associated with toxicity. If specific heavy metals accumulate to toxic levels as a consequence of evapotranspiration, for example, use of reclaimed water could eventually result in diminished growth rate and greater cost for achievement of output objectives.

To provide a framework and rationale for related experiments in this area, a simplified model for algal growth and resource (nutrient and water) use is provided in the following chapter. Also the economics and feasibility of an algal biodiesel industry based on wastewater resources are exposed to the extent possible. The metals and wastewater

toxicities are evaluated for the cultivation of the specific algal species, and the compositions of the algal fatty acids are compared for algae grown in the presence and absence of wastewater constituents.

#### **1.4 Role of ammonia and carbonates in scavenging hydroxyl radicals generated during megasonic irradiation of wafer cleaning solutions**

Advanced oxidation processes (AOPs) are being used in water/wastewater treatment for disinfection and to polish waters containing trace organic pollutants. Usually during the process, highly reactive chemical radicals, such as hydroxyl radicals ( $\bullet\text{OH}$ ) are being formed, and they can react indiscriminately with residual organics in wastewater effluent.

A better understanding of the hydroxyl radical generation and scavenging can be achieved by reviewing traditional AOPs. Research shows that  $\bullet\text{OH}$  has a high reduction potential of 2.86V (Muruganandham et al., 2014), thereby making it a highly oxidizing species. This property has been used by typical AOPs to degrade organic effluents present in waste water. In addition to effluents, wastewater often contains compounds such as carbonates, bicarbonates, alcohols that are known to scavenge  $\bullet\text{OH}$ . Although scavenging of  $\bullet\text{OH}$  is undesirable in AOPs but they could serve as a harbinger to identifying optimum cleaning conditions in the semiconductor industry. To date, only a limited number of studies have been carried out for determining the role of carbonate scavenging hydroxyl radicals for cleaning (Keswani et al., 2014).

In integrated circuit (IC) production industry, particulate contamination is a major cause for yield loss (Kern, 1990). The removal of particulates and residues from wafer

and photomask surfaces is typically achieved by megasonic cleaning. Acoustic streaming and cavitation play an integral role in particle removal when exposed to a megasonic field. It is of importance to be able to characterize cavitation as it not only helps understand the mechanism behind particle removal, but also contributes to feature damage. Cavitation manifests itself in two forms, namely, stable and transient. Since damage is predominantly caused by the presence of transient cavities, the scope of discussion will be limited to understanding its effects. Implosion of transient cavities results in the generation of temperatures and pressures of about 5,000-10,000 K and a few hundred bars, respectively (Suslick et al., 1999). Damage could occur as an effect of the resulting high pressures in the vicinity of the features and enhanced by etching due to generation of highly reactive radicals such as  $\bullet\text{OH}$  at high temperatures. This study focuses on developing effective means to identify megasonic cleaning solutions that could result in scavenging of  $\bullet\text{OH}$ , thereby possibly reducing damage to the fragile features.

Several earlier studies have shown that dissolved carbon dioxide ( $\text{CO}_2$ ) in DI water significantly suppresses the intensity of sonoluminescence (SL) (Kumari et al., 2011a). Increasing concentrations of  $\text{CO}_2$  (aq) in air containing DI water not only caused a significant decrease in the number of breakages to line structures during megasonic cleaning, but also decreased lengths of the line breakages at all power densities up to 2.94  $\text{W}/\text{cm}^2$  (Kumari et al., 2011b). The ability of dissolved  $\text{CO}_2$  to protect against feature damage correlated well with its ability to suppress SL in megasonic irradiated DI water. The issue with using soluble gases such as  $\text{CO}_2$  is that it shifts the pH towards more

acidic conditions, while typical cleaning solutions in the semiconductor industry are alkaline in nature.

A novel chemical method has been established based on the results for *in situ* release of CO<sub>2</sub> from aqueous NH<sub>4</sub>HCO<sub>3</sub> solutions through pH shift (Kumari et al., 2014). The study suggested that about 130 ppm of CO<sub>2</sub> (aq) generated by NH<sub>4</sub>HCO<sub>3</sub> was sufficient for complete suppression of SL generation in water at an acoustic frequency ~1 MHz and the effect was independent of power density. Further investigations on cavitation behavior were conducted by adding NH<sub>4</sub>HCO<sub>3</sub>/NH<sub>4</sub>OH into megasonic cleaning system (Kumari et al., 2014). This system serves as a novel means for controllable generation of CO<sub>2</sub> (aq) over an extended pH range (4.0–8.5) and shows complete suppression of SL signal. Therefore this method could possibly be used as an effective means to reduce wafer damage without compromising megasonic cleaning efficiency.

Nowadays, several methods are being used for characterizing the intensity of transient cavitation, which include the detection the SL signal, potassium iodide or ferrous ions dosimetry, electron spin resonance spectroscopy and fluorescence spectrometry (Kohno et al., 2011; Rooze et al., 2013). Terephthalic acid dosimetry has been selected to measure the generation rate of hydroxyl radicals in alkaline cleaning solutions at acoustic frequencies of ~ 1 MHz (Keswani et al., 2014) in our study.

Chemical formulations used for megasonic cleaning process typically contain hydroxides, peroxides, carbonates and others which can affect particle removal efficiency and feature damage. Previous research work (Han et al., 2013; Kumari et al., 2011a; Kumari et al., 2014) indicates that cavitation behavior has been characterized only in a

limited range of power density ( $\sim 0.1$  to  $2.94 \text{ W/cm}^2$ ). The aim of this work was to investigate the role of ammonia/carbonate/bicarbonate system generated from different chemical combinations in regulating the oxidation power of megasonic cleaning solutions through the scavenging of hydroxyl radicals in power density range of  $2\text{-}8 \text{ W/cm}^2$ , by using the detection method of terephthalate dosimeter.

## 1.5 References

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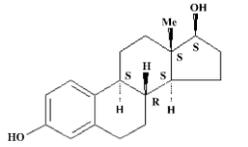
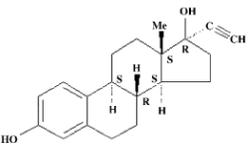
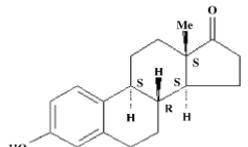
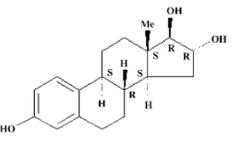
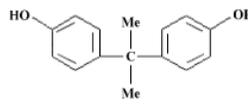
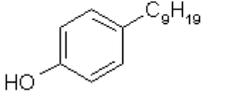
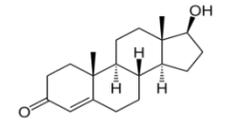
## CHAPTER 2 FATE OF ESTROGENS/ANDROGENS DURING SLUDGE DIGESTION

### 2.1 Introduction

Population growth, urbanization, industrialization, and associated changes in agricultural and other land-use practices have led to changes in the necessity for and objectives of municipal wastewater treatment. As never before, growth and environmental objectives stand in conflict. During the past decade, there was much progress toward understanding the occurrence, fate and toxicology of trace organic pollutants that enter the environment in treated wastewater. More than any other groups of trace organics, endocrine disrupting compounds (EDCs) have attracted the attention of environmental professionals and the public.

EDCs interfere with normal endocrine function via several mechanisms, affecting the health, growth, and reproduction of a wide range of organisms. Estrogenic chemicals include (i) natural steroidal estrogens that are produced by most vertebrates and even some plants, e.g., estrone ( $E_1$ ),  $17\beta$ -estradiol ( $E_2$ ) and estriol ( $E_3$ ), and (ii) anthropogenic compounds, such as ethinylestradiol ( $EE_2$ ), the main active ingredient in oral contraceptives, and alkylphenols (APs), which are the degradation products of nonionic surfactants. The chemical structures and parameters of representative EDCs are provided (Table 2.1).

Table 2.1 Properties of common wastewater EDCs.

Compound	Formula	Structure	Molecular Weight	Water solubility (20°C) (mg/l)	Log Dow at pH=7*[1]	Relative potency (YES <sup>S</sup> bioassay)
17β-Estradiol (β E <sub>2</sub> )	C <sub>18</sub> H <sub>24</sub> O <sub>2</sub>		272.39	13	4.15	1.0
17α-Ethinylestradiol (EE <sub>2</sub> )	C <sub>20</sub> H <sub>24</sub> O <sub>2</sub>		296.40	4.8	4.11	1.32
Estrone (E <sub>1</sub> )	C <sub>18</sub> H <sub>22</sub> O <sub>2</sub>		270.37	13	3.62	0.20
Estriol (E <sub>3</sub> )	C <sub>18</sub> H <sub>24</sub> O <sub>3</sub>		288.39	13	2.53	0.01
Bisphenol A (BPA)	C <sub>15</sub> H <sub>16</sub> O <sub>2</sub>		228.29	300	3.64	-
4- <i>n</i> -nonylphenol (4- <i>n</i> -NP)	C <sub>15</sub> H <sub>24</sub> O		220.35	5.43	6.14	2×10 <sup>-4</sup>
Testosterone	C <sub>19</sub> H <sub>28</sub> O <sub>2</sub>		288.42	23.4	3.18	-

\*- Log Dow: pH-dependent *n*-octanol–water distribution ratio. \$- Yeast Estrogen Screen.

[1]- data from SciFinder database.

Octanol-water partition coefficient ( $K_{ow}$ ) is defined by US EPA as “a coefficient representing the ratio of the solubility of a compound in octanol (a non-polar solvent) to its solubility in water (a polar solvent)” (<http://www.epa.gov/oust/cat/tumgloss.htm>). The higher the  $K_{ow}$  value, the more non-polar or hydrophobic the compound.  $\log K_{ow}$  is generally used as a relative indicator of the tendency of an organic compound to sorb to soil organics.

The octanol-water distribution ratio ( $D_{ow}$ ) is a very important parameter for studying the fate of trace organic compounds in the environment, and it is closely correlated with  $K_{ow}$ . For acids, octanol-water partition coefficients at pH 7 ( $D_{ow}$ ), were calculated for compounds using the following expression,

$$D_{ow} = K_{ow} \times \frac{1 + 10^{-pKa}}{1 + 10^{pH-pKa}}$$

The above equation reflects an assumption that the ionized species does not partition into the octanol phase at either the pH for which  $D_{ow}$  is estimated (usually pH 7), or at pH 0, which is the reference value for  $K_{ow}$ .

$\log D_{ow}$  values are generally inversely related to aqueous solubility and (among compounds of a single class) directly proportional to molecular weight. This parameter is especially important in wastewater treatment, because hydrophobicity determines the degree to which compounds are likely to be found in the liquid phase and, to an extent, present in wastewater effluent. Conversely, hydrophobic compounds tend to be absorbed onto suspended solids and sludges that are destined for land disposal (Ahel et al., 1994a; Drewes et al., 2005).

Wells (2006) defined low  $K_{ow}$  values as  $<500$  ( $\log K_{ow} < 2.7$ ), midrange values as  $500 \leq K_{ow} \leq 1000$  ( $2.7 \leq \log K_{ow} \leq 3.0$ ), and high  $K_{ow}$  values as greater than 1000 ( $\log$

$K_{ow} > 3.0$ ). Other researchers found it useful to consider compounds with a log  $K_{ow}$  less than 1 as highly hydrophilic, and compounds with a log  $K_{ow}$  above 3 to 4 as highly hydrophobic (Kirk et al., 2002; Patureau and Trably, 2006).

More than 70,000 chemicals are believed to have endocrine disruptive potential (Gillesby and Zacharewski, 1998). Per Table 2.1, most of the EDCs have high  $D_{ow}$  values. The hypothesis that EDCs will partition to sediments or soils is supported by modeling data ( $E_2$ ,  $E_1$  and  $EE_2$ ), and results from previous research (Lai et al., 2000).

Estrogenic compounds enter the environment in a variety of ways. The majority of the natural estrogens come from the feces and urine of humans and livestock. Food and many consumer goods contain or degrade to yield EDCs (Song et al., 1999; Stroheker et al., 2003). In addition, environmental EDCs are among the by-products created by such processes as the incineration of biological materials, industrial waste and chlorine bleaching of paper products. Agriculture can also be a source of EDCs. Based on extensive research, the main source of EDCs in surface waters, oceans and sediments appears to be the discharge of the effluents and sludge from sewage treatment plants (Chang et al., 2011).

The presence of wastewater-derived contaminants in surface water can result from inadequate wastewater treatment by publicly owned treatment works. Once released to surface waters, bioactive chemicals can exert the activity for which they were originally intended on non-target organisms, including biota and even humans. Furthermore, they can resist drinking water treatment processes. There is ample motivation to optimize wastewater treatment processes for their removal.

EDCs can cause reproductive disturbances among humans and wildlife at exceptionally low concentrations. An early report speculated that chronic exposure to EDCs is responsible for decreased sperm counts in human males (Sharpe and Skakkebaek, 1993). Also there have been numerous observations of abnormal sexual development in wildlife. For example, research by Jobling et al. (1996) suggested that male fish located downstream of wastewater treatment plant outfalls sometimes produce vitellogenin, a precursor protein for egg yolk production. Histological alterations, abnormal ratios of sex steroids, and fish having both sex-specific gonads are appearing with greater frequency. Physiological studies have demonstrated that even very subtle effects on the endocrine system can result in changes in growth, development, reproduction or behavior that can affect the organism itself, or even their offspring (Kidd et al., 2007). Purdom et al. (1994) showed that 10 ng/L EE<sub>2</sub> in water can induce the synthesis of vitellogenin in immature cyprinids, or in rainbow trout at concentration as low as 0.1 ng/L EE<sub>2</sub>. In another study, life-cycle exposure of the fathead minnow to low concentrations of EE<sub>2</sub> produced concentration-related impacts on growth, development, sexual development, and reproductive health as low as 1.0 ng/L (Lange et al., 2001).

EDCs can interact with endocrine systems to disrupt normal endocrine functions in several ways: As hormonal agonists they bind to endocrine receptors, inducing estrogen-dependent cell responses. EDCs can also bind to natural hormonal receptors and prevent normal responses (antagonism), interfere with the way natural hormones are synthesized, metabolized, or regulate (Filali-Meknassi et al., 2004). A number of human organs and tissues show estrogen receptor expression including the brain, immune system, cardiovascular system, lung, mammary glands, liver, kidneys, reproductive tract,

adipose tissue, and bone (Mueller, 2004). The tissues affected by EDCs can develop diseases including cancers (Clemons and Goss, 2001; Yager and Davidson, 2006).

Controlling sources of EDCs is very difficult. Both humans and animals excrete natural estrogens. Women excrete 10-100  $\mu\text{g}$  of estrogens per day, depending on the phase of their menstrual cycle (Tyler et al., 1998). During pregnancy, excretion of estrogens is much higher than normal, reaching up to 6000  $\mu\text{g}/\text{d}$  (Johnson and Williams, 2004). Also some of the surfactants and pharmaceuticals that contribute to wastewater estrogenic activity are broadly used throughout the population and in agriculture. The cost for finding product alternatives is sometimes high, so that source control measures cannot be rapidly implemented.

The increasing numbers and improved performance of wastewater treatment plants worldwide result in the production of large amounts of sludge and biosolids (Gron, 2007), much of which is landfilled or added as fertilizer on agricultural land. Of the more than 7 million tons of sewage sludge produced in the United States in 2004, about 50% was applied to land as fertilizer or soil amendment, and 45% was disposed of in landfills or as landfill cover (North East Biosolids and Residuals Association, 2007). Since sewage sludge is a by-product of wastewater treatment, it inevitably contains a wide range of trace organic contaminants, particularly those that are relatively hydrophobic. EDCs such as  $\text{E}_2$ ,  $\text{EE}_2$ , alkylphenols (APs) and alkylphenol polyethoxylates (APnEOs) are major contributors to the estrogenic activity present in sewage sludge (Brunner et al., 1988; Lee et al., 2004; Petrović and Barceló, 2000).

Removal mechanisms and biotransformation pathways are different for each group of estrogenic compounds. Removal strategies are shaped by compound-specific

physical and chemical properties such as hydrophobicity and biodegradability. The majority of steroidal estrogens in human urine and animal feces are present as sulfate/glucuronide conjugates, most of which are bio-transformed to their free estrogens by fecal bacteria in wastewater collection systems before they enter municipal wastewater treatment plants (WWTPs) (Johnson and Sumpter, 2001). However, a portion of the overall deconjugation process occurs during sludge treatment (Ternes et al., 1999a; Ternes et al., 1999b). Both biological transformations and sorption mechanisms contribute to the removal of estrogens during sewage treatment. Bacteria from activated sludge and nitrifying activated sludge can biodegrade estrogens effectively (Lee and Liu, 2002; Muller et al., 2010; Ternes et al., 1999b). However, because estrogens are fairly hydrophobic (Table 2.1), sorption results in the transfer of some estrogens to solids that are produced and separated during conventional wastewater treatment (Andersen et al., 2003; Braga et al., 2005; Muller et al., 2008).

Only a few studies have dealt with the treatment of estrogens in sludge, essentially due to the difficulty of measuring these compounds at  $\text{ng g}^{-1}$  levels in a complex matrix such as sludge (Kuster et al., 2004). Muller et al. (2010) found that hydrophobic properties of the estrogens influence their respective tendencies to adsorb to wastewater sludge. In a few cases, estriol ( $\text{E}_3$ ) was present at concentrations higher than other natural estrogens in wastewater but relatively low concentrations in sludge. Plant-scale anaerobic digestion is fairly inefficient at removing most estrogenic compounds (<40%) and the concentrations of  $\text{E}_2$  and  $\text{EE}_2$  can actually increase during final dewatering (Muller et al., 2010).

Nitrifying wastewater treatment plants have proven to be relatively efficient for removal of estrogenic activity (Clara et al., 2005a; Clara et al., 2005b; Svenson et al., 2003). Efficient degradation of EE<sub>2</sub>, for example, is possible using nitrifying activated sludge (Vader et al., 2000). In pure culture, *Nitrosomonas europaea*, a well-studied nitrifying bacterium, is capable of co-oxidizing other organic compounds in the presence of ammonia nitrogen (Chang et al., 2002; Keener and Arp, 1994). Shi et al. (2004) observed the biodegradation of estrogens in nitrifying mixed cultures derived from activated sludge and in pure cultures of *N. europaea*. Therefore, removal of some EDCs is highly probable when WWTPs are designed and operated for nitrification. The biological transformation of trace organic compounds also depends on the age of the activated sludge (Ternes et al., 2004). A trace pollutant's affinity to the bacterial enzymes in the activated sludge influences the pollutant's transformation or decomposition. Biological decomposition of various compounds increases with the age of the sludge. The bacterial community may become more diversified with increasing sludge age (longer residence time of microorganisms), possibly because slow-growing bacteria eventually reach relevant numbers (Carballa et al., 2004). Biotransformation is likely due to bacterial cometabolic activity, since steroidal compounds are not present in high enough concentrations to support substantial microbial growth or to motivate species selection in mixed cultures.

Alkylphenols (APs) are the estrogenic metabolites of alkylphenol polyethoxylate (APnEOs). About 200,000 tons of APnEOs are produced each year in the United States, and they are widely used as detergents; wetting and dewetting agents in paper and textile processing; and for the production of paints, resins, cleaners and degreasers (Soares et al.,

2008). There are reports on the degradation of nonylphenol (NP) by various biochemical mechanisms (Soares et al., 2003; Tanghe et al., 1998). In wastewater treatment process, APnEOs follow a complex biodegradation pathway (Ahel et al., 1994a) illustrated in Figure 2.1. The initial step in the degenerative pathway is to sequentially remove ethoxy groups from respective ethoxylate chains. Then, depending on whether aerobic or anaerobic conditions prevail, the short-chain APnEOs are carboxylated to make alkyphenoxy acetic acid (AP1EC or AP2EC), or ethoxy groups are further eliminated to produce AP1EO or AP2EO. Under anaerobic conditions, only the latter process is possible, and further anaerobic biotransformations are slow because of the presence of the benzene ring and their limited water solubility of these compounds.

Hydrophobicity of APnEOs increases with decreasing ethoxy chain length. As a result, shorter chain compounds tend to sorb onto the solid phase, and compounds with longer chains are more likely to appear in final effluent of WWTPs (John et al., 2000). Nonylphenol and octylphenol are more hydrophobic than APnEOs, with log  $D_{ow}$  values of 6.14 for 4-*n*-NP, and 5.63 for 4-OP. For this reason, APs, especially 4-*n*-NP, sorb to sludges, making the compounds more resistant to biodegradation (Tomohiko Isobe et al., 2001).

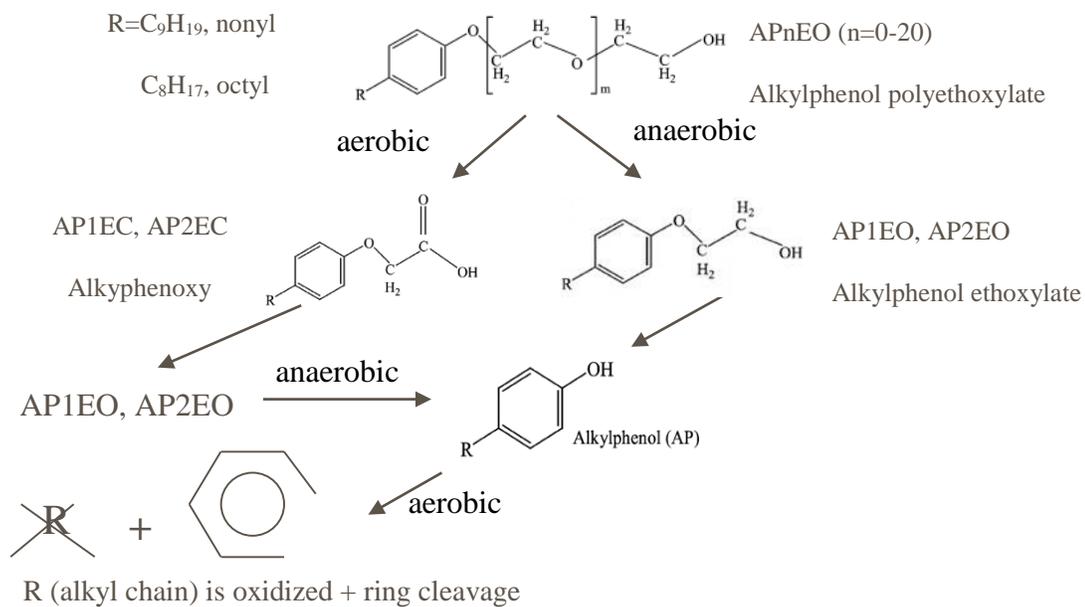


Figure 2.1 Aerobic and anaerobic biotransformation pathways of APnEOs. (Ahel et al., 1994a)

## 2.2 Objectives

The main goals for this research project are to:

1. Evaluate the combined effects of sequential anaerobic/aerobic digestion on the estrogenic/androgenic activity of sludge using bench-scale bioreactors to simulate full scale, two-stage digestion.
2. Monitor concentration changes of some major EDCs during two-stage digestion.
3. Find operational conditions (temperature, oxygen loading rate, sludge retention time) that favor removal of estrogenic/androgenic activity during aerobic sludge digestion.

## 2.3 Hypotheses

The following hypotheses motivate the experimental design:

1. Major EDCs tend to sorb to the solid phase in sludge suspensions. This hypothesis is suggested by the relative hydrophobicity of EDCs (Williams et al., 2003; Yu et al., 2004), so that the investigation of estrogenic/androgenic activity and the concentration changes of EDCs in sludge solids is a major objective.
2. Estrogenic/androgenic activity and concentration of EDCs of the sludge liquid phase are negligible compared with their counterparts in the solid phase.
3. Estrogenic activity in raw sludge increases during anaerobic digestion due to deconjugation of natural estrogens or removal of ethoxy groups from alkylphenol ethoxylates, but decreases during subsequent aerobic digestion steps. This hypothesis is suggested by research on alkylphenols and natural estrogen degradation pathways (D'Ascenzo et al., 2003; Ying et al., 2002b).

4. EDC removal and removal of total estrogenic/androgenic activity during aerobic sludge digestion will be a function of operational variables such as temperature, solid retention time (SRT) and air flow rate.
5. Concentrations of NP and OP will increase during anaerobic digestion and decrease during aerobic digestion.
6. Nitrification/denitrification during aerobic digestion can modulate changes in the concentrations of EDCs, thus altering the estrogenic/androgenic activity of the sludge solid.

## **2.4 Experimental**

### **2.4.1 Simulation of sequential anaerobic and aerobic sludge digestion**

Bench-scale anaerobic and aerobic digesters were operated sequentially. Mixed sludges (primary and waste activated sludge,  $v:v=1:1$ ) from the Tres Rios Wastewater Reclamation Facility (WRF) were provided to the anaerobic digester. Anaerobically digested sludge was then further digested aerobically. Changes in estrogenic activity, androgenic activity and concentrations of specific estrogens during simulated anaerobic/aerobic digestion were measured. Steady conditions were reestablished following each operational discontinuity — it was anticipated that a period of time on the order of three hydraulic retention times was necessary before renewing data acquisition. Steady performance was evaluated based on total solids (TS), volatile solids (VS) concentration of all reactors, as well as methane production rate for the anaerobic digester.

Operational conditions that were varied in the aerobic digesters included temperature (25-55°C), hydraulic retention time (HRT, 2.5-20 days) and air flow rate (1-

2 liter/min). In order to compare the estrogenic activity changes for different aerobic digestion retention times, four aerobic digesters with different temperatures were set up in parallel, and maintained at 25, 35, 45 and 55°C, respectively. Airflow rate of 1.0L/min and 2.0L/min were applied. SRTs used for these digesters were 20-, 10-, 5- and 2.5-day in this study. The twenty-day maximum SRT was selected representing retention time most frequently employed in the aerobic digestion practice (20-30 days) (Metcalf & Eddy, 2003).

#### **2.4.2 Measuring estrogenic/androgenic activity and major EDCs concentrations**

In order to determine the effects of sequential digestion on micropollutant concentrations, sludge samples from the reactors were periodically extracted for analysis. Estrogenic/androgenic activity was measured using an *in vitro* biological assay, for which protocols were developed previously in our lab. NP and OP were directly analyzed by gas chromatography–mass spectrometry (GC-MS). There are several studies on the analytical methods for E<sub>2</sub>, EE<sub>2</sub> and other estrogens, using GC-MS methods to measure derivatized compounds in the sample (e. g., (Hibberd et al., 2009; Labadie and Budzinski, 2005; Li et al., 2012)) and liquid chromatography–mass spectrometry (LC-MS) can be used to analyze the extracted samples directly (Heisterkamp et al., 2004). To the extent possible, the alkylphenols were measured and correlated to whole-sample estrogenic activity in order to establish the primary sources of estrogenic activity at each stage of treatment.

#### **2.4.3 The link between nitrification and EDC degradation**

Nitrification is expected during aerobic digestion, at least under high SRT/high air flow conditions. To investigate the possible link between nitrification and EDC transformations, nitrogen transformation data were developed and examined for

correlations with EDC loss or reduction in total estrogenic activity. This work may also provide better ideas on setting the operation configurations of sequential anaerobic/aerobic digesters that are used to manage the efflux of trace organic contaminants into the environment with biosolids that are used to condition soils.

## **2.5 Materials and methods**

### **2.5.1 Sampling site and procedure**

Raw sludge from the Tres Rios WRF was used as the microbial seed to feed the lab-scale anaerobic digester. As needed, a mixture of primary and wasted activated sludge (1:1, v/v) was collected from Tres Rios WRF (25 million gallon per day treatment capacity) in Tucson. Samples were collected in 4-liter amber borosilicate glass bottles (previously muffled at 550°C for 5 h) and transported back to the lab on ice. Subsequently, raw sludge was stored at 4°C pending use as reactor feed.

### **2.5.2 Semi-continuous reactor setup for sludge treatment**

Different process configurations were evaluated using lab-scale semi-continuous stirred tank reactors (semi-CSTR). A 20-liter Microgen Bench-Top Fermenter (Model SF-116, New Brunswick Scientific) was used to simulate anaerobic digestion (Figure 2.2). It was filled with 16 L of raw sludge, with a 4 L headspace, and it was airtight except for a biogas outlet on top of the reactor that was chilled to avoid water loss. This gas exhaust port was connected to a gas collector, which was filled with sodium hydroxide solution (NaOH 2% w/w in water), in order to react with CO<sub>2</sub> generated from the anaerobic digester. Methane (CH<sub>4</sub>) production was then measured by displacing liquid in the gas collector. HRT (same as SRT) for the anaerobic digestion process was 20 days. Reactor pH was maintained at 6.3 ~ 7.8 by the addition of alkali (2M NaOH) or hydrochloric acid (1M HCl). Every two days, 1.6 L of anaerobically digested sludge (ten percent of the sludge) was withdrawn and replaced with the same volume of mixed undigested raw sludge (semi-batch operation).

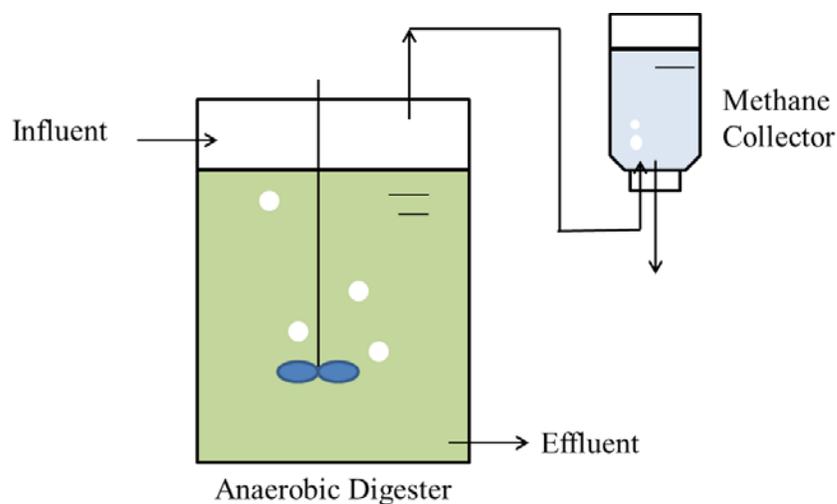


Figure 2.2. Schematic of the anaerobic digester used to treat sludge.

Four 2-L Virtis Omni-Culture™ Fermenters (glass reactors) were set up in parallel in the chemical fume hood to act as semi-batch aerobic digesters following anaerobic digestion. They were set up at different temperatures and they were fed with ambient air at selected flow rates (Figure 2.3). Anaerobically digested sludge from the bench-scale fermenter was added to the aerobic digesters to initiate experiments at 25, 35, 45 and 55°C. Samples were collected periodically from both the anaerobic and aerobic digesters for analysis when the reactors were stabilized. HRTs ranging from 2.5 to 20 days, and air flow rates of 1.0 or 2.0 L/min were used for aerobic digestion in order to evaluate the impact of different process configurations on the removal of sludge estrogenic/androgenic activity and target EDCs. The pH of these aerobic digesters was maintained at 6.3 ~ 7.8 as well.

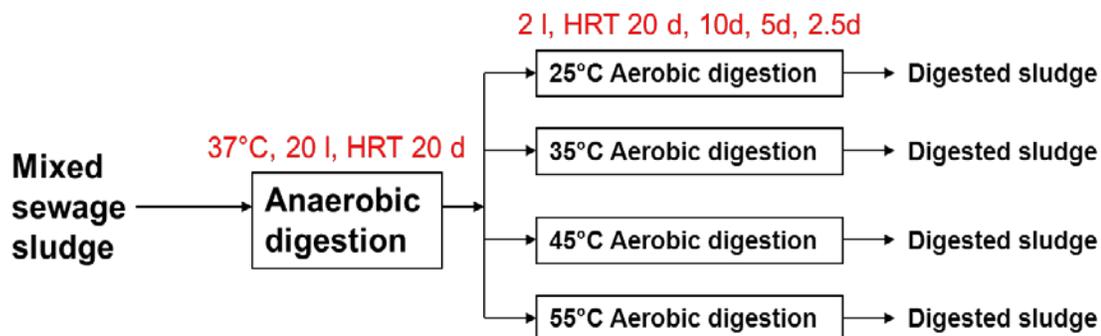


Figure 2.3 Schematic of the reactor systems used to treat sludge.

### 2.5.3 Sample characterization

Usually, a startup time of three or more SRTs was required for reactor re-stabilization following changes to the operating parameters (SRT and air flow rate). Periodically pH and solid removal were measured to evaluate reactor performance and stability. Total solids were determined gravimetrically after a 48-h drying period (10 mL samples) at 60°C (VWR Sheldon 1350G Oven). Volatile solids were measured gravimetrically (by difference) after burning the samples in Thermolyne 6000 Muffle Furnace (Thermolyne Corporation) at 550°C for 5 h, following the standard method (American Public Health Association, 2005). The rate of CH<sub>4</sub> gas production was another indicator for the anaerobic digester stability. Once the reactors were stable, samples were taken and stored at -20°C pending extraction and analyses.

Methane concentration from the headspace of the anaerobic digester was determined by gas chromatography (GC) using an HP5890 series II system (Agilent Technologies, Palo Alto, CA) equipped with a flame ionization detector (FID). The GC was fitted with a DB-FFAP column (J&W Scientific, Palo Alto, CA). The temperature of the column, injector port and detector was 140, 180 and 250°C, respectively. The carrier gas was helium at a flow rate of 9.3 mL min<sup>-1</sup> and a split flow of 32.4 mL min<sup>-1</sup>. Samples

for measuring methane content (100  $\mu\text{L}$ ) in the headspace were injected into the GC column using a pressure-lock gas syringe.

#### **2.5.4 Sample extraction and cleanup methods**

Fractionation of sludge samples was carried out to separate estrogenic compounds from sources of toxicity in sample concentrates. Separation of organic compounds based on hydrophobicity is widely used in chemical analyses. In the reactor sample extraction, organic compounds were separated from most polar to the least by eluting materials retained on C-18 resin cartridge using a series of eluent that increased in methanol/water ratio ( $v/v$ ) (20%, 50%, 80% and 100% methanol).

Raw sludge and digested sludge samples were centrifuged using a Beckman J2-21 centrifuge for 30 min, at 17,000 RCF (relative centrifugal force) to separate the liquid and solid phases. The liquid phase was then filtered using Whatman GF/F glass-fiber filters (pore size 0.7 $\mu\text{m}$ ), and 10ml was frozen for the analysis of nitrogen species (nitrite, nitrate and ammonium).

Sludge solids were dried to constant weight in VWR Sheldon 1350G Oven at 60°C. After drying, solids were homogenized with a mortar and pestle. A microwave accelerated reaction system (MARS, CEM Corporation, Matthews, North Carolina) was used to extract hydrophobic compounds from the resultant solid phase. One and a half gram samples of dried sludge were placed in Xpress<sup>®</sup> Teflon vessels along with 15 mL methanol. The microwave apparatus was operated at 70°C for 60 min. Upon completion, the solvent was collected, and Teflon vessel was rinsed with two 5 mL aliquots of methanol, which were collected and transferred to the same borosilicate glass vial. The

extract was dried under a gentle stream of nitrogen to 2 mL, and resuspended in 200 mL NanoPure water.

The mixture was processed through reverse-phase C-18 disks (octadecylsilane, Empore™, 3M, Eagan, Minnesota). The purpose of this step was to clean the extract by removing particulates and hydrophilic compounds. The hydrophobic compounds were retained on the C-18 disc throughout the extraction process. When dictated by our experimental objectives, adsorbed organics were separated via differential elution in a series of 15 mL methanol/water mixtures consisting of 20, 50, 80 and 100% (v/v) methanol. Half of the eluate fractions were combined for the chemical analysis of EDCs such as NP, OP, E<sub>2</sub> and EE<sub>2</sub>. The other half of the eluate fractions were evaporated to near dryness under gentle N<sub>2</sub> flow in a nitrogen evaporator system (Oa-Sys™ and N-Evap™ 112, Organomation Associates, Inc., Berlin, Massachusetts) and rehydrated in sufficient NanoPure water for yeast estrogen/androgen screen bioassay.

### **2.5.5 Yeast estrogen/androgen screen bioassay**

The yeast estrogen screen (YES) and yeast androgen screen (YAS) assays were used for the analysis of estrogenic and androgenic activity in samples. These bioassays are based on the procedure developed by Routledge and Sumpter (1996). Each sample concentrate was 2-fold serially diluted across 10 wells of a 96-well micro-titer plate (Corning Life Sciences). In positive controls, refreshed cells were added to serial dilutions of standard hormone preparations (EE<sub>2</sub> or testosterone plus NanoPure water) in the 96-well plates. Final hormone concentrations in positive controls ranged from  $3.9 \times 10^{-11} \text{M}$  to  $2.0 \times 10^{-8} \text{M}$  for YES (EE<sub>2</sub>), and from  $2.0 \times 10^{-10} \text{M}$  to  $1.0 \times 10^{-7} \text{M}$  for YAS (testosterone). Cell suspensions were then incubated for 24 hours at 32°C in the presence

of sample dilutions or standard preparations for additional growth and hormone-dependent gene expression. After that, 50 $\mu$ L of cycloheximide/CPRG (chlorophenol red  $\beta$ -D-galactopyranoside) solution was added to each test well. Following an additional 24-hour incubation at 32°C, color was measured ( $A_{570}-R \times A_{630}$ ) using a UV-VIS micro-plate reader (ELx800, Bio-tek Instruments, Inc.), in which R (usually around 1.1) is the ratio of optical densities (OD) at 570nm and 630nm in negative controls. Turbidity of each well (OD<sub>630</sub>) was also recorded in order to study the cytotoxicity effects of the samples.

Results of representative positive control experiments are provided as in Figure 2.4. The hormone-dependent absorbance data are expressed as a percentage of the highest value obtained in each test. EC<sub>20</sub> value is the concentration of EE<sub>2</sub> or testosterone that produced 20% of the maximal test response. Positive control curves were generated and EC<sub>20</sub> values were estimated with each batch of samples analyzed in order to minimize variation arising from the condition of the test species and growth medium components.

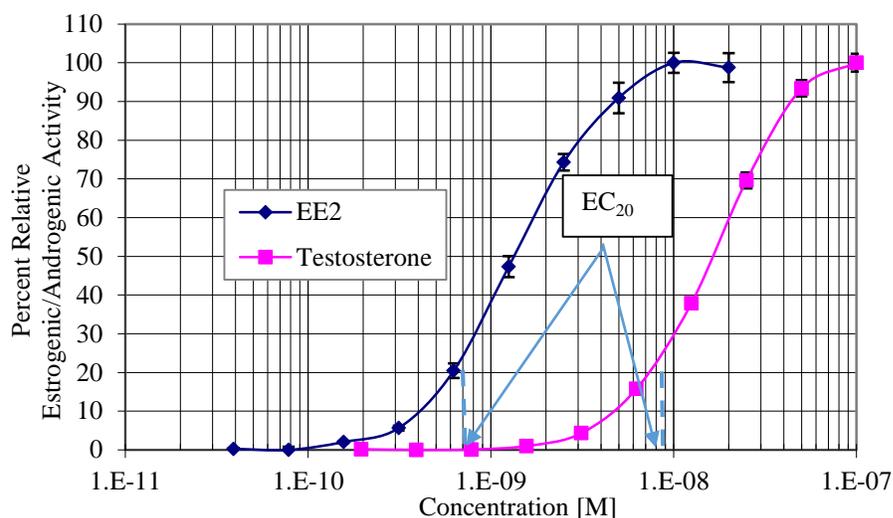


Figure 2.4. Positive control response curve for the YES and YAS reporter-gene bioassay. Error bars represent  $\pm$  one standard deviation in test response (n=8).

To convert estrogenic activities in samples to equivalent EE<sub>2</sub> concentrations (EEQ), respective EC<sub>20</sub> values were divided by sample concentration factors necessary to produce the level of response indicated, e.g. 20% of the highest response observed in the positive control.

$$EEQ(nM) = \frac{EC_{20}(nM)}{CF}$$

A practical quantitation limit (PQL) for the assays was arbitrarily adopted as the EC<sub>20</sub> value for EE<sub>2</sub> divided by the sample concentration factor in the YES procedure (normally 200).

Below the PQL was the method detection limit (MDL), defined as the EE<sub>2</sub> concentration that produced a test response equal to 3 times the standard deviation in ( $A_{570} - R \times A_{630}$ ) in hormone-free (blank) test wells, again divided by the sample concentration factor. That is,

$$MDL(nM) = \frac{EA_{3 \times SD} \times EC_{20}(nM)}{EA_{20} \times CF}$$

where EA<sub>3×SD</sub> is the percent estrogenic activity of three-standard deviation values above background, EA<sub>20</sub> is 20%, and CF is the sample concentration factor. For instance, a typical EC<sub>20</sub> value for EE<sub>2</sub> is 0.2 nM, with a standard background noise of 3% relative estrogenic activity (EA<sub>3×SD</sub>), and the environmental samples are commonly concentrated 200-fold (CF). This equates to MDL of 0.15 pM.

The limit of quantitation (LOQ) is the EE<sub>2</sub> equivalent concentration at which environmental samples can be assigned a numerical value according to equation of calculating EEQ. This is defined as the EC<sub>20</sub> concentration divided by the maximum sample concentration:

$$LOQ(nM) = \frac{EC_{20}(nM)}{CF_{max}}$$

Most often, the estrogenic activity of the sample lies between the detection limit and the quantitation limit. In this case, the estrogenic activity used is that which produced the greatest response at a given sample concentration factor. This value is labeled as an estimate in the following chapters:

$$EEQ(nM, estimate) = \frac{EA_{sample} \times EC_{20}(nM)}{EA_{20} \times CF}$$

If the sample reached EC<sub>10</sub> (EA<sub>sample</sub> = 10%) at a concentration factor of 200×, and the EC<sub>20</sub> for EE<sub>2</sub> is 0.2 nM, the estimated EEQ is 0.5 pM.

For estimation of the androgenic activity and method sensitivity, testosterone equivalents are calculated in the same manner. And the same principles apply.

### 2.5.5.1 Quality control of the YES assay

In order to convert the measurement of the optical density (A<sub>570-R</sub>×A<sub>630</sub>) into estrogenic activity, the value of each single sample was compared with EC<sub>20</sub> from the standard curve of that batch of YES bioassay. One reason is that the value of EC<sub>20</sub> was stable through the bioassay process. The other reason for using this number was based on the actual estrogenic activities of some samples (A<sub>570-R</sub>×A<sub>630</sub>), which could only reach a small number of optical densities, and the highest percentage it could get was between zero and twenty percent when showed up on the dose-response curve. EC<sub>20</sub> was used as the base for estimating the actual estrogenic activities under such circumstances, for it is relatively close to the measured number than EC<sub>50</sub> values (Figure 2.5).

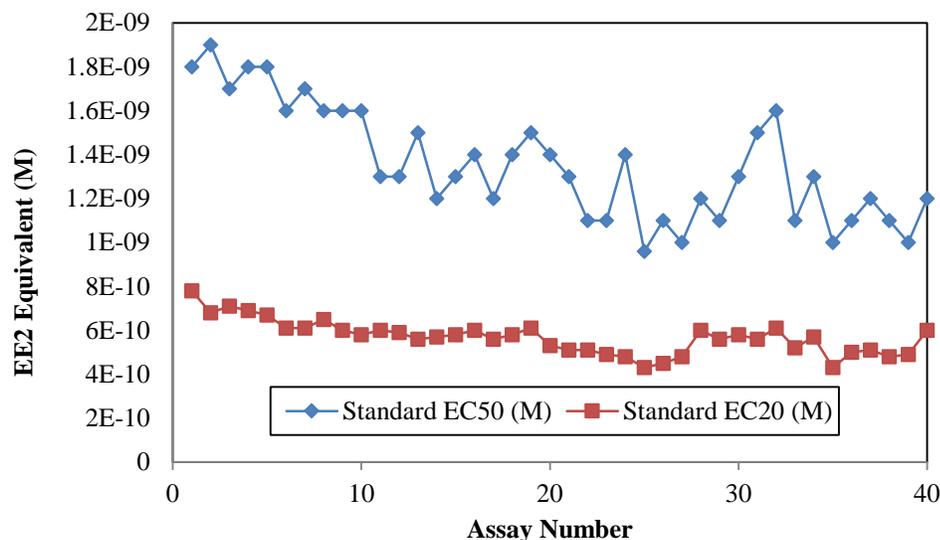


Figure 2.5 Comparison of EC<sub>20</sub> and EC<sub>50</sub> values of the YES bioassays.

### 2.5.6 Gas chromatography–mass spectroscopy

An Agilent 6890 gas chromatograph coupled to an Agilent 5973 mass-selective detector (Agilent Technologies, USA) was employed for the GC-MS analysis of NP and OP. Separation of compounds was accomplished on a fused silica column (HP5-MS, 30 m × 0.25 mm i.d., 0.25 μm film thickness, Agilent Technology). The oven temperature program was maintained at 90°C for 1 min, then rising at 15°C min<sup>-1</sup> to 220°C and held for 2 min. The temperature was then ramped at 15°C min<sup>-1</sup> to 240°C, and 10°C min<sup>-1</sup> to 280°C. Helium was used as the gas carrier at a constant flow of 1 mL min<sup>-1</sup>. The inlet, transfer line and ion source were set at 250, 280 and 230°C, respectively. Sample injection (1 μL) was in splitless mode. The mass detector was operated in the electron ionization mode using the selected ion monitoring (SIM) method. The quantification ions used were as follows: mass-to-charge ratio (m/z) 135 for NP, m/z 107 for OP. The limit of detection (LOD) and limit of quantification (LOQ) were defined as three times and ten times the standard deviation of the blank methanol solution (Komori et al., 2004).

### **2.5.7 Ion chromatography**

Levels of nitrate, nitrite and ammonium were indicators of nitrification in reactors. They were determined by ion chromatography (IC) using Dionex-200 system (Dionex, Sunnydale, CA). Nitrite and nitrate are separated using isocratic conditions with an Ion Pac AS16 analytical column (4×250 mm). The eluent used was composed of 10 mM NaOH. Ammonium ion was separated on an Ion Pac CS16 analytical column (5×250 mm), with eluent composed of 30 mM methanesulfonic acid (MSA). The injection volume was 25 µl and the eluent flow-rate was 1.0 ml/min. Analytes were detected by suppressed conductivity detection.

### **2.5.8 Volatile fatty acids analytical method**

The concentration of volatile fatty acids (VFA, i.e, acetic, propionic, butyric and valeric acids) in liquid samples was determined by gas chromatography using an HP5290 Series II system (Agilent Technologies, CA) equipped with a flame ionization detector (FID). DB-FFAP capillary column (J&W Scientific, Palo Alto, CA) was used for the GC-FID analysis. The temperature of the column, the injector port and the detector were 140, 180 and 275°C, respectively. Samples collected for VFA measurements were centrifuged at 10,000 rpm for 10 min, and the liquid phase was collected and stored at -20°C until analysis.

### **2.5.9 HPLC fractionation of reactor samples**

After microwave-accelerated extraction of 1.5 gram solid samples, the extract in solvent were collected, dried down and resuspended in 0.5 ml methanol. Liquid chromatographic separation of the purified sediment sample fractions was performed using the Agilent 1200 HPLC system (Agilent Technologies, USA). The column used for

fractionation was a Phenomenex Luna Phenyl-hexyl column (5  $\mu\text{m}$ , 150 mm  $\times$  4.60 mm, Phenomenex, Torrance, CA, USA). Samples (100 $\mu\text{L}$ ) were injected into the HPLC system.

A mobile phase gradient elution program with solvent A (acetonitrile) and solvent B (water) was applied at a starting flow rate of 0.2 ml/min, and gradually increased to 0.4 ml/min over 15 min. The gradient program of the solvents started at 5% acetonitrile (95% water), staying constant for five minutes, and then increased linearly to 100% acetonitrile (no water) during minutes 5 to 40. This condition was held until 60 min. From 60 min to 70 min, the elution program returned to its initial condition. The column temperature was maintained at 30°C. A Bio-Rad fraction collector (Model 2110) was connected to the HPLC system for sample separation at two-minute intervals. Fractionated samples were collected in pre-muffled glass test tubes and stored at 4°C for further biological screening assay and chemical analysis.

#### **2.5.10 Identification of EDCs by liquid chromatography tandem mass spectrometry**

EDC target analytes were measured by ultra performance liquid chromatography – quadrupole time-of-flight mass spectrometry (UPLC-QToFMS) using negative mode electrospray ionization. The equipment consisted of an Ultimate 3000 Rapid Separation Liquid Chromatography System (RSLC; Dionex, Sunnyvale, CA) with UV detection controlled by DCMS Link, and a TripleTOF mass spectrometer controlled by Analyst TF 1.6 software (AB Sciex, Framingham, MA) with a calibrant delivery system to maintain accurate mass. Electrospray Ionization conditions were as follows: ionspray voltage floating 4500 V, nitrogen curtain gas at 30 and ion source gases 1 and 2 at 50, interface heater temperature at 700 °C, and declustering potential at 80 V.

Liquid chromatographic separation of auto injected samples (10  $\mu$ l) was achieved using a Phenomenex Luna Phenyl-hexyl column (5  $\mu$ m, 150 mm  $\times$  4.60 mm, Phenomenex, Torrance, CA, USA) with a reversed phase binary mobile phase solvent system consisting of aqueous and acetonitrile phases at a constant flow rate of 0.25 ml  $\text{min}^{-1}$ : 0-5 min isocratic 5% LCMS grade J.T. Baker acetonitrile (VWR Intl.) and 95% LCMS grade Omnisolve water (VWR Intl.), 5-40 min increasing to 99% acetonitrile and 1% water, 40-60 min isocratic 99% acetonitrile and 1% water, and then back to isocratic 5% acetonitrile and 95% water from 61-66 min.

Information-dependent acquisition (IDA) triggered product ion experiments were run as follows: a 0.1 s accumulation time survey scan over mass range 35–1000 followed by 6 dependent product ion scans each with 0.1 s accumulation times over mass range 35–1000 (total cycle time 0.75 s) with a collision energy spread from 20-50, dynamic background subtraction, 20 counts per second threshold, and high resolution mode.

Semi-quantitative target analyte amounts were determined by integrating parent ion chromatograms with AB Sciex MultiQuant 2.1 software. A 0.01 mass unit window was used to extract a chromatogram centered around the following  $m/z$  values: 4-octylphenol (205.1598), 4-*n*-nonylphenol (219.1753), estrone (269.1548), 17 alpha-ethynylestradiol (295.1703).

### **2.5.11 Statistical methods**

In this project, Student's t-test statistical method was used to compare between two sets of data if they are significantly different from each other.

Two-way ANOVA (analysis of variance between groups) followed by Tukey's post-hoc test method was applied to statistical assessment of relationships between the

dependent variables such as estrogenic/androgenic activity changes, concentrations of EDCs, and nitrogen species changes, with the independent variables, i.e., settings of the aerobic digesters (temperature, SRT and airflow rate).

It is considered that the difference is significant at 0.05 level ( $p < 0.05$ ) in all cases study. Statistical Package for the Social Sciences software (IBM SPSS, Version 20) was employed for the data analytical work in this study.

## **2.6 Results and discussion**

Full-scale sludge digestion was simulated using bench-scale sequential anaerobic/aerobic bioreactors. The primary independent variables in this study were HRT, temperature and air flow rate during aerobic digestion.

### **2.6.1 The performance of the sludge digesters**

The stability of anaerobic digester and aerobic digesters in each different stage was evaluated using the data from periodic measurements of pH, total solid (TS) and volatile solid (VS). For the stability of anaerobic digester, methane production rate was also measured.

#### **2.6.1.1 Monitoring pH of the bioreactors**

Anaerobic and aerobic digestions were operated under neutral pH conditions (pH 6.3 ~ 7.8). In anaerobic digestion, methanogenic activity will be slower if the pH is less than 6.3 or higher than 7.8, inhibiting biogas production (Leitão et al., 2006). The optimal aerobic digestion process requires the same pH range as well (Ugwuanyi et al., 2005). Immediately after starting the anaerobic digester, the pH of the system varied rapidly, sometimes decreasing slightly below 6.5. The pH was adjusted to the acceptable range by adding sodium bicarbonate in the digester, after which it became steady.

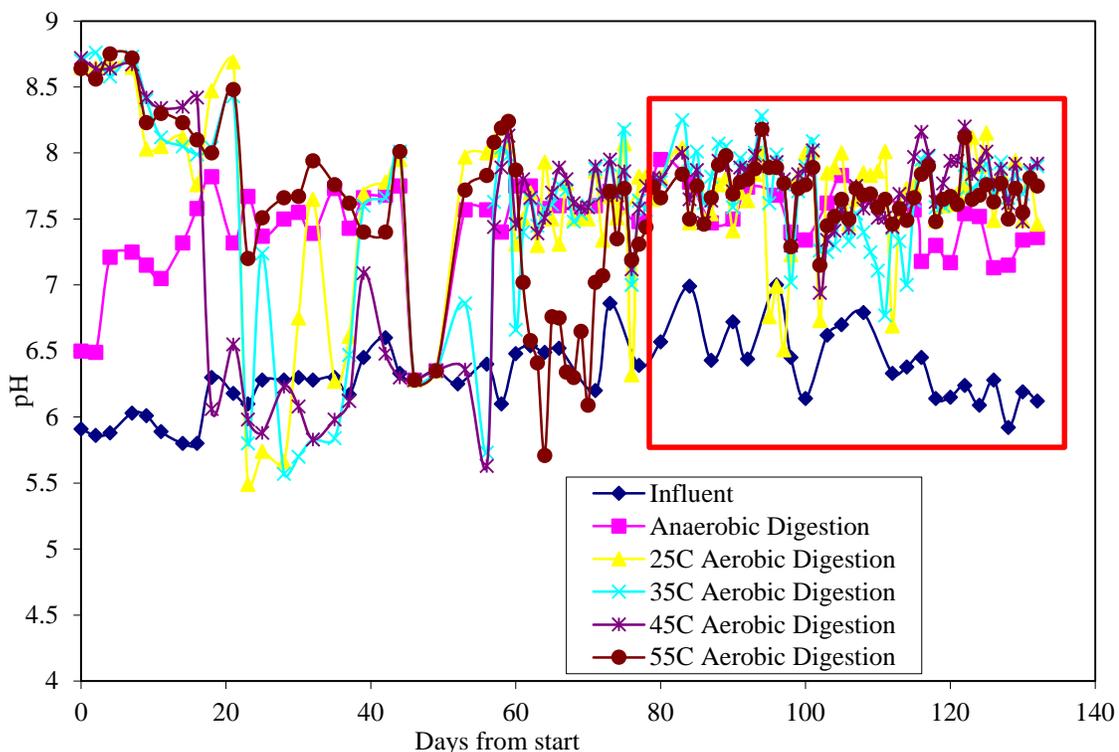


Figure 2.6 Monitoring of pH changes for the sludge digestion reactors. (HRT= 20 days for both the anaerobic and aerobic digesters)

Figure 2.6 illustrates the startup stage of the anaerobic/aerobic digesters for the 20-day HRT. Stabilization of both anaerobic and aerobic digesters took about 60 days, or about three times the sludge age in these digesters. The stability of the anaerobic process is important since balance between several microbial populations must be maintained. The hydrolysis and fermentation phases have the most robust organisms allowing them to maintain activity over a relatively broad range of pH. During loading, however, acid production can overwhelm the buffering capacity, lowering the pH outside acceptable limits for the methanogens. When this happens, methane production stops and the acid levels rise to the tolerance level of the acid formers.

Since the HRT of the anaerobic digester was constant at 20 days in this project, only the HRT of the aerobic digesters was changed for the study. After each stage, an

additional three hydraulic retention times was required for aerobic reactors restabilization (results not show), before the pH of the aerobic digesters was again steady.

### 2.6.1.2 Sludge solid reduction during digestion process

Another criterion for the stabilization of the anaerobic and aerobic digestion processes was the stabilization of solid content in the reactors. Total solid and volatile solid contents were measured semi-continuously during periods of reactor operation (Figure 2.7).

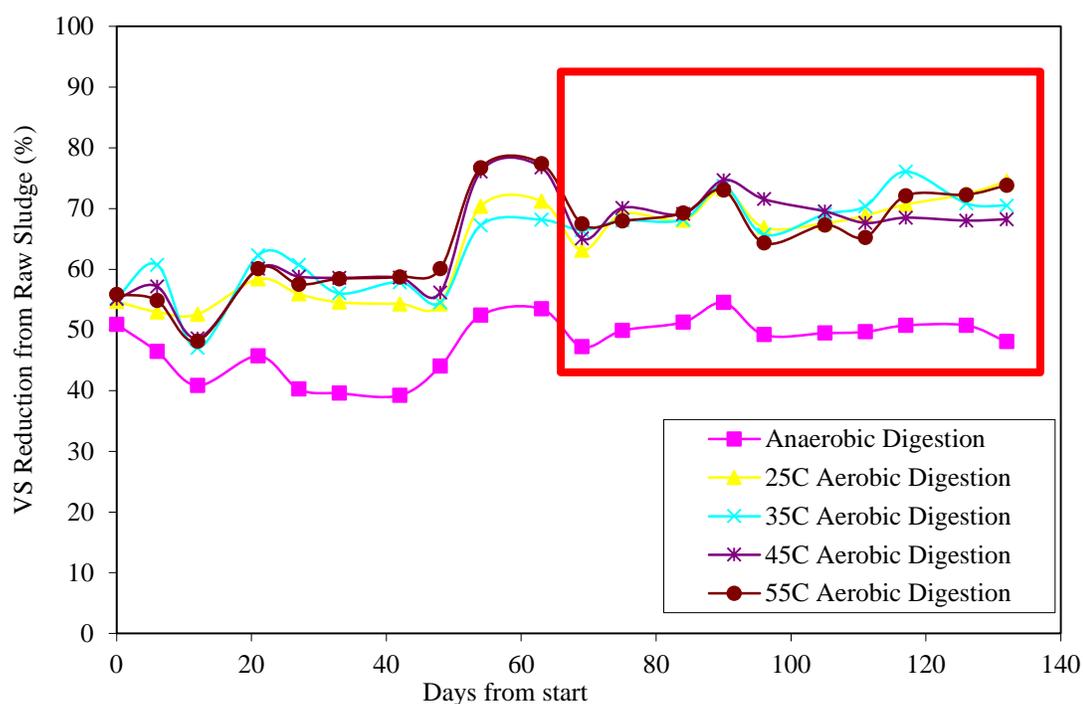


Figure 2.7 Monitoring of volatile solid reduction ratio for the sludge digestion reactors. The period of stable operation is highlighted. (HRT= 20 days for both the anaerobic and aerobic digesters)

Per Section 2.6.1.1, stabilization of anaerobic/aerobic digesters required a period equal to  $\sim 3 \times$  the HRT to reestablish steady conditions following perturbations. This conclusion was verified from the measured solids reduction before and after digestion.

Anaerobic digestion reduced volatile solids by ~50%. During aerobic digestion process, another portion of the original sludge volatile solid content was destroyed (Figure 2.8).

Two-way ANOVA statistical test was used for determine which independent variable (HRT or temperature) was important for the reduction of solid in the aerobic digestion process. The results suggest that HRT of the aerobic digesters plays an important role in the reduction of the VS content of the sludge, VS reduction ratios of 20-day and 10-day HRT are statistically larger than that of 5-day and 2.5-day HRT, but for 20-day and 10-day HRT, there are no differences between their reduction ratios. When comparing the effects of temperature, differences among reactor performances at temperatures from 25-55°C were not statistically significant by using the same statistical method.

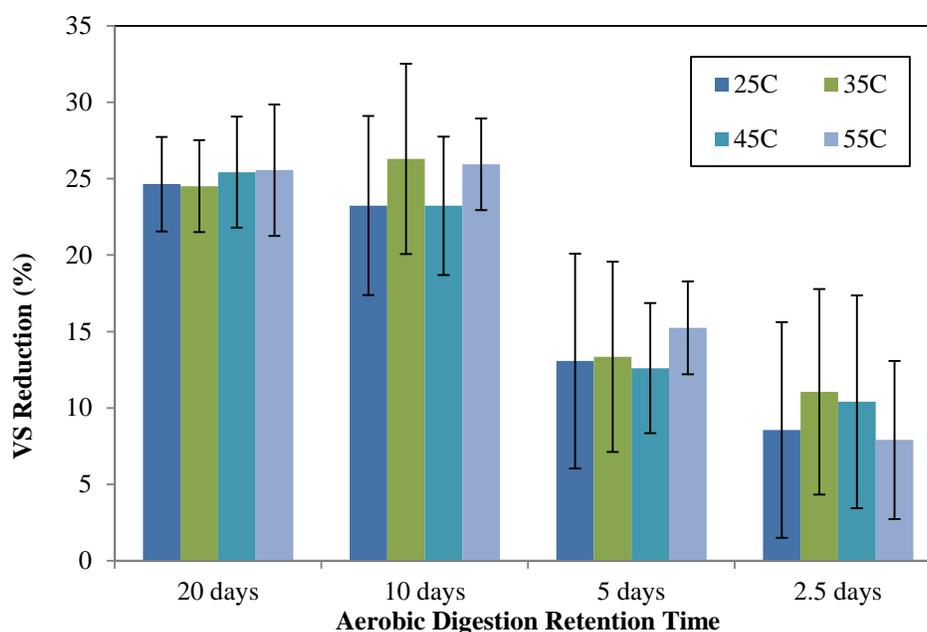


Figure 2.8 Comparison of volatile solids reductions following a bench-scale aerobic digestion sequence—as a function of detention time and temperature during aerobic digestion.

In two-stage (anaerobic/aerobic) digestion, a large fraction of the organic matter originally present is eliminated before the sludge reaches the aerobic digester. Therefore, the residual organic matter from the anaerobic digester has a lower oxygen demand than otherwise. Studies performed by Park et al. (2006) and Kumar et al. (2006) showed more VS reduction in combined anaerobic/aerobic or aerobic/anaerobic sludge digestion processes than in a single (anaerobic) digestion process with the same overall detention time. In another study by Parravicini et al. (2006), residual VS degraded significantly when anaerobic sludge was further stabilized by aerobic digestion. Later Parravicini et al. (2008) reported that post-aerobic digestion (6d SRT, 36°C) of mesophilic anaerobically digested sludge (30d SRT) reduced the organic solids an additional 16%. These studies showed that after anaerobic digestion, some organic fraction in the sludge remains undegraded, and available for further degradation under aerobic conditions.

Use of aerobic digestion following anaerobic digestion resulted in additional VS destruction. Results suggested that the longer HRT of aerobic digesters could lead to greater destruction of volatile solids (Figure 2.8). The results agreed qualitatively with previous research (Subramanian et al., 2007). At the same air flow rate, the longer retention time led to more degradation of the volatile solids (HRT=20 and 10 days). When the HRT dropped to  $\leq 5$  days, the reduction of the VS was only around 10% for the additional aerobic digestion process. Again, the temperature of the aerobic digestion process had little or no effect on the degradation of VS.

Another important characteristic investigated here was the ratio of volatile solids to total solids. Based on the measurement of VS and TS for raw sludge and anaerobic/aerobic digested sludge, the ratio of VS/TS was about 0.90 for the raw sludge.

After anaerobic digestion, the ratio decreased to about 0.70, and following aerobic digestion the ratio dropped to around 0.60. The further stabilization of solids observed here suggests that anaerobic/aerobic digestion can improve sludge quality characteristics and stability prior to sludge disposal in landfill or use as a soil amendment.

### **2.6.1.3 Methane production from the anaerobic digester**

To further evaluate reactor operational stabilities, the methane production rate from the anaerobic reactor was measured. Biogas was produced during anaerobic digestion. Methane ( $\text{CH}_4$ ) and carbon dioxide ( $\text{CO}_2$ ) normally account for much more than ninety percent of the total gas by volume. A fixed amount of methane produced per unit VS fed is an indication of stable operation (Figure 2.9) (Tanaka et al., 1997), and variations in the methane to VS ratio over time can indicate poor performance of the digester. Here, the  $\text{CH}_4$  production rate was about 12.4 mL/g VS·day, in the same range as other studies of this nature (Mata-Alvarez et al., 2000; Tanaka et al., 1997).

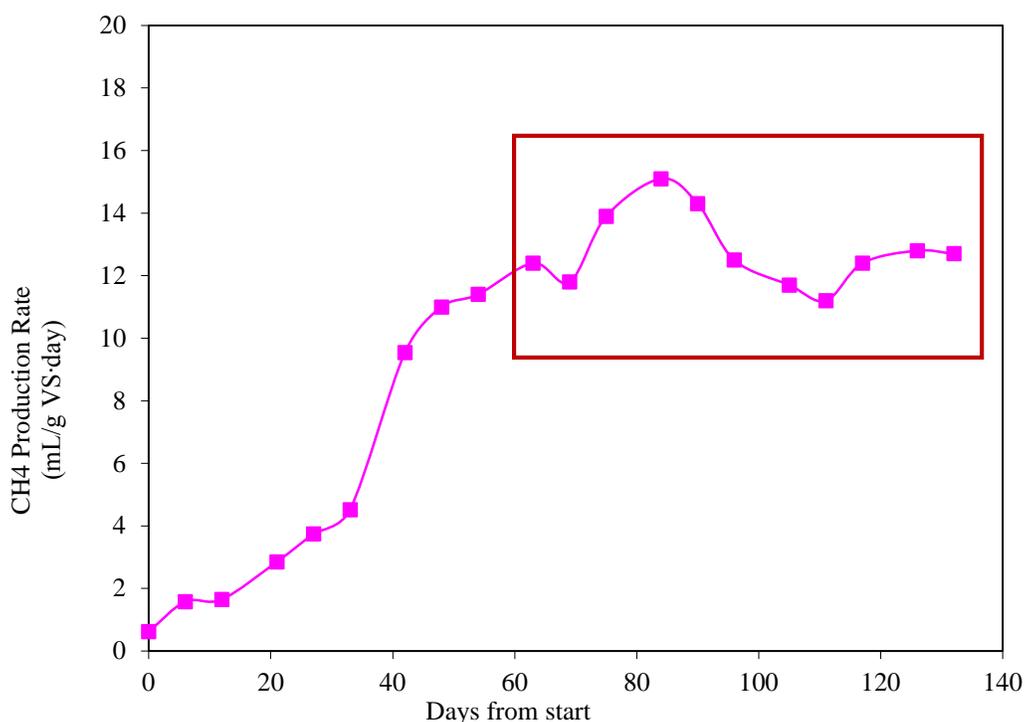


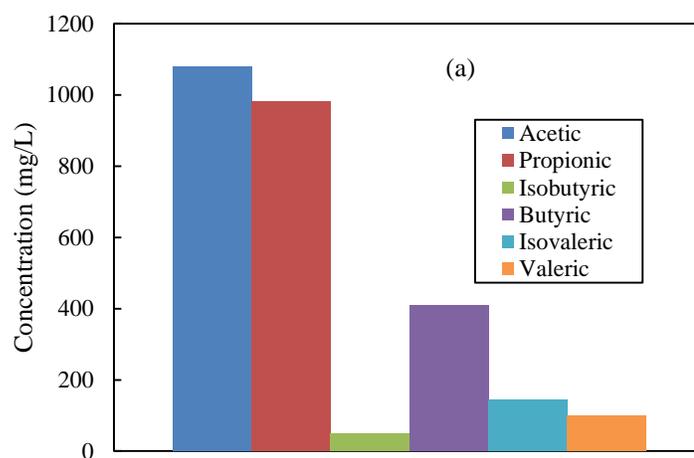
Figure 2.9 Methane production rate during anaerobic digestion—representative (start up) period of operation @ HRT = 20 days and T = 37 °C. (Stabile performance is again indicated after about three detention periods.)

#### 2.6.1.4 VFA removal from the anaerobic digester

Volatile fatty acids (VFAs) profiles were monitored for the raw sludge and anaerobic digested sludge when the anaerobic digester was already stabilized by other parameters, such as solid reduction, pH and methane production rate. Typical profiles of VFAs in the samples are shown in Figure 2.10. From the results, it is clear that during the anaerobic digestion process, the levels of volatile fatty acids were decreased when comparing each single VFA. For longer chain VFAs, such as isobutyric, butyric, isovaleric and valeric acids, they were almost 100% removed by the process since their concentrations in the anaerobic digester samples were below the detection limits

(0.5mg/L). For shorter chain VFAs, propionic acid was 99% removed, and acetic acid was 98% removed after the sludge going through anaerobic digestion process.

In anaerobic digester, organic compounds in the raw sludge, such as lipids and proteins, were first being converted to soluble compounds (fatty acids and amino acids), and then in the acidogenesis step, these molecules would be further digested into VFAs. Then the longer chain VFAs were converted into shorter chain VFAs. Finally, methanogens degrade these products to methane and carbon dioxide (Metcalf & Eddy, 2003). The results of VFAs profile indicate the anaerobic digestion process in our reactor was very efficient by consuming almost all the VFAs from the raw sludge, and the methane production was stable as explained in Section 2.6.1.3.



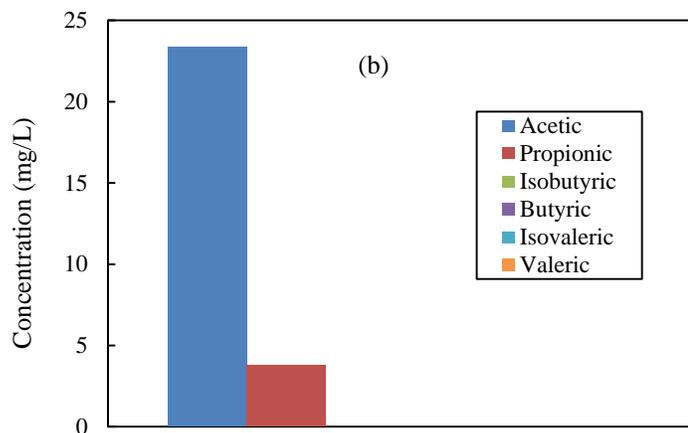


Figure 2.10 VFA profiles in (a) raw sludge and in (b) anaerobic digester sludge.

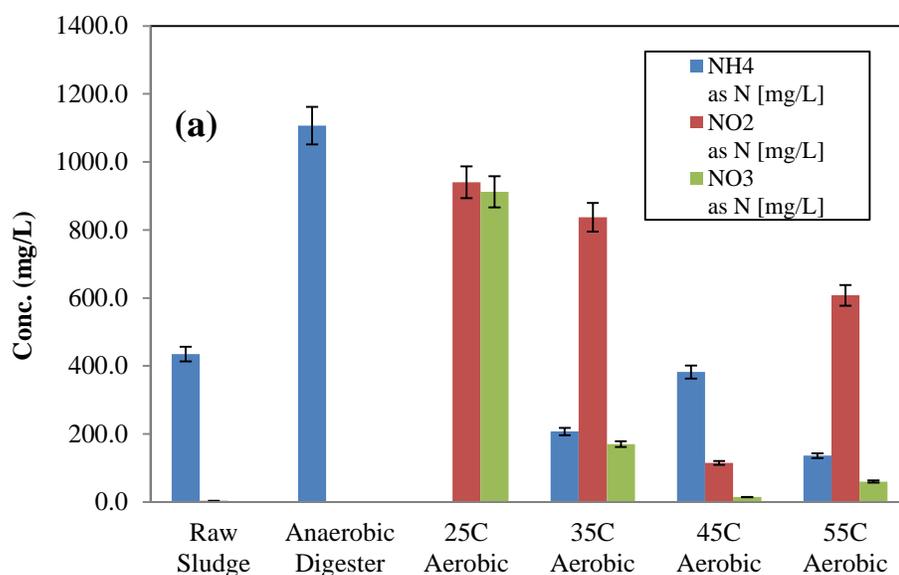
### 2.6.1.5 Nitrogen species—changes during the sludge digestion process

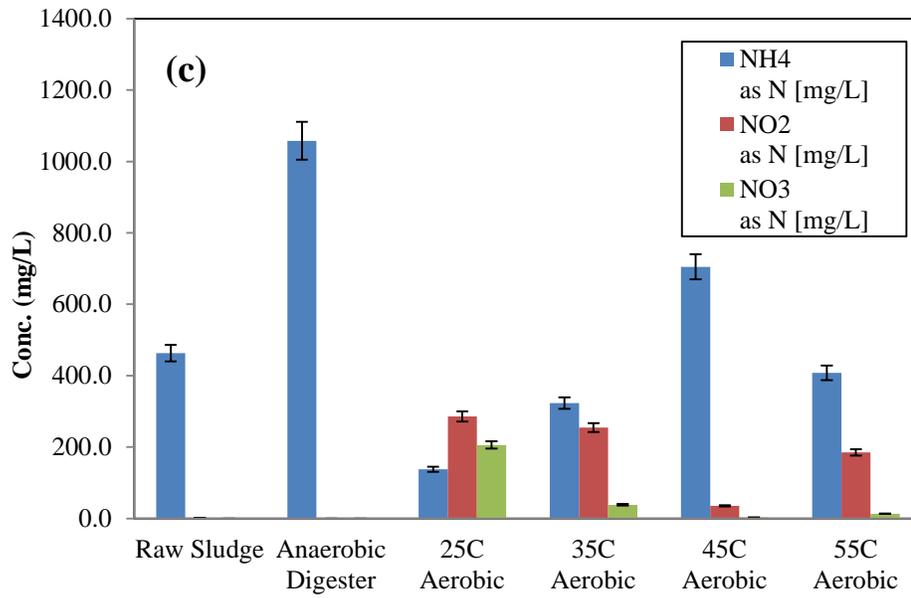
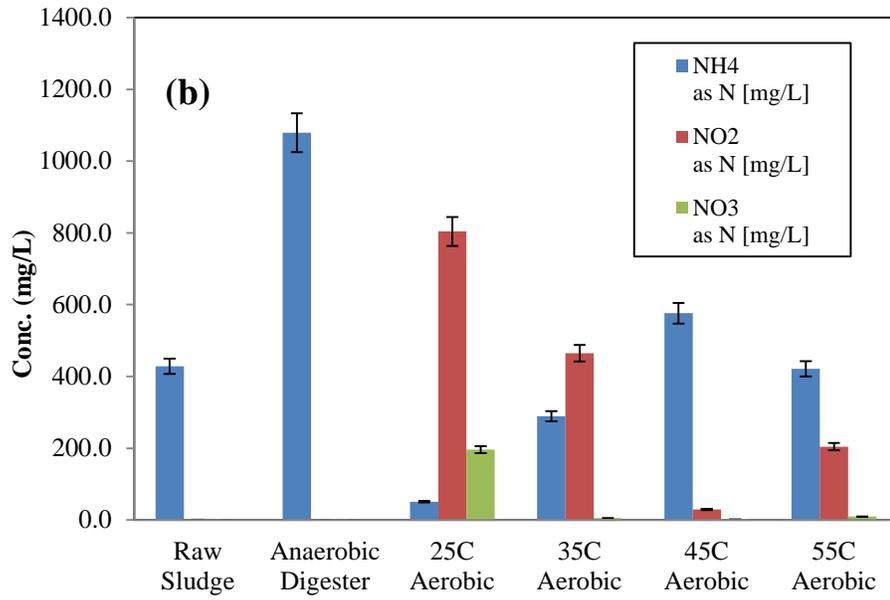
During anaerobic sludge digestion, ammonium nitrogen is released via the degradation of macromolecules such as proteins. Anaerobic digestion can release up to 50% of sludge-bound nitrogen (Chen et al., 2008). The centrate, which is the side stream generated from anaerobic digested sludge dewatering process, is commonly recycled to the headwork of the wastewater treatment due to high ammonium content (on the order of a g/L) adding to the nutrient loading and oxygen demand in the plant. Ammonia levels above 0.2 mg/L can cause mortality in several fish species (Dehedin et al., 2013), suggesting that  $\text{NH}_3$  recycling may increase the need for in-plant nutrient control measures.

Nitrification is a biological process in which ammonium ion ( $\text{NH}_4^+$ ) is oxidized to nitrite ( $\text{NO}_2^-$ ) and then nitrate ( $\text{NO}_3^-$ ) using molecular oxygen as electron acceptor. This process is commonly employed in wastewater treatment to reduce oxygen demand and toxicity to fish and other aquatic organisms caused by elevated levels of ammonia in wastewater effluent (Metcalf & Eddy, 2003). It has frequently been observed that

nitrifying systems exhibit higher removal efficiencies for trace organic compounds compared to non-nitrifying systems (Andersen et al., 2003; Khanal et al., 2006).

In this study, nitrification was encouraged in the subsequent aerobic digestion process, both for nutrient removal and for better removal of estrogenic/androgenic compounds. It was hypothesized that since nitrification occurs at longer SRTs during aerobic digestion, nitrifying bacteria may play a role in trace organic compound removal. Suarez et al. (2010) investigated the removal of different classes of trace organics by varying SRT as a design parameter. Removals of many compounds were directly related to SRT during aerobic digestion.





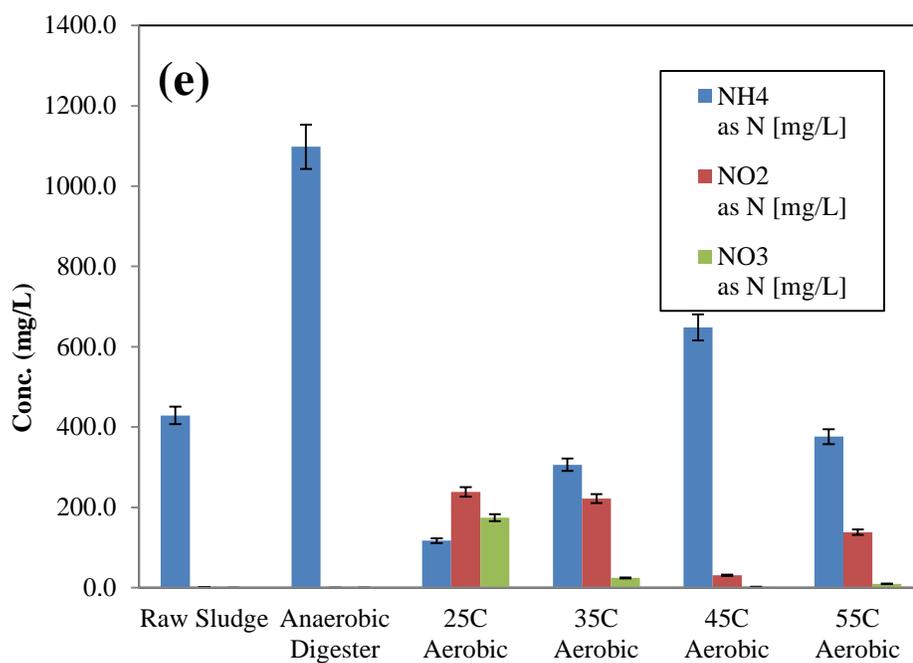
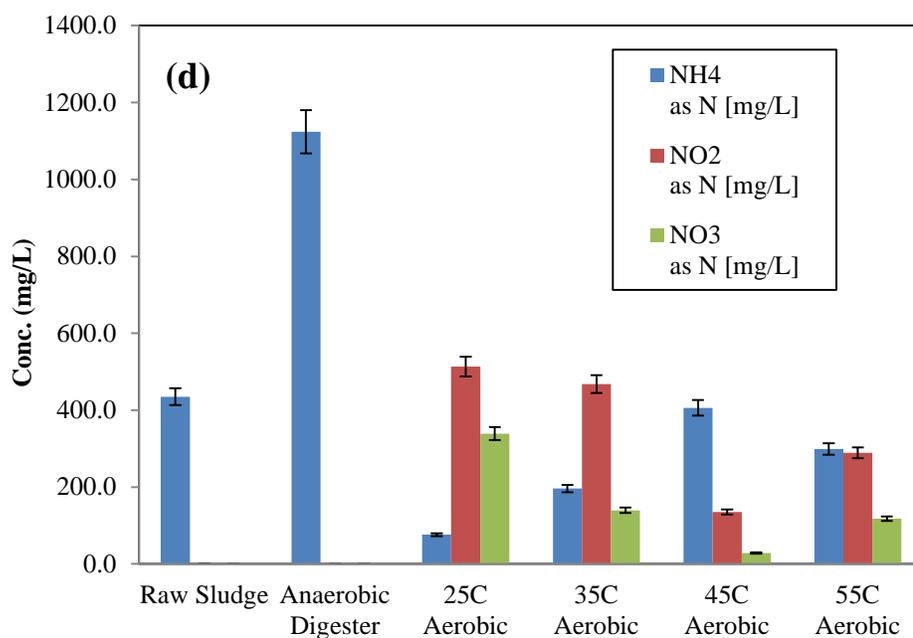


Figure 2.11 Comparison of ammonium, nitrite and nitrate concentration during the digestion process.

(Average number and standard deviation calculated from five sets of samples for each condition).

Retention time: (a) SRT=20 days, (b) SRT=10 days, (c) SRT=5 days. (d) SRT=5 days and (e) SRT=2.5 days.

Airflow rate: a, b and c: 1.0L/min; d and e: 2.0L/min.

Anaerobic digestion converts organic nitrogen in the sludge to ammonia nitrogen, as evidenced by the increase in ammonia nitrogen between raw sludge and anaerobically digested sludge (Figure 2.11). Nitrification was expected during stage-two aerobic digestion if the SRT was long enough and temperature did not preclude the activities of nitrifying bacteria.

The relationship between the changes of nitrogen species (dependent variable) and several independent variables was being investigated in this study. Two-way ANOVA statistical method was proposed for the data analysis. In order to fit all three parameters used in the aerobic digesters (temperature, SRT and airflow rate) in the statistical model, two independent variables were proposed: one was temperature, and the other was the air load applied to the digester, calculated from the product of SRT and airflow rate (Table 2.2). For the dependent variable, which represented the degree of nitrification/denitrification in these aerobic digesters, was expressed in the following form:

$$\text{Change of Nitrogen (\%)} = \frac{[NO_3^-] + [NO_2^-]}{[NH_4^+]_{Anaer} - [NH_4^+]_{Aero}} \times 100\%$$

In this equation, the numerator represents the total concentrations of nitrite and nitrate detected in aerobic digesters by nitrification/denitrification process. The denominator represents the decrease of ammonium concentration in the aerobic digesters from the anaerobic digested sludge.

Table 2.2 Calculation of air volume applied to the aerobic digesters.

Solids Retention Time (days)	Airflow rate (L/min)	Air loading (m <sup>3</sup> )
20	1.0	28.8
10	1.0	14.4
5	1.0	7.2
5	2.0	14.4
2.5	2.0	7.2

Two-way ANOVA statistical test results were listed in Table 2.3. From the results of post-hoc analysis using Tukey's test for air volume applied to the aerobic digester (Table 2.3a), the ratio of nitrite and nitrate to ammonia change was getting higher with increasing air load, reaching a maximum at the air loading of 28.8 m<sup>3</sup> (20 day SRT). There were substantial differences for the nitrification process between different air volumes applied to the aerobic digesters, since the significance of these results were all less than 0.0005 when they were compared with each other. The nitrification process can still happen in the aerobic digesters if the SRT was less than 20 days, but to a less extent, according to the concentrations of nitrite and nitrate measured in the solution.

The influence of different temperatures for aerobic digesters were also being studied (Table 2.3b). The ratios of [NO<sub>2</sub><sup>-</sup>] and [NO<sub>3</sub><sup>-</sup>] to the change of [NH<sub>4</sub><sup>+</sup>] in the aerobic digester at 25 °C were significantly higher than the levels present at other higher temperatures (The sequence was: 25 °C > 35 °C > 55 °C > 45 °C), under the condition of each different air volume applied to these aerobic digesters (Figure 2.12). When comparing the ratios of nitrogen species, in a few cases the number had already exceed 100%, especially for 25 °C and 35 °C reactors running at the highest air loading condition (28.8 m<sup>3</sup>). In the aerobic digesters, organic nitrogen was converted to inorganic form [NH<sub>4</sub><sup>+</sup>] first, and then the condition in the digesters favored the nitrification process

due to the power of nitrifying microorganisms. Anoxic conditions existed around the sludge particles, which were probably caused by the air circulation conditions in the reactor. Part of the nitrate ion was converted into nitrite under that circumstance.

In all cases,  $\text{NO}_2^-$  level in the 25 °C aerobic digester was higher than the other three digesters, and  $[\text{NH}_4^+]$  was much lower in the effluent of the 25 °C reactor, suggesting that denitrification process happened in this reactor. Others have noted a similar dependence of nitrifying activity on temperature (Novak et al., 2011).

One exception was the aerobic digester set at 45 °C. From the comparison of this reactor with the other three aerobic digesters, the ratio of nitrogen did not change with the air volume in the reactor, and it did not follow the trend with the other three aerobic digesters. The reason was probably due to the quantity of microbes in this reactor was not enough for the nitrification process, or even it was not the optional condition for the growth of nitrifying bacteria in this bioreactor.

Table 2.3 Two-way ANOVA statistical results for the nitrogen change in the aerobic digesters.

(a) Dependent Variable: Change of Nitrogen (%)

Air volume	Air volume	Mean Difference	Std. Error	Sig.
7.20	14.40	-22.7884*	1.44388	<0.0005
	28.80	-61.1697*	1.76839	<0.0005
14.40	7.20	22.7884*	1.44388	<0.0005
	28.80	-38.3813*	1.76839	<0.0005
28.80	7.20	61.1697*	1.76839	<0.0005
	14.40	38.3813*	1.76839	<0.0005

\*. The mean difference is significant at the 0.05 level.

(b) Dependent Variable: Change of Nitrogen (%)

temperature	temperature	Mean Difference	Std. Error	Sig.
25.00	35.00	27.6718*	1.82638	<0.0005
	45.00	76.2563*	1.82638	<0.0005
	55.00	48.6243*	1.82638	<0.0005

35.00	25.00	-27.6718*	1.82638	<0.0005
	45.00	48.5845*	1.82638	<0.0005
	55.00	20.9525*	1.82638	<0.0005
45.00	25.00	-76.2563*	1.82638	<0.0005
	35.00	-48.5845*	1.82638	<0.0005
	55.00	-27.6320*	1.82638	<0.0005
55.00	25.00	-48.6243*	1.82638	<0.0005
	35.00	-20.9525*	1.82638	<0.0005
	45.00	27.6320*	1.82638	<0.0005

\*. The mean difference is significant at the 0.05 level.

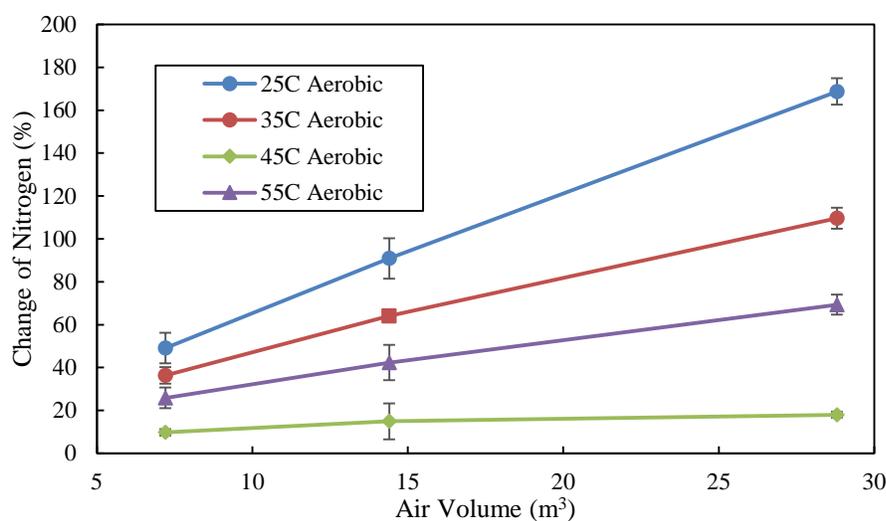


Figure 2.12 Comparison of nitrogen species changes in aerobic digesters with temperature and air loading.

Air volume applied to these reactors can also play an important role in the nitrification/denitrification process. In the aerobic digester at 25 °C, when the retention time was 20 days and the airflow rate was 1 L/min (highest air loading),  $[\text{NH}_4^+]$  decreased from ~1200 mg/L to below the detection limit (0.25mg/L). And when the SRT increased from 5 days to 20 days,  $[\text{NO}_2^-]$  and  $[\text{NO}_3^-]$  increased with air loading to this reactor. A similar trend was observed in the other three aerobic digesters.

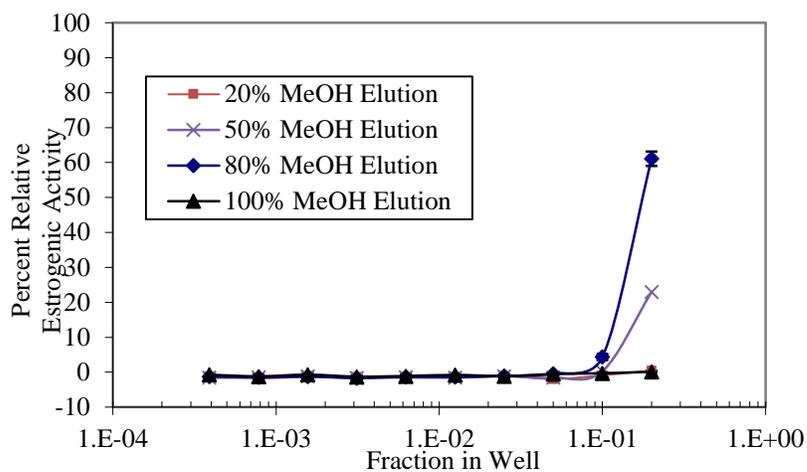
At shorter SRTs (5 days and 2.5 days), with the increase of the airflow rate from 1.0 L/min to 2.0 L/min, the ratio of nitrite and nitrate was increasing. It is apparent that higher airflow rates can encourage nitrification, particularly at low SRT values and the maintenance of higher levels of molecular oxygen in the reactors. It is speculated at this point that higher dissolved oxygen levels will result in greater removal of EDCs including most of the important estrogens.

## 2.6.2 Yeast estrogen screen results

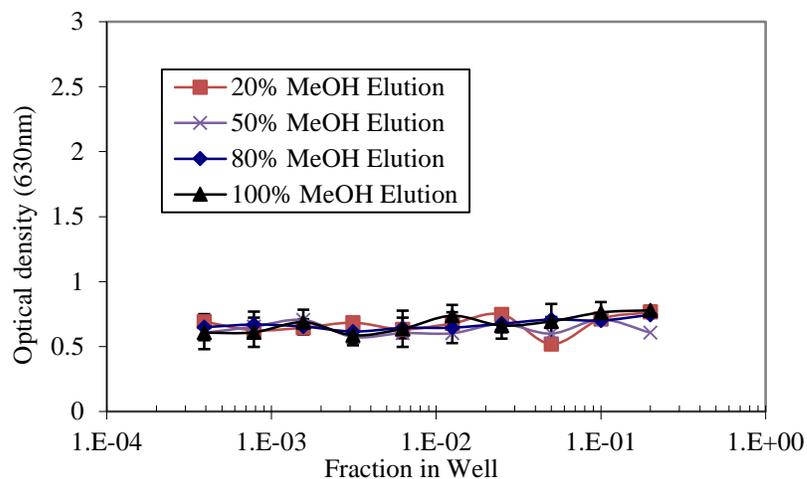
Raw sludge, anaerobically digested and aerobically digested sludge were extracted, and estrogenic activities of the samples were measured using the yeast estrogen screen (YES) bioassay.

### 2.6.2.1 Single sample estrogenic activity measurement

A typical set of sample measurements (solid phase) plot is shown in Figure 2.13-- Figure 2.15. The active fractions in the raw sludge were in 50% and 80% (v/v) methanol, with the 80% methanol fraction being the most estrogenic. The other two fractions, 20% and 100% methanol fractions, did not show estrogenic activity. All of these fractions had no toxic effects on the yeast cell growth.



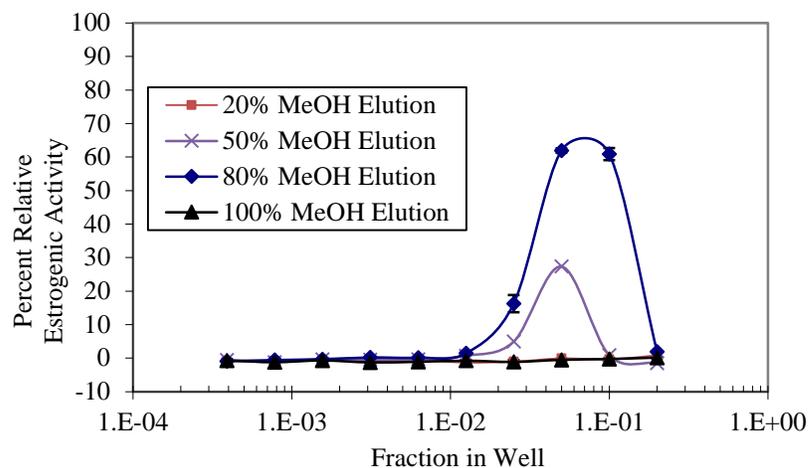
(a) Estrogenic response of sample fractions



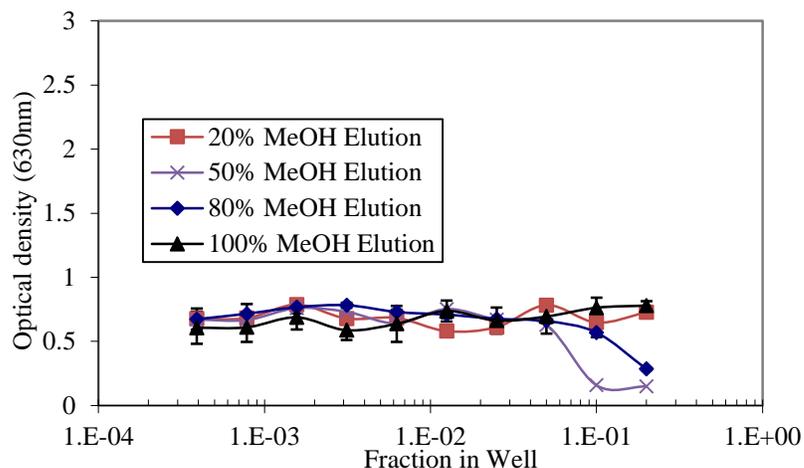
(b) Effects of sample concentration on yeast growth (toxicity)

Figure 2.13 Estrogenic response in the YES assay for raw sludge samples collected from Tres Rios WRF (November 5, 2011)

In general, samples collected from anaerobic digester had a higher estrogenic response than the raw sludge. In this set of samples, both the 50% and 80% methanol fractions showed higher estrogenic activities than their corresponding fractions in the raw sludge. Reduced growth of the yeast cells was observed in 50% and 80% methanol fractions at higher concentrations in the serial dilutions, while yeast cell growth in the 20% and 100% methanol fractions was unaffected by sample toxicity. Again, the 20% and 100% methanol fractions produced no estrogenic response. Since this result was consistent, measurement of estrogenic activity in the 20% and 100% fractions was eventually discontinued.



(a) Estrogenic response of sample fractions

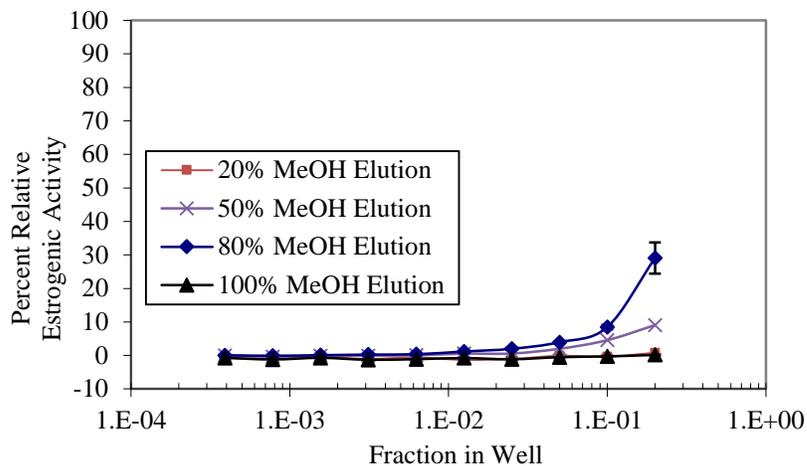


(b) Effects of sample concentration on yeast growth (toxicity)

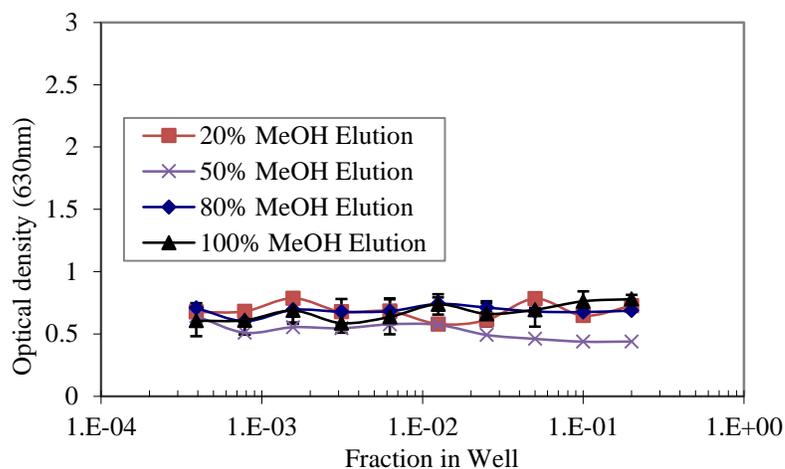
Figure 2.14 Estrogenic response in the YES assay for samples collected from anaerobic digester (SRT=20 days)

Aerobic digestion generally reduced estrogenic activity in sludge samples. For example, the results from YES assay shown for purposes of illustration (Figure 2.15) are from the aerobic digester maintained at 35 °C, and were collected on the same day as the anaerobic digested sludge sample. From Figure 2.15, it is evident that the estrogenicity of the aerobic reactor sample was less than that of samples taken out from the mesophilic anaerobic digester. It is likely that some estrogenic compounds were degraded via aerobic

digestion, or compounds with stronger estrogenic activity were converted to less potent compounds.



(a) Estrogenic response of sample fractions



(b) Effects of sample concentration on yeast growth (toxicity)

Figure 2.15 Estrogenic response in the YES assay for samples collected from 35°C aerobic digestion (SRT=20 days)

Samples from all sources produced the same distribution of estrogenic activity in sample fractions obtained by differential elution. That is, the 50% and 80% methanol fractions dominated the overall estrogenic activities in all samples. This was likely due to the hydrophobicity of the extractable estrogenic compounds in sludge samples.

Furthermore, the fraction eluted in 80% methanol was generally more estrogenic than the 50% fraction. It seems apparent that aerobic treatment did not dramatically alter the distribution of hydrophobilities among residual estrogens and estrogen mimics.

Compounds causing cytotoxicity also appeared in the 50% and 80% methanol fractions, indicating that responsible compounds were hydrophobic.

For subsequent analyses, the estrogenic activity represents the sum of estrogenic activities in the 50% and 80% methanol fractions. The 20% fraction was generated, nonetheless, in order to remove the hydrophilic toxic compounds that might contribute to sample toxicity, ahead of generation of the 50% and 80% fractions.

#### **2.6.2.2 Cytotoxicity effects of samples during YES/YAS assay**

Fairly extensive research reviewed by Kortenkamp (2007) suggests that the effects of multiple estrogens or androgens that are present simultaneously in a single sample can be approximated using an additive model. The validity of additive modeling has been verified in laboratory studies with different multi-component mixtures of selected estrogens using a variety of *in vitro* and *in vivo* assays.

However, actual environmental samples typically consist of not only estrogenic/androgenic substances but also estrogen antagonists, androgen antagonists and a variety of endocrine inactive chemicals that cannot be separated in the extraction and cleanup steps. These compounds can interfere with the normal cell signaling system of YES/YAS bioassays. As a consequence, the bioassay-based assessment of the actual estrogenic/androgenic activity of such complex mixtures, as well as the applicability of concentration addition models, remains subject to question. Such interferences have recently been put forward as one of the main reasons an inability to better predict *in vitro*

and *in vivo* estrogenic/androgenic activity of wastewater samples from the results of exhaustive chemical analysis (Thorpe, 2006).

Interference from non-estrogenic/androgenic chemicals may result in either a reduction or an increase of biological response in the yeast cells. Three types of interferences are of major relevance: On one hand, toxicity masking and antagonistic modulation tend to lower the observable estrogenic response. On the other hand, synergistic modulation results in greater activity than predicted from chemical analyses. If toxicants are present at sufficiently high concentrations, estrogenic/androgenic response may be reduced or masked completely due to cytotoxicity among the test cells or organisms.

Methods for estimating sample cytotoxicity were adapted from Teske (2009), using light scattering ( $A_{630}$ ) data obtained during the YES/YAS bioassays. Because there is much less variability in the toxicity readings of an environmental sample compared to estrogenic response,  $A_{630}$  values of samples were compared to those of the negative control series using Student's t-test. The onset of toxicity was apparent when the sample dilution series produced values that were significantly lower than those of the negative controls ( $p < 0.05$ ).

For the raw sludge collected from the Tres Rios WRF,  $OD_{630}$  values in cell cultures after 24-hour exposures to organic fractions from differential elution using 20, 50, 80 and 100% methanol in water were constant over the entire YES dilution series (Figure 2.13). That is, there was no statistically significant difference between growth in sample-amended wells in the 96-well plates and cell growth in the negative controls. In this case, sample cytotoxicity fell below the measureable limit.

For the extracts from the anaerobic digested sludge, reduced growth of the yeast cells was observed in 50% and 80% methanol fractions at higher concentrations in the serial dilutions (Figure 2.14), suggesting that sample toxicity increased in the more hydrophobic fractions. In 50% methanol fraction, the onset of cytotoxicity was observable at an overall concentration factor of 0.1. And in the 80% fraction, the same concentration factor was toxic to yeast cells in the bioassay. But the difference between cytotoxicity for these two fractions was significant when comparing these two fractions. It was probably due to different non-estrogenic compounds existing in these two fractions, since the hydrophobicity in these two fractions would be different based on the composition of the eluting solution. The cells may undergo necrosis, in which they lose membrane integrity and die rapidly as a result of cell lysis. The cells can experience a decrease in cell viability, or the cells can activate a genetic program of controlled cell death (Carmona-Gutierrez et al., 2010).

Second-stage aerobic digestion tended to reduce the toxicity caused by the anaerobically digested sludge (Figure 2.15), irrespective of temperature, indicating that toxicants can be aerobically transformed into less toxic products.

The same cytotoxicity pattern was observed during measurements supporting the YAS bioassay. This was expected since the strains of yeast in the two bioassays are essentially identical.

### **2.6.2.3 Comparison of estrogenic activity in extracts of sludge solid and liquid phases**

From Section 2.6.2.1, the results showed that estrogenic compounds extracted from the solid phase of the sludge samples were distributed primarily in the 50% and

80% methanol eluent fractions, suggesting that the primary contributors to estrogenic activities were hydrophobic in character. Liquid phase estrogenic compounds were also extracted for measurement of estrogenic activity and compared to solid phase results (Figure 2.16).

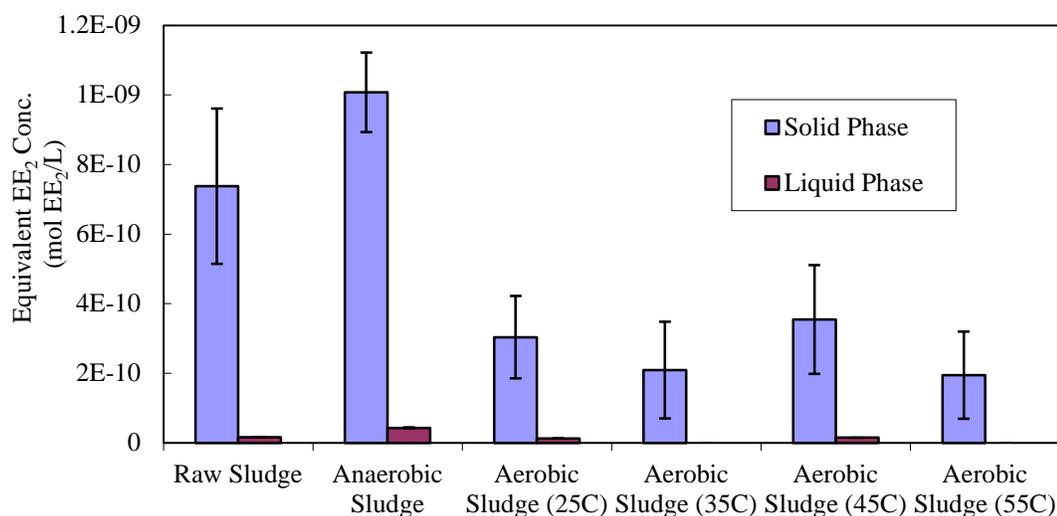


Figure 2.16 Comparison of estrogenic activities in solid and liquid phases in various sludge types as a function of treatment.

In each case, the SRT for aerobic sludge digestion was 5 days. Numbers of independent samples are as indicated ( $n=4$ ). Error bars indicate  $\pm 1.0\sigma$ .

Four sets of samples derived from the aerobic digesters (SRT=5 days,  $n=4$ ) were analyzed for estrogenic activity of both the solid and liquid phases. All measurements were converted into units of mole per liter of sample volume (mol EE<sub>2</sub>/L) using total solid (TS) data in each reactor in order to compare liquid- and solid- phase levels of activity. Results indicate that the contribution of liquid-phase estrogenic activity was always much less than that of sorbed, extractable chemicals. Only about two percent of the total estrogenic activity was present in the liquid phase of the raw sludge samples (Figure 2.16). This value increased to ~5% after mesophilic anaerobic digestion. During aerobic digestion, the fractional estrogenic activity of the liquid phase again decreased

(Table 2.4). The average percent reductions in the EE<sub>2</sub> equivalent concentration during aerobic digestion were 88%, 95%, 49% and 91% at 25, 35, 45 and 55°C (five-day SRT). This conformed to the summary observations of others and probably resulted from the biodegradation of estrogens (Hernandez-Raquet, 2013; Teske and Arnold, 2008). Based on these observations, the liquid-phase contribution to overall estrogenic activity was neglected in subsequent work.

Table 2.4 Comparison of estrogenic activities in solid and liquid phases of sludge samples (SRT=5 days, n=4).

Sample Name	Solid phase estrogenic activity (mol EE <sub>2</sub> /L)	Liquid phase estrogenic activity (mol EE <sub>2</sub> /L)	Ratio (liquid/solid)
Raw sludge	$(7.38 \pm 2.23) \times 10^{-10}$	$(1.60 \pm 0.15) \times 10^{-11}$	2.2%
Anaerobic digested sludge	$(1.01 \pm 0.11) \times 10^{-9}$	$(4.30 \pm 0.21) \times 10^{-11}$	4.3%
Aerobic digested sludge (25°C)	$(3.04 \pm 1.18) \times 10^{-10}$	$(1.28 \pm 0.16) \times 10^{-11}$	4.2%
Aerobic digested sludge (35°C)	$(2.09 \pm 1.39) \times 10^{-10}$	$(1.02 \pm 0.17) \times 10^{-12}$	0.57%
Aerobic digested sludge (45°C)	$(3.55 \pm 1.56) \times 10^{-10}$	$(1.51 \pm 2.13) \times 10^{-11}$	4.2%
Aerobic digested sludge (55°C)	$(1.95 \pm 1.25) \times 10^{-10}$	$(3.50 \pm 0.25) \times 10^{-12}$	1.8%

#### 2.6.2.4 Comparison of estrogenic activity in extracts of raw and anaerobic digested sludge

As mentioned in Section 2.6.2.1, the results showed that estrogenic activity increased after the sludge coming out from the anaerobic digester. Since for each suite of samples, raw sludge and anaerobic digested sludge were always being measured, the comparison of these two groups of samples is illustrated in Figure 2.17.

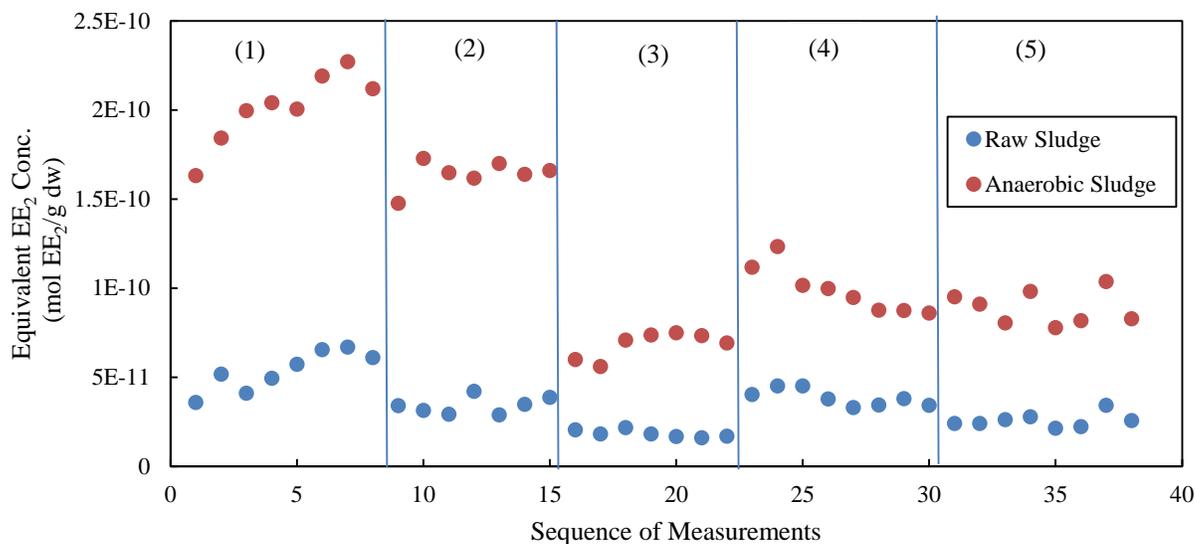


Figure 2.17 Comparison of estrogenic activity in raw sludge and anaerobic digested sludge.

Separate lines indicate different batch of raw sludge were used for the digestion process. Different settings for aerobic digesters: (1) SRT=20 days; (2) SRT=10 days; (3) SRT=5 days; (4) SRT=5 days; (5) SRT=2.5 days.

Airflow rate settings: (1)-(3): 1 L/min; (4) and (5): 2L/min.

As observed from the graph, the overall increase in estrogenic activity during anaerobic digestion is again apparent. Student's t-test was used for the comparison of these two groups of estrogenic activity, and the results suggest that there are significant difference between them ( $p < 0.0005$ ). Potential explanations include the generation of more potent estrogens via anaerobic transformations. Some compounds, such as NPnEOs are biotransformed to nonylphenol (NP), nonylphenol monoethoxylate (NP1EO), and nonylphenol diethoxylate (NP2EO), which are more estrogenic than their parent compounds (Routledge and Sumpter, 1996; Vethaak et al., 2005). These daughter compounds may then be degraded via aerobic digestion (Figure 2.1). It is also possible that conjugated (e.g., sulfonated) forms of other estrogens were deconjugated during anaerobic digestion, increasing the overall estrogenic activity in sludge.

Due to the short storage period of grabbed raw sludge samples from the Tres Rios WRF and the long waiting time for aerobic digesters to reach their stable operational status between each different setting, for different SRTs of the aerobic sludge digestion, different batches of raw sludge were used. Results indicate that estrogenic activity in sludge solid part was increased after the standard mesophilic anaerobic digestion process (unit in mol EE<sub>2</sub>/g dry weight), by about a factor of five when this study was at 20- and 10-day SRT periods. But for the 5-day SRT period, the estrogenic activity after the anaerobic digestion was only increased to three times of the activity of the raw sludge solid samples in average. One reason was probably due to that the properties and chemical compositions of raw sludge from municipal wastewater treatment plant were different from one batch to another, or even seasonal variations, especially the endocrine disrupting compounds (Harries et al., 1996; Jin et al., 2008). It can be proved by the measurement of different batches of raw sludge, in which the estrogenic activity in the raw sludge fed into the anaerobic digester in the SRT 5-day (aerobic digestion) period was only half when comparing with that of the other two batches sludge here (Figure 2.17). The variation of the raw sludge resulted from the fluctuation during the wastewater treatment process (Metcalf & Eddy, 2003).

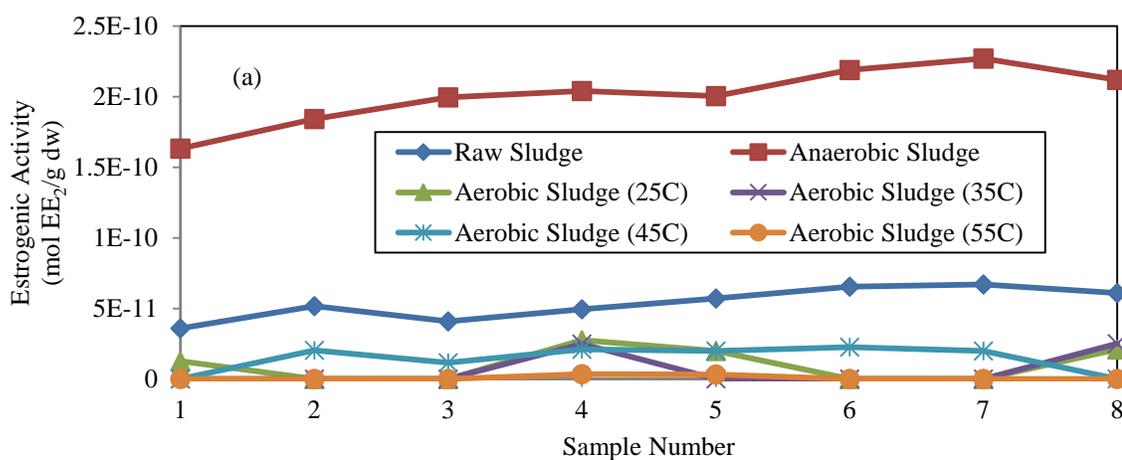
#### **2.6.2.5 Effect of operational variables on estrogenic activity during aerobic digestion**

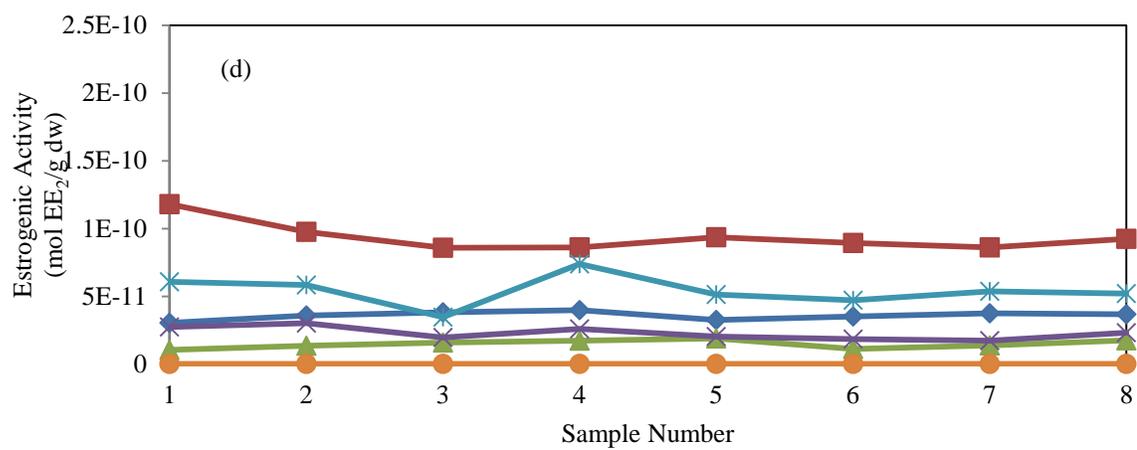
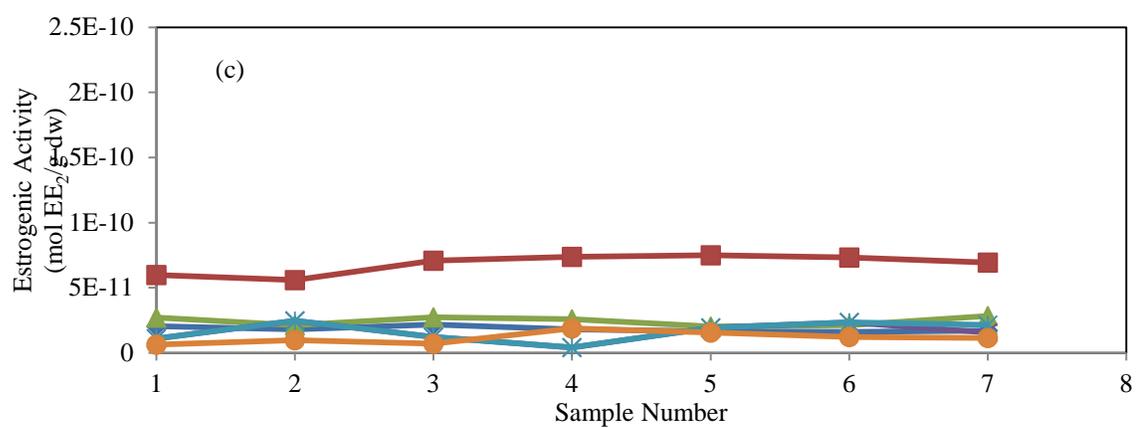
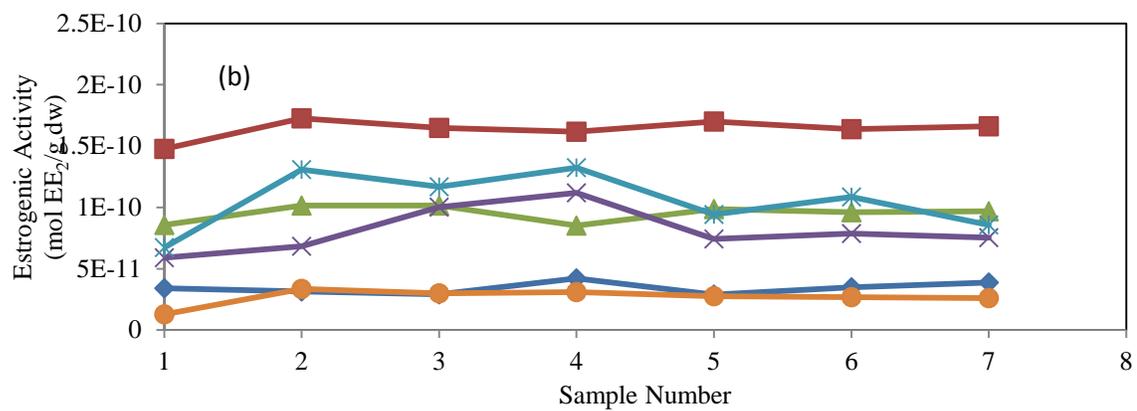
From the setup of the aerobic digesters, independent variables for this work include temperature, sludge retention time and airflow rate in the aerobic digesters. But as discussed in Section 2.6.1.5, two-way ANOVA statistical method was proposed for analyzing the relationship between the reduction ratio of estrogenic activity (dependent variable) and independent variables. In order to simplify the problem, same as in Section

2.6.1.5, two independent variables was proposed: one was the temperature of the aerobic digesters, and the other was the air loading volume applied to these digesters, which was calculated by multiplying SRT and airflow rate (Table 2.2). For the dependent variable, it was calculated by the following equation, which represented estrogenic activity removal ratio by the aerobic digestion process from the anaerobic digested sludge:

$$\text{Estrogenicity Removal (\%)} = \frac{[\text{Estrogenicity}_{anae}] - [\text{Estrogenicity}_{aero}]}{[\text{Estrogenicity}_{anae}]} \times 100\%$$

Under each setup condition of the aerobic digesters, seven to eight suites of sludge samples were collected, extracted and analyzed in order to compare the change of estrogenic activity during the digestion process (Figure 2.18). Estrogenic activities have been corrected by the average  $EC_{20}$  value of all batches of YES bioassay for these tests. Estrogenic activities in the raw sludge, anaerobic digested sludge and sequential aerobic digested sludge samples were stable during each sampling period of the SRT settings, which indicates there was no outlier for the statistical analysis. It also proved that the reactors are running at the stable status from another perspective of view.





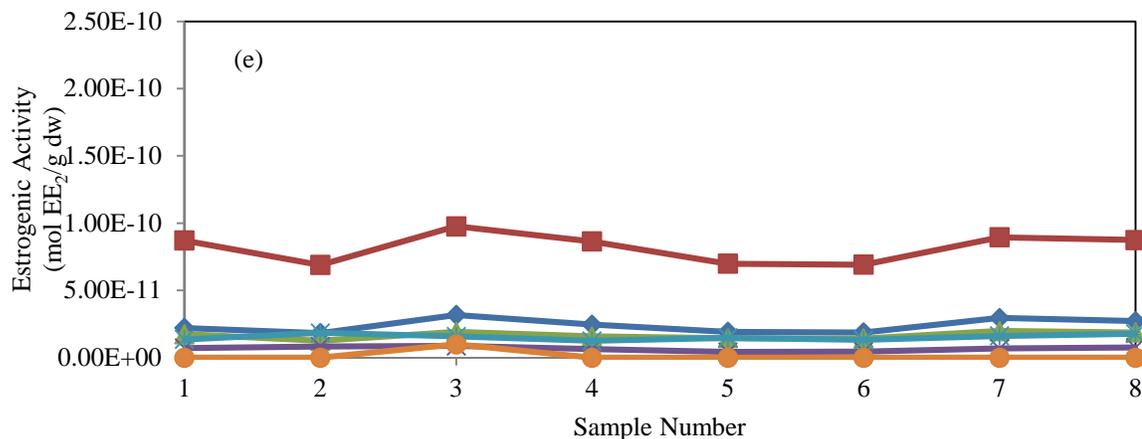


Figure 2.18 Estrogenic activities in solid phase of samples change with different aerobic digester settings.

Different settings for aerobic digesters: (a) SRT=20days; (b) SRT=10days; (c) SRT=5days; (d) SRT=5days; (e) SRT=2.5days.

Airflow rate settings: (a)-(c): 1 L/min; (d) and (e): 2 L/min.

Two-way ANOVA statistical analysis was performed for the comparison of different settings of aerobic digesters. For the correlation between air loading and the estrogenic activity reduction ratio of reactors, the results were shown in Table 2.5. The difference of estrogenic activity reduction ratios was statistically significant for all the cases ( $p < 0.0005$ ). The reduction ratio was positively correlated to the air volume applied to these aerobic digesters. For all the temperature settings (from 25 to 55 °C), the estrogenic activity reduction ratio increased with the increasing air volume at different degree, which means that air volume is an important factor to the degradation of estrogenic compounds in the aerobic digesters. More air the sludge exposed to in the aerobic digester, the better removal efficiency for estrogenic activity it can achieve, while other operational parameters maintained the same. At the highest air loading to the digesters (28.8 m<sup>3</sup>), for all the reactors at different temperatures, the removal ratios were very close to each other (92-100%). This result indicates that if air loading to the aerobic digesters are large enough, the nitrification process would occur in the system because

there are enough oxygen act as electron acceptors (Kumar et al., 2006). As discussed in Section 2.6.1.5, under these circumstances, the estrogenic compounds would have been degraded by the nitrifying bacteria in these reactors.

Table 2.5 Two-way ANOVA statistical results for the nitrogen change in the aerobic digesters.

(a) Dependent Variable: Reduction of estrogenic activity (%)

Air volume	Air volume	Mean Difference	Std. Error	Sig.
7.20	14.40	-16.3142*	2.12900	<0.0005
	28.80	-30.6921*	2.55258	<0.0005
14.40	7.20	16.3142*	2.12900	<0.0005
	28.80	-14.3780*	2.55258	<0.0005
28.80	7.20	30.6921*	2.55258	<0.0005
	14.40	14.3780*	2.55258	<0.0005

\*. The mean difference is significant at the 0.05 level.

(b) Dependent Variable: Reduction of estrogenic activity (%)

temperature	temperature	Mean Difference	Std. Error	Sig.
25.00	35.00	-5.2011	2.67522	0.215
	45.00	7.6166*	2.67522	0.026
	55.00	-19.5479*	2.67522	<0.0005
35.00	25.00	5.2011	2.67522	0.215
	45.00	12.8176*	2.67522	<0.0005
	55.00	-14.3468*	2.67522	<0.0005
45.00	25.00	-7.6166*	2.67522	0.026
	35.00	-12.8176*	2.67522	<0.0005
	55.00	-27.1645*	2.67522	<0.0005
55.00	25.00	19.5479*	2.67522	<0.0005
	35.00	14.3468*	2.67522	<0.0005
	45.00	27.1645*	2.67522	<0.0005

\*. The mean difference is significant at the .05 level.

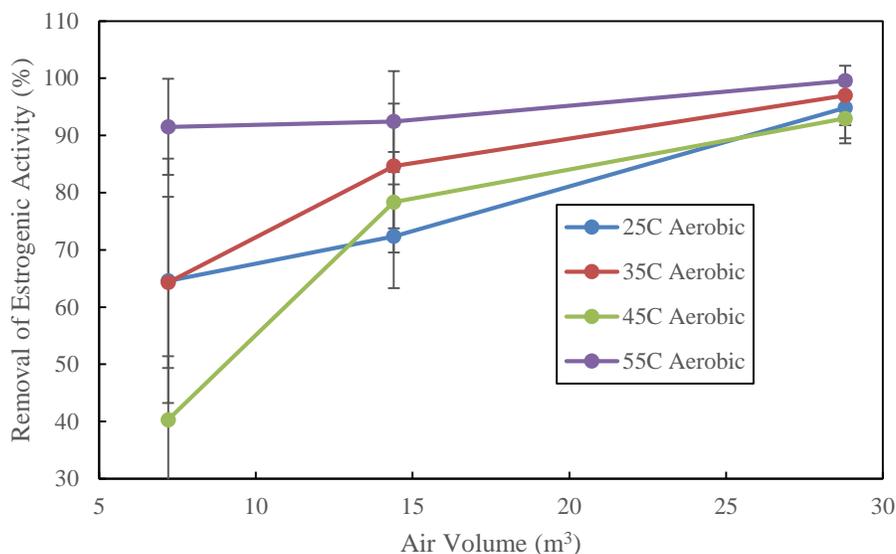


Figure 2.19 Comparison of estrogenic activity reduction ratios under different aerobic digester conditions.

Another important factor for the degradation of estrogenic compounds in the aerobic digesters is temperature (Table 2.5b). The reactor maintained at 55 °C was more effective on reducing estrogenic activity in the sludge. When comparing with the other three digesters, the difference is statistically significant ( $p < 0.0005$ ). For 25 °C and 35 °C digesters, there are no significant difference between their performances on the degradation of estrogenic compounds in the sludge. But these two were better than the reactor setting at 45 °C ( $p < 0.05$ ).

As observed from Figure 2.19, among these four temperatures, thermophilic (55 °C) aerobic digestion was the best one reducing estrogenic activity regardless of what the air loading to the bioreactor they were set to (removal ratio around 90-100%). The high temperature and community characteristic of thermophilic bacteria may have contributed to the reduction of estrogenic activity in this reactor (Banat et al., 2000). Estrogenic activity even fell below the YES assay detection limit ( $1.30 \times 10^{-13}$  mol EE<sub>2</sub>/g dw) at 55 °C during aerobic digestion of 20-day SRT (air volume=28.8 m<sup>3</sup>). As indicated in the

statistical results, the estrogenic activity of sludge samples in the 55 °C aerobic digester dropped the most among four bioreactors. For aerobic digesters operated at the other three temperature settings, estrogenic activities decreased as well, but not as much as the level changes in the bioreactor operating at 55 °C under the same air loading condition at 7.2 and 14.4 m<sup>3</sup>.

At temperature of 25, 35 and 55 °C, the reactors followed the trend: with the temperature increased, the estrogenic activity reduction ratio increased as well. But for the bioreactor set at 45°C, at the lowest air loading, the removal ratio was even lower than all the other three temperatures. The main reason for this phenomenon was probably due to the nitrification process in this reactor was not as strong as the other three digesters under the same air loading condition, which has been discussed in Section 2.6.1.5.

### **2.6.3 Comparison of nonylphenol and octylphenol concentrations during different sludge treatment processes**

Gas chromatography–mass spectrometry (GC-MS) was used to measure the concentrations of nonylphenol and octylphenol as a function of aerobic digester conditions. The contributions to total sample (equivalent) estrogenic activities were also calculated. For that purpose, an additive model was assumed.

Sample preparation (extractions) were carried out in the same manner as extractions for measurement of total estrogenic activity (MAE and C-18 SPE cleanup procedures). After extraction, different fractions of the sample were then combined and resuspended in methanol for GC-MS analysis.

### 2.6.3.1 Nonylphenol concentrations comparison

Before analyzing nonylphenol in samples by GC-MS, pentafluorobenzophenone (10 $\mu$ L of 1.0g/L in methanol), was added as an internal standard to 1mL NP standard series or extracted sludge samples prepared for the GC-MS analysis. A typical chromatogram containing both the NP standard and internal standard is provided as Figure 2.20.

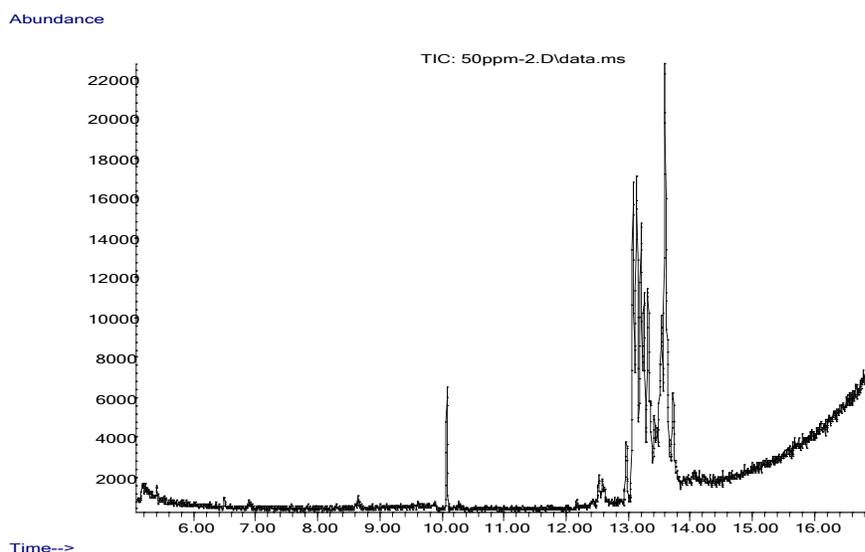


Figure 2.20 Chromatogram showing the internal standard, pentafluorobenzophenone, at 10 minutes, and the nonylphenol standard, between 13 and 14 minutes. (Here nonylphenol consisted of a mixture of isomers of 4-nonylphenol.)

There are approximately 20 isomers in technical grade 4-nonylphenol, although up to 102 individual isomers have been detected (Moeder et al., 2006; Tanaka et al., 1997). The multiple eluting peaks at 13-14 minutes in the chromatogram (Figure 2.21) correspond to individual isomers of nonylphenol. These isomers were monitored under the selected ion monitoring (SIM) mode on GC-MS. Here, five peaks are visible for the  $m/z$  ratio 135, which is representative for NP (Santos et al., 2007). No attempt was made to identify specific isomers. To determine the total concentration of nonylphenol in standards or samples, the total area of all the isomers was integrated.

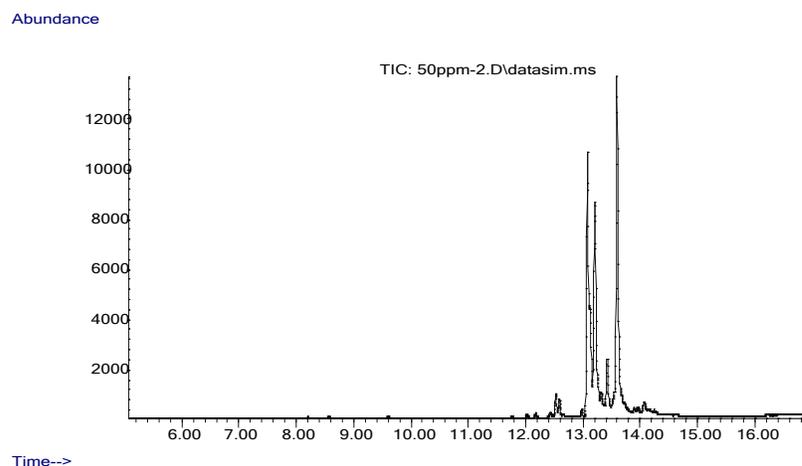


Figure 2.21 Chromatogram of the nonylphenol standard (50ppm), between 13 and 14 minute. ( $m/z = 135$ )

This type of analysis was adequate for measuring nonylphenol in the calibration range of 0.25 to 50 mg/L (Figure 2.22). The response was linear in this range, with the  $r^2$  value of 0.999. A calibration curve was run with each batch of measurements.

Nonylphenol in sludge samples are rarely found at these high levels after extractions, but was concentrated prior to analysis (Section 2.5.4). Sample concentrations of total solids (TS) were used to normalize the measured concentrations based on solids content (units of ug/kg dw).

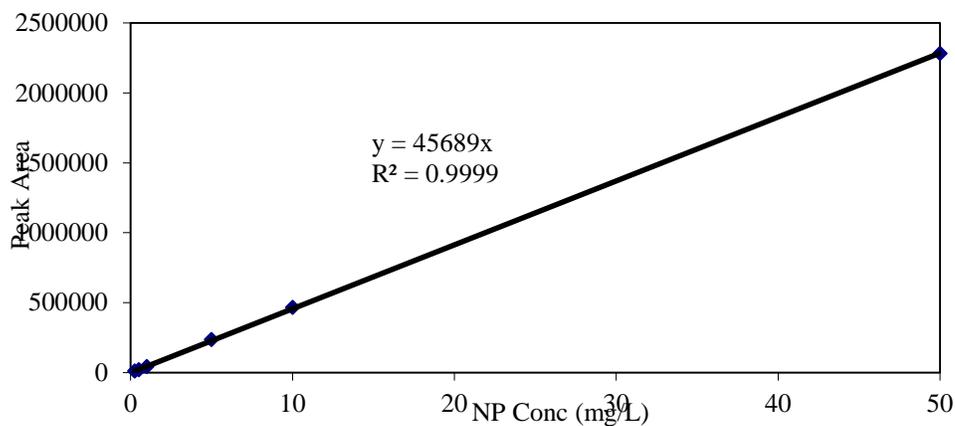


Figure 2.22 Standard curve for nonylphenol using GC-MS. The curve is linear in the range of 0.25 to 50 mg/L.

Liquid phase NP concentrations in sludge samples, separated by centrifugation, were lower than the detection limit (data not shown); hence, solid phase NP values represent total sample concentrations. Concentrations of nonylphenol under different digester conditions were as shown (Figure 2.23). For each condition, four batches of raw sludge, anaerobic digested and sequential aerobic digested sludge were measured and averaged.

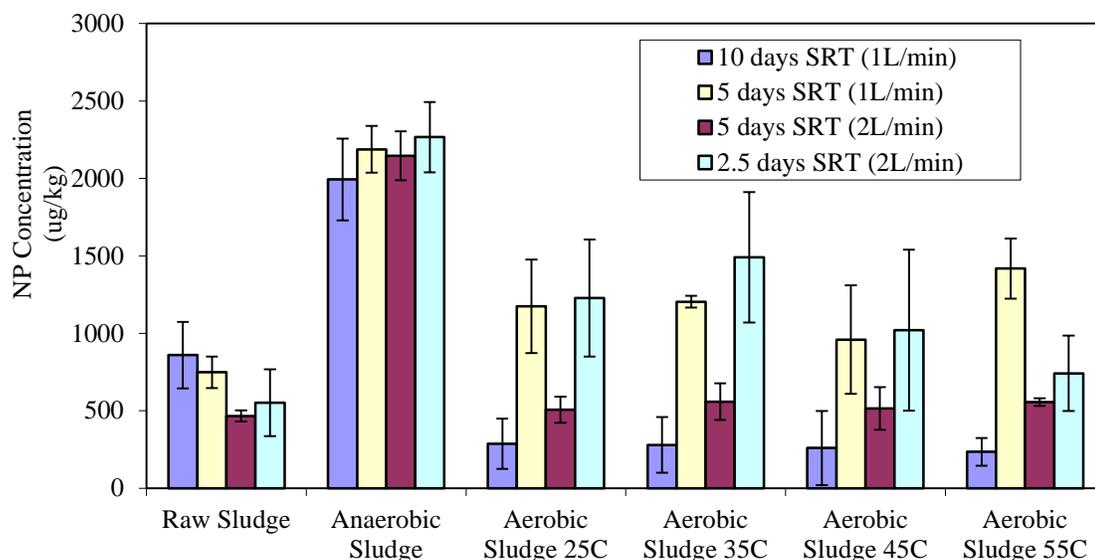


Figure 2.23 Nonylphenol concentration comparison for different sludge retention time and different airflow rate in aerobic digestion.

Numbers of independent samples are as indicated ( $n=4$ ). Error bars indicate  $\pm 1.0\sigma$ .

The concentration of nonylphenol in the solid phase of the sludge increased by a factor of about four during the anaerobic digestion process, compared with raw sludge. But when a reduction of about 50% total solid in the sludge was taken into account, the concentration of nonylphenol in the whole sample was increased to two fold of the original samples of the raw sludge. This is probably due to biodegradation of nonylphenol ethoxylates (NPnEOs) during anaerobic digestion.

The distribution of NPnEOs and its metabolites probably evolves through the wastewater treatment process. Long-chain NPnEOs are hydrophilic compounds which tend to stay in the liquid phase. However, as the ethoxylated chain is shortened, losing their hydrophilic moieties, they became more hydrophobic. Consequently, the compounds NP1EO, NP2EO, and NP, displaying high octanol/water distribution ratios ( $\log D_{ow}$  between 5.63 and 6.14) (Chen et al., 2008), will be sorbed onto the hydrophobic sludge solid fraction. This could account for the observed increase in the solid phase NP as a consequence of anaerobic sludge digestion (Banihani, 2009).

During aerobic digestion, nonylphenol transformations were directly related to both SRT and air flow rate, or the independent variable—air volume (air flow rate times SRT) (Figure 2.24), as we defined in the previous section. Student's t-test was performed to compare the nonylphenol reduction ratio under two different air loading rates, and the results suggest that the total air loading may be a fairly good predictor of nonylphenol reduction. It is indicated by similarities in the performances of all these four bioreactors running at different temperatures, for the 10-day SRT/1.0 LPM (L/min) reactor and the 5-day SRT/2.0 LPM reactor. The performances of the 5-day/1.0 LPM and the 2.5-day/2.0 LPM reactors were also similar for all temperatures.

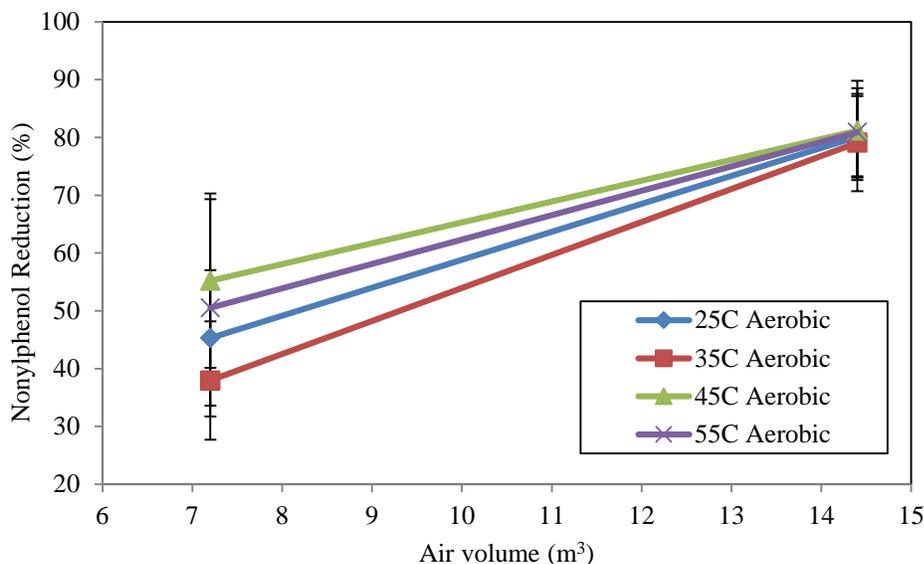


Figure 2.24 Comparison of nonylphenol reduction ratios under different aerobic digester conditions.

Further work is necessary to establish cause and effect. That is, it is possible that nonylphenol removal was correlated with removal of DOC or degree of nitrification, among other things, but insufficient evidence has been obtained to distinguish among the possibilities. Effluent concentrations of NP were always lowest in the 25 °C and 45 °C reactors. The database is considered to be too limited to draw firm conclusions in this area.

### 2.6.3.2 Octylphenol concentrations comparison

Octylphenol (OP) and NP differ in structure only in terms of the lengths of their respective alkyl chains. Both of them can be formed from the anaerobic degradation of alkylphenol ethoxylates. Octylphenol also represents a large number of isomeric compounds. The octyl group may be branched or linear, originating the 2-, 3- or 4-position of the benzene ring. However, only 4-octylphenol (CAS No. 1806-26-4) is currently available commercially both in Europe and the United States (Jin et al., 2013). As such it was the target compound for studying the fate of octylphenol in this research.

A GC/MS chromatogram (Figure 2.25) corresponding to an OP standard SIM mode ( $m/z = 107$ ) is provided.

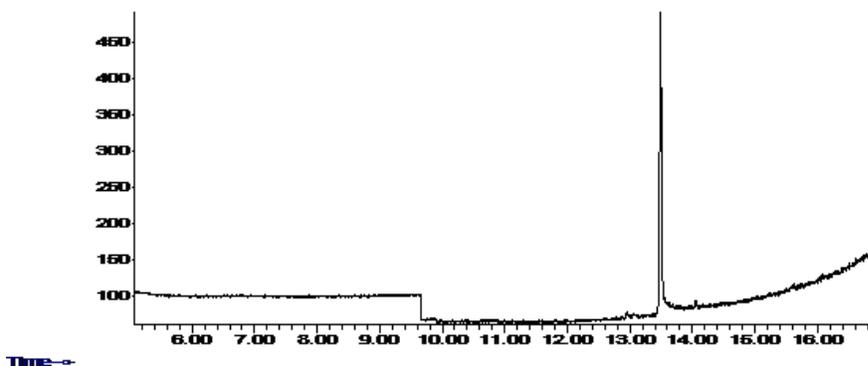


Figure 2.25 Chromatogram of the octylphenol standard (10ppb), between 13 and 14 minute ( $m/z = 107$ ).

Similar to the detection of NP, GC-MS analysis is suitable for OP measurement in the concentration range 10 - 250  $\mu\text{g/L}$  (Figure 2.26). The response was linear in that range, with an  $r^2$  value of 0.992. Samples were extracted and concentrated in the same way as NP, and the results were normalized based on sample solids content (reported as  $\mu\text{g/kg dw}$ ).

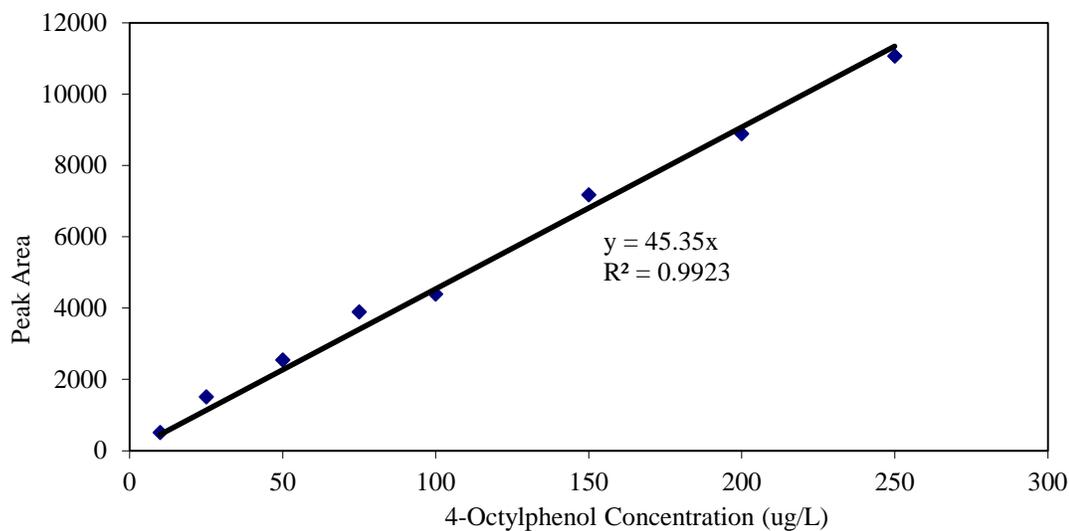


Figure 2.26 Standard curve for octylphenol using GC-MS. The curve is linear in the range of 10 to 250  $\mu\text{g/L}$ .

OP is hydrophobic ( $\log D_{ow}=5.63$ ) (Ying et al., 2002b) and tends to sorb on organic solids during sludge anaerobic digestion. Like NP, concentrations of OP in the liquid phase of sludge samples were below the detection limit ( $2 \mu\text{g/L}$ ); hence, data reported here were OP concentrations in the solid phase of sludge samples. The reported OP concentrations increased by a factor of 4-5 during anaerobic digestion, due in part to the destruction of organic solids. The transformation of ethoxylated species was probably also a factor.

Results at each reactor condition (Figure 2.27) represent the average of four independent samples. Not surprisingly, the fate of OP during anaerobic and aerobic digestion steps was similar to that of NP. The concentration of OP increases about four times during mesophilic anaerobic digestion, probably due in part to the degradation of octylphenol ethoxylates (OPnEOs).

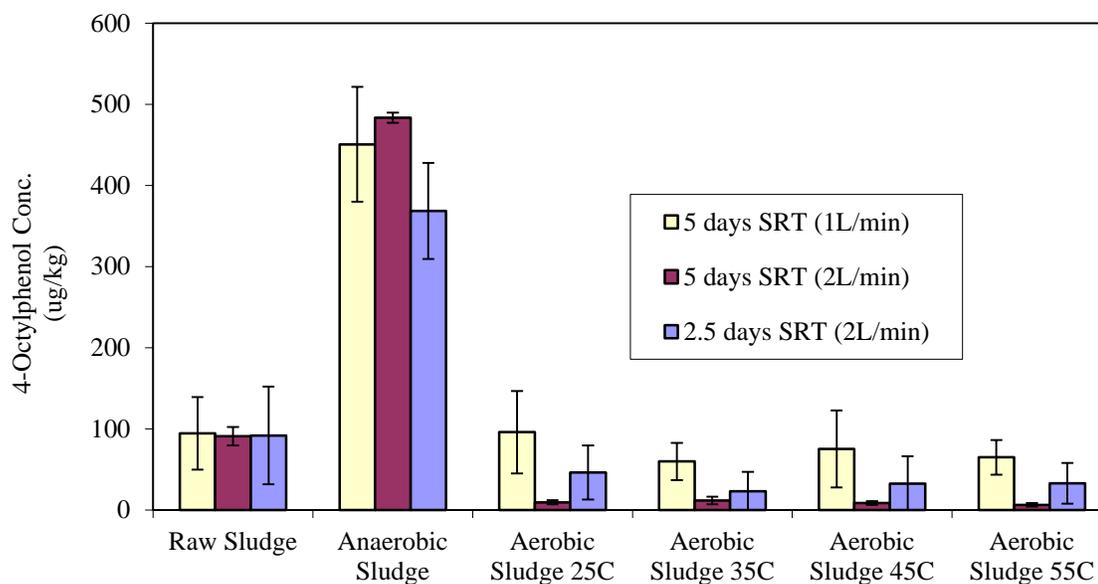


Figure 2.27 Octylphenol concentration comparison for different sludge retention time and different airflow rate in aerobic digestion.

Numbers of independent samples are as indicated ( $n=4$ ). Error bars indicate  $\pm 1.0\sigma$ .

At different SRTs (5 and 2.5 days) and aeration rates (1.0 and 2.0L/min), loss of OP was again directly related to oxygen loading, although the correlation was imperfect. The concentration of NP was much higher than that of OP in the sludges analyzed. This is in general agreement with reports from others (Mak and Chen, 2004), probably because the global production and household use of NPnEOs is much higher than OPnEOs (Sharma et al., 2009).

### **2.6.3.3 Contribution of nonylphenol and octylphenol to total estrogenic activities**

A suite of compounds, including three estrogenic steroid hormones 17 $\beta$ -estradiol (E<sub>2</sub>), estrone (E<sub>1</sub>), and estriol (E<sub>3</sub>), the synthetic hormone 17 $\alpha$ -ethynylestradiol (EE<sub>2</sub>), the hormone mimics NP, 4-OP and the androgenic hormone testosterone were tested for their estrogenic activities using the yeast estrogen screen. The most potent estrogenic compounds were EE<sub>2</sub> and E<sub>2</sub> (Figure 2.28). E<sub>1</sub> and E<sub>3</sub>, which are the metabolic by-products of E<sub>2</sub>, were less estrogenic. The surfactant degradation products NP and 4-OP were three orders of magnitude less estrogenic based on selective EC<sub>20</sub> values (Table 2.6). The least estrogenic compound was testosterone. The main difference between testosterone and other compounds is the lack of aromatic ring at ring position A. The results are comparable to those obtained by others (Conroy, 2006; Routledge and Sumpter, 1996).

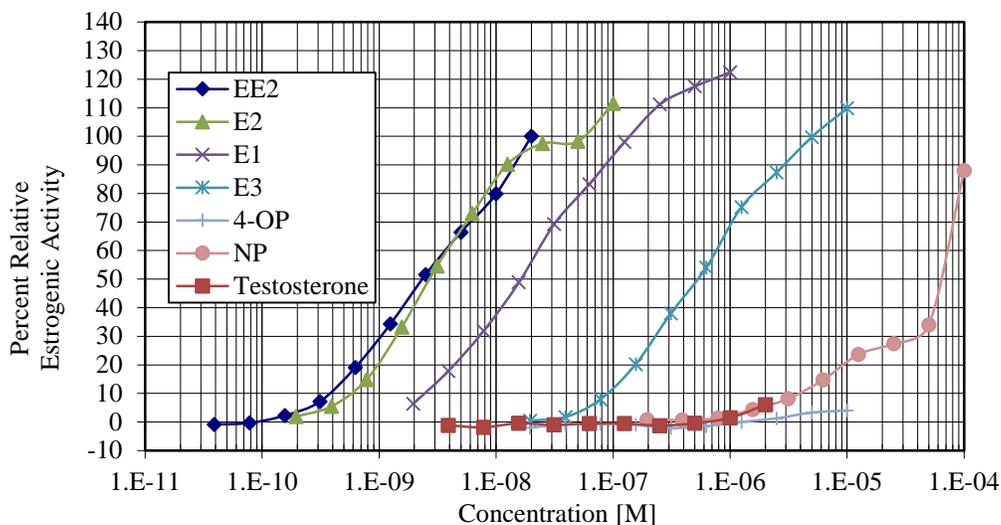


Figure 2.28 Yeast estrogen screen results. Response to known estrogens and estrogen mimics. Data were normalized to the minimum and maximum absorbance values of EE<sub>2</sub> in the positive controls.

Table 2.6 Estrogenic activities of EDCs relative to EE<sub>2</sub>.

Compounds	EC <sub>20</sub> (mol/L)	Relative Estrogenic Activity
E <sub>2</sub>	9.00E-10	1.43
EE <sub>2</sub>	6.30E-10	1
E <sub>1</sub>	4.20E-09	1/7
E <sub>3</sub>	1.50E-07	1/240
4-NP	9.00E-06	1/14,300
4-OP	2.00E-04	1/300,000
Testosterone	n/a	n/a

Specific estrogenic activities of NP and OP were 1/14,300 and 1/300,000 times that of EE<sub>2</sub> (Table 2.6). These factors were used to calculate the contributions of OP and NP to the measured total sample estrogenicity using the analytical results reported above (Table 2.7).

Table 2.7 Calculation of NP and OP contributions to the total estrogenic activities of sludge samples.

	Sample Total EC20 (mol/g dw)	NP (mol /g dw)	NP Equivalent EC20 (mol /g dw)	NP Estrogenic Activity Percentage (%)	OP (mol /g dw)	OP Equivalent EC20 (mol /g dw)	OP Estrogenic Activity Percentage (%)
<b>Raw Sludge</b>	3.61E-11	2.12E-09	9.53E-13	<b>2.64%</b>	4.3E-10	1.43E-15	<b>0.004%</b>
<b>Anaerobic Sludge</b>	9.33E-11	9.74E-09	4.38E-12	<b>4.70%</b>	2.05E-09	6.82E-15	<b>0.007%</b>
<b>25C Aerobic</b>	1.52E-11	2.30E-09	1.03E-12	<b>6.82%</b>	4.36E-10	1.45E-15	<b>0.010%</b>
<b>35C Aerobic</b>	2.27E-11	2.54E-09	1.14E-12	<b>5.02%</b>	2.72E-10	9.07E-16	<b>0.004%</b>
<b>45C Aerobic</b>	5.38E-11	2.33E-09	1.05E-12	<b>1.95%</b>	3.43E-10	1.14E-15	<b>0.002%</b>
<b>55C Aerobic</b>	1.32E-11	2.52E-09	1.14E-12	<b>8.60%</b>	2.96E-10	9.85E-16	<b>0.007%</b>

From the conversion of NP and OP to estrogenic activity, and comparison with the total estrogenic activity in the same sludge sample, it is concluded that the estrogenic activity of NP was much higher than that of OP in each sample, and that the contribution of OP to total estrogenicity of a sample is probably negligible (always  $\leq 0.01\%$ ).

Although the contributions of NP to sample estrogenicity were, in general, about three orders of magnitude higher than those of OP, all NP contributions account for  $\leq 10\%$  of total sample estrogenic activity. It is concluded that neither NP nor OP was the primary source of estrogenic activity in the digester samples. More work (bioassay-directed HPLC fractionation) is needed to identify the major estrogenic compounds present.

### 2.6.4 Yeast androgen screen results

Relatively little is known about the fates of androgens and androgen mimics in the environment. Most reports have been focused on the fates of individual androgens during conventional wastewater treatment, with particular attention to influent-to-effluent removal efficiency (Bradley et al., 2008; Sumpter and Jobling, 2013). There have been few measurements of concentrations in sludge or biosolids.

The level of androgenic activities measured in raw sludge and anaerobic digested sludge are shown in Figure 2.29. It is clear that the level of androgenic activity in raw sludge was low—below the assay detection limit ( $3.0 \times 10^{-13}$  mol testosterone/g dw) in most samples. After mesophilic anaerobic digestion, however, androgenic activity increased to levels on the order of  $10^{-9}$  mol testosterone/g dw. Androgenic activity was dramatically enhanced during anaerobic digestion.

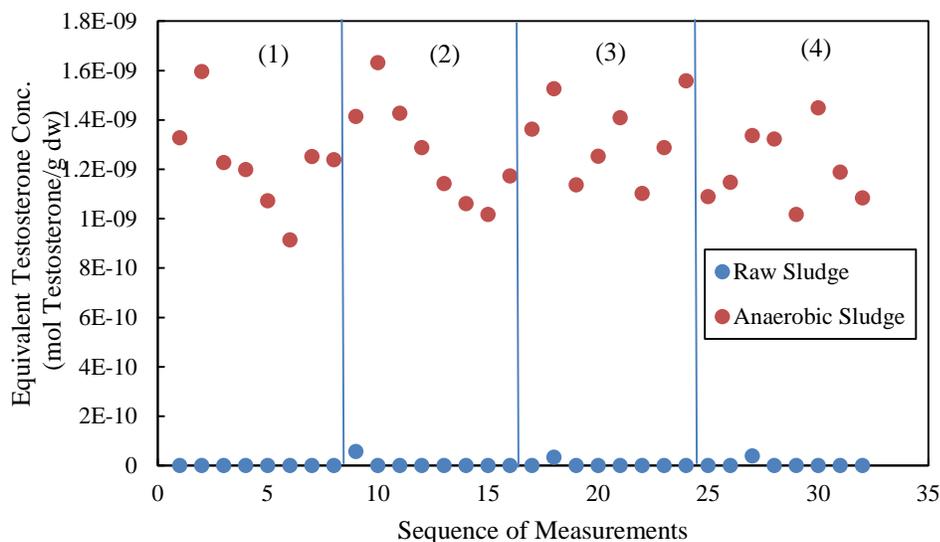


Figure 2.29 Comparison of androgenic activity in raw sludge and anaerobic digested sludge. Separate lines indicate different batch of raw sludge were used for the digestion process.

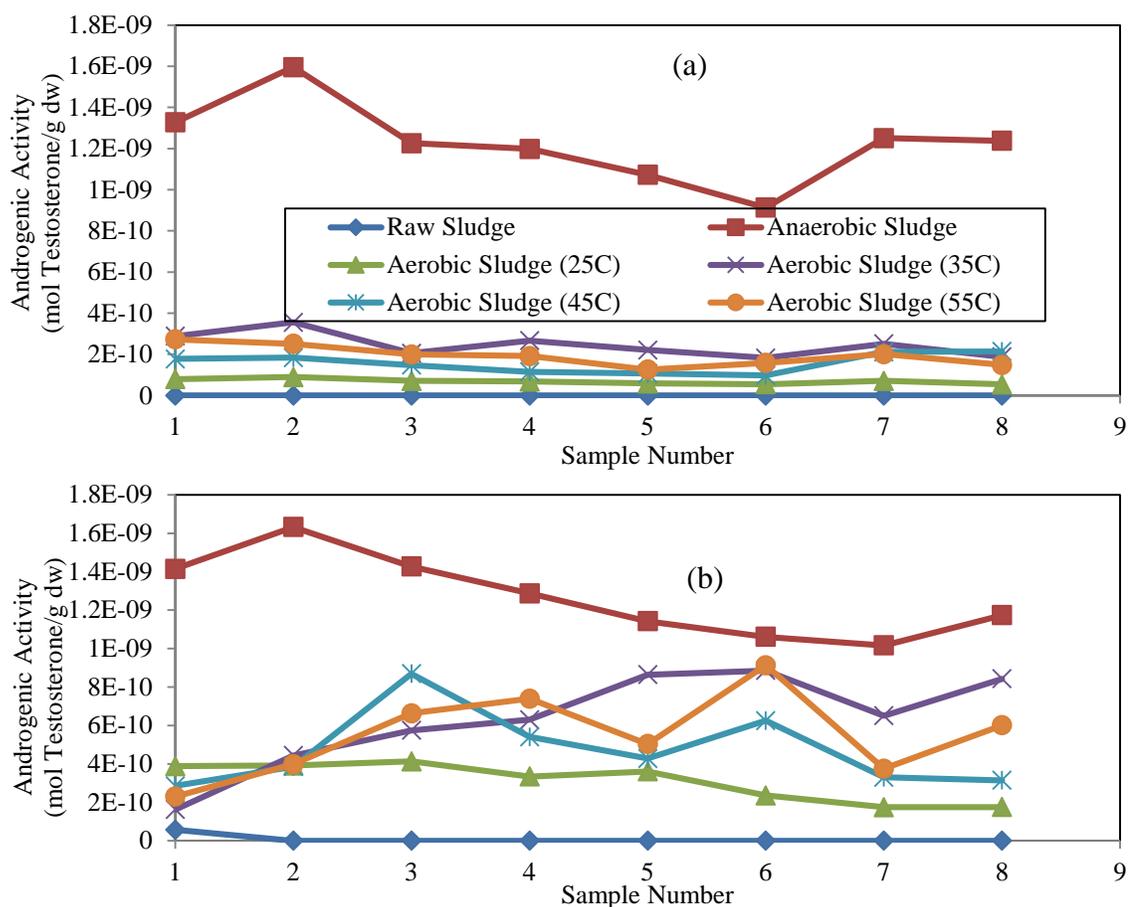
Different settings for aerobic digesters: (1) SRT=10 days; (2) SRT=5 days; (3) SRT=5 days; (4) SRT=2.5 days.

Airflow rate settings: (1) and (2): 1 L/min; (3) and (4): 2 L/min.

The fact that androgenic and estrogenic activities both increased during anaerobic digestion is unremarkable inasmuch as the structures of natural androgens and estrogens are very similar. All are C18 steroids, made up of by 18 carbon atoms distributed in three hexagonal rings and one pentagonal ring. The androgenic chemicals are hydrophobic as well, with moderate affinities for organic solids ( $\log K_{ow} = 2.4-4$ ) (Lai et al., 2000). Androgenic activity in the liquid phase of sludge samples was negligible in relation to sorbed activity. Although a detailed mechanism is beyond the scope of this work, it is evident that the anaerobic digestion process has a profound effect on sample androgens, perhaps converting relatively inert, conjugated forms to active androgens. Only unsupported speculation is possible at this point.

Comparing with estrogenic activity results of the anaerobic digestion process, the level of androgenic activity is almost an order of magnitude greater than the estrogenic activity equivalent detected in the same anaerobic digested sludge sample (performing YES and YAS assays for the same sample). Since the quality of municipal wastewater is adversely affected by anthropogenic activities, one possible explanation for this phenomenon is that the circulating concentrations of androgens in men and women are higher (nmol/L level) than the concentrations of estrogens (pmol/L level) from the evidence of the medical research (Melmed et al., 2011; Secreto et al., 1983). Hence, concentrations of androgen or androgen mimics in raw sewage and effluents might be expected to be higher than the concentrations of estrogens, which agreed with what have been discovered in this project and the results of chemical analytical work done by the other research group (Chang et al., 2011).

One further step was needed in the sludge treatment process for degrading these androgenic compounds which were generated in the solid phase of the anaerobic digested sludge. Four parallel aerobic digesters at different temperatures were set up as described in Section 2.5.2. The changes of androgenic activities after the aerobic digestion from different settings is showed in Figure 2.30.



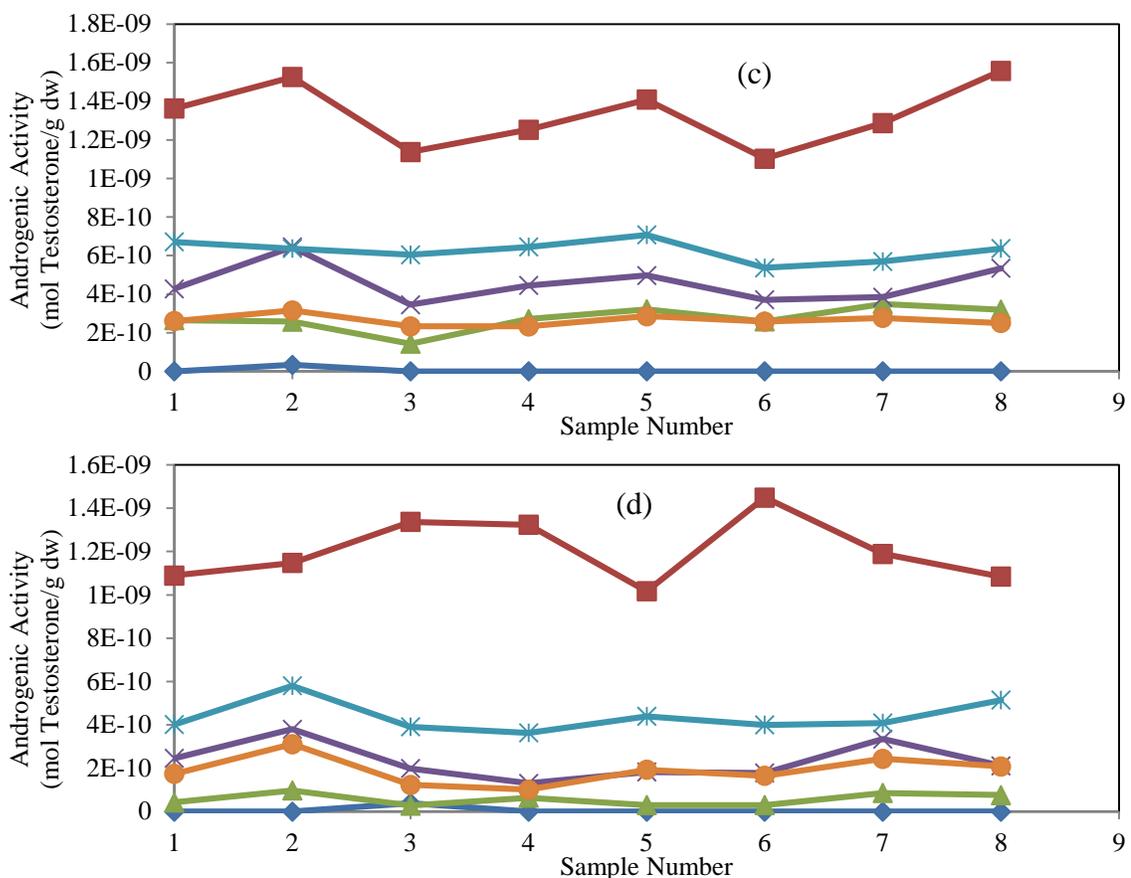


Figure 2.30 Androgenic activities in raw, anaerobically digested and sequentially digested sludges. Activities are presented as equivalent concentrations of testosterone and normalized in terms of the sludge dry weight.

Different settings for aerobic digesters: (a) SRT=10 days; (b) SRT=5 days; (c) SRT=5 days; (d) SRT=2.5 days.

Airflow rate settings: (a) and (b): 1 L/min; (c) and (d): 2 L/min.

Two-way ANOVA as used for the analysis of estrogenic activity change was applied to the androgenic activity as well. The independent variables are still air loading and temperature set to these aerobic digesters, while the dependent variable is the reduction ratio of the androgenic activity from the anaerobic digested sludge during the aerobic digestion process (same equation as the calculation for estrogenic activity reduction). The results of post-hoc Tukey analysis are showed in Table 2.8. From the results, it is clear that the reduction ratio at the temperature of 25 °C were better than the

other three aerobic digester at higher temperatures (Figure 2.31). Unlike estrogen inactivation was effective at 55 °C, androgenic activity was most effectively removed at 25 °C. When the air loading decreased to 7.2 m<sup>3</sup>, androgenic activity still decreased, but by noticeably less than during the 14.4 m<sup>3</sup> air loading to the reactors. The performance of the 25°C reactor remained superior to those of the other three digesters.

Table 2.8 Two-way ANOVA statistical results for the nitrogen change in the aerobic digesters.

Dependent Variable: Reduction ratio of androgenic activity (%)

temperature	temperature	Mean Difference	Std. Error	Sig.
25.00	35.00	17.8875*	3.77540	<0.0005
	45.00	19.9953*	3.77540	<0.0005
	55.00	10.5762*	3.77540	0.030
35.00	25.00	-17.8875*	3.77540	<0.0005
	45.00	2.1078	3.77540	0.944
	55.00	-7.3113	3.77540	0.218
45.00	25.00	-19.9953*	3.77540	<0.0005
	35.00	-2.1078	3.77540	0.944
	55.00	-9.4191	3.77540	0.066
55.00	25.00	-10.5762*	3.77540	0.030
	35.00	7.3113	3.77540	0.218
	45.00	9.4191	3.77540	0.066

\*. The mean difference is significant at the .05 level.

The loss of androgenic activity during aerobic digestion was similar to the loss of estrogenic activity under comparable circumstances. At a stable air loading of 14.4 m<sup>3</sup> and 7.2 m<sup>3</sup>, the androgenic activity reduction ratio of the aerobic digesters was being compared by Student's t-test method. The result indicate that the removal of androgenic activity was directly related to air loading (product of SRT and aeration rate) during

aerobic digestion, which was similar to the results of estrogenic activity removal by the reactors.

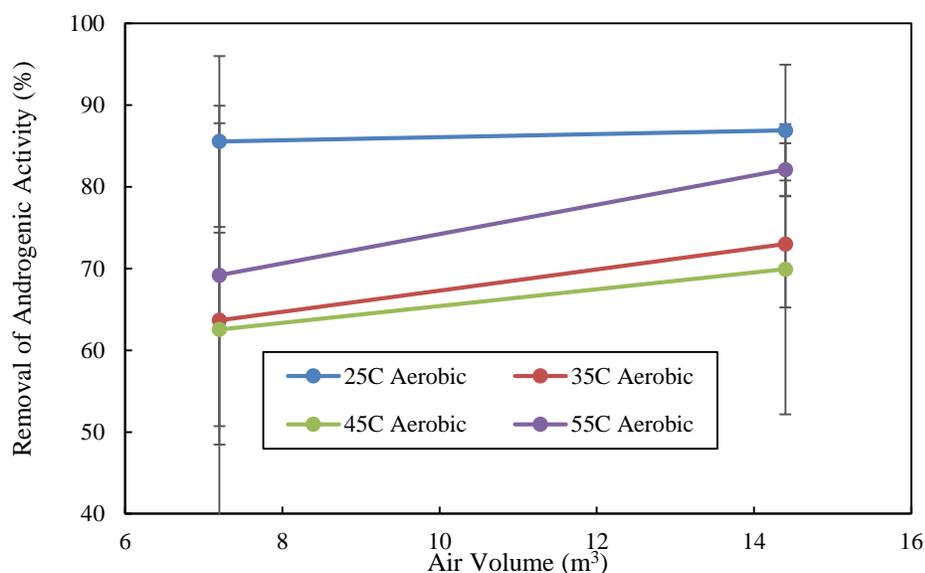


Figure 2.31 Comparison of androgenic activity reduction ratios under different aerobic digester conditions.

There have been relatively few reports on the fates of specific androgens during wastewater treatment. Esperanza et al. (2007) measured the concentration of testosterone and androstenedione in a pilot-scale WWTP with aerobic sludge digestion. Complete removal of these compounds was observed in the sludge and final effluent. Another survey was done in Canada by Lee et al. (2004), in which they measured testosterone in the influent of four WWTPs in Toronto area at concentration levels ranging from 24 to 180 ng/l. They reported effluent testosterone concentrations below the detection limit of 1 ng/l.

## **2.6.5 Separation of estrogenic/androgenic chemicals in sludge: fractionation and biological screening**

### **2.6.5.1 HPLC fractionation and biological screening**

As discussed in Section 2.6.2.2, the cytotoxic effects of non-estrogenic/androgenic chemical mixtures in a single sludge sample can inhibit the growth of exposed yeast cells during YES/YAS measurements. Thus toxicity in the biological assay can conceal the estrogenic/androgenic response to the sample, and the measured activity underestimated that of the sample.

To illustrate, results of YES and YAS assays on the same anaerobically digested sludge sample are shown (Figure 2.32 and Figure 2.33). From the figures, it is clear that the estrogenic/androgenic response of yeast cells to the 50% and 80% methanol fractions was depressed, at least over a part of the curves, by sample toxicity. This is apparent from depressed growth ( $A_{630}$  values that the highest sample concentrations in the 96-well plates).

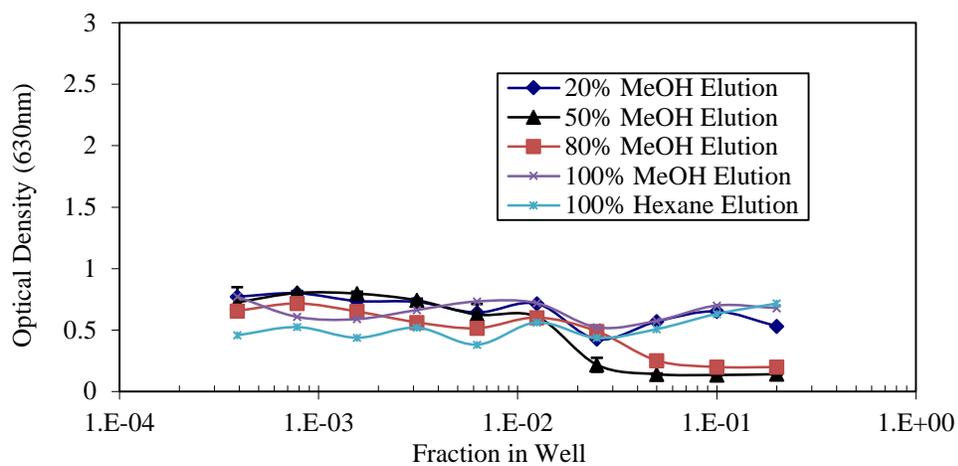
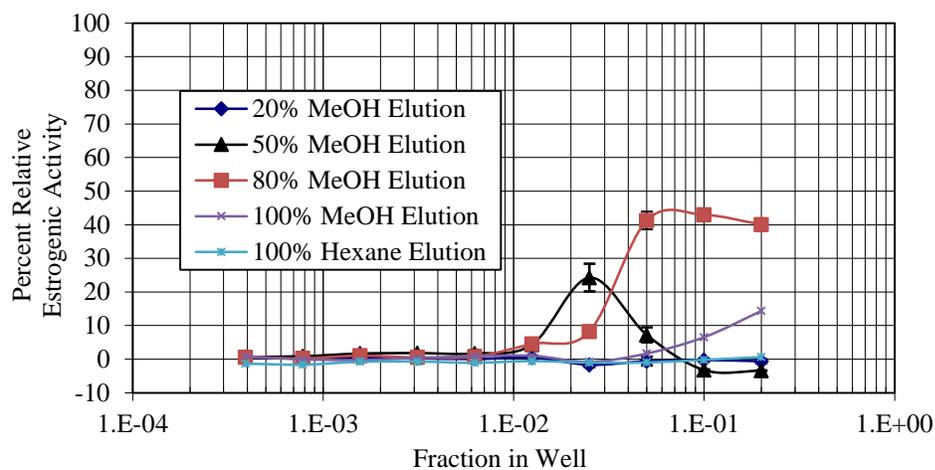
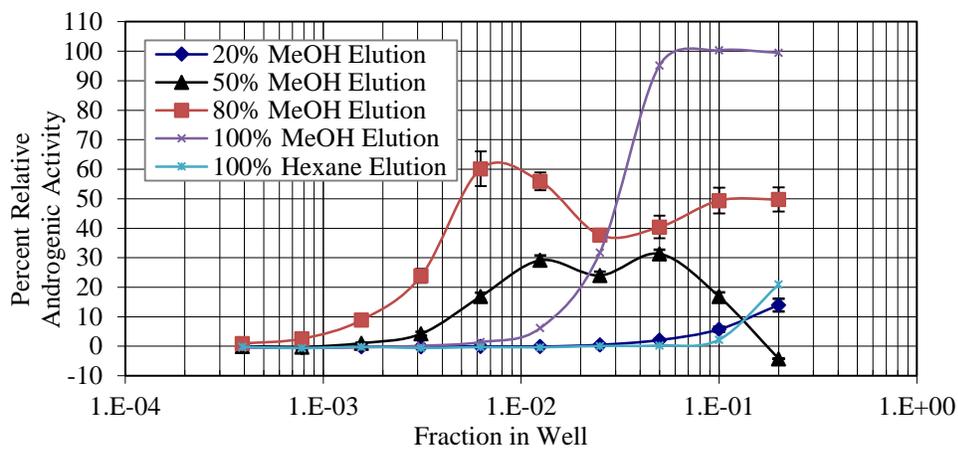


Figure 2.32 Estrogenic response in the YES assay for samples collected from anaerobic digester (January 20, 2012)



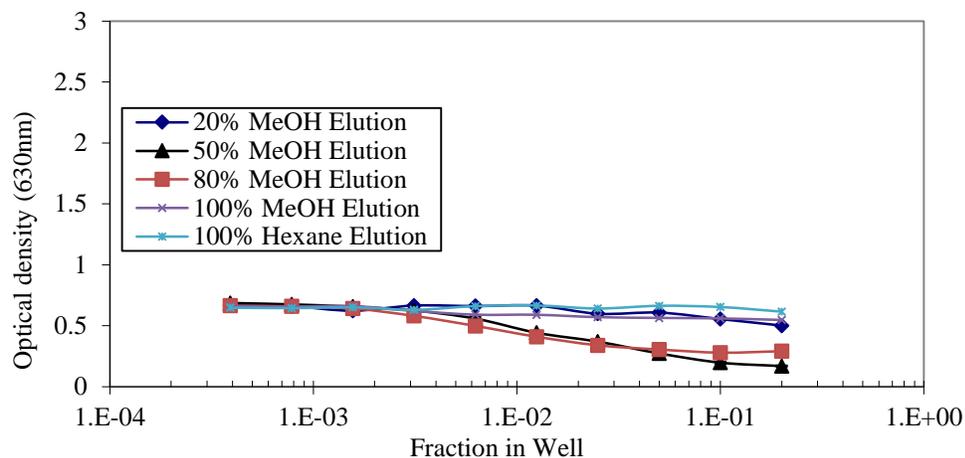


Figure 2.33 Androgenic response in the YAS assay for samples collected from anaerobic digester (January 20, 2012)

In order to eliminate or minimize sample toxicity and to improve the accuracy of estrogenic/androgenic activity measurements, HPLC separations were employed prior to measurements of estrogenic and androgenic activities in HPLC fractions. Samples were first extracted, dried and resolubilized 100% methanol. HPLC fractionation followed the method described in Section 2.5.9. Figure 2.34 shows a typical reverse-phase HPLC chromatogram so obtained.

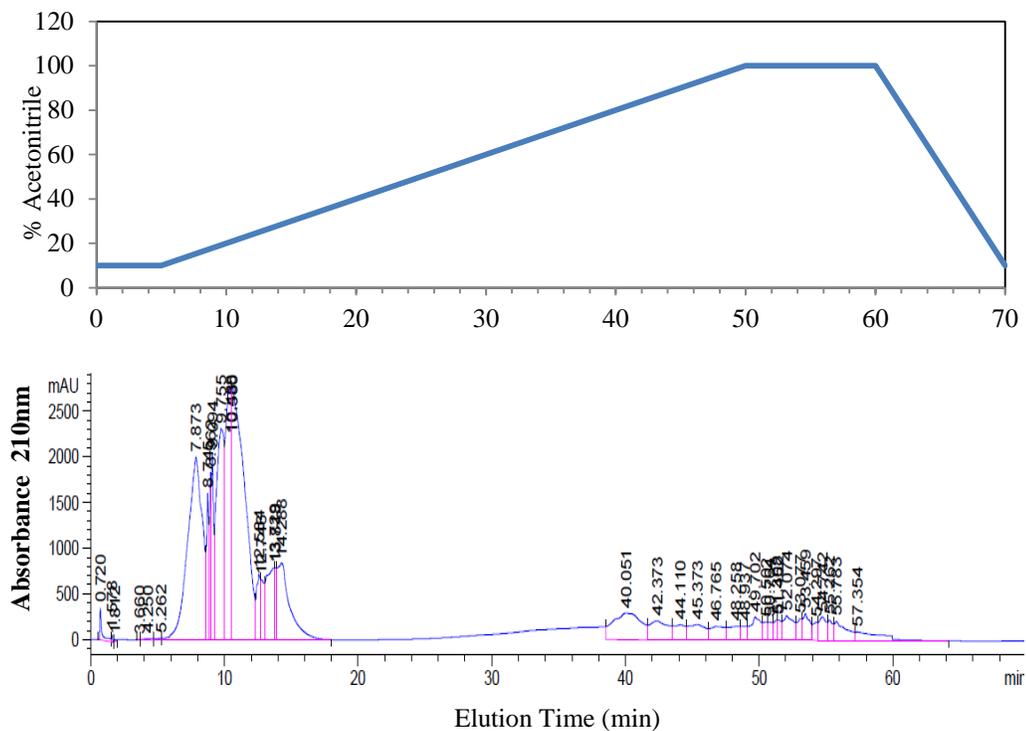


Figure 2.34 HPLC chromatogram derived from an extract of anaerobically digested sludge using reverse-phase HPLC.

(a) elution profile—acetonitrile in water, (b) UV absorbance profile— $\lambda = 210$  nm.

As expected, the UV absorbance profile ( $\lambda = 210$ nm) indicates that the sample contained a highly complex mixture of organic compounds and potential toxicants.

Eluent fractions were collected every two minutes, and the estrogenic/androgenic activity of each single fraction was screened using a simplified version of the YES and YAS biological screening assays.

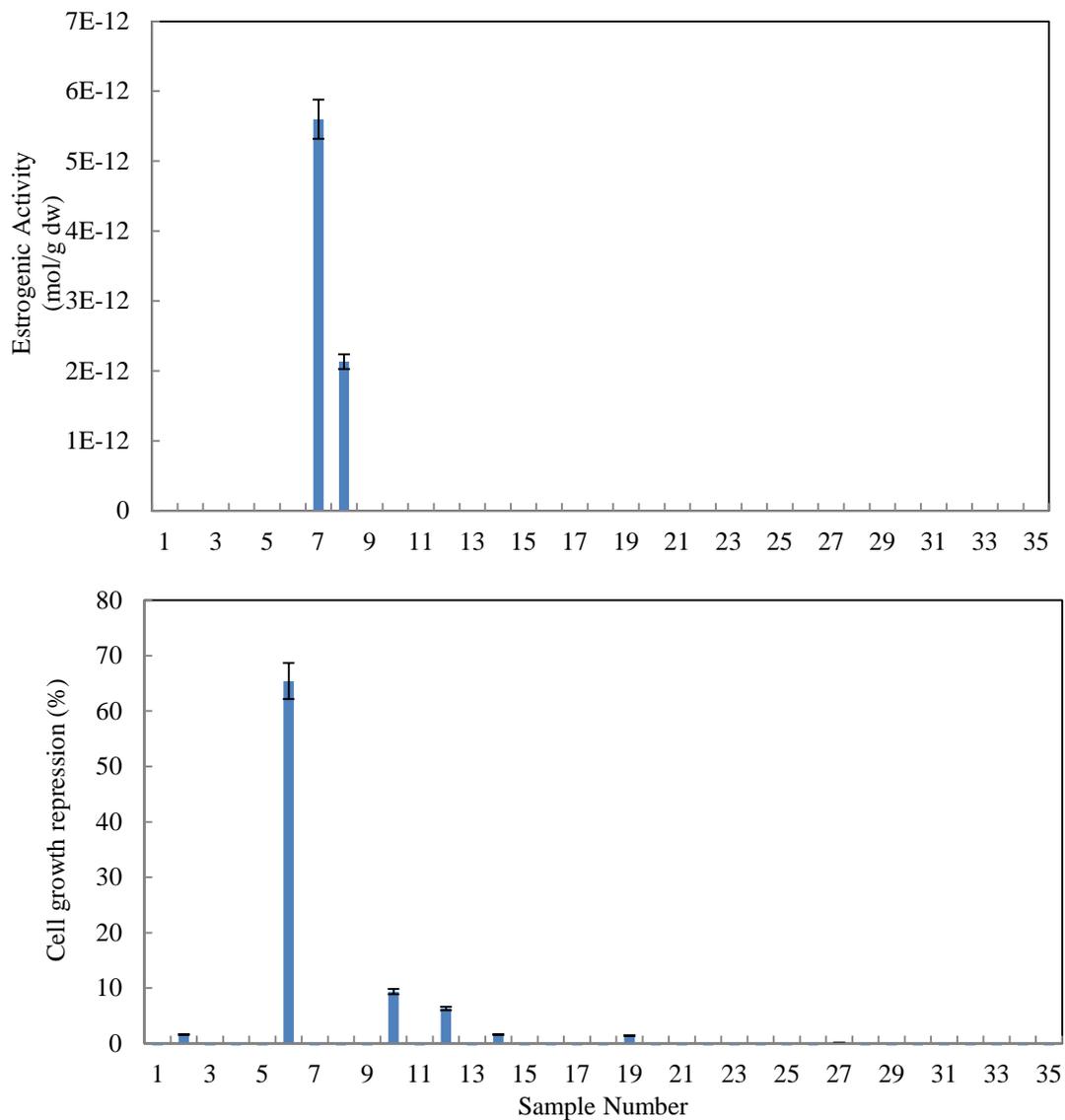


Figure 2.35 Estrogenic activity and toxicity profiles produced from whole extract fractionation using reverse-phase HPLC separation starting with a sample from the anaerobic digester.

Sample numbers refer to fractions collected every two minutes (a) estrogenic activity profile. (b) toxicity effects of sludge sample fraction to the cell in the YES assay.

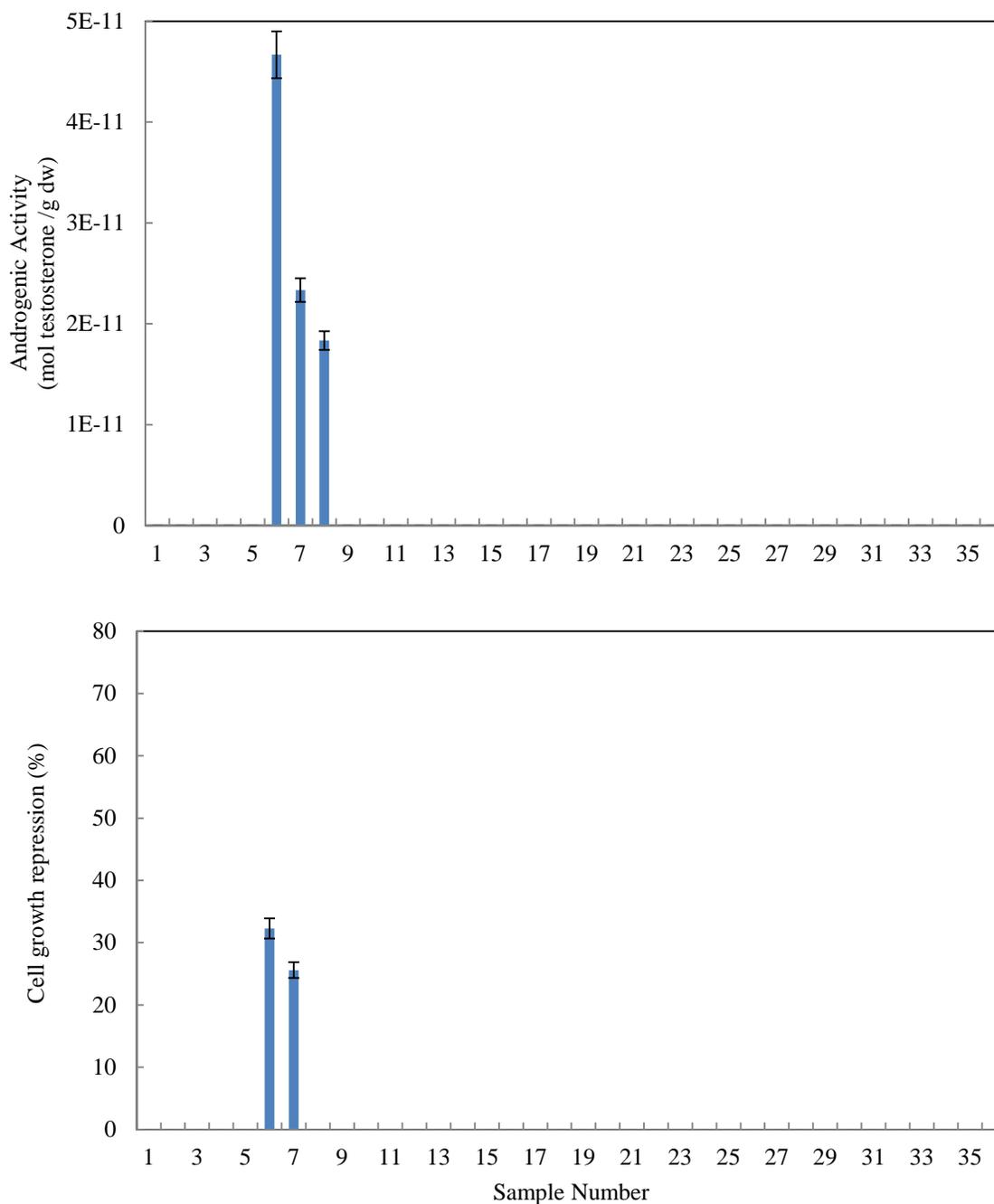


Figure 2.36 Androgenic activity and toxicity profiles produced from whole extract fractionation using reverse-phase HPLC separation starting with a sample from the anaerobic digester. Sample numbers refer to fractions collected every two minutes (a) androgenic activity profile. (b) toxicity effects of sludge sample fraction to the cell in the YAS assay.

Figure 2.35 and Figure 2.36 illustrate the results for the YES/YAS assays for the same sample of anaerobically digested sludge. Estrogenic and androgenic activities are apparent in several fractions (13 - 16 min for estrogenic activity, and 11 - 16 min for androgenic activity). The results suggest that the estrogenic/androgenic response in the biological screen is not caused by a single compound, but perhaps a suite of compounds with modestly different hydrophobic/hydrophilic properties. The elution profiles of androgenic and estrogenic activities were similar, although not exactly the same. When samples were spiked with NP and OP prior to separations those compounds eluted in fractions 21 and 22, showing again that these compounds did not contribute heavily to sample estrogenic activity.

When comparing the results of fractionation and the whole sample YES/YAS, the sums of these additive fractions for the estrogenic and androgenic activities were only count about 24% and 20% of the whole sample, respectively. It is possible the concentration factors were not large enough for the HPLC fractionation work, and some of the estrogenic/androgenic response were too weak to show up in the bioassay. And since the cytotoxic fraction showed up both in the YES/YAS response fractions (11-12 min), it can certainly mask the actual response of that fraction in the yeast cells.

The toxic responses of yeast cells in the YES and YAS assays to the same sample were different. Fraction 6 (11 to 12 min) was toxic to yeast cells in both YES and YAS bioassays. But in the YES assay, additional fractions proved to be modestly toxic to the yeast strain. This may have resulted from differences in the responses of the two cell lines. That is, the cells used in the YES bioassay may be more vulnerable to chemical

toxicity than cells used in YAS assay. Alternatively, toxicity results representing < 10% inhibition of cell growth may be simply noise in the turbidity measurements.

#### **2.6.5.2 Identification of estrogenic compounds by liquid chromatography tandem mass spectrometry**

From Section 2.6.5.1, it is impossible to determine whether the EDC activity within the fraction was due to a highly abundant component or whether it was due to some other minor components that did not show up in the HPLC chromatograms. Consequently, liquid chromatography tandem mass spectrometry (LC-QToF) was used with the responsive fractions in order to identify responsible compounds. Raw sludge, anaerobic digested sludge and the sequential anaerobically/aerobically digested sludge (35 and 55 °C) samples were all tested in this manner. Several known estrogens were spiked into controls prior to HPLC separations.

When running LC-MSMS, the authentic standard compounds provided information that was applied to the interpretation of data derived from samples. Figure 2.37 represents the information-dependent acquisition (IDA survey) of a standard set of estrogens. For each standard compound (NP, OP, E<sub>1</sub>, EE<sub>2</sub>), information can be derived from the IDA survey by the extracted-ion chromatogram (XIC). As observed from the chromatograph, retention time was different for each compound based on its hydrophobicity.

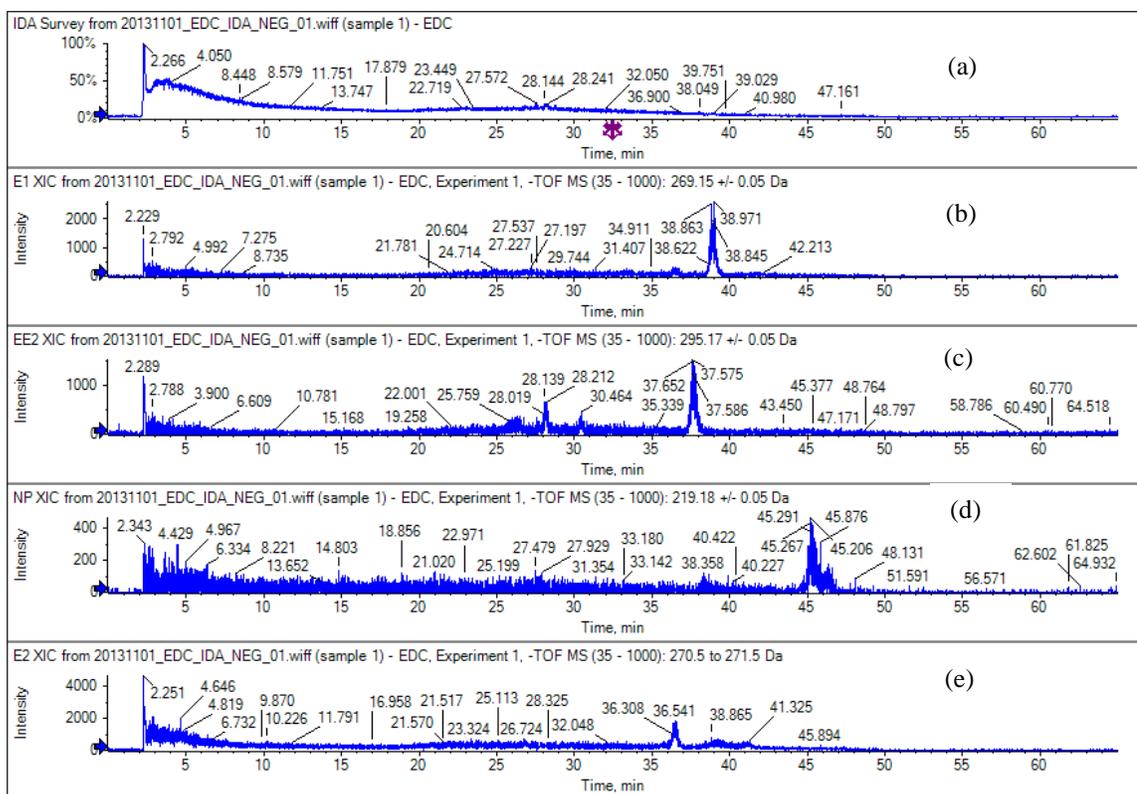


Figure 2.37 EDC standard chromatographs for comparison with the samples.

(a) Information-dependent acquisition chromatogram (IDA survey) of the standards; (b) Extracted-ion chromatogram (XIC) for E<sub>1</sub>; (c) XIC for EE<sub>2</sub>; (d) XIC for NP; (e) XIC for E<sub>2</sub>.

The same principle was applied to the analysis for the sludge fractions. As of each fraction running through the LC-MSMS, the XIC information was extracted from the IDA survey as well for the known estrogens. In this comparison work, the peak values for each known compound (Figure 2.37) were summarized and compared, and the distribution of these compounds in the fractions in each sample was being compare.

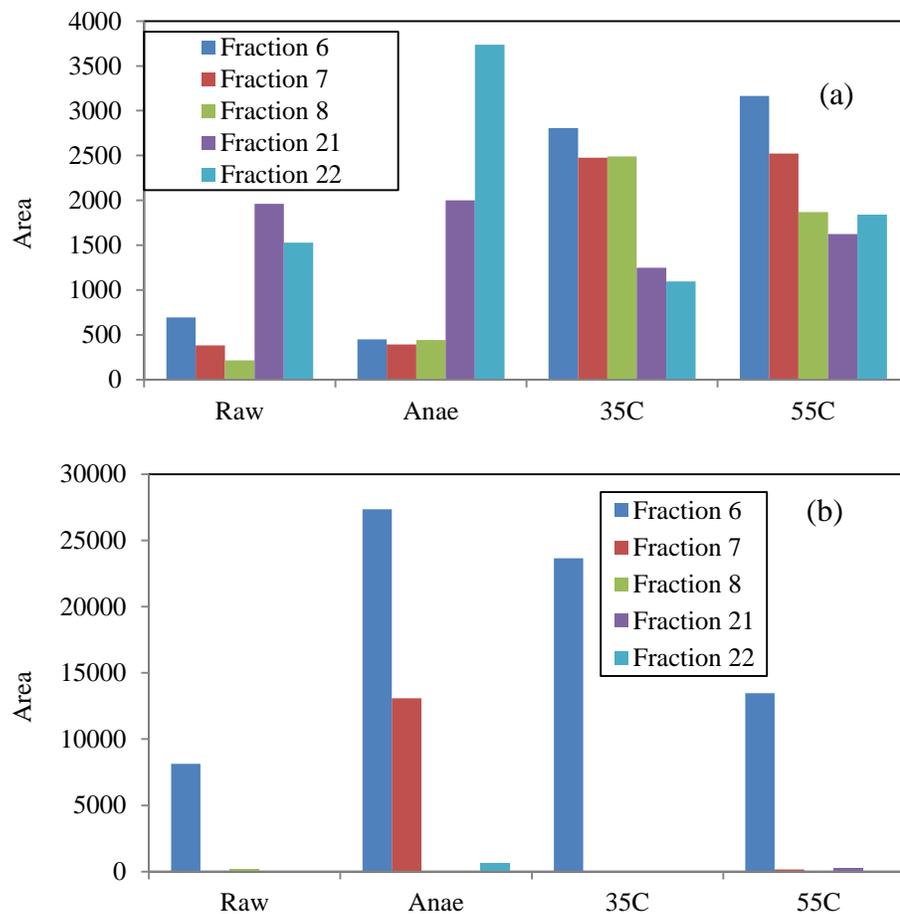


Figure 2.38 Comparison of area for OP and NP distribution in the fractions in a suite of samples.

(a) Octylphenol; (b) Nonylphenol.

The results for OP and NP distribution among different fractions are displayed in Figure 2.38. As discovered from the distribution of OP, for raw sludge and anaerobic digested sludge, the majority was in more hydrophobic fractions (Fraction 21 and 22). But following aerobic digestion process, the distribution of OP among the fractions changed, as the main part was in the first several fractions, and the areas for OP in the last two fractions decreased. This is probably because the biological degradation of OP during the aerobic digestion process, and OP was being eluted from the LC separation with other compounds in the same sample as well.

For the result of NP in the fractions of a sample, it can conclude that this compound was mainly distributed in the front end fractions, which was opposite to its high hydrophobicity. It was possibly due to the reason that in the sludge samples, NP was co-eluted with other compounds early in the fractionation process. But as the LC-QToF analytical work, NP was being well separated from the other chemicals in the fractionated samples. Also from the result for comparing the areas of NP among these samples, it can be seen that the concentration of NP increased during the anaerobic digestion. After the sequential aerobic digestion, its concentration decreased for the same reason we mentioned in Section 2.6.3.

E<sub>1</sub> and EE<sub>2</sub> were measured only in Fraction 7 of the aerobically digested sample (55°C), and E<sub>2</sub> did not show up in all the fractions we tested. It is probably due to the concentrations of these compounds were low in these HPLC fractions, or they were low in the original concentrated extracted samples from the sludge. A further study should be carried out for getting better results in this analytical work, in order to identify the main responsible components for the estrogenic/androgenic activity of the sludge samples.

## 2.7 Conclusions

Sewage sludge contains a wide range of trace organic contaminants, particularly those that are relatively hydrophobic. In this project, the combined effects of sequential anaerobic/aerobic digestion on residual trace organics were evaluated, concentrating on chemicals that are responsible for observed estrogenic/androgenic activity in biosolids. Full-scale sequential digestion was simulated using bench-scale bioreactors in which the primary independent variables were retention time, temperature, and oxygen loading during aerobic digestion.

Results included treatment-dependent changes in estrogenic and androgenic activities. Standard mesophilic anaerobic digestion increased the total estrogenic and androgenic activities of sludge, and the androgenic activity in the anaerobic digested sludge was much higher than the estrogenic activity.

Our study results showed that an additional step of aerobic digestion after the anaerobic digestion process in sludge treatment is very useful, lowering both estrogenic and androgenic activity in biosolids that may be destined land application, possibly due to the nitrification/denitrification in the aerobic digestion. The effectiveness of aerobic treatment was directly related to all three independent variables. Furthermore, the thermophilic (55 °C) aerobic digestion was much more effective at reducing estrogenic activities, while androgenic activity was most effectively removed at 25 °C. At relatively short detention times (5 and 10 days). Doubling the airflow rate from 1.0 L/min to 2.0 L/min at the 5-day retention time further improved reactor performance.

Concentrations of NP and OP were increased after anaerobic digestion due to the degradation of NPnEOs in the sludge. During the aerobic digestion, levels of trace

organic compounds NP and OP were also decreased, which was related to the air loading to the reactors. NP only counted for less than 10% of the estrogenic activity, while the contribution of OP was negligible. Further work for identifying the major components of the estrogenic/androgenic activities based on HPLC fractionation and chemical analytical method was needed.

Results suggest that conventional sludge treatment processes can be designed and operated to better remove estrogenic activity in sludge. This is particularly important when digested sludge is utilized as a soil amendment.

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## CHAPTER 3 FATE OF TRACE ORGANICS IN WATERS AND SEDIMENTS OF AN EPHEMERAL STREAM

### 3.1 Introduction

For more than thirty years, it has been known that pharmaceuticals, personal care products and other trace organic contaminants survive conventional wastewater treatment and persist in the environment to varying degrees (Ahel et al., 1994a; Kolpin et al., 2002; Richardson and Bowron, 1985). More than any other group of trace organics in municipal wastewater, endocrine disrupting compounds (EDCs) have attracted the attention of health professionals and the public (Heberer, 2002; Sumpter and Jobling, 2013; Teske and Arnold, 2008). EDCs interfere with normal endocrine function by blocking signaling/response (antagonists) or stimulating inappropriate activity (agonists). Chemicals present in municipal wastewater that promote estrogenic response include natural steroidal estrogens such as estrone ( $E_1$ ),  $17\beta$ -estradiol ( $E_2$ ) and estriol ( $E_3$ ), and anthropogenic mimics such as  $17\alpha$ -ethinylestradiol ( $EE_2$ ) and alkylphenols (APs) (Routledge and Sumpter, 1996; Teske and Arnold, 2008). All these compounds share similar chemical structure: presence of phenolic rings, similar molecular weight, mild to high hydrophobicity.

Early evidence of endocrine disrupting activity in treated wastewater arose from elevated incidence of intersex characteristics (compromised gonadal growth, spermatogenesis, sperm motility, and fertilization success) among male fish in rivers of the United Kingdom (Jobling et al., 1998; Purdom et al., 1994).  $E_2$  and  $EE_2$  elicit physiological changes in continuously exposed organisms at levels that are routinely measured in conventionally treated municipal wastewater (Sanchez et al., 2011). Factors

that influence the removal of endocrine disrupting compounds and other trace organic contaminants during wastewater treatment have been widely studied. Simulations of conventional treatment have not, however, been entirely successful in predicting chemical-specific removal efficiencies during municipal wastewater treatment (Rojas et al., 2010).

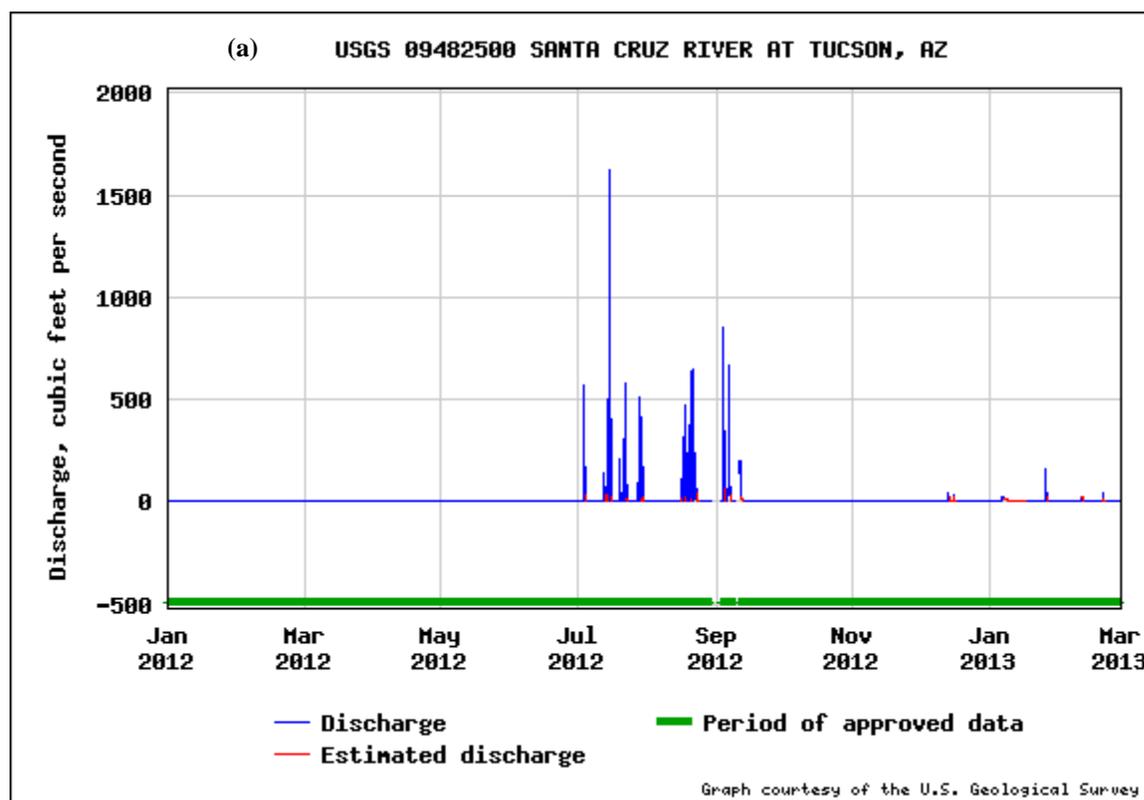
Gross et al. (2004) studied the fates of trace organics in an effluent dominated stream and wetland in southern California, measuring attenuations of ibuprofen, gemfibrozil and chlorinated tris-propylphosphates as well as the conversion of alkylphenol polyethoxylates to APs. Fono et al. (2006) measured the attenuations of ethylenediamine tetraacetate (EDTA), gemfibrozil, ibuprofen, metoprolol, naproxen, and total adsorbable organic iodide in the Trinity River, an effluent dominated stream in Texas. Although the iodated compounds were conserved, others decreased in concentration by 60-90% during two weeks of travel over 500 km. In a recent Arizona survey, Chiu and Westerhoff (2010) found that surface water concentrations of sucralose, sulfamethoxazole, acetaminophen, cotinine, dilantin, caffeine, DEET and oxybenzone exhibited seasonal trends. Oxybenzone and sucralose concentrations were generally higher in summer months due to increased recreational use of surface water resources.

Candidate mechanisms for the attenuation of trace organics during in-stream transport include biodegradation, photolysis and adsorption on riverbed sediments. Conditional rate constants representing relative compound biodegradability have been based on either direct measurement (Salveson et al., 2010) or moiety-specific contributions to biological transformation rates (Proctor and Gamble, 1999). The contribution of removal on organic solids should be directly related to compound

hydrophobicity, as reflected by octanol/water partitioning ( $K_{ow}$  or  $D_{ow}$ ) (Snyder et al., 2003; Ying et al., 2002a). It is expected that, other factors being equal, compound hydrophobicity would lead to accumulation in river sediments due to both sedimentation and the retention of contaminants during infiltration. Direct and indirect photolysis also contribute to in-stream compound disappearance (Watts and Linden, 2008; Wols and Hofman-Caris, 2012). Direct photolysis occurs when a compound undergoes transformation as an immediate result of light absorption. This is envisioned as a two-step process in which the absorption of light energy promotes the compound to an excited state, after which either chemical transformation occurs or the excited compound devolves to its original ground state. The ratio of transformation reactions to photons absorbed is the reaction quantum yield. Indirect photolysis is initiated through light absorption by other chemicals, leading to production of reactive intermediates such as hydroxyl radicals that react chemically with the target compound (Wols and Hofman-Caris, 2012).

In the vicinity of Tucson, AZ, the Santa Cruz River (SCR) is an effluent dependent stream (Figure 3.1). Tucson's two major wastewater treatment plants, Agua Nueva Water Reclamation Facility (WRF) and Tres Rios WRF together discharge ~50,000 AFY (acre-feet per year) ( $6.17 \times 10^8 \text{ m}^3/\text{year}$ ) of municipal wastewater effluent to the SCR (Figure 3.2). Galyean (1996) estimated that about 90% of the treated wastewater effluent discharged to the Santa Cruz River infiltrates within a 25-mile (40 km) length downstream from the Agua Nueva WRF. In a 1999-2000 nationwide survey of United States surface waters, the US Geological Survey measured some of the highest in-stream concentrations of trace organics at a sampling point in the lower SCR, several miles

downstream from the Tucson outfalls (Kolpin et al., 2002). The rainfall pattern in southern Arizona typically includes brief periods of heavy precipitation in the summer months that motivate equally brief periods of high stream flow (Figure 3.1). It has been shown that these events tend to scour sediments in the SCR, redistributing deposits that accumulate over the remainder of the year (Lacher, 1996). It is expected that high flow conditions in the river will produce discontinuities in time-dependent sediment concentrations of trace organic contaminants, although this has not been confirmed via direct measurement.



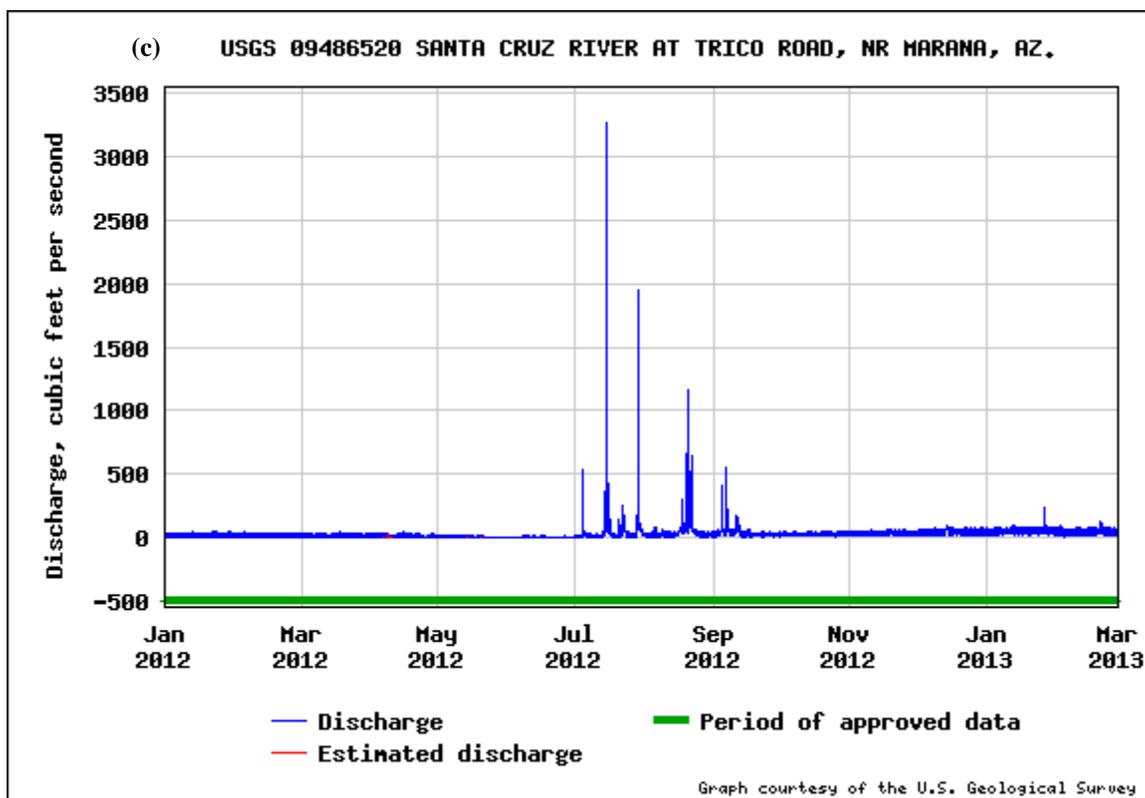
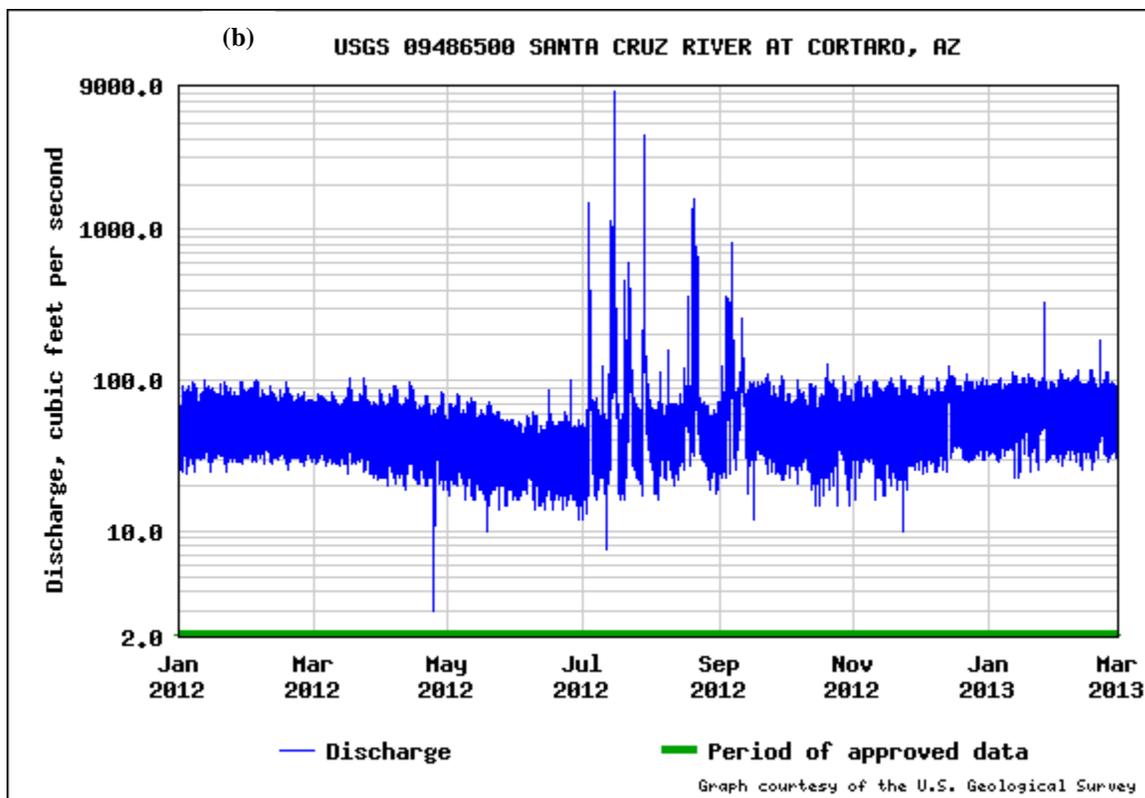


Figure 3.1 Record of flows at Tucson area USGS gaging stations on the Santa Cruz River during the period of study—February 2012 to February 2013.

For station locations, see Figure 3.2. Comparison shows that (i) the river is dry upstream of the Agua Nueva WRF (a) except during rainfall events and (ii) nearly 60% of the flow measured at Cortaro Road (b) infiltrates in the riverbed between Cortaro Road and Trico Road (c).

In this work, concentrations of trace organic contaminants of wastewater origin and extractable estrogenic activity were measured in SCR surface waters, groundwater samples obtained from monitoring wells proximate to the SCR and riverbed sediments. The monitoring program and supplementary controlled experiments using local wastewater effluent, were designed to test hypotheses regarding mechanisms of stream purification. The work is timely in light of process improvements recently completed at the Agua Nueva WRF and Tres Rios WRF with consequent improvements in both effluent and environmental quality. The data provide a baseline against which the effects of process improvements can be measured. The hypotheses related to natural stream purification mechanisms follow:

- Biodegradable organic contaminants, including those that contribute to estrogenic activity, are rapidly attenuated with distance and time of travel in the Santa Cruz River. It is noted that dilution is not a candidate mechanism for contaminant attenuation, since the Santa Cruz River is effluent dependent except during infrequent periods of rainfall/runoff.
- The primary mechanisms of contaminant attenuation in the river are related to physical/chemical properties of the contaminants including biodegradability, hydrophobicity and susceptibility to indirect or direct photolysis.

- Hydrophobic contaminants accumulate in riverbed sediments during dry weather periods and are periodically scoured from the bottom during storm-related periods of high flow.

## 3.2 Materials and methods

### 3.2.1 Chemicals and Reagents

All chemicals (Table 3.1) were reagent grade or better and used as obtained. Bisphenol A, salicylic acid and tris (2-chloroethyl) phosphate (TCEP) were from TCI America (Portland, OR). *N,N*-diethyl-meta-toluamide (DEET), ibuprofen, triclosan and perfluorooctanesulfonic acid (PFOS) were from Alfa Aesar (Lancashire, UK). Iopromide was from USP (Rockville, MD). Tonalide was purchased from Toronto Research Chemicals (Toronto, CA). Estrone, carbamazepine, ciprofloxacin, sulfamethoxazole, 4-nonylphenol and 4-octylphenol were from Sigma-Aldrich (St. Louis, MO). Sucralose was from AK Scientific (Union City, CA). Perfluorohexadecanoic acid (PFHxDA) was from Matrix Scientific and meprobamate was from Cerilliant (Round Rock, TX). Compounds were selected based on their recognized presence in conventionally treated wastewater effluent (from previous studies) and the existence of sufficiently sensitive analytical methods. Together, they provide a broad range of physical/biochemical characteristics, allowing us to investigate hypothesized cause-effect relationships governing contaminant fates in surface waters.

Table 3.1 Relevant physical/chemical properties for trace organics measured in the SCR and riverbed sediments samples (Compounds in italics were measured in the sampling events of 2011).

Compound	Usage	Log D <sub>ow</sub> at pH=7 <sup>[1]</sup>	k <sub>OH</sub> (10 <sup>9</sup> M <sup>-1</sup> s <sup>-1</sup> )	P <sub>B</sub> <sup>[8]</sup>	Log K <sub>AW</sub> <sup>[9]</sup>
17β-Estradiol (β E2)	Steroid hormone	4.15	14.1 <sup>[2]</sup>	0.1058	-8.827
17α-Ethinylestradiol (EE2)	Estrogen	4.11	10.3±0.7 <sup>[2]</sup>	0.0354	-9.489
Estrone (E1)	Steroid hormone	3.62	26 <sup>[3]</sup>	0.1074	-7.809
Estriol (E3)	Steroid hormone	2.53		0.1237	-10.265
4- <i>n</i> -nonylphenol (4- <i>n</i> -NP)	Detergents	6.14	11.0±0.2 <sup>[4]</sup>	0.5086	-2.857
4-Octylphenol	Detergents	5.63	14.0±0.2 <sup>[4]</sup>	0.5025	-3.735
Atenolol	B-blockers	-2.09	7.10 <sup>[5]</sup>	0.2349	-16.252
Atrazine	Herbicide	2.64	2.30±0.14 <sup>[2]</sup>	0	-7.016
Benzophenone	Photo initiator	3.21		-0.0737	-4.101
Benzotriazole	Drug precursor	1.42		0.3935	-5.221
Bisphenol A	Plasticizer	3.64	8.00±3.11 <sup>[2]</sup>	0.1559	-9.427
<i>Carbamazepine</i>	<i>Anticonvulsant</i>	<i>1.89</i>	<i>8.02±1.90<sup>[2]</sup></i>	<i>0.0364</i>	<i>-8.355</i>
Caffeine	Stimulant	-0.63	6.40±0.71 <sup>[2]</sup>	0.0521	-8.835
<i>DEET</i>	<i>Insect repellent</i>	<i>2.42</i>	<i>5<sup>[5]</sup></i>	<i>0.4437</i>	<i>-6.07</i>
Dexamethasone	Anti-inflammatory and immunosuppressant	2.03		0.0015	-5.534
Diclofenac	Nonsteroidal anti-inflammatory drug (NSAID)	1.77	8.38±1.24 <sup>[2]</sup>	0.0029	-9.714
Diltiazem	Calcium channel blockers	2.98		0.0267	-14.453
Diphenhydramine	Antihistamine	1.25	5.42 <sup>[2]</sup>	0.0313	-6.82
<i>Fluoxetine</i>	<i>Anti-depressant</i>	<i>1.15</i>	<i>9.0±0.6<sup>[13]</sup></i>	<i>0</i>	<i>-5.439</i>
Gemfibrozil	Blood lipid regulator	2.07	10 <sup>[6]</sup>	0.6123	-6.313
Ibuprofen	Nonsteroidal anti-inflammatory drug (NSAID)	0.94	7.04±0.52 <sup>[2]</sup>	0.1521	-5.207
Meprobamate	Anxiolytic	0.7		0.314	-8.121
Naproxen	Nonsteroidal anti-inflammatory drug (NSAID)	0.73	8.61 <sup>[2]</sup>	0.3447	-7.858
Norgestrel	Hormonal contraceptives	3.37		0.0469	-7.502
PFBS	Surfactant	-1.81		0	-3.23
PFDA	Surfactant	4.15		0	2.011
PFD <sub>o</sub> A	Surfactant	5.61		0	3.452
<i>PFOA</i>	<i>Surfactant</i>	<i>2.69</i>	<i>0.01<sup>[13]</sup></i>	<i>0</i>	<i>0.57</i>
<i>PFOS</i>	<i>Surfactant</i>	<i>1.01</i>	<i>0.01<sup>[13]</sup></i>	<i>0</i>	<i>-0.347</i>

PFHxDA	Surfactant	8.53		0	6.334
Prednisone	Immunosuppressant	1.57		0.1452	-7.937
Primidone	Anticonvulsant	0.83	6.70 <sup>[2]</sup>	0.4211	-8.101
Propylparaben	Preservative	2.88		0.8344	-6.584
Simazine	Herbicide	2.28	2.90±0.28 <sup>[2]</sup>	0	-7.414
Sucralose	Artificial sweetener	0.23	1.50±0.1 <sup>[10]</sup>	0.004	-16.787
Sulfamethoxazole	Antibiotic	-0.22	5.82±1.99 <sup>[2]</sup>	0.006	-10.408
Testosterone	Steroid hormone	3.18		0.1514	-6.841
Triclocarban	Antibiotic	6.07		0.0017	-8.733
Triclosan	Antibiotic	5.28	5.40 <sup>[10]</sup>	0.0187	-6.69
Trimethoprim	Antibiotic	0.27	6.30±0.85 <sup>[2]</sup>	0.0172	-12.01
TCEP	Flame retardant	-5.91	2.00 <sup>[7]</sup>	0.0193	-3.871
TCPP	Flame retardant	2.53	27.1	0.0013	-5.613
Tonalide	Aromatic musk	5.06	4.72 <sup>[14]</sup>	0.0737	-2.245
Iopromide	Contrast medium	-2.66	3.30 <sup>[2]</sup>	0	-26.388

Log D<sub>OW</sub>: n-octanol–water distribution ratio at pH 7; k<sub>OH</sub>: hydroxyl radical reaction rate constant; P<sub>B</sub>: Probability of Biodegradation; K<sub>AW</sub>: air-water partition coefficient

<sup>[1]</sup> Data from SciFinder

<sup>[2]</sup> (Wols and Hofman-Caris, 2012)

<sup>[3]</sup> (Nakonechny et al., 2008)

<sup>[4]</sup> (Ning et al., 2007)

<sup>[5]</sup> (Song et al., 2008)

<sup>[6]</sup> (Razavi et al., 2009)

<sup>[7]</sup> (Machairas, 2004)

<sup>[8]</sup> Calculated by Biowin6 (MITI Non-Linear Model). US EPA; Estimation Program Interface (EPI) Suite. Ver. 4.1. Jan, 2011. Available from, as of Feb 25, 2013

<sup>[9]</sup> Calculated by KOAWIN. US EPA; Estimation Program Interface (EPI) Suite. Ver. 4.1. Jan, 2011. Available from, as of Feb 25, 2013.

<sup>[10]</sup> (Dodd et al., 2009)

<sup>[11]</sup> (Toth et al., 2012)

<sup>[12]</sup> (Lam et al., 2004)

<sup>[13]</sup> (Hyunwoong, 2010)

<sup>[14]</sup> (Ward, 2010)

Isotopically labeled internal standards were from three sources. <sup>13</sup>C<sub>4</sub>-PFOA, <sup>13</sup>C<sub>4</sub>-PFOS, <sup>13</sup>C<sub>2</sub>-PFHxA, <sup>13</sup>C<sub>4</sub>-PFBA were from Wellington Laboratories (Ontario, Canada). <sup>13</sup>C<sub>6</sub>-diclofenac, primidone-d<sub>5</sub>, Ibuprofen-d<sub>3</sub>, sulfamethoxazole-d<sub>4</sub>, ciprofloxacin-d<sub>8</sub>, triclosan-d<sub>3</sub> and iopromide-d<sub>3</sub> were from Toronto Research Chemicals (Toronto, Canada). Gemfibrozil-d<sub>6</sub>, 4-nonylphenol-d<sub>4</sub>, estrone-d<sub>4</sub>, salicylic acid-d<sub>3</sub>, DEET-d<sub>7</sub>, carbamazepine-d<sub>10</sub> and bisphenol A-d<sub>16</sub> were from C/D/N Isotopes (Quebec, Canada).

Stock solutions of target analytes and internal standards were prepared in 50% (v/v) water/MeOH. Calibration and fortification solutions were prepared by successive dilution of stocks to 10 µg/L in methanol. Liquid chromatography solvents were of the highest purity available. Methanol, acetonitrile and formic acid were purchased from EMD Chemicals (Gibbstown, NJ). Methyl *tert*-butyl ether (MTBE) and ammonium hydroxide were from Fisher Scientific. LCMS reagent water was obtained from J.T. Baker.

### **3.2.2 Sample collection and preparation**

Sample collection vessels and all glassware were cleaned with methanol and rinsed three times with ultrapure water, then heated in Thermolyne 6000 Muffle Furnace (Dubuque, IA) at 550 °C for 5 h prior to use. For the study of effluent infiltration along the lower Santa Cruz River in spring and summer 2011, surface water sampling along the lower SCR was performed three times at twelve locations along the 37 km study reach (Figure 3.2). Groundwater samples near the river were obtained from a series of eleven monitoring wells owned by the Pima County Regional Wastewater Reclamation Department (PCRWRD).

Liquid samples were filtered through 0.7 µm glass fiber filters (PALL Corporation) before deuterated internal standards were added. Samples were then extracted using Oasis HLB SPE cartridges (Waters). HLB sorbents were first conditioned by stepwise addition of 5 mL of MeOH, 5 mL of MTBE and 5 mL of water. A half gram of EDTA was dissolved in 1 L of each water sample before it was loaded onto the SPE sorbent at 10 mL/min using a Dionex™ AutoTrace™ 280 Solid-Phase Extraction (SPE) instrument (Thermo Scientific). Following the sorption step, sorbents/adsorbates were

dried under nitrogen gas for 40 min before they were sequentially eluted with 3 mL of MeOH, 3 mL of 5% NH<sub>4</sub>OH in MeOH, 3 mL of ACN and 3 mL of MTBE. The combined eluents were evaporated to about 50 µL and re-dissolved in 1 mL of 50% water/methanol for liquid chromatography tandem mass spectrometric (LC/MS) analysis.

Surface waters and riverbed sediment samples were collected four times at specific Santa Cruz River locations (Figure 3.2) during 2012-2013. Locations were chosen based on ease of access, position in the river (0-37 km extending northwest from the Agua Nueva Water WRF outfall) and the condition of riverbed sediments. At each sampling point, 4-L surface water samples were collected. The monitoring program was designed to provide samples before (June 22, 2012), during (July 18, 2012) and after (October 13, 2012) the summer period (July-September) of heavy precipitation. A fourth set of samples was collected after a winter rainfall event (February 3, 2013). At each sampling site/time, one riverbed surface sediment sample and two sediment core samples (5 cm in diameter) were collected. These were subsequently used to produce sediment samples hereinafter referred to as surface sediment (0-2.5 cm), top core sediment (0-5.1 cm) and bottom core sediment (10.2-12.7 cm). Samples were stored at 4 °C and were extracted on the day after they were obtained.

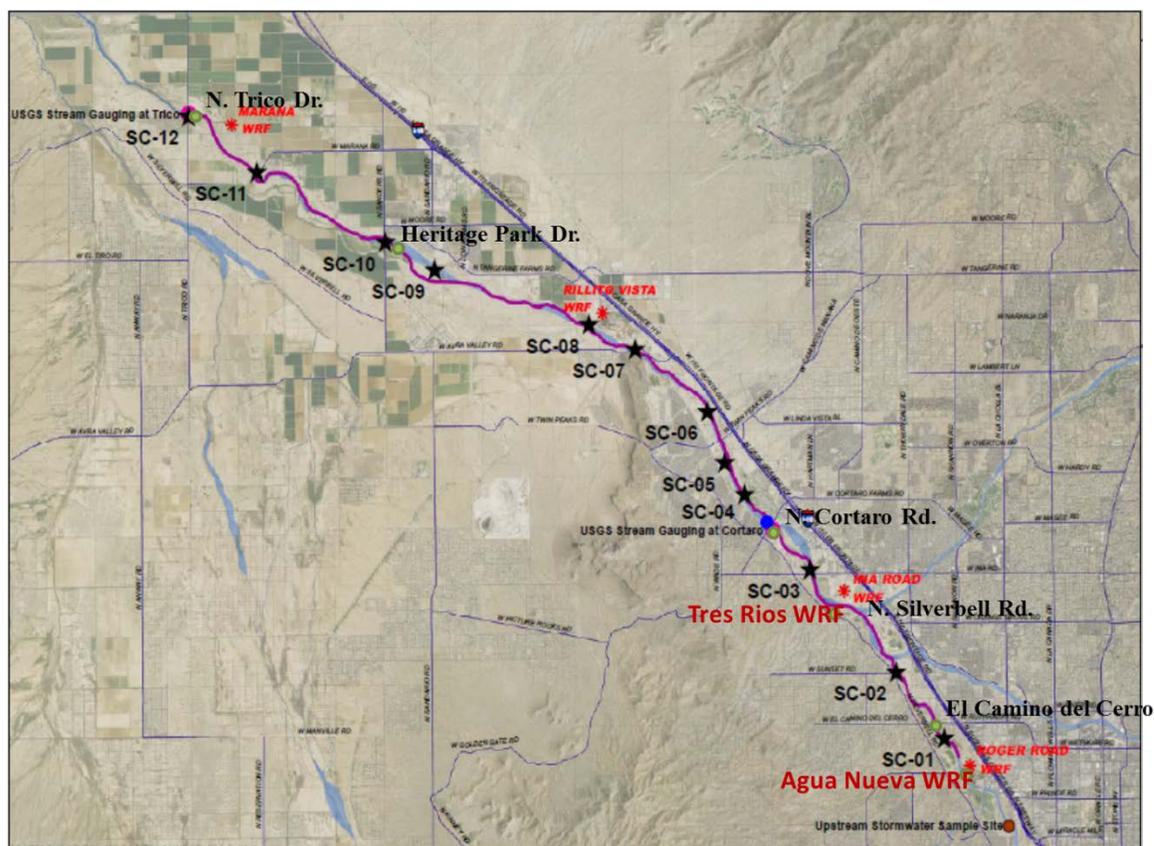


Figure 3.2 Wastewater treatment facilities and sampling sites along the Santa Cruz River. Black stars: sampling sites in the year of 2011. Green dots: sampling sites in 2012-13.

Two liters of each surface water sample were filtered through a 0.7  $\mu\text{m}$  Whatman GF/F glass fiber filter (GE Healthcare). The pre-weighed filters and non-filterable solids were dried for 48 h at 60  $^{\circ}\text{C}$  and reweighed to establish suspended solids concentrations before organics were extracted at 70  $^{\circ}\text{C}$  for 60 min via microwave-assisted extraction (MAE) using a Microwave Accelerated Reaction System (MARS, CEM Corporation) (Lopez-Avila et al., 1994; Sanchez-Prado et al., 2010; Sparr Eskilsson and Björklund, 2000). For riverbed sediment extractions, about 3 g of dried sediment were conditioned overnight in 15 mL methanol in Teflon microwave vessels ahead of MAE extractions (70  $^{\circ}\text{C}$  for 60 min). Extracts were poured off and saved after which MARS vessels and residual sediments were rinsed twice with 5 mL methanol. The pooled extracts were then

dried under a gentle stream of N<sub>2</sub> gas before residual organics were re-dissolved in 1 mL methanol. In order to clean the extracts, the methanol containing extracted organics was diluted to 250 mL with Nanopure water before the mixture was slowly passed under vacuum through 47mm Empore™ Extraction Disks (octadecylsilane, 3M, Eagan, Minnesota). Retained organics were separated by sequential elution in a stepwise methanol gradient using 15 mL washes consisting of 20%, 50%, 80% and 100% (v/v) methanol in water. Half of each eluent mixture was added to a single 40-mL glass vial and stored at 4 °C for further processing. The other half was dried under nitrogen and redissolved in Nanopure water for analysis of estrogenic activity. To eliminate bacterial contamination, samples for measurement of estrogenic activity were autoclaved at 121 °C for 10 min prior to analysis (Littlehat, 2007).

### **3.2.3 Yeast estrogen screen (YES) bioassay method**

The YES bioassay, based on that of Routledge and Sumpter (1996) was used to measure estrogenic activity in sample extracts. The test organism was a recombinant strain of *Saccharomyces cerevisiae* in which the DNA sequence of the human estrogen receptor (hER- $\alpha$ ) was integrated into the yeast genome. The yeast strain also contains expression plasmids carrying estrogen-responsive sequences (EREs) controlling transcription of the reporter gene *lacZ* (encoding  $\beta$ -galactosidase). In the presence of estrogen,  $\beta$ -galactosidase is synthesized in the cells and then secreted into the medium, where its activity is measured colorimetrically. Sample extracts were serially diluted across 96-well micro-titer plates (Costar). In positive controls, refreshed cells were added to serial dilutions of standard hormone (EE<sub>2</sub>) preparations. Cell suspensions were incubated for 24 h at 32 °C in the presence of either the prepared extracts or known

concentrations of EE<sub>2</sub> for growth and hormone-dependent gene expression. After that, 50 µL of cycloheximide (4mg/mL)/CPRG (chlorophenol red β-D-galactopyranoside, 0.4 mg/mL) solution was added to each test well. Following an additional incubation for 24 h at 32 °C, color was measured ( $A_{570} - R \times A_{630}$ ) using a UV-VIS micro-plate reader (Bio-tek Instruments, Inc.), where A is absorbance, and R is the ratio of optical densities at 570 nm and 630 nm in negative controls. Color was converted to total sample estrogenic activity using results of the contemporary positive control. To compare standards and samples from one event to another, data were normalized as follows:

$$\text{Percent relative estrogenic activity} = \frac{A_{\text{sample}} - A_{\text{background}}}{A_{\text{maximum}} - A_{\text{background}}} \times 100\%$$

In the equation,  $A_{\text{maximum}}$  and  $A_{\text{background}}$  are the maximum and the negative control optical density at 570 nm for the standard curve (EE<sub>2</sub> solution), and  $A_{\text{sample}}$  is the optical density for the river sample. The percent relative estrogenic activity for the sample was the estrogenic activity indicated relative to the standard curve run along with the data set. That is, the range of response observed in the standard was used to normalize the data from the sample.

A practical quantification limit (PQL) for the assays was arbitrarily adopted as the EC<sub>20</sub> value for EE<sub>2</sub> divided by the highest sample concentration factor tested in the YES procedure (normally 400). Below the PQL was a method detection limit (MDL). The MDL was the EE<sub>2</sub> concentration that produced a test response equal to 3 times the standard deviation in ( $A_{570} - R \times A_{630}$ ) in hormone-free (blank) test wells, divided by the highest concentration factor tested.

### 3.2.4 Gas chromatography–mass spectroscopy

An Agilent 6890 gas chromatograph and Agilent 5973 mass-selective detector were used for GC-MS analyses of nonylphenol (NP) and octylphenol (OP). Compounds were separated on a fused silica column (HP5-MS, 30 m × 0.25 mm i.d., 0.25 μm film thickness, Agilent). The oven temperature program used was: 1 min at 90 °C, ramp at 15 °C min<sup>-1</sup> to 220 °C, hold for 2 min, ramp at 15 °C min<sup>-1</sup> to 240 °C, followed by 10 °C min<sup>-1</sup> to 280 °C. Helium was the carrier gas at a constant flow rate of 1 mL min<sup>-1</sup>. The inlet, transfer line and ion source were at 250, 280 and 230 °C, respectively. Sample injection (1 μL) was in splitless mode. The mass detector was operated in the electron ionization mode using selected ion monitoring (SIM) method. Quantification ions were as follows: m/z 135 for NP and m/z 107 for OP. The limit of detection (LOD) and limit of quantification (LOQ) were defined as three times and ten times the signal-to-noise ratio (Komori et al., 2004).

### 3.2.5 Liquid chromatography–mass spectrometry

An Agilent 1290 Infinity LC System and Agilent 6460 Triple Quadrupole LC/MS system with both positive and negative electrospray ionization were used for analyses of other trace organic contaminants. Multiple-reaction monitoring mode was used for fragmentation data, monitoring both quantifiers (highest response product ion) and qualifiers (next highest product ion) for each compound measured. Calibration curves contained at least five points, with  $R^2 > 0.99$  for linear fits. LOD and LOQ were defined the same as in the GC-MS analytical work. When surrogate (isotope) recovery was less than 5%, concentrations were flagged as non-detectable due to disappearance during extraction.

### 3.2.6 Photodegradation of estrogenic activity in secondary effluent

Milli-Q water (18 M $\Omega$ ) was prepared using a Millipore water purification system (Billerica, MA). Secondary effluent from the Agua Nueva WRF was spiked with EE<sub>2</sub> (0.32 nM). Irradiation experiments were done in direct sunlight in cloud-free weather. Five glass photoreactors, containing 600 mL Milli-Q water or secondary effluent plus EE<sub>2</sub> were set up in parallel and sacrificed at 0, 0.5, 1, 2 and 4 h for analysis via excitation-emission matrix (EEM) fluorescence spectroscopy and the YES bioassay (estrogenic activity). Dark controls were set up and sacrificed after four hours for the same set of measurements.

Fluorescence of secondary effluent samples was characterized by a PerkinElmer LS 55 fluorescence spectrophotometer. The inner filter effect (IFE) caused by light absorption and reabsorption of light absorbing compounds was corrected by a mathematical model that takes into account the absorption spectrum of the sample (MacDonald et al., 1997). A Thermo Scientific Genesys 10s UV-Vis spectrophotometer was employed to obtain the absorption spectra of test samples.

EEM fluorescence data obtained from the fluorescence spectrophotometer corresponded to scan ranges of emission and excitation wavelengths of 280 to 400 nm. Scan speed was set at 600 nm/min.

### 3.2.7 Statistical analyses

Statistical assessment of relationships between compound-specific fractional removals during transport in the Santa Cruz River and compound physical/chemical parameters was carried out using both simple linear regression analysis and a non-parametric approach (Spearman correlation and Pearson's correlation) that are available

in Statistical Package for the Social Sciences (IBM SPSS statistics, Version 20, release 20.0.0.1). Spearman's correlation measures strength of association between variables that are not necessarily linearly related, and is relatively insensitive to outlier values. The Pearson correlation coefficient is a measure of the strength and direction of association that exists between two variables.

Backward multiple linear regression method was also used for analyzing the relationship between the properties of these chemicals and the fate of the trace organics in the river and sediment. This method eliminates independent variables one by one from the model, until one or more independents which are statistically correlated to the dependent variable appear.

### **3.3 Results**

#### **3.3.1 Trace organics and estrogenic activity in the Santa Cruz River**

A suite of twelve dissolved trace organic compounds (TOrcs) were measured over the same reach of the SCR during three sampling campaigns in 2011, while p-nonylphenol and p-octylphenol were measured separately during a February 2013 sampling event. A list of the twelve compounds measured can be found in Table 3.3.

For the sampling campaigns in 2011, the twelve compounds measured were detected in the effluents of both the Agua Nueva WRF and Tres Rios WRF and at the twelve sampling points along the Santa Cruz River. A complete set of sampling results from the three sampling periods is provided in Table 3.2. TOrcs present at the highest concentrations were sucralose, sulfamethoxazole, trimethoprim and DEET. Of these compounds, DEET, trimethoprim, and iopromide were substantially higher in effluent from the Agua Nueva WRF than in the Tres Rios WRF effluent, whereas the reverse was true for primidone and fluoxetine. There is some indication, however, that wastewater treatment efficiency at Tres Rios WRF may have been superior to that of the Agua Nueva WRF, accounting in part for observed differences in effluent concentrations of trace organics.

Table 3.2 Dissolved concentrations of trace contaminants at sampling sites in the Lower Santa Cruz River surface.

Sampling point	Carbamezapine	DEET	Sulfamethoxazole	TCEP
<b>Roger Rd Outfall</b>	343.5 ± 22.2	786.4 ± 127.5	1308.9 ± 436.4	326.1 ± 24.7
<b>SC-1R</b>	419.1 ± 122.4	764.0 ± 135.0	1446.4 ± 477.5	319.9 ± 13.5
<b>SC-2R</b>	321.9 ± 149.5	739.2 ± 95.9	1390.0 ± 548.1	352.0 ± 52.8
<b>Ina Rd Outfall</b>	354.4 ± 25.2	346.6 ± 168.1	775.0 ± 409.8	318.9 ± 39.3
<b>SC-3R</b>	369.4 ± 90.7	511.9 ± 174.5	1420.1 ± 657.5	325.5 ± 44.4
<b>SC-4R</b>	316.4 ± 77.8	308.4 ± 6.3	759.5 ± 96.9	306.5 ± 5.5
<b>SC-5R</b>	390.7 ± 103.7	415.0 ± 96.4	1152.8 ± 329.5	323.5 ± 42.3
<b>SC-6R</b>	365.6 ± 75.3	467.2 ± 222.6	1095.0 ± 283.6	342.5 ± 70.3
<b>SC-7R</b>	389.4 ± 108.4	454.3 ± 149.8	1207.1 ± 403.5	329.0 ± 66.7
<b>SC-8R</b>	398.1 ± 98.7	474.7 ± 23.9	1398.4 ± 437.8	288.6 ± 3.9
<b>SC-9R</b>	399.6 ± 126.5	399.3 ± 80.8	1460.8 ± 483.6	282.4 ± 35.6
<b>SC-10R</b>	434.0 ± 113.9	401.0 ± 96.5	1545.6 ± 296.2	301.8 ± 21.9
<b>SC-11R</b>	445.7 ± 139.6	367.9 ± 131.7	1571.7 ± 483.4	278.0 ± 8.9
<b>SC-12R</b>	361.9 ± 105.7	319.9 ± 126.8	1522.6 ± 589.0	300.0 ± 29.5

Sampling point	Tonalide	Fluoxetine	Trimethoprim	Primidone
<b>Roger Rd Outfall</b>	39.2 ± 4.8	41.9 ± 5.5	547.4 ± 174.5	109.7 ± 40.7
<b>SC-1R</b>	28.6 ± 7.6	29.7 ± 1.9	596.8 ± 167.3	112.9 ± 37.7
<b>SC-2R</b>	20.8 ± 7.0	25.2 ± 4.0	465.7 ± 300.4	121.0 ± 32.1
<b>Ina Rd Outfall</b>	28.7 ± 8.1	70.7 ± 19.9	310.4 ± 71.6	160.6 ± 15.5
<b>SC-3R</b>	23.5 ± 5.9	35.4 ± 4.5	381.8 ± 199.0	137.0 ± 39.4
<b>SC-4R</b>	23.2 ± 3.1	49.7 ± 13.4	247.9 ± 139.8	166.3 ± 33.3
<b>SC-5R</b>	22.4 ± 2.4	40.2 ± 19.9	323.8 ± 127.6	156.8 ± 20.3
<b>SC-6R</b>	21.0 ± 3.4	34.4 ± 15.4	318.4 ± 145.4	152.0 ± 7.1
<b>SC-7R</b>	18.6 ± 1.8	35.1 ± 22.2	347.6 ± 139.1	148.0 ± 14.2
<b>SC-8R</b>	17.9 ± 1.6	24.9 ± 4.8	242.4 ± 71.8	177.4 ± 26.3
<b>SC-9R</b>	20.3 ± 7.5	23.6 ± 10.2	329.9 ± 189.9	172.9 ± 19.0
<b>SC-10R</b>	18.8 ± 5.1	20.1 ± 7.2	313.7 ± 126.7	175.2 ± 17.0
<b>SC-11R</b>	20.3 ± 7.4	14.0 ± 5.9	275.7 ± 147.7	178.4 ± 10.7
<b>SC-12R</b>	15.2 ± 5.8	11.9 ± 4.6	208.9 ± 158.4	160.2 ± 17.6

Sampling Point	Iopromide	Sucralose	PFOS	PFOA
<b>Roger Rd Outfall</b>	239.1 ± 68.0	2804.3 ± 991.3	19.9 ± 23.2	11.0 ± 8.0
<b>SC-1R</b>	229.9 ± 40.4	2726.6 ± 687.1	11.9 ± 13.0	5.9 ± 1.2
<b>SC-2R</b>	204.1 ± 137.5	3029.0 ± 968.0	37.3 ± 49.6	14.0 ± 7.6
<b>Ina Rd Outfall</b>	96.2 ± 49.3	3886.5 ± 1924.8	9.2 ± 4.2	11.8 ± 2.5
<b>SC-3R</b>	180.2 ± 92.5	4661.9 ± 2822.1	15.6 ± 14.6	12.2 ± 5.5
<b>SC-4R</b>	104.5 ± 86.6	4632.8 ± 1495.3	18.3 ± 14.6	9.5 ± 2.5
<b>SC-5R</b>	137.1 ± 54.1	4091.6 ± 1094.6	18.0 ± 19.8	11.2 ± 3.4
<b>SC-6R</b>	81.5 ± 52.5	3690.8 ± 1451.7	19.3 ± 24.0	13.2 ± 7.2
<b>SC-7R</b>	88.0 ± 35.9	3647.6 ± 1141	21.2 ± 24.8	10.9 ± 2.6
<b>SC-8R</b>	90.0 ± 52.2	3584.8 ± 1021.1	11.7 ± 4.7	7.6 ± 4.4
<b>SC-9R</b>	77.0 ± 33.3	3956.3 ± 1071.6	18.0 ± 13.8	11.2 ± 1.4
<b>SC-10R</b>	59.9 ± 41.2	3983.0 ± 789.9	12.8 ± 10.3	10.9 ± 1.7
<b>SC-11R</b>	81.9 ± 31.7	3996.5 ± 808.5	10.3 ± 2.6	10.4 ± 1.1
<b>SC-12R</b>	72.2 ± 25.9	3214.0 ± 197.5	8.1 ± 3.9	9.5 ± 3.7

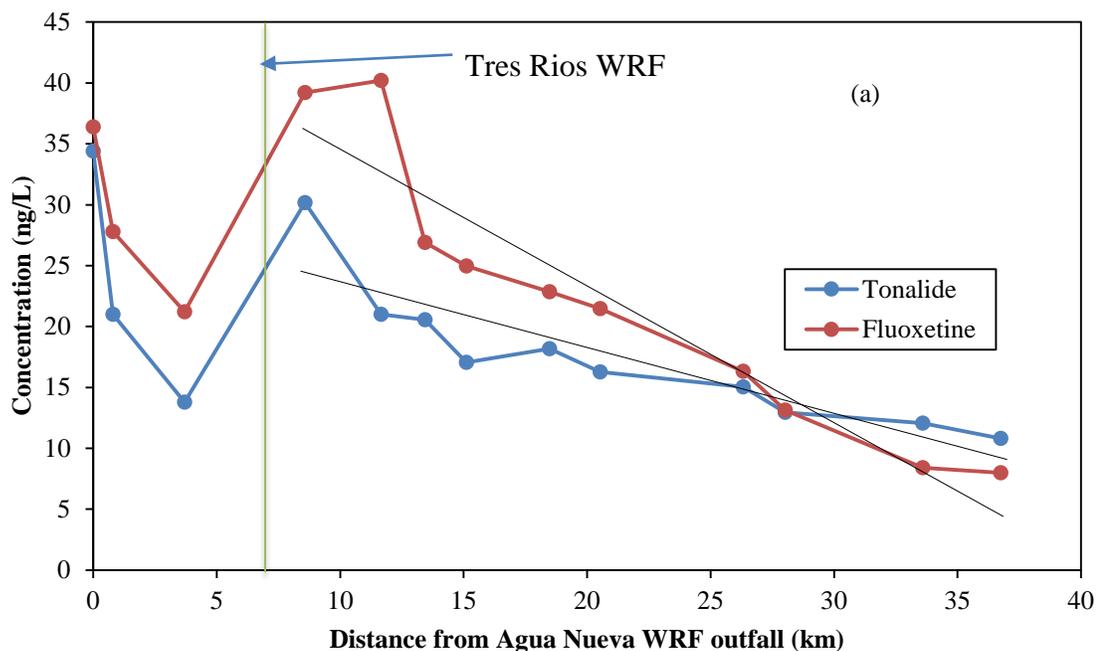
Note to Table 3.2: The positions of sampling points are shown in Figure 3.2. Values represent averages plus/minus one standard deviation (ng/L) based on three sampling campaigns during 2011.

Since Tres Rios WRF is located about 7.5 km downstream of the Agua Nueva WRF, and the contribution of TOxCs from the secondary effluent to the SCR is not negligible, only the fate of contaminants starting from the Tres Rios WRF to the end of our survey area is being considered. Based on one-time measurement of these chemicals, profiles of contaminant concentration with distance downstream from the Tres Rios WRF (Figure 3.3a and b) indicate that tonalide, fluoxetine, iopromide, sucralose and PFOS appear to have been attenuated during surface transport along the 37-km reach of the river that was sampled. In contrast, the conserved compounds were: carbamazepine, DEET, sulfamethoxazole, TCEP, trimethoprim, primidone, PFOA. In-stream dissolved concentrations of PFOA were too variable to support speculation regarding compound attenuation. This may be due to the fact that concentration of PFOA was particularly variable in the Agua Nueva WRF and the Tres Rios WRF effluents.

Concentrations of NP and OP in the SCR were measured during the February 2013 sampling event. In filtered river samples, NP concentrations ranged from 244-484 ng/L (Figure 3.3c), which was smaller than the concentration of NP in the river at Cortaro Road measured by Kolpin et al. (2002). For OP, concentrations spanned the range 8.6-101 ng/L, although only one stream sample was above 50 ng/L. This was similar to the experiences of Kolpin et al. (2002). Concentration profiles of NP and OP followed the same trends, first decreasing below the Agua Nueva WRF and then fluctuating modestly with distance in the stream reach immediately below the Tres Rios WRF outfall. The data suggest that the gradual increase of NP and OP in dissolved concentrations may

arise from the in-stream conversion of alkylphenol polyethoxylates and carboxylates (APnEOs and APnECs) to the more stable AP forms, as observed by others (Ahel et al., 1994b; Gross et al., 2004). In the river water samples, NP was about 10-fold more concentrated than OP in all samples, probably result of the widespread use of nonylphenol ethoxylates (NPnEOs) in industrial, agricultural, and domestic applications (Giger et al., 1984). Although in-stream loss was not observed here, previous work has shown that NP is biodegraded under aerobic conditions during wastewater treatment and in receiving waters (Soares et al., 2008).

Attenuation and persistence trends in the position-dependent concentrations were examined using linear regression analysis (examples are given in Figure 3.3). When measured values decreased significantly with distance ( $p \leq 0.05$  with negative slope in the concentration vs. distance fit), the potential mechanism(s) of attenuation became the subject of investigation.



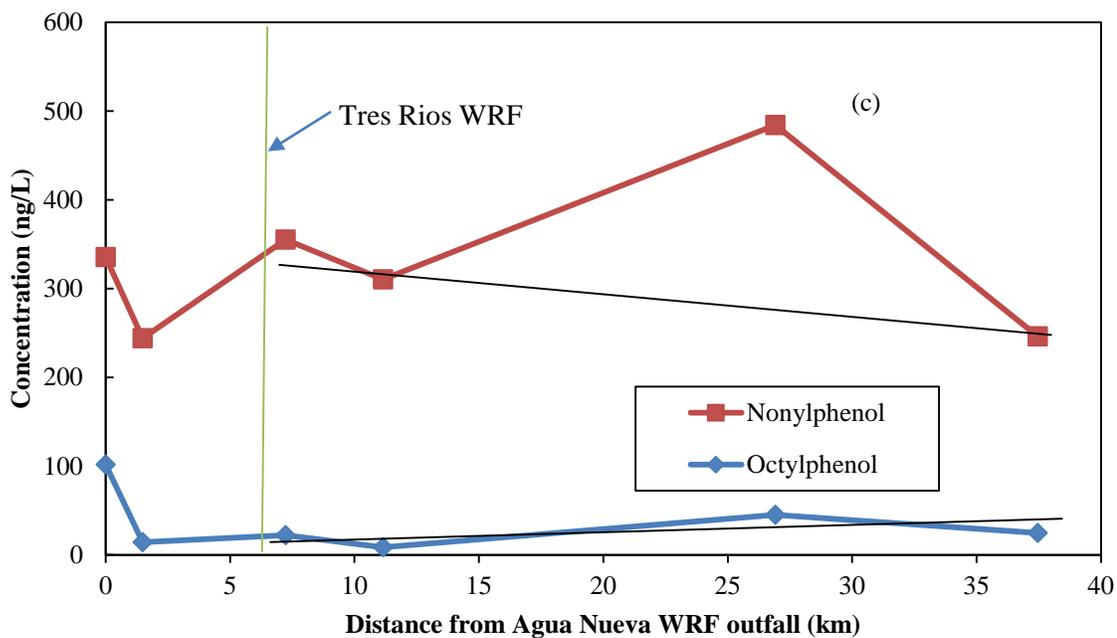
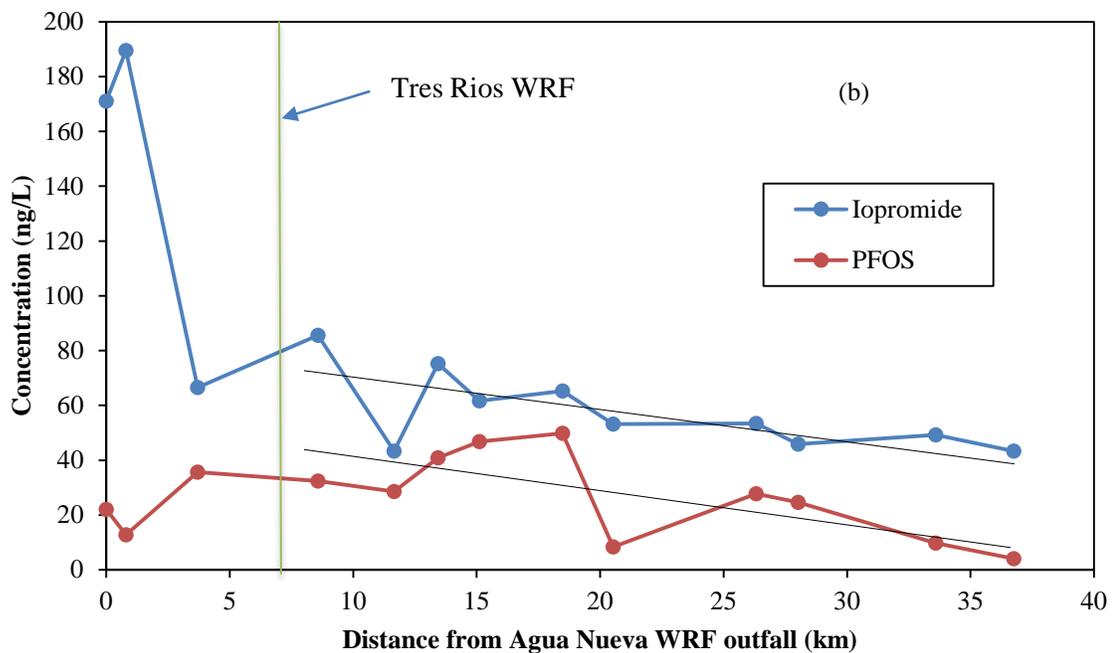


Figure 3.3 Results of linear regression analysis (solid straight lines) of in-stream TOxC concentration versus distance traversed in the Santa Cruz River downstream from the Tres Rios WRF.

Regression data are shown in Table 3.3.(a) tonalide and fluoxetine; (b) iopromide and PFOS; (c) p-nonylphenol and p-octylphenol.

Table 3.3 Results of simple linear regression analysis ( $C=B_0X+B_1$ , where C is concentration and X is distance from the Tres Rios WRF outflow) in the Santa Cruz River liquid samples (values that are statistically significant are written in bold italics).

Compound	<b>B<sub>0</sub></b> (ng/ L km)	<b>B<sub>1</sub></b> (ng/L)	<b>R<sup>2</sup></b>	<b>p (significance)</b>
Carbamazepine	0.610	280.76	0.077	0.437
DEET	4.780	352.45	0.533	0.016
Sulfamethoxazole	7.629	879.61	0.175	0.229
TCEP	0.897	275.58	0.171	0.236
<b><i>Tonalide</i></b>	<b><i>-0.523</i></b>	<b><i>24.03</i></b>	<b><i>0.789</i></b>	<b><i>0.001</i></b>
<b><i>Fluoxetine</i></b>	<b><i>-1.123</i></b>	<b><i>36.375</i></b>	<b><i>0.901</i></b>	<b><i>&lt;0.0005</i></b>
Trimethoprim	-1.961	184.11	0.248	0.143
Primidone	-0.194	179.57	0.011	0.777
<b><i>Iopromide</i></b>	<b><i>-1.001</i></b>	<b><i>70.30</i></b>	<b><i>0.451</i></b>	<b><i>0.033</i></b>
<b><i>Sucralose</i></b>	<b><i>-94.153</i></b>	<b><i>6321.6</i></b>	<b><i>0.580</i></b>	<b><i>0.010</i></b>
<b><i>PFOS</i></b>	<b><i>-1.145</i></b>	<b><i>41.836</i></b>	<b><i>0.468</i></b>	<b><i>0.029</i></b>
PFOA	-0.123	11.879	0.062	0.448
4- <i>n</i> -Nonylphenol	-1.009	362.52	0.020	0.859
4-Octylphenol	0.532	18.172	0.244	0.506

Candidate mechanisms for TO<sub>r</sub>C attenuation in the river include biodegradation, photolysis and sorption on bottom sediments or suspended solids. Since none of the TO<sub>r</sub>Cs monitored was considered to be volatile (based on air-water partition coefficient  $K_{AW} < 10^{-5}$ , Table 3.1), and each survived conventional wastewater treatment, volatilization was neglected as a mechanism of attenuation during in-stream transport. Relationships were investigated by correlating the normalized slopes of curves representing compound dissolved concentration vs. transport distance in the SCR with specific compound properties using simple linear regression analysis, Spearman correlation and Pearson's correlation statistical methods.

Pearson's correlation and Spearman correlation based on rank-ordered values of normalized slopes ( $-1/C_0 \times dC/dx$ ) and different independent variables ( $\log D_{OW}$  and  $K_{OH}$ ) did not produce statistically significant correlations (Table 3.4), which indicates that

these two independent variables are not the major cause for the degradation of trace organics in the stream. For probability of biodegradation ( $P_B$ , calculated by Biowin6 in Estimation Program Interface (EPI) Suite, US EPA), another important variable for the fate of the trace organics in the stream, the correlation coefficients in both Pearson's and Spearman models are negative, and they are statistically significant. The result suggests that it is inversely correlated to the degradation of the target compounds in the SCR, some compounds degraded in the river was not caused by the biodegradation mechanism, or there were not microbes in the river responsible for the degradation of these compounds.

Table 3.4 Comparison of Pearson and Spearman correlations between the normalized slope of the compound-specific trajectory of in-stream concentrations removal and the fate parameters indicated. (Only sampling points below Tres Rios WRF for one sampling event were taken into account)

Independent variables:	simple linear regression		Pearson		Spearman	
	$D_0$	$D_1$	r	p	$\rho$	p
$\log D_{ow}$	-0.719	6.456	-0.139	0.635	-0.081	0.782
$P_B$	-0.242	12.401	-0.618	0.019	-0.627	0.016
$k_{OH} (M^{-1} s^{-1})$	-0.508	8.038	-0.113	0.702	-0.123	0.675

Pearson r values and p statistics represent results of simple linear regression using compound-dependent expected removals over the reach, as the dependent variable and each of the independent variables listed.  $D_0$  and  $D_1$  values represent the slopes and intercepts of linear regression lines derived using data of normalized slope vs. indicated parameter from one sampling event.

A multiple linear regression method was also adopted for the statistical analysis of the relationships between the three independent variables and the fate of the trace organics in the river (Table 3.5). From the results, it is clear that  $k_{OH}$  and Log Dow were not the main contributors to the degradation of these compounds, and these two components were removed from the regression one by one based on their significance. For the probability of biodegradation ( $P_B$ ), the model keeps it as a statistically significant

component to the degradation of trace organics, but still has a negative correlation with the chemical degradation. The statistical results (Table 3.4 and Table 3.5) suggest that there is some other mechanism existing for the responsibility of the trace organics removal in the stream, opposite to our hypothesis, in which we proposed that the hydrophobicity of the compound, hydroxyl radical reaction rate and biodegradability play important roles for the removal of TOrcs in the river.

Table 3.5 Multiple linear regression statistical results for correlation between the physical/chemical properties and the normalized removal ratios of trace organics. (Dependent variable in the model: normalized slope)

Model	Unstandardized Coefficients		Standardized Coefficients	significance	
	B	Std. Error	Beta		
1	(Constant)	9.463	6.420		0.171
	Log Dow	1.415	1.475	0.274	0.360
	P <sub>B</sub>	-0.330	0.115	-0.844	0.017
	k <sub>OH</sub>	0.720	1.191	0.160	0.559
2	(Constant)	12.234	4.364		0.017
	Log Dow	1.586	1.406	0.307	0.283
	P <sub>B</sub>	-0.309	0.106	-0.791	0.014
3	(Constant)	12.401	4.411		0.016
	P <sub>B</sub>	-0.242	0.089	-0.618	0.019

### 3.3.2 In-stream attenuation of estrogenic activity

Estrogenic activity was measured in the SCR during each of four sampling campaigns (2012-2013) to indirectly establish probable attenuation mechanisms (Figure 3.4a). The measured estrogenic activity in Agua Nueva WRF effluent was almost two orders of magnitude higher than levels that feminize continuously exposed fathead minnows (*Pimephales promelas*) (Lange et al., 2001). Just 1.50 km below the Agua Nueva WRF outfall, remaining estrogenic activity was ~20-25% of measured levels in

Agua Nueva WRF effluent (Figure 3.4a). Travel time from the outfall to that point is estimated at one hour. Despite the rapid attenuation of estrogenic activity, however, measured values exceeded concentrations necessary to disrupt sexual differentiation and reproduction in continuously exposed organisms throughout a distance of ~10 km below the outfall. Tres Rios WRF effluent did not increase the total estrogenic activity of the stream at the plant outfall, which is located about 8 km below the Agua Nueva WRF. These results suggests that, when fully implemented, nutrient management steps at the Tres Rios WRF will dramatically improve secondary effluent quality and in-stream environmental quality. At a distance of ~15 km downstream of the Agua Nueva WRF, estrogenic activity fell below the detection limit, estimated as  $1.5 \times 10^{-13}$  M (equivalent concentration of EE<sub>2</sub>). Log D<sub>ow</sub> values for estrogenic compounds in wastewater lie in the range 3-4, suggesting that attenuation of their aqueous-phase concentrations during wastewater treatment and receiving waters may be due, at least in part, to sorptive partitioning on organic-rich solids (Drewes et al., 2005; Lee and Liu, 2002). However, estrogenic activity was measured in extracts from suspended solids in SCR samples (Figure 3.4b) and in no river sample did the contribution of sorbed estrogenic activity exceed 20% of total estrogenic activity, and estrogenic activity in extracts derived from non-filterable material was usually a much smaller fraction of total activity. Consequently, it seems unlikely that sorption/sedimentation is a major mechanism for reduction of total estrogenic activity with travel distance in the SCR.

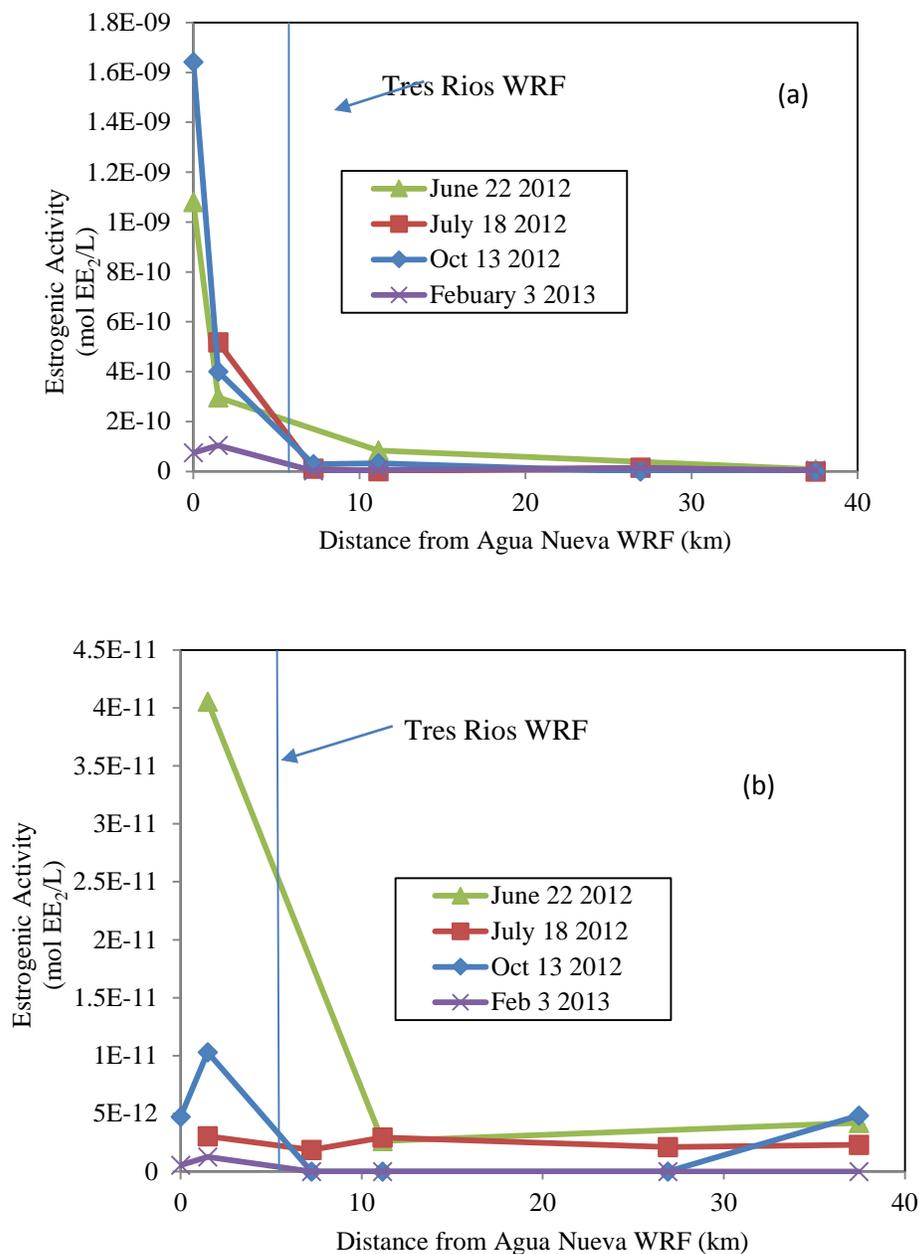


Figure 3.4 (a) aqueous-phase and (b) suspended solid estrogenic activity as a function of position in the lower Santa Cruz River.

Dates shown are those of sample collection. Suspended solids were isolated from river water samples by filtering through 0.7  $\mu\text{m}$  Whatman GF/F glass fiber filters.

As discussed above, NP and OP concentrations in the river decreased much more slowly than did total estrogenic activity. Furthermore, representative factors to convert

the concentrations of NP and OP to their equivalent concentrations of EE<sub>2</sub> are  $7.0 \times 10^{-5}$  and  $3.3 \times 10^{-6}$ , respectively (Routledge and Sumpter, 1996). Thus, in samples taken close to the treatment plants, it is unlikely that APs contributed significantly to total sample estrogenic activity (Table 3.6).

Table 3.6 Contributions of NP and OP to the total estrogenic activity in the liquid samples.

Calculations are based on the relative estrogenic activities of single estrogens in the YES test (Conroy, 2006), and an additive method for total estrogenic activity.

Sampling point	Distance from Agua Nueva WRF outfall (km)	Total estrogenic activity (M)	NP equivalent activity (M)	Percent NP for estrogenic activity	OP equivalent activity (M)	Percent OP for estrogenic activity
Agua Nueva WRF effluent	0	$7.51 \times 10^{-11}$	$3.05 \times 10^{-13}$	<b>0.41%</b>	$3.57 \times 10^{-15}$	<b>0.048%</b>
El Camino del Cerro	1.5	$1.05 \times 10^{-10}$	$2.21 \times 10^{-13}$	<b>0.21%</b>	$5.08 \times 10^{-16}$	<b>0.005%</b>
N Silverbell Rd	7.2	$2.67 \times 10^{-12}$	$3.22 \times 10^{-13}$	<b>12.1%</b>	$7.89 \times 10^{-16}$	<b>0.296%</b>
N Cortaro Rd	11.1	$5.33 \times 10^{-12}$	$2.82 \times 10^{-13}$	<b>5.3%</b>	$3.03 \times 10^{-16}$	<b>0.057%</b>
Heritage Park Dr	26.9	$1.33 \times 10^{-11}$	$4.39 \times 10^{-13}$	<b>3.3%</b>	$1.59 \times 10^{-15}$	<b>0.120%</b>
N Trico Rd	37.5	$4.44 \times 10^{-12}$	$2.23 \times 10^{-13}$	<b>5.0%</b>	$8.75 \times 10^{-16}$	<b>0.197%</b>

### 3.3.3 Phototransformation effects

To investigate the roles of photolysis in the in-stream reduction of estrogenic activity in the SCR, batch reactors containing either wastewater effluent from the Agua Nueva WRF or Milli-Q water were spiked with 0.32 nM EE<sub>2</sub> and exposed to sunlight on the University of Arizona campus for a period of four hours (10 am to 2 pm). A dark control was maintained over the same period, and a second control consisted of effluent without supplementary EE<sub>2</sub>. Samples were withdrawn for separation and measurement of total estrogenic activity at the start of the experiment and after 0.5, 1.0, 2.0 and 4.0 h of exposure. In addition, excitation-emission matrix (EEM) measurements were carried out on each sample. Preliminary tests designed to measure recovery of estrogenic activity

from wastewater spiked with EE<sub>2</sub> showed that the extraction procedure adopted recovered essentially all of the EE<sub>2</sub> added plus the estrogenic TORCs present in the original water. Ground level spectral irradiance data during a series of solar irradiation experiments (Figure 3.5) were obtained from SMARTS (Simple Model of the Atmospheric Radiative Transfer of Sunshine), a spectral irradiance simulation program that requires geographical information and atmospheric conditions for the experiment site and date as input parameters (Gueymard, 1995; Kaskaoutis and Kambezidis, 2008). In this study, atmospheric conditions were obtained from satellite data (OMI/Aura and MODIS) provided by Giovanni, a NASA database (Gueymard, 2004). On the dates of the experiments (between June 20, July 29 and August 13, 2013), the noontime ground level global spectral irradiances were indistinguishable.

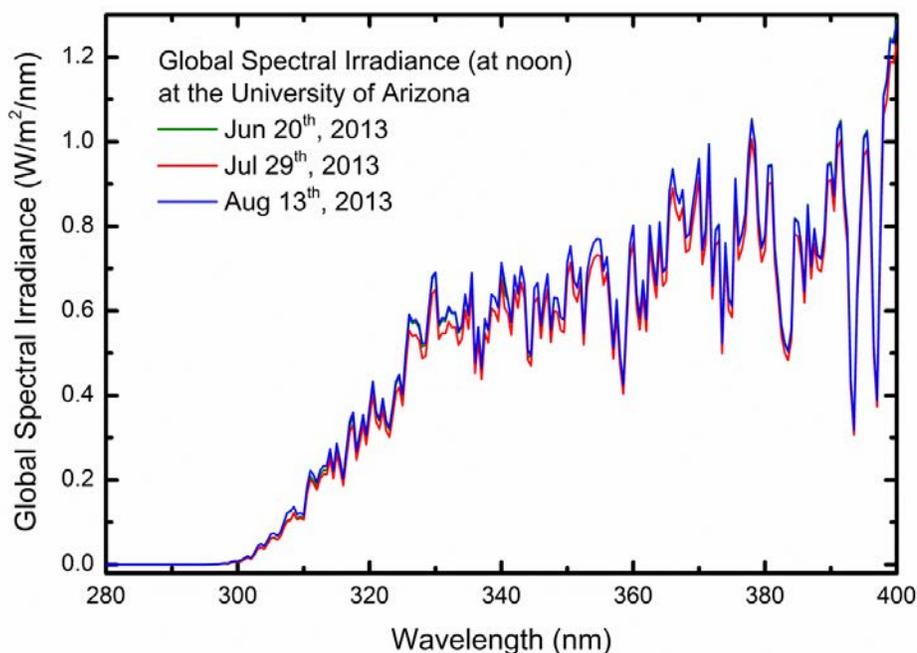


Figure 3.5 Global spectral irradiance for the Tucson area and the dates of sunlight photolysis experiments.

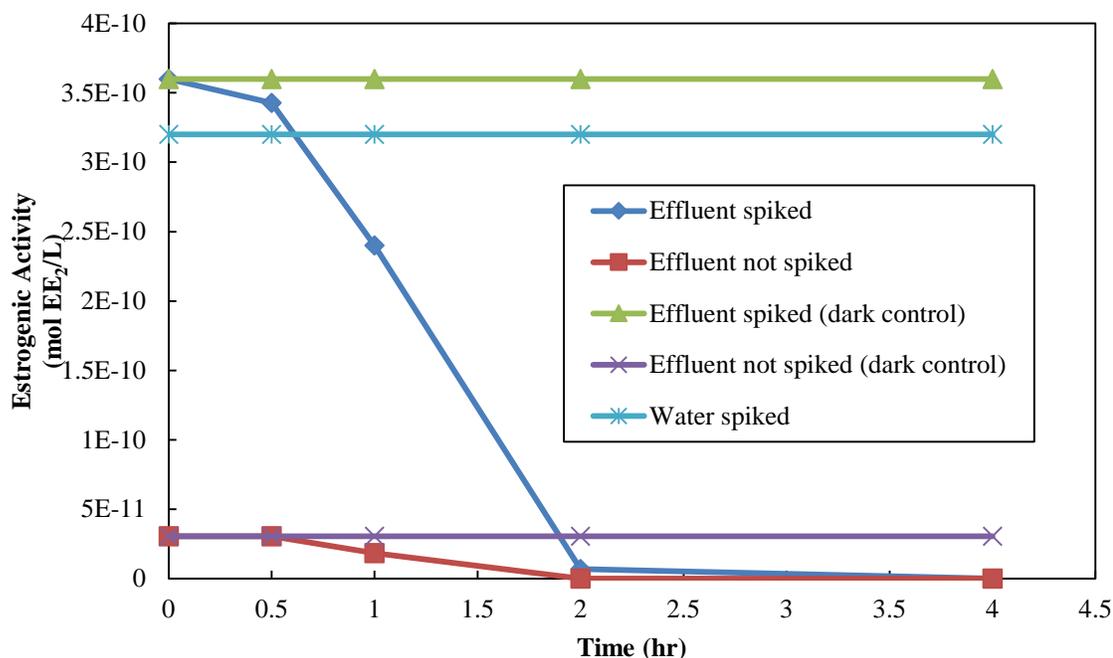


Figure 3.6 Estrogenic activity changes in Agua Nueva WRF secondary effluent during sunlight photolysis experiments. Results are shown for original samples and samples spiked with 0.32 nM EE<sub>2</sub>.

In both the unspiked effluent control and the reactor containing effluent plus 0.32 nM EE<sub>2</sub>, total estrogenic activity decreased below the method detection limit during the 4-hour period of solar light exposure (Figure 3.6). The dependence of fractional removals on time in the spiked and unspiked effluent was similar. Half-lives for total estrogenic activity in the reactors were about 0.75 h. Estrogenic activity was essentially unchanged, however, in dark controls and in Milli-Q water spiked with EE<sub>2</sub>. Results indicate that direct photolysis is not an important mechanism for destruction of EE<sub>2</sub>, and perhaps the steroidal hormones as a class of chemicals, under most environmental conditions, including those encountered in the SCR. The decrease in estrogenic activity of spiked and unspiked samples suggests that indirect photolysis is responsible for the results obtained in the batch reactors, and it may be an important mechanism for the rapid

attenuation of estrogenic activity with distance in the SCR. Indirect photolysis depends on the excitation of chromophores in the water sample, in this case on the irradiation of residual dissolved organics in wastewater effluent by UV and visible components of sunlight. Subsequent reactions that are independent of sunlight can produce a variety of aggressive radicals and hydrogen peroxide (Jasper and Sedlak, 2013). Photolytic pathways leading to production of hydroxyl radicals can also begin with sunlight irradiance of aqueous-phase nitrate and nitrite ions and various iron complexes (Blough and Zepp, 1995). A number of previous investigators have established the importance of dissolved organics in wastewater in similar photolytic reactions (Leech et al., 2009; Lin and Reinhard, 2005). The resistance of estrogenic activity to attack via direct photolysis may be due to lack of light absorbance by the steroidal hormones at  $\lambda > 310$  nm. Most energy in the solar spectrum is focused in the visible and infrared ranges ( $\lambda > 380$  nm; Figure 3.5) (Rosenfeldt and Linden, 2004).

Involvement of wastewater effluent DOC in sunlight-activated photolytic reactions is apparent in fluorescence EEM measurements (Figure 3.7). EEM peaks in the Agua Nueva WRF effluent sample have been assigned to humic-acid-like (upper peak) and fulvic-acid-like (lower peak) substances (Chen et al., 2003). Attenuation of these peaks over the four-hour period of experimentation is apparent in the exposed reactors but not in the dark control.

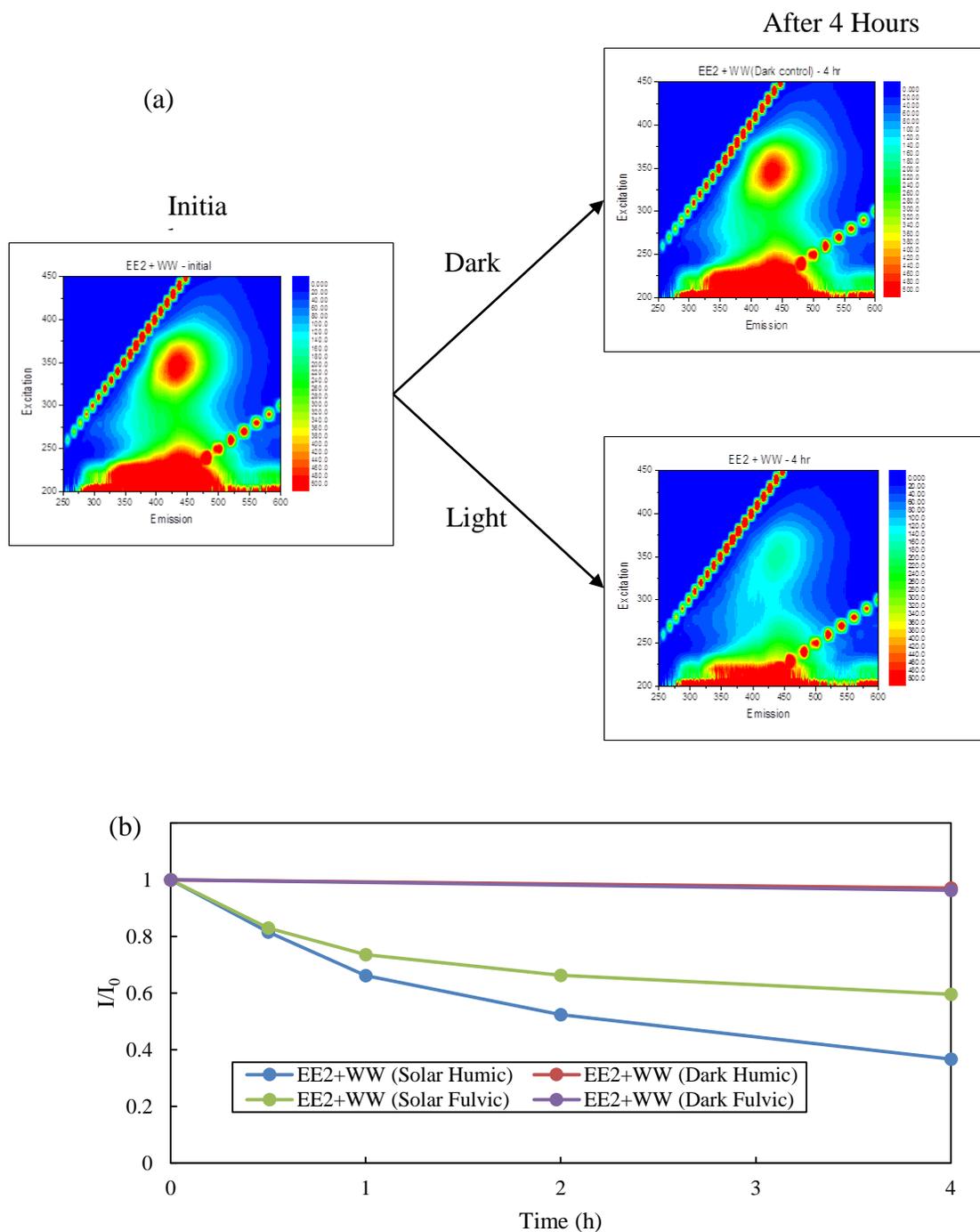


Figure 3.7 Fluorescence Excitation–Emission Matrix test results for the Agua Nueva WRF secondary effluent spiked with EE<sub>2</sub>.

(a) EE<sub>2</sub> does not contribute significantly to the high intensity regions in the EEM maps. The humic (top in a) and fulvic (bottom in a) peaks were attenuated after four hours in sunlight but not in the dark.

(b). In this figure,  $I$  represents fluorescence intensity, obtained by integrating the EEM contours over a region of 5 nm around the maximum intensity point.

### 3.3.4 Trace organics in monitoring wells along the SCR

Twelve monitoring groundwater wells along the SCR (Figure 3.2) were sampled during three sampling campaigns in 2011. Eight out of the twelve target TOrCs (Table 3.3, excluding NP and OP) were detected (Table 3.7). The compounds not detected in these monitoring wells were DEET, TCEP, fluoxetine, and trimethoprim. There were substantial well-specific differences in TOrC concentrations, with the highest levels generally in wells SC-3 and SC-5. Quanrud et al. (2004) showed that samples from SC-3 and SC-5 contained a substantially higher fraction of water derived from the infiltration of wastewater using measurements of boron isotope ratios, which indicated that the SC-3 sample consisted entirely of SCR infiltrate. Trace organic compound concentrations in the wells were generally at least an order of magnitude lower than respective aqueous-phase concentrations in SCR samples, and sulfamethoxazole and iopromide were about two orders of magnitude lower. Candidate mechanisms for compound attenuations include biodegradation, sorption and/or dilution with native ground water. Exceptions included PFOS, PFOA, sucralose and carbamazepine, which were present at similar or higher concentrations in the wells than in river samples. In common, these four compounds have relatively low biodegradation rates, and low log Dow as well (Table 3.1), so that they were not be biodegraded or being retained by the sediments of the river, and tend to accumulate in the groundwater by infiltration process.

Table 3.7 Concentrations of TOxCs in the Lower Santa Cruz River monitoring wells.

Sampling point	Carbamazepine	Sulfamethoxazole	Primidone	Iopromide
SC-1W	191.2 ± 3.5	7.9 ± 0.1	134.4 ± 2.9	<MRL
SC-2W	268.5 ± 55.3	15.2 ± 6.6	142.3 ± 10.4	1.4 ± 1.3
SC-3W	369.1 ± 110.3	260.8 ± 81.1	184.9 ± 0.4	22.7 ± 4.1
SC-5W	312.0 ± 96.4	55.0 ± 43.6	172.4 ± 33.5	2.0 ± 0.5
SC-6W	374.5 ± 2.6	31.0 ± 1.5	227.8 ± 3.0	<MRL
SC-7W	259.2 ± 57.2	62.1 ± 1.0	193.4 ± 4.0	1.4 ± 0.3
SC-8W	202.4 ± 0.6	13.2 ± 2.3	59.5 ± 2.8	<MRL
SC-9W	7.5 ± 0.4	9.0 ± 1.9	9.2 ± 0.1	<MRL
SC-10W	12.3 ± 0.2	6.4 ± 0.1	12.4 ± 0.9	<MRL
SC-11W	196.0 ± 6.3	12.8 ± 0.9	74.2 ± 1.9	<MRL
SC-12W	329.6 ± 3.1	50.0 ± 28.5	43.3 ± 0.7	<MRL

Sampling point	Sucralose	PFOS	PFOA	Tonalide
SC-1W	307.8 ± 5.4	13.2 ± 0.1	9.5 ± 0.6	5.9 ± 0.5
SC-2W	322.1 ± 160.8	55.1 ± 4.5	12.6 ± 5.9	18.4 ± 4.8
SC-3W	1180.4 ± 158.8	20.5 ± 0.7	7.9 ± 3.3	109.0 ± 61.9
SC-5W	3470.2 ± 1014.7	34.2 ± 5.7	10.6 ± 2.7	18.4 ± 19.2
SC-6W	609.0 ± 17.3	40.2 ± 0.4	17.8 ± 0.7	71.6 ± 6.8
SC-7W	1808.9 ± 57.5	49.3 ± 7.5	12.3 ± 9.4	92.4 ± 29.8
SC-8W	109.4 ± 21.7	65.7 ± 2.1	14.8 ± 0.3	1.8 ± 0.3
SC-9W	94.7 ± 31.4	8.9 ± 1.3	2.5 ± 0.3	4.9 ± 0.1
SC-10W	71.0 ± 1.2	8.3 ± 0.6	2.5 ± 0.1	8.0 ± 3.4
SC-11W	379.9 ± 23.6	47.3 ± 1.7	10.9 ± 0.3	12.4 ± 0.9
SC-12W	179.1 ± 47.0	79.7 ± 4.1	13.9 ± 0.1	1.1 ± 0.5

Values represent averages plus/minus one standard deviation. (unit: ng/L)

<MRL means less than method reporting limit, a value that is arbitrarily set at 9:1 the signal-to-noise ratio.

### 3.3.5 Trace organics in riverbed sediments

Trace organic compounds were measured once in extracts derived from riverbed sediments during the February 2013 sampling event. A number of these chemicals are hydrophobic and poorly biodegraded (Table 3.1) and therefore likely to accumulate in riverbed sediments. Among the thirty-eight trace organic analytes, only fourteen were present in sediment extracts at concentrations above their respective detection limits (concentrations for these twelve compounds are listed in Table 3.8).

Table 3.8 Concentrations of TOxCs in the Lower Santa Cruz River sediment samples from February 2013 sampling event (unit: ng/g dw).

Sampling point	Nonylphenol	Octylphenol	Caffeine	Trimethoprim	PFOS	TCPP	Benzophenone
Agua Nueva WRF	101.46	9.64	67.6	0.52	0.89	2.83	4.51
El Camino del Cerro	85.55	8.05	19.5	0.72	0.16	2.73	23.2
N. Silverbell Rd	110.30	3.01	16.0	0.53	0.11	2.75	10.3
N. Cortaro Rd	117.73	3.16	5.87	0.26	0.10	1.63	12.5
Heritage Park Dr	254.10	5.43	7.50	0.36	0.05	1.38	5.29
N. Trico Rd	148.12	2.77	4.54	0.31	0.11	1.30	3.89

Sampling point	Benzotriazole	Diclofenac	Bisphenol A	PFDA	Triclocarban	Carbamazepine	Triclosan
Agua Nueva WRF	0.24	0.56	0.24	0.19	3.72	0.39	3.68
El Camino del Cerro	3.39	<MRL	1.25	0.08	2.41	0.11	2.73
N. Silverbell Rd	0.08	0.26	1.13	0.08	0.92	0.39	0.57
N. Cortaro Rd	0.18	0.16	0.70	<MRL	1.00	0.02	1.49
Heritage Park Dr	0.33	<MRL	0.17	0.06	0.54	0.02	<MRL
N. Trico Rd	0.20	0.16	0.42	0.07	1.00	0.02	0.62

<MRL means less than method reporting limit, a value that is arbitrarily set at 9:1 the signal-to-noise ratio.

When comparing with the results from the river liquid phase, only five of the fourteen detected compounds were also detected in the liquid phase. The concentrations of these compounds as a function of distance from the Agua Nueva WRF are shown in Figure 3.8. As observed from the results, four out of the five compounds show a decreasing trend through the river study range; only NP appeared to be stable across the range of our study area. Our statistical analysis (Table 3.3) of river water shows that, of these five compounds, only PFOS exhibits a definite decreasing trend with distance.

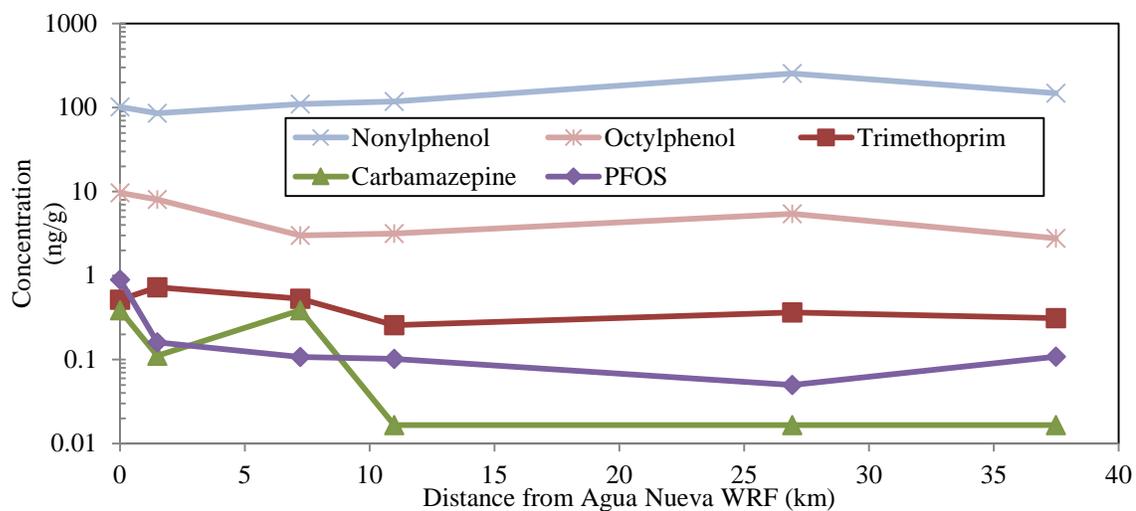


Figure 3.8 Observed trace organic concentrations in SCR sediment vs. distance in the February 2013 sampling event.

Concentrations are in ng of compound per g of dry sediment. Measurements correspond to surface sediment samples from the riverbed (0-2.5 cm depth).

Here, we are concerned with the mechanisms responsible for the presence of trace organics in the sediment, as related to the river liquid phase. Multiple linear regression method was used for the statistical analysis of the relationships between four independent variables (distance from the Agua Nueva WRF, biodegradation probability,  $k_{OH}$  and  $\log D_{ow}$ ), using as dependent variable the ratio of TO<sub>OC</sub> concentration in the river liquid phase and sediment phase. From the statistical results (Table 3.9), it is clear that the model eliminated distance and  $k_{OH}$  as relevant independent variables, and kept the biodegradation probability and  $\log D_{ow}$  as trend determinants, indicating a decrease in the liquid/sediment ratio was related with the decrease of biodegradation probability and increase with  $\log D_{ow}$ . The results suggest that some of the compounds with higher biodegradability were absorbed onto the riverbed sediment while compounds with lower hydrophobicity may have been degraded in the river by a mechanism other than

biodegradation, such as indirect photolysis, leading to relatively low concentrations in the liquid phase while preserving their presence in the sediments.

Table 3.9 Backward linear regression statistical results for correlation between the physical/chemical properties and the ratios of trace organics in the river liquid phase and sediment phase.

Model	Unstandardized Coefficients		Standardized Coefficients	Significance	
	B	Std. Error	Beta		
1	(Constant)	-3875.694	2568.085		0.144
	Distance	128.899	75.969	0.261	0.102
	P <sub>B</sub>	-651.621	210.854	-2.430	0.005
	K <sub>OH</sub>	351.981	264.419	0.240	0.195
	Log Dow	5451.953	2124.203	2.062	0.017
2	(Constant)	-2888.150	2494.825		0.258
	Distance	128.899	77.089	0.261	0.106
	P <sub>B</sub>	-689.241	212.031	-2.571	0.003
	Log Dow	6139.451	2090.825	2.322	0.007
3	(Constant)	-1128.887	2336.137		0.633
	P <sub>B</sub>	-689.241	218.969	-2.571	0.004
	Log Dow	6139.451	2159.240	2.322	0.008

A comparison of NP and OP concentrations in the SCR water and sediments, where the two compounds are both present at comparable concentrations throughout the river area explored, indicate that the sorption mechanism may play an important role on the fate of alkylphenols in the river due to their relatively high hydrophobicity (Tomohiko Isobe et al., 2001). Also biodegradation of their parent compounds APnEOs can contribute to observed moderate concentrations increase of these compounds in river sediment samples as well (Ahel et al., 1994a). Concentrations of these two compounds in the surface layer (0-2.5 cm) of riverbed sediment (85-254 ng/g for NP and 2.8-9.6 ng/g for OP) are comparable with other studies in other effluent-dependent streams (Tomohiko Isobe et al., 2001; Liu et al., 2004).

After NP, caffeine was observed to be the next most abundant compound in the riverbed sediments, while BPA, PFOS and PFDA were observed at values near their detection limits. Concentrations of compounds observed in the sediments did decrease with distance. Caffeine had the highest initial concentration, while the prefluorinated compounds had the lowest observed concentration, vanishing to below detection after approximately 10 km downstream of the Agua Nueva WRF (Table 3.9).

For the general trend of observed chemicals in the riverbed sediment samples, some are persistent with distance downstream, such as carbamazepine, TCPP, trimethoprim, bisphenol A, perfluorinated surfactants (PFOS, PFOA and PFDA) and diclofenac. And for other chemicals, such as caffeine, benzophenone, benzotriazole, triclosan and triclocarban, the concentrations in the sediment samples decreased along the river.

Most Compounds that persist in sediments are hydrophobic, with high  $\log D_{ow}$  values (Table 3.1), which suggests that they tend to be absorbed onto the riverbed solid when they were released from the Agua Nueva WRF, and the sorption mechanism may be important for the fate of this type of chemicals. Carbamazepine, in studies done in other rivers, was observed to persist as well (Tixier et al., 2003; Yamamoto et al., 2009). TCPP is structurally similar to TCEP, which was observed to persist in liquid phase samples in our study and elsewhere (Fries and Puttmann, 2001; Yoon et al., 2010). The  $\log D_{ow}$  of TCPP indicates that it is hydrophobic as well, so this is not surprising to see it consistently appearing in the sediment samples along the river.

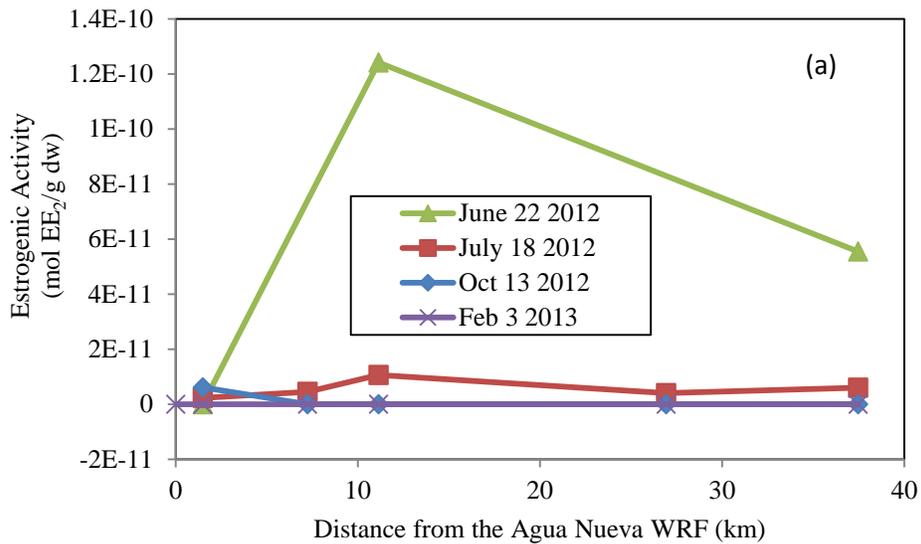
### 3.3.6 Estrogenic activity in riverbed sediments

The total estrogenic activities in the top layer (0-2.5 cm) and lower layer (10.2-12.7cm) of riverbed sediments were tested for each sampling campaign during 2012 and 2013. They were generally higher before the intense summer rainfall period (June 22) than immediately after (July 18). Subsequent rainfalls preceding the last two sampling events seem to have effects on further reduction of estrogenic activity in the sediment samples to under detection limit when using YES bioassay ( $< 1.33 \times 10^{-12}$  mol EE<sub>2</sub>/g dw) (Figure 3.9). The data suggest that intense rainfall/runoff events are capable of attenuating sediment estrogenic levels both at the surface and lower layers, probably through sediment scour and redeposition. Lacher (1996) studied the effects of river flows on sediment quality in the Santa Cruz River, and found a general increase in channel infiltration capacity following high flows in early March. During the summer months, they also observed a general increase followed by a progressive reduction in the infiltration capacity of the streambed indicated by the manner in which the limit of the effluent stream moved progressively downstream. While storm flows scoured the upstream portion of the study reach, they exhibited depositional behavior in the downstream end of the reach. This can explain the changes on the estrogenic activity through the reach in our study.

Level of estrogenic activity detected in the surface sediment before the first intense rain of monsoon season were 5× higher than the level in so-called bottom sediment at the sampling site near 11 km downstream of the outfall of Agua Nueva WRF (Figure 3.9b). But at the sampling site around 37 km, the estrogenic activity in the bottom layer of sediment was about two times higher than that of the surface layer, indicating

that estrogenic compounds were not degraded completely or, alternatively, that some estrogenic compounds deposit onto the sediment of the lower part of the river during dry periods.

Consistent with measurements of estrogenic activity in the surface layers of sediment, the high estrogenic activity of the lower layer was evident before the heavy rainfall/runoff season started. With increasing water flow during the wet season, soil particles in this layer were also disturbed and, then mixed with new particles with lower or no estrogenic activity.



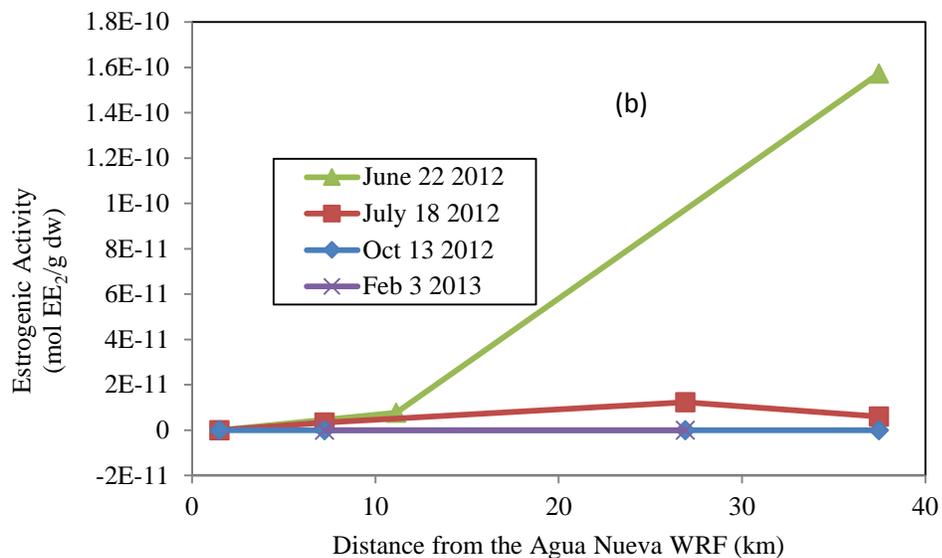


Figure 3.9 Estrogenic activities as a function of position and time in riverbed sediment samples taken from the Santa Cruz River.

(a) surface sediment (top 2.5 cm); (b) lower layer sediment (10.2-12.7 cm depth).

River scouring during high flows does occur and can influence the deep level of the river sediment (Williams et al., 1999). When comparing the estrogenic activities of samples collected at different times in our study, scouring can flush estrogenic activity from the surface sediments of the river channel that has accumulated during dry season, even to depths in excess of 10 cm. Resuspension of estrogens originally attached to the riverbed sediments in the water column reduces estrogenic activity to below YES bioassay detection limit, possibly indicating that these estrogenic compounds may be transformed to less estrogenic ones or be redistributed in the river and sediment.

### 3.4 Conclusions

Results obtained in this part of the research project suggest that dilution is not a candidate mechanism for TOrC attenuation, since the Santa Cruz River is effluent dependent except during infrequent periods of rainfall/runoff. Biodegradable organic TOrCs, including those that contribute to estrogenic activity, are rapidly attenuated with distance and time of travel in the Santa Cruz River. Also indirect photolysis of estrogenic compounds through the river might play an important role for the observation of estrogenic activity changes in the SCR.

Hydrophobic TOrCs may accumulate in river sediments during dry weather periods. Riverbed sediment quality is periodically improved through storm-related scouring during periods of heavy rainfall and runoff. For further research, mechanisms other than biodegradation, photolysis and hydrophobicity are needed to be further investigated for explaining the fate of the trace organics in this effluent dependent stream.

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## CHAPTER 4 CULTIVATION OF *NANNOCHLOROPSIS SALINA* IN WASTEWATER FOR BIOMASS AND LIPID PRODUCTION

### 4.1 Introduction

There are unresolved issues regarding the sustainability of an algal biofuels industry of even modest size (National Research Council, 2012) in light of related demands for water and macronutrients. To that end, it has been widely suggested that algae can be grown on treated municipal wastewater (Jiang et al., 2011; Kong et al., 2010; Min et al., 2011; Noüe et al., 1992; Wang et al., 2010) with attendant water quality benefits in the form of reduced nutrient loading in receiving waters.

From the perspectives of sunlight and land availability, no area in the United States is better suited for an algal biofuels industry than the Southwest. Conversely, no region is less appropriate based on consideration of water resources. The lower Colorado River basin is the only watershed in the country in which annual water consumption exceeds runoff (Christensen et al., 2004); the region is water stressed by any objective measure. In every major population center in the Southwest, reclamation and reuse of municipal wastewater is either contemplated or practiced, so that water resources management is based on consideration of water and municipal wastewater as a single resource. Previous studies have established both (i) the utility of water and nutrients in municipal wastewater for growth of microalgae (National Research Council, 2012) and (ii) water quality benefits attributable to the use of algae for wastewater treatment (Debelius et al., 2009; Li et al., 2011; Mallick, 2002; Wang et al., 2010). High rate algal treatment ponds are shallow raceway type oxidation ponds in which oxygen required for aerobic bacterial activity is provided via algal photosynthesis. Carbon, nitrogen and

phosphorus needed for algal growth are provided in part by bacterial decomposition of wastewater constituents (García et al., 2000). Furthermore, algae have been used to remove metals from wastewater, replacing physical/chemical treatments (Mehta and Gaur, 2005). For example, immobilized *Chlorella homosphaera* cells have been used to remove Cd, Zn and Au from wastewater (Costa and Leite, 1991), and *Spirulina* spp. (cyanobacteria) are efficient biosorbents for dissolved Cr, Cd and Cu (Chojnacka et al., 2005). The integration of algal biofuels production with wastewater treatment processes can also enhance the removals of trace organic contaminants such as triclosan and triclocarban, widely used antimicrobial agents that are incompletely removed by conventional wastewater treatment (Coogan et al., 2007). Hirooka et al. (2005) showed that the green microalga *Chlorella fusca* can treat bisphenol A and other phenolic compounds in wastewater, reducing estrogenic activity.

It seems likely that municipal wastewater will eventually find uses higher than replacement of evaporative losses from algae ponds. Even under extreme water-limited conditions, however, nitrogen and phosphorus requirements for algal growth might be satisfied using relatively low-flow, nutrient-rich side streams derived from wastewater treatment—without sacrificing the receiving water quality benefits derived from nutrient removal.

A number of trace metals that are essential to growth of microalgae inhibit cell growth or lipid production at higher concentrations (Cid et al., 1995; Monteiro et al., 2011; Stauber and Florence, 1987). Sensitivity to metals inhibition is highly species dependent, however, and correlates poorly with cell size, taxonomic classification and metals sorption capacity (Debelius et al., 2009). Lin et al. (2007) found that *Chlorella*

*pyrenoidosa*, a freshwater alga, was considerably more sensitive to Cd, Pb, Co, Ni, and Zn in continuous cultures than in batch tests. The US EPA has used algae to measure aquatic ecotoxicity (<http://cfpub.epa.gov/ecotox/>), noting the species-dependent disparities in sensitivity to a variety of trace organic contaminants that are frequently encountered in treated wastewater (Cleuvers, 2003; Walsh et al., 1987). Furthermore, it is possible that high levels of ammonia nitrogen in conventionally treated municipal wastewater inhibit algal growth, highlighting the needs for selection of tolerant species for biofuels production (Abeliovich and Azov, 1976; Källqvist and Svenson, 2003).

From the perspective of algal biofuels production, cell lipid content is as important as growth rate. Recent laboratory-based studies in which nutrients were provided from centrate derived from sludge dewatering measured lipid contents of 10-30% of cell dry weight (CDW) in small batch and semi-continuous algal cultures. Because centrates are usually recycled to the head end of municipal wastewater treatment plants, they increase the burden of nutrient treatment and disposal (Min et al., 2011; Wang et al., 2010). Although both studies showed that microalgae can be grown successfully on nutrients derived from nutrient-rich centrates, the primary purpose of their work was related to nutrient control as opposed to biodiesel production.

Here we will establish the potential economic savings associated with use of treated wastewater and associated macronutrients in the algal biofuels industry. The analysis is specific to the semiarid American Southwest, although findings should apply in other water stressed areas. For the hypothetical situation in which reclaimed water is reserved for uses higher than biofuels production, but macronutrients can be conveniently separated in treatment plant side streams, the value of recovered nutrients and reduction

in treatment cost for nutrient control are taken as benefits. Potential impediments to the use of wastewater nutrients for growth of metals tolerant microalgae are explored experimentally. Finally, a strategy is presented with which the advantages of growing microalgae in the semiarid Southwest might be realized despite significant problems arising from water scarcity.

## 4.2 Objectives

The purpose of this research project was to exploit the use of treated municipal wastewater and/or nutrient rich side streams derived from municipal wastewater treatment as sources of water and nutrients for algal growth. The rationale for this line of investigation follows.

- There is nutrient value in secondary effluent that can be exploited. A representative value for total available nitrogen in secondary treated municipal wastewater effluent (absent denitrification) is 30 mg/L as N, and a representative total phosphate concentration is 3 mg/L (Metcalf & Eddy, 2003). The nutrient value of effluent suggests that wastewater can be used to defray, although perhaps not eliminate, nutrient costs for large scale growth of microalgae.
- Wastewater can reduce the demand of an algae industry for more conventional potable water resources—those that can be prepared for potable use at much lower cost. The value of wastewater use for algal growth is case specific, and any figure that is used for the purpose of economic analysis should be reexamined in a local or regional context. In an economic sense, use of reclaimed water displaces the most expensive water source necessary to satisfy potable demand. It follows

that the economic value of such practice (reclaimed water for algal growth) is equal to the marginal cost of water resource development for potable needs. In southern Arizona, for example, that value is at least as high as the cost of purchasing Colorado River water from the Central Arizona Project, estimated here at \$140/acre-foot, and probably much higher since water to western cities is heavily subsidized through federal water projects. Options for expanding the suite of water resources utilized in the lower Colorado River basin have been estimated to cost from \$1000-\$2000 per acre-foot or water provided (USBR, 2011). At that price it seems likely that alternative sources of water not usually earmarked for potable use must be employed if a viable algal biofuels industry is to evolve in the American Southwest.

- Use of treated wastewater for algal growth will greatly reduce the cost of wastewater treatment in areas such as Tucson, where nutrient removal is a condition for effluent discharge to a surface water. Pima County is in the process of retrofitting Tucson's major wastewater treatment facilities for nitrogen and phosphorus control. The cost will be on the order of a billion dollars for nutrient control in ~50 MGD of treated wastewater.
- When water is discharged from algae-producing facilities, certain water quality characteristics may actually be improved. Nutrient removal is only the most obvious example. It is also possible that algae will take up metals and trace organics that survive conventional wastewater treatment. Water quality changes, however, may not be entirely favorable since salts will inevitably accumulate due to evapotranspiration during algal growth. Consequently, if water quality changes

that arise during algal growth can be established experimentally, they should not be taken as a potential economic benefit.

- The primary liability associated with use of reclaimed water for algal growth is associated with toxicity. If heavy metals accumulate to toxic levels as a consequence of evapotranspiration, for example, the repeated use of reclaimed water through recycling could eventually result in diminished growth rate and greater cost for achievement of algal output objectives.

To provide a framework and rationale for related experiments in this area, a simplified model for algal growth and resource (nutrient and water) use is provided here.

### **4.3 Hypotheses**

The following collection of hypotheses and postulates motivated the program of experiments and analysis conducted here:

- The American Southwest is ideal for development of an algal biofuels industry from the perspective of climate, if problems related to water availability can be solved.
- Treated municipal wastewater that is unsuitable for human consumption can be used to satisfy water needs for growth of microalgae.
- The same waters carry macronutrients in sufficient quantity to significantly offset the cost of fertilizer for growth of microalgae and prevent an unnecessary competition for fertilizers between the biofuels industry and more conventional agriculture practices.

- Metals levels in most conventionally treated municipal wastewaters will not impede the growth of at least some metals-tolerant microalgae at either (i) levels present in wastewater effluent or (ii) concentrates in waste streams derived from the treatment of wastewater such as centrate or pressates from sludge dewatering.
- Metals toxicity is likely to be greater in continuous cultures of microalgae than in batch cultures since continuous culture methods produce very low concentrations of growth limiting nutrients.
- Even when salts (and metals) accumulate in waters used for algal growth due to evaporation and recycling, select algal species will be capable of unimpeded growth and lipid production.
- There are no other, as yet unknown, toxicants in treated wastewater that will eventually impede the growth of a wastewater-dependent algal biofuels industry.

#### **4.4 Algal photobioreactor modeling**

##### **4.4.1 Algal bioreactor configuration**

It should be recognized that the objectives of rapid growth and lipid production may be incompatible. For this reason, a two-reactor production scheme is used as the basis for the illustration that follows. The first reactor would be operated to maximize the rate of algal growth, probably under light-limited conditions, without consideration of lipid production. In the second reactor, conditions could be maintained to promote lipid production. Here we analyze only the process of algal growth (reactor #1), leading to an estimate of the demand for wastewater effluent and savings in the forms of reduced nutrient demand and reduction in potable water use.

The algal growth reactor is modeled as a plug-flow reactor (with evaporative loss) in which a fraction of the effluent (R) is recycled. Actually, however, water is simply recirculated around the looped raceway. With each pass around the raceway, a portion of the water is withdrawn for separation of algae via a process to be identified later such as dissolved air flotation or centrifugation. Separation efficiency isn't much of an issue, so the separator will likely be selected to minimize energy utilization while achieving the separation targets. The design of this component of the algae production process is beyond the scope of the analysis, however. Enough algae will be recirculated in the raceway reactor at each pass to render the growth rate zero-order in biomass concentration (probably due to light-limited growth) and eliminate the need to maintain plug flow in the reactor in order to maximize the growth rate. That is, if growth is zero-order in biomass concentration, the performances of a PFR and well mixed reactor (or any intermediate reactor form) would be similar in terms of biomass production rate.

#### **4.4.2 Estimation of nutrient and water savings**

In the exercise that follows, we will size a hypothetical algal growth reactor to produce 1 metric ton of dry algal mass per day, then find the nutrient and water savings that attend use of secondary effluent as a water source. Results will eventually yield an annual savings, subject to the several assumptions incorporated into the analysis along the way and specific to the suite of parameters chosen to represent operating conditions. We start by making water and salt balances around the reactor (Figure 4.1). More importantly, the exercise illustrates the need for data to drive the model and motivates the related experimental program.

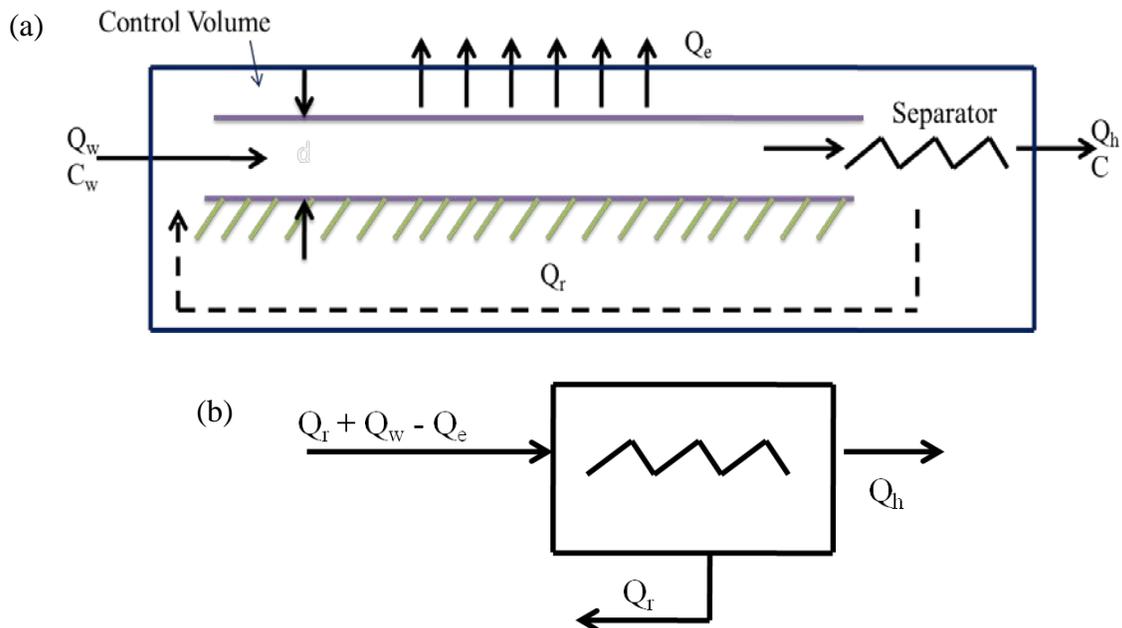


Figure 4.1. Control volume representations for water and salt mass balances on a hypothetical algal raceway.

(a) The raceway itself. The salt concentration (TDS) of water coming into the reactor is  $C_w$  and the salt concentration in water leaving the reactor is  $C$ .

(b) Water balance on the algal separation process at the exit of the biomass growth reactor.

#### 4.4.2.1 Balances and algal productivity/reactor size

Under steady conditions:

$$Q_w = Q_e + Q_h \quad (1)$$

where  $Q_w$  is the rate of water addition per unit surface area of reactor [m/d],

$Q_e$  is the rate of evapotranspiration from the reactor [m/d], and

$Q_h$  is the rate of water loss to Reactor #2 for algae harvesting [m/d].

A second water balance applies to the (unidentified) algae separation device (Figure 4.1b).

$$1-R = Q_h / (Q_r + Q_w - Q_e) \quad (2)$$

where  $Q_r$  is the rate at which water returns to Reactor #1 from the separator [m/d], and  $R$  is the recycle ratio [ ].

A salt balance under steady conditions in the raceway yields:

$$Q_h \times C = Q_w \times C_w \quad (3)$$

where  $C$  is the total dissolved solids concentration leaving the raceway, and  $C_w$  is the TDS concentration in the wastewater source.

Solving the set of three algebraic equations simultaneously:

$$Q_h = C_w \times Q_e / (C - C_w) \quad (4)$$

$$Q_w = C \times Q_e / (C - C_w) \quad (5)$$

$$Q_r = R / (1 - R) \times C_w / (C - C_w) \times Q_e \quad (6)$$

The reactor surface area is selected to satisfy an arbitrary algal production requirement. That is,

$$P \times A = 10^6 \text{ g/d} \quad (7)$$

where  $A$  is the reactor surface area [m<sup>2</sup>], and

$P$  represents algal productivity [g/m<sup>2</sup>-d].

To go further, it is necessary to use preliminary estimates of various process parameters such as nutrient yield coefficients. In reality these must be established experimentally and are used here only to justify the related experimental program. Estimating  $P$  at 10.0 g/m<sup>2</sup>-d (Shen et al., 2009), the required surface area of the field-scale reactor for production of 1 MT per day of dry biomass is  $1.0 \times 10^5$  m<sup>2</sup>.

#### 4.4.2.2 Savings due to wastewater utilization

Since  $Q_w$ , the rate of consumption of treated wastewater, is a function of the steady reactor total dissolved solids (TDS) concentration (Equation 5), it is necessary to select a value for  $C$  in order to calculate water demand. For illustration, it is assumed that saltwater algae can tolerate TDS levels in the range  $20,000 < C < 40,000$  mg/L. Under those circumstances,  $C \gg C_w$ , making  $Q_w \approx Q_e$ . For freshwater algae, salinity limitations could increase water demand considerably.

Cost savings due to wastewater use is proportional to  $Q_w$ . Here, commercial nitrogen and phosphorus costs are taken as \$1.1/kg-N and \$3.3/kg-P (USDA, <http://www.ers.usda.gov/data-products/fertilizer-use-and-price.aspx>), and the potential annual fertilizer cost savings are:

$$A\$_N = Q_w \times A \times C_N \times V_N \times 365 \quad (8)$$

where  $A\$_N$  is the annual savings in nitrogen fertilizer cost [\$/yr],

$C_N$  is the concentration of available nitrogen in wastewater—here, 0.03 kg/m<sup>3</sup>, (Metcalf & Eddy, 2003), and

$V_N$  is the unit cost of nitrogen fertilizer (above).

Similarly,

$$A\$_P = Q_w \times A \times C_P \times V_P \times 365 \quad (9)$$

where  $C_P$  is the concentration of available phosphorus in wastewater—here, 0.003 kg/m<sup>3</sup>, (Metcalf & Eddy, 2003), and

$V_P$  is the unit cost of phosphate fertilizer (above).

Finally,

$$A\$_W = Q_w \times A \times V_w \times 365 \quad (10)$$

where  $A\$_W$  is the annual savings in water [\$/yr], and

$V_w$  is the marginal cost of freshwater provision, here \$0.14/m<sup>3</sup>

(<http://cms3.tucsonaz.gov/water/rates>).

Taking the net annual evaporation rate in Tucson, Arizona as 1.53 m/yr (AZ Water), the annual cost of water necessary for production of a MT of biomass per day in the semiarid Southwest (saltwater alga) would be ~\$21,400/year. Clearly, however, water savings from wastewater use is only available in areas where reclaimed water is not already earmarked for other purposes or is valued at < \$0.14/m<sup>3</sup>. Savings on nutrients could be as much as ~\$5,200 per year for nitrogen and ~\$1,700 per year for phosphate, assuming that (i) wastewater effluent is the sole source of make-up water and (ii) the entire nutrient content of the effluent source is necessary for algal growth (see below). Thus the total annual savings attributable to the use of reclaimed water in this scenario could be as high as \$28,300, of which more than 75% would be attributable to water replacement. The exercise can easily be modified to assess the value of reclaimed water use for algal growth in areas that differ in terms of water cost and net evaporation rate or for growth of a freshwater algal species with limited salt tolerance.

To calculate the total nutrient demand (and cost) to produce a MT of algae per day, yield coefficients are necessary. Experimental evidence (Figure 4.8) indicates that the ratio of cell dry weight produced to nitrogen consumed by the saltwater species *N. salina* is ~20. It follows that production of 1 MT of dry algal biomass would consume 50

kg of available nitrogen, or about four times the nitrogen in effluent necessary to replace evaporative water loss. If the entire nitrogen requirement for growth is provided as centrate from sludge dewatering, the savings from nitrogen replacement would be ~\$20,000 per year.

The full potential savings on phosphate fertilizer is approached similarly. Here, the ratio of P/N (wt/wt) for balanced algal growth is estimated at  $31/(14 \times 16) = 0.14$ , where 16 is the molar ratio of nitrogen to phosphorus in algal biomass (Masters and Ela, 2008). Thus, the yield coefficient for growth on phosphorus is estimated at 143 (mass CDW/mass P consumed), and the rate of phosphorus demand to produce a MT of CDW per day would be 7.0 kg/d (5.6× the mass of P in effluent required to replace evaporative loss) for a savings of ~\$8,500 per year.

Table 4.1 Summary of annual cost savings attributable to use of wastewater effluent and/or nutrients for algal growth in a biofuels industry.

Hypothetical case	Water replacement <sup>a</sup>	Nitrogen value <sup>b</sup>	Phosphorus value <sup>c</sup>	Total savings
Effluent containing N/P to replace evaporative loss <sup>d</sup>	\$21,400	\$5,200	\$1,700	~\$28,300
Nitrogen/phosphorus from nutrient-rich side stream; no effluent <sup>e</sup>	\$0	\$20,000	\$8,500	~\$28,500
Effluent to replace evaporative loss and N/P from sidestream <sup>f</sup>	\$21,400	\$20,000	\$8,500	~\$49,900

Figures in this table are specific to the southwestern US and growth of a saltwater alga to yield one MT of dry biomass per day.

<sup>a</sup>Replacement of water loss due to net evaporation (1.53 m/yr) from ponds designed to produce 1MT of dry algal biomass per day. The assumed value of water replaced was \$0.14/m<sup>3</sup>.

<sup>b</sup>Represents the value of wastewater nitrogen used for growth of 1 MT/day of microalgae CDW. The assumed cost of nitrogen fertilizer was \$1.10/kg-N.

<sup>c</sup>The value of wastewater phosphate used to grow 1 MT/day of microalgae CDW. The assumed cost of phosphate fertilizer was \$3.30/kg-P.

<sup>d</sup>Case involves the use of conventionally treated municipal wastewater effluent to replace evaporative water loss. Nitrogen and phosphorus values arise from residual nitrogen (30 mg/L-N) and phosphate (3 mg/L-P) in effluent.

<sup>e</sup>Values represent the entire costs of nitrogen and phosphorus fertilizers to grow 1 MT of CDW. Yield factors for growth on nitrogen and phosphorus were 20 and 143 (mass CDW/mass nutrient), respectively.

<sup>f</sup>In this case effluent containing residual N, P was used to replace evaporative loss, and additional nitrogen was obtained from nutrient-rich centrate to complete cell requirements for N, P.

In summary, if wastewater effluent is used to replace water lost to evaporation and nutrient requirements for algal growth are entirely satisfied from wastewater nitrogen and phosphorus, the entire demand for water and macronutrients would be met from waste with a related savings of about \$50,000 per year (Table 4.1). For perspective, a metric ton of algal cell dry weight is expected to yield perhaps 300 kg of biofuel. At a specific gravity of 0.85, this amounts to ~34,000 gallons of biofuel per year, and at \$4/gal, the product value is ~\$136,000 per year. Use of effluent or effluent plus centrate from municipal wastewater treatment could decrease biofuel production costs by \$0.83 to \$1.47 per gallon of product.

#### **4.4.3 Experimental design**

Expected savings from the use of wastewater effluent and nutrients derived from wastewater treatment will be realized only if algal cells can grow and generate lipids efficiently in waters containing concentrated wastewater components. Reactor operation as outlined is sure to concentrate compounds that are conserved through evaporation and recycle. Accumulated compounds would likely include, for example, metals with limited nutrient value and persistent trace organics. The experimental program that follows was designed to identify problems associated with water reuse for algal growth when municipal wastewater effluent is used to replace evaporative loss or centrate is used as a source of nutrients. Specifically, experiments were designed to test the following ideas:

- When municipal wastewater effluent or a nutrient-rich side stream derived from wastewater treatment serves as water or nutrient source for growth of microalgae, the resultant growth medium can be recycled, essentially without limit.

- Microalgae readily grow and produce lipids on residual nutrients in conventionally treated wastewater or nutrient-rich side streams.
- Metals or other toxicants that accumulate due to water recycling will not inhibit lipid production among metals-tolerant species.
- Metals toxicity is no greater among cells grown in continuous culture than in batch cultures.

Experiments build on an already considerable body of literature related to the growth of microalgae on wastewater effluent and the toxicity of metals to algae.

Contributions specific to this work pertain to (i) the use of nutrient rich side streams derived from wastewater treatment (e.g., sludge dewatering) and (ii) investigation of metals toxicity when metals are presented in combination and concentrated many fold in order to simulate evaporative water loss. Finally, a strategy is presented with which the advantages of growing microalgae in the semiarid Southwest might be realized despite significant problems arising from water scarcity.

#### **4.5 Materials and methods**

Methods are provided for lab-scale experiments with a sea water algal species, *Nannochloropsis salina*—CCMP 1776. Our primary interest was in establishing the utility of municipal wastewater or concentrated waste streams derived from the treatment of wastewater as a source of nutrients and water for the production of algal biofuels. It was also necessary to look for evidence of toxicity in the form of growth inhibition when

cells were grown on treated wastewater or the concentrated waste streams. A description of methods common to the majority of experiments follows.

#### **4.5.1 Algae strain and culture conditions**

The marine microalga *N. salina* (CCMP 1776) was obtained from Bigelow Laboratory for Ocean Sciences and maintained in f/2-Si artificial seawater media (Guillard and Ryther, 1962). Instant Ocean synthetic sea salts (Aquarium Systems, Mentor, OH) were added to achieve a salinity of 3.5%, with make-up water added as needed during experiments to maintain constant water volume. For batch experiments, reactors were inoculated at 1:10 (v/v) from the stock, to achieve an initial cell dry weight of ~0.05 mg/L, and grown at room temperature. Flasks were continuously supplied with air that was sometimes supplemented with 5% CO<sub>2</sub> as a carbon source. Culture pH was maintained between 7.0 and 8.5. For all experiments, light was provided by four T5- HO fluorescent tubes (54 W/tube) (Spectralux, USA) positioned above the flasks using a 12 h: 12 h light: dark cycle.

Algal biomass concentrations were monitored as optical density ( $\lambda=750$  nm). Measurements were converted to CDW and ash free dry weight (AFDW) based on conversion factors of 0.3225 g CDW/L-absorbance unit, and 0.3055 g AFDW/L-absorbance unit).

#### **4.5.2 Algal metal toxicity tests**

##### **4.5.2.1 Algal metals toxicity tests in batch experiments**

For batch toxicity tests, *N. salina* was grown at room temperature in 250 ml Erlenmeyer flasks. Metals were selected for study based on their relative concentrations in treated municipal wastewater (Table 4.2), including copper, zinc, cobalt, lead, and

nickel. Metal stock solutions were prepared from CuSO<sub>4</sub>, ZnCl<sub>2</sub>, CoCl<sub>2</sub>, PbCl<sub>2</sub> and NiSO<sub>4</sub> salts and passed through 0.22 µm filters before they were added to the f/2-Si artificial seawater media at target concentrations. Two types of batch metal toxicity experiments were carried out: (i) combined-metal experiments in which metals were provided in proportion to their concentrations in Tucson municipal wastewater—that is, metal levels were varied jointly to maintain proportions present in treated wastewater; (ii) copper or zinc alone. Metal concentrations selected were relevant to algal growth in previous work (Lin et al., 2007; Stauber and Florence, 1987).

Table 4.2. Comparison of continuous flow metals toxicity (EC<sub>50</sub> values) to metals levels in effluent or centrate from the Tres Rios WRF in Tucson, AZ.

Metal	EC <sub>50</sub> from Scientific Literature (µg/L)	Measured Concentration in Local Wastewater Effluent (µg/L)	Measured Concentration in Local Centrate Wastewater (µg/L)
Copper	50.2 <sup>a</sup>	17.5	23.6
Zinc	240 <sup>b</sup>	24.8	23.4
Cobalt	520 <sup>b</sup>	n/a	2.23
Lead	680 <sup>b</sup>	1.53	0.60
Nickel	410 <sup>b</sup>	13.1	7.40

<sup>a</sup> Stauber and Flourence, 1987.

<sup>b</sup> Lin *et al.*, 2007.

#### 4.5.2.2 Algal metal toxicity tests in 96-well plates

These were straightforward experiments designed to investigate the feasibility of pursuing metals and other toxicity objectives in batch cultures grown in individual wells of a 96-well micro plate (Corning). In this way, the reproducibility of inhibition data and toxicant concentration effects could be pursued simultaneously, taking advantage of the multiple wells and ability of plate scanning devices to conveniently gather optical density data from all wells at essentially the same time. Success of the procedure depended on

the ability of growth in the wells of the plates to faithfully mimic that of growth in other types of cultures (here batch cultures in glass flasks). Growth was monitored via changes in optical density at 680 nm using a scanning plate reader. Values were not immediately comparable to those obtained spectrophotometrically (above) due to differences in light pathlength.

#### **4.5.2.3 Zinc Toxicity Tests in CSTR**

*N. salina* was grown in continuous culture at room temperature in a 2-liter bioreactor. Based on previous experiments and growth kinetics in batch cultures, a hydraulic detention time of 4 days was selected to avoid the possibility of washout that is unrelated to metals toxicity. The growth medium was as described previously (f/2-Si medium). In order to establish steady conditions conveniently, a modified operation was adopted in which one-fourth of the well mixed reactor contents was replaced with fresh growth medium daily. This was generally done by slowly adding the replacement medium and allowing the displaced volume of reactor contents to be removed at an equal rate in order to maintain a constant volume. Lamps were positioned around the reactor in order to provide light, and the reactor contents were supplemented with air containing 5% CO<sub>2</sub>. Every two days, the cell density in the reactor was measured. Steady conditions were established prior to metal addition. Zinc was added to the normal growth media from low to high concentration (constant for one or two weeks) until the optical density of algae decreased. In order to retain enough algae in the reactor, zinc concentrations were again decreased, allowing the algal specific growth rate to increase and avoiding washout. Steady conditions were established before the influent level of zinc was again increased.

### 4.5.3 Cultivation *N. salina* using wastewater

Two types of wastewater, secondary effluent and centrate, were used for testing whether the algal species could grow or not due to the toxic effects in them. For secondary effluent collected from Tres Rios Wastewater Reclamation Facility (WRF) in Tucson, AZ, vibratory shear enhanced processing (VSEP) system was applied to get the water filtered, and the concentrated brine was collected for algae growth experiment, which was five times concentrated for the metals and salts comparing with original wastewater effluent.

The other type of wastewater used for this study was a nutrient-rich centrate (Table 4.3), derived by dewatering anaerobically digested sludge, was collected from Tres Rios WRF and used as the nutrient source for cultivation of microalgae. Centrate is generated in this manner is characterized by high carbon, nitrogen and phosphate levels. Centrate was filtered through 0.45 $\mu$ m and 0.22 $\mu$ m filters for sterility before it was used for the growth on *N. salina*. Different percentages of centrate were combined with normal growth media (f/2-Si medium), or tap water compensated with enough Instant Ocean salt to provide an equivalent salt level, in 1L flasks. Air supplemented with 5% CO<sub>2</sub> and sufficient light were provided, as described previously.

Table 4.3. Chemical composition of centrate from sludge dewatering. Analyses were conducted on filtrate after passage through a 0.22  $\mu$ m filter.

<b>Parameters</b>	<b>Concentration</b>
Total Organic Carbon (mg/L)	359
Total Nitrogen (mg/L)	563
F <sup>-</sup> (mM)	0.383
Cl <sup>-</sup> (mM)	227.8
Br <sup>-</sup> (mM)	0.341
NO <sub>3</sub> <sup>-</sup> (mM)	0.285
Total PO <sub>4</sub> (mM)	0.158

$\text{SO}_4^{2-}$  (mM)

7.78

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#### 4.5.4 Microtox tests

In order to identify the responsible part of wastewater toxicity to algae growing, Microtox acute toxicity test method was adopted in this project. It is a standardized toxicity test system which is rapid, sensitive, reproducible, ecologically relevant and cost effective. It is recognized by US EPA and used throughout the world as a standard test for aquatic toxicity testing. This test uses a bioluminescent marine bacterium *Vibrio fischeri*, by measuring its bioluminescence at 490 nm.

The bacteria were exposed to serial dilutions of the secondary effluent or centrate wastewater, which was used in the algal growth tested. *V. fischeri* is sensitive to pH and temperature (should below 10°C). When running the toxicity test, pH should be kept between 6 and 8. For this reason, the pH of all the wastewater samples tested was well adjusted before the assay. The reduction in intensity of light emitted from the bacteria is measured along with standard solutions and control samples, using a Microtox Model 500 analyser (Microbics Corp., Carlsbad, California). The change in light output and concentration of the toxicant produced a dose/response relationship. The results were then normalized, and if possible, the EC<sub>50</sub> (concentration producing a 50% reduction in light) was calculated.

Centrate and secondary effluent used in the algae growth were tested for the toxicity. Empore C18 (3M) solid phase extraction disks were employed for the separation of the hydrophilic part and hydrophobic organics of the water. In this way the responsible part for wastewater toxicity could be identified, whether it is the inorganic metals, or the organic compounds.

#### **4.5.5 Algal lipid extraction — microwave assisted extraction (MAE).**

Traditional algal lipid extraction method was based on Guillard and Ryther (1962) method. It involved several steps of phase separation using centrifuge and time consuming. Also the technique of handling samples may vary from person to person. On the contrary, The MAE extraction method avoids use of dangerous solvents, and numerous samples can be processed simultaneously. Algae were totally dried before they were ground to fine powder using mortar and pestle. The powdered algae were poured into MARS Xpress vessels (0.5g maximum for each vessel) of the CEM Microwave Accelerated Reaction System (MARS, CEM Corporation, Matthews, North Carolina). Ten mLs of pre-mixed chloroform/methanol (2:1 v/v) was added to each vessel and held at room temperature for at least two hours. The suspensions were then heated via microwave to 70°C and held for 60 minutes. Vessel contents were then cooled at room temperature for at least 15 minutes before they were removed from the vessels. Each container was dried in order to obtain as estimate of lipid dry weight. Among the drawbacks of the MAE method is the inability to separate lipids from other cell components during extraction. That shortcoming was circumvented here by comparing FAME profiles following esterification and following alternative, more traditional extraction steps.

#### **4.5.6 Fatty acid analysis**

The algal lipid extracts were transesterified in 3N methanolic HCl (80°C, 10 min). After drying under N<sub>2</sub> and redissolving in dichloromethane, the fatty acid methyl esters (FAMES) were analyzed by Agilent 7890A/5975C GC/MS System (gas chromatography–mass spectrometry). Separation of compounds was accomplished on a

fused silica column (Supelco Omegawax 250, 30 m × 0.25 mm I.D., 0.25 μm). The oven temperature program was 2 min at 50°C, increasing at 3°C min<sup>-1</sup> to 250°C, and maintained at the high temperature for 15 minutes. The inlet and detector were set at 250 and 260°C, respectively. Sample injection (1 μL) was in splitless mode. Helium was used as the gas carrier at a constant flow of 1 mL min<sup>-1</sup>. Individual peaks were identified by comparison with retention times of known standards. Fatty acid content as a percentage of algal dry weight was determined by dividing each of the identified fatty acid components by the dried biomass weight.

## **4.6 Results and discussion**

### **4.6.1 Algal metals toxicity tests**

#### **4.6.1.1 Algal metals toxicity tests in batch experiment**

The metals investigated as potential toxicants included those present at the highest concentrations in regional municipal wastewaters. Metals and their respective EC<sub>50</sub> values (obtained using a particularly sensitive algal species) were as illustrated (Table 4.2). When available, the table includes measured values in effluent from the Tres Rios WRF. Initial experiments involved *N. salina* in batch cultures containing five-metal combinations at concentrations representing multiples of their respective EC<sub>50</sub> concentrations—from 1-100× the EC<sub>50</sub> values in the table. Subsequent work in this area was designed to determine which metal species was the predominant source of observed toxicity.

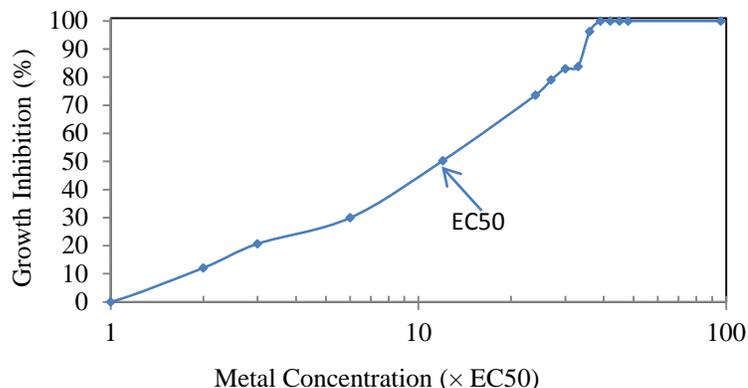


Figure 4.2 Growth of *N. salina* in standard medium supplemented with mixtures of the Table 4.2 metals.

Metals concentrations were multiples (horizontal axis) of the EC<sub>50</sub> values given in Table 4.2.

Fifty percent inhibition of the *N. salina* growth rate was observed in the culture amended with the Table 4.2 metals at 11× their respective EC<sub>50</sub> values (Figure 4.2). That is, zinc and copper were present at concentrations near mg/L levels—exceptionally high relative to their typical concentrations in regional municipal wastewater effluent (Table 4.2). The total copper concentration, for example, was > 30× and the concentration of total zinc was > 100× their respective values in Tres Rios WRF effluent. Results indicate that metals levels in most municipal wastewaters will not inhibit algal growth when wastewater is used as a nutrient source for biofuels development.

Additional experiments were carried out involving single-metal additions to determine the primary metals responsible for the observed toxic effects (Figure 4.3). Results suggest that zinc alone can account for the toxicity observed in the mixtures. That is, zinc at ~11× its reported EC<sub>50</sub> value, with no other metal added, was capable of reducing the growth rate of *N. salina* by about 50 percent. When copper was tested on its own, a 300-fold increase above the EC<sub>50</sub> was necessary to produce the same degree of inhibition of cell growth rate.

The bottom line findings for this work were that, among the Table 4.2 metals, zinc seems to have been responsible for the observed metals toxicity in the mixture. However, the zinc  $EC_{50}$  in these experiments was  $\sim 100x$  the concentration in Tucson's municipal wastewater. Without going into greater depth, it appears that metals levels in most municipal wastewaters will not inhibit algal growth when wastewater is used as a nutrient source for biofuels development. However, it remains possible that *N. salina* is a particularly metals-tolerant species. Furthermore, some component of treated wastewater is capable of inhibiting algal growth when wastewater comprises a large fraction of the growth medium volume.

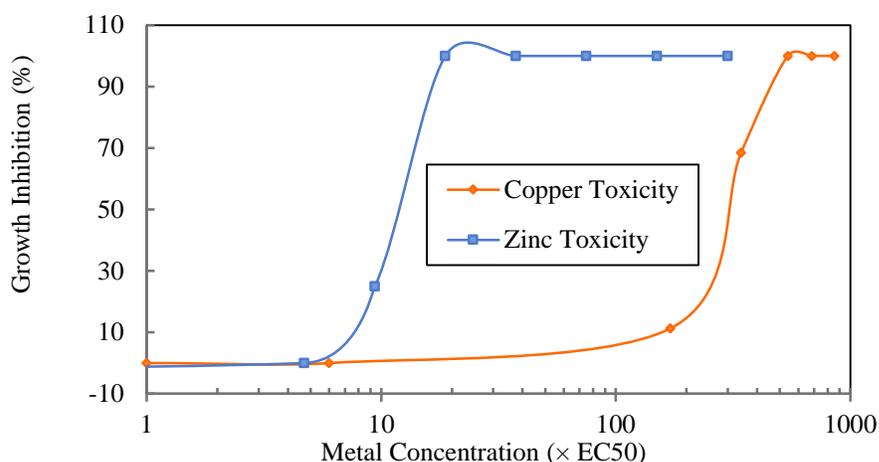


Figure 4.3. Copper and zinc toxicity experiments. Values on the horizontal axis represent multiples of  $EC_{50}$  values shown in Table 4.2.

#### 4.6.1.2 Algal metal toxicity test in 96-well plates

Additional experimental results indicated that metals inhibition experiments could be conveniently run in 96-well plates without significant loss of sensitivity. Results of zinc toxicity tests are shown in Figure 4.4. *N. salina* in plates and flask were growing side-by-side in a closed shaker incubator under the same growth condition. Data suggest that growth in the plates was at least as fast as growth in the flask, indicating that

inhibition work can be pursued, with much greater speed and convenience, in 96-well plates. Also there is a dose dependent trend for zinc toxicity to the algae. As observed from the graph, the algae growth is restrained at concentrations greater than 18mg/L (which equals to  $75 \times EC_{50}$ ) Zn in growth media, which is comparable to the result with the  $EC_{50}$  values got from other flask batch experiment.

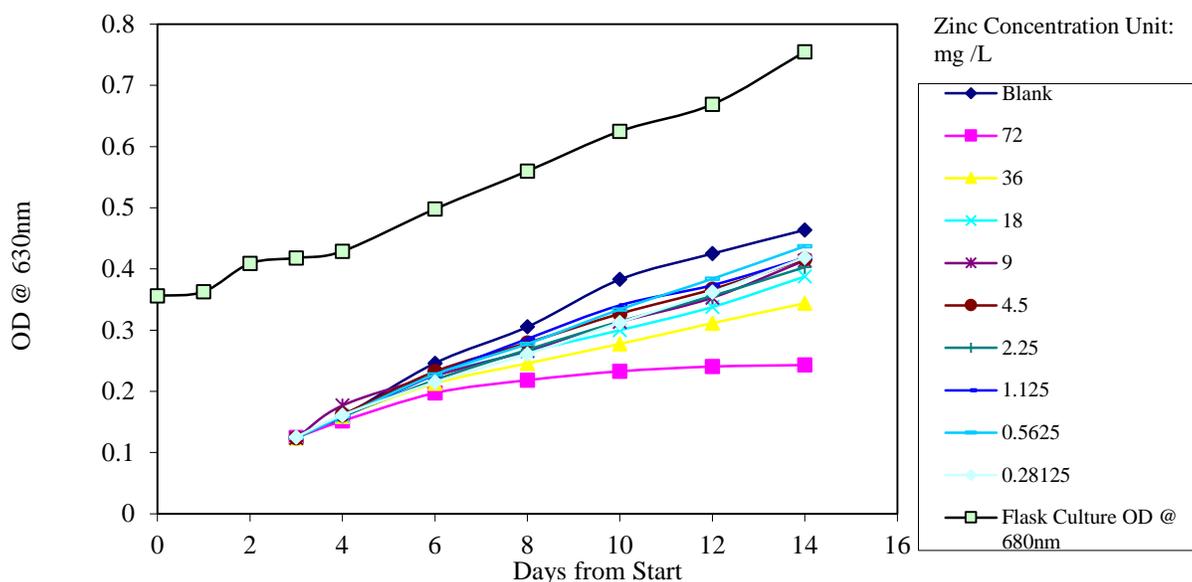


Figure 4.4 Zinc Toxicity Test in 96-well plates for *N. salina*.

Copper toxicity test was also performed in 96-well plate. Results are shown in Figure 4.5. The data suggest that for copper concentration greater than 39.69mg/L, which equals to  $790 \times EC_{50}$  value reported, the algae would stop growing after seven days. As the copper concentration in the media get higher, algae cell density becomes lower. Compared the results with zinc toxicity test, it could be concluded that zinc is more responsible for the algal toxicity. The results indicate that zinc alone is responsible for the metal toxicity to *N. salina*, which is identical to the results from the traditional flask cultural method.

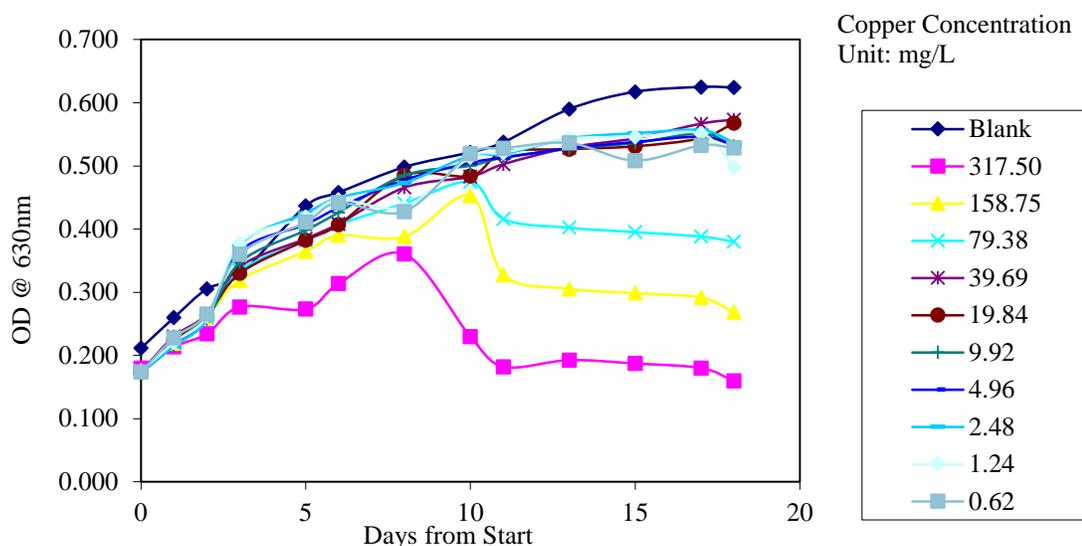


Figure 4.5 Copper Toxicity Test in 96-well plates for *N. salina*

#### 4.6.1.3 Algal metal toxicity test in CSTR

The above metal toxicity tests led to selection of zinc for CSTR toxicity experiments. Steady conditions were indicated by the cell density ( $A_{680}$ ) over a period of about three weeks (Figure 4.6). At that point, 720  $\mu\text{g/L}$  of zinc was added to the reactor influent. This represents  $3\times$  the reported  $\text{EC}_{50}$  for Zn in continuous culture. It was expected that a near-steady reactor concentration of Zn approaching 720  $\mu\text{g/L}$  would be established after 12 days beyond the point of addition of zinc. The concentration of Zn increased each time when optical density restablized following an increase in Zn concentration. Doubling the total soluble zinc concentration from 5.7 mg/L to 11.5 mg/L ( $48\times \text{EC}_{50}$ ) resulted in rapid loss of culture density, leading toward cell washout. The actual  $\text{EC}_{50}$  was calculated for the Zn on the algae in the CSTR, it was about 7.5 mg/L. The  $\text{EC}_{50}$  value of the continuous test was much more than that of the batch test results, indicating that algae in the batch test was more sensitive than that of the continuous test.

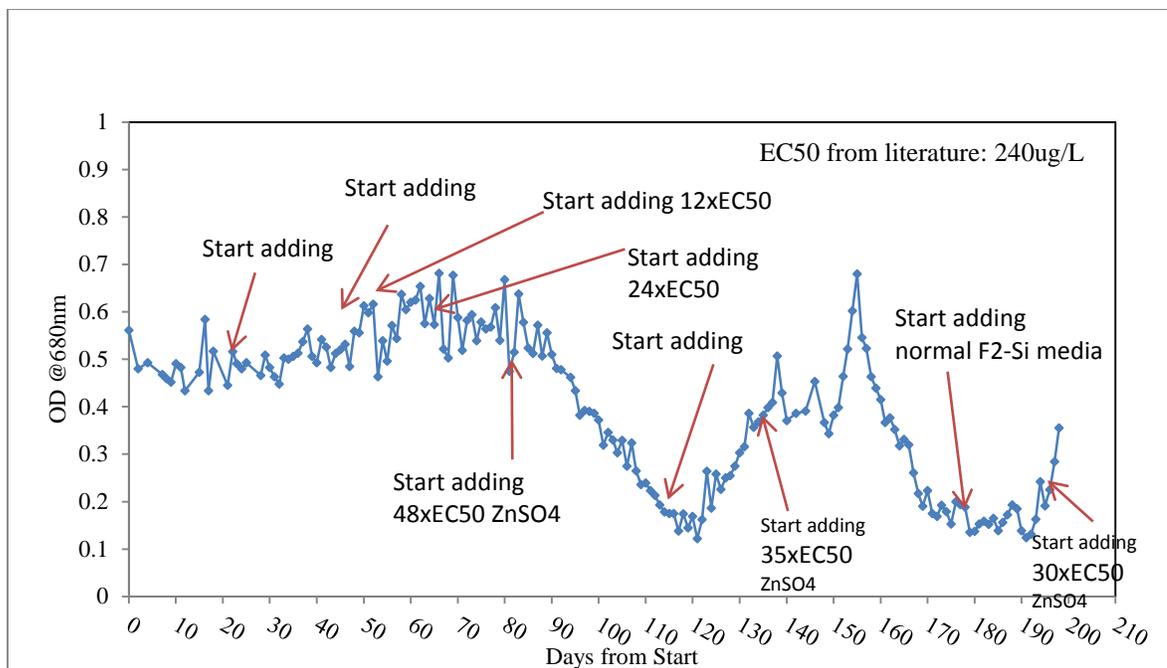


Figure 4.6. Time dependent cell density as a function of the influent Zn (II) concentration in a continuous culture of *N. salina* CCMP 1776.

The hydraulic detention time in these experiments was 4 days.

#### 4.6.2 Nitrogen yield during batch growth of *N. salina*

This experiment was to provide useful information on the yield factor of nitrogen and phosphorus for algae grow in normal medium. *N. salina* was growing in batch cultures, varying only the amount of available nitrogen (nitrate) added to the f/2-Si standard growth medium. Algal cell dry weight (CDW) concentrations at the onset of stationary growth were then plotted as a function of the initial  $\text{NO}_3^-$ -N concentration. The slope of the straight-line relationship was taken as the yield factor in units of grams dry weight per gram nitrogen (Figure 4.7 and Figure 4.8).

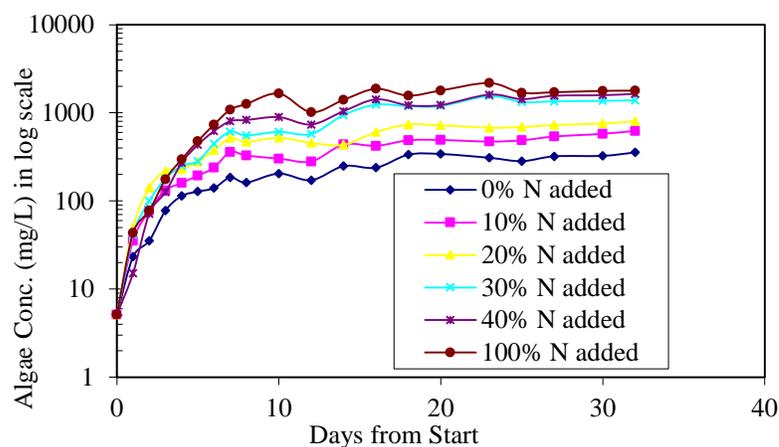


Figure 4.7. Algal growth in batch cultures as a function of initial available nitrogen level. The complete medium contained  $8.84 \times 10^{-4}$  M nitrogen.

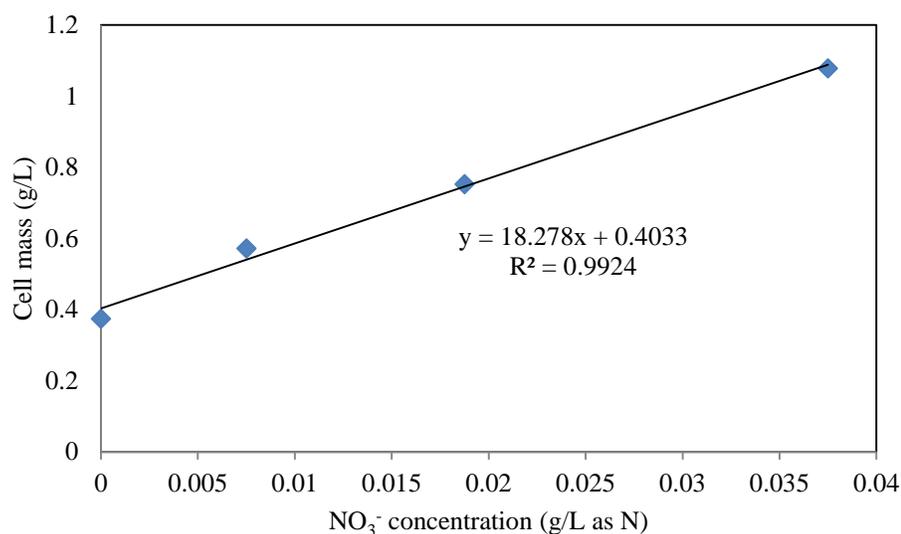


Figure 4.8. Terminal algal CDW as a function of initial nitrate concentration. The slope of the graph is about 20:1 for a nitrogen yield of 20 grams CDW/gram N consumed.

Results indicate that a nitrogen yield factor on the order of 20 g CDW/gN can be expected under conditions of nitrogen starvation. This is about the same range compared with the value predicted based on the reported stoichiometry for major constituents of algal biomass ( $C_{106}H_{263}O_{110}N_{16}P$ , Chemical formula of algae) (Masters and Ela, 2008), which leads to an estimated biomass/available nitrogen ratio of 15.8. Since we lack the

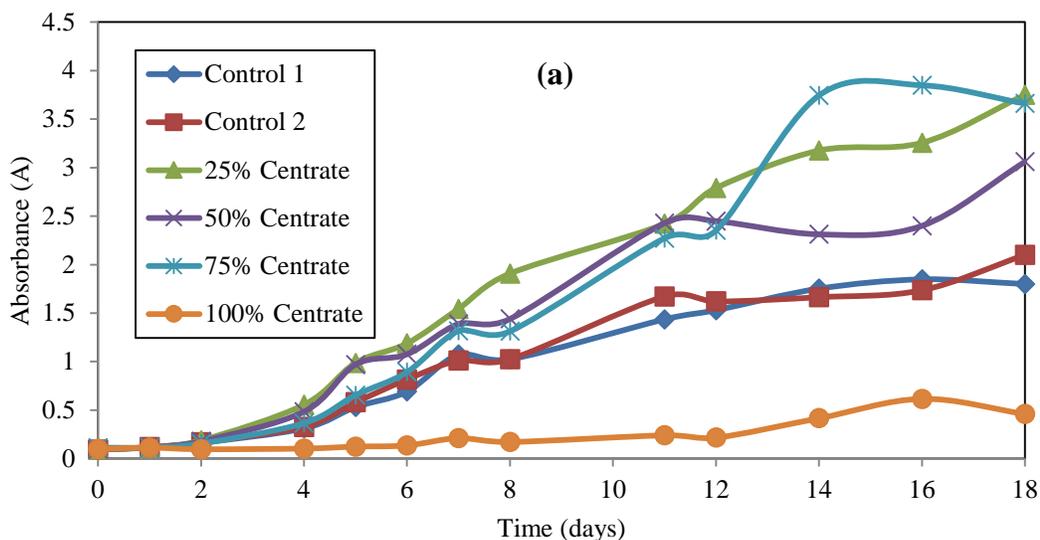
data with which to develop an empirically based yield coefficient for phosphorus, our estimate of N/P ratio for algal growth is based on the stoichiometric representation shown. Under those circumstances, the ratio of N/P demand for balanced algal growth is about 7.0 wt/wt and the phosphorus-based yield factor would be  $112 \times 7 = 784$ g CDW/g available P. These values are used subsequently to estimate the consumption of available nitrogen and phosphorus for algal production.

#### **4.6.3 Growth of microalgae on wastewater nutrients**

To determine whether *N. salina* was capable of growth without inhibition on wastewater nutrients, either effluent concentrates (as described in Section 4.5.3) or centrate produced via sludge dewatering was added to the f/2-Si medium minus the normal sources of nitrate and phosphate. Effluent or centrate fractions of the total liquid volume ranged from 0 - 100%. Assuming that effluent constituents were completely retained during the reverse osmosis concentration step, levels provided in algal cultures ranged from 0-100% of their respective levels in Tres Rios WRF effluent. Salts were added to maintain ionic strength at  $\geq 25\%$  of that in the f/2-Si medium in all cultures. Relative growth rates and depressed terminal optical densities were taken as indications of nutrient exhaustion (Figure 4.9).

The addition of centrate derived from biosolids dewatering to the standard f/2 medium (minus N and P) increased both the rate and extent of growth of *N. salina* at addition ratios ranging from 5-75% v/v (Figure 4.9a). Algal growth was inhibited only in the culture containing 100% centrate, a level that is unnecessarily high for the provision of macronutrients.

Wastewater effluent from IRWPCF proved to be much more toxic, depressing growth rates at levels of effluent constituent throughout the range of addition—as low as 10% of levels expected in effluent (Figure 4.9b). The normal growth medium with no wastewater effluent showed the best results indicating that the wastewater effluent was not a reliable source for media replacement. The higher the concentration the more toxic the wastewater effluent seems to be for the *N. salina* species. The toxicity for this wastewater effluent can be due to toxicity within the source or a lack of nutrient (shown in the microtox toxicity test). Because the level of toxicity was directly related to the level of effluent constituents in these cultures, reduction in growth was apparently due to toxicity as opposed to exhaustion of an essential nutrient.



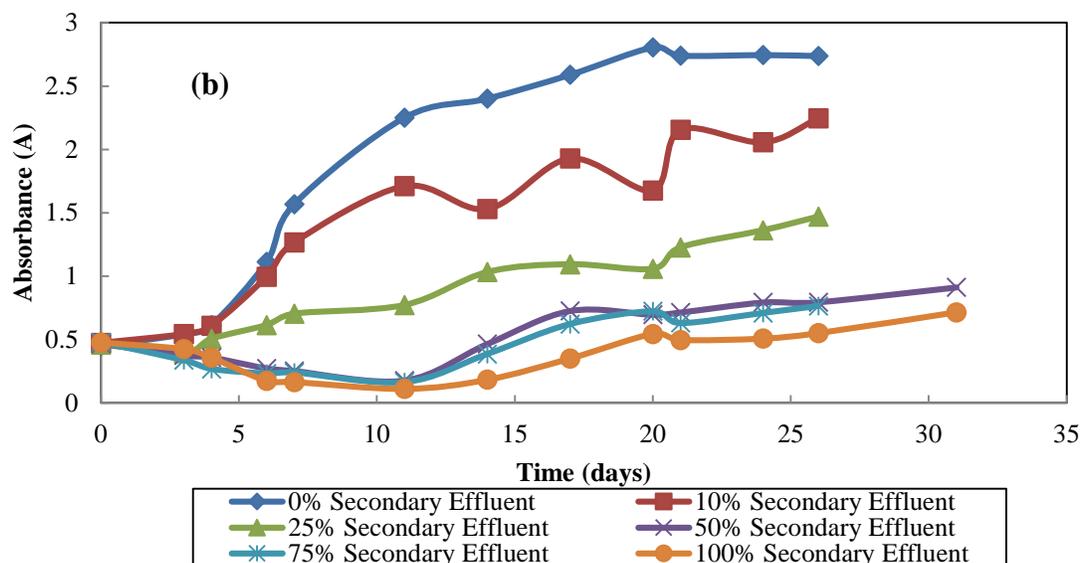


Figure 4.9. Batch growth of *N. salina* in wastewater combined with growth media. (a) f/2-Si medium supplemented with centrate derived from sludge dewatering at the Tres Rios WRF (controls represent cultures grown without centrate in the standard growth medium) and (b) mixtures of f/2-Si medium and Tres Rios WRF effluent ( $v/v$  additions as indicated).

#### 4.6.4 Microtox acute toxicity test results

For the microtox toxicity test, centrate and pre-concentrated secondary effluent from Tres Rios WRF in the algae cultivation was evaluated (Figure 4.10). Results showed that centrate was toxic to the bioluminescent marine bacterium *V. fischeri*. But the toxicity was not strong enough for getting the EC50 calculated. When removing the hydrophobic organic compounds from the centrate by using C-18 resin, the toxicity of the remaining part of the centrate disappeared. As the hydrophobic organics kept on the resin were redissolved in water (1/4.5 of the original wastewater volume), the concentration of these organic compounds increased to 4.5 fold of the original sample. The results showed that this part was toxic to the bacterial growth. It followed the same trend as the whole water sample. Considering the result, it indicates that the main responsible part for the

toxicity to algal growth was the hydrophobic organic part, which was separated out by C-18 resin, not the metals in the hydrophilic portion.

Four dilutions of the pre-concentrated secondary effluent used in the algae cultivation were also measured in the Microtox test. As described in the separation of the centrate, the secondary effluent ran through C18 resin to remove hydrophobic organic compounds, in order to compare the toxicity in the inorganic and organic part of the water. Results showed that both the whole sample and the hydrophilic part are not toxic in this assay. Hydrophobic part, which has been concentrated to 40 times from the original sample, shows toxic effect in the assay. The result showing lack of algal growth with this water source is not because of the toxicity. It may be due to the lack of nutrient instead.

Taken as a whole, Microtox test results suggest that the source of observed toxicity in the centrate and treated wastewater is hydrophobic in nature. It is possible, for example that toxicity results from interference with the chemiosmotic function of cell membranes due to the accumulation of hydrophobic uncouplers—although this remains an untested hypothesis. It is less likely that either metals or ammonium ion contributed substantially to the toxic response.

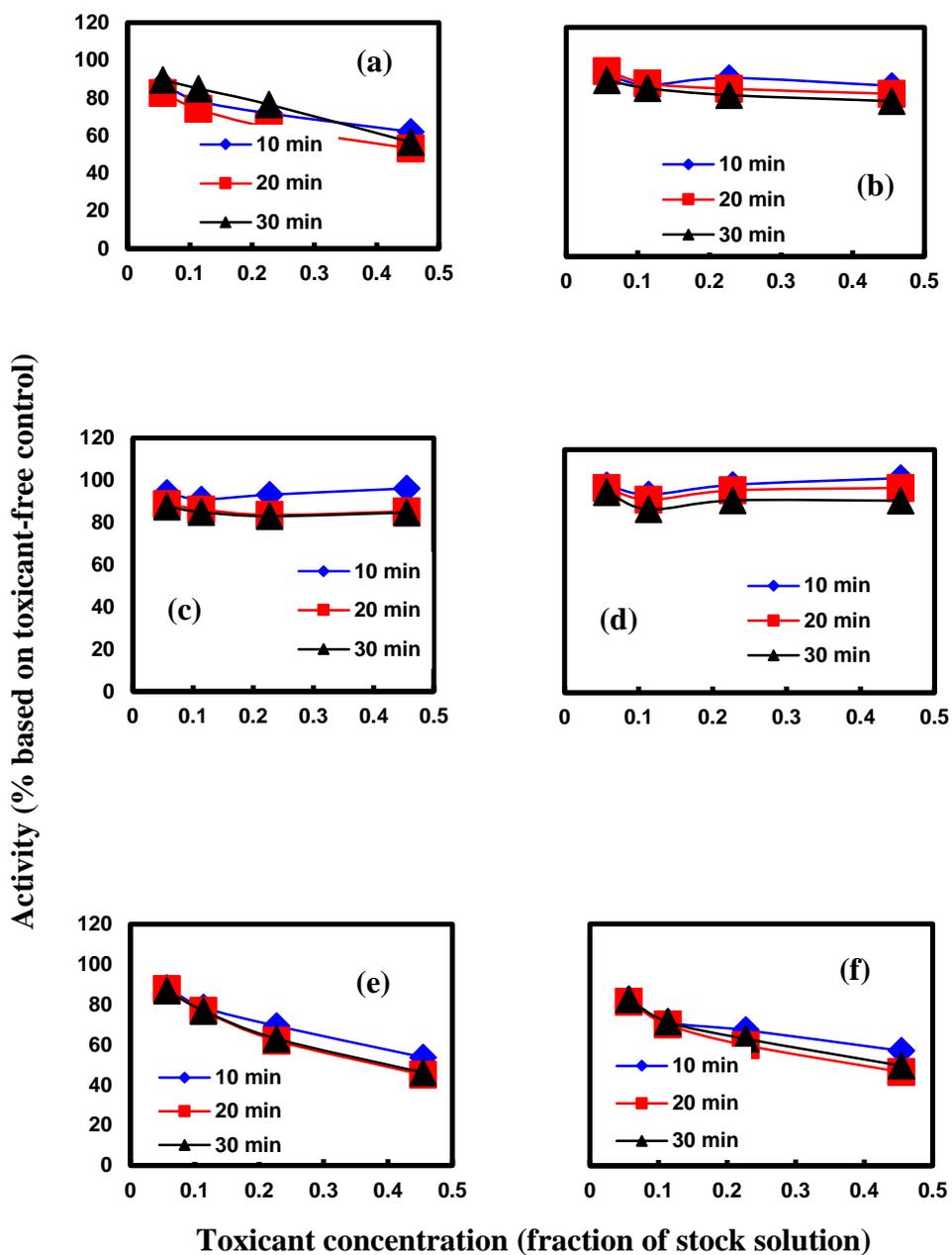


Figure 4.10. Results of Microtox tests on centrate (left) and 5 $\times$  concentrated secondary effluent (right) from Tres Rios WRF. Unfractionated samples (a and b); hydrophilic fractions (unretained on C-18 resin, c and d); and hydrophobic fractions (retained on C-18 resin, e and f). The times indicated are exposure times prior to measurement of luminescence.

#### 4.6.5 Comparison of efficiencies on lipid extraction methods

The comparison was based on FAME-derived profiles of methyl ester fatty acids following extraction and transesterification. The MAE method avoids use of dangerous solvents and numerous samples can be processed simultaneously. Among the drawbacks of the MAE method is the inability to separate lipids from other cell components during extraction. That shortcoming was circumvented here by comparing FAME profiles following extraction and transesterification. Method comparison was based on extractions of a split sample from the same batch of pilot scale photobioreactor (PBR) where fresh water algae species *Chlorella protothecoides* (UTEX 250) was growing. The esterified fatty acids were compared via GC-FID analysis of the two extracts. The MAE extraction method (in methanol) was at least as effective as the traditional chloroform extraction procedure and much less time consuming.

Traditional (multiple chloroform steps) and microwave assisted extraction procedures proved about equally capable of extracting lipids from algae (based on three traditional lipid extracted samples and four MAE samples), as evidenced by comparison of FAME profiles following esterification steps (Figure 4.11 and Figure 4.12).

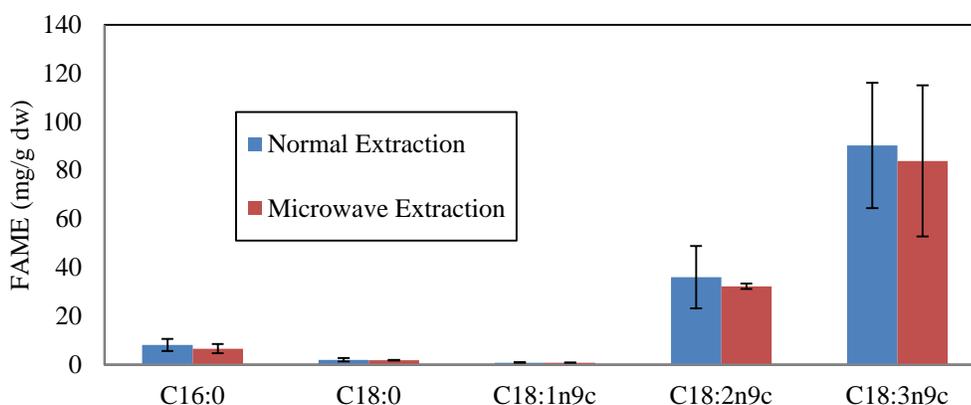


Figure 4.11 Comparison of peak areas for abundant fatty acid products of esterification reactions.

Lipid extraction from a split culture using (i) traditional chloroform extraction or (ii) a MAE protocol to separate lipids.

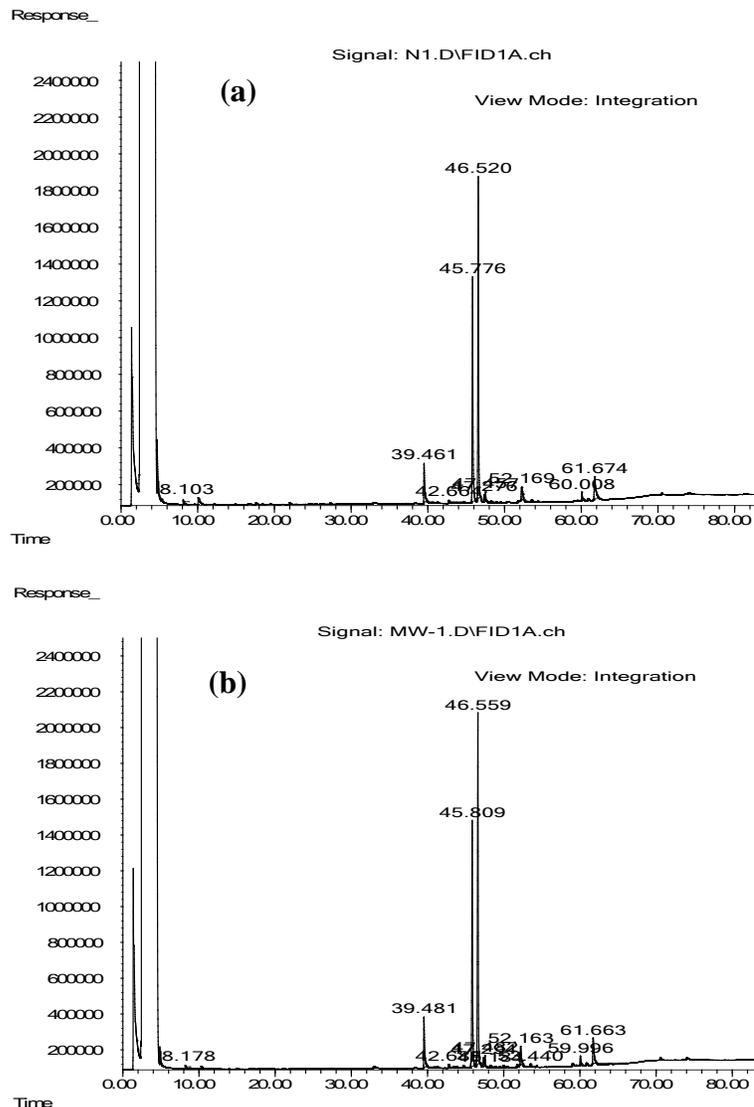


Figure 4.12. Chromatograms corresponding to FAME analyses for fatty acid content. These are from a split culture extracted in (a) chloroform using traditional way and (b) using an MAE protocol. Results are essentially identical.

#### 4.6.6 Lipid composition comparison in normal growth medium with wastewater

Since salt water algal species *N. salina* could grow better when the centrate was added to the normal f/2-Si medium, the quality of lipids produced from the algae in

centrate and the normal growth media was compared. After the microwave assisted extraction and transesterification process, the lipid extracts were injected into the GC/MS system.

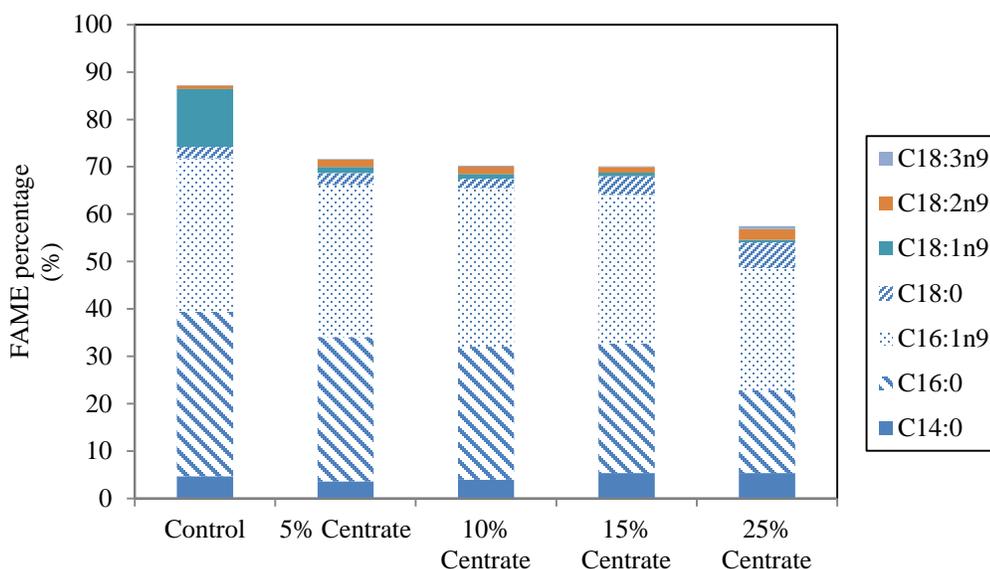


Figure 4.13. FAME profiles for *N. salina* grown in mixtures of f/2 medium and digester centrate.

All values are percentages of the total mass of fatty acid methyl esters present in transesterified whole cell extracts. Total values less than 100% indicate that some of the FAME components could not be identified using available authentic standards.

From the percentage of the each component of the FAME profile, only minor changes were apparent in the lipid compositions of the cells grown in centrate (Figure 4.13). Centrate addition at any level virtually eliminated production of fatty acid C18:1n9 and led to the production of several fatty acids that could not be identified based on retention times using the suite of authentic standards available here. In each case the main FAME components (C16:0 and C16:1n9) were the same. The result suggests that centrate can be used as a source of nutrient for algal growth.

#### 4.6.7 Sustainability issues

The algal biofuels industry cannot depend on the same regional water resources and sources of macronutrients (N, P) that are required for conventional agriculture (Clarens et al., 2010; Handler et al., 2012; Pfromm et al., 2011). The use of recycled wastewater or other impaired water resources can increase the sustainability of algal biodiesel production (Handler et al., 2012; Pittman et al., 2011), although not without limit. It is relatively easy to show that unless seawater is the source of water for microalgae growth, water reuse is requisite to an algal biofuels industry of meaningful scale. We start the analysis of algal biofuels sustainability issues by reinforcing that point.

Land requirements for algal biofuels production can be estimated from assumptions regarding microalgae productivity, mass conversions, and lipid energy content. Using Table 4.4 values and assuming that (i) lipid conversion to biodiesel is one-to-one on a mass basis and (ii) the energy contents of biodiesel and gasoline are roughly equivalent, the total pond surface required to satisfy 5% of the nation's demand for transportation fuel (33 million metric tons of petroleum per year; NRC, 2012) would be ~25,000 km<sup>2</sup>, or a land area slightly larger than Lake Erie or Maryland.

If a saltwater alga is grown for this purpose using fresh water to replace evaporative losses, recall that water consumption would be about equal to the net rate of evaporative loss. A representative net evaporation rate in the semiarid Southwest is ~1.5 m/yr, so that the annual rate of water loss/replacement would be ~37.5 km<sup>3</sup> per year (32 million acre feet per year) or about 2× the average flow in the Colorado River. Use of a freshwater alga for the same purpose would require substantially more water to maintain

TDS concentrations within tolerable limits for growth. The use of water on that scale in the Southwest can be ruled out unless a previously untapped water resource can be identified for which there is no higher use than biofuels development. Brackish groundwater, for example, might serve that purpose in the Southwest, although the magnitude and distribution of that resource is not completely known. More likely, a large-scale biofuels industry must be located in an area that is less water stressed or where seawater can be tapped as a water source.

Table 4.4. Effluent and nutrient needs for algal biodiesel production to satisfy 5% of annual US demand for transportation fuels

<b>a. Assumptions</b>		
<b>Parameters</b>	<b>Numbers</b>	<b>References</b>
Petroleum demand in the U.S.	965 million MT/year	(U.S. Department of Energy)
Petroleum demand for transportation	643 million MT/year	= 2/3 Petroleum demand
5% of petroleum demand for transportation (arbitrary)	33 million MT/year	calculation
Algal production	10 g CDW/m <sup>2</sup> day	(Shen et al., 2009)
Lipid yield	33% of algal CDW	(Pal et al., 2011)
Nitrogen yield	20 g CDW/g N	(empirical)
Phosphorus yield	140 g CDW/g P	7 times N (Masters and Ela, 2008)
Nitrogen concentration in Wastewater	30 mg /L	(Metcalf & Eddy, 2003)
Phosphorus concentration in Wastewater	3 mg /L	(Metcalf & Eddy, 2003)
Per capita wastewater production rate	100 gal /day	(U. S. EPA)
Tucson net water evaporation Rate	1.53 m/year	(AZ Water)
<b>b. Calculations</b>		
Algae biomass demand for 5% transportation fuel	100 million MT / year	calculated
Area demand	26,700 km <sup>2</sup> 6.60 million acres	= biomass demand / algal productivity
Water demand (evaporation)	33.0 million acre-ft / year	= area × net evaporation rate

Nitrogen demand	5.0 million MT / year	= biomass demand / nitrogen yield
Wastewater demand for nitrogen	120 billion gallons / day	= nitrogen demand / N conc. in wastewater
Population for nitrogen demand	1.20 billion per capita equivalent	= wastewater demand for nitrogen / wastewater produced per capital
Phosphorus demand	0.70 million MT/ year	= biomass demand / phosphorus yield
Wastewater demand for phosphorus	168 billion gallons/ day	= phosphorus demand / P conc. in wastewater
Population for phosphorus demand	1.68 billion per capita equivalent	= wastewater demand for phosphorus / wastewater produced per capita

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Nitrogen and phosphorus requirements can be approached similarly—i.e., by estimating the N, P requirements for sufficient microalgae to satisfy 5% of the nation’s requirements for transportation fuels and then translating those requirements to equivalent flows of municipal wastewater effluent. Per Table 4.4, the nitrogen and phosphorus requirements would be ~6 million MT/yr and ~1 million MT/yr, respectively. If these are provided from wastewater without nutrient recovery and reuse, nutrient demands translate to a wastewater flow of  $1.90 \times 10^6$  AFY or roughly 5× the municipal wastewater created by the entire population of the United States. It is apparent that nutrient recovery and reuse will be necessary. That conclusion is supported by the National Research Council (2012) which stated “...with current technologies, scaling up production of algal biofuels to meet even 5% of U.S. transportation fuel needs could create unsustainable demands for energy, water, and nutrient resources...”

Taking this one step further, it follows that energy will have to be invested in order to accelerate nutrient recovery and recycle from lipid extracted algae. It is first postulated that nutrients can be recovered from lipid extracted algae via anaerobic digestion. Early experiments (not shown) indicate that cell disruption via sonication

enhances nutrient (and methane) recovery during the bench-scale digestion starting with intact microalgae. Here we calculate an energy input that might be acceptable for the purpose of cell disruption. The data and assumptions used follow:

- The ratio of CDW/N = 20 (wt/wt) and CDW/P = 143 among intact microalgae.
- Cell lipid content is 33 percent.
- Dewatering produces a sidestream containing algae for digestion that is 2% (dry) solids, half of which is carbon.

The analysis is arbitrarily based on a biomass production rate of 1 MT/day, so that the corresponding volume rate of flow to the hypothetical digester is 50 m<sup>3</sup>/day, and the lipid production rate is 333 kg lipid/day. If the energy density in algal biofuel is equivalent to that of gasoline ( $44.4 \times 10^6$  J/kg), the available energy from 1 MT/d of microalgal growth would be  $333 \times 44.4 \times 10^6 = 14.8 \times 10^9$  J/d, a portion of which could be used for cell disruption for nutrient and energy recovery in a sustainable biofuels industry. Here we assume that the allowable energy expense for that purpose is just 10% of the energy liberated, or  $1.48 \times 10^9$  J/d. Others have assumed that the energy return on investment must be  $\geq 3$  for fuel resource sustainability (NRC, 2012).

To add perspective, normalizing this rate of energy input to the 50 m<sup>3</sup>/d of water that must be subjected to some form of treatment shows that  $1.48 \times 10^9$  J/d would be sufficient to heat water at that flow rate by only about 7°C. Methods that require or result in excessive heating are clearly infeasible. Whether or not a method can be developed to efficiently recover nitrogen and phosphorus from lipid extracted algae with this sort of energy input remains to be determined.

## 4.7 Conclusions

*Nannochloropsis salina*, a saltwater species selected on the basis of its growth rate and over-production of lipid, readily tolerates metals at levels present in Tucson area wastewater. Among the metals present at relatively high concentration, including zinc, copper, cobalt, lead and nickel, zinc was the primary source of observed toxicity in combinations of metals concentrated beyond those levels present in wastewater effluent. Nevertheless, the zinc EC<sub>50</sub> for *N. salina* grown on normal growth media was ~100× the level normally found in treated Tucson municipal wastewater. Zinc was no more toxic to *N. salina* grown in continuous culture than it was in batch growth experiments. Metal toxicity tests results suggest that wastewater metals will not degrade algal growth rates, at least for metals tolerant species, at reasonable levels of accumulation that will follow from water recycling in the biofuels industry. Metals toxicity experiments were successfully conducted in 96-well plates, potentially increasing the convenience of toxicity experiments of this type.

This research program described has shown that the economic and environmental sustainability of a meaningful algal biofuels industry requires use of CO<sub>2</sub> and fertilizer nutrients that are not derived from fossil fuels and which do not reduce the availability of fertilizer for agriculture. Recycling water or using otherwise impaired water can further increase the sustainability of biodiesel production from algae. One kg of biodiesel requires 3726 kg water, 0.33 kg nitrogen, and 0.71 kg phosphate if freshwater is utilized (Yang et al., 2011). Therefore, the use of wastewater as the source of water and nutrients is requisite to the development of algal biofuel technology in Arizona and other parts of the semiarid Southwest. Straightforward calculations indicate that without nutrient

recovery and reuse, the supply of municipal wastewater cannot satisfy large scale biofuel nutrient requirements. In their recent report entitled *Sustainable Development of Algal Biofuels in the United States* (2012) the National Research Council of the National Academies concluded: “...with current technologies, scaling up production of algal biofuels to meet even 5% of U.S. transportation fuel needs could create unsustainable demands for energy, water, and nutrient resources...” Identification of alternative water and nutrient sources is necessary to make algal biofuels a sustainable energy resource. Municipal wastewater is among the most promising sources of water and nutrients (nitrogen, phosphorus, and trace elements) for algal growth. However, annual production of 39 billion liters of algal biofuel, which is equivalent to 5% of annual U.S. demand for transportation fuels, requires at least 123 billion liters of water, 6 million metric tons of nitrogen (N) and 1 million metric tons of phosphorus (P). Without recycling, it would take over 1×, 4×, and 5× the entire US population, respectively, to generate sufficient wastewater to provide that much water, N and P. Therefore, nutrient and water recycling/reuse became fundamentally critical for microalgae to be a sustainable energy source.

This project has led to the following preliminary conclusions:

- The saltwater alga *N. salina* grows well in mixtures of growth media (minus macronutrients) and either treated municipal wastewater or centrate derived from the dewatering of digested sludge. However, secondary effluent wastewater content > 50% v/v wastewater was detrimental to algal growth, as was centrate at > 75% v/v. The cells can satisfy their requirements for nitrogen, phosphorus and other nutrients from either source.

- Metals in treated wastewater are unlikely sources of growth inhibition at the concentrations initially present. Metals accumulation due to wastewater recycling and evaporation could become a problem at very high recycle ratios, on the order of 0.98-0.99. However, the statement may belong to one of the more metals tolerant algal species, *N. salina*.
- Although treated wastewater or nutrient rich sidestreams derived from that treatment may be well suited as a source of water and nutrient for algal growth, in the American Southwest and probably throughout the world, there is simply not enough municipal wastewater to satisfy the water, nitrogen and phosphorus demands of an algal biofuels industry of virtually any meaningful scale. Nutrient requirements can be provided from wastewater, however, if nutrients are efficiently recovered from lipid extracted algae. If a lipid based biofuel product is rejected in favor of another form of energy recovery from algae such as methane production from biomass, the nutrient recovery objective will still have to be satisfied.
- Based on these results, it seems that any strategy to satisfy a meaningful fraction of transportation energy demand from algal biofuels will depend on identification of a massive, underutilized water source—probably seawater—and a source of nutrients that does not result in competition with traditional agriculture for fertilizer. Nutrient-rich streams derived from the treatment of wastewater can provide such a source if and only if nutrients are effectively recovered and reused following biofuel generation.

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## CHAPTER 5 ROLE OF AMMONIA AND CARBONATES IN SCAVENGING HYDROXYL RADICALS GENERATED DURING MEGASONIC IRRADIATION OF WAFER CLEANING SOLUTIONS

### 5.1 Introduction

Particulate contamination is a major cause for yield loss in integrated circuit (IC) manufacturing industry, and it has become a concern in the semiconductor chip manufacturing process (Kern, 1990). The removal of particulates and residues from wafer and photomask surfaces is typically achieved through liquid chemical formulations aided by a sound field in the MHz frequency range (Keswani et al., 2009).

During acoustic irradiation of liquid during megasonic cleaning process, two types of mechanisms are known to occur, namely acoustic streaming and acoustic cavitation (Gale and Busnaina, 1999). Acoustic streaming refers to time independent motion of liquid due to viscous attenuation. In acoustic cavitation, air bubbles can be formed during rarefaction cycles of the sound wave either continuously oscillate over many cycles (stable bubbles), or they can grow in size and collapse in a few cycles eventually (transient bubbles). The flow generated from stable bubbles is very important for particulate removal in the megasonic cleaning process, while transient bubbles are believed to be responsible for damages on the surface of the wafer. It is known that high temperature of a few thousand degree can be reached inside the bubble during its collapse, excitation of various species, as well as the formation of radicals, such as hydroxyl radicals ( $\bullet\text{OH}$ ) occurs at the same time (Keswani et al., 2014; Kohno et al., 2011).

It has been reported that dissolved gases play an important role in controlling the physical and chemical properties of the oscillating acoustic cavitation bubbles (Kang et al., 2014; Rooze et al., 2013). And the most important factors which determine the cavitation intensity are the type and concentration of gas dissolved in water. The intensity of sonoluminescence (SL), which is the phenomenon of light release when liquid is irradiated by sound waves of sufficient intensity, is a sensitive indicator of cavitation events (Gale and Busnaina, 1999; Kumari et al., 2011a). It has been used widely as a good indicator for the extent of feather damages on patterned wafers. The intensity of SL in noble gas saturated water was studied by Didenko et al. (2000). They found that at a given power density (20 kHz and 70 W/cm<sup>2</sup>), SL signal followed the sequence of Xe>Kr>Ar>Ne>He. Kang et al. (2014) studied the change of acoustic bubble growth rate in DI water at 0.83MHz by three dissolving gases, and the results suggest that the particulate removal efficiency on the Si wafer and the damage to the photoresist (PR) line patterns were related to the gas bubble growth rate (H<sub>2</sub>>N<sub>2</sub>>Ar), among which the transient cavitation behavior can be reduced by Ar gas due to its lower acoustic bubble growth rate under such condition.

Until recently, carbon dioxide (CO<sub>2</sub>) was found to be an exceptional gas which can suppress the intensity of SL (Kumari et al., 2011a) while most other gases help increase SL signal. It has been discovered that increasing concentration of CO<sub>2</sub> (aq) in air saturated DI water not only made a significant decrease in the number of breakages to line structures on wafer during megasonic cleaning process, but also decreased lengths of the line breakages significantly, at all power densities up to 2.94 W/cm<sup>2</sup> (Kumari et al., 2011b). Also the ability of dissolved CO<sub>2</sub> to protect against feature damage correlated

well with its ability to suppress SL in ultrasound irradiated DI water. Carbon dioxide in the form of  $\text{CO}_2$  (aq) is not only incapable of SL generation, but also a potent inhibitor of SL generation when comparing with other gases (Ar, Air,  $\text{O}_2$ ,  $\text{N}_2$ ). A novel chemical method has been established based on the above results for *in situ* release of  $\text{CO}_2$  from  $\text{NH}_4\text{HCO}_3$  in the carbonate/bicarbonate equilibration in deionized water through pH shift (Kumari et al., 2011a), which suggested that about 130 ppm of  $\text{CO}_2$  (aq) generated by  $\text{NH}_4\text{HCO}_3$  was sufficient for a fully suppression of SL generation in water under all tested conditions of power density and duty cycle of acoustic energy at the frequency 0.93 MHz. A further step for the research has been taken for the suppression of transient cavitation by adding  $\text{NH}_4\text{HCO}_3/\text{NH}_4\text{OH}$  into megasonic cleaning system (Kumari et al., 2014), and a novel means for controllable generation of  $\text{CO}_2$ (aq) over an extended pH range (4.0–8.5) has been proved to be beneficial to the suppression of SL signal, which can be applicable in reducing wafer damage without compromising megasonic cleaning efficiency.

Nowadays, several methods are being used for characterizing the intensity of transient cavitation, which include the detection the SL signal, potassium iodide or ferrous ions dosimetry, electron spin resonance spectroscopy and fluorescence spectrometry (Kohno et al., 2011; Rooze et al., 2013). A more applicable technique is that radicals being trapped by other species, and converted into species which can be detectable by fluorescence spectroscopy. Terephthalate dosimeter is one of such systems which can be applied in the measurement of hydroxyl radicals in the ultrasound field. This method was originally developed for radiochemical work (Armstrong et al., 1963), and then was being widely used for the detection of radical production in other area, such

as medical ultrasound field (Kohno et al., 2011; Price et al., 1997). The ferrous ion system was found to be useful at higher concentrations, whereas at lower concentration of hydroxyl radicals, they can be accurately monitored by the terephthalate dosimeter (Price et al., 1997). The suitability of terephthalate dosimeter for the measurement of concentration of hydroxyl radicals generated in biologically relevant reactions was examined by Barreto et al. (1994). Recent study using terephthalate dosimeter technique on the effect of various solution parameters on hydroxyl radical generation rate in alkaline cleaning solutions of the semiconductor industry has been investigated (Keswani et al., 2014). It has been proved that the generation rate of hydroxyl radicals can be measured by the formation of 2-hydroxyterephthalic acid in alkaline aqueous solutions in the pH range of 7.2–11.2 as a function of transducer power density, solution temperature and pH and type of alkali used. Greater generation rate of radicals were related to higher power density and higher bulk solution temperature. It showed higher radical generation rate in Ar saturated solution than air saturated solution, while the concentration of  $\text{OH}\cdot$  generated from the  $\text{CO}_2$  saturated solution and ammonium solution (pH 11.2) was almost zero.

In reality, chemical formulations used for megasonic cleaning process typically contain hydroxides, peroxides, carbonates and others which can affect particle removal efficiency and feature damage. This paper is focused on using the detection method of terephthalate dosimeter, in order to investigate the role of carbonate/bicarbonate system generated from different chemical combination in regulating the oxidation power of megasonic cleaning solutions through the scavenging of hydroxyl radicals.

## 5.2 Materials and methods

All solutions were prepared using high resistivity de-ionized water (18 M $\Omega$ -cm). Ammonium carbonate, sodium carbonate, sodium hydroxide, potassium ferricyanide and potassium chloride of purity greater than 99.9 % were purchased from Fisher Scientific Inc. Terephthalic acid and 2-hydroxy terephthalic acid greater than 99.9 % purity was procured from Sigma Aldrich Inc. Ammonium hydroxide (29%, VLSI grade) was purchased from Honeywell Inc. Megasonic experiments were performed in Mini-meg<sup>®</sup> tank (PCT Systems Inc.) of volume ~ 4.5 liters and consisting of 125 cm<sup>2</sup> transducer affixed at the bottom with an operating frequency of 1 MHz (Keswani et al., 2014) and power density range of 0.1-8 W/cm<sup>2</sup>. Hydroxyl radical ( $\bullet$ OH) measurement experiments were conducted in alkaline solutions (pH adjusted using NH<sub>4</sub>OH or NaOH) containing 75  $\mu$ M terephthalic acid (TA) for fluorescence spectroscopy detection (FluoroMax 4, Horiba Inc.). The solutions were stirred for 30 minutes prior to the addition of NH<sub>4</sub>HCO<sub>3</sub>/NaHCO<sub>3</sub> salts. The alkaline pH of the solution was necessary to achieve complete dissolution of TA. The terephthalic acid reacts with the generated  $\bullet$ OH (due to megasonic field) to form 2-hydroxyterephthalic acid which undergoes emission (425 nm) when excited at a wavelength of 318 nm. Experiments were conducted over a period of 3 minutes during which the concentration of hydroxyl radicals (measured every 30 s) increased linearly with time. Solutions of varying concentrations (0.1-25  $\mu$ M) of 2-hydroxyterephthalic acid were used to obtain the calibration curve of fluorescence intensity versus concentration of 2-hydroxyterephthalic acid, which was later used to calculate the amount of  $\bullet$ OH released during megasonic exposure. All hydroxyl radical

measurement experiments were conducted using air saturated solutions unless stated otherwise.

Chronoamperometry measurements were conducted in argon saturated solutions containing 50 mM  $K_3[Fe(CN)_6]$  and 100 mM KCl in the absence and presence of  $NH_4OH$ . Microelectrode was used as a sensor to characterize cavitation behavior. The microelectrode setup consisted of three electrodes, working (25  $\mu m$  diameter Pt disc), reference and counter (500  $\mu m$  diameter Pt wires). Details of this set up are available elsewhere (Keswani et al., 2013). During the experiments, the working electrode was maintained at a constant potential of -0.6 V (versus Pt ref.) by means of Gamry Interface 1000 potentiostat and current was measured as a function of time in the absence and presence of megasonic field at a sampling rate of 1000 samples/s.

### **5.3 Results and discussion**

#### **5.3.1 Effect of ammonia, bicarbonates and carbonates on scavenging of hydroxyl radicals**

This section describes the effect of added constituents like carbonates, bicarbonates and ammonia on the  $OH^\bullet$  scavenging rate. Preliminary mass and charge balance calculations were done to determine the equilibrium concentrations of both the dissociated and dissolved species. Table 5.1 shows the equilibrium concentrations in solutions containing varying concentrations of  $NH_4HCO_3/NH_4OH$ ,  $NaHCO_3/NaOH$  and  $NaOH$ . The pH of the solutions typically varies between 8.6-12.4 depending upon the chemical formulation. Hydroxyl radical measurements were conducted under the conditions given in the table, the results of which are discussed later. The reason behind using solutions of  $NH_4HCO_3/NH_4OH$  and  $NaHCO_3/NaOH$  was to eliminate the effect of

ammonia scavenging in the presence of carbonates/bicarbonates. It could be seen that for a particular chemical formulation (NaHCO<sub>3</sub>/NaOH) as the pH increased from 8.6-12.4, the concentration of carbonate in the solution also increased.

Table 5.1 Theoretical calculations of release of different ions into air saturated solutions of varying concentrations of ammonia and carbonates (All concentrations are in mM and partial pressures are in atmospheres)

Type	pH	NH <sub>4</sub> OH	NH <sub>4</sub> <sup>+</sup>	HCO <sub>3</sub> <sup>-</sup>	CO <sub>3</sub> <sup>2-</sup>	H <sub>2</sub> CO <sub>3</sub>	CO <sub>2</sub> aq.	NH <sub>3</sub> (gas)	CO <sub>2</sub> (gas)
0.2M NH <sub>4</sub> HCO <sub>3</sub> , 40mM NH <sub>4</sub> OH	8.8	0.038	0.2	1.95E-1	3.7E-3	2.3E-6	1.4E-3	6.3E-4	4E-2
Type	pH	NaOH	Na <sup>+</sup>	HCO <sub>3</sub> <sup>-</sup>	CO <sub>3</sub> <sup>2-</sup>	H <sub>2</sub> CO <sub>3</sub>	CO <sub>2</sub> aq.	NH <sub>3</sub> (gas)	CO <sub>2</sub> (gas)
0.2M NaHCO <sub>3</sub> , 7.3mM NaOH	8.6	0	0.207	1.9E-1	4.3E-3	1.9E-6	1.1E-3	0	3.2E-2
0.2M NaHCO <sub>3</sub> , 0.2M NaOH	10.4	0	0.4	0.82E-1	1.2E-1	1.32E-8	7.8E-6	0	2.3E-4
0.2M NaHCO <sub>3</sub> , 0.33M NaOH	12.4	0	0.53	1.4E-3	2E-1	2.2E-12	1.3E-9	0	3.8E-8
25mM NaOH	12.3	0	0	2.2E-5	2.4E-3	4.3E-14	2.5E-11	0	7.4E-10

In their previous work, Keswani et al. have demonstrated the effect of ammonia concentration on the rate of generation of hydroxyl radicals in aqueous solutions subjected to megasonic field (Keswani et al., 2014). It was shown that at an acoustic frequency of ~ 1 MHz and power density of about ~ 2 W/cm<sup>2</sup>, as the concentration of ammonia was increased from about 1:100000 to 1:100 (NH<sub>4</sub>OH(29%):H<sub>2</sub>O by volume) in air saturated solutions, the generation rate of •OH reduced significantly from about 0.17 μM/min to almost zero. This reduction in the •OH generation was attributed to the scavenging of hydroxyl radicals by ammonia (•OH + NH<sub>3</sub> = •NH<sub>2</sub> + H<sub>2</sub>O) and a NH<sub>3</sub>(aq.) concentration of 75 mM was found to be sufficient for complete scavenging of •OH. Interestingly, in 1:10000 ammonia solutions when air was replaced with carbon dioxide gas (Balachandran et al., 2014), the measured •OH generation rate dropped from 0.12 μM/min to zero even though NH<sub>3</sub> (aq.) concentration was much lower (few tens of nM)

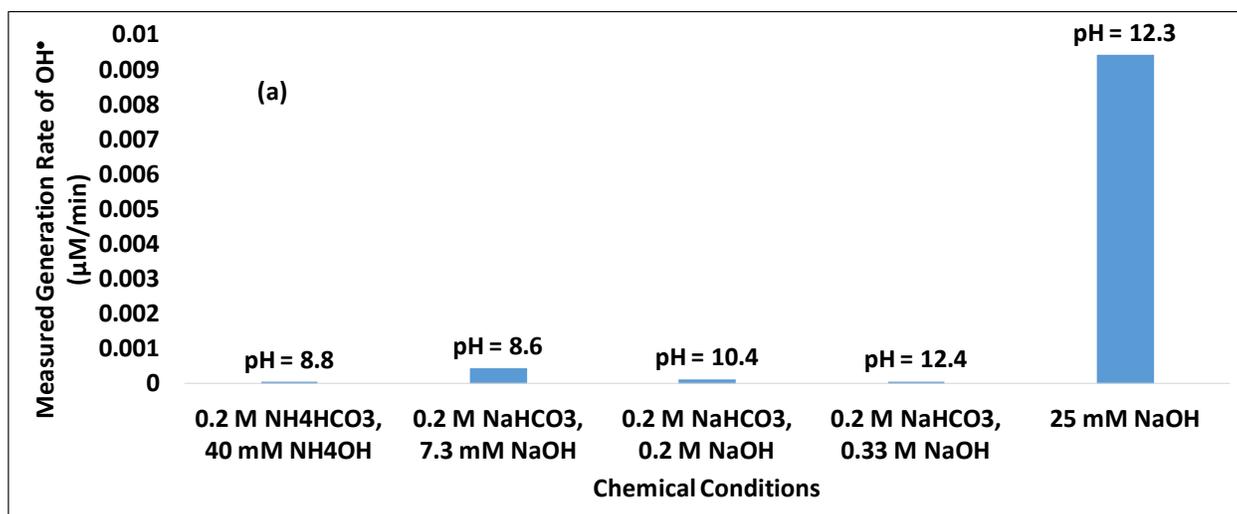
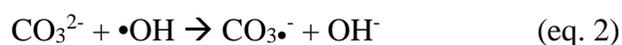
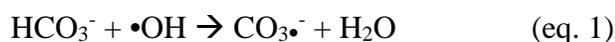
in these solutions. It may be noted that CO<sub>2</sub> saturated 1:10000 ammonia solutions contain CO<sub>2</sub> (aq.), HCO<sub>3</sub><sup>-</sup>(aq.) and CO<sub>3</sub><sup>2-</sup>(aq.) ions, in the concentration of 35 mM, 0.7 mM, and 1.7 nM, respectively (calculated using equilibrium and charge conservation equations). It is known that dissolved carbon dioxide in the form of CO<sub>2</sub> (aq.) can reduce the generation of OH• due to lower  $\gamma$  (polytropic index) value of CO<sub>2</sub> compared to air that results in lower transient bubble temperature during collapse.

Bicarbonate and carbonate ions, when present in appreciable concentrations, can also scavenge hydroxyl radicals, which can reduce the measured rate of generation of OH•. In order to investigate the role of these ions in scavenging of OH•, experiments were conducted using ammonium or sodium bicarbonate solutions with varying concentrations of NH<sub>3</sub>(aq.), CO<sub>2</sub>(aq.), HCO<sub>3</sub><sup>-</sup>(aq.) and CO<sub>3</sub><sup>2-</sup>(aq.) achieved by adjusting the pH of the solutions using ammonium hydroxide or sodium hydroxide. Figure 5.1a and (b) show the rate of generation of •OH in ammonium hydroxide and sodium hydroxide solutions of different total carbonate levels and pH exposed to ~ 1 MHz field at two different power densities of 2 and 8 W/cm<sup>2</sup> respectively. The desired carbonate levels were achieved by addition of ammonium bicarbonate or sodium bicarbonate to the solutions. The concentration of various species present in these solutions is displayed in Table 5.1. It may be noted from Figure 5.1a that in 0.2 M NH<sub>4</sub>HCO<sub>3</sub>/40 mM NH<sub>4</sub>OH solution at pH 8.8 where the NH<sub>3</sub>(aq.) and CO<sub>2</sub>(aq.) concentrations were calculated to be about 38 and 1.4 mM respectively, the measured rate of •OH generation was negligible. The concentrations of NH<sub>3</sub>(aq.) and CO<sub>2</sub>(aq.) in this solution was lower than that in NH<sub>4</sub>OH<sub>(aq.)</sub>/air<sub>(g)</sub> and NH<sub>4</sub>OH<sub>(aq.)</sub>/CO<sub>2(g)</sub> solutions, respectively, where complete OH• scavenging was observed. However, the bicarbonate/hydroxide solution contains

substantial levels of bicarbonate ions (~ 200 mM), which is likely contributing to significantly reduced concentration of  $\bullet\text{OH}$ . To confirm this effect, further experiments were conducted using sodium bicarbonate solutions of varying pH (8.6-12.4) where no aqueous ammonia was present and  $\text{CO}_2$  (aq.) concentrations were very small (nanomolar to millimolar). The pH of these solutions was adjusted using sodium hydroxide solutions of concentrations in the range of 7.3-330 mM. At moderately alkaline pH (~8.6), most of the carbonate ions are in the form of  $\text{HCO}_3^-$  while as the pH increases, the ratio of bicarbonate to carbonate ions decreases and at the experimental pH of 12.4, almost all of the bicarbonate ions are deprotonated to carbonate ions. This is reflected in Table 5.1, where the concentration of bicarbonate and carbonate ions in the sodium bicarbonate solutions of pH values 8.6, 10.4, 12.4 was calculated to be 190, 82, 1.4 mM and 4.3, 120, 200 mM respectively. The results in Figure 5.1 indicate that the measured rate of generation of  $\bullet\text{OH}$  in sodium bicarbonate solutions was lower than  $0.001 \mu\text{M}/\text{min}$  clearly suggesting that bicarbonate and carbonate ions act as good scavengers of hydroxyl radicals. In order to ensure that the scavenging effect was not related to the high alkaline pH of these solutions, hydroxyl radical measurements were also conducted in 25 mM sodium hydroxide solution of pH 12.3, which did not contain any ammonia or carbonates. The rate of generation of  $\bullet\text{OH}$  in this solution was measured to be significant (~ 0.16  $\mu\text{M}/\text{min}$ ) and much higher than bicarbonate based solutions ruling out any possibility of the role of alkaline pH in reducing the concentration of  $\bullet\text{OH}$ .

At higher power density of  $8 \text{ W}/\text{cm}^2$ , the results of which are illustrated in Figure 5.2b, the rate of generation of  $\bullet\text{OH}$  remains low (~ $0.001 \mu\text{M}/\text{min}$ ) for ammonium bicarbonate/ammonium hydroxide solutions suggesting that the combined concentration

of  $\text{NH}_3(\text{aq.})$  and  $\text{HCO}_3^-$  in these solutions (38 and 195 mM respectively) is sufficient to completely scavenge the hydroxyl radicals. This was also the case for sodium bicarbonate/sodium hydroxide solutions (pH = 12.4) where significant concentration (>200 mM) of carbonate ions was present indicating that these ions by themselves can act as effective  $\bullet\text{OH}$  scavengers when available in reasonable concentrations. Interestingly, in sodium bicarbonate/sodium hydroxide solutions of pH 8.6, the concentration of  $\text{HCO}_3^-$  was 190 mM and the rate of  $\bullet\text{OH}$  generation was 0.014  $\mu\text{M}/\text{min}$ . This  $\bullet\text{OH}$  rate was more than an order of magnitude higher than that seen in solutions of pH of 12.4. These results indicate that the carbonate ions are likely better scavengers of hydroxyl radicals than the bicarbonate ions. Possible scavenging reactions of bicarbonates and carbonates with  $\bullet\text{OH}$  are shown below as equations 1 and 2 (Adams et al., 1965; Buxton and Elliot, 1986). The reaction rate constant for the carbonate reaction ( $2\text{-}4.2 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ ) was observed to be higher than that for bicarbonates ( $8.5 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ ) (Adams et al., 1965; Buxton and Elliot, 1986), thereby showing that the results are in line with published data.



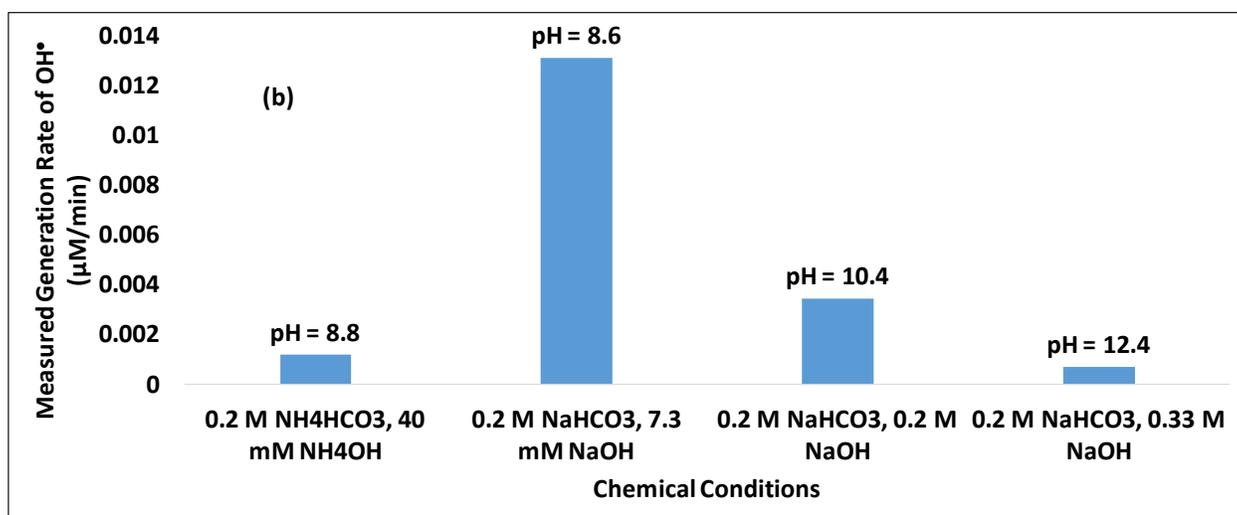


Figure 5.1 Rate of generation of hydroxyl radicals for different bicarbonate formulations subjected to an acoustic field at a frequency of 1 MHz and power density of (a) 2 W/cm<sup>2</sup> and (b) 8 W/cm<sup>2</sup>

### 5.3.2 Effect of ammonia on cavitation behavior

The effect of added NH<sub>4</sub>OH was further investigated by means of using a microelectrode. Microelectrode setup was configured to detect mass transfer phenomena that results from transient cavitation. As shown in Figure 5.2, chronoamperometry measurements were conducted both in the presence and absence of added NH<sub>4</sub>OH (1:100 NH<sub>4</sub>OH(29%):DI by vol.) at an acoustic frequency of ~ 1 MHz and power density of 8 W/cm<sup>2</sup>. Experiments were performed in argon saturated solutions as cavitation is more pronounced under these conditions (Keswani et al., 2013) and also, the effect, if any, of the added NH<sub>4</sub>OH could be explicitly observed. As seen from the Figure 5.2 (a) and (b), in the absence of an acoustic field, a baseline current of about -0.45 μA was observed in both cases. In the presence of an applied megasonic field and in solutions containing no added NH<sub>4</sub>OH (Figure 5.2a), several (about 8 or so) discrete current peaks of magnitude greater than ~ 1 μA were observed. These peaks occur as a result of collapse of transient cavities (Keswani et al., 2013). Figure 5.2b shows the cavitation behavior in the presence

of 1:100  $\text{NH}_4\text{OH}$  solution and clearly indicates a significant reduction in number of transient cavities in the presence of an applied acoustic field. The presence of  $\text{NH}_4\text{OH}$  could result in two effects, namely, (1) cushioning of transient cavitation by means of dissolved  $\text{NH}_3$  gas present in solution and (2) scavenging of  $\bullet\text{OH}$  by  $\text{NH}_3$ . The solubility of  $\text{NH}_3$  in water at  $25^\circ\text{C}$  is about 500,000 ppm (Sherwood, 1925) while that for argon is about 60 ppm thereby suggesting that ammonia could enter the cavity and act as a suppressor for cavitation. A similar effect was observed in the case of solutions saturated with  $\text{CO}_2$  (solubility = 1500 ppm) where the transient cavitation was almost absent (Keswani et al., 2013). Another important factor while quantifying transient cavitation is the polytropic index ( $\gamma$ ) of the gas present in the solution. In the case of  $\text{NH}_3$ , the polytropic index is about 1.3 (same as  $\text{CO}_2$ ), thereby indicating the possibility of weaker collapses in the presence of  $\text{NH}_3$ . Therefore, in the presence of  $\text{NH}_4\text{OH}$ , there is a significant suppression of transient cavitation, which could be reducing the generation of hydroxyl radicals in addition to their scavenging by ammonia.

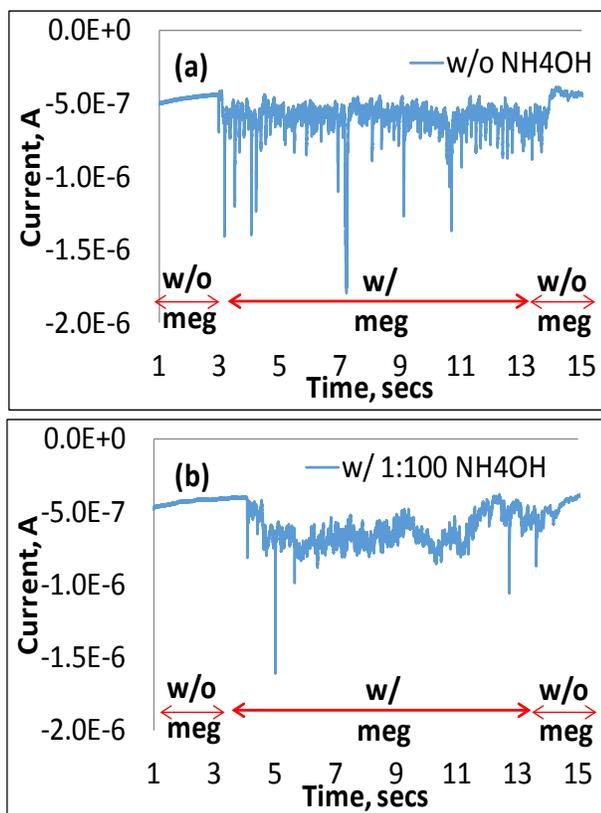


Figure 5.2 Chronoamperometry data obtained from microelectrode studies in 50mM  $K_3[Fe(CN)_6]$  and 100mM KCl solution at an acoustic frequency of 1 MHz and power density of 8 W/cm<sup>2</sup> in the (a) absence and (b) presence (1:100) of added  $NH_4OH$

### 5.3.3 Effect of bulk solution temperature on generation of hydroxyl radicals

In semiconductor cleaning, solutions of varying temperatures are used. Solution temperature plays an important role in modulating cavitation as well as scavenging of radicals by altering the rate constants of participating reactions. Figure 5.3 shows the effect of varying the bulk solution temperature on the rate of generation of  $OH^\bullet$  at different power densities of 2, 4 and 8 W/cm<sup>2</sup> in solutions containing 0.2M  $NaHCO_3$  and 7.3 mM  $NaOH$ . At 8 W/cm<sup>2</sup>, as the bulk temperature was increased from 10 to 30°C, the  $OH^\bullet$  generation rate increased from 11 nM/min to 17 nM/min. Similar increase in  $\bullet OH$  generation rate was observed at 4 W/cm<sup>2</sup> while at 2 W/cm<sup>2</sup>, the change in rate of  $\bullet OH$  generation with temperature was insignificant. Increasing the solution temperature gives

rise to a complex cavitation behavior (Keswani et al., 2014). One of the important effects is that the solubility of gases decreases with increasing temperature thereby decreasing the amount of dissolved gas in solution. At lower temperatures of 10°C, the solubility of air is higher (Keswani et al., 2014), thereby possibly providing greater cushioning effect as more gas enters the cavity in comparison to that at higher temperatures. Both vapor pressure and surface tension of water, which are affected by bulk temperature, will influence transient cavitation (Young, 2004). All these parameters in combination likely lead to conditions that result in an increase in transient cavitation and consequently generation of hydroxyl radicals with bulk temperature. It may be noted that the rate of scavenging reaction of hydroxyl radical by terephthalic acid may also be impacted by solution temperature, which will be reflected in the measured rate of generation of  $\bullet\text{OH}$ . However, investigation of this effect is outside the scope of the current work.

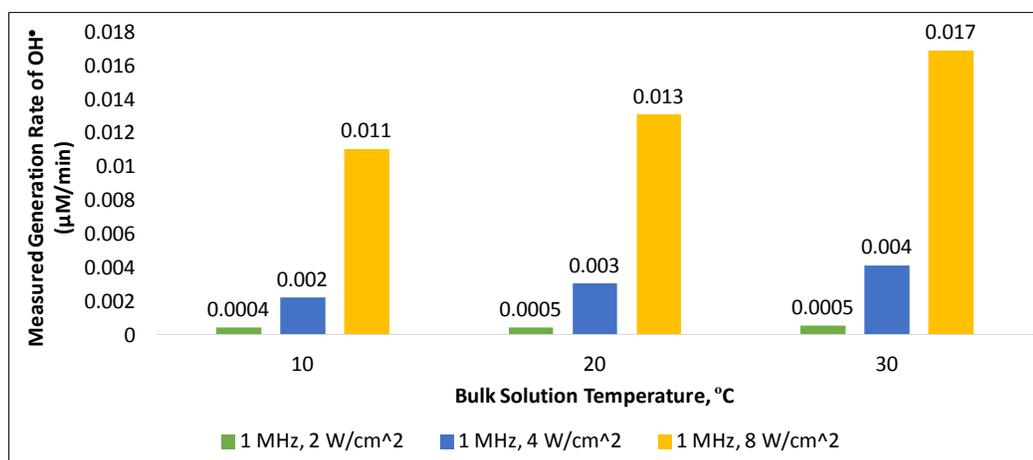


Figure 5.3 Rate of generation of  $\bullet\text{OH}$  as a function of varying temperature at different power densities at an acoustic frequency of  $\sim 1$  MHz. Chemical formulation: 0.2M  $\text{NaHCO}_3$ , 7.3mM  $\text{NaOH}$ , pH = 8.6

## 5.4 Conclusions

Generation rate of hydroxyl radicals was measured in various chemical formulations irradiated with an acoustic field of  $\sim 1$  MHz and at different power densities

(2 and 8 W/cm<sup>2</sup>). The results showed that in ammonia based formulations, the •OH generation rate was insignificant (2 nM/min) even at high megasonic power densities of 8 W/cm<sup>2</sup>. The lower rate of •OH generation in ammonia solutions was attributed to scavenging of •OH by aqueous ammonia as well as reduced transient cavitation in these solutions. For solutions containing bicarbonates and carbonates it was established that complete scavenging of •OH was achieved in the presence of carbonate ions at a concentration of ~ 200 mM. Further, carbonate ions were shown to be better scavengers of hydroxyl radicals than the bicarbonate ions. The study on the effect of bulk solution temperature revealed that the rate of generation of •OH at a power density of 8 W/cm<sup>2</sup> increased with increased in temperature from 10-30°C suggesting increase of transient cavitation with temperature.

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