Project Title: Bioassessment Of Fish Populations Up- And Downstream Of Two Public Wastewater Treatment Plants And Investigation Of The Possible Presence Of Estrogenic Chemicals In The Effluent

Project Period: June 1, 2007 – November 1, 2007

Introduction

Treatment of domestic wastewater typically involves three stages, called primary, secondary and tertiary treatment. First, the solids are separated from the wastewater stream by screening. Then dissolved organic matter is progressively metabolized and converted into a solid mass by bacteria. The objective is complete breakdown into simple environmentally benign molecules such as carbon dioxide, water, nitrates, sulfates and biomass (sludge). The sludge is sterilized and disposed of. The treated water is disinfected and discharged into a stream, river, or other water body.

If the wastewater is not treated adequately, either due to too short a residence time in the plant or other reasons, organic chemicals can be incompletely removed by the process and will be present in the "treated" wastewater that is discharged. These chemicals may have undesirable effects on organisms living in the receiving waters. In recent years, it has become apparent that some of the compounds frequently present in treated wastewater can act like hormones or interfere with hormones and thus act as "endocrine disruptors." Investigations worldwide have detected bioactive estrogens in waters receiving treated wastewater (Snyder et al. 2001, Gomes et al. 2003, Cargouet et al. 2004, Sheahan et al. 2002, Hemming et al. 2004, Kolodziej et al. 2003, Tilton et al. 2002, Murk et al. 2002, Sarmah et al. 2006, Lishman et al. 2006, Snyder et al. 2001, Murk et al. 2002, Sheahan et al. 2002, Tilton et al. 2002, Gomes et al. 2003, Kolodziej et al. 2003, Cargouet et al. 2004, Hemming et al. 2004, Sarmah et al. 2006, Lishman et al. 2006). Municipal wastewater is a complex mixture of natural and synthetic organic chemicals. The most active endocrine disrupting chemicals (EDCs) that are commonly detected in treated wastewater include the natural hormones 17β-estradiol (estrogen, E2), its breakdown product, estrone (E1), and 17α-ethinylestradiol (the synthetic estrogen used in birth control pills, EE2). Other non-steroidal organic chemicals have been shown to possess estrogenic activity, but are much weaker than the steroid hormones. These include the degradation products of nonionic surfactants, such as alkylphenolpolyethoxylates, and plasticizers, such as bisphenol A (Barber et al. 2000).

EDCs can disrupt the sexual development and functioning of aquatic organisms and impair their ability to reproduce successfully (Tyler et al. 1998, Jobling et al. 1998,McLachlan and Arnold 1996,Sumpter 2005). Also, if the water is withdrawn downstream for human drinking water, it is unlikely that these compounds will be removed by the treatment process which typically filters and sterilizes the water, but does not removed dissolved organic molecules. In this situation, the EDCs can pose a threat to human sexual development and functioning.

The objective of the project is to assess the status of fish populations in Five Mile Creek (Jefferson County, Alabama) upstream and downstream of two wastewater treatment plants. These data will allow us to determine whether the WWTPs have any discernible effects on the overall "health" of the fish populations. In addition, we will evaluate one species of fish (largescale stoneroller) for evidence of endocrine disruption. If such evidence is found, water samples will be analyzed so that we can identify the hormonally active substance(s) in the effluent.

Study Sites

The study sites are shown in Figure 1.The Five Mile Creek WWTP is located west of Coalburg Road at Five Mile Creek Bridge in Fultondale. The plant receives sewage flow from Tarrant City, Inglenook, Lewisburg, Roebuck, Center Point, Grayson Valley, the southern end of Pinson Valley, Fultondale, and southern Gardendale. The plant was placed in operation in 1978. Its average design capacity was upgraded to 20 million gallons per day (MGD) in 1993. The estimated population generating wastewater flows to this plant is approximately 42,300 persons. The treatment process utilizes a modified conventional activated sludge process (http://jeffco.jccal.org/portal/page?_pageid=314,54239,314_54247&_dad=portal&_schema=PORTAL, accessed 5/16 2007).

The Prudes Creek plant is located at 500 Fifth Street NE in Graysville. The plant serves the Cities of Graysville and Adamsville. The plant was constructed in 1988 with an average design capacity of 0.6 MGD. The estimated population generating wastewater flows to this plant is approximately 905 persons. The treatment process utilizes an extended aeration activated sludge process and ultraviolet disinfection (http://jeffco.jccal.org/portal/page?_pageid=314,54263,314_54271&_dad=portal&_schema=PORTAL, accessed 5/16/2007).

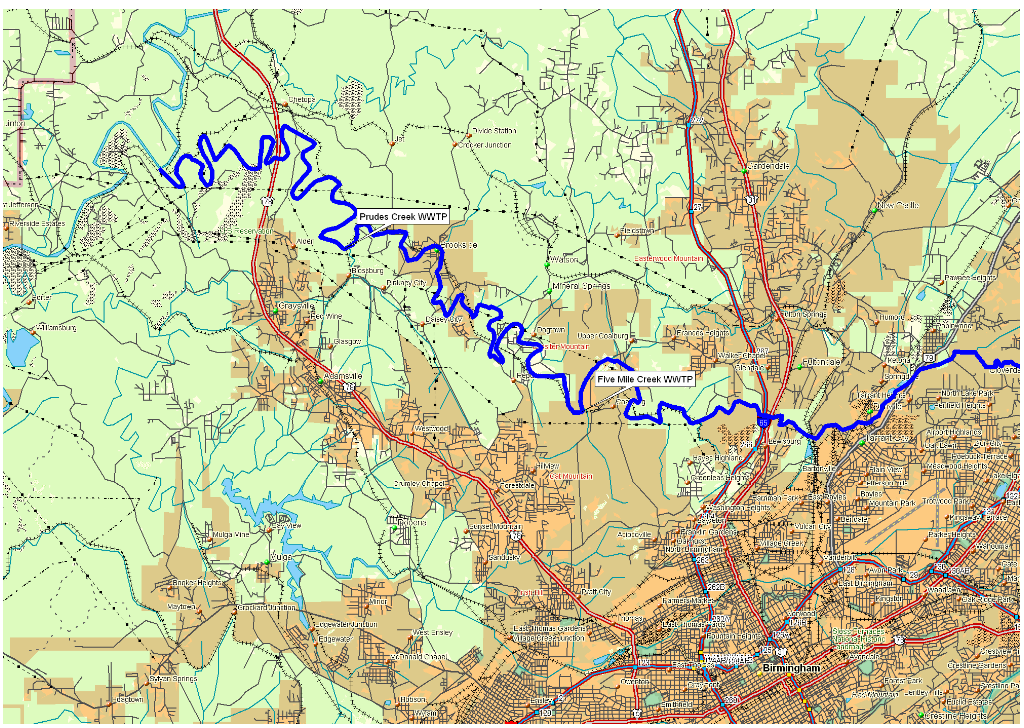

Figure 1. Wastewater treatment plants on Five Mile Creek.

Fish collections will be made immediately upstream and downstream of these WWTPs.

Bioassessment Protocol

Fish bioassessments will be performed in the early summer (June) and again in the autumn (October) of 2007.

The ichthyofaunal (fish) community responds to habitat/water quality alteration (Barbour et al. 1997, Karr 1981). Fish are considered to be better indicators of longer-term stresses than the other major group often used in bioassessment: macro-invertebrates, since fish generally live longer than most aquatic invertebrates. Additionally, since fish communities include species representing a variety of trophic levels, they tend to integrate effects on lower levels. Thus, fish community structure reflects overall environmental health of the aquatic ecosystem.

Sampling - We use two complementary methods for collecting fish: electrofishing and seining. The reach is first sampled by electrofishing with a Smith-Root backpack electrofisher. We sample for 30 minutes. The fish are identified and enumerated immediately after the electrofishing sample is completed. The fish are then released back into the stream. We then return to the bottom of the reach and re-sample it by seining. The fish collected by seining are identified and enumerated immediately after the sample is completed and released. Although electrofishing is viewed as the most effective method for sampling stream fish (Barbour et al. 1997), it is somewhat biased toward collection of larger fish (Wiley and Tsai 1983) and is not as good as seining in collecting open water schooling fish (pers. obs.). A combined effort, with both electrofishing and seining, provides a more representative sample of the fish community (Onorato et al. 1998). Sampling always begins at the downstream boundary of the sampling reach and proceeds upstream. All geomorphic channel units and instream habitat features are sampled.

Processing - Fish are held in buckets a manner consistent with minimizing stress, i.e. water is changed regularly and fish are not overcrowded. Immediately after the 30-min. collecting effort is completed, all fish that have been captured are identified to species and enumerated. Any fish of uncertain identity is anesthetized in MS-222 and preserved in 10% formalin for keying out in the laboratory. All other fish are released in good condition back into the stream.

Fish Data Summarization - Data summary will employ the Index of Biotic Integrity (IBI), a fish assemblage assessment approach developed by Karr (1981). For comparative purposes, we will use a slight modification of the IBI that was developed by O'Neil and Shepard (2000) for central Alabama. This IBI uses 12 metrics to assess biological integrity based on abundances, and the taxonomic and trophic composition of the fish community. The metrics used are: total number of fish species (decreases with habitat degradation); number of darter species (a relatively disturbance–sensitive group); number of sunfish species (decrease with degradation of pools and instream cover); number of minnow species (commonly most of fish biomass in small streams; an indication of diversity); number of sucker species or bottom–feeding species (an indication of a trophic level group); number of intolerant species (as defined by Shepard et al. [Shepard et al. 1997]; an indication of disturbance); proportion of individuals as sunfish (goes up with moderate disturbance, as sunfish are relatively tolerant); proportion of individuals as insectivorous minnows (decreases as insect community decreases due to siltation); proportion of individuals as top carnivores (indicates trophic abundance shifts); number of individuals collected per hour of effort (evaluates abundance); and proportion of individuals with physical anomalies. Additionally, several standard species diversity and richness indices will be calculated (e.g., Shannon-Wiener, etc.).

Stream Characteristics

An evaluation of habitat quality is an important component of the assessment of the ecological integrity of a site. The alteration of the physical structure of the habitat is one of the major factors from human activities that degrade aquatic resources. Habitat, as structured by instream and surrounding topographical features, is a major determinant of aquatic community potential. Both the quality and quantity of available habitat affect the structure and composition of resident biological communities (Barbour and Stribling 1991). We will follow EPA-recommended procedures for habitat assessment of high gradient streams, as outlined in the Revision to Rapid Bioassessment Protocols for Use in Streams and Rivers (Barbour et al. 1997). We have used these procedures in our previous studies. Stream characterization includes:

Watershed features - documentation of predominant surrounding land use / land cover (determined using GIS technology), local watershed nonpoint source pollution, soil type and local watershed erosion.

Riparian vegetation: documented as the dominant type and species.

Instream features - reach length, stream width, reach area, stream depth, stream velocity, streamflow volume (annual range), canopy cover, leaf area index, solar radiation, high water mark, proportion of reach represented by each of the stream morphological types (riffle, run, pool), whether or not the stream is channelized and whether a dam is present.

Aquatic vegetation - The general type and relative dominance of aquatic plants are documented. Besides being an ecological assemblage that responds to perturbation, aquatic vegetation provides refugia and food for aquatic fauna.

Water Quality

A number of water quality characteristics are measured at the time a bioassessment is performed. These include temperature (°C), conductivity (µS), dissolved oxygen (µg/L), pH, and turbidity. In addition evidences of pollution (or lack thereof) are noted, such as oil slicks on the water surface or odors from water or substrate.

Endocrine Disruption Study

We will focus on the largescale stoneroller (*Campostoma oligolepis*, Figure 2) for this portion of the study. This small minnow (~22 mm, total length) is very abundant throughout Five Mile Creek, and we will be able to obtain samples of them at all four collection sites.

Figure 2. Largescale stoneroller, *Campostoma oligolepis* (R. Angus photo).

At each sample site (spring and fall), we will collect 20 live stonerollers and bring them back to the lab at UAB. The fish will be lethally anesthetized (MS-222, 300 mg/L) and the gonads and liver removed. The gonads will be immediately inspected under a stereomicroscope and preliminarily identified as ovaries, testes, or intersex, based on external appearance.

Gonads - The gonads will be preserved in Bouin's fixative, prepared for histology. Thin sections will be made from the center of each gonad and stained with eosin and hematoxylin. The stained sections will be mounted on slides and examined with a compound microscope. We will be looking for intersex (mixed ovarian and testicular tissue) gonads. These have been found in other fish (e.g. European roach, largemouth bass) in other rivers (Jobling et al. 1998, Tyler and Routledge 1998, Vigano et al. 2001, van Aerle et al. 2001, Fahrenthold 2006, Bjerregaard et al. 2006) and are indicative of endocrine disruption. Ovotestes typically result when genetic males are exposed to estrogens during the period of reproductive development.

Liver – RNA will be extracted from liver and the amount of message for the vitellogenin gene will be measured using a very sensitive molecular technique called real time reverse transcriptase polymerase chain reaction (PCR). Vitellogenin is a yolk protein precursor normally synthesized by females when they are producing eggs. The gene is turned on by estrogen and is normally not expressed in males. The presence of vitellogenin mRNA in males is a biomarker of exposure to environmental estrogen(s) (Denslow et al. 1999). Conversely, if females are exposed to chemicals with androgenic (male hormone) activity, vitellogenin gene expression can be inhibited (Stanko and Angus 2007).

Water Analyses

Water sample will be analyzed using two methods: HPLC, which separates the organic compounds in the sample chromatographically, and the yeast estrogen screen (YES) assay, a highly sensitive cell-based assay that detects compounds with estrogenic activity.

HPLC - If intersex male stonerollers are observed, water samples collected up- and downstream of the WWTPs will be analyzed for estrogenic chemicals. The analyses will involve the use of high performance liquid chromatography (HPLC) techniques. The water will be passed through a C18 solid phase extraction column which will remove the organic chemicals from the water. The columns will then be extracted into a small volume of acetonitrile (an organic solvent), producing a much more concentrated solution of the chemicals than was in the original water sample. The organic compounds are then separated via HPLC using an acetonitrile gradient. The most frequently observed estrogens in treated domestic wastewater are estradiol (estrogen), ethynylestradiol (the synthetic estrogen in birth control pills), and estrone (a breakdown product of estrogen). These can be identified in HPLC chromatograms by comparing their retention times to those of known standards (Figure 3).

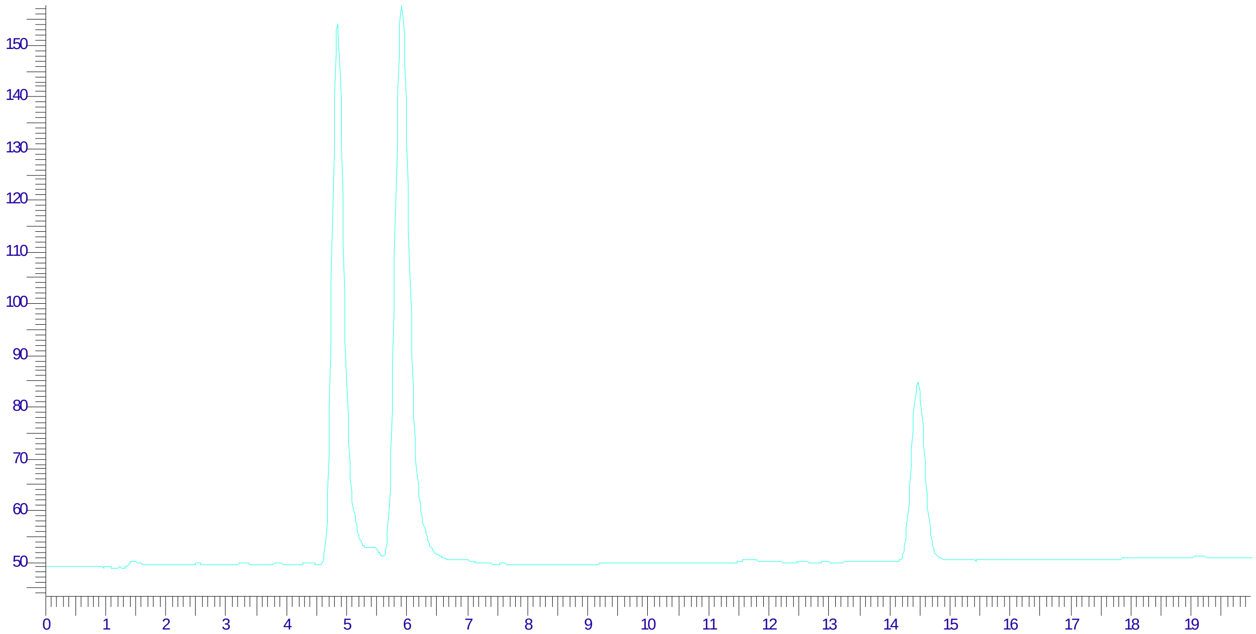

Figure 3. HPLC chromatogram of a water sample containing estradiol, ethynylestradiol and progesterone, a steroid precursor. The horizontal axis is retention time (minutes); the vertical axis is in absorbance units at 235 nm.

Yeast estrogen screen - The YES assay utilizes genetically engineered baker's yeast cells (*Saccharomyces cerevisiae*) containing a constitutively expressed gene for the human α estrogen receptor and an estrogen response element coupled to the *E. coli* β-galactosidase reporter gene (Arnold et al. 1996). The assay has two parts: first, cells are incubated overnight in a 96-well cell culture plate in the presence of the compound to be tested. Different wells contain different concentrations (or dilutions) of the test compound, triplicate replicates are done of each. If the compound binds to the estrogen receptor, the complex will in turn bind to the estrogen response element and stimulate expression of the β-galactosidase gene. After the incubation period, the cells are placed in a buffer which lyses the cells and which contains the chromogenic β-galactosidase substrate O-Nitrophenyl β-D-galactopyranoside (ONPG). If the cells have produced β-galactosidase, the enzyme will cleave the ONPG and produce a yellow product. The amount of product is quantified by reading the absorbance at 405 nm. The absorbance is proportional to the estrogenic activity of the compound with which the cells were incubated and, with appropriate controls, can be stated as E2 equivalents. The concentration of E2 in water that can have endocrine disrupting effects (<5 ng/L in whole life exposure studies) has been determined (Fenske et al. 2005). Water samples to be screened for estrogenic activity with the yeast estrogen screen assay will be prepared as for HPLC, except that after drying under nitrogen, the samples will be reconstituted in dimethylsulfoxide (DMSO), since methanol is toxic to living cells. The extraction process concentrates the organics in the water sample by a factor of 2,000. Arnold et al. were able to detect E2 in the yeast estrogen screen assay at concentrations down to 0.01 nM. This back calculates to an ability to detect E2 in the original water sample at a concentration of 0.15 ng/L.

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BUDGET

	Cost Categories	Estimated Costs
1	Salaries and Wages A. Principal Investigators	
	R. Angus (1 month)	$9,435.00
	B. Other Professional Staff No.0, Man Months:0	$0.00
	C. Graduate Students 1 Master's student (12 months)	$14,400.00
	D. Others (Undergrad.) No. 0, Man Months: 0	$0.00
	TOTAL SALARIES AND WAGES	$23,835.00
2	Fringe Benefits (24.4%, PIs only)	$2,302.14
3	Supplies	$4,400.00
4	Equipment	$0.00
5	Subcontracts or Consultants	$0.00
6	Travel	$485.00
7	Other Direct Costs	$0.00
8	Total Direct Costs	$31,022.14
9	Indirect Costs@ 45% MTDC	$13,959.96
10	TOTAL ESTIMATED COST	$44,982.10