SEASONAL RELEASE OF ESTROGENICALLY ACTIVE MUNICIPAL WASTEWATER: AN ASSESSMENT UTILIZING *IN SITU* BIOMARKERS

Fred Tilton, Daniel K. Schlenk, and William H. Benson
Environmental Toxicology Research Program/ECHR and Department of Pharmacology
School of Pharmacy, University of Mississippi, University, Mississippi

INTRODUCTION

The survival of a species depends upon the ability of individual organisms to develop and reproduce. This biological success relies on a delicate control of the endocrine system during critical windows of development for reproductively competent organisms to arise. A perturbation in the endocrine system may have far reaching consequences to the organism and the population within the affected aquatic ecosystem (Arcand-Hoy 1998). Researchers in the U.K. and the U.S. have reported observations in fish implicating reproductive and developmental toxicity as a result of exposure to effluents from municipal wastewater treatment plants (WWTPs). By far, the most extensive investigation of municipal WWTPs has occurred in the U.K., where intersex was recently observed in feral roach populations in river systems that receive estrogenically active wastewater from WWTPs (Jobling 1998). Prior to these findings, induction of a critical phospolipoprotein in female egg production, vitellogenin (Vtg), was measured in several caged male fish in these same U.K. river systems (Harries 1996, 1997). In male fish, Vtg has been shown to be induced in a dose dependent manner with exposure to 17-B-estradiol (E2) and Vtg has become widely used as a biomarker of estrogen exposure (Purdom 1994).

Pharmaceutical natural and synthetic estrogens are specifically designed or isolated to supplement endogenous levels of E2 in human females. The synthetic estrogen, ethinyl estradiol (EE), is prescribed to over 10 million U.S. women each year as the major constituent in oral contraceptives (IMS America 1995). In humans, EE is 30% more potent than the endogenous hormone, E2 (Arcand-Hoy 1998). Therefore, less EE is required to elict a similar effect to E2. This is not the case with conjugated estrogens used in estrogen replacement therapy which have potencies considerably less than E2. In view of this lower potency and the growing aged populations in the U.S., the amount of total conjugated estrogens by weight prescribed in the U.S. is several fold larger than the more potent EE (Arcand-Hoy 1998). These pharmaceutical products along with endogenous hormones are rapidly metabolized to more water soluble forms and excreted on a daily basis. When this diverse population of parent and metabolized estrogens, all of varying potencies, enter municipal WWTPs they are susceptible to the metabolic activity of microbial populations within a WWTP. Microbial aerobic degradation of organic matter in the activated sludge of WWTPs has the potential to de-conjugate E2 metabolites resulting in production of more biologically active compounds. Considering that a properly functioning WWTP will remove over 90% of the organic material entering WWTPs, microoganisms present in WWTPs may mineralize the metabolites to biologically less active or inactive forms (Operation of Municipal WWTP 1996). Recent German studies utilizing bench top activated sludge models demonstrated metabolism of estradiol 3-glucuronide to the biologically active compound, E2, by diluted activated sludge within a few hours. However, over a 24 hour period E2 was rapidly metabolized to the primary metabolite, estrone, followed by subsequent mineralization over the next several hours (Ternes 1999). Clearly, these benchtop studies illustrate the effects of active metabolic processes on estrogenic compounds entering WWTPs.

To date, estrogenically active wastewater has been reported to arise from several WWTPs in the U.S. and U.K. as demonstrated by studies utilizing caged and feral fish populations downstream of WWTP outfalls (Purdom 1994; Folmar 1996). The majority of studies have focused on the identification of estrogenically active wastewater downstream of WWTPs and do not reflect the seasonal variation that may exist. The focus of the present investigation was to identify and characterize the production of estrogenically active wastewater from two activated sludge treatment facilities in Lafayette County, Mississippi. U.S.A. over several seasons. Wastewater was screened, on a seasonal basis, utilizing a caged fish model placed within the effluent of two activated sludge treatment facilities for 21 days. These data demonstrate the seasonal release of estrogenically active wastewater and suggest that the release of this wastewater is dependent on the metabolic efficacy of the microbial populations within the WWTP.

MATERIALS AND METHODS

Site Description

Two municipal WWTP utilizing the activated-sludge process in Lafayette County, Mississippi, were selected for investigation. The two facilities process waste with a primary grit removal followed by vigorous physical agitation of the mixed liquor suspended solids in the aeration lagoon. From the lagoon, both plants have similar secondary clarifiers for sedimentation of material followed by chlorination and de-chlorination before releasing the wastewater into the receiving water (Figure 1). The activated sludge process is by far the most common method for the aerobic degradation of organic material entering WWTPs. A properly functioning facility is capable of removing over 90% of the biochemical oxygen demand from the water as a direct result of the metabolic activities of the microbial populations within the activated sludge lagoon. Due to the dependence on microbial populations there is potential for perturbations in the system because of environmental influences (e.g., rain events, temperature fluctuations, toxic dumps from WWTP users). The two facilities vary somewhat in the physical processes utilized, industrial inputs, and retention time of solids (Table 1). WWTP-A and WWTP-B are both located in the same watershed and contribute to tributaries of the Yocona River. The effluent of WWTP-A contributes approximately 50% of the volume of the small receiving creek that meanders several miles prior to entering a perennial tributary of Yocona River. The outfall for WWTP-B creates a small creek approximately 200 meters in length which flows directly into Yocona River. Observations made during the field study found an abundant wildlife population (feral channel catfish and smaller species) presumably utilizing the protected and shaded stream with a relatively constant flow and temperature.

Animal Model

Channel catfish were selected as the animal model, in part, because of the large aquaculture industry in and around the state of Mississippi and consequently catfish are abundant and readily available. Channel catfish also were desirable for use in the Southeastern United States because they are an endemic species in the turbid waters of the region. Channel catfish (*Ictalurus punctatus*) were obtained from the Fish Farming Experimental Laboratory, U.S. Department of Interior, National Biological Service, Stuttgart, Arkansas. Male channel catfish (12-18 months; N= 10; 200-400g) were segregated based on gender, by external examination. To control for disease or stress from handling following transport, fish were held for 10 to 14 days prior to being placed in the field.

In Situ Exposure

Fish were transported to each site and held for 21 days in 2' x 1.5' vinyl wire cages in the pre-chlorinated waste streams, as well as, the effluent of each WWTP. During this period fish were not fed, however, catfish normally experience long periods of fasting and did have the ability to consume organic matter in the wastewater streams. As a reference, fish were held within cages in a laboratory flow-through stream, Bramlett Pond (University of Mississippi Biological Field Station) and a small suburban creek with characteristics (flow, depth, water quality) similar to the effluent streams. On day 21, fish were anesthetized by cervical dislocation, weighed and blood samples were collected from the caudal vein (ventral on the tail) with an unheparinized needle and syringe. Livers were excised and weighed and frozen on dry ice. Livers were stored at -80C until analysis. Collected blood was allowed to clot on ice followed by centrifugation to separate serum. Serum samples were stored at -80C until analysis. Gonads were examined and weighed.

Biological Indices

Hepatic-somatic index (HSI) and gonad weight reported as percent of total body weight was used to identify tissue level effects from exposure to effluents. Serum Vtg levels were determined by use of a competitive enzyme linked immunosorbant assay (ELISA) with monoclonal antibodies specific for channel catfish (Goodwin et al. 1992). Ethoxyresorufin-O-deethylase activity (EROD) from heptic microsomes was measured utilizing a fluorescence detection method modified for 96-well plate (Hahn et al. 1996). Cytochrome P4501A1 activity was determined utilizing a direct ELISA and a monoclonal channel catfish antibody (Rice and Schlenk 1998).

RESULTS

Vitellogenin and GSI

Serum Vtg was significantly elevated in two of four seasons studied at WWTP-A (Figure 2). Spring 1997 and Fall 1997 serum Vtg levels were 5-fold to 6-fold above reference values. At WWTP-A, fish were unable to be kept alive in the effluent because of an upstream contaminant. All the fish placed at this site would generally die within 7 days above and below the outfall. Fall 1997 serum Vtg showed considerable variability in a response ranging from 7ug/mL to 122 ug/mL. During this study period, there were three separate major rain events that caused combined sewer overloads (CSO). There was no significant changes in the gonad weight of fish during any of the seasons studied at both

WWTPs (data not shown).

Serum Vtg was significantly elevated 2- to 4-fold above reference values in Fall 1996 and Spring 1997 relative to controls (Figure 3). Interestingly, in Fall 1997, the season with abundant rainfall, there was no detection of Vtg at WWTP-B and the fish in the effluent perished under the turbulent conditions of the CSO. At both WWTPs the summer Vtg induction while detectable was not significant compared to reference values.

Hepatic Tissue Indices

Hepatic microsomal EROD activity were significantly elevated in Fall 1996 and Spring 1997 at WWTP-A (Figure 4) and WWTP-B (Figure 5). There also was significant differences between the pre-chlorinated site (Pre-Cl_2) and the effluent site in Fall 1996 and Spring 1997 at WWTP-B (Figure 5). Effluent toxicity prevented any observation of this trend at WWTP-A. Both sites had no significant elevations of EROD activity in the Summer 1998 study period. The most significant elevation of CYP1A1 content occurred during Fall 1997, the season of abundant rainfall, where apparent dilution of the stream contaminants allowed fish to be kept alive during the entire 21 day study period (Figure 6). The HSI was significantly elevated at both sites in WWTP-A in Fall 1997 (data not shown). The most consistent elevation of CYP1A1 was at WWTP-B which was elevated 1- to 2-fold above reference values in all seasons, except Fall 1997.

DISCUSSION

Comparison of serum Vtg induction at WWTP-A (Figure 2) and common activated sludge-WWTP parameters, Sludge Age and Fecal Coliform (Figure 8) demonstrate a correlation between the efficacy of the metabolic activity of activated sludge and the seasonal release of estrogenically active wastewater. Sludge Age is determined by the ratio of activated sludge, measured as total suspended solids (TSS), in the aeration lagoon (Kg per volume) and the TSS entering the facility (food to microorgainsms) in the influent (Kg in flow volume/day) (Mississippi Operation and Training Manual 1994). Therefore, when the amount of TSS entering the facility decreases, Sludge Age rises. When Sludge Age rises, the microorganisms become less efficient as a result of lack of nutrients. An extended period of elevated Sludge Age can result in the wasting of sludge out of the WWTP resulting in increases in effluent fecal coliform. In Fall 1996, serum Vtg (1.5-fold induction) was not significantly elevated (Figure 2). Sludge Age at this time increased from 40 days to 80 days indicating an aging microbial population, however, fecal coliform in the effluent was relatively low and therefore the efficacy of the metabolic activity of the microbial population was relatively stable resulting in an insignificant increase in Vtg (Figure 8). This was not the case for Spring 1997, when for 3 months prior to the study period, Sludge Age was elevated 80 to 120 days and Vtg was significantly induced 6-fold above reference values. Furthermore, in the month following the study period, WWTP-A experienced a major sludge wasting event where fecal coliform levels in the effluent went out of compliance for that WWTPs effluent permit. Conversely, in Summer 1998 Sludge Age and fecal coliform were considerably low and Vtg induction at WWTP-A was at its lowest levels for all seasons studied (1-fold induction). Interestingly, Vtg induction in Fall 1997 (4.8-fold induction) does not appear to correlate with the previous trends described. However, closer examination of the Sludge Age shows a rising Sludge Age starting in August 1997 which was interrupted by the large rainfall in October 1997. This abundant rainfall resulted in several CSO and an increase in fecal coliform release. Furthermore, Vtg induction during this season was unusual in that several fish showed levels of induction at or below the levels observed in the reference fish. Only 4 fish (of 10) showed induction of Vtg signifcantly above reference values. This phenomenon has been observed in our laboratory (Thompson et al. 1999) and by other researchers in the U.K. with aqueous exposure to E2 at the threshold level of induction (Routledge 1998). Therefore, the rain in Fall 1997 could have diluted the TSS in the aeration lagoon such that Sludge Age does not accurately reflect the metabolic efficacy of the microbes in the lagoon. Furthermore, the rain events during the Fall 1997 season as reflected in the significant CYP1A1 induction (Figure 6) possibly exposed the fish to contaminants carried into the waste stream from rain run-off. This illustrates the need for chemical characterization, such that, the contaminants for the Vtg induction, may vary according to the environmental factors that influence the influent entering WWTPs.

Generally speaking there was a constant source of CYP1A1 inducers found in both facilities. Traditional contaminants such as PAH's and PCB's, are both inducers of CYP1A1 and have been identified in WWTP effluents in several studies (Kosmala 1996). While chemical characterization would be necessary to identify the presence of co-planer contaminants they are most likely the contributing constituent of the induction observed in the present study. Laboratory studies have demonstrated a down-regulation of CYP1A1 with exposure to increasing concentrations of E2 (Stegaman 1994). This was not explicitly observed in this field study most probably due to the complex mixture of substances in WWTP effluent. However, the largest induction of Vtg in Spring 1997 (as well as the

largest induction in the entire study Figure 2) did correspond to the only season in which CYP1A1 was not significantly induced at WWTP-A (Figure 6). This trend was not observed at WWTP-B where significant CYP1A1 induction (Figure 7) appears to correlate with induction of Vtg (Figure 3).

The estrogenic response in channel catfish appears to be significantly less than that observed in other studies utilizing rainbow trout (Harries 1996,1997; Routledge 1998). Preliminary evidence in our laboratory indicate channel catfish to be the most insensitive of several fish species studied in terms of Vtg response to E2 exposure. Recent controlled laboratory studies exposing channel catfish to aqueous E2 resulted in the development of a biological dose-response curve (Figure 9). From this curve, preliminary data suggests that the responses observed in the field correlates to an Estradiol Equivalents between 650 to 723 ng/L of E2. Utilizing a monoclonal antibody for E2 and ELISA techniques, 10ng/L E2 was measured in water sampled from both WWTPs. The difference between the biological response and that measured using ELISA could reflect the contribution of several estrogenically active compounds including EE and E2 metabolites such as estrone and estriol. Clearly, further analytical work needs to be done in order to understand the difference between the biological response and the chemically measured value.

In summary, the two WWTP under investigation were found to release estrogenically active wastewater on a seasonal basis. This seasonal release appears to be, in part, dependent on the metabolic efficacy of microbial populations within the activated sludge lagoons. While the release of estrogenically active wastewater is not constant there is potential for releases of estrogenically active wastewater, particularly at WWTP-B, such that fish populations are exposed at critical periods of reproduction and development. The biological impact of the levels of estrogenic activity measured in this study needs to be understood in greater detail. Further study into the correlation between Sludge Age and Fecal Coliform to estrogenically active wastewater release is needed; however, the ability of municipal WWTP operators to control the release of estrogenically active wastewater with existing monitoring techniques could prove to be most beneficial.

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Table 1. SITE DESCRIPTION

Treatment Facility	Type	Population Equivalents $\frac{\text{BOD, Influent}}{60g\ \text{Total Suspended Solids}}$	Flow (MG/day)	Industrial Input (%)	Solids Retention Time (Days)
WWTP A	Extended-Air Activated Sludge	3,300	0.5-0.8	0	1
WWTP B	Oxidation-Ditch Activated Sludge	5,000	1.3-1.6	<10	2

WASTEWATER TREATMENT PLANT R-1

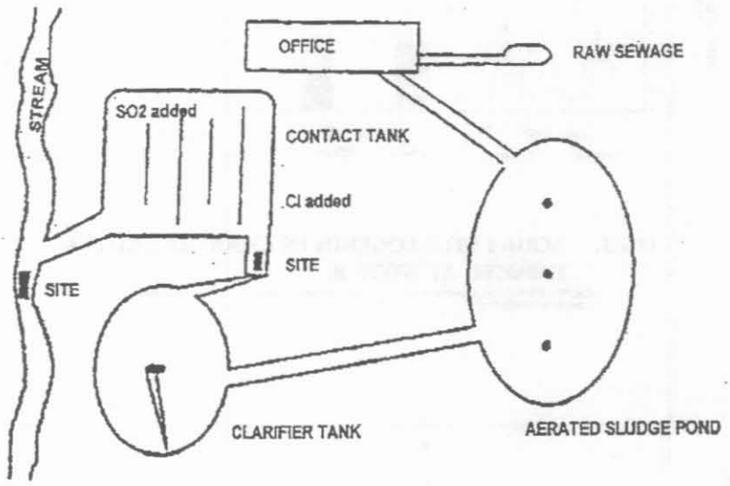

FIG 1. SCHEMATIC DIAGRAM OF WWTPS UNDER STUDY

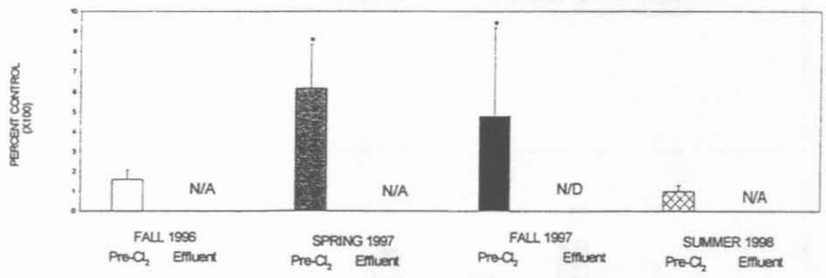

FIG. 2 SERUM VITELLOGENIN IN CHANNEL CATFISH EXPOSED AT WWTP A.

* DENOTES STATISTICAL SIGNIFICANCE FROM REFERENCE SITE N/A - fish did not survive N/D - no detection

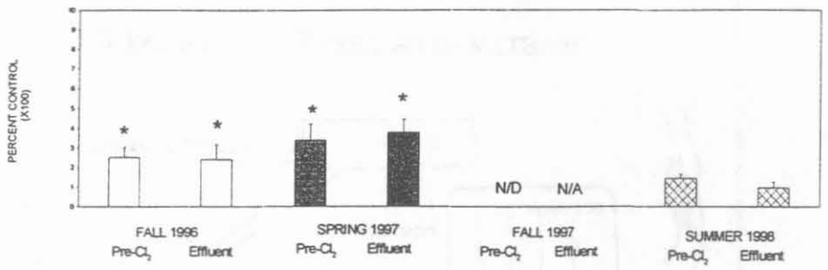

FIG 3. SERUM VITELLOGENIN IN CHANNEL CATFISH EXPOSED AT WWTP B.

* DENOTES STATISTICAL SIGNIFICANCE FROM REFERENCE SITE N/A - fish did not survive N/D - no detection

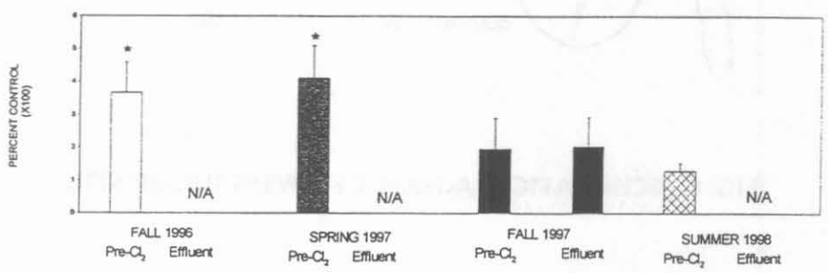

FIG. 4 HEPATIC EROD ACTIVITY IN CHANNEL CATFISH EXPOSED AT WWTP A.

* DENOTES STATISTICAL SIGNIFICANCE FROM REFERENCE SITE N/A - fish did not survive

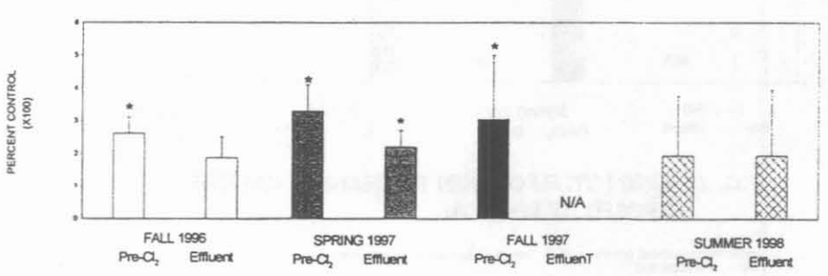

FIG 5. HEPATIC EROD ACTIVITY IN CHANNEL CATFISH EXPOSED AT WWTP B.

* DENOTES STATISTICAL SIGNIFICANCE FROM REFERENCE SITE N/A - fish did not survive

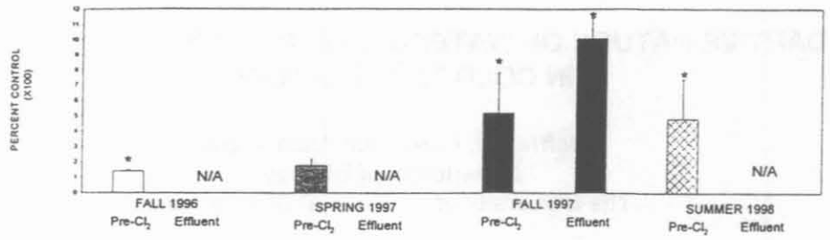

FIG. 6 MICROSOMAL CYP1A1 CONTENT IN CHANNEL CATFISH EXPOSED AT WWTP A.

* DENOTES STATISTICAL SIGNIFICANCE FROM REFFERENCE SITE N/A - fish did not survive

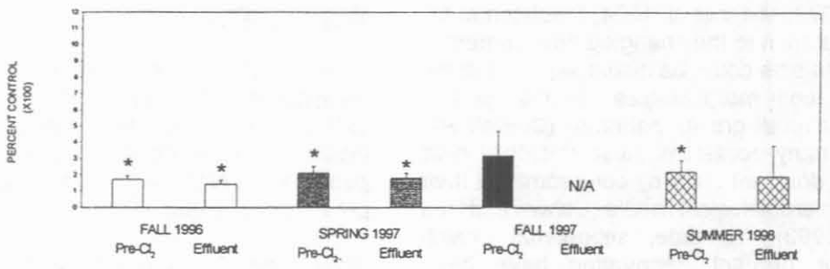

FIG 7. MICROSOMAL CYP1A1 CONTENT IN CHANNEL CATFISH EXPOSED AT WWTP B.

* DENOTES STATISTICAL SIGNIFICANCE FROM REFERENCE SITE N/A - fish did not survive

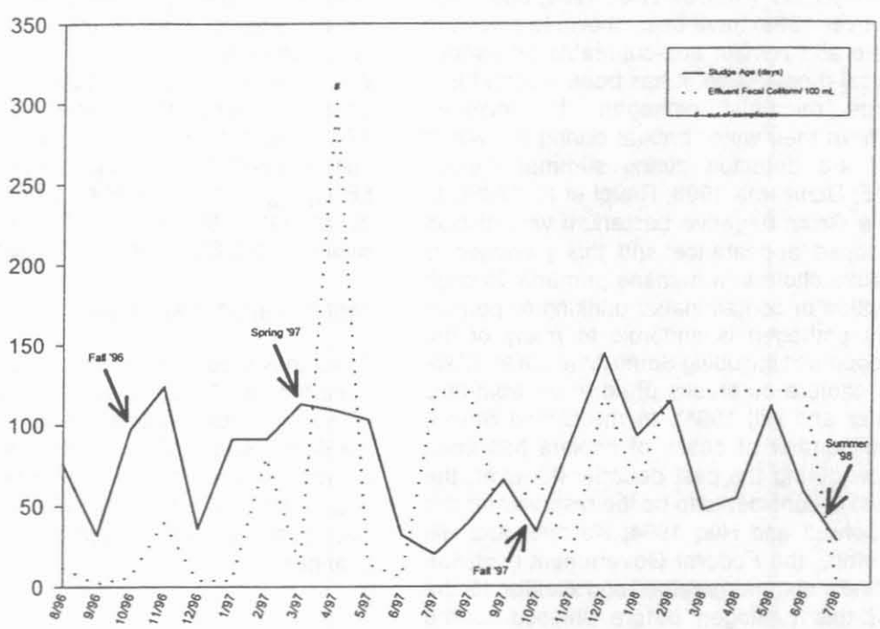

FIG 8. TREND CHART FOR SLUDGE AGE AND EFFLUENT FECAL COLIFORM COUNT FOR WWTP A.