

## PRELIMINARY OBSERVATIONS OF ESTROGENIC ACTIVITY IN SURFACE WATERS OF THE MYAKKA RIVER, FLORIDA

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**ABSTRACT:** *Environmental pollutants capable of disrupting endocrine function and impairing hormonally regulated processes such as animal reproduction pose significant threats to the health of aquatic wildlife populations. Unfortunately, broad assessments of endocrine-disrupting activity are difficult to perform for large aquatic ecosystems because of the substantial number of hormonally active contaminants and the expense of conducting specific chemical analyses for each of these compounds. This report describes pilot attempts to detect the presence of endocrine-disrupting pollutants in the surface waters of southwest Florida estuaries using the E-SCREEN, a short-term, cell culture bioassay that measures the occurrence and abundance of estrogen-mimicking substances by their ability to induce proliferation in estrogen-sensitive, MCF-7 human breast cancer cells. Using this technique, estrogenic activity was detected in multiple sites in the Myakka River, a major tributary of the Charlotte Harbor estuary. Estrogenic substances appear to occur primarily near areas of significant residential development in the lower portions of this river system. Therefore, it is likely that these contaminants enter the Myakka River via input of stormwater runoff and/or unprocessed sewage, perhaps from overburdened or damaged septic systems.*

**Key Words:** Endocrine disruption, E-SCREEN, xenoestrogens, pollution, Myakka River, Charlotte Harbor, Sarasota County, Charlotte County, Florida

DURING the past decade, there has been growing concern about the environmental impact of man-made pollutants capable of interacting with the vertebrate endocrine system and impairing hormone-regulated processes, in particular, animal development and reproduction (see National Research Council, 1999 for review). These “endocrine disrupting compounds (EDCs),” as they are commonly referred to, include a number of common anthropogenic pollutants, such as synthetic hormones used in human contraceptives, natural hormones used to promote growth of livestock, organochlorine pesticides, industrial chemicals (e.g., polychlorinated biphenyls), byproducts of pulp and paper production (e.g., dioxins), and breakdown products of plastics and detergents. Few regions in the world have received as much attention as the state of Florida, with regards to the potential effects of these compounds on aquatic wildlife. In fact, exposure to elevated concentrations of EDCs has been linked with reproductive anomalies in several species residing in Florida rivers and lakes including the American alligator (*Alligator mississippiensis* [Daudin]) (Semenza et al., 1997; Guillette et al., 1999; Gunderson et al., 2004), brown bullhead catfish

(*Ameriurus nebuosus* Lesueur) (Gallagher et al., 2001), largemouth bass (*Micropterus salmoides* Lacepède) (Orlando et al., 1999; Sepúlveda et al., 2001; 2002), and mosquitofish (*Gambusia holbrooki* Baird and Girard) (Bortone and Cody, 1999; Parks et al., 2001; Toft et al., 2003). Exposure to some of these pollutants, organochlorine pesticides in particular, has also been implicated as a possible cause of genital disorders in the endangered Florida panther (*Puma concolor coryi* Bangs) (Facemire et al., 1995). Given these concerns, it is important to determine the identities, sources, and concentrations of EDCs in Florida waters so that policies to reduce the release and ecological impacts of these compounds can be developed.

Due to the large number of potential environmental EDCs and the expense of conducting chemical-specific measurements for each of these compounds, it is generally difficult to perform broad assessments of EDC concentrations in large-scale aquatic ecosystems. However, recent studies have demonstrated that a cost-effective approach for identifying elevated concentrations of EDCs in environmental matrices is to use short-term, cell culture bioassays to pre-screen samples for EDC activity prior to performing more specific chemical analyses (Körner et al., 1999; Oh et al., 2000; Furuichi et al., 2004; Schiliró et al., 2004; Soto et al., 2004; Leusch et al., 2005). One of the most commonly used bioassays in such studies is the E-SCREEN, a cell culture technique that is capable of detecting the presence and concentration of compounds that mimic the natural hormones, estrogens, by their ability to stimulate proliferation of estrogen-dependent MCF-7 human breast cancer cells (Soto et al., 1995). Using this method, estrogenic activity has been detected in rivers bordering rural sites in central Korea (Oh et al., 2000) and areas downstream of cattle feedlots in the Elkhorn River, Nebraska (Soto et al., 2004). Based on these studies, this technique shows promise as an effective approach for screening EDC activity in Florida rivers and estuaries, which may accumulate estrogenic compounds as a result of agricultural activity, stormwater runoff, and wastewater discharge.

The present study describes recent efforts to screen for EDC activity in estuarine waters in southwest Florida, a rapidly developing coastal area. In particular, the E-SCREEN bioassay was used to characterize the presence and distribution of estrogenic substances in surface waters of the Myakka River, a tributary of the Charlotte Harbor estuary. This project was a component of a larger study focused on identifying the ecological threats that EDCs pose to the Charlotte Harbor ecosystem and its resident wildlife.

**MATERIALS AND METHODS**—Surface water samples were obtained from 15 sites along the length of the lower Myakka River between Sarasota and Charlotte counties during a 2-day period in September 2004 (Fig. 1). The sampling period occurred one month after a major hurricane (Hurricane Charley) passed to the east of the Myakka River. Water samples were collected in pre-cleaned, 1-L amber glass bottles and held on ice until returned to the laboratory. Samples were stored at 4°C for a maximum of 2 weeks until processed for extraction of active components.

Samples were measured to 1 L and filtered through 10- $\mu$ m stainless steel wire mesh for removal of particulate matter. Afterwards, samples were transferred to 2-L glass separatory funnels and active components extracted following methods described in Soto and co-workers (2004). Briefly, each sample was extracted three times with 60 mL of dichloromethane (DCM) with shaking for 2 min. Water and DCM fractions were allowed to separate for 10 min, after which DCM fractions were filtered through a stemmed funnel filled to a depth of  $\sim$ 2 cm with solvent-rinsed sodium sulfate. Filtered extracts were

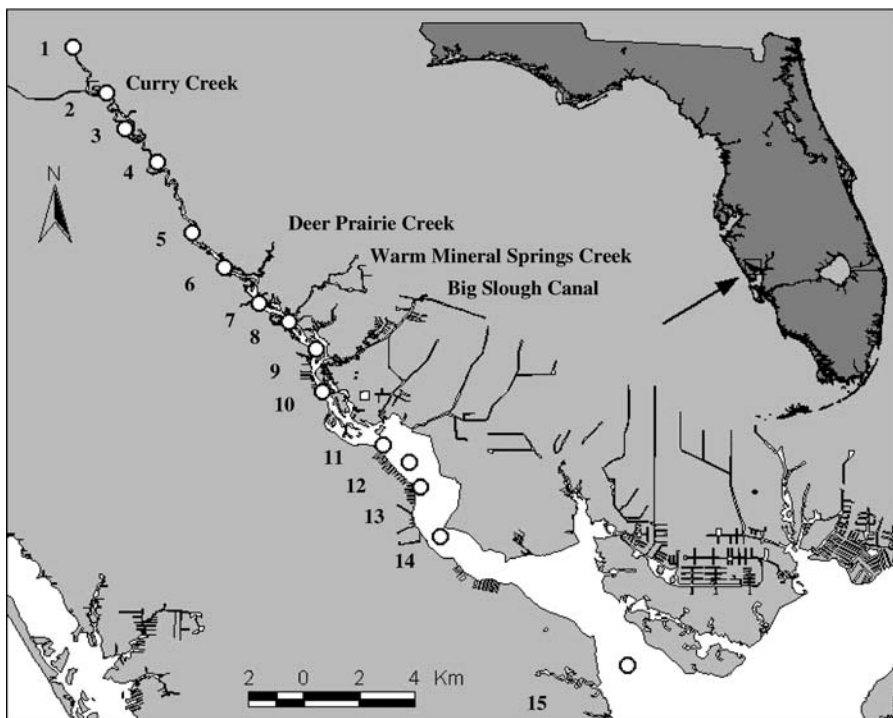


FIG. 1. Map of 15 water collection sites within the central to lower Myakka River, Florida. Nearby tributaries are identified for reference.

combined in a single 500-mL glass bottle and concentrated to a volume of 1–1.5 mL using a RapidVap N<sub>2</sub> evaporation system (Labconco, Kansas City, MO). Samples were then transferred to 1.5-mL glass vials and evaporated to near dryness using a gentle stream of nitrogen. Extracts were solvent-exchanged with 1 mL dimethyl sulfoxide (DMSO) and nitrogen was applied for an additional hour to remove residual DCM. Extracts were stored at –20°C until used in the E-SCREEN assay.

Estrogen-sensitive MCF-7 BOS human breast cancer cells (see Villalobos et al., 1995 for a comparison of MCF-7 cell lines) were kindly provided by Drs. A.M. Soto and C. Sonnenschein, Tufts University (Boston, MA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.5 I.U./mL penicillin, 50 µg/mL streptomycin (Mediatech, Inc, Herndon, VA), 2.25% sodium bicarbonate, 0.584% L-glutamine, 0.01% sodium pyruvate, and 5% heat inactivated fetal bovine serum (FBS) at 37°C with 5% CO<sub>2</sub> and 95% air under saturating humidity.

A miniaturized E-SCREEN assay was developed using the reduction of MTT [(3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide)] as a specific indicator of cell proliferation (Tian et al., 2002). Cells were harvested with phosphate buffered saline (PBS) containing 0.25% trypsin and 0.03% EDTA, re-suspended in 5% FBS DMEM, and plated onto 96-well plates at an initial density of 5,000 cells/well in 200 µL of medium. Following a 24 hour period during which cells adhered to plates, medium was replaced with 100 µL phenol red-free DMEM containing 5% FBS previously treated with dextran-coated charcoal (Sigma-Aldrich, St. Louis, MO) to minimize endogenous estrogenic activity. Cells were exposed to one of four dilutions (0.1–100 pM in DMSO, Fig. 2) of 17β-estradiol (E<sub>2</sub>, Sigma-Aldrich) or one of four dilutions of sample extracts (equivalent to 1.25–10 µL of river water) for a period of 5 days. Controls were exposed to 0.1% DMSO, the concentration used in all treatment assays. All exposures were performed in triplicate.

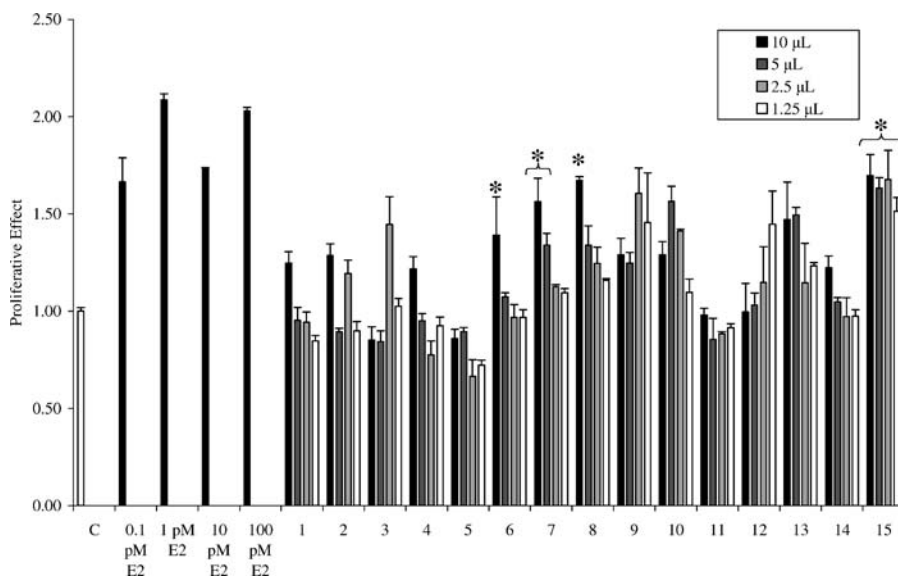


FIG. 2. Cell proliferation in 100- $\mu$ L cultures of MCF-7 cells exposed to DMSO (control, C), 0.1–100 pM 17 $\beta$ -estradiol (E<sub>2</sub>), and extract representing the equivalent of 1.25–10  $\mu$ L of river water obtained from sites 1–15 in the Myakka River. Bars represent means  $\pm$ SE. \*Cells exposed to extracts from sites 6 (10  $\mu$ L), 7 (5–10  $\mu$ L), 8 (10  $\mu$ L), and 15 (1.25–10  $\mu$ L) exhibited proliferative responses significantly greater than that observed in hormone-free controls (ANOVA with Bonferroni correction,  $P < 0.05$ ).

Following the culture period, 20  $\mu$ L of 5 mg/mL MTT prepared in 10 mM phosphate buffered saline (PBS) was added to each well. After an incubation period of 4 h, 100  $\mu$ L of a solubilizing solution (0.01 N HCl/10% SDS in PBS) was added to each well and allowed to react overnight. The following day, optical density of wells was measured at 570 and 630 nm using a microplate reader. MTT is reduced by metabolically active cells to an insoluble purple formazan product, which is released into the culture supernatant by solubilization. Therefore, the degree of color reaction in a microplate well is proportional to cellular activity.

The proliferative effect (PE) of E<sub>2</sub> and water extracts was calculated to characterize estrogenic activity (Körner et al., 1999; Oh et al., 2000). The PE is level of cell proliferation induced by E<sub>2</sub> or water extracts, respectively, relative to that in estrogen-free controls and was calculated as:

$$PE = OD_{\text{estradiol or water extracts}} / OD_{\text{control}} \quad (1)$$

where  $OD_{\text{estradiol or water extracts}}$  refers to the optical density measurements of samples exposed to E<sub>2</sub> or water extracts and  $OD_{\text{control}}$  refers to the mean optical density of DMSO-exposed controls. Measurements of PE were grouped by sample and compared with the estrogen-free control by one-way ANOVA with Bonferroni error protection to detect the presence of significant estrogenic activity. Since the goal of the larger study was to “pre-screen” numerous (>200) water samples for evidence of water-borne xenoestrogens, the relative potency or estradiol equivalency factor (EEF; Körner et al., 1999) of samples was not calculated because it requires the use of >4 dilutions of each extract and a greater amount of cells than that regularly available. However, EEF measurements will be calculated for sites in which estrogenic activity is consistently detected and presented in future reports.

**RESULTS**—Significant estrogenic activity was detected in 4 of the 15 water samples obtained from the Myakka River (ANOVA,  $P < 0.05$ ; FIG. 2). Together,

these samples appeared to represent two general locations in which elevated concentrations of estrogenic compounds were present. The first region included sites 6, 7, and 8, and lies between two tributaries, Deep Prairie Creek and Warm Mineral Springs Creek. This region lies near the city of Nort Port and is one of few portions of the river within Sarasota County that has experienced a significant degree of coastal development (Southwest Florida Water Management District [SWFWMD], 2004). The second region included site 15 and is located within Charlotte County in the southernmost portion of the river. This region is adjacent to the city of Port Charlotte and is also an area of significant urban development (SWFWMD, 2004). No other sites appeared to possess detectable levels of estrogenic compounds.

**DISCUSSION**—The results presented in this study demonstrate the presence of significant hormonal activity in portions of the Myakka River within Sarasota and Charlotte Counties. Elevated concentrations of estrogenic substances were detected in multiple water samples obtained from two regions of the river during late summer-early fall, a period of increased precipitation and high river discharge. However, little evidence of estrogenic activity was observed in other locations in the central to lower portions of the river. These findings suggest that estrogen mimics pose low risks to the ecological health of the Myakka River in comparison with other tributaries of the Charlotte Harbor estuary in which widespread estrogenic activity has been consistently detected (e.g., Caloosahatchee River, Cox and Gelsleichter, unpublished data). Nonetheless, future research may be necessary to identify the substances that contribute to estrogenic activity in certain portions of the river given that samples from these regions are occasionally capable of inducing potent biological responses in the E-SCREEN assay.

The presence of significant estrogenic activity in sites 6–8 and 15 is consistent with what is known regarding land usage in these areas (SWFWMD, 2004). The first region is adjacent to a major urban roadway (US 41) and a large residential subdivision in Nort Port, and likely receives nontrivial amounts of both residential discharge and stormwater runoff. The second location is situated near the most developed portion of the entire sampling area, which has been reported to contain a high density of on-site sewage disposal systems (Lipp et al., 2001). In contrast, the region between the northernmost sampling site and sites 6–8 is largely undeveloped because Sarasota County has designated virtually its entire portion of the river a conservation area. The region between sites 6–8 and 15 is also bordered by conservation lands and is only sparsely populated.

Based on the land use patterns described above, wastewater-related compounds likely contribute greatest to estrogenic activity present in the central and southern Myakka River. This category of EDCs includes human-excreted hormones (both natural and synthetic), detergent metabolites, and plasticizers, which together represent nearly 80% of the total organic wastewater contaminants commonly detected in U.S. streams and rivers (Kolpin et al., 2002). Organochlorine pesticides may also be responsible for estrogenic activity in these regions based on the high per acreage application rates of some of these compounds (e.g., endosulfan) in the southwest Florida region. Anabolic agents excreted by livestock (Soto et al., 2004) presumably

contribute little to the total estrogenicity of the mid- to lower portions of the river. However, these compounds may be more prevalent in surface waters to the north of the sampling area, where agricultural activity is more concentrated.

Although estrogenic activity in the lower Myakka River is present in areas of significant residential development, its abundance in surface waters may not be directly linked with seasonal changes in human activity. This premise is based on previous studies that have demonstrated elevated concentrations of fecal indicator organisms and human enteroviruses in the surface waters of the lower Myakka River primarily during the less-populated, wet season (Lipp et al., 2001). During such periods, these compounds enter the riverine environment more substantially via increased stormwater runoff and backflow of flooded and/or damaged sewerage systems (Euripidou and Murray, 2004). In consideration of these results, the authors hypothesize that estrogenic contaminants may pose their greatest and, perhaps, only threat to the Myakka River ecosystem during periods of flooding associated with increased precipitation and extreme weather events (e.g., tropical storms, hurricanes). This would be especially true for the lower segment of the sampling area, which experienced significant chemical contamination just prior to September 2004 due to flooding and structural damage caused by Hurricane Charley. Interestingly, an increase in the abundance of fecal organisms may also influence the estrogenic activity of surface waters because they are capable of re-converting inactive conjugates of natural and synthetic estrogens excreted by humans back to their non-conjugated, active form (Ying et al., 2002).

In conclusion, this study has demonstrated the presence of estrogenic EDCs in surface waters of the lower Myakka River near regions of high residential development. Identification of the specific contaminants that contribute to hormonal activity in this region will be necessary in future studies based on the potential health risks that these chemicals pose to wildlife populations. However, given the limited presence of xenoestrogens in this river system, EDC screening in other areas of the Charlotte Harbor ecosystem that consistently possess evidence of estrogenic contamination may be of greater concern at this time. To this end, the present study has also demonstrated the value of the E-SCREEN bioassay as a useful technique for pre-screening Florida waters for evidence of these harmful, yet rarely measured contaminants.

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