

# Proceedings of the Endocrine Disruptors in the Environment - A U.S. EPA Science To Achieve Results (STAR) Progress Review

NOVEMBER 16, 2008  
TAMPA MARRIOTT WATERSIDE HOTEL  
TAMPA, FL



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## **EXECUTIVE SUMMARY**

For other references also see:

U.S. EPA Endocrine Disruptors Program 2006 STAR Progress Review Workshop:  
<http://es.epa.gov/ncer/publications/workshop/endocrineworkshp71306.html>

Fate and Effects of Hormones in Waste From Concentrated Animal Feeding Operations (CAFOs) Workshop:  
[http://es.epa.gov/ncer/publications/workshop/08\\_20\\_07\\_cafo.html](http://es.epa.gov/ncer/publications/workshop/08_20_07_cafo.html)

# Endocrine Disruptors in the Environment: A U.S. EPA Science To Achieve Results (STAR) Progress Review

Tampa Marriott Waterside Hotel  
700 S Florida Avenue, Tampa, FL 33602  
(813) 221-4900

November 16, 2008

## Agenda

**Meeting Purpose:** The U.S. Environmental Protection Agency (EPA) and the National Center for Environmental Research (NCER) is holding a public meeting in conjunction with the SETAC North America Annual Meeting to review progress on characterizing sources of exposure and environmental impacts of endocrine disruptors. This meeting provides the opportunity for researchers to discuss recent findings, common methodological issues, and upcoming investigations on endocrine disruptors in the environment.

- 8:00 a.m. – 8:30 a.m.**                    **Registration**
- 8:30 a.m. – 8:45 a.m.**                    **Welcome**  
*Susan Laessig, EPA, NCER*
- Introduction—Endocrine Disruptors in the Environment**  
*Elaine Francis, National Program Director for Endocrine Disruptors Research*
- 8:45 a.m. – 10:30 a.m.**                    **Assessing Aquatic Exposure to Endocrine Disrupting Chemicals (EDCs)**
- 8:45 a.m. – 9:15 a.m.                    **Methods To Measure Indicators of Exposure in Real-World Aquatic Environments**  
*Jim Lazorchak, EPA, National Exposure Research Laboratory*
- 9:15 a.m. – 9:45 a.m.                    **Developing Rapid Assessment Tools To Evaluate the Biological Effects of Complex and Biologically Active Mixtures**  
*Heiko Schoenfuss, St. Cloud State University*
- 9:45 a.m. – 10:30 a.m.                    **Development of Receptor- to Population-Level Analytical Tools for Addressing EDC Exposure in Wastewater-Impacted Estuarine Systems**  
*Lee Ferguson, University of South Carolina*
- 10:30 a.m. – 10:45 a.m.**                    **Break**
- 10:45 a.m. – 12:15 p.m.**                    **Innovative Methods for Rapid Detection**
- 10:45 a.m. – 11:15 a.m.                    **Systems Approach To Assessing Cumulative Exposure to EDCs**  
*Gerald LeBlanc, North Carolina State University*
- 11:15 a.m. – 11:45 a.m.                    **Integrated Microfluidics, Bioreporting, Separations, Vibrational Spectroscopy, and Microcantilever Nanomechanical Evaluation of EDCs (LTG-2)**  
*Michael Sepaniak, University of Tennessee*
- 11:45 a.m. – 12:15 p.m.                    **Rapid Detection of Trace Endocrine Disrupting Chemicals in Complex Mixtures: A Full-Spectrum Deconvolution Technique With a UV-Transparent Passive Concentrator**  
*Tohren Kibbey, University of Oklahoma*
- 12:15 p.m. – 1:15 p.m.**                    **Lunch (on your own)**

**1:15 p.m. – 3:15 p.m.**

**Fate and Effects of Hormones in Animal Waste—Cattle, Swine, and Poultry**

1:15 p.m. – 1:45 p.m.

**Integrated EPA Studies on EDCs in CAFOs**

*Vickie Wilson*, EPA, National Health and Environmental Effects Research Laboratory

1:45 p.m. – 2:15 p.m.

**Persistence and Discharge of Hormones From Tile-Drained Fields Receiving Animal Wastes and Their Impact to Aquatic Organisms**

*Linda Lee*, Purdue University

2:15 p.m. – 2:45 p.m.

**Effects of Cattle Manure Handling and Management Strategies on Fate and Transport of Hormones in the Feedlot and the Field**

*Daniel Snow*, University of Nebraska, Lincoln

2:45 p.m. – 3:15 p.m.

**Transport and Transformation of Natural and Synthetic Steroid Hormones at Beef Cattle and Dairy Concentrated Animal Feeding Operations (CAFOs)**

*Edward Kolodziej*, University of Nevada, Reno

**3:15 p.m. – 3:30 p.m.**

**Break**

**3:30 p.m. – 5:30 p.m.**

**Fate and Effects of Hormones in Animal Waste—Cattle, Swine, and Poultry (*continued*)**

3:30 p.m. – 4:00 p.m.

**Assessing Occurrence, Persistence, and Biological Effects of Hormones Released From Livestock Waste**

*Jocelyn Hemming*, University of Wisconsin, Madison

4:00 p.m. – 4:30 p.m.

**An Integrated Approach To Developing a Total Facility Estrogen Budget at a Swine Farrowing CAFO**

*Seth W. Kullman*, North Carolina State University

4:30 p.m. – 5:00 p.m.

**Fate of Hormones in Waste From Concentrated Broiler Feeding Operations**

*Miguel L. Cabrera*, University of Georgia

5:00 p.m. – 5:30 p.m.

**Environmental Transport, Fate, and Ecological Effects of Steroids From Agricultural Application of Poultry Litter and Watershed Scale Evaluations of Existing and Novel Management Strategies**

*Lance Yonkos*, University of Maryland

**5:30 p.m.**

**Adjournment**

**6:30 p.m.**

**Optional Dinner (Jackson's Bistro; 601 S. Harbour Island Boulevard; Tampa, FL 33602; Phone: 813-277-0112)**

U.S. Environmental Protection Agency (EPA)  
**Endocrine Disruptors in the Environment:**  
**A U.S. EPA Science To Achieve Results (STAR) Progress Review**

**Tampa Marriott Waterside Hotel**

**November 16, 2008**

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# **INTRODUCTION**





## U.S. Environmental Protection Agency Endocrine Disruptors Research Program

### **ENDOCRINE DISRUPTORS IN THE ENVIRONMENT**

In 1996, the U.S. EPA initiated an Endocrine Disruptors Research Program to provide the scientific information needed to reduce or prevent unreasonable risks to humans and wildlife from exposures to chemicals that interfere with the function of the endocrine system. An endocrine disruptor or endocrine disrupting chemical (EDC) is an exogenous substance that causes adverse health effects in an intact organism, or its progeny, secondary to changes in endocrine function. The objective of EPA's EDC Research Program is to improve our knowledge and understanding of endocrine disruptors in the environment so that we can improve our methods of risk assessment and risk management.

Progress in the last decade of EPA research has linked endocrine effects in wildlife to exposure to organotin, organochloride pesticides, polychlorinated biphenyls (PCBs), and steroid hormones. Current research efforts are characterizing EDCs in discharges from wastewater treatment plants, concentrated animal feeding operations (CAFOs), and pulp and paper mills. Critical to these studies is the development of methods to detect and measure hormones and endocrine activity. Based on preliminary observations, EPA initiated an effort to assess the fate and effects of hormones, primarily estrogens and androgens, and determine the environmental impact of current CAFO waste management strategies. EPA scientists are working cooperatively with university researchers through the Science To Achieve Results (STAR) grants program to develop the next generation of methods, determine fate and transport of EDCs in the environment, and assess the impact of EDCs from identified sources.

Future research efforts will continue to complement research programs conducted at other federal agencies, in other countries, and by industry. Important areas for future research include interpreting data from prioritization and screening assays, developing risk assessment approaches for cumulative effects of mixtures, and assessing impacts on ecosystem health. EPA is well positioned to continue by providing research results for improving our understanding of endocrine disruptors, their impacts on human health and the environment, and risk management options.

EPA's EDCs Research Program consists of research conducted by EPA's national laboratories and supported through the STAR competitive peer-reviewed grants program. Since 1996, EPA has spent more than \$130 million on EDCs research. Of that total, more than \$95 million has supported research in EPA's laboratories and approximately \$35 million has supported extramural research. Those expenditures have funded 59 grants at 37 institutions in 25 states. The research is answering specific pressing questions about the effects on wildlife and human populations, epidemiologic approaches, modeling low-dose effects, methods to estimate environmental exposure and occurrence, and fate and effects of hormones in animal waste.

# **ASSESSING AQUATIC EXPOSURE TO ENDOCRINE DISRUPTING CHEMICALS (EDCs)**

## **Methods To Measure Indicators of Exposure in Real-World Aquatic Environments**

*Jim Lazorchak<sup>1</sup>, Robert Flick<sup>1</sup>, Adam Bailes<sup>1</sup>, David Bencic<sup>1</sup>, and Gary Ankley<sup>2</sup>*

<sup>1</sup>National Exposure Research Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, Cincinnati, OH; <sup>2</sup>National Health and Environmental Effects Research Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, Duluth, MN

Most of what is known about the implications of endocrine disrupting chemicals (EDCs) in the environment is somewhat anecdotal. There are numerous reports of gonadal histological abnormalities (Potomac, United Kingdom Rivers), alterations in sex ratios (Boulder Creek, Colorado), and high vitellogenin protein levels in fish below wastewater treatment plants. There has been some linear regression modeling describing the relationship between estrogens and relative fecundity linked to population models to predict trajectories of decreases in population size. However, there remains no clear and direct field measured linkage between observed abnormalities and population-level effects. In the case of the Potomac, community-level measures such as indices of biotic integrity showed no relationships to ova-testis in smallmouth bass. One recent study in 2007, in which a whole lake was dosed with ethynylestradiol, did show a collapse of fathead minnows, pearl dace, and lake trout populations over a 5-year study in which the lake was dosed for 3 years with ethynylestradiol. Indicators were measured in this study from the molecular level to the whole animal level to link such measures to population effects. Only weight-of-evidence relationships could be drawn from this study. Some of the molecular level indicators, however, did show an indication of estrogen exposure before the Catch Per Unit Effort (CPUE) fish collections showed significant reductions in population size. Understanding when and how exposures occur is a critical factor that impacts the ability to understand the potential impacts of EDCs. This presentation will provide an overview of some recent approaches for the detection and characterization of EDC exposures using several ongoing small-scale studies. In addition, we will discuss some experimental approaches aimed at linking real-world exposure and effects due to EDC exposures.

## **Developing Rapid Assessment Tools To Evaluate the Biological Effects of Complex and Biologically Active Mixtures**

*Heiko Schoenfuss*

St. Cloud State University, St. Cloud, MN

The aim of this study is to test the hypothesis that mixtures of estrogenic chemicals will have adverse effects on the reproductive health of exposed aquatic organisms that cannot solely be accounted for by the summation of individual effects. In a series of exposure experiments, we assessed the effects of estrogens from several classes of known or suspected endocrine disrupting compounds (natural/synthetic estrogens, alkylphenols, pharmaceuticals, and personal care products) singular and in increasingly complex mixtures on several tiers of the aquatic trophic cascade. We assessed the effects of these compounds on the base of the aquatic food chain (diatoms) and an intermediate consumer (*Daphnia*). Results indicate that the primary producer community can be affected by classes of estrogenic compounds in a fashion that appears to be estrogen receptor independent. For example, the nutritional value of diatoms diminishes in the presence of alkylphenols, but is unaffected by natural and synthetic estrogens. Several classes of estrogenic compounds also increase the fecundity of daphnids in 21-day exposure experiments (synthetic estrogen). Furthermore, we have determined that daphnids are unable to distinguish between food sources tainted by exposure to estrogenic compounds (estrogens, alkylphenols), thus increasing the likelihood of exposure. In larval fathead minnows, exposure to natural estrogens and pharmaceuticals decrease their ability to respond to a threat stimulus with an effective evasive maneuver. This effect is manifested differentially when larvae are tested after embryonic or early larval exposure and is enhanced if the animal was exposed to compound mixtures. Adult male fathead minnows responded to mixtures of natural and synthetic estrogens more strongly than to single compound exposures. Adverse effects included vitellogenin induction, the inability of exposed males to compete for nest sites, and occurrence of intersex. Although the responses to exposures at environmentally relevant concentrations did exhibit greater complexity than would have been predicated from a simple mode-of-action response, effects were robust across experiments and frequently across endpoints, including changes in brain neurotransmitter concentrations. The observed effects at the base, midpoint, and apex of the trophic cascade suggest that biologically active compounds in environmentally relevant mixtures can impact the reproductive potential of aquatic vertebrates.

## Development of Receptor- to Population-Level Analytical Tools for Assessing EDC Exposure in Wastewater-Impacted Estuarine Systems

P. Lee Ferguson<sup>1</sup> and G. Thomas Chandler<sup>2</sup>

<sup>1</sup>Department of Chemistry and Biochemistry, University of South Carolina, Columbia, SC;

<sup>2</sup>Arnold School of Public Health, University of South Carolina, Columbia, SC

**Objectives:** This project is aimed at the production of new tools for identifying and quantifying endocrine-active contaminants in complex environmental mixtures and for defining endocrine disruptor exposure in sensitive estuarine systems. The work complements and extends previous and current research on the development of targeted, quantitative analytical methods and bioassays for specific classes of endocrine disrupting chemicals (EDCs) in environmental samples. The specific objectives of the proposed research are to:

- (1) Develop nuclear hormone receptor-affinity extraction techniques as tools for isolating EDCs from complex wastewater mixtures.
- (2) Apply these methods in combination with high-performance mass spectrometry for activity-directed analysis of EDCs in wastewater and estuarine receiving waters on the South Carolina coast.
- (3) Utilize sensitive vertebrate (zebrafish) and invertebrate (copepod) EDC-exposure laboratory bioassays to link exposure measurements (above) to biological effects.
- (4) Apply novel biomolecular endpoints to assess EDC exposure in field populations of sensitive meiobenthic invertebrates in wastewater-impacted estuarine environments.

**Approach:** Our research plan will address these objectives through development of novel bioanalytical methods and application of bioassays already in use by the investigators. Specifically, we will utilize commercially available estrogen, androgen, and thyroid hormone and custom ecdysone/ultraspiracle recombinant nuclear hormone receptors to construct bioaffinity-extraction columns for identifying, concentrating, and purifying receptor-binding EDCs from two important wastewater discharge regimes impacting the South Carolina coast (surface water discharge and land application). Isolates will then be analyzed using HPLC-MS/MS (quadrupole/time-of-flight and triple quadrupole) and GC-MS for both target (e.g., estrogens, alkylphenols, hydroxylated PCBs, and bisacylhydrazine insecticides) and non-target EDCs. *In vivo* activity of receptor-isolated EDCs will then be examined by sensitive laboratory endocrine assays, including vitellogenesis in male fish and copepods, and population/molecular endpoints in copepods, to provide a link between measured EDCs and biological effects. Finally, as a field validation of analytical and laboratory bioassay results, we will utilize biomolecular endpoints (ecdysteroid and lipovitellin titres) to assay EDC exposure in field populations of estuarine meiobenthic copepods proximal to a coastal golf course receiving wastewater irrigation.

**Expected Results and Benefits:** The proposed work will, for the first time, provide activity-based analytical tools for assessing potential EDC exposure in complex environmental mixtures. An important component of our proposed work is the linkage between mechanistic analytical methods (defining *potential* exposure) and sensitive laboratory and field bioassays (defining *actual* exposure). Further, our focus on both vertebrate (estrogen, androgen, and thyroid) and invertebrate (ecdysteroid) EDC pathways will provide the scientific community with a broad set of tools for performing holistic studies of endocrine disruption potential in complex mixtures. Finally, our focus on wastewater-impacted salt-marsh systems is novel and will generate new scientific knowledge related to potential EDC exposure in these environments.

# **INNOVATIVE METHODS FOR RAPID DETECTION**

## **Systems Approach To Assessing Cumulative Exposure to EDCs**

*Gerald A. LeBlanc*

Department of Environmental and Molecular Toxicology, North Carolina State University,  
Raleigh, NC

The goal of this program is to develop an approach for evaluating exposure to endocrine disrupting chemicals based on changes in the expression profile of endocrine-related genes in *Daphnia magna*. Specific objectives are to develop quantitative PCR methods for the detection and quantification of specific target gene products, expose daphnids to a series of endocrine-active and non-active chemicals, and evaluate changes in gene expression profiles that may be indicative of exposure to specific classes of endocrine toxicants. Fourteen gene products were identified for use in the assay that function either as regulatory factors or terminal products along juvenoid and ecdysteroid signaling cascades. Induction of the hemoglobin genes HB1 and HB2, along with the transcription factors HR3 and ECRb, was the most consistent indicator of exposure to juvenoid agonists. Chemicals in the ecdysteroid training set elevated mRNA levels of the transcription factors HR3 and E75, and suppressed mRNA levels of ECRb as well as the hemoglobin HB2 and vitellogenin VTG2 gene products. Temporal differences in the modulation of gene expression were documented for transcription factors along the ecdysteroid signaling cascade. These differences must be considered when interpreting the significance of changes in expression profile of these genes resulting from chemical exposure. Chemicals with putative juvenoid activity included atrazine and triclosan, and effects of cyperoteron acetate on gene expression suggested anti-juvenoid activity. None of the chemicals evaluated provided strong evidence for having ecdysteroidal activity, but several altered the gene expression profile consistent with anti-ecdysteroidal activity. Results demonstrate that a suite of as few as six genes can be used to distinguish chemicals having juvenoid, anti-juvenoid, ecdysteroidal, and anti-ecdysteroidal activity. This detection system has limited utility in the assessment of exposure to vertebrate-relevant endocrine disrupting chemicals (i.e., estrogens, androgens). Unexpectedly, the detection system also was found to be effective in assessing exposure to chemicals that elicit oxidative stress.

This work was supported by EPA grant RD-832739.

## **Integrated Microfluidics, Bioreporting, Separations, Vibrational Spectroscopy, and Microcantilever Nanomechanical Evaluation of EDCs (LTG-2)**

*Michael Sepaniak*

University of Tennessee, Knoxville, TN

Our work serves to answer the following scientific questions: (1) Can modern  $\mu$ -fluidic platforms and approaches facilitate EDC analysis via sample manipulation, concentration, and chemical separations? (2) Can bioassays be reliably performed using surface immobilized receptor proteins as nanomechanical responders in microcantilever array (MCA) transducers? (3) Will advances in substrates and techniques improve the analytical performance of Surface Enhanced Raman Spectroscopy (SERS) to reliably obtain information-rich EDC spectra? (4) Will the analytical figures of merit (sensitivity, selectivity, precision, etc.) of these techniques, singularly and integrated, enhance the effectiveness of endocrine disruptor detection above the currently available method?

The interaction between biologically significant ligands with bioreceptors (e.g., antibodies or nuclear receptor proteins) immobilized on one side of the MC surface causes an apparent surface stress, resulting in static bending (nanomechanics) of the cantilever, which we detect by an optical beam bending technique. The nature of the MC surface and the method by which the bioreceptor is immobilized influence these performance metrics and, hence, we have recently conducted optimization studies involving these conditions. In our work, we demonstrate the exquisite sensitivity (pico-M range) and selectivity for cantilevers with surface immobilized  $\beta$ -ER,  $\alpha$ -ER, and TRG proteins. In this manner, relevant bioaffinity interactions are combined with physical science principles to sense and screen EDCs and TDCs without living cell/animal involvement.

Potential benefits of using multiplexed  $\mu$ -fluidics with SERS nonmetallic particles flowing through them include performing parallel, high-throughput, and sensitive detection/identifications of single or various analytes under easily manipulated conditions. The work that will be described utilizes individually addressable  $\mu$ -channels for high-throughput qualitative and quantitative SERS studies and rapid performance optimization. Reduced dimension fluidic devices should be effective for extractions and separations to enhance detectability and selectivity. In addition to the nanoparticle SERS approach, we are fabricating unique substrates with rationally designed periodic and biologically inspired aggregate-like structures using sophisticated electron beam lithography and other nanofabrication techniques. The performance of these substrates will be shown. The benefits to EDC analysis are that substrates with more “hot” regions may lead to better sensitivity and the ability to uniformly fabricate that will improve reproducibility, issues that have hindered SERS development.

Reasonable progress has been made with integrating separations with SERS, bioreporter elements (receptor proteins) with nanomechanics on MCs, and integrating extraction/concentration with these techniques. A particularly exciting integration we are pursuing is the first-time use of *multiple* bioreceptor proteins in a single MCA system to simultaneously monitor endocrine and thyroid disrupting chemicals and assess the effects of these and other nuclear receptors significant to their synergistic effects on living systems.



The enhanced science and products of this research will reduce uncertainty in risk assessment/management by:

- Improving the existing technology and attacking the problem of EDC exposure and activity monitoring with an arsenal of informative tools such as MCAs, SERS, and chemical separations.
- Clarifying and elucidating which chemicals, and in what combinations, can mimic or inhibit endocrine signaling molecules.
- Adding speed and in-field portability to these analytical techniques.

## **Rapid Detection of Trace Endocrine Disrupting Chemicals in Complex Mixtures: A Full-Spectrum Deconvolution Technique With a UV-Transparent Passive Concentrator**

*Tohren C.G. Kibbey and David A. Sabatini*

School of Civil Engineering and Environmental Science, University of Oklahoma, Norman, OK

Suspected endocrine disrupting chemicals (EDCs) have been detected in many natural waters, and their presence is a potential concern for human and ecological health. Screening waters for EDCs to assess risk can be complex and time consuming, as their low concentrations typically require labor-intensive extraction steps and analyses using specialized laboratory equipment. This project has focused on the development of a method for rapid, low-cost detection of EDCs in natural waters to allow more waters to be tested on a routine basis. The method is based on an ultraviolet-transparent polymer, which can be used as a passive sampler to pre-concentrate EDCs and other dissolved compounds. The polymer itself is then used as an optical cell that can be scanned using a low-cost fiber optic spectrophotometer. A deconvolution method is applied to the spectrum to quantify partitioned components; measured partition coefficient and rate information is then used to determine aqueous concentrations.

Research results show that the method is capable of detecting individual compounds in highly complex aqueous mixtures, and has detection limits that are appropriate for environmental detection of a wide range of different compounds for screening applications. Results of tests of the method will be described, along with implications for application of the method to environmental detection of EDCs.

**FATE AND EFFECTS OF HORMONES IN  
ANIMAL WASTE—CATTLE, SWINE, AND POULTRY**

## Integrated EPA Studies on EDCs in CAFOs

Vickie Wilson

National Health and Environmental Effects Research Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, Research Triangle Park, NC

Preventing and controlling water pollution from concentrated animal feeding operations (CAFOs) is a major priority for the U.S. Environmental Protection Agency (EPA) under the Clean Water Act. CAFOs annually produce more than 500 million tons of animal waste that can pose substantial risks to the environment and public health (EPA, 2003). Currently, very little is known about the fate, transport, exposures, and environmental effects resulting from exposures to the natural steroidal and synthetic hormones in generated waste and potential discharges from CAFOs. The extent of such exposures and their potential impact on human and ecosystem health is largely unknown. However, effluents from both the United Kingdom and the United States have been shown to possess endocrine disrupting activity of sufficient potency to adversely impact reproduction in some fish species. Additionally, there is some evidence that CAFO waste may have substantial potential to introduce hormonally active materials, including high-potency natural and synthetic steroids, into surface and groundwaters, as well as to terrestrial systems (through manure applications).

To address this need, a team of scientists from across EPA's Office of Research and Development (ORD) has been assembled to conduct integrated research concerning hormones in CAFOs. The group includes researchers from the National Health and Environmental Effects Research Laboratory (NHEERL), the National Exposure Research Laboratory (NERL), and the National Risk Management Research Laboratory (NRMRL). Recently, ORD has partnered with EPA grantees through several cooperative agreements to expand ongoing research. The overall goal of these studies is to ascertain whether or not EDCs, primarily estrogens and androgens, contribute to unacceptable risks associated with CAFO waste. Specific goals of the integrated ORD project are multiple, including:

- Develop robust *in vitro* and analytical methods to identify and quantify compounds responsible for endocrine (e.g., androgenic, estrogenic) activity of complex CAFO discharges. This includes providing ORD, EPA's Office of Water, and the regions with evaluated and optimized *in vitro* assays and screening approaches for the evaluation of a variety of effluents.
- Identify ecologically relevant biomarkers in aquatic species (primarily fish) of exposure to estrogenic/androgenic CAFO discharges through the use of state-of-the-art genomic approaches.
- Evaluate the environmental fate, transport, and metabolism of CAFO-derived EDCs relative to occurrence in surface and groundwaters.
- Assess possible ecological impacts of EDCs from CAFOs using a combination of laboratory and field studies.
- Evaluate the capability of existing risk management technologies for CAFOs to reduce exposure to EDCs.
- Characterize the magnitude and extent of the impact of hormones released by CAFOs and determine the impact of current CAFO waste management strategies on the fate and effects of hormones.

One focus of the integrated project, which also will be the primary topic of this presentation, is participation in several collaborative projects using *in vitro* transcriptional activation and binding assays. Two of the goals of this work are to aid in characterization of the estrogenicity and/or androgenicity of environmental effluent samples and to further the understanding of the interactions (i.e., additive, synergistic, antagonistic) of the components of these environmental mixtures to properly assess the risk that they may pose.

## **Persistence and Discharge of Hormones From Tile-Drained Fields Receiving Animal Wastes and Their Impact to Aquatic Organisms**

Linda S. Lee, M. Sepúlveda, C. Jafvert, H. Gall, B. Khan, B. Jenkinson, and S. Sassman

Purdue University, West Lafayette, IN

Evidence indicates that concentrated animal feeding operation (CAFO) waste may have the potential to introduce hormonally active compounds, such as natural and synthetic steroids, into surface and groundwaters and to terrestrial systems through manure applications. There is still little known, however, on hormone persistence in manure-applied fields and subsequent attenuation of hormone release from manure-treated fields, the contribution of tile-drained fields on hormone loads to aquatic systems, and the impact of hormone mixtures relevant to animal wastes on aquatic species. This uncertainty is particularly important to EPA as regulatory efforts to control unacceptable impacts associated with CAFOs are implemented. The primary goal of these studies is to quantify the load and evaluate the effect on aquatic species of natural and synthetic steroids entering the environment through tile-drain discharge of agricultural fields under beef manure broadcasting, subsurface injected swine manure, and lagoon effluent application.

Intensive field monitoring studies are being conducted at the Water Quality Field Station (WQFS) and the Animal Science Research and Education Center (ASREC), both part of Purdue University's infrastructure. We are taking flow-weighted samples over time of drainage at the WQFS from eight plots (each  $\approx 520 \text{ m}^2$ ) fertilized with subsurface injection of swine manure (four in the spring prior to planting and four in the fall after harvest). At the ASREC site, we are taking flow-weighted samples over time at four tile drains: one draining a 200 ha under beef and dairy lagoon effluent irrigation; one draining a 60 ha field fertilized with beef and dairy solids; one draining 160 ha fertilized with beef and dairy solids as well as irrigated with beef and dairy lagoon effluent; and one draining 150 ha fertilized with subsurface injection of swine manure and irrigated with swine and poultry lagoon effluent; and at three sites in the stream (Marshall Ditch) receiving this tile-drain discharge.

Water samples from both drains and stream stations are being analyzed for  $17\alpha$ - and  $17\beta$ -estradiol and trenbolone, and related metabolites (estrone, estriol, trendione, and androstendione) using LC/MS along with traditional water quality measurements. In addition, a subset of samples from both drains and streams are being analyzed for nitrates, phosphate, and dissolved organic carbon; and a subset of stream samples is being analyzed for pesticides used on the associated fields (atrazine, acetochlor, and related metabolites). Chemical and flow data are being compiled to produce chemographs that will be evaluated in light of management activities, landscape details, and weather conditions. Laboratory support studies evaluating the persistence of trenbolone isomers and metabolites in soils show that half-lives ( $t_{1/2}$ ) under optimal temperature and moisture conditions are less than 0.5 days and 4 days, respectively. However,  $t_{1/2}$  increases with increasing soil concentrations and when weather conditions result in dry or lower temperature soil conditions, in agreement with observed hormone concentrations (although low) in tile drain effluent several weeks after application in early spring during which there was no rain. Additional laboratory studies detailing mechanisms controlling persistence and impact in support of the field-scale studies will be presented, including the moisture and temperature effects on  $17\alpha$ - and  $17\beta$ -trenbolone persistence in soil, and stereo-specific sorption of  $17\alpha$ - and  $17\beta$ -estradiol and trenbolone isomers.

Fish sampling to evaluate the potential impact of CAFOs on fish communities was performed at (1) Marshall Ditch, which is bordered by crop production (corn and soybean) and receives field runoff treated with manure from beef and dairy CAFOs; (2) Box Ditch, which is solely crop production, but has drainage from Marshall Ditch; and (3) Ghost Creek (control site), which runs through a recreational area and is bordered by small shrubs and tree cover. Fish data revealed lower species richness and fish numbers in both agricultural ditches compared to that of the control. In addition, the reproductive condition of creek chubs (*Semotilus atromaculatus*), measured as gonad wet weights and later corroborated by histological examination, was decreased in fish sampled from the agricultural ditches. Throughout the summer months, water levels declined in the agriculture ditches and an increase of temperatures occurred. Algal blooms were observed in both ditches along with severely reduced levels of dissolved oxygen throughout the summer. This resulted in an even higher decline in fish species richness and abundance. In comparison, the control site retained higher dissolved oxygen levels, higher total numbers of fish, and greater species richness. These results imply that current ditch management practices have negative impacts on aquatic fauna.

## **Effects of Cattle Manure Handling and Management Strategies on Fate and Transport of Hormones in the Feedlot and the Field**

*Daniel Snow<sup>1</sup>, Shannon Bartelt-Hunt<sup>1</sup>, Bill Kranz<sup>1</sup>, Terry Mader<sup>1</sup>, Charles Shapiro<sup>1</sup>, David Shelton<sup>1</sup>, Simon van Donk<sup>1</sup>, Tian Zhang<sup>1</sup>, David Tarkalson<sup>2</sup>, and Steve Ensley<sup>3</sup>*

<sup>1</sup>University of Nebraska, Lincoln, NE; <sup>2</sup>Agricultural Research Service, U.S. Department of Agriculture, Kimberly, ID; <sup>3</sup>Iowa State University, Ames, IA

This research focuses on the occurrence, fate, and transport of exogenous and endogenous hormones during management of cattle manure produced at four concentrated animal feeding operations through manure application to crop land and conservation buffers. The research objectives include: (1) comparison of hormone concentrations in manure in cattle feedlots under different management strategies, (2) measurement of the effects of manure handling on the stability and availability of hormones, (3) comparison of the effects of land application strategies on the fate and transport of hormones, and (4) determining if grasses fertilized with manure will assimilate hormones. The central hypothesis is that hormones in cattle manure will persist and accumulate in soil, but the fate and transport of hormones will be affected by the waste management and handling strategies. Five research tasks are underway to help meet the objectives of this research. Two feedlot studies have been conducted to help quantify hormone levels in cattle feedlots under different management strategies. Approximately 1,200 feedlot surface and runoff samples have been collected for analysis to quantify steroid hormone concentrations.

Manure collected from the feedlot studies has been collected separately for a comparison of the effects of stockpiling and composting on steroid hormone levels. Stockpiled and composted manure from the first feedlot study was used for a comparison of the effects of tillage practices on the loss of steroid hormones in cropland runoff using rainfall simulators. Stockpiled manure has been applied to conservation buffers and grasses sampled to investigate the potential for compound uptake. Stockpiled and composted manure has been applied to irrigated wheat test plots instrumented with monolithic lysimeters to determine steroid hormone leaching potential. Methods for extraction and analysis of steroid hormone analysis using liquid chromatography-tandem mass spectrometry (LC/MS) in both aqueous and solid samples have been developed and validated. Sample analysis is underway. Laboratory studies using radioisotope labeled steroid hormones and feedlot samples are underway to help identify potential chemical and microbial transformation products, degradation rates, and leaching potential under aerobic and anaerobic conditions. A survey task will begin early next year to sample four existing feedlots and crop fields receiving manure to examine the occurrence of hormones in cattle manure under different climatic and management conditions.



## **Transport and Transformation of Natural and Synthetic Steroid Hormones at Beef Cattle and Dairy Concentrated Animal Feeding Operations (CAFOs)**

*David Sedlak<sup>1</sup>, Edward Kolodziej<sup>2</sup>, and Thomas Harter<sup>3</sup>*

<sup>1</sup>University of California at Berkeley, Berkeley, CA; <sup>2</sup>University of Nevada, Reno, Reno, NV; <sup>3</sup>University of California at Davis, Davis, CA

**Objectives:** This project will assess the occurrence, fate, and transport of synthetic steroid hormones used for beef cattle production and endogenous steroids produced by cattle and cows from concentrated animal feeding operations (CAFOs). We hypothesize that the most important pathways for steroid releases from CAFOs are the discharge of contaminated stormwater runoff and migration in groundwater recharged through animal waste lagoons and animal feeding areas.

**Approach:** The fate of synthetic hormones will be evaluated at research and commercial beef cattle feedlots located in California, Colorado, and Iowa. Synthetic steroid hormones will be evaluated using a sensitive new analytical method that will be developed by modifying existing analytical methods. To quantify the relationship between growth hormone treatment and surface water releases, stormwater runoff samples will be collected at two research feedlots where hormone administration rates and waste handling procedures are rigorously controlled. Additional insight into steroid hormone fate and transport will be obtained by collecting samples at locations throughout two research feedlots and several full-scale commercial beef cattle CAFOs. The fate of endogenous steroid hormones will be evaluated through the collection of samples at dairy CAFOs located in California. The potential for transport of steroid hormones via surface water pathways will be assessed through the collection of samples of stormwater runoff, animal waste lagoons, and other sites that could release steroids during rainstorms. The potential for transport of steroid hormones in groundwater will be evaluated through the collection of groundwater downgradient of leaking animal waste lagoons and a series of tile drains that integrate groundwater recharged over large CAFOs. These results will be complemented by the development of a transport model and soil column studies with synthetic and endogenous steroids.

**Expected Results:** Research conducted as part of this project will provide the U.S. Environmental Protection Agency (EPA) with a better understanding of the relative importance of surface and groundwater pathways for steroid hormone releases from CAFOs. The results also will help EPA develop policy guidelines for agricultural practices designed to minimize steroid hormone releases from CAFOs.

## **Assessing Occurrence, Persistence, and Biological Effects of Hormones Released From Livestock Waste**

Jocelyn Hemming, Martin Shafer, Terence Barry, and James Schauer

University of Wisconsin, Madison, Madison, WI

**Objectives:** The overall goal of this project is to determine the presence, persistence, and biological effects of natural and synthetic hormones that may be released into the environment from concentrated animal feed operations (CAFOs), and to evaluate the effects of different animal waste disposal practices on the fate and activity of these compounds. This research will help to evaluate whether CAFO waste is an important source of endocrine disrupting chemicals in the environment. The specific objectives are to:

- (1) Identify and quantify the suite of estrogenic, androgenic, and progesteronic compounds associated with various types of intensive animal farming.
- (2) Characterize the environmental transport and fate of natural and synthetic steroid hormones that accompany discharges and the disposal of animal wastes from CAFOs in Wisconsin.
- (3) Evaluate how various animal waste handling/management strategies (e.g., lagoon storage and spraying of liquid manure vs. deep-stacking and field application of solid manure) impact the transport, fate, potential exposure, and associated effects of steroid hormones discharged from CAFOs.
- (4) Investigate the ecological effects associated with steroid hormones in animal waste from CAFOs using reproductive, developmental, and gene expression endpoints in fathead minnows.

**Approach:** The objectives of this study will be achieved by sampling representative cattle, dairy, swine, and poultry operations. Waste loading will be estimated by measuring hormones in manure and urine. Transport/fate will be monitored in several environmental and engineered reservoirs such as tile drains, groundwater, soils, waste storage facilities, and field-applied manure slurry. Steroids will be analyzed using LC-MS-MS. To evaluate potential biological activity of the CAFO waste, a suite of molecular- and cellular-based bioassays (e.g., E- and A-screens and transfected yeast assays) will be used on extracts of collected samples. These bioassay results will be compared with compounds measured in the samples to determine if specific compounds present in CAFO waste samples can account for the endocrine activity measured by cellular/molecular bioassays. Additionally, partial life-cycle fathead minnow assays will be conducted using a suite of developmental and reproductive endpoints. The whole animal effects will be correlated with specific molecular- and cellular-based bioassays to identify biomarkers of CAFO-associated endocrine disruption.

**Expected Results:** The project will determine the potential for environmental release of hormones under various waste management practices, degradation of hormones and associated activities in these practices, the persistence of residuals in transport from CAFOs, and the relative importance of different natural and synthetic hormones to the biological activity of CAFO wastes and effluents. The results will assist CAFO operators to optimize management practices that mitigate environmental problems associated with hormones discharged from CAFOs, and help regulators with risk assessment as biologically relevant chemicals will be identified, quantified, and ranked. Ultimately, the proposed project will help protect sensitive aquatic environments, native species, and humans from wastes associated with CAFOs.

## **An Integrated Approach To Developing a Total Facility Estrogen Budget at a Swine Farrowing CAFO**

Seth W. Kullman<sup>1</sup>, Karl G. Linder<sup>2</sup>, Kenneth H. Reckhow<sup>2</sup>, and Michael T. Meyer<sup>3</sup>

<sup>1</sup>North Carolina State University, Raleigh, NC; <sup>2</sup>Duke University, Durham, NC;

<sup>3</sup>U.S. Geological Survey, Lawrence, KS

**Objectives:** Little information is available regarding the concentration, release, fate, and transport of estrogenic compounds in animal waste treatment and storage facilities. Naturally occurring estrogens in animal wastes present an emerging risk to terrestrial and aquatic environments through their potential release and action as endocrine disruptors. Given the trend in agriculture toward concentrated animal feeding operations (CAFOs) and the extensive volume of waste generated, the potential for environmental impact cannot be overstated. Large data gaps include: operation-specific generation, and concentrations and fate of these hormones, their conjugates, and metabolites throughout CAFO facilities. Specifically, little data have been generated evaluating estrogen loads from different swine operations such as farrowing and finishing facilities. We must develop quantitative information regarding: reproductive status and estrogen excretion by individual animals, the stability of estrogens in open pit holding lagoons, and mobility of estrogens to surface waters following spray field application of swine waste as fertilizer. To address these data gaps, we will focus on a swine farrowing CAFO based on its operational units. By creating a hierarchical structure, assessments of estrogen fate will be “parameterized” and used for input into a Bayesian network model.

**Experimental Approach:** As such, we propose two specific aims: (1) establish a “total facility estrogen budget” based on composite measurements of natural estrogenic compounds throughout a swine farrowing CAFO; and (2) develop a Bayesian network model that will characterize causal relationships for a total facility estrogen budget in a probabilistic manner. Our experimental approach is designed as “proof-of-principle” to test the hypothesis that a mass balance for total estrogen equivalents from swine CAFOs can be predicted based on quantitative input and modeling of estrogen concentrations throughout each facility.

**Anticipated Results:** The anticipated result of this project is prioritization of operational practices in regard to waste management strategies and contributions of total estrogens to the environment. With this knowledge, we will be well poised to predict and determine the overall contribution of estrogenic compounds originating from different swine operations. This will aid in developing a comprehensive understanding of the fate and movement of these compounds, their putative impact on surrounding environments, and ultimately, the impact of these agricultural practices on local and regional watersheds.

## **Fate of Hormones in Waste From Concentrated Broiler Feeding Operations**

*Miguel L. Cabrera<sup>1</sup>, Brian D. Fairchild<sup>1</sup>, Peter G. Hartel<sup>1</sup>, Sayed H. Hassan<sup>1</sup>, David E. Kissel<sup>1</sup>,  
David E. Radcliffe<sup>1</sup>, William K. Vencill<sup>1</sup>, and Dinku Endale<sup>2</sup>*

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Each year, the U.S. poultry industry produces about 9 billion broilers with a generation of approximately 13 million Mg of broiler litter, a mixture of excreta and bedding material. Most of this litter is used to fertilize grasslands because it contains N, P, and K, but in addition to plant nutrients, broiler litter contains the sex hormones 17 $\beta$ -estradiol and testosterone. One of the objectives of our research is to determine concentrations of 17 $\beta$ -estradiol, estrone, and testosterone in different classes of broiler litter. For that purpose, selected samples of broiler litter received by the Agricultural and Environmental Services Laboratory of the University of Georgia are being analyzed for hormones. Approximately 150 samples have been analyzed to date, and preliminary results indicate that testosterone tends to decrease as the number of flocks increases from 1 to 5. Also, higher hormone concentrations are being detected in caked litter when compared to regular litter. A second objective of our research is to evaluate the effect of stacking broiler litter on hormone concentrations. Two stack houses have been sampled at the time of litter storage and 4 weeks later. Preliminary results obtained from the first stack house suggest that broiler litter stacking may reduce the concentration of testosterone in the bottom 0-30 cm of the stack. A third objective is to evaluate the transport and decomposition of radiolabeled hormones mixed with broiler litter and applied to soil. Laboratory studies were initiated to study the decomposition of estradiol and testosterone in broiler litter mixed with four different soils and incubated at -0.03, -0.75, and -1.5 MPa, each at 10, 20, and 30°C. Even after 105 days of incubation, the percentage of estradiol mineralization was low (< 11%) regardless of soil series, soil water potential, and soil temperature. The percentage of mineralization increased with increasing water content for all soil series to a maximum of 10 percent. In some soils, estradiol decomposition increased with temperature, whereas in other soils it tended to decrease with temperature. Preliminary results suggest that the decomposition of testosterone is proceeding at a faster rate than that of estradiol. Our fourth objective is to evaluate the effect of runoff occurring at different times after broiler litter application on the concentration of hormones in surface runoff from grassed plots. Rainfall simulation was carried out at 0, 1, 2, or 4 weeks after broiler litter application, and runoff water was collected and analyzed for hormones. Preliminary results show elevated concentrations of testosterone with respect to control plots. The fifth objective is to evaluate the effect of mechanical aeration on hormone concentrations in runoff from large field plots. Broiler litter was applied to these plots at 5 Mg/ha in April 2008, but no runoff has occurred since the application. A new broiler litter application is planned for late October 2008.

## Environmental Transport, Fate, and Ecological Effects of Steroids From Agricultural Application of Poultry Litter and Watershed Scale Evaluations of Existing and Novel Management Strategies

Lance T. Yonkos and Daniel J. Fisher

Wye Research and Education Center, University of Maryland, Queenstown, MD

**Objectives:** Poultry manure is used abundantly on the Delmarva Peninsula and elsewhere as organic fertilizer, usually for producing corn as an animal feed. Water quality impacts associated with runoff of poultry manure-associated nutrients have previously been demonstrated. The impacts of various emerging contaminants of concern, such as fecal steroids and arsenicals, are less well understood. Our research investigates the presence of these contaminants in poultry manure and their transport from fields to receiving waters under competing agronomic cropping strategies. In addition, we examine, via laboratory controlled research field and *in situ* watershed investigations, the impacts of poultry manure-associated fecal steroids on aquatic species.

**2008 Research Activities:** During the spring of 2008, corn was grown on adjacent approximately 35-acre fields using either *No-Till* or *Turbo-Till* cropping strategies. Poultry manure was applied to each field at a rate of 3.0 tons/acre. Runoff from these fields was collected for quantification of nutrients, androgenic and estrogenic steroids, estrogenicity, and arsenic. Laboratory-reared male fathead minnows were screened for VTG mRNA induction and the presence of VTG in plasma following exposure to field runoff presumed to contain poultry manure-associated fecal steroids. Male largemouth bass were collected from regional lakes that receive substantial agricultural runoff following spring manure application. These fish were screened for VTG protein in plasma and for VTG mRNA. Testes were examined histologically for intersex (e.g., testicular oocytes) or other evidence of endocrine disruption.

**Preliminary Results:** Preliminary chemistry results indicate that nutrient runoff differed between tillage practices. Exposure to manure-influenced field runoff induced minimal to moderate plasma VTG concentrations in approximately 50 percent of exposed male fathead minnows. Although field collected male largemouth bass did not have demonstrably elevated VTG levels, approximately 40 percent had rare to infrequent oocytes present within testicular tissue. The significance of these findings along with preliminary chemical analyses will be discussed.

## **Endocrine Disruptors in the Environment: A U.S. EPA Science To Achieve Results (STAR) Progress Review**

**Tampa Marriott Waterside Hotel  
Tampa, FL**

**November 16, 2008**

### **Final Executive Summary**

#### **OVERVIEW**

The Endocrine Disruptors in the Environment: A U.S. EPA Science To Achieve Results (STAR) Progress Review was held November 16, 2008, in Tampa, Florida. The workshop was sponsored by the National Center for Environmental Research (NCER), which is part of EPA's Office of Research and Development (ORD). Scientists from academic and government sectors assembled to discuss research addressing endocrine disruptors in the environment and their effects on human and environmental health. The meeting provided an opportunity for grantees in the EPA-funded STAR Program to present their research and interact with EPA staff and others conducting endocrine disrupting chemicals (EDCs) research. The meeting also permitted a chance to review related research being conducted in ORD laboratories. Approximately 50 individuals attended the meeting.

#### ***Welcome***

**Susan Laessig, EPA/ORD/NCER**

Dr. Susan Laessig welcomed the participants and explained that EPA has awarded five grants to support research to develop new exposure methods to detect, measure, and track EDCs in the environment. These projects began in 2005 and are now in their third year. Seven grants have been awarded to characterize and assess the impacts of hormones in livestock waste from large animal feeding operations. These projects began in 2007 and are now in their second year. To better leverage resources and improve and advance the research, four of the projects are cooperative agreements between the STAR grantees and EPA collaborators.

#### ***Introduction to EPA's Endocrine Disruptors Research Program***

**Elaine Francis, EPA/ORD/National Program Director for the Endocrine Disruptors Research Program**

Dr. Elaine Francis welcomed the participants and provided an overview of the research in EPA's EDCs Research Program. As a whole, EPA's EDCs Research Program is addressing three key long-term goals (LTGs) as identified in the Agency's Multi-year Plan (MYP) for Endocrine Disruptors. LTG 1 seeks to provide a better understanding of the science underlying the effects, exposure, assessment, and management of endocrine disruptors. LTG 2 aims to determine the extent of the impact of endocrine disruptors on humans, wildlife, and the environment. The goal of LTG 3 is to support the Agency's screening and testing program. Most of the research projects being presented at this meeting fall under LTG 2. Overall funding for the EDCs Research Program has been reduced in recent years, with STAR grant funding completely eliminated in 2005. There is much Congressional interest in the research conducted in the EDCs Research Program. There are many opportunities for improved communication and partnerships. STAR researchers are encouraged to propose/organize/participate in targeted workshops, serve as advocates for the research being conducted and its funding, keep ORD informed of the use of their research products, inform ORD before the release of major publications, provide updated

biosketches and other information as requested, and assist ORD by agreeing to present a poster at an EPA Board of Scientific Counselors (BOSC) review or at the Annual EPA Science Forum.

## **ASSESSING AQUATIC EXPOSURE TO ENDOCRINE DISRUPTING CHEMICALS (EDCs)**

### *Methods to Measure Indicators of Exposure in Real-World Aquatic Environments*

**James Lazorchak, EPA/ORD/National Exposure Research Laboratory**

Few studies have successfully tied EDC exposure and effects work in the laboratory to changes in wildlife in the field. To date, only two studies have shown population effects on wildlife from pharmaceuticals. A field study showed effects in different types of fish following exposure to ethynylestradiol (EE2), including higher *Vtg* expression in males, impaired reproduction, and population declines. In another study, fathead minnows (FM) were exposed to river water that received runoff from dairies and feedlots. Male FMs were found to be producing *Vtg*, suggesting a biologically significant exposure to estrogens. Histology at 1 month was inconclusive but suggested that the male FMs were no longer producing sperm. The next research steps include using an array of measurements to determine sources, duration, and possible contaminants and concentrations. Studies in Ohio and Indiana have shown possible androgen exposure in FMs in water downstream from concentrated animal feeding operations (CAFOs) and animal feeding operations (AFOs). Many different substances were found in these waters, making it difficult to determine the cause(s) of any changes in the fish. Future questions to be addressed include: How do we use laboratory-derived data to set protective standards given the paucity of data showing population effects? What endpoints should we use to measure EDC exposures? Do we really need to show population effects of EDCs? What are the new thresholds? Endpoints? The level of protection? How do we address the exposure soup?

### *Exposure Measurement Tools for Endocrine Disrupting Chemicals in Mixtures*

**Heiko Schoenfuss, St. Cloud University**

The primary goal of this research project is to assess the hypothesis that mixtures of estrogenic compounds will have adverse effects on aquatic vertebrate reproductive fitness that can be rapidly assessed by quantifying alterations in the neuroendocrine system of fish. This will be accomplished by assessing the synergistic interactions of mixtures; assembling a broad, biologically relevant effects matrix; and developing neuroendocrine endpoints for rapid assessment. Researchers began by determining the concentration of various chemicals in effluent from two different samples of treated wastewater. Diatoms, *Daphnia magna*, and FMs were then exposed to chemicals. Controlled experiments showed that diatom health was affected by alkylphenols but not by estrogens, and that diatoms differentially bind alkylphenols. Daphnids were exposed to different mixture concentrations, but the researchers saw no effects on molting frequency, survival, production of male daphnids, or embryonic development. When the daphnids were exposed to diatoms that had been previously exposed to nonylphenol, the nonylphenol had an indirect, food-mediated effect on *Daphnia* (increased molting frequency). Predator avoidance studies showed that embryo and larvae performance was adversely affected after 12 days of exposure. Adult FMs were then exposed and compared with control males to determine the effects of exposure on *Vtg*, secondary sex characteristics, nest holding, and intersex. Estrogen effects were additive, but mixture responses at times contradicted the single exposure responses. Neuroendocrine endpoints were then analyzed before nest competition in male FMs; estrone exposure had an effect while estradiol and ethynylestradiol showed no effects. Limited effects were seen after mixture exposure. After the nest competition, the same compounds showed different effects. Thus, it is crucial to consider how the social hierarchy of the fish resulting from reproductive competition affects their response to chemicals. Validation of these studies in the field has begun.

## **Discussion**

A participant asked Dr. Schoenfuss what endpoint was used to determine intersex. Dr. Schoenfuss replied that the endpoint used was multiple clearly definable oocytes in testicular tissue. Another participant asked if the researchers planned to expand the trophic experiments to FMs. Dr. Schoenfuss said that this was the plan for the upcoming year. Another participant asked what the concentrations of the individual estrogen compounds were in the mixture. Dr. Schoenfuss responded that there was 50 µg/L of each compound in the mixture. A participant asked if the researchers found that nonylphenol was binding to glass. Dr. Schoenfuss said that he had no definitive answer to this question. He explained that his team had measured the mucous layer surrounding the diatoms and found that at higher levels of exposure, the mucous layer was removed by alkylphenols. If the alkylphenols are removing the mucous layer, it is likely that they are binding to the remaining glass shell.

### ***Development of Receptor- to Population-Level Analytical Tools for Assessing Endocrine Disruptor Exposure in Wastewater-Impacted Estuarine Systems*** **Lee Ferguson, University of South Carolina**

Grand challenges for assessing EDC exposure in the aquatic environment include assessing identity, sources, and exposure levels; and determining which components of complex mixtures contribute to EDC activity. HPLC-MS/MS is considered the gold standard for analyzing EDCs because of its high sensitivity and amenability to polar compounds, but there is a need for broadband, sensitive, mode-of-action based methods for surveying exposure to EDCs in mixtures. This research project is working to develop nuclear hormone receptor-affinity extraction techniques for isolating EDCs from complex wastewater mixtures. These methods will be applied in combination with high-performance mass spectrometry for activity-directed analysis of EDCs in wastewater and estuarine receiving waters on the South Carolina coast. Sensitive vertebrate (fish) and invertebrate (copepod) EDC exposure laboratory assays will be used to link exposure measurements to biological effects. Novel biomolecular endpoints will be developed and applied to assess EDC exposure in sensitive meiobenthic invertebrates in wastewater-impacted estuarine environments. Nuclear receptor activity is implicated in many EDC modes of action and these proteins form the basis of many common EDC screening tools. Using the standard HPLC-MS/MS method to test water samples, the researchers found high levels of estrogens in certain places, but the samples also included a lot of noise. Using the effluent extract after immunosorbent cleanup showed much clearer results from HPLC-MS/MS. The researchers identified a purified, soluble estrogen receptor ligand-binding domain that is stable and competently binds to estrogens. A fluorescence polarization ligand-binding assay was used to verify that this substance maintained binding affinity for a number of different estrogens and zenoestrogens. A number of environmental contaminants were found to bind to the estrogen receptor. Testing this method in the field, estrogenic compounds were successfully separated from the mixture. Future plans include integrating the mysid shrimp ecdysone receptor/ultraspiracle heterodimer (EcR/USP) into receptor-affinity extraction methods and fluorescence-polarization EDC screening.

## **Discussion**

A participant asked how dynamic the relationship between the ligand and the receptor was. Dr. Ferguson responded that their experiments showed that both estradiol and nonylphenol were able to successfully bind to the receptor.



## **INNOVATIVE METHODS FOR RAPID DETECTION**

### ***Systems Approach to Assessing Cumulative Exposure to Endocrine Disrupting Chemicals*** **Gerald LeBlanc, North Carolina State University**

Existing measures to assess the influence of cumulative exposure to EDCs by conventional analytical chemistry or reporter gene approaches are insufficient because of the complex nature of EDC mixtures, which can elicit toxicity via a range of mechanisms. To address this limitation, the current study is examining cumulative exposure to EDCs by taking a systems approach that assesses multiple endocrine signaling circuits simultaneously. This approach will incorporate toxicokinetic and toxicodynamic actions and also will provide information on mechanisms of action (MOAs) and interactivity. The main objective is to develop an approach for evaluating exposure to EDCs based upon changes in the expression profile of endocrine-related genes in a whole organism. Expression of targeted genes will be evaluated by quantitative real-time PCR to identify genes of interest, sequence genes, generate primers, sequence PCR products, and optimize all procedures. To date, 25 nuclear receptors have been identified in the *Daphnia magna*. Daphnids were exposed to a number of chemicals suspected to affect endocrine activity. Experiments have shown that differential expression of a suite of at least six genes can be used to assess exposure of daphnids to EDCs. This exposure detection system has application for assessing exposure to several classes of EDCs. The gene battery also holds promise for the assessment of exposure to chemicals that elicit toxicity via the generation of free-oxygen radicals.

#### **Discussion**

A participant asked if *Daphnia* genes change over the 3-day molt cycle. Dr. LeBlanc replied that they do change. The animals in the time course experiments were all the same age and the data were corrected for changes in the level of gene expression in the untreated animals.

### ***Microfluidic Systems for Bioreporting, Separations, Vibrational Spectroscopy, and Microcantilever Sensing of EDCs***

**Michael Sepaniak, University of Tennessee**

The goal of this project is to develop a fully integrated microfluidic device for the detection of EDCs. The research accomplishments to date include: optimization of protein receptor immobilization on microcantilevers (MCs), analysis for EDCs using endocrine receptor functionalized MCs, and analysis of thyroid disrupting chemicals using thyroid hormone transport protein functionalized MCs. Representative EDCs have been surveyed for surface-enhanced Raman spectroscopy (SERS) response signatures using nanocomposite substrates, but sensitivity was not adequate. The researchers demonstrated the possibility of creating advanced substrates using a direct electron beam lithography (EBL) procedure; sensitivity was more controlled, but still not adequate. Next, nanotransfer printing and a stamp and repeat process were used to address the scalability limitation of EBL; this approach was successful. Future work will include pursuing further improvements in “hot” substrates with several novel approaches. The enhanced science and products of this research should reduce uncertainty in risk assessment/management by: improving the existing technology and attacking the problem of EDC exposure and activity monitoring with an arsenal of informative tools; clarifying and elucidating which chemicals, and in which combinations, can mimic or inhibit endocrine signaling molecules; and providing faster and more portable analytical techniques to facilitate screening and characterization of samples in the field. Future directions include functionalizing the MC arrays and developing field-deployable systems.

#### **Discussion**

A participant asked Dr. Sepaniak if this approach could differentiate between different types of estrogens. Dr. Sepaniak said that it could not.

***Rapid Detection of Trace Endocrine Disrupting Chemicals in Complex Mixtures: A Full-Spectrum Deconvolution Technique With a UV-Transparent Passive Concentrator***

**Tohren Kibbey, University of Oklahoma**

This research is aimed at developing a rapid, inexpensive screening method that can detect EDCs and other related compounds. Traditional analyses are labor-intensive and costly because they rely on extraction steps and on specialized laboratory equipment. A low-cost approach would allow more waters to be screened and these results could be used to prioritize testing using more complex and costly methods. The method being evaluated uses a UV-transparent polymer that concentrates chemicals and acts as an optical cell for detection. Preliminary partitioning results showed Dow Corning Sylgard 184 Silicone Encapsulant (PDMS) to be the most promising polymer candidate, so further tests were conducted with PDMS. Detection limits for the chemicals tested were found to depend on partition coefficients (higher  $K_{PW}$  = better detection), UV absorbance (more chemospheres typically equal better detection), and polymer thickness (thicker = better detection). Estimated detection levels were high enough for most of the chemicals with the exception of estrone and estradiol. Field testing showed no interference from higher turbidity. Dissolved organic carbon (DOC) in landfill water appeared to influence the spectrum slightly, but did not seem to interfere with detection. Future work includes measuring more basis spectra in PDMS, revisiting polymer sampler design, quantitatively assessing uncertainty and the effects of unknown compounds, exploring other deconvolution methods, exploring multi-polymer deconvolution to increase selectivity, and conducting preliminary field testing.

**FATE AND EFFECTS OF HORMONES IN ANIMAL WASTE—CATTLE, SWINE, AND POULTRY**

***Integrated EPA Studies on EDCs in CAFOs***

**Vickie Wilson, EPA/ORD/National Health and Environmental Effects Research Laboratory (NHEERL)**

CAFO wastes include both synthetic steroids and natural hormones and often end up in water bodies. Unlike human waste, CAFO wastes are often unregulated and receive minimal treatment. Studies have shown adverse reproductive effects in FMs exposed to trenbolone, a steroid that is commonly used in CAFOs. EPA assembled a research team to characterize occurrence and ecological impacts of estrogenic and androgenic chemicals from CAFO waste. The research approach includes developing and validating *in vitro* and analytical methods to identify and quantify compounds responsible for endocrine activity in CAFOs across the United States; assessing the ecological impacts using a combination of laboratory and field studies; identifying ecologically relevant biomarkers in aquatic species using genomic approaches; evaluating the environmental fate, transport, and metabolism of CAFO-derived EDCs in surface and groundwaters; and evaluating the capability of existing risk management technologies for CAFOs to reduce exposure to EDCs. The researchers have encountered a number of issues with effluent testing in *in vitro* assays and have devised or are working on solutions. Studies conducted to date have shown that the level of EDCs can vary greatly at different places in a single watershed; thus, a single point assessment is not sufficient. *In vitro* assays appear to be good tools for bioactivity-directed analytical chemistry and for overall assessment of hormonal activity. Estrogens were identified more often than androgens. The group will continue to address other issues and will ultimately study the ecological impact of EDCs.

**Discussion**

A participant noted that the seven androgens in the mixture effects experiment and the dose-response curves seemed to fall into two groups and asked Dr. Wilson if she could explain this. Dr. Wilson explained that this could be because the androgens are normalized to the trenbolone on the same plate. Another participant asked if any researchers were studying atmospheric transport of EDCs. Dr. Wilson said that her laboratory has found androgenic activity in some products of combustion. Dr. Francis commented that she knew of some nitrogen-focused research that is being done.

***Fate of Hormones in Tile-Drained Fields and Impact to Aquatic Organisms Under Different Animal Waste Land Application Practices***

**Linda Lee, Purdue University**

The Purdue team is working in collaboration with EPA to quantify the contribution of tile-drained agricultural fields under different manure and lagoon effluent application practices to hormone loads in aquatic systems. To do so, animal wastes were applied to two field sites using three different methods. Initial sampling proved to be much more time consuming than anticipated. A more efficient sampling method is being implemented and QA/QC of current hormone and flow data is ongoing. The group also is working to assess hormone persistence in fields under different manure and lagoon effluent practices, studying the persistence in surface soils and stream sediments, tracking average hormone concentrations in manure and lagoons 3 months after beef implanting, performing laboratory-based degradation studies quantifying the effect of temperature and moisture content on trenbolone sorption, and supporting sorption studies. Data collected to date have shown that degradation appears reversible in soil and in the tissues of unintended receptors such as fish. The group also is working to evaluate the impacts of these hormone loads (relevant levels and mixtures) on aquatic organisms. The researchers are studying fish and turtles to identify reproductive effects from EDCs. Laboratory studies have shown changes in gene expression in FM larvae exposed to both potent and weak estrogens, showing that the effects on young FMs may be different than the effects on adult FMs. Field studies showed that fish abundance decreased throughout the year, especially at the CAFO sites and higher species richness was seen at the control site. Creek chubs from the site closest to the CAFO were either immature or had very undeveloped gonads compared to the other sites. The researchers are developing quantitative PCR techniques for evaluating changes in gene expression for this species. For the turtle studies, the researchers developed a semi-quantitative vitellogenin assay; plasma samples will be used to quantify sex hormones.

**Discussion**

Dr. Francis noted that there were more females than males further downstream from the Box Site and wondered if this was significant. Dr. Lee responded that she did not know the reason for this. Sampling will be repeated over the next year, which will help the researchers identify trends.

***Effects of Cattle Manure Handling and Management on Fate and Transport of Hormones in the Feedlot and on the Field***

**Daniel Snow, University Nebraska–Lincoln**

The objectives of this project were to: quantify hormones in various stages of the manure processing pathway in cattle feedlots; determine the effects of different handling practices of cattle feedlot wastes on the stability and availability of hormones; determine the effects of different land application strategies on the fate and transport of hormones in vadose zone soils; and determine if grasses grown in conservation buffers assimilate hormones. Much of the work to date has focused on quantifying the differences in cattle waste as influenced by handling practices such as stockpiling, composting, and runoff retention basins. Two feedlot studies have been completed in an attempt to identify and quantify products from biological degradation. Additional project tasks included: determination of the effect of tillage on hormone levels in field runoff using five manure sources, quantification of the uptake of hormones in manure to selected grass species commonly used in buffer strips, and determination of the fate and transport of hormones in vadose zone soil by examining leaching. Hormones were extracted and analyzed using microwave-assisted solvent extraction, automated solid-phase extraction, and liquid chromatography in tandem with mass spectrometry. In the preliminary results from feedlot runoff samples, few samples contained detectable levels of synthetic growth promoters. However, natural steroid hormones (an average of  $\ll 0.5$  ppb of androsterone, 4-androstenedione, estrone,  $17\beta$ -estradiol, and progesterone) were detected in both treatment and control pens. The amount of steroid hormones in the feedlot pen surface samples (feces) was comparable to that in the runoff samples, but no steroid hormones

were detected in freshly scraped samples, and trace levels of synthetic hormone metabolites were detected in pen surface samples collected after implanting.

## **Discussion**

A participant asked if no chemicals at all were found in the samples. Dr. Snow responded that in the first feedlot runoff samples, no synthetic hormones were detected; however, the team did find substantial levels of endogenous hormones, specifically estrogens and progesterone. Most hormones were found in the solids.

### ***Transport and Transformation of Natural and Synthetic Steroid Hormones at Beef Cattle and Dairy CAFOs***

**Edward Kolodziej, University of Nevada, Reno**

This research project focused on the transport pathway through which steroid hormones move from the CAFO to receiving waters to evaluate risks to aquatic organisms in the receiving waters. The research method involved a complex filtration process that moved a water sample through several steps until a derivatized steroid compound could be isolated and analyzed via GC/MS/MS. EPA's NHEERL tested samples for estrogenic and androgenic activity. Estrone was found in 88 percent of the samples. However, little correlation was noted between the various sites, or between the amount of hormones present and the hormone activity found. Florisil cleanup has improved sample quality, and hypothesis driven sampling methods may be implemented in the future. Rainfall simulator test plots were studied to determine if occurrence and transport could be predicted at the field sites, and whether rangelands could be used to study CAFOs, since they have a direct link to surface waters, have little dilution, are flood irrigated, and are subject to variable manure management. A significant amount of steroid hormones was found to be transported off of these test plots when tested at 5, 20, 40, and 60 minutes. Future research will focus on groundwater testing and modeling. Tile drain and groundwater samples will be collected. Researchers will work to identify the most effective soil column/subsurface transport experiment and determine if surrogates can predict where hormones will be detected. The growth in the organic and natural beef markets has resulted in a significant decrease in the use of steroid implants in California. As a result, there is likely a much smaller amount of synthetic steroids in waters in California today compared to 10 years ago.

## **Discussion**

A participant asked if Dr. Kolodziej knew of any studies on physiological effects in wildlife that measure hormone concentrations in nanograms per liter. Dr. Kolodziej responded that the number used for a predicted null effect of estrogen is approximately 1 nanogram/liter estradiol equivalent. The 1-10 nanogram/liter level is the break point where effects in aquatic organisms can be expected.

A participant noted that Dr. Kolodziej had stated that estrone was found in 88 percent of the field samples collected, but he did not say that they were typically above the predicted no effect concentration (PNEC); the PNEC for estrone and estradiol, however, would be different. What was the typical concentration he was referring to? Dr. Kolodziej responded that only 10 to 15 percent of the samples were above PNEC. There was a 5:1 difference between estradiol equivalency of estrone and estradiol, so estrone was prevalent, but often below PNEC. The participant was concerned that the PNEC has not yet been established for all hormones; she thought that the levels were probably a lot higher. She asked why EPA did not find a correlation with their bioassays if they were running them on unconcentrated samples. Dr. Wilson noted that the samples were eluted in 10 mL of solvent and then further concentrated. In the concentration process, during which the solvent was dried and resuspended, it is possible that less than 100 percent of the hormones present were redissolved. After the extraction, there still is potential for interference. It is possible that EPA lost some of the activity due to the process. Now that EPA is

conducting research with estradiol equivalents, it would be useful to revisit the data using some of the new testing techniques to find more specific levels. The participant asked specifically about 17 $\alpha$ -estradiol. Dr. Wilson responded that EPA usually ran tests on 17 $\beta$ -estradiol so she did not have that data, but she expected that 17 $\alpha$ -estradiol would be slightly less potent.

***Assessing Occurrence, Persistence, and Biological Effects of Hormones Released From Livestock Manure***

**Jocelyn Hemming, University of Wisconsin at Madison**

The overall project goal is to determine the presence, persistence, and biological effects of natural and synthetic hormones that may be released into the environment from CAFOs, and evaluate the effects of different animal manure handling practices on the fate and activity of these compounds. The specific objectives include: identifying and quantifying the suite of estrogenic, androgenic, and progestogenic compounds associated with animal farming; characterizing the environmental transport and fate of natural and synthetic steroid hormones that accompany animal manure from farms in Wisconsin; evaluating how various animal manure handling/management strategies impact the transport, fate, potential exposure, and associated effects of steroid hormones discharged from farms; and investigating the ecological effects associated with steroid hormones in animal manure from farms using reproductive, developmental, and gene expression endpoints in FMs. A variety of soil and water samples were taken from currently operating private farms. Hormones of interest included natural and synthetic estrogens, androgens, and progestins. Data collection is ongoing, but results to date suggest that rain events and snow melts are likely important in hormone mobilization. Compared to other samples, snow melt samples had the highest concentrations of progesterone. E-screen bioassays were performed to determine preliminary potency of the target compounds. Results to date show that increasing the concentration decreases proliferation; 17 $\beta$ -trenbolone was found to be the most potent compound in the E-screen assay. Results from transgenic yeast assays did not have adequate sensitivity. FM assays did not show a significant effect of diluted manure on reproduction, but high doses of trenbolone acetate increased mortality in FM embryos. Future work will include continued sampling; finalizing extraction and clean-up steps for solid samples; characterizing the target hormone suite with HPLC-MS/MS and hormonal activity with *in vitro* bioassays; and investigating the effects of other steroids associated with CAFO effluent, especially on FM oocyte maturation, embryonic development, and shorter exposures on gonad development and gene expression.

***An Integrated Approach to Developing a Total Facility Estrogen Budget at a Swine Farrowing CAFO***

**Seth Kullman, North Carolina State University**

This project is testing the hypothesis that a mass balance for total estrogen equivalents from swine CAFOs can be predicted based on quantitative input and modeling of estrogen concentrations throughout a CAFO facility. Operational elements that may impact hormone concentration, fate, and transport also will be identified. Livestock farms are a putative source of natural steroid hormones in the environment. In North Carolina, swine farming generates about 19 million tons of waste per year and this amount is expected to grow. Estrogens are excreted in swine urine and feces. Most of the swine waste in North Carolina is held in lagoons and then applied to fields as fertilizer. This project aims to provide an estimate of total estrogen production and composition, identify the fate and stability of estrogens in lagoons, assess off-site transport of estrogens following spray field applications, and develop a Bayesian network model to characterize causal relationships for a total facility estrogen budget in a probabilistic manner. A kickoff meeting was held in April 2008. To date, researchers have identified a new field site and developed a sampling strategy for this site; developed analytic procedures; completed phase 1 of sampling; and started model development. Future work includes further refining the Bayesian model, beginning phase 2 of sampling in the lagoon, and beginning phase 3 sampling of the spray fields.

***Fate of Hormones in Waste From Concentrated Broiler Feeding Operations***  
**Miguel Cabrera, University of Georgia**

The State of Georgia produces 2 million tons of broiler litter each year. The first objective of this project is to determine the variability of hormone concentrations in broiler litter. Findings to date show hormone concentrations to be higher in caked litter (as compared to whole litter), and increasing the number of flocks from one to three appears to increase hormone concentrations in caked litter but not in whole litter. Broiler litter samples were taken from two different houses and were examined to determine if the age of the broiler had an effect on hormone levels. Samples from both houses showed little to no effect on hormones based upon the age of the broiler. The second project objective is to determine the effect of storing broiler litter in stack houses on hormone concentrations. Two of five houses have been sampled to date and preliminary results from one stack house show an approximate 50 percent reduction in all hormones after 4 weeks. The third project objective is to evaluate the effect of soil temperature and water content on the decomposition of estradiol and testosterone in soil when mixed with broiler litter. Preliminary results show that water content and temperature interact to affect the mineralization of estradiol. Ten percent of estradiol was decomposed in 100 days. Preliminary results for testosterone show that the mineralization in the Sedgelyield soil (poorly drained) is high even under relatively dry conditions. Forty percent of testosterone was decomposed in 20 days. The fourth objective involves comparing estradiol and testosterone transport in intact and disturbed columns using stock solutions of hormones mixed with broiler litter. Preliminary work on this objective has recently begun. Researchers also are evaluating the effect of runoff occurring at different times after litter application on hormone concentrations in runoff. Analysis of the data has begun, but results are not yet available. The effect of grassland aeration on concentrations of hormones in runoff from large field plots also will be evaluated. Broiler litter has been applied twice to aerated soil, but there have not yet been rains to stimulate runoff.

**Discussion**

Dr. Kolodziej asked if the researchers had measured any androgens besides testosterone. Dr. Cabrera said that they had not yet tested other androgens; the researchers are currently working to determine if it is possible to clean the samples adequately to search for other androgens. Dr. Kolodziej noted that the literature shows androgen levels higher than those in the data presented and asked Dr. Cabrera to explain this discrepancy. Dr. Cabrera said that for one of the stack houses sampled, preliminary data show much higher levels of testosterone than the data presented today. The samples with the high levels of androgens are the ones that have not been cleaned out for years; this may be due to accumulation of androgens over the years.

***Transport, Fate, and Ecological Effects of Poultry Manure-Associated Contaminants***  
**Lance Yonkos, University of Maryland**

The goals of this controlled field runoff study were to: compare contaminants in runoff under competing cropping strategies; investigate the persistence of contaminants in receiving waters; and investigate the contaminants' effects on fish. Two research fields treated with poultry litter were studied, one that was turbo tilled, and one that was not tilled. Because steroids are not widely used in poultry farming, the focus was on determining the presence of the endogenous hormones estradiol, estrone, estriol, and testosterone. Runoff was collected from both fields, and water in a retention pond was sampled for 3 weeks. After a rain event, fish in the retention pond died quickly, and the ammonia level was found to be more than 6 mg/L (taken directly from the no till runoff flume it was over 30 mg/L.) FMs then were exposed to both fresh and preserved water from the flume and the receiving pond for 4 to 19 days. Plasma *Vtg*, estrone, and estradiol were measured. In an additional test, largemouth bass (LMB) were collected from six lakes in the Delmarva Peninsula and examined for testicular oocytes. Preliminary results indicate that: turbo till reduces lateral transport of nutrients (and probably other contaminants) from fields; poultry litter-associated runoff is estrogenic; exposure to nearly lethal runoff and surface water treatments induced only

moderate levels of *Vtg*; and incidence of testicular oocytes in field-collected LMB was 33 to 100 percent, but severity was very minor. More conclusions can be made after additional data are gathered and analyzed.

## **Discussion**

A participant asked if pesticides, versus ammonia, were ruled out as a cause of the fish kill. Dr. Yonkos responded that he did not measure for pesticides. The only pesticide applied to the field is Roundup®, which persists for approximately 2 days and would have been applied 3-4 weeks prior to the runoff event.

A participant noted that corn is planted in the area, and atrazine is a pesticide commonly used on corn. Dr. Yonkos responded that atrazine is not used on these fields, but is in use in some places in the Delmarva Peninsula. Dr. Yonkos stated his belief that it is ammonia that killed the fish in less than 5 hours rather than pesticides, given that the ammonia concentration was extremely high.

A participant noted that there are similar fields in North Carolina, and there is a problem with historical applications of arsenic from 50 to 60 years ago. Dr. Yonkos responded that the concentration of arsenic in poultry litter is approximately 40 mg/kg, which is well below the acute toxicity level.