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BIOANALYTICAL ANALYSIS OF NATURAL ESTROGENS AT CONCENTRATED ANIMAL FEED OPERATIONS AFFECTING NORTH CAROLINA RIVER WATERS

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ABSTRACT

Riverine and coastal regions of North Carolina (NC) are severely impacted by chemical stressors including agricultural, industrial and urban contaminants. More recently attention is directed towards potentially hazardous effects of hormonally active compounds termed endocrine disruptors (EDCs). Sources of EDCs are multiple and include SWT effluent, leakages from landfills, private septic systems or as runoff from spray irrigation or discharges from Concentrated Animal Feed Operations (CAFOs). These stressors are typical of those faced by surface waters and coastal estuaries wherein the pan watershed impacts of multiple stressors affect water quality, biotic diversity, and ultimately ecosystem integrity and human health. In this study we have examine endocrine activity and reproductive effects of natural estrogens originating from CAFO lagoon and surface water extracts. Three specific aims were conducted 1. Identification of large-scale feeding operations within the Neuse River basins as models for assessment of estrogenic activity from CAFO waste sites. 2. Analysis estrogenic activity of CAFO water extracts using quantitative in vitro and in vivo bio-analytical analysis and 3. Assessment of sub-lethal reproductive effects of CAFO extracts as an indicator of multigenerational, population level disruption. As community development, industry and agricultural practices grow around riverine and marine environments of NC, great concern is generated for impacts on water quality. Water is the most important global resource and reliable assessment of water quality is essential for human and environmental health, safety and security. Deployment of validated bioanalytical tools enables regulatory and health authorities to make decisions based on sound scientific data rather than formulating strategies based on precautionary principles. Results from this study will form proof of principle for development of effective monitoring techniques for natural estrogens in riverine and coastal environments; and, aid in the development of best management practices for improving water quality and source water protection.

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SUMMARY AND CONCLUSIONS

Recently, attention has been directed towards the potentially hazardous effects of natural estrogens from Concentrated Animal Feed Operations (CAFOs). Steroidal estrogens are excreted in urine and feces of all farm animals. The extent of excretion is a function of age, sex, health, reproductive status and many additional factors such as diet that differ between animal types (Hanselman et al., 2003). Although the hormone content of waste has not been systematically studied, a relatively large total mass of hormones is released yearly given the estimated 291 billion pounds of manure generated annually in the United States (USEPA, 2001). Little information is available regarding the concentration, release, fate and transport of estrogenic compounds in animal waste treatment and storage facilities. Additionally there exist large data gaps in our understanding of the occurrence, concentration and fate of these hormones, their conjugates and metabolites throughout CAFOs.

To address these data gaps we have examined the biological activity of natural estrogens from two identified CAFO's in a selected NC waterways. Here a two tiered approach was taken. Tier one incorporated one in vitro and one in vivo bio-analytical assays for identifying substances which have the potential to induce an estrogenic response. Tier two examined, reproductive effects using an aquatic in vivo assay employing the medaka fish model. Our analysis demonstrated that swine lagoons possess a variable, but consistently high levels of estrogenic activity. Adjacent secondary waterways both below CAFOs examined additionally exhibit estrogenic activities above background levels. However, these activities varied with both season and environmental events including spray application of lagoon contents and weather events. Lastly, exposure of fish to the predominant swine estrogen, 17β-Estradiol, resulted in significant reproductive abnormalities including reduced fecundity, fertility and embryo hatching success.

While the data generated in this report is preliminary, it is anticipated that continued efforts form this analysis will result in prioritization of operational practices in regards to waste management strategies and minimization of off-farm estrogen outputs to aquatic environments. With this knowledge we will be well poised to predict and determine the over all contribution of estrogenic compounds originating from specific swine farrowing and finishing operations. This will aid in developing a comprehensive understanding of the fate and movement of these compounds, their putative impact on surrounding environments and the ultimately the impact of these agricultural practices on local and regional watersheds

1.0 INTRODUCTION

1.1 Background

U.S. surface waters are subjected to increasing anthropogenic stress coupled with natural stressors such as: salinity, dissolved oxygen, temperature, nutrient inputs and physical energy. Additionaly, river and coastal waters/sediments are the ultimate recipients of an array of pollutants released in municipal and industrial point discharges, and carried in urban and agricultural non-point runoff. As such, many aquatic regions throughout the US have been impacted by multiple chemical stressors. These stressors have imposed adaptive pressures upon resident organisms and in some instances altered community structure and biotic diversity. More recently, attention has been directed towards the potentially hazardous effects of hormonally active substances. Sources of these compounds are multiple arising from sewage treatment effluent, leakages from landfills, private septic systems or as runoff from spray irrigation or discharges from Concentrated Animal Feed Operations (CAFOs).

Animal production in the U. S. has become increasingly specialized using large scale feeding operations for hogs, cattle and poultry. To date, over 238,000 U.S. feeding operations produced over 291 billion pounds of manure per year with over half produced by CAFOs (www.cdc.gov) (USEPA, 2001). North Carolina is home to a very large and growing livestock and poultry industry including cattle, hogs, pigs, broilers, chickens and turkeys. The NC Department of Agriculture and Consumer Services reports that NC livestock operations retained over 9.9 million hogs ranking second nationally only to Iowa. In response to the nationwide proliferation of CAFOs and potential for impact on environmental health, the US EPA has mandated that CAFOs obtain discharge permits and develop nutrient-management plans designed to keep animal waste from contaminating surface- and groundwater (USEPA, 2003). While a great deal of attention has been given to questions of nutrient loading and dispersion of volatile organics form CAFOs, little information is available regarding the fate, mobilization and environmental impact of natural and synthetic hormones from these facilities. To date, a few studies have demonstrated the presence and/or effects of natural and synthetic hormones in aquatic ecosystems below CAFOs (Soto et al., 2005; Orlando et al., 2004; Durhan et al., 2005). These anthropogenic pressures have potential to lead to long-term degradation of water quality including eutrophication, algal blooms, anoxic/hypoxic conditions, and acute/chronic toxicity.

As community development, industry and agricultural practices grow around riverine and marine environments, they generate great concern for impacts on water quality possibly jeopardizing health and productivity of these critical systems. Water is the most important resource worldwide and reliable assessment and protection of water quality will provide tremendous benefit for human and environmental health, safety and security. Also, economic benefits are derived from maintaining high water quality standards including reduced water treatment costs, tourism and recreation, and potential commercial impacts. Problems occurring in NC aquatic systems are typical of those faced by surface waters and coastal estuaries. When faced with new water quality problems (such as those posed by CAFO's), the combined impacts of multiple stressors of diverse origins may assume critical proportions. Cumulative impacts of multiple stressors affect water quality, biotic diversity, and ultimately both ecosystem function and human health. Results from water quality assessments will form an effective means to evaluate, prioritize and establish best management practices for improving water quality and source water protection. This project's deliverables provides sound scientific basis on which community and government concerns regarding the impact of CAFO derived hormone release to aquatic environments can be addressed. Ultimately defined water quality testing will facilitate planning and management requirements for livestock including regulation and control of emerging contaminants released into aquatic ecosystems.

1.2 Specific Aims

Little information is available regarding the concentration, release, fate and transport of estrogenic and androgenic 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 at low concentrations (10-100 ng L^-1). Given the trend in agriculture toward concentrated animal feeding operations and the extensive volume of waste generated, the potential for environmental impact cannot be overstated. The precedent has been established that these facilities contribute to natural free-and conjugated estrogens in urine and fecal wastes (Raman et al., 2004). However, there exist large data gaps in our understanding of the occurrence, concentration and fate of these hormones, their conjugates and metabolites throughout CAFOs. Specifically, little data has been generated to determine estrogen loads from differing swine operations such as farrowing and finishing facilities, whether holding lagoons are effective barriers for retention of estrogen containing wastes, and if anaerobic/aerobic holding lagoons effectively contribute to the degradation and or immobilization of these compounds. Additionally, little is know about release and transport of estrogens from swine operations to groundwater aquifers from leaching of holding lagoons, surface runoff following spray field applications of waste as fertilizer or large scale release following flooding with intensive weather events. The necessity to understand potential impact of CAFOs on local and regional watersheds is unprecedented. A clear understanding of agricultural outputs such as nitrogen and phosphorous in addition to emerging contaminants such as natural and synthetic animal hormones is necessary to make sound scientific decisions for reliable assessment and protection of water quality.

To address these data gaps we have examined the biological activity of natural estrogens from two identified CAFO's in a selected NC waterway. Here a two tiered approach was taken. Tier one incorporated one in vitro and one in vivo bio-analytical assays for identifying substances which have the potential to induce an estrogenic response. Tier two examined, reproductive effects using an aquatic in vivo assay employing the medaka fish model. While the data generated in this report is preliminary, it is anticipated that continued efforts form this analysis result in prioritization of operational practices in regards to waste management strategies and minimization of off-farm estrogen outputs to aquatic environments. With this knowledge we will be well poised to predict and determine the over all contribution of estrogenic compounds originating from specific 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 the ultimately the impact of these agricultural practices on local and regional watersheds.

Three specific aims were conducted:

Specific Aim 1. *Identify two large-scale feeding operations within the Neuse River basin as models for assessment of natural estrogenic activity from CAFO waste discharges*.

Specific Aim 2. *Determine estrogenic activity of CAFO water extracts using quantitative in vitro and in vivo bio-analytical analysis.*

Specific Aim 3. *Assess sub-lethal reproductive effects of CAFO extracts on surrogate fish as an indicator of multigenerational, population level disruption*.

2.0 FIELD SITE DESCRIPTION

Field site A is in northeastern Wayne County, about 9 miles north of Goldsboro, NC. The farm houses a large farrowing operation, with the farm facilities. A waste lagoon is located adjacent to the hog houses and both the liquid and solid fractions of the wastes are applied as fertilizer to nearby fields. The site drains into Nahunta Swamp, a channelized stream, which is a tributary of Contentnea Creek which in turn, flows into the Neuse River. As part of previous investigations at this site by the U.S. Geological Survey, the North Carolina Department of Natural Resources, and North Carolina State University, a series of wells and piezometers were installed along the perimeter of a field downgradient from the hog houses and lagoon. This field is used for production of various crops typically corn, soybeans, and wheat. Wastes from the lagoon are routinely applied to this field. Well installations include three well clusters installed along a transect from the top of the field to the edge of the stream, a pair of wells along the upgradient corner of the field, and three piezometers two of which are along Nahunta Swamp and one which is at the upper edge of the field. Well depths range from less than 10 to more than 60 feet. There is no vegetative buffer between the field and Nahunta Swamp.

Soils in the field range from well drained along the upper edge of the field to poorly drained at the lower edge. Tile drains reportedly were installed to facilitate drainage in the field and two outfalls can be accessed for collection of water samples when Nahunta Swamp is wadeable. Flow has been observed at both outfalls on a year-round basis. Previous investigations have indicated that the tile drains intercept a large part of the nitrogen applied to the field. The shallow aquifer under the field is a mixture of fine, medium, and coarse sand. Thickness of this aquifer ranges from about 10 m upper edge of the field to about 1 m near Nahunta Swamp. This shallow aquifer is underlain by a confining unit of dark gray clayey silt about six feet thick. Below the confining layer is a brown and gray medium-grained sand aquifer. Water-level data at the site indicate that the head is similar in both aquifer systems. This site was part of an investigation of the contribution of confined animal feeding operations to antibiotics in the environment as well as studies of antibiotic resistance by personnel from the U.S. Environmental Protection Agency, the U.S. Geological Survey, and the University of North Carolina at Chapel Hill School of Public Health. Water chemistry and microbiological data are available for samples from the wells, tile drain outfalls, piezometers, Nahunta Swamp, and the lagoons for various periods from 1996 through 2002.

Filed Site B is in the Contentnea Creek sub-basin of the Neuse River and lies south of the confluence of the Sandy Run and Middle Swamp headwater streams in the 110 km2 Middle Swamp watershed. The site encompasses a first-order drainage, known locally as Plum Tree Branch that drains to Sandy Run a third-order stream. Since 1995 the site has been operated as a CAFO finishing facility housing both a wastewater holding facility (lagoon) and a spray field waste treatment field. A waste lagoon is located adjacent to the hog houses and both the liquid and solid fractions of the wastes are applied as fertilizer to nearby fields. This field is used for production of various crops typically corn, soybeans, and wheat. Geologically, field site 2 lies in the Coastal Plain, a landscape characterized by a series of progressively younger paleoshorelines and intervening terraces that step down in elevation and age toward the coast and into drainages (Tesoriero et al., 2005). Landforms form are typical for the costal plain with poorly drained, upland wet flat headwaters adjacent to well to moderately drained upland dry flats and valley side slopes. The hydrogeologic framework for field site 2 indicates the presence of a shallow groundwater system of near-surface aquifers and confining units.

3.0 RESEARCH PLAN

3.1 Specific Aim 1*. Identify two large-scale feeding operations within the Neuse River basin as models for assessment of natural estrogenic activity from CAFO waste discharges.*

3.1.1 Results and Discussion

In approaching a sampling plan for assessing the biological activity of EDC's from CAFO's in natural waters, various sites around the state were considered. We first contacted state and academic experts, and examined data from USGS, NC Department of Agriculture and Consumer Services and NC DENR to determine appropriate areas to base our study. We identified sites along the Cape Fear and Neuse Rivers, that appeared to house numerous large scale feeding operations, and provided candidate sources for hormonally active compounds. These potential "hot spots" and our rationale for selection were discussed and a Technical Review Committee (TRC) (see below) was formed to assist in final selection of the study sites. During this first meeting, the investigators presented proposed site data, gathered and outlined specific characteristics of each potential sampling site. The group then finalized the study site locations for the research. Based on available resources and our working knowledge of the types of bioanalytical assays planed, it was propose to investigate two sites as part of our study. Sites chosen were based upon the following criteria: proximity to Neuse River Basin-tributaries, type of swine operation (farrowing or finishing), repeated accessibility to site and accessibility to sampling areas, established large open air waste lagoons, spray application of waste slurries on agricultural land plots and cooperation of facility operators. Based on these criteria two field sites were chosen one farrowing and one finishing facilities both of which were considered indicative of similar types of swine facilities throughout the region. Both facilities were located within the Neuse River basin and located within counties ranked in the top five hog -producing counties in North Carolina (Table 1). Both sites have previously been used in large-scale EPA projects for assessment of phosphate fate and transport, ammonia emissions and assessment of antibiotic residues.

A sampling plan was established and consisted of a collection of samples every 3 months incorporating each season (May-spring, August-summer, November-fall and February-winter). Samples were collected directly from retaining ponds at feedlots with a high density of penned animals and from secondary streams located adjacent to the waste lagoons. Sampling sites were chosen with the aid of a Technical Review Committee comprised of a panel of water quality experts from the academic community and the NC Division of Environmental Health Table 2.

Table 1. *Facility information*

Site No.	Name	Nature of operation	County in NC	# Of animals	Annual waste generated (gallons/yr)	Geographic Co-ordinates
1	Private Swine Operation A	Integrator	Greene	~5000	2,300,000	35°31'37" 77°33'46
2	Private Swine Operation B	Integrator	Wayne	~6,500	3,000,000	35º 30´59˝ 77º 55´ 23˝

Source*: www.scorecard.org (2005), data from 1997 Census of Agriculture.*

Table 2. *Technical Review Committee*

Name	Affiliation
Seth Kullman	Duke University, Nicholas School of the Environment
Karl Linden	Duke University, Pratt School of Engineering
David Hinton	Duke University, Nicholas School of the Environment
Kenneth Rechow	Duke University, Nicholas School of the Environment
Gerald LeBlanch	NC State University, Environmental, Molecular Toxicology
Robert Rice	NC State University, Agricultural Engineering
Barbara Grimes	NC Department of Environmental and Natural Resources

3.2 Specific Aim 2*. Determine estrogenic activity of water samples using quantitative in vitro and in vivo bio-analytical analysis.*

3.2.1 Background

Substantial development of biological methods to assess the endocrine like activity of water including drinking water, ground water, surface water and wastewater has been achieved. These methods are finding increasing utility as environmental screening tools due to the analytical challenges in detecting low levels of hormonally active compounds in complex water matrices. Bioanalytical methods are a necessary adjunct and in some cases, replacement, for chemical analysis due to the ability to detect biological activity at ultra low concentrations. Bioassays also provide an assessment of integrated exposure (e.g. synergistic and/antagonistic effects), as the effect of chemical mixtures cannot always be inferred from their concentrations. In keeping with this general principle North Carolina Department of Water Quality opted to conduct biological assays using Ceriodaphnia as their principal mechanism for evaluation of municipal and industrial effluents.

In addition to the above, numerous in vitro assays have been developed for assessment of the direct action of environmental estrogens including: reporter gene assays, hormone receptor ligand binding assays, cell proliferation assays, and analysis of estrogen responsive genes in cell lines are used. To date, many of these assays have been successfully used to determine water quality analysis. However, while effective for screening purposes, the major criticism of in vitro testing is that they may not reliably predict endocrine modulating activity in vivo. This concern is due to inherent differences between in vitro and in vivo testing systems. Thus, to fully examine endocrine modulating activity, it has been suggested that a complimentary battery of in vitro and in vivo assays be utilized.

Herein we use a complementary set of in vitro and in vivo bio-analytical assays representing different levels of biological organization to examine the biological activity of natural estrogens from large scale feed lots in a selected North Carolina waterway. Appropriate design and implementation of toxicity assays facilitates assessment of risk posed by these compounds and was used as a screening procedure for phase two of this study where chronic sub-lethal reproductive effects were evaluated. Our experimental protocol is designed to assess estrogenic potency and each assay differs with respect to mechanism and functionality,

providing a breadth of information on estrogenic activity. Additionally, each assay is chosen based on sensitivity, cost, and time required to establish effect.

3.2.2 Methods

Sample Collection: Water was collected quarterly at each site, including water samples from retaining ponds at feedlots with a high density of penned animals and from streams located just below effluent outfall that receive runoff. Sample vol. = 2.0 L were collected by immersion of acid/solvent washed glass bottles into each sample matrix. Samples were stored at 4˚C until extracted.

Sample extraction*:* All samples were passed through Millipore glass fiber pre-filters, pH adjusted and concentrated by solid phase extraction (SPE) with Empore C18 disks. Stream samples: 500mL of raw water was filtered using a 0.45μm glass filter. Following filtration, filtrate is acidified to pH to 2-3. Waters were extracted using solid phase Empore filter disks previously conditioned with acetone/methanol. Organics fractions were eluted in 30 ml of methanol. Sample were dried with a steady stream of nitrogen and resuspended in 0.5 mL of methanol. Lagoon samples are treated same as others however, samples are centrifuged at 24,000g for 20 minutes prior to pre filtration to remove suspended solids. Dilutions of this concentrated extract were made between 1000X and 0.01X and examined by YES, VTG and reproductive analysis.

Yeast Estrogen Screen: Yeast cells were provided by Dr. Sumpter at Brunel University in U.K. The YES assay was performed as previously described in Chen et al. (2005). Briefly, 100 μl of sample extract was added to 96 deep-well plates (1ml capacity/well, VWR, West Chester, PA). The plate was placed in the hood for several hours to evaporate methanol in the extract (only for 1000 fold concentrates) and the dried extract was resuspended in 100μl of deionized (DI) water. Serial dilutions (11) were made from the sample extracts using DI water as a diluent. All dilutions of sample extract were performed in duplicate. 17β-estradiol (E2) (0-100nM) was made in DI water in duplicate and served as a positive control. DI water was the blank control. 300 μl of diluted yeast solution at an optical density of 0.07-0.08 at 630 nm was added to each well in the plates. Plates were then covered with sterilized papers and incubated at 30˚C for 3 days with gentle shaking. After 3 days of incubation, 400μl of 0.1% (w/w) ortho-nitrophenyl-beta-D-galactopyranoside (ONPG) substrate was added to each well for 30 minutes of enzyme/substrate reactions. β-galactosidase subsequently catalyzed the cleavage of ONPG; resulting in colorimetric response (see below) at 420 nm. 200μl of 1 M sodium carbonate was added to stop the reaction. Plates were centrifuged at 3000 rpm for 10 minutes. 100μl of supernatant was taken from each well and then transferred to a new 96 well microtiter plate to determine O.D at 420nm and 630 nm. All values were presented as OD_{420} minus OD_{630}.

Data Analysis for YES assay*: T*he YES assay provides an assessment of estrogenic activity reflecting the degree of "estrogenic" contaminants (ligands) in a given sample. Results are given as estradiol equivalents (EEQ's) see *Equation (1)*, which relates environmental samples with the 50% maximal activity observed with stock concentrations of 17-β Estradiol (E2) in laboratory water. In order to estimate EC50 values for each sample, serial dilutions of concentrated extracts were made and a dose response calculated. All water samples were split into two sub samples and YES dose response curves determined in duplicate. Sigmoid concentration-response curves of E2 and all samples were fitted to a symmetric logistic function (based on Hill equation) using software Prism (GraphPad, San Diego, USA). The responses of the standard and the samples were expressed as percent of maximum response evoked by E2 as (R-

Rctl)/(Rmax-Rctl). Effective concentrations to half maximum response (EC50) for the E2 standard or each sample were then fitted again with the software. (EEQ's) were calculated using the ratio of EC50 for E2 to EC50 for each sample.

***Vitellogenin assay**:* Adult sexually mature medaka were exposed by static renewal immersion in 2 L rearing baths spiked with dilutions of environmental extracts. Rearing media (50%) is replaced bi-daily to control for water quality. Fish are fed a casein-based, purified diet and environmental conditions are rigorously maintained by partial submersion of beakers into a tabletop tank system maintained at 25^{o}C under a 16hr light/8 hr dark photoperiod. Exposure durations are for 7 d (based on previous results for maximal VTG production). Non-spiked ERM plus ERM-MeOH were used as a negative control and rearing media spiked with 17β- estradiol served as a positive control. Following exposures, serum was collected extracted from medaka tail vein, immediately placed in ERM containing 1mM PMSF to ensure inhibition of protease activity. Quantification of vitellogenin was determined by ELISA as described in Arukwe et al. 2001, using a commercially available ELISA to medaka VTG Biosense Co. (Bergen, Norway). Quantitative evaluation of estrogenic potential generated from ELISA data will is determined relative to hormone free control. Results are reported as μg VTG/ml or as percent of ERM control. General statistical analysis was performed using the S-PLUS statistical analysis program

3.2.3 Results and Discussion

Sampling design: Analysis of estrogenic activity was determined at two private swine operations listed. Our general strategy was to sample several sites within each farm and make assessments to the mobility of estrogenic compounds between waste lagoons and the adjacent tributaries. Waters and waste slurry samples were taken from each of the four indicated times and processed for extractions and estrogenic analysis using the in vitro (YES assay) and in vivo (VTG assay).

Table 3. *Sampling period*

Sample period	Season
Sample set 1	Spring 2005
Sample set 2	Summer 2005
Sample set 3	Fall 2005
Sample set 4	Winter 2006

Our sampling protocol for both swine operations included collecting water samples form adjacent tributaries or secondary waterways 1. Up-stream of the facility, 2. Down-stream from the facility and 3. Directly from the waste lagoon. From operation A, additional samples were initially collected from a drainage ditch where run off from lagoon spray field application had collected or from a stagnant swamp approximately one half mile down stream of swine operation A. All samples were collected in well cleaned, alcohol washed, amber containers and stored at 4°C after collection from their respective sites.

Sampling and analysis of swine waste presented several challenges including development of appropriate sample extraction methodologies and assessment of estrogenic activity. An initial component of this study was to identify an appropriate protocol for extraction and analysis of water and waste slurry samples for use in both the in vitro yeast estrogen screen and in vivo VTG assay. Initially, three different approaches were used for sample preparation including 1. Direct analysis of raw water (no extraction or concentration), 2. Freeze

drying of water samples and waste slurries and 3. Solid phase extraction. Our results in phase one of the study demonstrated that solid phase extraction is the most robust method for both in vivo and in vitro analysis (data for methods 1 & 2 not shown). We thus continued with this method for extraction for all samples collected during each sampling period. (SPE) results in reproducible recovery of estrogenic contaminants and minimized water matrix interference including TOC, alkalinity and other water parameters, which can significantly influence our biological assays. Additionally SPE enables concentration of samples up to 1000X enhancing dose response analysis. For quantitative assessment of estrogenic activity, we employed a two-tiered evaluation model, using both in vitro (Yeast estrogen screen-YES) and an in vivo (medaka vitellogenin-mVTG-ELISA) bio-analytical analysis. This strategy encompasses mechanistically diverse assay tools to fully evaluate the biological activity of environmental water/slurry samples.

Yeast Estrogen Screen: The Yeast Estorgen Screen (YES) is a reliable and consistent mechanism used to biologically assess estrogenic activity from environmental matrices (Jobling et al., 1997; Hugget et al., 2003,). The assay is based upon the development of a recombinant yeast strain engineered to contain the human estrogen receptor (hER) and estrogen response elements (ERE) coupled to *lac* reporter gene. Since the ERE is linked to a reporter gene *lac*-Z, the enzyme β-galactosidase is secreted into the medium when ER ligands (such as natural estrogens) bind and transactivate the ER/ERE complex and initiate expression of *lac*-Z gene (Routledge and Sumpter, 1996). β-galactosidase subsequently catalyses the cleavage of ortho-nitrophenyl-beta-D-galactopyranoside (ONPG) resulting in a colorimetric response at 405 nm (Huggett et. al., 2003). YES data is presented as a functional molar equivalent of the physiological estrogen 17β-Estradiol. This provides a mechanisms to compare relative estrogenic activities to a known standard and is expressed as the estrogen equivalence quotient.

$$EEQ = \frac{EC_{50}[E2]}{Dilution\ Factor\ for\ 50\%\ response\ of\ sample} \qquad \text{Equation 1}$$

YES data for lagoons samples consistently demonstrated that waste catchments contain high levels of estrogenic activity with EEQ values ranging between 1.33E-7 to 6.25E-10 (note EC 50 for 17β-estradiol 2.0E-9) Table 4. This suggests that natural estrogens such as estradiol are excreted, mobilized and bioavailable in lagoons during animal farrowing and rearing practices. Adjacent tributaries and creeks exhibited seasonal variability in estrogenic activity, which coincide with spray field application of aqueous lagoon wastes. This fact however was highly dependent upon land application practices and environmental conditions including rain events. Determination of estrogen equivalents demonstrated a significant difference between farrowing and finishing facilities with the farrowing facility consistently generating higher estrogen equivalents (EEQ) values.

We suspect that higher activity in the farrowing facility is due to the synchronization of sow and gilts into estrus and lactation with resultant high concentrations of estrogens excreted daily. The farrowing facility additionally contains a larger number of animals. However, until we determine specific operational parameters including: flow rates of aqueous and solid waste into the lagoon, totals numbers of animals reared, total number of animals in estrus and total volume of holding lagoons we cannot draw definitive conclusions regarding operational differences. We additionally observed seasonal variation in estrogenic activity in lagoons and creek beds, with fall and winter months being one to two orders of magnitude lower than that observed in spring and summer. Changes in activity are most likely a result of different rearing and operational

practices between summer and fall months. Additional consideration for this change in estrogen activity could include nutrition practices, animal management, and animal behavior, which are distinct within the rearing process.

Table 4. *YES Assay: EEQ values by Sampling Period and Time of Year*

EEQ Values				
Sample	Summer 2005	Fall 2005	Winter 2005	Spring 2006
Site A: Upstream	2.68E-11	6.52 E-11	1.34 E-12	4.52 E-13
Site A: Lagoon	6.58 E-11	1.42 E-09	1.29 E-09	2.02 E-07
SiteA: Downstream	3.42 E-12	4.12 E-11	5.93E-10	9.86E-13
Site B: Upstream	2.30E-12	7.70 E-11	9.12 E-11	5.93 E-12
Site B: Lagoon	1.02 E-08	1.02 E-10	4.22E-08	3.73E-11
Site B: Downstream	1.43E-11	7.03E-12	1.41E-10	6.34 E-
Environmental concentrations of E2: ~1.0 E-13				

Vitellogenin Assay: Estrogenic activity was also measured by a complementary VTG assay. The VTG assay is an aquatic in vivo assay that addresses limitations associated with in vitro testing, specifically differences in pharmaco/toxicokinetics and multiple modes of hormone action. (Nimrod and Benson 1998; Thompson et al., 2000). Vitellogenin is a phospholipoprotein synthesized in response to ER receptor stimulation in the livers of oviparous female organisms. Since expression is relegated predominantly to the female, the appearance of vitellogenin in the male is indicative of abnormal hepatic ER activation. Recent studies initiated by the PI's and other laboratories demonstrated that specific environmental estrogens readily induce these reproductive proteins making them ideal markers for endocrine disruption (Arukwe et al., 2001; Arukwe et al., 1997). VTG production is demonstrated to be extremely sensitive to estrogens (down to the 0.0007 nM range) and it is highly applicable for the aquatic medium.

The Vitellogenin bioassay was conducted for 2 sampling periods Fall 2005 and Spring 2006 enabling quantification of vitellogenin synthesized in male medaka fish exposed to CAFO lagoon samples. As demonstrated in figures 1 and 2 exposure to 1X and 0.5X lagoon extracts demonstrate the potential capacity of estrogenic samples contained in the lagoon samples, to induce vitellogenin synthesis in male fish. For both sampling periods where VTG assay was performed, lagoon samples induced vitellogenin levels equal to or greater than levels induced by the positive standard 17-β Estradiol (1μg/L). The standard concentrations were 5-100 times greater than background environmental concentrations previously observed. This is an indication of the bioavailability of natural estrogenic compounds present in lagoon samples. Additionally these data demonstrate that the concentrations of estrogens in CAFO holding ponds are nearly 4-80 times greater than those typically found in the environment.

One difficulty with this approach however is water quality. Extracts of lagoon samples were consistently dirty, containing an unknown viscous substance which, significantly impaired water quality. As such our assay we limited to acute (up to 7 days) fish exposures. Fish were monitored several times a day for basic health or visible signs of toxicity. Even following rigorous monitoring and extended dilution up to (0.1X), fish exposed to lagoon extracts from field site B (Fall 2005) remained toxic and fish did not survive. As such data for VTG expression during this time period is only provided for filed site A. In spring of 2006 the 1X lagoon extracts exhibit toxicity with one fish out of six replicates surviving. VTG expression in both lagoons A and B remained high with the 0.5X extracts and response was equal to or higher than that

observed with the positive control 17β-Estradiol. To remedy the problem with toxicity, new extraction and clean up methods are being implemented. Thus more chronic exposure can be conducted with lower dilutions of extract. It was initially assumed that induction of VTG in male medaka was due to the presence of natural estrogens in the lagoon extracts. Typically swine excrete estrogens in their urine (Moore et al., 1982; Palme et al., 1996) and several studies have demonstrated that estrogen concentration (estradiol, estrone, estriol) varies with reproductive stage, non-pregnant sows having the lowest and near term sows the greatest (Choi et al., 1987; Vos et al., 1999). Estimates in the United States suggest that swine contribute approximately 2.7 Mg estrogen per year (Hanselman et al., 2003). However, our YES analysis of MeOH extracted feed exhibited strong estrogenic activities demonstrating dietary intake and possible excretion of phytoestrogens may contribute to the estrogenic activities assayed in the lagoons. The occurrence and fate of these compounds through each facility has not been evaluated and is a subject of interest in our laboratory.

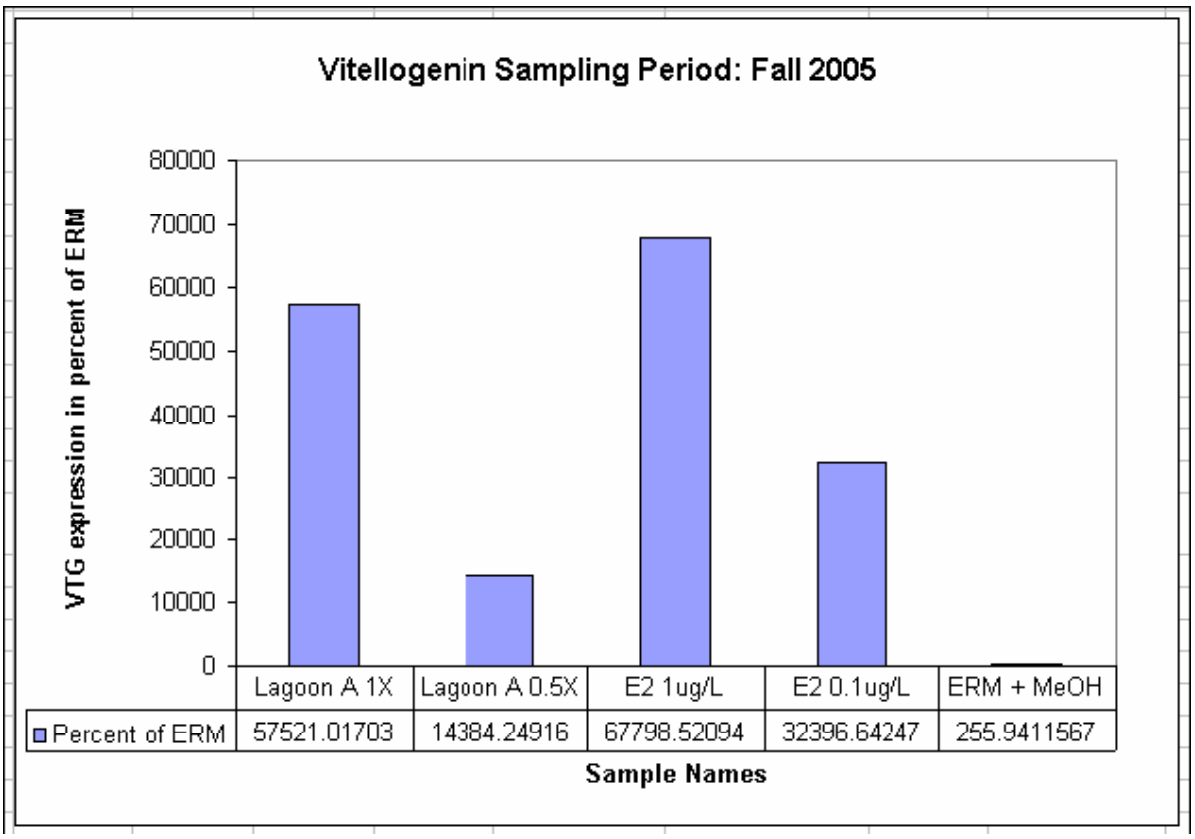

Figure 1. Vitellogenin expression as percent of negative control for Fall 2005 Sampling period

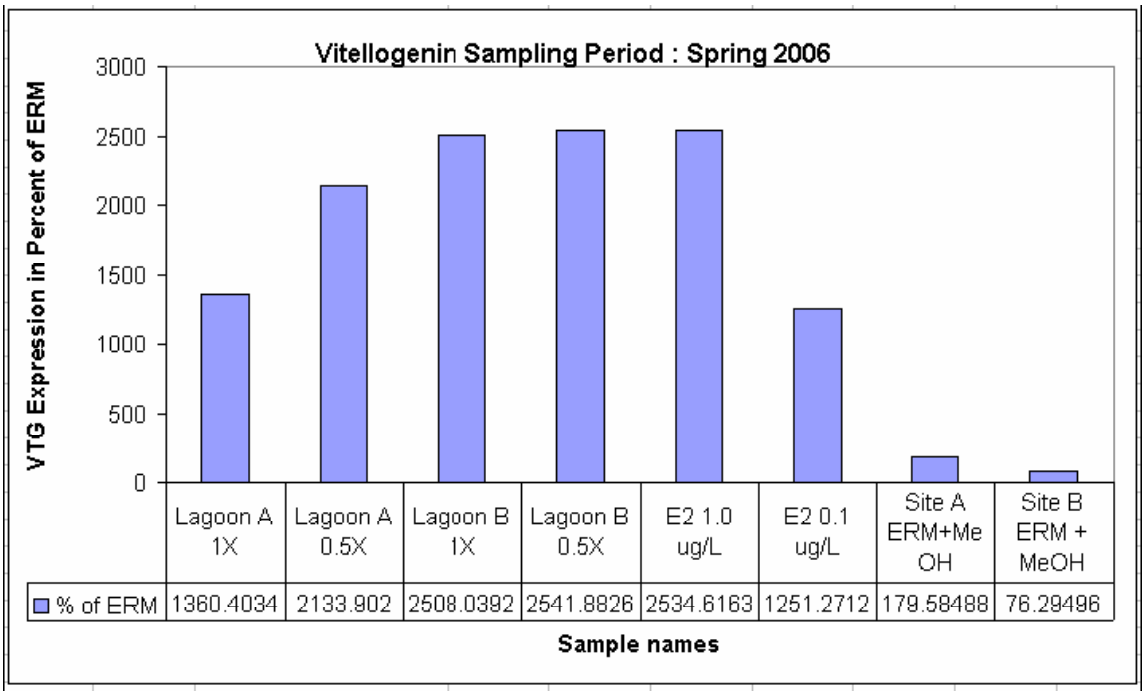

Figure 2. Vitellogenin expression as percent of negative control for Spring 2006 Sampling period

3.3 Specific Aim 3. *Assess sub-lethal reproductive effects of CAFO extracts on surrogate fish as an indicator of multigenerational, population level disruption*

3.3.1 Background

Here we have employed the small aquarium fish medaka (*Oryzias latipes*) to determine whether reproductive impairment is associated with exposure to estrogenic-estrogen contaminated water. This model, developed over the past 10 years in our laboratory group, is a highly standardized and calibrated in-vivo model of reproductive toxicity and endocrine disruption (Teh and Hinton, 1998; Cooke and Hinton, 1999; Koger et al., 1999). By rigorous control of temperature and lighting, reproductive capacity of breeding pairs is defined prior to exposure. Following exposure endpoints include: fertility, fecundity, hatching success, and embryo development of resultant offspring. This model has been used in a number of studies by USGS (Papoulias et al., 2000), US EPA (Arcand-Hoy and Benson, 1998) and various universities (Koger et al., 1999; Patyna et al., 1999; Cheek et al., 2001) focusing on endocrine disruption and reproductive effects, and has been recommended as model of choice for this type of work (Arcand-Hoy and Benson, 1998). In this aim we address the *so what* question, "Does exposure to natural estrogens in surface waters result in reproductive impairment"?

3.3.2 Methods

Chemicals: 17β-Estradiol (98% purity, Sigma-Aldrich) was used to prepare nominal stocks (0.004, 0.04, and 0.4 mg/ml) in dimethyl sulfoxide (DMSO). The stocks were stored at room temperature in the dark.

Test animals: **O**range-red medaka (*Oryzias latipes*) was used for this study. These animals reach sexual maturity at 3 months of age. Approximately six month old fish were collected from the laboratory's colony, which is maintained under recirculating aquaculture conditions.

Breeding experiment*:* The breeding experiment consisted of three time periods: pre-exposure, male exposure, and post-exposure. Throughout all three time-periods, egg production, fertilization rate, and hatching success were monitored.

Pre-exposure. Newly reproductive (5-6 months old, 85 females, 30 males) adult medaka were separated by sex, and kept under re-circulating conditions for one week. Fish were placed randomly in breeding groups consisting of three females and one male (25 breeding groups total). Each breeding group was maintained in a 2-L glass beaker with 1800 mL of fresh, well-aerated ERM, covered with organdy mesh. The water was renewed by 75% daily. Each breeding group received approximately 2 mg/day dry food (Otohime, Reed Mariculture, Campbell, CA, USA) and ~1 mL brine shrimp from lab culture twice per day. Beakers were arranged randomly in isolation and maintained at 23^{o}C on a 14:10 light:dark cycle for 12 days. Eggs were collected from each beaker about one hour after the lights came on every morning. For each breeding group, eggs were counted and fertilization rate determined under a dissecting microscope. During this period, fertilized eggs from all beakers were pooled and placed in a 100 x 15 mm polystyrene Petri dish in an incubator at 26^{o}C and 16:8 light:dark cycle. After the pre-exposure period, each breeding group was randomly assigned to one of four treatments (DMSO control, 0.1 ppb E2, 1.0 ppb E2, or 10.0 ppb E2). The fecundity and fertilization rate of the groups were then tested to ensure that there were no statistical differences among groups prior to exposure.

Male exposure. The male from each breeding group was removed and placed individually in 500-mL beakers. During the male exposure period, the females were maintained in the same manner, as were the full groups during pre-exposure, including the monitoring of fecundity and fertilization rate. Each male exposure beaker was filled with ERM spiked with the appropriate treatment in the manner described previously (DMSO control, 0.1 ppb E2, 1.0 ppb E2, or 10.0 ppb E2). The males were fed approximately 0.5 mg/day dry food and brine shrimp daily. The spiked water was renewed by 50% every other day. Males were exposed for 14 days at 26^{o}C and 16:8 light:dark cycle.

Post-exposure. Males were returned to the appropriate beaker and conditions were maintained in the same manner as in the pre-exposure period, including the 75% water renewal. For each breeding group, fecundity and fertilization rate were recorded daily for 14 days in addition to days 17 and 20 of the post exposure period. Each day, the fertilized eggs from each group were kept in individual 60 x 15 mm polystyrene Petri dishes (one dish/breeding group/day) in an incubator at 26^{o}C and 16:8 light:dark cycle until hatching. The number of hatchlings and day of hatch were recorded for each batch of eggs collected from each breeding group.

3.3.3 Results and Discussion

Our protocol assesses the reproductive impact of natural estrogens by following defined procedures developed in our laboratory (Koger et al., 1999). In this study we chose to use a model compound 17β-Estradiol, in place of actual lagoon extracts due to the observed toxicity of the extracts in the VTG assay (see Specific Aim 2). Several studies, including our own, demonstrate that swine waste lagoons contain high concentrations of natural estrogens including 17β-estradiol, estrone and estriol. While these studies do not completely recapitulate

the complexities of actual lagoon waste, here we develop a quantitative reproductive assessment of a known lagoon component. Thus replicate breeding pairs included control and exposed male orange red medaka were exposed to 17β-estradiol for a 14 d duration, followed by an assessment of fertility, fecundity and hatching success.

Fecundity. Figure 3 shows the average total number of eggs produced for each of the four treatments. During both the pre-exposure and exposure periods there were no statistical differences between the treatment groups. The females did continue to produce eggs in the absence of the males, but at a greatly reduced rate. During the post-exposure period, the three E2 treatments resulted in a dose-dependent decrease in the average total number of eggs produced by the breeding groups.

Fertilization rate. The average percentage fertilization for each treatment is shown in Figure 4. During both the pre-exposure and male exposure periods there were no statistical differences between the treatment groups. Fertilized eggs were collected in a limited number of beakers for as many as 4-5 days after removal of the male. However, all groups achieved zero percent fertilization by six days after removal of the male. After return of the males, fertilization immediately returned to 90-95% for both the control and 0.1 ppb E2 treatment groups. The 1.0 E2 treatment group stayed at about 65-70% fertilization for the entire post-exposure period. The 10.0 E2 treatment group initially achieved about 50% fertilization, but the fertilization rate dropped throughout the post-exposure period to about 15-20%.

Hatching. The percentage of fertilized eggs that hatched for each treatment group is shown in Figure 5. The lower two doses of E2 were not significantly different from the control group (84.0% hatched). However, percentage hatching was only 50% in the 10.0 E2 treatment group. Time to hatch was slightly delayed only in the 10.0 E2 treatment group. Offspring of control fish took 10.0 days to hatch, on average, whereas offspring of fish in the highest treatment averaged 10.3 days to hatch.

Based on our previous studies we expect exposure to natural estrogens to result in reduced numbers of eggs per clutch (fecundity) and diminished fertilization success (fertility). Any reduction in fecundity, fertility, and viable offspring produced and extension of time needed to attain sexual maturation is regarded as potential for population level effect. Such response is interpreted as an impairment of recruitment of young of the year fishes to the population (Diekmann et al., 2004).

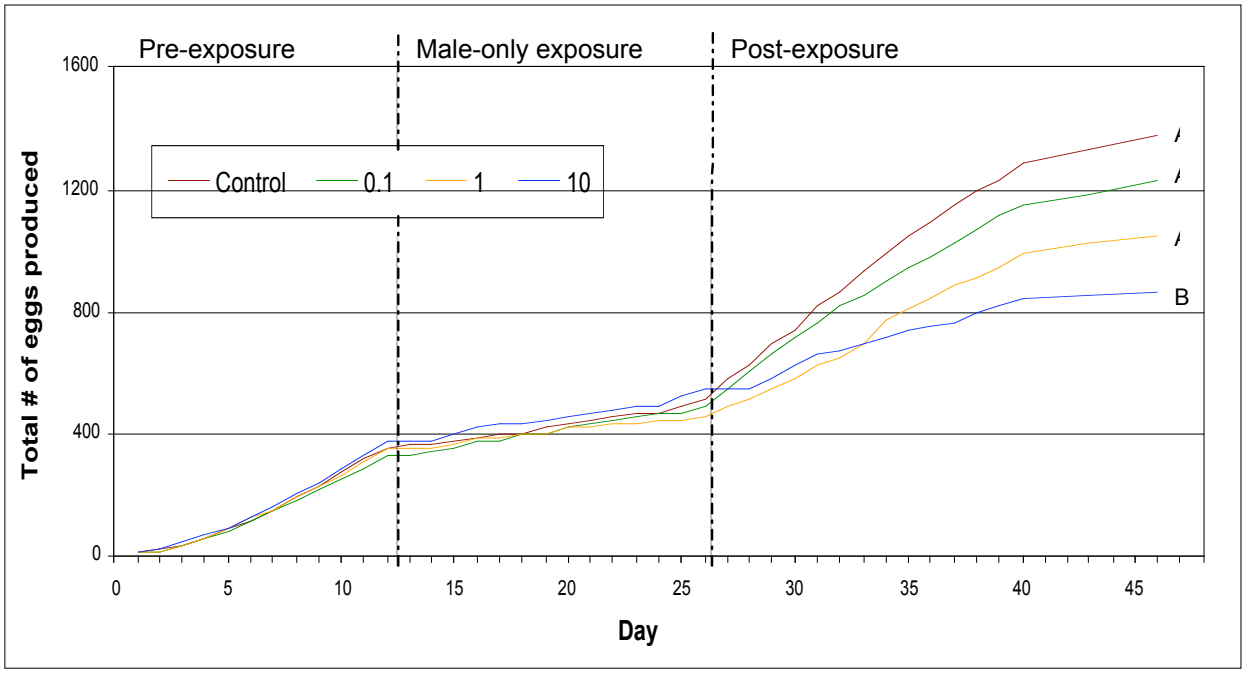

Figure 3. Average total number of eggs produced by day for medaka breeding groups during three periods: pre-exposure, male exposure to four treatments (DMSO, 0.1 ppb EE2, 1.0 ppb EE2, 10.0 ppb EE2), and post-exposure breeding. For lines followed by different letters, the total number of eggs produced was statistically different ($p < 0.05$).

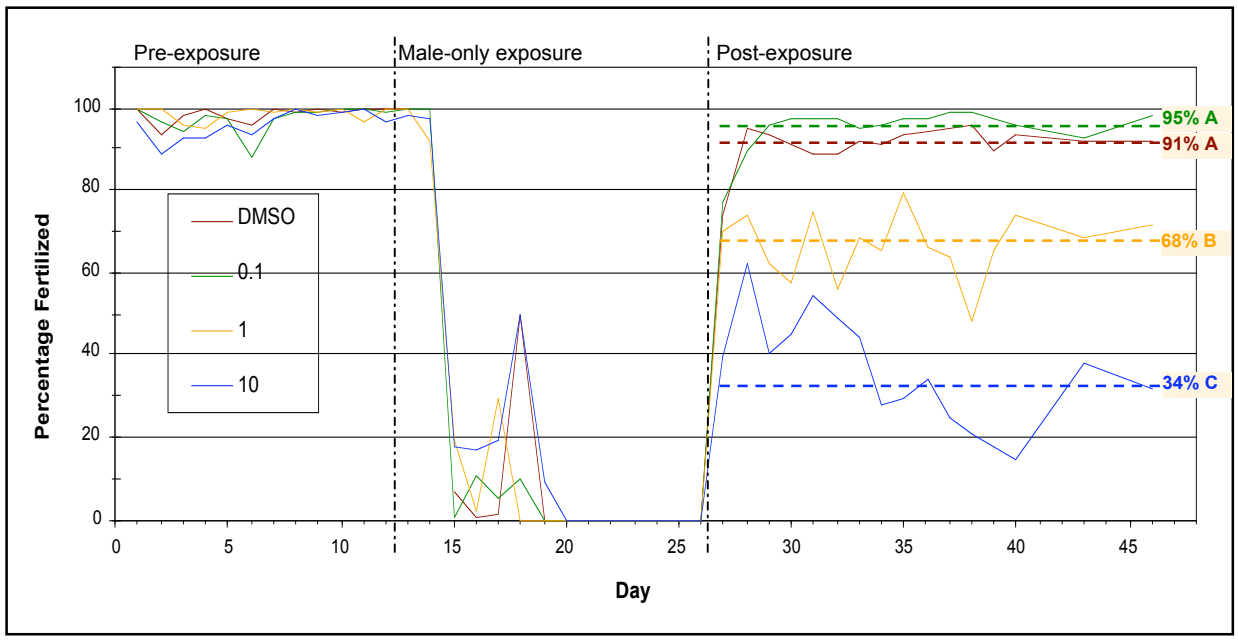

Figure 4. Percentage of eggs fertilized for medaka breeding groups during three periods: pre-exposure, male exposure to four treatments (DMSO, 0.1 ppb EE2, 1.0 ppb EE2, 10.0 ppb EE2), and post-exposure breeding. Dashed lines indicate the mean percentage fertilized during the 14-day post-exposure period for each treatment. Dashed lines followed by different letters are statistically different ($p < 0.05$).

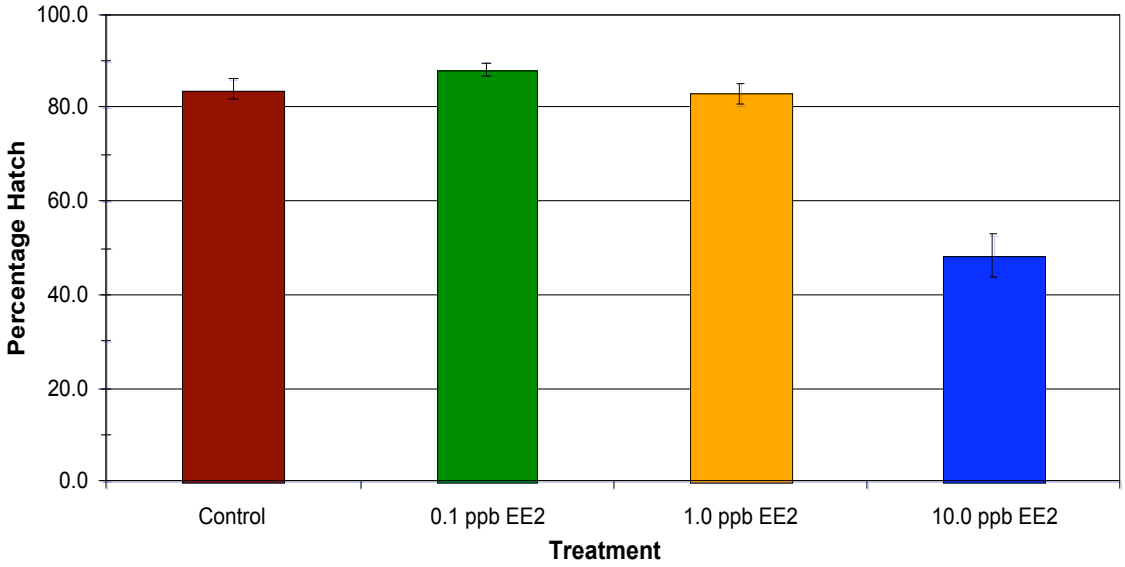

Figure 5. Mean percentage of fertilized eggs that successfully hatched for medaka breeding groups wherein males were exposed to four treatments (DMSO, 0.1 ppb EE2, 1.0 ppb EE2, 10.0 ppb EE2) for 14 days. Bars marked by different letters are statistically different ($p < 0.05$)

Few studies have assessed the influence of estrogen on adult male reproductive capacity in fish. Using the fathead minnow, (Filby et al., 2007; 2006) thoroughly examined target genes involved in sex steroid action, sex steroid synthesis, and sexual development and reproduction. This study found that a model estrogen ethinyl estradiol (EE2) induced gonadal gene expression alterations distinctive from the gene expression under controlled conditions. In some instances gene expression changes appeared to be linked to activation of the testicular estrogen receptor. As with previous studies exposure to EE2 induced classic feminized phenotypic effects. Previous studies examining the effects of estrogen on male reproductive function in medaka have also demonstrated that exposure of male fish to estrogen results in impaired reproductive function (Balch et al., 2004; Kang et al., 2001; Tilton et al., 2005). Male medaka exposed to 10ng/L EE2 from hatchlings to adults had a decrease in copulations and fertilization rate when paired with unexposed females and increased gonadal intersex (Balch et al., 2004). Adult medaka exposed to 500ng/L EE2 for 14 day showed a decrease in egg production, fertilization and hatching rate but an increase in egg production at 0.2ng/L (Tilton et al., 2005). This suggests that low concentrations of EE2 stimulate reproduction, while higher concentrations inhibit it. This study paired exposed males and females together as breeding groups, therefore, male or female specific effects leading to the decrease in reproduction seen cannot be conclusively deciphered. These results indicate the need for further understanding of the molecular mechanisms and pathways involved in the disruption of both male reproduction following exposure to environmental estrogens. Additionally more apical endpoints need to be evaluated in the context of differing exposure scenarios. While it appears that acute exposures to high concentrations of environmental estrogens have significant effects on reproduction, little has been determined regarding long term, chronic exposures to low environmentally relevant concentrations.

3.0 CONCLUSIONS AND RECOMONDATIONS

Steroidal estrogens are excreted in urine and feces of all farm animals. The extent of excretion is a function of age, sex, health, reproductive status and many additional factors such as diet that differ between animal types (Hanselman et al., 2003). Although the hormone content of waste has not been systematically studied, a relatively large total mass of hormones is released yearly given the estimated 291 billion pounds of manure generated annually in the United States (USEPA 2001). Swine excrete estrogens predominantly in their urine (Moore et al., 1982; Palme et al., 1996) and several studies have shown that estrogen concentration (estradiol, estrone, estriol) varies with reproductive stage, non-pregnant sows having the lowest and near term sows the greatest (Choi et al., 1987; Vos et al., 1999). To date, the use of estrogen excretion data for calculation of mass estrogen flux is uncertain. However, estimates in the United States suggest that swine contribute approximately 2.7 Mg estrogen per year (Hanselman et al., 2003). In today's farming practices swine facilities are usually arranged based on operational function. Farrowing facilities consist of sows and gilts that are often synchronized into estrus through the use of steroidal cocktails like PG600 containing a mixture of equine chorionic gonadotrophin and human chorionic gonadotrophin (Estill et al., 1999). This technology has enabled farmers to coordinate large-scale reproductive efforts and facility operations. Many CAFO's now incorporate these practices and maintain a highly organized facility where individual sows and gilts are penned based on their position in the reproductive cycle. In future studies these practices create an ideal opportunity to assess how temporal changes in operational practices impact natural hormone excretions and output to waste holding facilities. As such, farrowing facility maintain an organized structure where groups of animals are clustered and housed according to their reproductive phase including pre estrus, estrus, and lactation

From the perspective of waste management, most swine facilities in NC have adopted the open pit lagoon method for waste management and disposal. This entails collection of solid and liquid wastes from confined animal holding facilities, consolidation in large lagoons and periodic drainage of waste as spray field fertilizer to agricultural fields in accordance with state regulations. This method of waste management was originally incorporated based upon relative ease and lack of affordable, alternate waste disposal technologies. Over many years however questions have been raised regarding impact of this waste management strategy on environmental health. Initially concern was generated with regard to the release of nutrients, nitrogen and phosphate, then volatile organics and now natural steroidal hormones. Current novel technologies are in fact being develop to address these issues (Smithfield agreement). Several of which are recognized as promising mechanisms to effetely modify waste management practices with low environmental impact. However, implementation of these technologies will be costly and slow. New market- based incentives are being established to off set the costs but it is predicted that full deployment will take many years. This means open pit holding lagoons, as a waste management strategy, will continue for the foreseeable future.

Several studies, including our own, demonstrate that swine waste lagoons contain high concentrations of natural estrogens including 17β-estradiol, estrone and estriol. To date however, the fate of these estrogens in holding lagoons has not been fully established. Specifically, there are data gaps in our understanding of the transformation and mobility of these compounds in these waste catchments in addition to potential impacts on aquatic biota. Critical to this evaluation are several questions regarding the conversion of excreted 17β-estradiol to estrone and estriol, the stability of gulcuronide and sulfate conjugates and the partitioning of these compounds to either the solid or aqueous phase of the waste slurry. In future studies it

will be necessary to thoroughly characterize the stability and fate of estrogens in the holding lagoons. As the lagoons are the largest reservoir of estrogens at the CAFO, they represent an essential factor and critical determinant for environmental impact.

Also of concern is the mobilization of natural hormones to adjacent first and second order streams and aquifers. Where as the environmental fate of nitrogen is governed by volatilization, assimilation by crops, runoff, and infiltration into groundwater system, (Glasgow et al., 2000; Tesoriero et al., 2005), little is known regarding fate of natural estrogens in this process. CAFO's may have a substantial effect on estrogen loading to streams based on the amount of waste produced and applied to spray fields. Preliminary results from our pilot study demonstrate that adjacent first and second order streams exhibited seasonal variability in estrogenic activity, which coincided with spray field application of aqueous lagoon wastes. This fact however was highly dependent upon land application practices and environmental conditions including rain events. While highly conditional, these results suggest that CAFOS may be contributing to this activity. Often lagoons and spray fields are located adjacent to secondary and tertiary water ways and lagoon slurries are regularly applied during both the growing and off seasons as a means of adding fertilizer and controlling for waste accumulation. It is estimated that hundreds of gallons of lagoon slurry are applied per acre. It is proposed that the dominant pathway for estrogen transport from spray fields is surface runoff. However, transport to and from ground water is certainly possible and further characterization of transport is a necessary component to a complete assessment of the mobility of these compounds. To date however there is little data to quantify the environmental release rates of hormones from CAFO's. One study found approximately 1.3 μg/l estradiol in runoff from land applied with poultry litter (Nichols et al., 1997). Other studies demonstrated that runoff from a field receiving poultry waste contained up to 3.5 μg/l estradiol (Finlay-Moore et al., 2000; Burnison et al 2003). The mobility of estrogens following applications of animal waste is not well understood. The observation that estrogens are found in streams receiving runoff from agricultural plots as opposed to the waters above suggest that this practice may be contributing. Other factors that promote runoff to surface waters are likely to include geological factors such as plot slope, soil porosity or permeability, and proximity to surface waters. (USEPA, 2001). Thus further investigation to the major mechanisms governing fate and transport of these compounds needs to be established.

The source of estrogenic activity however remains equivocal and merits further examination on an operation specific basis. Results for this preliminary study provoke a myriad of questions regarding the occurrence, fate and distribution and biological effect of estrogenic compounds in reference to waste management at CAFO facilities. These questions are the major thrust of our laboratory and consistent with the objectives and goals of the WRRI community to maintain health and productive aquatic environments throughout North Carolina.

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Smithfield agreement:
http://www.cals.ncsu.edu/waste_mgt/smithfield_projects/smithfieldsite.htm#top

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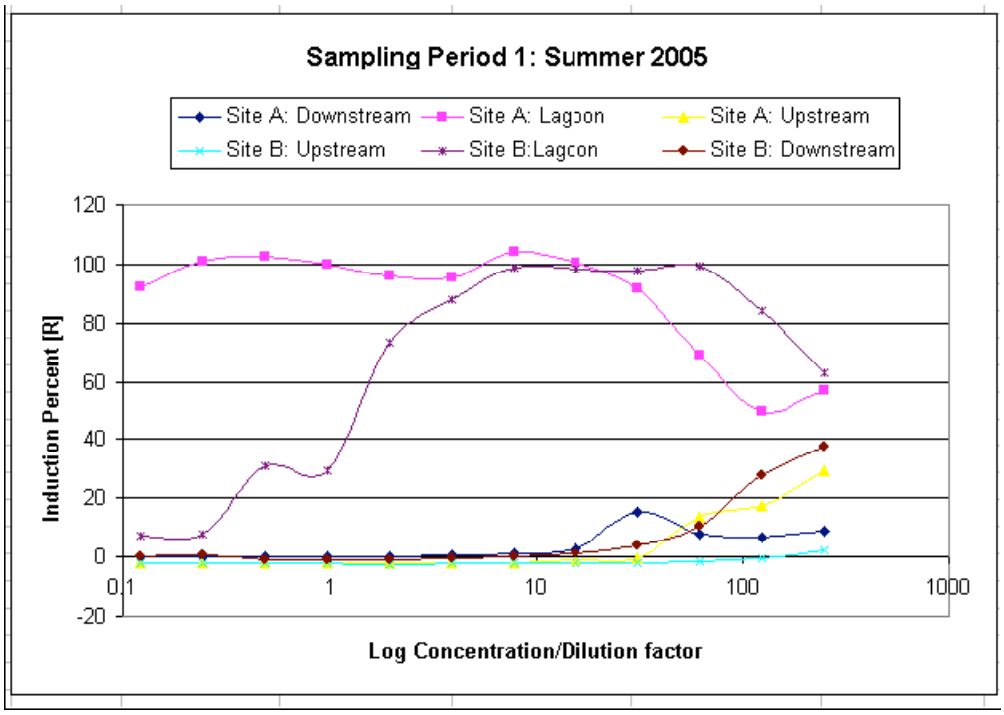
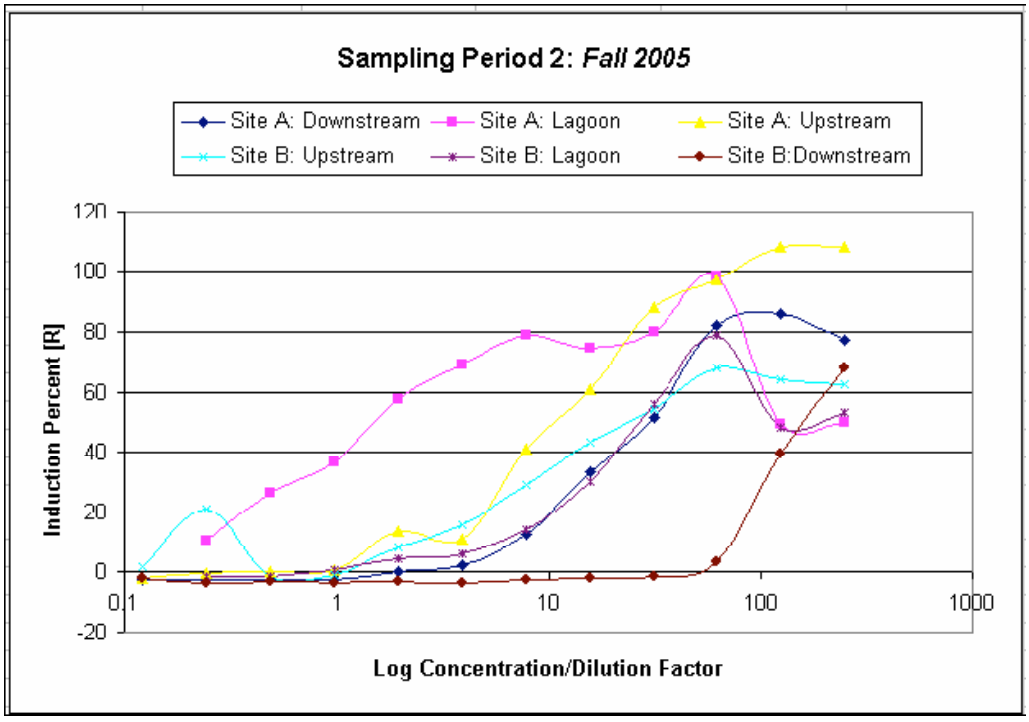
APPENDIX A:YEAST ESTROGEN DATA

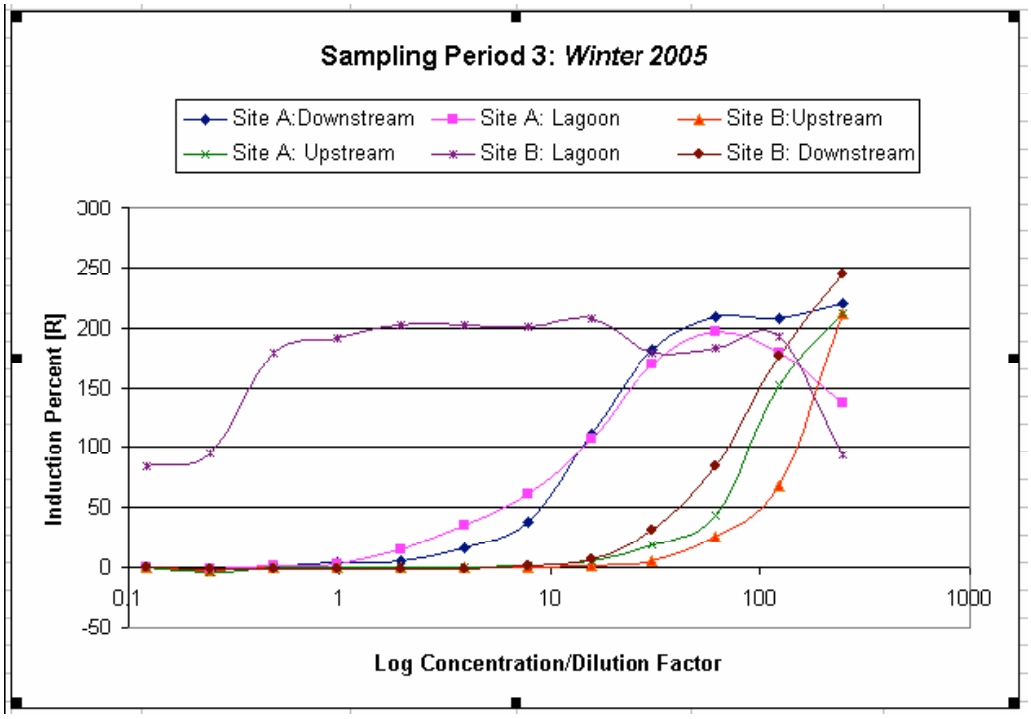
A1. Yeast estrogen screen (YES) for Sampling Period 1: Summer 2005



A2. Yeast Estrogen Screen (YES) for Sampling Period 2: Fall 2005



A3. Yeast Estrogen Screen (YES) for Sampling Period 3: Winter 2005



A4. Yeast Estrogen Screen (YES) for Sampling Period 4: Spring 2006