REPORT

Title: Biological Treatment of Wastewater Contaminated with Estrogenic Compounds

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Abstract:

Currently there are no surface or groundwater regulations to address the concentrations of natural and synthetic hormones resulting from animal waste treatment processes and domestic wastewater treatment effluents. These emerging contaminants are endocrine disrupting compounds. Estrogen concentrations as low as 10 ng/L in wastewater have been known to alter the sex of fish. Biological treatment of estrogenic water will be studied in this research. Wastewater from various relevant sources will be collected and screened for organisms that are capable of degrading hormones. Enrichment culture based methods will be used during the initial screening process. Potential bacterial species capable of degrading hormones will be isolated, genomic DNA from the isolates will be extracted, and sequenced. Then various environmental factors that control and govern the biodegradation of hormones will be studied to design biological treatment processes. The results from this study will be presented here.

Problem and Research Objectives:

Emerging contaminants such as steroid hormones are not currently under regulation, yet field and lab studies show a potential for endocrine disruption in the aquatic environment (Tyler et al., 1998). Research was done 45 years ago to study the degradation of estrogenic compounds in the environment (Stumm-Zollinger and Fair, 1965). This study concluded that there would not be high enough concentration in the environment to cause a problem (Stumm-Zollinge and Fair, 1965). However, recent studies have shown that in streams that have concentrations as low as 10 ng/L can disrupt the endocrine system of fish, causing feminization of male fish in some cases (Hutchinson et al., 1999; Jobling et al., 1998; Lai et al., 2002; Tyler et al., 1998; Yu et al., 2007). The natural estrogens consist of estrone (E1), 17β-estradiol (E2), and estriol (E3), where the synthetic estrogen compounds include ethinylestradiol (EE2) and mestranol (MeEE2).

Wastewater effluents from Wastewater Treatment Plants (WWTPs) and Confined Animal Feeding Operations (CAFOs) are major estrogen contributing sources in the terrestrial environment. Municipal WWTPs discharge effluent with estrogen concentrations from 0.2 to 10 ng/L (Bartonti et al., 2000; Ternes et al., 1999). The manure produced in CAFOs can have E2 between 114 and 522 ng/g on a dry weight basis (Shemesh and Shore, 1994). Streams receiving discharged wastewater have up to 93 ng/L of E2 present (Finlay-Moore et al., 2000; Kolpin et al., 2002).

Typical WWTP processes are able to decrease the estrogen concentrations to ng/L levels (Bartonti et al., 2000; Ternes et al., 1999). Studies on dissipation of estrogens in municipal WWTP have documented that microbial degradation is the primary fate process, occurring in the activated sludge treatment (Gaulke et al., 2008; Ivanov et al., 2009; Muller et al., 2009; Pauwels et al., 2008; Shi et al., 2004; Yu et al., 2007). However, there are only few studies on the removal mechanisms of estrogens from CAFO wastewater (Chen et al., 2008; Khanal et al., 2006) and few studies have focused on the presence of estrogen compounds in water bodies near CAFOs (Arnon et al., 2008; Shemesh and Shore, 1994; Soto et al., 2004).

Texas Commission for Environmental Quality (TCEQ) which closely follows the Environmental Protection Agency (EPA) guidelines do not currently regulate surface waters as to the levels of estrogenic compounds that are discharged into streams. Current treatment, sorption and degradation, at WWTPs will lower the concentration but additional treatment is needed to get the concentration to a concentration level that does not have adverse affects.

Estrogens from CAFO waste are making their way in to the environment. In Arkansas, five springs with recharge zones that include pastures fertilized with poultry waste were sampled for fecal coliform, *E. coli*, and E2 (Peterson et al., 2000). Concentrations of E2 ranged from 6 to 66 ng/L in the sampled springs. Animal waste is one of the predominant sources of estrogenic compounds in the environment (Khanal et al., 2006; Hutchins et al., 2007; Raman et al., 2004).

US EPA regulates CAFO waste management. When CAFOs are discharging effluent they must apply for a National Pollutant Discharge Elimination (NPDES) permit (USEPA 2008). This permit does not specify limits of estrogenic compounds. CAFOs that are not discharging effluent must design an open containment system for the liquid waste that ensures discharge will not occur. Generally, CAFO waste treatment includes settling and separating the solid material from the liquid wastewater. The solid material is often land applied or composted. The supernatant goes to a series of aerobic or anaerobic lagoons lined with plastic or clay lining. Usually the lagoon liners are designed to allow for permissible infiltration. Estrogens if present in the storage wastewater can infiltrate below the liners. A study conducted in Israel documented estrogen concentrations at 32 m below a CAFO waste lagoon (Arnon et al., 2008).

After treated in the waste lagoons, the CAFO wastewater can be used to irrigate surrounding fields. Studies have been conducted on the transport of estrogens in soils and water bodies (Casey et al., 2003, 2005; Lee et al., 2003; Lim et al., 2007). Concentrations of estrogenic compounds were higher in the Fall than in the Spring for a river in Israel (Barel-Cohen et al., 2006). Lee et al. (2003) studied the sorption of E2 in soil and found a strong association between E2 and soil. Lee concluded that estrogenic compounds in soil were likely to be transported with surface runoff. Appropriate treatment of CAFO wastewater is crucial to minimize the exposure of estrogenic compounds to the aquatic environment from runoff and infiltration.

Increased residence time in CAFO lagoons is a viable option for removing estrogens. But further research should be conducted to study the factors that influence the removal of estrogens in CAFO wastewater (Zheng et al., 2008). Temperature affects microbial degradation of estrogenic compounds. However, most of the studies on the effect of temperature on estrogen degradation were conducted in soil or solid waste in CAFOs (Colucci et al., 2001; Hakk et al., 2005; Hemmings and Hartel, 2006; Jacobsen et al., 2005; Stumpeand Marschner. 2007). Optimum soil temperature for mineralization of estrogens was found to be between 30 and 37°C (Colucci et al., 2001). Accumulation of E2 can occur in agriculture soils because the mineralization rate is low (Stumpe and Marschner. 2007).

Even though there are several studies on fate, transport, and removal of nutrients in dairy CAFO wastewaters, there are only limited studies on degradation of estrogens in wastewaters resulting from dairy CAFOs (Cho et al., 2000; McNab et al., 2007; Singleton et al., 2007). Few studies conducted on degradation of estrogens in poultry or swine lagoons. Hemmings and Hartel (2006) found that mineralization of estrogens in poultry litter generally increased as temperature decreased. Jacobsen et al. (2005) found that microorganisms in swine slurry degraded 17β-estradiol when added to agricultural soil at 30°C. Aerobic composting of poultry manure decreased the water-solubility of 17β-estradiol over time (Hakk et al., 2005). Similar studies on the degradation of estrogens in dairy CAFO lagoons are needed.

In this research, the effects of temperature and pH on the aerobic degradation of 17β-estradiol in CAFO wastewater were studied. The overall objective was to determine the

effect of temperature and pH on microbial degradation of 17β-estradiol by an isolated estrogen degrading bacterium and mixed cultures from CAFO wastewaters.

- Hypothesis 1: As the temperature decreases, the removal percentage of 17β-estradiol by estrogen degrading bacteria will decrease.

- Hypothesis 2: As the pH of the wastewater is more acidic, the removal percentage of 17β-estradiol by estrogen degrading bacteria will decrease.

Materials/Methodology:

Estrogen-degrading microorganism. Estrogen degrading organism isolated and sequenced by a screening study previously done by Sullivan (2010) will be used in the experiments. The isolate was confirmed as *Sphingobacterium* sp. For the batch degradation study, this isolate was grown in mineral medium (0.4 g $Na_2HPO_4 \cdot H_2O$, 0.25 g KH_2PO_4, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.5 g $(NH_4)_2SO_4$, 0.25 g yeast extract, 2.5 mL trace element solution) (Haiyan et al., 2006) dissolved in 500 mL de-ionized water with 3 mg/L of 17β-estradiol eluted in ethanol. The culture was grown in 1 L conical flasks, kept on a rotary shaker at 100 rpm and incubated at 35˚C for two days.

Mixed culture from CAFO wastewater. Wastewater from two different CAFOs will be taken from the primary lagoon.

Water microcosms. Nine autoclaved 500 mL Pyrex® bottles sealed with sterilized cotton plugs were used as lab-scale bioreactors. Three bottles contained 450 mL of wastewater collected from an aerobic treatment lagoon of CAFO A. Three bottles were filled with autoclaved wastewater collected from the same lagoon treatment; and three bottles were filled with autoclaved deionized water. All bottles were spiked with 3 mg/L of 17β-estradiol. One milliliter of the mineral media enriched with *Sphingobacterium sp.* was added to the three bottles with wastewater and three bottles with autoclaved deionized water while the three bottles with autoclaved wastewater served as control for this biodegradation study. The same experimental design was repeated with wastewater collected from aerobic lagoon of CAFO B.

The effect of pH was studied by setting each bioreactor to acidic, neutral, and then alkaline pH while maintaining at a constant temperature. At each of the different pH values, the temperature was kept at 5°C, 10°C, and 25°C to observe the effect of temperature on the microbial degradation of 17β-estradiol. The study was done over a 20 day period for each temperature.

Chemicals. All chemicals used in this study including 17β-estradiol were obtained from (Fisher Scientific, Houston, TX).

17β-Estradiol Analysis. A specific immunoassay (Abraxis, Warminster, PA) was utilized in this study to determine 17β-estradiol concentrations. The analysis was conducted according to manufacturer's recommendation. Briefly, a solution of estradiol antibody and magnetic particles are added to both samples and standards in test tubes,

vortex, and then reacts for 30 min at room temperature before adding estradiol enzyme conjugate. The conjugate reacts for 90 min at room temperature and then the magnetic separator holding the test tubes is inverted to separate the particles. A washing solution is added and the separator rack is inverted again. Then, a coloring reagent is added to the test tubes and reacts for 20 min at room temperature. Finally, the stopping solution is added to each tube. After 15 min, optical density (OD) of the solutions in test tubes is measured using a photometer set at 450 nm. A standard curve is generated and used to determine 17β-estradiol concentrations in samples and controls. The pH as well as the OD at 600 nm was measured at each 17β-estradiol concentration measurement. Optical density at 600 nm measures the bacterial cell growth.

Preliminary Study. Four autoclaved 500 mL Pyrex® bottles sealed with sterilized foam plugs were used as lab-scale bioreactors. All bottles contained 250 mL of autoclaved deionized water and were spiked with 3 mg/L of 17β-estradiol. The bottles were covered with aluminum foil to prevent photo-degradation of 17β-estradiol. One milliliter of the mineral media enriched with *Sphingobacterium sp.* was added to two of the bottles while the other two bottles served as control for this biodegradation study. The pH of each microcosm was measured and recorded. Two bottles (one sample and one control) were incubated at temperature 5˚C and continuously stirred at 100 rpm. The other two bottles (one sample and one control) were incubated at temperature 35˚C, continuously stirred at 100 rpm. The concentrations were measured using Abraxis immunoassay kit five times over 12 days.

Principal Findings:

There have been several technical problems encountered. Initially, a Gas Chromatography-Mass Spectrometry (GC-MS) was used to determine the concentrations of 17β-estradiol. Training on the GC-MS was obtained and a method for analyzing 17β-estradiol samples was tested. Contaminants in the column were detected and consistent standards could not be analyzed to obtain a standard curve. An ELISA kit was obtained and used to measure 17β-estradiol concentrations. The basics of the kit were learned and a specific photometer was purchased to measure the absorbance for the small sample volume. The preliminary study described in this proposal was conducted and the data was recorded. However, the absorbance values of standards did not follow a linear trend when plotted. New standards were obtained from Abraxis along with the certificate of analysis that included the standard curve that should be expected. Following the suggestion from Abraxis, the standards were allowed to reach room temperature before running the assay. Again, absorbance values of standards did not fit the certificate of analysis standard curve. This research is still ongoing.

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