ABSTRACT

Title of Dissertation: POULTRY LITTER-INDUCED ENDOCRINE DISRUPTION: LABORATORY AND FIELD INVESTIGATIONS

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Nearly 1.6 billion lbs. of poultry litter are generated annually as a by-product of the Delmarva poultry industry. Disposal via application to fields as fertilizer results in runoff of poultry litter-associated contaminants (PLACs) into receiving waters. Of particular concern are natural steroid hormones 17 β-estradiol (E2) and testosterone (T), responsible for gender differentiation and development of reproductive structures. The objective of this research project was to assess the potential for endocrine disruption (ED) in fish populations on the Delmarva Peninsula as a result of agricultural litter application.

The investigation included 5 laboratory and 2 controlled field exposures of fish *(Pimephales promelas*, *Cyprinodon variegatus* and *Fundulus heteroclitus)* to water-soluble PLAC. Laboratory assays involved 21-d exposures of adult male and mixed gender larval/juvenile fish to negative, positive (E2) and solvent (EtOH) control treatments, and to one or several environmentally relevant PLAC solutions. Effects on gonads were assessed histologically and plasma and whole-body homogenate vitellogenin (Vtg) levels were measured as a gauge of estrogenicity. Results were used to determine PLAC lower-effects thresholds. Litter application on research fields allowed

comprehensive monitoring of runoff over entire planting seasons. Environmental persistence and transport were investigated by measuring PLAC in litter prior to field application and subsequently in runoff and receiving waters. Controlled field exposures involved caging of mature male *P. promelas* in surface waters receiving litter-amended runoff.

Laboratory PLAC exposures routinely induced Vtg in male *P. promelas* with response generally dose-dependent. Induction in *F. heteroclitus* only occurred at the highest tested PLAC concentration while *C. variegatus* were unresponsive at any tested concentration. All three species produced considerable Vtg in response to the E2 positive control. Gonadosomatic index was unaffected in adult fish, but gamete maturity appeared inversely related to PLAC concentration. PLAC exposure caused a pronounced feminization in *P. promelas* exposed as larvae (3 – 24 dph) but not exposed as juveniles (36-57 dph). Steroid concentrations (E2 and T) in field runoff were substantial (up to 350 ng E2/L). E2 in receiving waters had an environmental persistence of weeks to months and in one instance exceeded lower effects thresholds identified in the laboratory. ED was not evident in *P. promelas* caged within receiving waters. However, exposure in the laboratory to agricultural runoff (frozen and renewed daily) induced substantial Vtg in adult male *P. promelas.*

A digital morphometric method for quantifying the proportion of mature sperm within testicular epithelium was found to be accurate and reproducible and should prove applicable with minimal modification to a variety of small fish species. Also, the small fish caging system developed for this study (floating baskets within protective barrels) proved dependable for *in situ* experiments in a variety of locations.

POULTRY LITTER-INDUCED ENDOCRINE DISRUPTION: LABORATORY AND FIELD INVESTIGATIONS

by

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PREFACE

EXECUTIVE SUMMARY

The Delmarva Peninsula, consisting of eastern Maryland, most of Delaware, and the portion of Virginia east of the Chesapeake Bay, is one of the most densely concentrated poultry producing areas in the US. The region generates 600 million birds and 1.6 billion lbs of manure (or litter) annually. Excessive land application of poultry wastes has precipitated severe water quality problems in surface and ground waters throughout the region. Impacts include harmful algal blooms, decreases in water clarity, widespread anoxia and declines in submerged aquatic vegetation (SAV). Pollutants and pathogens in poultry litter traditionally linked to environmental degradation include nutrients and protozoan, bacterial and viral agents. In addition, recent attention has turned toward various non-traditional *poultry litter-associated contaminants* (PLAC). Included are feed additives (e.g., trace metals, antibiotics), poultry house/bedding material impurities (e.g., metals, pesticides) and fecal/urinary steroids (e.g., estrogenic and androgenic hormones).

In most vertebrates, sex steroids, specifically 17 β-estradiol (E2) and testosterone (T), are responsible for gender differentiation, development of reproductive structures and stimulation of breeding behaviors. They are released naturally in poultry urine and feces and persist at high concentrations (e.g., ≤ 904 ng/g E2 and ≤ 670 ng/g T on a dry weight basis) and for prolonged durations (> 2 years) in litter. Studies conducted previously on the Delmarva Peninsula and elsewhere have demonstrated the transport of E2 from poultry litter-amended fields to surface and ground waters at levels sufficient to

warrant environmental concern. However, limitations in our knowledge of PLAC dynamics in natural systems and our incomplete understanding of endocrine disruption (ED) as a consequence of PLACs exposure make any assessment of risk premature. The study was undertaken to provide information on some of these areas of uncertainty by quantifying poultry litter-associated steroid levels in natural waters and by exposing fish to PLAC in controlled settings. Major objectives of the study were:

1) Determine whether PLAC are capable of inducing endocrine disrupting effects in fish.

   H_0: A PLAC exposure concentration exists that will cause endocrine disrupting effects in fish.

2) Determine what PLAC concentrations and exposure durations are required to elicit such effects.

   H_0: A PLAC exposure level exists below which exposure does not produce endocrine disrupting effects in fish.

3) Determine whether PLAC reach surface waters via runoff from agricultural fields following "standard" litter application practices in sufficient quantities to produce these effects.

   H_0: Application of poultry litter to agricultural fields will introduce PLAC to receiving waters at concentrations capable of inducing effects.

4) Determine if fish in receiving waters are adversely impacted by exposure to PLAC in agricultural field runoff.

   H_0: PLAC entering receiving waters as a result of poultry litter application to agricultural fields will induce endocrine disruption in resident fish.

METHODS

These objectives were investigated in a research project comprised of laboratory and field components. The laboratory portion of the project included a series of bioassays in which freshwater and estuarine fish were exposed to aqueous PLAC

solutions. In all, five assays were performed, three with the freshwater fathead minnow *(Pimephales promelas)* and two with the estuarine sheepshead minnow *(Cyprinodon variegatus)* and mummichog *(Fundulus heteroclitus)*. The assays used accepted biological measures of endocrine disruption to investigate the sensitivity of selected test species to endocrine active contaminants in poultry litter. Tissues, especially gonads, were assessed histologically to investigate degenerative/developmental effects of litter exposure. Plasma and whole-body homogenate vitellogenin (Vtg) levels were measured to gauge the utility of vitellogenesis as a biomarker of poultry litter-associated estrogenic exposure. Effects-thresholds and dose-response relationships were investigated by exposing test animals to complex contaminant mixtures in dilution series' spanning the environmentally relevant range. Multiple life stages (larval, juvenile and adult) were employed to investigate the implications of age on contaminant sensitivity. Aims of laboratory assays were two-fold: (1) to identify the endocrine related toxicological endpoints most sensitive to PLAC exposure, and (2) to determine the suitability of the selected test species as sentinels of deleterious effects on resident fish populations.

Application of poultry litter on research fields at the University of Maryland Wye Research and Education Center (UMD-WREC) in 2000 and again in 2002 allowed specific monitoring of runoff over entire planting seasons. Measurement of steroids in litter prior to application and subsequently in field runoff and a receiving pond demonstrated persistence and movement of PLACs from fields into receiving bodies. Fish *(P. promelas)* were caged within the pond receiving litter-influenced agricultural runoff to investigate the "real world" effects of PLAC exposure on laboratory-reared animals. Finally, *P. promelas* were exposed in the laboratory to field runoff (frozen at

the time of collection) to investigate PLAC effects under controlled conditions. Endocrine endpoints developed in laboratory assays were employed to assess effects in field-exposed animals.

RESULTS & DISCUSSION

Laboratory Results

Results from laboratory assays clearly demonstrate that endocrine disrupting effects in *P. promelas* can result from exposure to PLACS at environmentally relevant concentrations. Plasma Vtg was induced to >40,000 μg/mL in mature male *P. promelas* exposed to aqueous poultry litter solutions. The 21-d LOEC for Vtg induction in male *P. promelas* occurred in a PLAC treatment with 40 ng/L E2 (3 of 8 exposed fish had appreciable plasma Vtg). Histological examination of testes revealed reductions in mature gametes (based on proportion of area occupied by spermatozoa/spermatids) but only following 21 d exposure to the highest treatment with a litter derived E2 level of 192 ng/L. The digital morphometric method, designed to quantify the proportion of testicular epithelium comprised of mature gametes (spermatozoa and spermatids) proved accurate and reproducible and should be applicable with minimal modification to a variety of small fish species.

Larval *P. promelas* exposed to litter-derived contaminants for 21 d (beginning at 3 dph) showed perhaps the most consequential effects when examined histologically (at 60 dph). The proportion of females (definitive based on presence of oocytes and presumed based on presence of an oviduct-like structure) exceeded 92% (24 of 26) in a poultry litter treatment containing E2 at 74 ng/L. Genotypic male fish either underwent a

gender reversal (becoming phenotypic females) or were sufficiently feminized to express a distinctly female oviduct-like structure. This result was dose-dependent, occurring to lesser extents, 72% and 55% female, in PLAC treatment with E2 levels of 45 ng/L and 18 ng/L, respectively. This result did not occur in juvenile *P. promelas* exposed to PLAC from age 36 – 57 d. Future tests with larvae should be directed at determining the age(s) at which *P. promelas* are sensitive to gender biasing effects and the minimum concentration and duration of exposure required to induce such effects. Also, exposed fish should be grown to maturity to investigate the permanence and consequence of developmental effects that occur in adolescence.

Unlike *P. promelas*, *C. variegatus* and *F. heteroclitus* were not sensitive to PLAC. While a positive control treatment (100 ng E2/L) readily induced Vtg in adult males of both estuarine species, *C. variegatus* were completely non-responsive to poultry litter-derived E2 >140 ng/L and *F. heteroclitus* were only minimally responsive (moderate Vtg induction 1 of 7 fish). A very modest increase in whole-body homogenate Vtg in larval *C. variegatus* from the positive control treatment suggests that, at this early age, this species is not particularly well suited as an indicator of exogenous estrogenic exposure.

Field Results

Detection of considerable E2 in runoff from litter-amended research fields (≤350 ng/L) and in the receiving pond (≤83 ng/L) clearly demonstrated the transport of PLACs to surface waters following rain events. Concentrations in field runoff were dependent on agronomic practices (e.g., *No-Till* vs. *Conventional-Till*) and intensity and duration of

rainfall. Because the *No-Till* research field required less precipitation to initiate runoff than did the *Conventional-Till* field, a small rain event (3.0 cm) that transported a concentrated pulse of water-soluble PLAC (E2 of 208 - 350 ng/L) from the *No-Till* field to the *Research Pond* did not produce any runoff from the *Conventional-Till* field. In a larger rain event (5.89 cm) initial E2 concentrations in *No-Till* runoff were high (190 ng/L), but decreased quickly (<100 ng/L) as a consequence of dilution. E2 transported in runoff from the *No-Till* field during this event was 2.7% of total field-applied E2. Runoff from *Conventional-Till* started later (after more dilution), was lesser in volume (because of soil infiltration) and, carried a lower E2 load (42 ng/L). Only 0.26% of litter-applied E2 was transported via runoff from the *Conventional-Till* field.

Resulting E2 levels in the research pond (receiving *No-Till* runoff) were a function of runoff volume and contaminant concentration, such that a high runoff volume of moderate E2 concentration *(Spring 2000)* resulted in pond E2 levels of 60 - 83 ng/L, whereas, a small runoff volume with highly concentrated E2 *(Spring 2002)* resulted in a pond E2 level of ~ 30 ng/L. Reduction of aqueous E2 in the pond (presumably through microbial degradation, adsorption to solids, deposition, etc) was sufficiently slow in 2000 that levels remained above 30 ng/L for the duration of the 21 d caged fish exposure. The resulting average E2 level for the *in situ* exposure interval was 50 ng/L, higher than the 21 d LOEC of 40 ng/L identified in laboratory assays. Nevertheless, 21 d *in situ* exposure did not induce vitellogenesis in mature male *P. promelas*.

However, 21 d exposure of mature male *P. promelas* to rations of litter-influenced field runoff (thawed and renewed daily, E2 = 147 ng/L) produced plasma Vtg levels

>3,000 µg/mL, confirming that environmental contaminants resulting from standard agricultural practices are capable of causing endocrine disruption in aquatic animals.

CONCLUSIONS

1. Exposure to poultry litter-associated contaminants (PLACs) at environmentally relevant concentrations caused endocrine disrupting effect in mature male fathead minnow. The most sensitive indicator of endocrine disruption, detection of plasma Vtg, was observed to levels >40,000 µg/mL in adult male fish exposed in the laboratory to aqueous extracts of poultry litter. Vitellogenesis occurred in >40% of fish exposed for 21 d to a treatment with poultry litter-derived17 β-estradiol (E2) of 40 ng/L.

2. Gender differentiation in larval fathead minnow showed dose-dependent sensitivity to PLAC exposure. The proportion of female/feminized fish exceeded 90% following a 21 d exposure to a treatment with poultry litter-derived E2 of 74 ng /L. Male fish either underwent gender reversal or were feminized to the point of developing an oviduct-like structure. The result occurred to a lesser degree in a treatment with 45 ng E2/L (74% ♀), and less still with E2 at the 18 ng/L detection limit (55% ♀).

3. Sheepshead minnow and mummichog were not sensitive to endocrine disruptive effects of poultry litter. Male sheepshead minnow were completely non-responsive to all tested PLAC treatments and male mummichog were only minimally responsive to the highest tested treatment.

4. Substantial quantities of poultry litter-derived E2 can be transported to surface waters via runoff from agricultural fields. The amount transported is a function of the initial E2 concentration in litter, the frequency, volume and intensity of precipitation and

the agronomic practices employed. Fields under "No-Till" management practices can lose up to 10 times more E2 than fields employing conventional tillage. At most, E2 transported to surface waters in runoff amounts to only several percent of total field-applied E2. Maximum measured E2 concentrations in runoff from *No-Till* and *Conventional-Till* fields were 350 ng/l and 42 ng/L, respectively.

5. Poultry litter-derived E2 can enter surface waters via field runoff and persist for weeks to months at environmentally relevant concentrations. For example, E2 in the *Research Pond* was increased to >60 ng/L by introduction of field runoff and required nearly 2 months to return to pre-runoff levels. The average E2 concentration for the 21 d post-runoff interval was 50 ng/L, higher than the 21 d LOEC of 40 ng/L identified in the laboratory.

6. Runoff from poultry litter-amended agricultural fields was capable of causing endocrine disruption in mature male fathead minnow. Preserved field runoff (collected and frozen) was sufficiently estrogenic to induce vitellogenesis in male fish exposed in the laboratory (21 d). However, dilution and/or natural "aging" (i.e., microbial degradation, particulate adsorption) sufficiently reduced the poultry litter-associated estrogenicity within the receiving body, that vitellogenesis in adult male fathead minnow was not evident following 21 d *in situ* or laboratory exposures.

8. The morphometric method developed to quantify proportions of mature gametes in histological slides of fathead minnow testes proved accurate and reproducible and should be applicable with minimal modification to a variety of small fish species.

9. The caging system developed for this study, using floating baskets within protective barrels, proved sufficiently adaptable for *in situ* exposure of small to medium

size fish in a variety of locations. The system works at depths of 0.5 to 4 meters, is robust enough to tolerate strong currents during high flow periods and is tamper resistant to discourage vandalism.

DEDICATION

This dissertation is dedicated to my parents, James and Tonya, my brother, Jate, and especially to Jennifer Pitz, whose support, confidence and perpetual patience made this effort possible. Thanks for everything.

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LIST OF ABBREVIATIONS

11-KT	11-Ketotestosterone
2,4-D	2,4-Dichlorophenoxyacetic acid
2,4,5-T	2,4,5-Trichlorophenoxyacetic acid
ac	Acre
ACHE	Acetylcholine esterase
ACTH	Adrenocorticotropin hormone
AFO	Animal feeding operation
AhR	Aryl hydrocarbon receptor
AR	Androgen receptor
BPA	Bisphenol A
CAFO	Confined animal feeding operation
CI	Condition Index
CRH	Corticotropin-releasing hormone
DDT	Dichloro-diphenyl-trichloroethane
dph	days post-hatch
E1	Estrone
E2	17 β-estradiol
E3	Estriol
EDC	Endocrine disrupting chemical
EE2	17 α-Ethinylestradiol
ELISA	Enzyme linked immunosorbent assay
EtOH	Ethanol
FSH	Follicle stimulating hormone
γ-HCH	γ-Hexachlorocyclohexane (Lindane)
GH	Growth hormone
GnRH	Gonadotropic releasing hormone
GSI	Gonadosomatic Index
GTH	Gonadotropic hormone
HPG	Hypothalamus-Pituitary-Gonad axis
LH	Luteinizing hormone
$LiqN_2$	Liquid nitrogen
LOQ	Limit of quantitation
MDE	Maryland Department of the Environment
MDL	Method detection limit
MS222	Tricaine methane sulphonate
MXC	Methoxychlor
NBF	Neutral buffered formalin
NOEL	No observable effect level
NRDC	Natural Resource Defense Council
OP	Octylphenol
PAH	Polycyclic aromatic hydrocarbons
PCB	Polychlorinated biphenyl
PGC	Primordial germ cell

PLAC	Poultry litter-associated contaminant
POP	Persistent organic pollutant
ppt	Parts per trillion
RIA	Radioimmunoassay
SHBG	Sex hormone binding globulins
T	Testosterone
TCDD	2,3,7,8-Tetrachlorodibenzodioxin (dioxin)
TRH	Thyrotropin-releasing hormone
Vtg	Vitellogenin
UMD-WREC	University of Maryland - Wye Research and Education Center
USEPA	United States Environmental Protection Agency
WHO	World Health Organization
XME	Xenobiotic metabolizing enzyme

CHAPTER I: INTRODUCTION

THE ENDOCRINE DISRUPTOR HYPOTHESIS

A Brief History of Endocrine Disruption

Scientists have long known that exposure to certain naturally occurring substances could induce a variety of developmental and reproductive consequences. Indeed people of antiquity ate specific foods, such as pomegranate and wild carrot, as a means of reproductive prophylaxis [Riddle, 1994]. Reproductive failure in Australian sheep was linked to grazing on abundant wild clover, the naturally occurring phytoestrogen formononetin, ultimately identified as the responsible agents by eliciting estrogenic activity [Bennet *et al*., 1946]. As early as 1950 researchers discovered that the synthetic organochlorine insecticide DDT was also a potent estrogen mimic capable of causing feminization (e.g., underdeveloped testes and failure to grow waddles and combs) when administered to young roosters [Burlington and Lindeman, 1950] and even to reduce sperm count after prolonged exposure in human male crop-dusters [Patlak, 1996]. Estrogenic activity was promoted by DDT despite the compound having little structural similarity to the primary vertebrate estrogens, 17 β-estradiol (E2), estrone (E1), and estriol (E3) *(Figure 1)*. Prescription of the synthetic estrogen diethylstilbestrol (DES), used for over 30 years to help prevent miscarriage, was discontinued in 1971 when found to dramatically increase rates of infertility and vaginal clear cell adenocarcinoma in daughters of users [Herbst *et al*., 1971]. Subsequent research identified an increased incidence of non-cancerous epididymal cysts among males exposed to DES *in utero* [Gill *et al*., 1979].

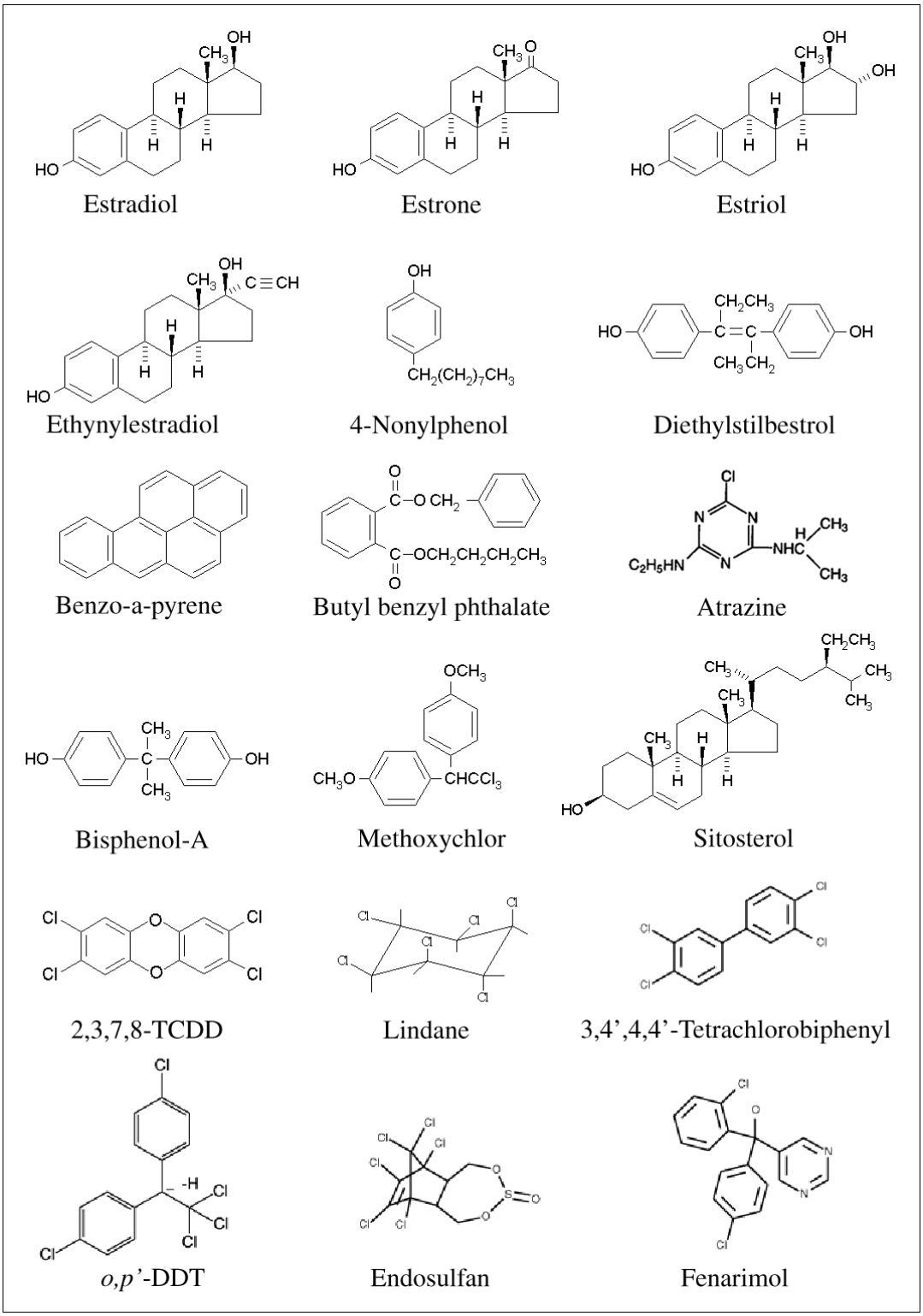


Figure 1. Molecular structures of natural sex steroids (17 **$**-estradiol, estrone, estriol), synthetic steroids (ethynylestradiol, diethylstilbestrol), and various other confirmed or suspected endocrine disrupting chemicals.

Reports were common in the 1970s linking reproductive and/or developmental impacts in wildlife to environmental contaminant exposures. Many involved hatching success in fish eating birds with the culprit contaminant identified as an intentionally dispersed pesticide or one of its metabolites (e.g., DDT, DDE, DDD) [Fry and Toone, 1981; Keith, 1966]. Because of high hydrophobicity and resistance to degradation, such compounds readily accumulate in biota and bio-magnify up food-webs. Thus, the most profound effects occur in climax predators [Norheim *et al*., 1992], such as the bald eagle *(Haliaeetus leucocephalus)*, which was in rapid decline until the 1975 US ban on DDT [Bowerman *et al*., 1995].

Since the 1970s, the number of suspected and/or confirmed compounds capable of endocrine disruption has risen dramatically *(Table 1)*. Contaminants implicated include intentionally released pesticides (e.g., 2,4-D; 2,4,5-T; DDT and metabolites; γ-HCH; methoxychlor; parathion; and others) [Amdur *et al*., 1991; Chowdhury, *et al*., 1987; Fry and Toone, 1981; Gray *et al*., 1989; Hayes and Laws, 1991; Rattner and Ottinger, 1992] and incidentally/accidentally discharged industrial process intermediates and/or by-products (PCBs; dioxins; furans; phthalate esters; alkylphenols; and trace metals) [ATSDR, 1991; Jobling *et al.,* 1996]. Some of the earliest effects attributed to endocrine disrupting chemical (EDC) exposure include abnormal thyroid function [Moccia *et al*., 1981; 1986], decreased fertility and/or hatching success [Kubiak *et al*., 1989; Leatherland, 1982; Mac *et al*., 1988; Reijnders, 1986; Shugart, 1980], feminization [Fry and Toone, 1981], masculinization [Davis and Bortone, 1992], and altered immune function [Erdman, 1988; Martineua *et al*., 1988]. Contaminants have been demonstrated to behave as hormonal agonists (mimics) or antagonists (blockers), competing for or

Table 1. Confirmed or suspected endocrine disrupting effects of various contaminants common to the aquatic

Class **Chemical**	**Source / Use**	**Endocrine Activity**
Alkylphenolics		
4-Nonylphenol	Surfactant intermediates	Weak estrogen agonist[1]
Octylphenol		
Bisphenol-A	Polycarbonate monomer	Weak estrogen agonist[2]
PAHs		
Benzo[a]pyrene	Fossil fuel combustion products	Weak ER agonist; strong AhR agonist[3]
Phenanthrene		
PCBs	Transformer oil	T3/T4 antagonist; Estrogen/androgen antagonist[4]
Phthalate esters:		
Butyl benzyl phthalate	Plasticizers	Weak estrogen agonist[5]
Di-n-benzyl phthalate		
Dioxins/furans	Industrial and waste incineration by-products	Strong AhR agonist[6]
Pesticides		
Atrazine	Herbicide	Reduced sensitivity to female priming hormones[7]
Vinclozolin	Fungicide	Androgen agonist[8]
Fenarimol	Fungicide	Aromatase inhibitor[9]
Methoxychlor	Insecticide	Weak estrogen agonist[10]
o,p'-DDT	Insecticide	Weak estrogen agonist[11]
p,p'-DDE	DDT metabolite	Androgen antagonist[8]

Table 1 (continued). Confirmed or suspected endocrine disrupting effects of various contaminants common to the aquatic environment.

Class **Chemical**	**Source / Use**	**Endocrine Activity**
Natural hormones		
17**$**-estradiol, estrone, estriol	Human and animal urinary/fecal steroids	Strong estrogenic and/or
Testosterone	from municipal effluent and agricultural runoff	androgenic activity
Synthetic hormones		
17α-ethynyl estradiol	Contraceptive steroids in municipal effluent	Strong estrogen agonist[12]
Phytoestrogens		
B-sitosterol	Plant sterols in pulp and mill effluent	Weak estrogen agonist[13]
Genistein		
Metals		
Lead	Battery production/recycling	Estrogen antagonist[14]
Mercury	Mine leachate	Thyroid hormone (ant)agonist[15]
Tributyltin	Marine antifoulant pint	Aromatase inhibitor[16]

[1] Jobling *et al.,* 1998; [2] Krishnan *et al.,* 1993; [3] Santodonato 1997; [4] Brouwer *et al.,* 1999; [5] Harris *et al.,* 1997; [6] Anderson *et al.,* 1996; [7] Moore and Waring 1998; [8] Kelce *et al.,* 1997; [9] Gray and Ostby 1998; [10] Hemmer *et al.,* 2001; [11] Fry and Toone 1981; [12] Folmar *et al.,* 2000; [13] MacLatchy *et al.,* 1997; [14] Ruby *et al.,* 2000; [15] Zhou *et al.,* 2000; [16] Matthiessen and Gibbs 1998.

inhibiting binding to hormone receptors [McLachlan, 1993]. Classes of animals in which these effects have been reported include fish [Jobling and Sumpter, 1993], amphibians [Gutleb *et al*., 1999], reptiles [Guillette *et al*., 1994], birds [Fry and Toone, 1981; Larson *et al*., 1996], and mammals (including humans) [Facemire *et al*., 1995] as well as members of numerous invertebrate phyla [DeFur *et al*., 1999; Ellis and Pattisina, 1990]. Theo Colborn, working for the W. Alton Jones Foundation and the World Wildlife Fund, together with several colleagues, was largely responsible for synthesizing the mounting evidence of chemical impacts on wildlife into the cohesive "Endocrine Disruptor Hypothesis" [Colborn *et al*., 1992; 1993; 1996].

Defining Endocrine Disruption

More than a decade since Theo Colborn, Frederick vom Saal and Ana Soto wrote "*Developmental effects of endocrine-disrupting chemicals in wildlife and humans*" [Colborn *et al*., 1993] the term endocrine disruptor has become virtually synonymous with any xenobiotic that modifies hormonal activity in a biological system. In this sense, endocrine disruption is not a toxicological endpoint *per se* but a functional change that may lead to adverse effect. With this is mind, definitions of endocrine disruption often differ depending on whether they involve only a perturbation of normal endocrine function, or require an ultimate adverse *in vivo* effect. The 1996 European Commission defined an endocrine disruptor as "an exogenous substance that causes adverse health effects in an intact organism, or its progeny, consequent to changes in endocrine function" [European Commission, 1996]. Kavlock *et al.* [1996] defined endocrine disruptors more precisely as "exogenous agents that interfere with the production,

release, transport, metabolism, binding, action or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes." In its 2002 *Global Assessment of the State-of-the-Science of Endocrine Disruptors* the World Health Organization (WHO) adopted the generic definition of an endocrine disruptor as:

> "an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations" [Damstra *et al*., 2002].

This final definition encompasses any alteration to the endocrine system, direct or indirect, that results in adverse effect to the organism or its offspring. Further, the definition acknowledges the potential for additive, synergistic, or ameliorative interactions between complex contaminant mixtures.

Endocrine Disruption: Fact or Fiction

Three decades before Theo Colborn and her collaborators popularized the concept of endocrine disruption, Rachel Carson opened Pandora's Box with her classic *Silent Spring* [Carson, 1962]. Meticulously researched, the book described how DDT entered the food chain and propagated by accumulation in fatty tissues to higher order predators, ultimately causing cancer and genetic damage. The book was unsettling to a public weaned on the belief that all technological advancement was for the greater good and that nature's resilience to human activity was insurmountable. Carson's work was immediately demonized by the chemical industry which attacked her professionally and personally, questioning both her scientific integrity and her very sanity. Thanks to careful and exhaustive preparation her work withstood the intense scrutiny and was

thoroughly vindicated after review by her scientific peers and a Presidential Advisory Committee [NRDC, 1997].

Carson had blown the whistle on our profligate disgorging of chemical concoctions onto the land and into the air and water. Through *Silent Spring* she was largely responsible for shifting the burden of proof of environmental harm from the opponents of unrestrained chemical use to the manufacturers of the chemical themselves. By melding insight, scientific rigor, and the strength of her prose, she connected with a receptive public and ignited a melee ultimately resulting, a decade later, in congressional passage of the Clean Air and Clean Water Acts. Heralded as triumphs of science over greed, three decades have passed and much of the glory of the Clean Air and Water Acts has been tarnished by delay, distraction and perpetual litigation.

Theodora Colborn's path of scientific inquiry followed a trajectory remarkably similar to that of Rachel Carson. Her 1996 book, *Our Stolen Future*, rekindled public concern for chemical effects on human health and the environment and stimulated research by government, academic and private institutions alike into contaminant effects on hormonal balance [Colborn *et al*., 1996]. Like Carson, she has faced strong opposition from industry, has testified before US Senate and House Committees, and has found her research largely corroborated by her peers. The scientific community has recognized Colborn's unique contributions by variously honoring her with the Society of Environmental Toxicology and Chemistry (SETAC) *Rachel Carson Award* in 2003 and the Center for Science in the Public Interest (CSPI) *2nd Annual Rachel Carson Award for Integrity in Science* in 2004. Both awards demand accuracy in the assembling of facts in defense of scientific positions, prize efforts to educate the public to more fully understand

the natural world and the threats that anthropogenic stressors have on that world, and applaud voicing the need for political change, even in the face of controversy [SETAC, 2005; CSPI, 2004].

From inception the endocrine disruptor hypothesis has come under attack from several fronts. First was the question of whether endocrine disruption was a new scientific paradigm, or simply an extension of existing classic environmental toxicology. Although semantic in character, this argument influenced the availability of research funds and the arena(s) in which researchers could compete for those funds. Beyond the semantic is the debate within and between scientific, regulatory and industrial communities concerning the relevance of anthropogenic contaminants on natural resources. This debate hinges on culpability, the outcome indicating whether there is a bill to pay, and if so, who is to pay it. As goes the argument by the few remaining "debunkers" of global warming, that anthropogenic sources of green-house gases pale by comparison to natural sources, and that nature has compensatory mechanisms, so goes the argument of the adversaries of endocrine disruption. They assert that endocrine bioactivity in xenobiotics tends to be many orders of magnitude less then that of endogenous hormones making the likelihood of impacts in the wild minuscule. Further, exposure to naturally occurring EDCs from plant sources over evolutionary time scales should produce defenses adequate to protect natural populations from EDCs of anthropogenic origin. Even more nuanced are challenges to data interpretation in which effects are purported to result from extremely low xenobiotic exposure concentrations or where atypical response-curves, often inverted u-shaped, are reported [vom Saal *et al*., 1997].

A Brief Review of the Endocrine System

A review of the vertebrate endocrine system is instructive in answering some of the charges against the endocrine disruptor hypothesis. The discussion that follows applies most specifically to teleost fish, though components mentioned have homologous tissues in most other vertebrate taxa. Endocrine tissues tend to be ductless glands, intimately associated with the vascular system and frequently incorporated into other organs. In teleost fish not less than 15 tissues have been identified throughout the body with endocrine activity: hypothalamus; anterior pituitary; saccus vasculosus; pineal; urophysis; thyroid; ultimobranchial; thymus; pseudobranchiae; chromaffin tissue; interrenal tissue; corpuscles of Stannius; endocrine pancreas (islets of Langerhans); enteroendocrine tissue; interstitial cells of the gonad; and justaglomerular cells of the kidney *(Figure 2)* [Groman, 1982; Harder, 1975]. By synthesizing and secreting hormones they function to regulate a complex array of biological processes affecting growth, development, reproduction, osmoregulation, metabolism, and much more *(Table 2)*. The pituitary, under neurosecretory control of the hypothalamus, regulates the functions of numerous other endocrine tissues, most notably gonad, thyroid, and adrenal, via feedback pathways or *axes*.

Reproduction is primarily controlled by the hypothalamus-pituitary-gonad or HPG axis. Despite broad differences in reproductive strategies among vertebrates, components of the HPG axis are remarkably well conserved across diverse species. Mammals, birds, reptiles, amphibians and fish all have the same primary components and most share homologous chemical signals that communicate between these components. Briefly, gonadotropic releasing hormones (GnRHs) are produced in the hypothalamus.

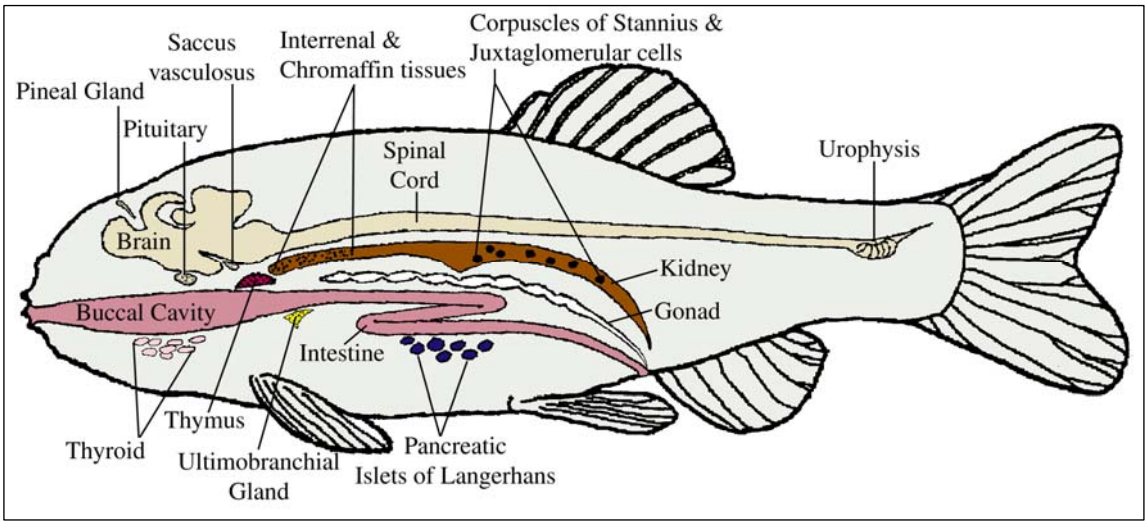


Figure 2. Organ systems and associated endocrine tissues in the fathead minnow, *Pimephales promelas*.

They are released into a portal system (most tetrapods) or simply diffuse (fish) the short distance between nerve endings and gonadotropes (neurosecretory cells) in the anterior pituitary. GnRHs bind to a specific receptor on the plasma membrane of gonadotropes and activate second messenger systems that regulate the synthesis and release of gonadotropic hormones (GTH I & II) into the circulation. Though varying in some species, GTH I is usually the functional equivalent to mammalian follicle-stimulating hormone (FSH) and acts to stimulate gametogenesis. GTH II is considered homologous to mammalian luteinizing hormone (LH). Its function in fish is less well understood, but is thought to play a role in final gamete maturation, ovulation and spermiation. GTHs function in gonads to influence sex steroid production within theca and granulosa cells in ovarian follicles and Leydig cells in testes of most species. Finally, sex steroids provide negative feedback to the hypothalamus and pituitary, inhibiting the release of additional GTHs thus completing the axis.

Table 2. Source, target and function of various endocrine glands/tissues in teleost fishes.

Source **Hormone**	**Target(s)**	**Action(s)**
Hypothalamus		
GnRH (Gonadotropin-releasing h.)	Pituitary	Releases GTH I & II
TRH (Thyrotropin-releasing h.)	Pituitary	Releases TSH
CRH (Corticotropin-releasing h.)	Pituitary	Releases ACTH
Pituitary		
GH (growth h.)	Liver; muscle; etc	Growth promotion
TSH (thyroid-stimulating h.)	Thyroid	Stimulate synthesis of thyroid hormones
GTH I (follicle-stimulating h.)	Gonad	Stimulate gonad development/gametogenesis
GTH II (luteinizing h.)	Gonad	Modulation of sex steroid production
ACTH (adrenocorticotropin h.)	Anterior kidney	Stimulate glucocorticoid secretion
Thyroid		
T4 (thyroxine); T3 (triiodothyronine)	Most tissues	Increase metabolism
Gonad		
Estrogen(s) & Androgen(s)	Primary/secondary sex structures	Stimulate development/gender differentiation
	Brain	Reproductive behavior
Pancreas - Islets of Langerhans		
Insulin	Liver; muscle; etc	Glycogen storage, glucose uptake, inhibit fat hydrolysis
Glucagon	Liver; adipose tissue; etc	Glycogen & lipid metabolism

Table 2. (continued). Source, target and function of various endocrine glands/tissues in teleost fishes.

Source **Hormone**	**Target(s)**	**Action(s)**
Chromaffin tissue		
Epinephrine/Norepinephrine)	Liver; muscle; heart	Stress response; glycogen breakdown to glucose
Interrenal tissue		
Corticosteroids	Circulation; gill; kidney	Carbohydrate & protein metabolism; stress response; etc
Corpuscles of Stannius		
Hypocalcin	Gill	Calcium homeostasis
Pineal		
Melatonin	Brain	Mediate circadian/seasonal rhythms
Ultimobranchial gland		
Calcitonin	Bone; gill; kidney	Calcium homeostasis
Urophysis		
Uropepsin I & II	Circulation; smooth muscle	Osmoregulation; blood pressure; smooth muscle contraction

During early development (fetal/neonatal), the endocrine axis becomes "programmed," establishing the feedback sensitivity of the hypothalamus and pituitary gonadotropes to steroidal inhibition. This determines the steroid levels that will be required to reduce GnRH or GTH secretion by these tissues. Notable distinctions in programming exist between males and females that effect temporal hormone cycles and gender specific breeding behaviors.

Given the complexities of the HPG regulatory system and the important role that sex steroids play in vertebrate life histories, opportunities for disruption by exogenous steroids are plentiful. Developmental stage at the time of exogenous steroid exposure is also critical to ultimate effect. For example, genotypic sex normally determines early gonad differentiation which leads to gonadal steroid production under HPG control, which in turn leads to phenotypic sex determination and differentiation of accessory sex structures and secondary sex characteristics including behavior. Impacts at early developmental stage can be permanent and catastrophic while exposure at later stages may cause temporary effects but are less likely to produce permanent morphological alterations.

Mechanisms of Action

Endocrine disruptors can act at multiple sites via multiple mechanisms of action. Some of the better understood mechanisms involve interferences with normal receptor-mediated hormonal processes. Probably the most studied mechanism of endocrine disruption is the ability of a chemical (other than the endogenous ligand) to bind a hormone receptor and trigger a response (i.e., agonist). Examples of estrogen receptor

(ER) agonists abound and include pesticides (DDT, methoxychlor), industrial by-products (bisphenol-A, nonylphenol), synthetic steroids (DES, EE2), and plant steroids (genistein). Effects of estrogen agonists can occur wherever ER occurs. Thus, hepatocytes can be induced to produce vitellogenin (Vtg), hypothalamic and pituitary receptors can be deceived into discontinuing GTH production, undifferentiated gonadal tissue can be feminized, and so on. Most estrogen agonists have lower ER affinities than those of the endogenous estrogens and thus require high exposure concentrations to produce qualitatively similar (but often less pronounced) responses. However, several synthetic steroids have ER affinities equal to or greater then that of E2, most notably 17 α-Ethynyl estradiol (EE2), the primary active component in human female oral contraception, [Folmar *et al*., 2000].

Many chemicals bind steroid receptors but do not trigger a response. They function as antagonists by blocking, competing with, or otherwise limiting access of the endogenous steroid to the receptor. In this way they can inhibit or even eliminate normal steroid mediated activity (e.g., gene transcription). One of the best examples involves vinclozolin, a dicarbomixide fungicide, and its metabolites (M1 and M2) which have antiandrogenic effects on the reproductive tract and within the hypothalamic-pituitary axis. Administration of vinclozolin to male rats resulted in elevated levels of LH indicating inhibitory binding to AR in the hypothalamus and/or pituitary [Monosson *et al*., 1999]. Similarly, vinclozolin exposure in peripubertal male rats delayed puberty and retarded development of androgen-dependent tissues indicating a competitive inhibition of steroid induced transcriptional activity [Monosson *et al*., 1999].

Several classes of endocrine disruptors operate via mechanisms other than

interference with receptor-mediate hormonal processes. These include chemicals that alter normal biosynthesis, transport, degradation, or excretion of steroid hormones. For example aromatase CYP450 is responsible for converting androgens (C_{19}) to estrogens (C_{18}). Therefore, inhibition of aromatase activity can lead to reductions in available estrogens. Pharmaceutical aromatase inhibitors (e.g., fadrozole) have been used in the treatment of estrogen-dependent breast cancer by exploiting this pathway as a means of reducing serum estrogen levels [Brodie *et al*., 1999]. Some environmental contaminants have been identified as aromatase inhibitors. Induction of imposex/intersex in gastropod mollusk species has been linked to tributytin (TBT) inhibition of aromatase activity leading to deficient estrogen levels and elevated androgens [Matthiessen and Gibbs 1998]. Similarly, the fungicide fenarimol has been found to inhibit mating behavior in male rats presumable by inhibiting conversion of androgens to estrogens in the brain [Hirsch *et al*., 1987].

The enzyme 5α-reductase is responsible for converting testosterone to dihydrotestosterone (DHT), a more potent androgen. In mammals DHT is involved in masculinizing external genitalia such that 5α-reductase inhibition during gestation causes profound alterations in prostate and genitalia of male offspring [Imperato-McGinley *et al*., 1992]. Complete inhibition of 5α-reductase produces incomplete feminization of external genitalia indicating that testosterone can partially compensate for the reduced DHT availability. One example of a 5α-reductase inhibitor is finasteride, a pharmaceutical developed to treat prostate cancer but used more commonly to treat hair loss in adult men - little wonder the ads tell women "warning, do not touch broken pills."

Another class of EDCs is aryl hydrocarbon receptor (AhR) agonists. Once inside

a cell, these compounds bind, often with high affinity, to the cytosolic AhR. This ligand-receptor complex is translocated to the nucleus where it binds DNA and initiates transcription of target genes including those for various Phase I and II xenobiotic-metabolizing enzymes (e.g., CYP450 monooxygenases, glutathione *S*-transferase, etc.) [Safe *et al*., 1991]. In addition, the AhR-ligand complex up- or down-regulates numerous other genes coding proteins involved in various critical cellular processes (e.g., hormone receptors, immune system receptors, protein kinases, etc.). One example, dioxin (TCDD), has been found to increase the rate of metabolism of sex steroids by up-regulating CYP450-monooxygenase and UDP-glucuronosyl transferase to increased levels of activity. As these enzymes systems are responsible for steroid hydroxylation and conjugation, respectively, up-regulation results in increased excretion and, therefore, reduced availability of circulating sex steroids [Anderson *et al*., 1996; Safe *et al*., 1991]. The influence of this mechanism is not limited to sex steroids. Dioxin has also been shown to facilitate elimination of thyroid hormone via increased thyroxine (T_4) glucuronidation and biliary T_4-glucuronide excretion [Van Birgelen *et al*., 1993]. Because the AhR has roles in multiple signal transduction pathways, agonists can induce a variety of biological effects at different life stages, in different species.

Several other potential mechanisms of endocrine disruption are less well understood. Sex steroids are lipid soluble and pass easily through membranes. So that they are not removed immediately from circulation >95% are bound by sex hormone binding globulins (SHBG) with only 2 - 3% free for biological activity [Rosner, 1990]. Modulation of liver synthesis of SHBG can potentially affect free available hormone levels. Likewise, chemicals with affinity for SHBG binding sites can compete with

steroids causing a net loss of un-bound steroids from circulation. EDCs could also act by disrupting membranes or otherwise inhibiting proper ion-channel function and thereby altering neural responses to stimuli including behavioral responses. Candidate EDCs include organophosphate and carbamate pesticides acting as acetylcholine esterase (ACHE) inhibitors and organochlorine pesticides like DDT obstructing axon sodium gate deactivation. Finally, neuroendocrine effects could also influence reproductive behavior. For example, female Atlantic salmon *(Salmo salar)* release a prostaglandin "priming pheromone" (PG F_{2a}) in urine when ovulated. Olfactory detection of the pheromone by male salmon leads to increased production of sex steroids and expression of milt. However, short-term exposure of male salmon parr to the herbicide atrazine (a photosynthesis inhibitor) dulls their olfactory sensitivity to PG F_{2a} and thus reduces their response to the priming effect of ovulated female urine [Moore and Waring, 1998].

Heresies of Endocrine Disruption

A basic tenet of classical toxicology, founded in pharmacological roots, is the assumption that for any given contaminant there must be a safe exposure level. An organism's ability to metabolize, sequester or otherwise compensate for exposure should provide sufficient protection such that a lower limit exists below which toxicity does not occur - *"Dosis facit venenum"* the "dose makes the poison" [Paracelsus,1493-1541]. Classical toxicology also assumes that biological responses always increase with increasing exposure such that the dose-response curve is monotonic. The rate of change of an effect (i.e., the slope) might change, and a threshold (i.e., asymptote) might be achieved, but the direction of the effect is always predictable. These basic assumptions

make toxicological studies fairly straight-forward. To characterize a compound's toxicity a toxicologists begins by exposing test animals to high doses/concentrations and works down until an exposure level is found where no effect is encountered, the proverbial "no observable effect level" (NOEL). At this point the researcher stops, assuming doses below the NOEL are of no further interest.

Recent results from low-dose experiments by Fred vom Saal and others challenge the classical paradigm. In a 1997 paper vom Saal *et al.* [1997] describe effects of *in utero* DES exposure on male mice. A maternal dose of 200 µg/kg produced a decrease in prostate weight compared to controls. However, doses of 0.02 to 2.0 µg/kg produced significant increases in prostate weight. The resulting dose-response was an inverted U, meaning data from the higher dose was not predictive of lower dose effects. Using a classic toxicological approach vom Saal would have stopped his investigation at 20 µg/kg, the NOEL, and not encountered the conflicting lower level effects. In the same study a similar inverted U dose-response curve was encountered when investigating the effects of fetal serum E2 levels on prostate development in male mice.

Next vom Saal and coworkers [Nagel *et al*.,1997] presented data demonstrating that maternal exposure to bisphenol A (BPA) at extraordinarily low levels (i.e. 2 - 20 µg/kg body weight) altered fetal mouse development such that male offspring experienced an increase in prostate weight when measured at 6 months. These results were profoundly important for two reasons: 1) they demonstrated the remarkable sensitivity of fetal development to low level hormone disruption; and 2) they showed that BPA affected mice at levels, when corrected for body size, comparable to the amount of BPA regularly ingested by people as a result of the using polycarbonate plastics in

common products. Publication of the results started a mild controversy.

Many questioned the validity of the response curves, doubting whether such low doses could actually cause significant biological effects. A possible explanation for vom Saal's atypical dose-response relationships is that at low levels the contaminants (DES, BPA) act as hormonal agonists such that their impact is mediated by interaction with estrogen receptors which stimulate prostate growth. Higher exposure levels, operating via other modes of action, eventually reach a point where damage to the prostate by other mechanisms overwhelms the subtle stimulatory effect.

Subsequent studies have produced substantial support for low-dose effects and unusual response curves [Christian and Gillies, 1999]. In defense of vom Saal's earlier results, Wetherill *et al*. [2002] found that a 1.0 nM exposure to BPA produced a larger impact on mouse prostate tumor proliferation than did a 100 nM exposure. Similarly, Cavieres *et al*. [2002] reported that 2,4-D, an off-the-shelf herbicide, increased fetal loss in mice. The curious result being that the largest reduction in litter size occurred at the lowest exposure dose, a level nearly an order of magnitude below the USEPA "maximum contaminant level" allowed in drinking water. Examples of inverted U type dose responses are also found in studies with lower animals. Oehlmann *et al*. [2000], examining the effects of octylphenol (OP) on the ramshorn snail *Marisa cornuarietis,* revealed a strongly non-monotonic dose-response curve on number of eggs/female and spawning masses/female where low and high OP levels had no effect but intermediate levels caused dramatic increases in both parameters.

In more recent work, vom Saal, with co-investigator Barry Timms [Timms *et al*., 2002] found *ip* injection of dioxin (1.0 µg/kg) to pregnant rats produced intrauterine

effects on serum E2 levels which altered prostate bud formation in male offspring. The nature of the analysis was rather complex employing computer serial reconstructions to describe morphometric parameters of prostate development and classifying fetuses according to the gender of their neighbors (e.g., 2M if between two males, 2F if between two females, etc.). Maternal exposure to dioxin at 1.0 ppb produced quantifiable and statistically significant alterations in fetal morphology. These alterations were dependent on an interaction with background levels of endogenous estradiol. The authors suggested that effects from dioxin exposure might also be mediated by simultaneous exposure to estrogenic chemicals such as those found in plastics (BPA), pesticides (2,4-D), and other common contaminants in human maternal diets. The amount of variation in serum E2 required to produce the observed differences ( ) = 20 pg/mL) indicate that only relatively small amounts of exogenous estrogen agonists (with high ER affinity) would be required to stimulate the effect.

Physiological systems don't necessarily follow linear dose-response relationships. Systems under the influence of chemical signals often function within narrow operational ranges; too little being deleterious, just enough being stimulatory, and too much being over-stimulatory or simply toxic for other reasons. The results are non-monotonic response curves. The growing body of literature on low-dose effects suggests that, where the endocrine system is concerned, many contaminants behave in a similar manner, most likely by moderating normal endocrine interactions. Because contaminants influence or mimic hormones which operate at exquisitely low levels, they themselves are also able to demonstrate an effect at similarly low levels.

ENDOCRINE DISRUPTORS IN THE AQUATIC ENVIRONMENT

Sources of Endocrine Disruptors

As mentioned previously, a broad array of natural and man-made substances are implicated as possible endocrine disruptors *(Table 1)*. Numerous point-source discharges of EDCs are well documented. Estrogenicity, as indicated by feminization of male fish, has been reported in receiving waters containing effluent from sewage treatment plants in the United States [Folmar *et al*., 1996; Goodbred *et al*., 1997] and Britain [Harries *et al*., 1997; Purdom *et al*., 1994]. Candidate compounds present in sewage outfalls and capable of hormonal activity include natural hormones excreted by humans and animals (e.g., E1, E2, E3, T, 11-KT, etc.) and synthetic substances commonly found in contraceptive pills (e.g., EE2) [Sheahan *et al*., 1994]. Industrial process wastes reaching receiving waters also contribute to endocrine disruption in wildlife. Environmentally persistent alkylphenolic compounds originating as surfactants in detergents are common in waste waters (e.g., nonylphenol and octylphenol) [Blackburn and Waldock, 1995]. Such chemicals have been shown to be weakly estrogenic causing feminization of male fish downstream of sewage treatment plants [Jobling and Sumpter, 1993]. In contrast, masculinized female fish have been found downstream of paper mills discharging bleached kraft mill effluents (BKMEs) [Howell *et al*., 1980]. High concentrations of phytosterols (plant estrogens), common in BKMEs, have been proposed as the masculinizing agents [Howell and Denton, 1989]. Sumpter *et al*. [1996] propose that dioxins, furans, and other synthetic poly-chlorinated aromatic compounds formed during the bleaching process may also be responsible for endocrine disruption by BKMEs.

Catastrophic impacts to wildlife have resulted from industrial accidents in which

high levels of persistent synthetic chemicals have been introduced to the environment. A marked decline in American alligator (*Alligator mississippiensis*) population around Lake Apopka, FL has been attributed to the 1980 spill of pesticides dicofol and DDT by the Tower Chemical Company. Guillette *et al*. [1994] documented elevated plasma estrogen concentrations and abnormal ovarian morphology in female alligators, and depressed plasma testosterone concentrations, abnormal testes, and small phalli in males. Woodward *et al*. [1993] postulated that a reduction in egg production and viability resulted in the subsequent decline in juvenile alligators.

Non-point source pollutants have also been identified as EDCs. Organohalide pesticides are typically stable and hydrophobic promoting environmental persistence and accumulation in sediments and biota after application. Surface and groundwater transport moves pesticides sorbed to sediment particles into aquatic environments where they are encountered by benthic organisms. Organochlorine contaminants ingested while sorbed to sediments are stored in fat reserves and bioaccumulate. Biomagnification leads to ever increasing contaminant burdens at successive trophic levels. Top predators (e.g., fish-eating reptiles, birds, and mammals) typically carry the greatest contaminant burdens [Norheim *et al*., 1992]. Egg laying animals pass contaminants to their offspring within lipid yolk reserves [Fry, 1995]. Mammals, including humans, pass high concentrations of contaminants in lipid rich breast milk to nursing infants [Smith, 1987; Meyer, 1989]. In both cases organisms are assailed with high concentrations of EDCs during sensitive developmental stages.

Animal Feeding Operations - AFOs

The 2002 US Census of Agriculture reports 400,000 animal feeding operations (AFOs) in the U.S. and a marked intensification of animal production occurring in the last 25 years [USDA, 2004a]. Of the primary contributors (beef, dairy, swine, and poultry) the poultry sector has experienced the most dramatic increase. Additionally, consolidation of AFOs into "factories" has resulted in ever increasing densities of animals and animal wastes in several regions of the US [Lander and Moffitt, 1996]. Nationally, farmed animals generate substantially more excreta then do humans [USDA, 2004b]. While human waste disposal is rigorously managed in the US, disposal of animal manure remains largely at the discretion of the producer. The primary means of disposal/utilization is application to agricultural land as organic fertilizer [Kellogg *et al*., 2000]. Because of high transportation costs, animal waste is typically hauled only short distances before field application. The result, in regions with dense AFOs, is manure application in excess of crop requirements and ultimately ecosystem enrichment and eutrophication [Lander and Moffitt, 1996]. Of US streams and rivers classified as impaired, 70% are degraded as a result of agricultural runoff [USEPA/USDA, 1998].

Poultry Industry on the Delmarva Peninsula

The Delmarva Peninsula, consisting of eastern Maryland, most of Delaware, and the portion of Virginia east of the Chesapeake Bay, is one of the most densely concentrated poultry producing areas in the US. The region generates 600 million birds and 1.6 billion lbs. of manure (or litter) annually [USDA, 2002]. Excessive land application of poultry wastes (i.e., manure, liquid processing plant effluent, etc) has

precipitated severe water quality problems in surface and ground waters throughout the region [Hamilton *et al*., 1993; Sims and Wolf, 1994; Staver *et al*., 1996; MDE, 1998].

Contaminants traditionally associated with poultry litter include: nutrients (e.g., nitrogen and phosphorus) [Kellogg *et al*., 2000]; protozoan (*Cryptosporidium*), bacterial *(Campylobacter, Salmonella)* and viral (avian influenza) pathogens [USEPA, 2004]; and trace metals (e.g., As, Cu, Se, and Zn) [Letson, 1996; Miller *et al.,* 1999]. The Maryland Department of the Environment (MDE) estimates that 93% of Maryland's impaired surface waters are degraded as a result of nutrient pollution [MDE, 1998]. Other water quality impacts associated with excessive manure application include harmful algal blooms, decreases in water clarity, widespread anoxia and declines in submerged aquatic vegetation (SAV) [Denver *et al*., 2004]. In a recent survey of SAV in Chesapeake Bay, scientists attributed a 30% decrease in Tangier Sound sea grasses to over-enrichment of the Pocomoke watershed [Peter Bergstrom, US Fish &Wildlife Service, Chesapeake Bay Field Office, personal communication]. Excess nutrients associated with AFO activities have also been linked to outbreaks of the dinoflagellate, *Pfiesteria piscicida* [Hughes *et al*., 1997]. Although not confirmed, evidence suggests that this toxic microbe contributed to fish kills in several Delmarva tributaries including the Pocomoke and Chicomicomico Rivers which are heavily impacted by nutrient runoff. Fish species observed with ulcers in association with exposure to *Pfiesteria piscicida* include the migratory striped bass *(Morone saxatilis)* and American eel *(Anguilla rostrata)* as well as such staple forage fish as Atlantic menhaden *(Brevoortia tyrannus)* and white perch *(Morone americana)* [Kane *et al*., 1998]. However, no cause and effect relationship between field exposure to *Pfiesteria* and ulcer development has been shown [Dykstra and Kane, 2000].

Recent concern has arisen over release to the environment of numerous non-traditional *poultry litter-associated contaminants* (PLACs). These include feed additives (e.g., trace metals; antibiotics), poultry house/bedding material impurities (e.g., metals; pesticides) and normal fecal/urinary steroid constituents (e.g., estrogenic and androgenic hormones). Poultry feed is augmented with essential micronutrients like Cu, Se, and Zn to satisfy dietary requirements. Organic arsenic (e.g., roxarsone) is also employed as a feed additive to increase weight gain, improve feeding efficiency, and control bacterial and parasitic diseases [Denver *et al*., 2004]. Eventually these feed additives are excreted and end up in poultry litter [Sims and Wolf, 1994]. Repeated application of litter to fields results in substantial accumulation of these metals. Analysis of surface waters and sediments in tributaries of the Pocomoke River, MD found elevated levels of As and Se thought to originate as poultry-feed additives and indicating transport from source (poultry feed) to sink (fresh, estuarine and coastal waters, sediments and biota) [Miller *et al*., 1999]. Antibiotics (e.g., tetracycline(s); Flavomycin) are routinely administered in poultry feed for digestion-enhancement, growth promotion and prophylactic control of bacterial infection. Ecological consequences of widespread antibiotic contamination are unclear but primary concerns include widespread development of antimicrobial resistance and potential alterations in microbial processes important to the healthy functioning of aquatic ecosystems [Wiggins, 1996; Kolpin *et al*., 2002; Hayes *et al*., 2004].

Natural Hormones in Poultry Litter

Finally, concerns have arisen over release to the environment of natural and synthetic endocrine disrupting chemicals (EDCs) as a result of agricultural activity. As

mentioned previously, many pesticides are potent EDCs, and repeated application of persistent varieties can result in substantial accumulations in sediment and biota [Miller *et al*., 1999]. More importantly, natural hormones produced by livestock, specifically 17 β-estradiol (E2), estrone (E1) and testosterone (T) can persist at high concentrations in animal manure. Poultry litter has been reported to contain up to 904 ng/g E2 and 670 ng/g T on a dry weight basis with concentrations varying according to gender, maturity, and reproductive status (i.e., broilers vs. laying hens) [Nichols *et al*., 1997; 1998; Shore *et al*., 1995]. Because poultry litter is so high in E2, the vertebrate estrogen responsible for development of the female reproductive tract and secondary sex characteristics, these processes may be negatively impacted in resident fish (and other aquatic biota) exposed to exogenous E2 in runoff from litter-amended fields. Previous studies on the Delmarva Peninsula [Shore *et al*., 1995] and elsewhere [Nichols *et al*., 1997; 1998; Finlay-Moore *et al*., 2000; Herman and Mills, 2003] have investigated the transport of E2 and/or T from poultry litter into surface and ground waters following application to fields and pastures. Shore *et al*. [1995] reported concentrations of 14 to 20 ng E2/L in a farm pond receiving runoff from poultry litter-amended agricultural fields. Herman and Mills [2003] found stream E2 concentrations as high as 120 ng/L in an instrumented 1.2-km^2 agricultural watershed in central Virginia, USA. Higher concentrations were observed early in the growing season (shortly after application of poultry litter) with values decreasing over the course of the summer and as a function of hydrological transport distance from the cropped fields. Runoff from small scale (1m x 3m) fescue plots amended with broiler litter was reported to contain E2 levels of 450 ng/L [Nichols *et al*., 1998]. Larger (0.8 ha) fescue plots produced E2 levels of 305 to 820 ng/L in runoff following amendment with

broiler litter [Finlay-Moore *et al*., 2000]. While these studies all report runoff of E2 from poultry litter-amended fields to surrounding receiving bodies they were not designed to investigate the link between poultry litter-associated contaminant exposure and endocrine disruption in resident fish within receiving streams and estuaries. If such a link exists, the magnitude of impact to the Delmarva Peninsula and other poultry industry-intensive watersheds could be substantial.

PROJECT OVERVIEW

Objectives and Hypotheses

The primary objective of this research project was to assess the likelihood of endocrine disruption in fish populations on the Delmarva Peninsula as a result of poultry litter application to agricultural fields. High levels of endocrine active compounds, especially sex steroids responsible for gender differentiation and development of reproductive structures, suggest that the potential for fish and other aquatic biota in receiving waters to be negatively impacted is substantial. However, the fore-going review of endocrine system related contaminant effects identified a number of problem areas in our understanding of endocrine disruption. Sensitivity to a given EDC is related to: species, gender, and age of the organism(s) in question; route, duration, and concentration of exposure of the particular contaminant(s); and the chemical and physical environment in which exposure occurs. Poultry litter contains numerous endocrine disrupting chemicals in varying concentrations. However, rates of introduction of these constituent contaminants to receiving waters are governed by litter application rates, tillage practices, field topography, and precipitation levels.

Current limitations in our knowledge make risk assessment based on the likelihood of exposure of a particular organism to a particular contaminant untenable. The present study was undertaken to provide information on some of these areas of uncertainty by quantifying poultry litter-associated contaminant (PLAC) levels in natural waters and by exposing fish to the contaminants in controlled settings. The major objectives of the study were:

1) Determine whether PLACs are capable of inducing endocrine disrupting effects in fish.

   H_0: Exposure to PLACs will cause endocrine disrupting effects in fish.

2) Determine what PLAC concentrations and exposure durations are required to elicit such effects.

   H_0: A PLAC exposure level exists below which exposure does not produce endocrine disrupting effects in fish.

3) Determine whether PLACs reach surface waters via runoff from agricultural fields following "standard" litter application practices in sufficient quantities to produce these effects.

   H_0: Application of poultry litter to agricultural fields will introduce PLACs to receiving waters at biologically relevant concentrations.

4) Determine if fish in receiving waters are adversely impacted by exposure to PLACs in agricultural field runoff.

   H_0: PLACs entering receiving waters as a result of poultry litter application to agricultural fields will induce endocrine disruption in resident fish.

These objectives were investigated in a research project comprised of laboratory and field components. The laboratory portion of the project included a series of bioassays in which freshwater and estuarine fish were exposed to aqueous solutions of poultry litter-associated contaminants (PLACs). In all, five assays were performed, three

with the fathead minnow, *Pimephales promelas*, and two with sheepshead minnow, *Cyprinodon variegatus*, and mummichog, *Fundulus heteroclitus*. The assays used accepted biological measures of endocrine disruption to investigate the sensitivity of the selected test species to endocrine active contaminants in poultry litter. Tissues, especially gonads, were assessed histologically to investigate degenerative/developmental effects of litter exposure. Plasma and whole-body homogenate vitellogenin (Vtg) levels were measured to gauge the utility of vitellogenesis as a biomarker of poultry litter-associated estrogenic exposure. Effects-thresholds and dose-response relationships were investigated by exposing test animals to complex contaminant mixtures in dilution series spanning the environmentally relevant range. Multiple life stages (larval, juvenile and adult) were employed to investigate the effects of age on contaminant sensitivity. Aims of the laboratory assays were two-fold: first, to identify the endocrine related toxicological endpoints most sensitive to PLACs exposure, and second, to determine the suitability of the selected test species as sentinels of deleterious effects on resident fish populations.

The field component of the project involved application of poultry litter on research fields at the University of Maryland - Wye Research and Education Center (UMD - WREC) during the 2000 and 2002 growing seasons. Measurement of contaminants in poultry litter prior to field application, in field runoff during rain events, and in receiving waters, was used to investigate the transport and environmental persistence of PLACs (especially steroids) from agricultural fields into receiving bodies. Finally fathead minnows were "caged" within an agricultural receiving pond to investigate the effects of "real world" PLAC exposures on laboratory reared animals

under controlled conditions. Endocrine endpoints used in laboratory assays were also employed to assess effects in field exposed animals.

Experimental fish exposures were designed with estrogens as the steroidal contaminants of primary concern. This was done for several reasons. First, exogenous estrogens, especially E2, have demonstrated efficacy at adversely affecting fish reproductive biology at low ng/L concentrations [Folmar *et al*., 2002; Purdom *et al*., 1994]. Second, poultry litter from numerous Delmarva sources consistently has higher levels of E2 than T [Yonkos *et al*., unpublished research]. Third, studies of poultry litter-associated steroids in natural waters consistently report E2 at biologically relevant levels [Finlay-Moore *et al*., 2000; Herman and Mills, 2003; Nichols *et al*., 1997; 1998]. And fourth, sensitive biomarkers of endocrine disruption are better developed for detecting estrogenic effects then androgenic effects [Gillesby and Zacharewski, 1998; Kime, 1999].

CHAPTER II: MATERIALS AND METHODS

POULTRY LITTER TEST MATERIAL

Poultry manure applied to agricultural fields as fertilizer, commonly called *poultry litter,* contains the mixture of feces, urine, bedding material (i.e., sawdust, peanut hulls, wood shavings, etc.) and feathers that accumulates within a poultry house during cultivation of 10 to 12 flocks of birds (~2 yr). During occasional "crust-outs," surface material is removed from the floor of houses and aggregated in storage sheds, often for several years, before use as fertilizer. Whole-house "scrape-outs" are performed approximately every two years with material trucked directly to end-users for immediate use or outside storage until needed.

Poultry litter is used as organic fertilizer to satisfy crop nutrient requirements. The predominant Delmarva crops are corn and soybeans. These, in turn, serve as feed for the substantial regional poultry industry. Crops may be grown using conventional or no-till practices as described in *Table 3.* Generally corn and soybeans are alternated during primary growing seasons with wheat and rye serving as winter cover crops. Poultry litter is applied as fertilizer prior to planting of corn and wheat, but not soybeans and rye.

A number of environmental contaminants not associated with poultry litter are applied to fields as a result of these common cropping practices. Various insecticides, fungicides and herbicides are used either individually or in combination to ensure high crop yields. For example, corn seeds may receive pre-treatment with the fungicide fludioxonil and nematicide metalaxyl prior to planting. At the time of planting additional seed treatments may include the insecticides lindane and diazinon and the fungicide

Table 3. Standard crop rotation characteristics for growing corn and soybeans on the Delmarva Peninsula using conventional and no-till* strategies.

Year	Season	Crop	Litter application	Planting strategy*	
				Conventional-till	No-till
1998	Spring	corn	yes	yes*	no*
98 - 99	Winter	wheat	yes	yes	yes
1999	Summer	soybeans (double crop)	no	no	no
99 - 00	Winter	rye	no	no	no
2000	Spring	corn	yes	yes	no
00 - 01	Winter	wheat	yes	yes	yes
2001	Summer	soybeans (double crop)	no	no	no
01 - 02	Winter	rye	no	no	no
2002	Spring	corn	yes	yes	no

* Planting strategies differ primarily in whether or not fields are tilled prior to the planting of corn. Other crops are managed similarly under both planting strategies.

Captan®. After planting the field may receive a pre-emergent treatment with the herbicide atrazine and insecticide methoxychlor. And finally, post-emergent treatment with the broad-spectrum herbicide Roundup® (glyphosate) is common when using Roundup Ready® crops (corn or soybean). Many of these compounds are persistent in soils and may reach surface waters either sorbed to suspended soil particles or, if sufficiently soluble, in surface runoff.

Poultry litter used in the first two fathead minnow laboratory assays *(WYE2000)* was collected from a ≥200 ton pile that originated from a whole-house "scrape-out" of a standard broiler operation on Maryland's eastern shore, delivered to the University of

Maryland - Wye Research and Education Center (UMD-WREC), Queenstown, MD, in the spring of 2000. Litter for the remaining fathead minnow assay and the sheepshead minnow and mummichog assay *(WYE2002)* was collected from a similar pile delivered to UMD-WREC in the spring, 2002. Both piles also served as fertilizer on research fields in controlled field exposures of fathead minnows to PLACs in runoff.

Prior to field application sub-samples of poultry litter were aggregated in ~40 kg batches, coarsely homogenized, then parceled into 4 L Ziploc® bags (~2.5 kg) for storage at -20EC until required. Samples for contaminant analysis were collected either directly from the pile(s) or as material was transported for field application. *WYE2000* samples, ranging from predominantly fine dry particles to predominantly moist clumps, were placed in pre-cleaned amber sample jars and immediately stored in a -20EC freezer before shipping to the US Geological Survey - Columbia Environmental Research Center (USGS-CERC) for analysis of trace metals, antibiotics, and historic and current-use agrichemicals. Analyses were conducted as part of a companion project with the US Fish and Wildlife Service - Chesapeake Bay Field Office (FWS-CBFO). Detailed analytical methods are provided in McGee *et al*. [2003]. Water-extractable E2 and T were determined via radioimmunoassay (RIA) for both litter sources according to the methods of Nichols *et al*. [1997; 1998]. Analyses were conducted at the Virginia Institute of Marine Science (VIMS), Gloucester Point, VA.

TEST SPECIES

The fathead minnow *(Pimephales promelas)* was used in laboratory exposures to water soluble PLACs in freshwater and in field caging experiments. Mature males were

grown from cultures maintained in-house at UMD-WREC or purchased from a biological supplier, Chesapeake Cultures, Inc., Hayes, VA, USA. Larval and juvenile fathead minnow were spawned from breeding groups of adult fish maintained in-house. Monthly reference toxicity tests (KCl) were employed to assess sensitivity of the cultures. Sheepshead minnow *(Cyprinodon variegatus)* and mummichog *(Fundulus heteroclitus)* were used in laboratory exposures to water soluble PLACs in estuarine waters. These species were selected because they reside near-shore, are non-migratory, and occur ubiquitously within estuarine portions of Delmarva watersheds [Hardy, 1978]. Adult male sheepshead minnow and mummichog were field collected from Decorsey Cove on the Wye River, Chesapeake Bay via minnow trap or seine, as necessary. Larval sheepshead minnow were purchased from Aquatic BioSystems, Inc, of Fort Collins, Colorado. Field collected animals were quarantined for a minimum of two weeks before initiation of any laboratory exposure. Advantages of all three species include ease of culturing, small size allowing use of adults in assays, and sexual dimorphism simplifying distinction of males and females.

BIOLOGICAL INDICATORS

Vitellogenesis

Sexually mature females of all oviparous species synthesize the egg yolk precursor Vtg [Sumpter and Jobling, 1995]. Synthesis occurs in the liver where production is regulated by the interaction of estrogens, predominantly E2, with the estrogen receptor. Because males maintain the ability to produce Vtg in response to estrogenic stimulation, detection of Vtg in male fish has been used as a biomarker of

exposure to estrogenic compounds of exogenous origin [Heppell *et al*., 1995; Denslow *et al*., 1999]. In addition, Vtg levels in immature animals are normally very low compared to mature females. Therefore, detection of Vtg in immature fish of either gender can be indicative of exogenous estrogenic exposure [Heppell *et al*., 1995].

Plasma Vtg collection and treatment. Adult fish of all species were anesthetized in 100 mg/L buffered tricaine methane sulphonate (MS222) [Sigma Chemical, cat # A-5040] before blood samples were collected into 70 µl heparinized microhematocrit tubes via incision into the caudal sinus *(Figure 3)*. Typically 40 to 70 µl of whole blood was obtained from each adult male specimen. Additional tubes were collected from prolific "bleeders" to investigate reproducibility of analytical results. Whole blood samples were centrifuged at 3000 g for 10 min [International Micro-Capillary centrifuge, Model *MB*] and resulting plasma was discharged into heparinized (35 USP/vial; Sigma Chemical Inc., cat # H-6279) and aprotinated (0.132 TIU/vial; Sigma Chemical Inc., cat # A-6279) 1.5 mL conical bottom cryovials for storage in liquid nitrogen until required for analysis. Bled fish were sacrificed for histological examination and GSI as described below.

Figure 3. Collection of whole blood from an adult male fathead minnow *(Pimephales promelas)* via incision into the caudal sinus.

Whole-body homogenate preparation. Juvenile fathead minnow and sheepshead minnow body homogenates were prepared by homogenizing whole bodies of individual fish in phosphate buffer at a ratio of 1:2 (wet weight:buffer volume), using an Ultra-Turrex T25 tissue homogenizer (Fisher Scientific, cat #14-259-68). Fish weights ranged from 150 - 400 mg for fathead minnow and from 25 - 135 mg for sheepshead minnow. Homogenates were centrifuged at 3000 g for 10 min [Eppendorf centrifuge, Model # 5415] with supernatant withdrawn and discharged into heparinized and aprotinated cryovials as described previously. Samples were stored in liquid nitrogen until required for analysis.

Condition Index

General health and somatic vigor of adult and juvenile fish was estimated by calculation of a condition index (CI): body wt expressed as a percent of the length cubed:

$$CI = (\text{body wt}/\text{lgth}^3) \times 100$$

where body weight and length were recorded in mg and mm, respectively. Typically mature male fathead minnow and mummichog CIs are ~1.0 (unitless measure) and sheepshead minnow CIs are ~2.0.

Gonadosomatic Index

Reproductive status of mature male fish was estimated by calculation of a gonadosomatic index (GSI): gonad weight, in this case testis wt, expressed as a percent of total body weight:

$$GSI = (\text{gonad wt}/\text{body wt}) \times 100.$$

Whole-body wet weight of individual fish was measured after anaesthetization but before bleeding and recorded to the nearest 0.01 g. After removal (via ventral incision from anus to isthmus and retraction of the left body wall) testis weights were recorded to the nearest 0.1 mg. Typical GSI values are in the range of 1 to 2% for mature male fathead minnow and mummichog and 0.5 to 1% for sheepshead minnow.

Histopathology

The occurrence of Vtg in male fish is well confirmed as an indicator of estrogenic exposure. However, induction of Vtg does not necessarily indicate an adverse health effect. Alterations in gonad structure as a consequence of chemical exposure provide a sensitive and arguably more meaningful endpoint of endocrine disruption. Environmental exposure of fish to municipal sewage treatment plant (STP) and pulp and paper mill effluents have been shown to cause reduced gonad growth, feminization of duct development in males, and alterations in germ cell development and gender assignment [Purdom *et al*., 1994; Harries *et al*., 1996; Larsson *et al*., 2000]. Because gonad development in fishes is especially plastic compared to other vertebrate classes, genotypic sex in many species is readily influenced by environmental exposure to sex steroid (ant)agonists [Shapiro, 1992; Baroiller *et al*., 1999]. Partial to complete sex reversal has been accomplished in more than 50 fish species by administration of sex steroids, agonists, antagonists and/or aromatase inhibitors [Devlin and Nagahama, 2002]. Such susceptibility of fish to exogenous steroids suggests the potential for catastrophic impacts to fisheries and natural communities and explains the rising worldwide concern about endocrine disruptors.

Histological evaluation of testes can identify cellular and tissue level alterations capable of impacting reproductive output and may provide insight into the mechanism of action of potential EDCs [Ankley *et al*., 2001]. Pathology in fish testes reported as resulting from EDC exposure include: degeneration/necrosis of germ cells and spermatozoa; hypertrophy and hyperplasia of interstitial and Sertoli cells; fibrosis within interstitium; inflammation comprised of eosinophilic granules and macrophage aggregates; and the occurrence of testis-ova [Miles-Richardson *et al*., 1999; Zillioux *et al*., 2001; Karels *et al*., 2003]. Configurational alterations in reproductive organ development have also been reported for male fish exposed to suspected estrogens during the period of gonadal differentiation. Prominent among these is the formation of an oviduct-like structure instead of the typical male vas deferens [Ankley *et al*., 2001; Gimeno *et al*., 1998; van Aerle *et al.,* 2002].

Adult male testis preparation. Testis sections from adult fish were examined via light microscopy for evidence of pathological change and to assess the relative maturity of gametes within germinal epithelium. After removal for calculation of GSI, testes were fixed for at least 48 h in 10% neutral buffered formalin (NBF) before routine histological preparation [Luna, 1968]. Briefly, tissues were dehydrated, embedded in paraffin, thick sectioned (5 μm; appropriate for light microscopy), mounted on glass slides, stained (H&E) and covered with glass cover-slips. Testes from the first fathead minnow assay were rough-cut transversely to yield segments from anterior, mid and posterior regions of each lobe. Testes from subsequent fathead assays and all sheepshead and mummichog assays were left whole and sectioned sagittally. Three sections/specimen, distributed equally within embedded tissue(s), were mounted on individual slides and archived for

subsequent analysis. The incidence and severity of testicular lesions was investigated via light microscopy by examining tissue sections at low (40x) and high (400x) magnifications (Olympus BH-2 research microscope).

Testis maturity ranking. A semi-quantitative method was used to assess testis maturity in adult male fish [Schmitt and Dethloff, 2000]. Briefly, fish were assigned ranks of 0 to 5 based on the proportion of germinal epithelium present relative to interstitial stroma and the degree of spermatogenic activity as indicated by proportion of gametes at various stages of spermatogenesis (e.g., spermatogonia, spermatocytes, spermatids and mature spermatozoa). A greater proportion of mature spermatozoa relative to less mature gametes was assumed to indicate a more advanced state of maturity. Multiple sections from discrete regions of tissue were assessed to determine ranks for individual fish with higher ranks indicating more advanced maturity. Treatment mean maturity indices were calculated as the average of individual maturity ranks. Additionally, an appraisal of reproductive competence, based on the presence and abundance of spermatozoa within collecting tubules was made for each specimen. Generally, animals possessing sperm within tubules at the time of sacrifice were deemed capable of spawning, those without were deemed incapable of spawning.

The qualitative nature of testis maturity ranking was considered inappropriate for statistical analysis. Application of morphometric methods can more accurately approximate the relative proportion of gametes at various spermatogenic stages and thus yield quantitative data appropriate for statistical analysis. Therefore, the previously described “qualitative” examination of tissue sections was employed to determine if more rigorous “quantitative” measures were warranted. Tissues from those assays in which

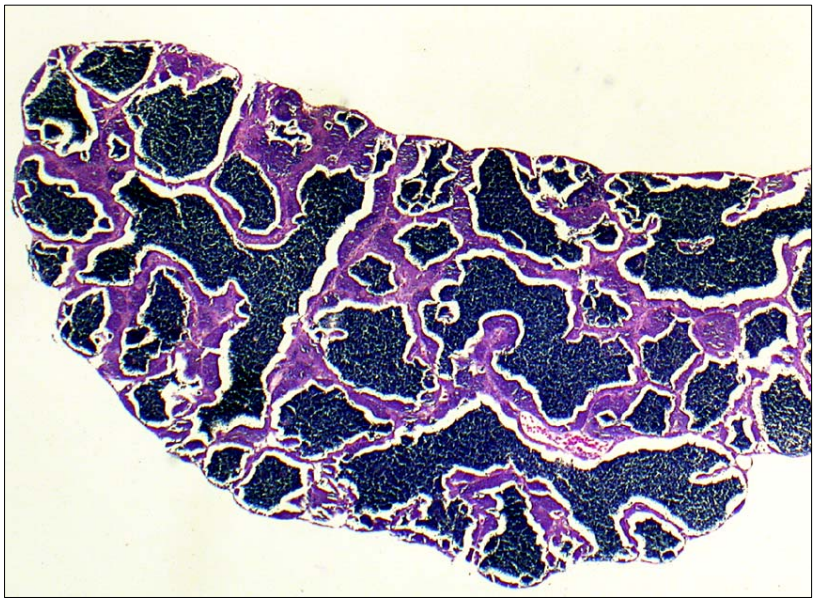
treatments appeared “qualitatively” distinct were further scrutinized using a simplified morphometric method.

Briefly, because of their large proportion of cytoplasm and relative lack of chromatin, spermatogonia and spermatocytes are eosinophilic and stain predominantly pink/red. In contrast, progressively more mature spermatids and spermatogonia with abundant dense chromatin and a near absence of cytoplasm are strongly basophilic and stain dark blue. Therefore, cysts within testis sections that are predominantly basophilic are presumed to be more mature than cysts occupied by eosinophilic (pink/red) cells. By exploiting this clear distinction in staining characteristics testis maturity and, hence, reproductive competence, was approximated as the proportion of basophilic material (i.e., spermatids and spermatozoa) to total germinal epithelium *(Figure 4)*.

Several steps were required to produce this ratio. Initially, multiple sections of tissue from each specimen were digitally photographed and images stored to disk. Digital traces were made of entire segments of interest and of basophilic regions within these segments (i.e., regions occupied by spermatozoa and spermatids). A digitizer tablet [Kurta IS/One] with pen-point input devise and image manipulation software [Adobe Photoshop ver 7.0; Adobe Systems Inc., San Jose, CA, USA] were used to make the traces. Area occupied by total and basophilic regions within traces was calculated automatically by SigmaScan Pro ver 5.0 image analysis software [SPSS Sciences, Chicago, IL, USA]. To avoid bias, image manipulations were performed blind, and only segments comprised predominantly of germinal epithelium and free of major histological preparation-related artifact were employed. Results from individual specimens were

calculated as the mean of all segments/fields analyzed. Treatment means only included data from individuals for which a minimum of three segments were analyzed.

(a)



(b)

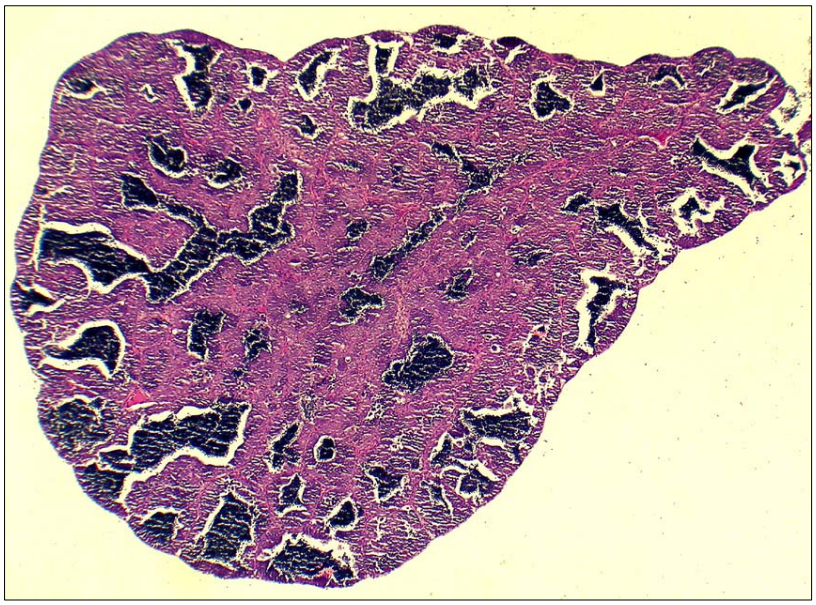
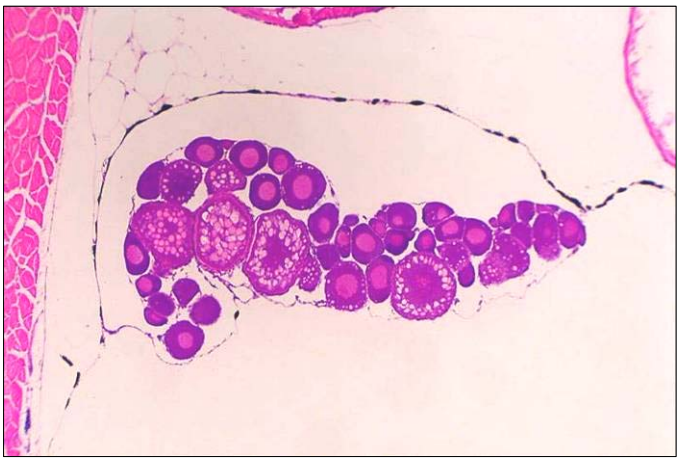


Figure 4. Examples of fathead minnow *(Pimephales promelas)* testis sections characterized by high ***(a)*** and low ***(b)*** proportions of spermatozoa and spermatids (dark blue regions) relative to total area. Morphometric analysis determined areas of sections ***(a)*** and ***(b)*** occupied by spermatozoa/spermatids to be 55.2% and 18.8%, respectively (H&E; 40x).

Gender identification of immature fathead minnow. Maintained at 25EC and adequately fed, primary oocytes can be distinguished in female fathead minnow as early 25 d post-hatch [van Aerle *et al*., 2004]. At 60 d post-hatch precocious females may have enlarged ovaries distended with various pre-vitellogenic oocyte types *(Figure 5a)*. However, yolk-vesicle formation and vitellogenesis are rare at this age. Cortical alveolar sex cell types typically first appear at ~90 d with vitellogenesis beginning at ~120 d [van Aerle *et al*., 2004]. Oviducts develop in fathead minnow prior to the onset of gametogenesis and can be readily distinguished in cross-section by the two clear points of mesenteric attachment of each ovary to the peritoneum *(Figure 5a & b)*. Males are difficult to distinguish definitively at 60 d. At this age in cross-section the testis typically appears as a compact "packet" of primordial germ cells (PGCs) and interstitial stroma suspended from the peritoneum by a single stout point of attachment *(Figure 5c)*. Prior to the onset of gametogenesis this packet will loosen to reveal cord-like structures surrounding cavities that precurse the seminiferous tubules.

After weighing and measuring for calculation of condition indices (CIs) fish were dissected along midventral lines from vent to pectoral girdle to facilitate penetration of fixative. After a minimum of 48 h in 10% NBF regions anterior to the pectoral girdle and posterior to the vent were removed and discarded leaving abdominal portions (with intact visceral mass) for histological preparation [Luna, 1968]. Groups of 4 similarly sized individuals were blocked in paraffin with anterior ends oriented downward. Multiple cross-sections were taken such that anterior, mid and posterior regions of the abdominal cavity were represented for each specimen. Sections were mounted on glass slides, stained (H&E), covered with glass cover-slips and archived for subsequent examination.

(a)



(b)

(c)

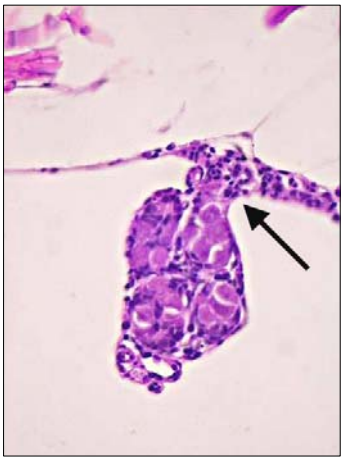


Figure 5. Gonad cross-sections from 60 d old fathead minnow *(Pimephales promelas)*; ***(a)*** female with many pre-vitellogenic oocytes (H&E; 40x); ***(b)*** female without apparent oocytes but with oviduct as demonstrated by two clear points of peritoneal attachment (arrows)(H&E; 250x); ***(c)*** presumptive male with single stout point of peritoneal attachment (arrow) (H&E; 400x).

During examination gonadal tissue was identified by location within the abdominal cavity and according to cellular characteristics. Sections were assessed for evidence of pathology and, where possible, to determine phenotypic gender. Gonads within tissue sections received one of four designations: ***(f)*** for female based on the clear presence of oocytes; ***(o)*** for the presence of a presumptive oviduct without attendant oocytes; ***(m)*** for male; and ***(i)*** for immature and/or indeterminate. Three sections were examined for each specimen, with evaluations performed "blind" to minimize investigator bias. A weight of evidence approach was employed with gender designations based on the most definitive cellular and morphological characteristics.

CHEMICAL ANALYSIS

17$-Estradiol and Testosterone Analysis

Concentrations of E2 and T (water-soluble fraction) were quantified by competitive radioimmunoassay (RIA) using methods modified from McMaster *et al*. [1992] and performed at the Virginia Institute of Marine Sciences (VIMS), Gloucester Point, VA, USA. Aqueous samples from negative, positive and solvent controls and from poultry litter treatments were analyzed directly without dilution or extraction. Briefly, the RIA depends on the competition between unlabeled and radiolabeled [^{3}H] steroid for limited steroid-specific antibody. Following incubation in tubes, centrifugation in the presence of a dextran/carbon solution pelletizes unbound steroid, allowing supernatant, containing antibody bound steroids (labeled and unlabeled), to be decanted into scintillation vials for determination of radioactivity via beta-counter. Resulting radioactivity is inversely proportional to the concentration of hormone in the

sample. Appropriate standards, blanks and controls are incubated to allow determination of a standard curve and calculation of steroid concentrations in unknown samples. As performed, the method detection limits (MDL) for E2 and T were 18.0 ng/L and 6.0 ng/L, respectively. As E2 and T concentrations of interest were often near the MDL, values reported as below MDL were estimated as ½ MDL (i.e., 9 ng/L for E2, 3 ng/L for T) for analytical, statistical and graphical purposes.

The relationship between conjugated (presumably non-bioactive) and un-conjugated (bioactive) steroids in water soluble PLACs was investigated using material from several poultry litter sources; *WYE2002* and 6 other whole-house scrape-outs of eastern shore broiler operations. Aqueous PLAC samples were prepared by adding deionized water (200 mL) to fresh litter (500 mg), mixing for 2 h with a wrist shaker (highest setting), then centrifuging at 5000g for 20 min. Supernatant was decanted and a portion stored at -20EC until analysis. Conjugated and unconjugated steroids in remaining sample aliquots were separated via aqueous:organic (liq:liq) extraction. Briefly, 1.0 mL of sample was vortexed with 5.0 mL ether and allowed to separate. After placing in liquid nitrogen ($LiqN_2$) the organic layer was decanted from the frozen aqueous layer. After thawing, a second volume of ether was added, vortexed, allowed to separate, then poured off and combined with the first. Organic samples were taken to dryness in a 50EC water bath and stored (-20EC) until ready for analysis, at which time they were reconstituted in 1.0 mL RIA buffer and analyzed as described above. Aqueous samples without extraction were presumed to contain both conjugated and unconjugated steroids while organic samples were presumed to contain only lipophylic unconjugated steroids.

Vitellogenin Quantification

Fathead minnow Vtg in plasma and whole-body homogenate samples was determined using a competitive enzyme linked immunosorbent assay (ELISA) method modified from Parks *et al*. [1999] and performed at VIMS. Briefly, the assay was performed by incubating unknown samples, standards, and blanks in individual tubes with a primary antibody, mouse monoclonal anti-carp Vtg [Cayman Chemical cat. #170115], for one hour before introduction to a 96-well plate pre-coated with fathead minnow Vtg and blocked with 5% BSA in carbonate buffer. Primary antibody attached to Vtg from samples was sequestered while remaining unbound antibody in solution was free to react with the Vtg well-coating. Only antibody attached to this plate-bound Vtg remained after three washings with a PBS/Tween 20 solution. Goat anti-mouse antiglobulin conjugated w/ horseradish peroxidase [Fisher Scientific cat. # OB0101-05] was introduced to wells and bound with the primary antibody/Vtg complex, where available. After a final wash only this Vtg antibody-antiglobulin complex remained attached to the wells. A TMB substrate [Pierce Chemical Company cat.# 34021], designed to degrade in the presence of horseradish peroxidase and cause a color change from clear to brilliant blue, was introduced to the wells. Appearance of an intense color change indicated the presence of significant conjugate and, therefore, a lack of Vtg in the original sample. Conversely, absence of a color change indicated a lack of conjugate and, therefore, the presence of significant Vtg in the original sample. Standards ranging from 23.4 ng/mL to 3000 ng/mL were made by serial dilution of a known Vtg stock and incubated in duplicate on each plate. Absorbance read at 450 nm on a 96-well plate reader allowed quantification of the color change, calculation of a standard curve, and

interpolation of unknown values.

Iterative runs of the assay during method validation indicate a MDL of 20 ng/mL with the linear portion of the standard curve lying between the lower limit of quantitation (LOQ; 60 ng/mL) and 750 ng/mL. Best results were achieved when samples were diluted such that Vtg concentrations fell within this linear portion of the standard curve. Typically, samples expected to contain very little Vtg (e.g., control treatments) received a 1:300 dilution providing a MDL and LOQ of 6 µg/mL and 18 µg/mL, respectively. Treatment samples where significant induction was anticipated (e.g., positive control treatments) receive a 1:120,000 dilution providing a MDL and LOQ of 2.4 mg/mL and 7.2 mg/mL, respectively. Values exceeding the linear portion of the curve required re-analysis with additional dilution. Samples with values falling between the MDL and the LOQ were either re-analyzed with less dilution if sufficient plasma remained, or else were reported with a notation indicating dubious accuracy. Results below the MDL were recorded as ½ MDL for subsequent statistical analyses (e.g., calculation of treatment means, SE, etc.). Similar competitive ELISA methods were used to measure mummichog and sheepshead minnow Vtg by the Molecular Biomarkers Core Facility, Biotechnology Program at the University of Florida, Gainesville, FL [Denslow *et al*., 1999; Folmar *et al*., 2000].

LABORATORY EXPOSURE PROTOCOLS

A series of laboratory assays was performed using a variety of test species exposed to control and contaminant treatments under several exposure regimes. In all, 5 separate assays were run, three with fathead minnow and two with sheepshead minnow

and mummichog. Because substantial evidence exists that low ng/L concentrations of exogenous steroidal estrogens can adversely affect reproductive biology in fish, a positive control treatment (100ng E2/L) was included in at least one assay with each test species. Endocrine disruption related endpoints included the induction of Vtg and alteration in gonadal histology and GSI. Additionally, survival and calculation of condition indices (CI) served as measures of the general health of test animals. *Table 4* summarizes information on exposure methods, treatments and biological endpoints for each assay. Detailed descriptions of test conditions for individual assays can be found in *Appendix 1*.

Generation of Exposure Treatments

Water-soluble PLAC treatments. Generation of PLAC exposure treatments was based loosely on methods of Nichols *et al*. [1997; 1998]. In general, litter was introduced to well water at a rate of 2.5 g dry weight/L, then mixed with sufficient vigor to maintain particulates in suspension for ~20 h. Exposure treatments were produced by dilution of this mixture following coarse (2.0 mm) filtration. For example, in the first fathead minnow assay a low treatment was the result of a six-fold dilution of the aqueous poultry litter mixture (equivalent to 417 mg dry litter/L well water) while a high treatment was the result of only a three-fold dilution (833 mg dry litter/L well water). The lack of fine filtration was intentional in an attempt to produce exposure treatments representative of complex environmental waters such as those resulting from agricultural runoff during rain-events. It was assumed that contaminants in treatments generated in this "proportional" fashion would themselves be proportional. Accuracy of this assumption was investigated by examination of daily measurements of various water quality

Table 4. Overview of freshwater and estuarine laboratory assay exposure protocols.

Assay #	Renewal Method	Life stage(s)	n	Exposure Duration (d)	Exposure Treatments	Biological Endpoints
Fathead Minnow Assay #1	Static w/ daily renewal	Adult male	10	21	-Control -Solvent Control -E2 Control -417 Litter -833 Litter	-Survival -Plasma Vtg -Gonad histology -GSI[a] -CI[b]
		Mixed larvae (1 dph)	25	21	-Control -Solvent Control -E2 Control -417 Litter -833 Litter	-Survival -Whole-body homogenate Vtg -CI
Fathead Minnow Assay #2	Flow-through w/ 8 daily vol. replacements	Adult male	6 6 7	4 9 21	-Control -104 Litter -208 Litter -417 Litter	-Survival -Plasma Vtg -Gonad histology -GSI -CI
		Mixed juvenile (36 dph)	40	21	-Control -Solvent Control -E2 Control -104 Litter -208 Litter -417 Litter	-Survival -Whole-body homogenate Vtg -Gonad histology -CI
		Mixed Larvae (3 dph)	40	21	-Control -Solvent Control -E2 Control -104 Litter -208 Litter -417 Litter	-Survival -Gonad histology -CI

Table 4 (continued). Overview of freshwater and estuarine laboratory assay exposure protocols.

Assay #	Renewal Method	Life stage(s)	n	Exposure Duration (d)	Exposure Treatments	Biological Endpoints
Fathead Minnow Assay #3	Static w/ daily renewal + one flow-through	Adult male	6 6 6	4 9 21	-Control -417 Litter 21 Day -833 Litter 4 Day -833 Litter 9 Day -833 Litter 21 Day -833 Litter Flow-Thru 21 Day	-Survival -Plasma Vtg -Gonad histology -GSI -CI
Sheepshead Minnow & Mummichog Assay #1	Static w/ daily renewal	Adult male	10	21	-Control -Solvent Control -E2 Control	-Survival -Plasma Vtg -Gonad histology -GSI -CI
		Mixed larval sheepshead minnow (3 dph)	25	21	-Control -Solvent Control -E2 Control -104 Litter	-Survival -Whole-body homogenate Vtg -CI
Sheepshead Minnow & Mummichog Assay #2	Static w/ daily renewal	Adult male	6	21	-Control -417 Litter -833 Litter -1,667 Litter	-Survival -Plasma Vtg -Gonad histology -GSI -CI

[a] Gonadosomatic Index (GSI)
[b] Condition Index (CI)

parameters (e.g., pH, conductivity, alkalinity, hardness) and by measurement of water soluble E2 and T.

Poultry litter mixtures were started daily for use as treatment renewal the following day. The volume of mixture produced varied according to the number of treatments, size of exposure vessels and treatment replacement method (static renewal vs. flow-through). Litter treatments in all assays were designated according to the ratio of dry litter (mg) to diluent water (L). Thus, an exposure treatment comprised of 833 mg poultry litter per 1 L of well water was designated *PLAC-833*, 417 mg litter/L well water was designated *PLAC-417,* etc.

Positive control and solvent control treatments. Initial assays with each test species and age class included an *E2 Control* treatment meant to investigate the "inducability" of estrogenic responses. The female hormone E2 was selected as positive control because it is known to persist in dry litter following application to fields and has been reported at environmentally relevant concentrations in receiving waters following runoff events [Shore *et al*., 1995; Nichols *et al*., 1997; Herman and Mills, 2003]. Further, induction of Vtg in male fish is widely accepted as a sensitive indicator of estrogenic exposure [Sumpter and Jobling, 1995; Denslow *et al*., 1999]. While not causally linked to a reduction in reproductive competence, Vtg induction in male fish has been found to correlate with reductions in GSI and pathological effects in testes [Miles-Richardson *et al*., 1999].

A nominal concentration of 100 ng E2/L, known to be effective at inducing Vtg in male fathead minnow [Sumpter and Jobling, 1995], was selected for the positive control. Because an ethanol (EtOH) vehicle was employed to place E2 into solution, a solvent

control treatment of equal EtOH concentration (0.002 mL EtOH/L) to that of the positive control was also employed. Positive and solvent controls were prepared by spiking diluent water and mixing thoroughly immediately prior to treatment renewal.

Fathead Minnow Assays

Treatment concentrations, exposure durations and methods of water renewal evolved in the fathead minnow laboratory assays as information from previous exposures was interpreted and new questions became germane. Briefly, the first assay was conducted using static exposures renewed daily for 21 d, the second was conducted flow-through and sampled at 4, 9 and 21 d, and the third employed a combination of the two water renewal methods and sample intervals. All three involved exposure of adult male fish to water-soluble PLACs diluted in aged, aerated and temperature adjusted well water. Additionally, mixed-gender larval and juvenile fish were exposed to aqueous poultry litter extracts in the first and second assays. Of the three assays, the first two used material from the *WYE2000* poultry source in generation of PLAC exposure treatments while the third used material from the *WYE2002* litter source. Steroidal (E2 and T) and metal contaminants were measured in both litter sources and *WYE2000* was further screened for pesticide (historic and current-use) and antibiotic contaminants.

Temperature was held constant at 25 ±1EC and a 16:8 light:dark cycle was maintained during acclimation, exposure and grow-out periods. Mature fish were fed crushed Tetramin® flake food twice daily (4% body weight/day). Larval and juvenile fish received live artemia nauplii twice daily with amounts increased sequentially to match feeding requirements of the growing fish. Excess food was siphoned from tank bottoms

during daily treatment renewals.

Fathead Minnow Assay I. The first fathead minnow assay involved continuously exposing groups of 10 sexually mature male fish (6 - 8 months old) and 25 mixed gender larval fish (1 dph at test initiation) to 5 assay treatments (*Control; E2 Control; Solvent Control; PLAC-417; PLAC-833*) for 21 days. Adult fish were held in 37 L all glass aquaria (34 L working volume) and larval fish were held in 10 L all glass aquaria (7.5 L working volume). Treatments were run static with 90% daily volume replacement. Litter concentrations were selected to assess the suitability of our toxicological endpoints for detecting endocrine disruption related effects while still maintaining environmental relevance. The low treatment was considered representative of an environmentally realistic exposure concentration, while the high was seen as a "worst-case scenario." An *E2 Control* treatment was also run to validate that our laboratory exposure system was capable of inducing endocrine associated effects such as have been reported previously [Ankley *et al*., 2001]. Finally, a *Solvent Control* containing 2.0 ppm ethanol (EtOH) was employed to ensure that the vehicle for placing E2 into solution (EtOH) was not responsible for confounding assay results.

Fathead Minnow Assay II. Having established in the previous assay that exposure to a *PLAC-417* treatment for 21 d was capable of inducing an estrogenic effect in male fish, the second assay was designed to explore the lower threshold at which PLAC-induced effects could be detected. Adult fish were exposed to multiple treatment concentrations for multiple exposure intervals to ascertain whether PLAC-induced effects were predominantly time or concentration dependent. Therefore, *Fathead Minnow Assay II* employed a series of three exposure concentrations with the high treatment, *PLAC-417*

equal in amount of water-soluble poultry litter to the low treatment from *Assay I*. The remaining litter treatments, prepared in dilution series, were *PLAC-208,* and *PLAC-104*. Batches of 19 sexually mature male fish (5½ months old) were placed in 37 L exposure aquaria (30 L working volume) and randomly sub-sampled at 4, 9 and 21 d intervals. Groups of 40 larval (3 dph at assay initiation) and 40 juvenile (36 dph at assay initiation) minnows (mixed gender) were also exposed for 21 d to the three PLAC treatments as well as positive (100 ng E2/L) and solvent (2.0 ppm EtOH) controls. Larval and juvenile exposures were conducted in 10 L aquaria (7.5 L working volume) with larvae segregated into mesh-sided baskets (1 mm) to minimize mechanical stress and protect them from larger juveniles. Exposure treatments were continually exchanged via a flow-through system providing 8 volume replacements/day. Following exposures half of the juvenile fish (58 dph at assay conclusion) were fixed for histological examination and half were reserved in liquid nitrogen for subsequent whole-body homogenate Vtg analysis. Larval fish were held under control conditions for an additional 36 d to an age of 60 dph, at which time they were fixed for histological examination.

Fathead Minnow Assay III. The final assay repeated the first by exposing adult male fish (5 - 6 month old) for 21 d in a static-renewal system to *PLAC-417* and *PLAC-833* treatments. Replicate *PLAC-833* treatments were sub-sampled at 4, 9 and 21 d to more thoroughly investigate the exposure duration/concentration relationship. Finally, an additional *PLAC-833* treatment was run flow-through for 21 d to explore differences in effects associated with water replacement method.

Sheepshead Minnow and Mummichog Assays

Two assays were performed with estuarine species. The first *(Sheepshead Minnow and Mummichog Assay I)* used sexually mature male sheepshead and mummichog (10/treatment) and mixed gender larval sheepshead (25/treatment; 3 dph at test initiation) exposed continuous for 21 d to *Control*, *E2 Control* (100 ng E2/L), and *Solvent Control* (2.0 ppm EtOH) treatments. The intent was to ascertain whether these species could be "induced" in a 21 d laboratory exposure to exhibit endocrine disruption related effects. The second *(Sheepshead Minnow and Mummichog Assay II)* was meant to quantify endocrine related effects resulting from exposure to PLACs. Mature male fish of each species (6/treatment) were exposed for 21 d to a series of poultry litter treatments (*PLAC-417*, *PLAC-833* and *PLAC-1667).* Because larval sheepshead showed little sensitivity to the *E2 Control* of the first assay they were omitted from the second.

In both assays adult male fish were held in 37 L all glass aquaria (30 L working volume). Sheepshead larvae *(Assay I)* were held in 10 L all glass aquaria (7.5 L working volume). Treatments were maintained static with gentle aeration and received daily renewal via siphoning and 90% volume replacement. Diluent, positive and solvent controls and PLAC treatments were prepared as described previously. Filtered (1 µm), aerated, temperature (25EC) and salinity (*Assay I:* 10‰; *Assay II:* 15‰) adjusted estuarine water from the Wye River on Maryland's eastern shore served as diluent. Temperature was held constant at 25 ±1EC and a 16/8 light/dark cycle was maintained during acclimation and exposure periods. Adults were fed crushed Tetramin® flake food twice daily (4% body weight/day). Larval fish received live artemia nauplii *ad libitum*. Excess food was siphoned from tank bottoms during daily treatment renewal.

FIELD INVESTIGATIONS

Controlled field studies were devised with two objectives in mind: first, to investigate the nature and quantity of PLACs that ultimately end up in receiving waters following application of litter as fertilizer to fields; and second, to identify any adverse effects exposure to these contaminants might have on resident aquatic resources. These studies were conducted during the 2000 and 2002 growing seasons using two adjacent ~35 acre agricultural research fields at the University of Maryland, Wye Research and Education Center (UMD - WREC) *(Figure 6a)*. The fields have been used continuously since 1984 for crop and soil studies and are fitted with discharge flumes for measuring runoff, piezometers for characterizing groundwater, and an automated weather station for recording regional meteorological information.

During the 2000 and 2002 planting seasons one field was cultivated under conventional-tillage practices of turning surface soil (~20 cm depth) into furrows before planting while the other was cultivated using no-till practices *(Table 3)*. Poultry litter was applied to each field at 3 ton/ac, a rate consistent with standard practices for production of corn, the dominant crop on the Delmarva Peninsula *(Figure 6b)*. Litter application and subsequent cropping of corn were performed by technicians at UMD - WREC with funding from Delmarva Poultry Industries, Inc., Maryland Grain Producers Utilization Board, and the Maryland Center for Agro-Ecology, Inc., organizations concerned with nutrient dynamics as they relate to agriculture and poultry litter application. A detailed history of agronomic practices on *Conventional-Till* and *No-Till* fields is provided by Staver [2004].

(a)

(b)

(c)

Figure 6. Research fields ***(a)*** at the University of Maryland - Wye Research and Education Center situated near the Wye River on Maryland's eastern shore; ***(b)*** poultry litter application on the *No-Till* field using a conventional manure spreader; ***(c)*** *Research Pond* providing retention of runoff from the *No-Till* field via a short grass raceway.

A *Research Pond* (25m x 75m x 0.67m) provides retention for runoff from the *No-Till* field, which eventually drains through a tidal marsh system into the Wye River *(Figure 6c)*. The *Conventional-Till* field drains along a grass and wooded raceway for several hundred meters before discharging into Quarter Creek, a small tidal tributary of the Wye River.

The first objective of the controlled field investigations was to gain an understanding of the transport and persistence of PLACs in natural waters. This was done by measuring known or suspected EDCs in poultry litter prior to field application and in runoff and receiving waters during and after rain events subsequent to litter application. Following litter application all rain events that produced runoff from *No-Till* and/or *Conventional-Till* fields for the remainder of the growing season(s) were characterized by sample collection via flow-actuated ISCO 3700 composite sampling devices located at field discharge flumes. Collection intervals were flow-dependent allowing generation of hydrographs (flow rate vs. time) for calculation of contaminant discharge loads. Fields under *No-Till* management tend to have less water retention capacity than those under *Conventional-Till*. As such, rain events tend to produce runoff more rapidly and more abundantly from *No-Till* fields than from *Conventional-Till* fields. Runoff samples were analyzed for nutrients and metals (UMD – WREC) and for E2 (VIMS).

The *Research Pond* provides retention for runoff from the *No-Till* field. Samples were collected from this pond using a manually actuated ISCO 3700 compositor during all post litter application runoff events. The compositor intake tube was positioned near the point of entry of runoff from the *No-Till* field. The device was started in anticipation

of rain-events with samples discarded if rain was insufficient to produce runoff. The insulated sample holding well within the compositor was iced whenever in operation to ensure sample preservation. After collection samples were frozen until submittal for steroid analysis.

The second objective of the controlled field investigations, to identify adverse effects of PLAC exposures on resident aquatic species, was addressed by caging fish in the *Research Pond* during and after the initial runoff event(s) subsequent to field litter application. The fish caging system consisted of 4 L floating mesh baskets housed within large (~150 L) cylindrical high-density polyethylene barrels attached to anchors via stainless steel cables *(Figure 7)* (detailed descriptions of cage materials, construction and deployment options are provided in *Appendix 2)*. The intake line for the *Research Pond* ISCO compositor was affixed to the fish-caging array so that water sampled for analysis would approximate actual fish exposures. A similar array was placed within a *Reference Pond,* located ~400 m from the *Research Pond.* This pond is similar in size and depth to the *Research Pond* but receives no agricultural surface water input.

Spring 2000 Controlled Field Investigation

Two hundred tons of poultry litter (*WYE2000*) from an eastern shore broiler operation were delivered to UMD - WREC over multiple days (2/29-3/3/00), placed in a common pile and covered. After preparation of research fields litter was applied on 5/8/00 and 5/9/00 at 3 ton/ac using a conventional spreader *(Figure 6b)*. Samples were collected from three discrete parts of the *WYE2000* poultry litter pile as batches were removed for field application. These were analyzed for nutrients (phosphate and nitrate)

(a)

(b)

(c)

Figure 7. Small fish deployment cages; ***(a)*** protective barrel and smaller floating baskets; ***(b)*** deployed barrel with lid removed to reveal arrangement of floating baskets; ***(c)*** fish cage deployed in the *Research Pond* at University of Maryland - Wye Research and Education Center with blue tarp to provide shade.

by staff at UMD – WREC, for trace metals, antibiotics, and current use agri-chemicals by the USGS – CERC, and for water-soluble steroidal contaminants (E2 and T) by technicians at VIMS (as described previously).

Fish caging barrels were deployed to the *Research* and *Reference Ponds* on 5/10/00 in anticipation of rain. Two small rain events occurred on 5/10/00 and 5/12/00 but produced no runoff from either field. In response to a severe thunderstorm forecast, mature male fathead minnow were placed in baskets and deployed to barrels in both ponds on 5/19/00. Two replicate baskets (5 fish/rep) were placed in each pond. Likewise, two replicate 10 gal aquaria each with 5 fish/rep were maintained in the laboratory as a control. A powerful thunderstorm (henceforth known as *"Event 1"*) dropped ~ 5 cm of precipitation between 0800 and 1100 on the morning of 5/22/00 and continued as a soft rain for the remainder of the afternoon (total *Event 1* precipitation was 5.89 cm). Runoff from the *No-Till* field to the *Research Pond* began shortly after 0930 and became very intense by 1130. Flow abated by 1300 but runoff continued until after midnight. *No-Till* discharge flume samples were collected from the inception of runoff until cessation on 5/23/00 at 0130. Because conventional till practices provide for substantial water retention within field furrows, runoff from the *Conventional-Till* field was less intense and did not begin until the morning of 5/23/00. Samples were collected from within the *Research Pond* over an 18 h period beginning 2 h prior to the initiation of runoff and continuing until the conclusion of the event. The *Research Pond* sampler was programmed to collect 125 mL aliquots every 30 min compositing groups of four into glass bottles such that individual bottles received 500 mL samples collected over 2 h intervals.

Fish deployed in the barrels had a 3 day acclimation period before *Event 1* initiated exposure to PLACs via runoff from the *No-Till* field. During the exposure period fish were fed Tetramin® flake food daily and observed for mortality and abnormal activity. Standard water quality parameters (temperature, dissolved oxygen, pH, conductivity and NH_3) were monitored and daily water samples were collected for E2 analysis. Fish were left *in situ* for 21 d after *Event 1* until retrieval on 6/12/00. Fish were held under control conditions in the laboratory for an additional 3 days before plasma collection and tissue fixation occurred on 6/19/00 (as described previously). Surface water from the *Research Pond* was collected daily during the 21 d fish exposure interval, then weekly for the remainder of the summer. Runoff from *No-Till* and *Conventional-Till* fields was also sampled during all rain events for the remainder of the summer.

Spring 2002 Controlled Field Investigation

WYE2002 poultry litter, also from an eastern shore broiler operation, was delivered to WYE - WREC on 3/19/02. Material from this litter pile was collected in a single large batch (~40 kg) at the time of delivery for subsequent use in laboratory assays. After homogenization sub-samples of this single batch were taken for analysis of nutrients, metals and steroids (as described previously). *WYE2002* was applied to the research fields at 3 ton/ac on 5/8/02. The first rain event after application (*Event 2*) occurred on 5/18/02 with rain falling from 0330 – 1030. Runoff from the *No-Till* field began at 0900 and continued until 2035. Runoff was collected from the discharge flume as described previously. In addition, 600 L of *No-Till* runoff was collected directly from the discharge flume between 0930 and 0945 via multiple bucket grabs and transferred to

4 L cubitainers for use in laboratory fish exposures. This material was transferred to a walk-in freezer and held at -20EC until needed. The 5/18/02 rain event did not produce runoff from the *Conventional-Till* field. It was not until 6/7/02 that a substantial thunderstorm produced any runoff from this field.

During *Event 2* surface water samples were collected from the *Research Pond* over a 15 h period beginning 1 h prior to the initiation of runoff and continuing until several hours after runoff had dissipated. The *Research Pond* sampler was programmed to collect 50 mL aliquots every 15 min compositing groups of four into glass bottles such that individual bottles received 200 mL samples collected over 1 h intervals. Theses samples were promptly frozen at -20EC in anticipation of subsequent steroid analysis.

Fish exposures in 2002 were similar to those of 2000. Barrels in *Research* and *Reference Ponds* each received 10 mature male fathead minnow (5/replicate basket). To avoid undue stress during the actual rain event fish were not deployed until 1930, near the conclusion of runoff into the *Research Pond.* In addition, 3 groups of fish were also placed in replicate baskets and maintained static in 37 L glass aquaria (34 L working volume) in the laboratory. These included: *Control/Lab* fish which received daily water renewals of aged aerated well water; *Pond/Lab* fish which received renewal with water "grabbed" daily from the *Research Pond;* and *Flume/Lab* fish which were exposed directly to the *No-Till* field runoff from *Event 2* by thawing batches of 6 cubitainers (24 L) for daily water renewal. Laboratory exposures were conducted at 23±1EC with a 16:8 light:dark cycle, and received gentle aeration. Fish in the ponds were subjected to ambient temperature and light cycles, but received aeration to avoid potential hypoxia related stress. Laboratory and field fish were both fed Tetramin® flake food daily and

observed for stress/mortality. Standard water quality parameters (temperature, dissolved oxygen, pH, conductivity and NH_3) were monitored daily and water samples grabbed from the *Research Pond* were sub-sampled for steroid analysis.

The initial design called for leaving fish *in situ* for 21 d after *Event 2* before retrieval on 6/8/02. However, the road adjacent to the *Research Pond* was resurfaced (oil and chip) on 6/4. Due to a 30% chance of rain fish were pulled from the ponds to avoid any chance of oiling. Therefore, the exposure duration for the 2002 field investigation was only 17 d.

STATISTICAL ANALYSIS

Statistical analyses were carried out using SAS and SigmaStat 3.0 computer programs [SAS Institute Inc., Cary, NC, USA and Jandel Scientific, San Rafael, CA, USA, respectively]. Prior to analysis, data were transformed as necessary (e.g., arcsine square root transformation of percent data) or to improve normality and homogeneity of variance [Sokal and Rohlf, 1987]. Unless otherwise noted data on GSI, Vtg, and morphometric histological assessments were tested by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison versus the control. Data not satisfying normality and/or homogeneity of variance requirements for parametric statistics subsequent to transformation were tested by Kruskal-Wallis one-way ANOVA on ranks with *post-hoc* Dunn's multiple comparison versus the control.

CHAPTER III: RESULTS

POULTRY LITTER-ASSOCIATED CONTAMINANTS

Analysis of litter samples revealed numerous contaminants including current use and historic pesticides, metals, PAHs, antibiotics and steroids. Detailed results of organic and elemental analyses are found in a USFWS report by McGee *et al*. [2003]. Of interest are trace amounts of the banned organochlorine pesticides, lindane, chlordane and DDT & metabolites (e.g., p,p'- DDT; p,p'-DDE), as well as the current use pesticides, acetachlor, metolachlor, diazinon, permithrin(s), λ-cyhalothrin, and the herbicide atrazine *(Table 5)*. Most were present in low ppb concentrations except atrazine at levels of 0.34 to 1.3 µg/g. Also of note was the antibiotic chlortetracycline found in two of three samples (1.2 and 1.6 µg/g dry weight).

Water-soluble E2 and T levels were measured at 108 ng/g and 34 ng/g, respectively, in the *WYE2000* litter source and 86 ng/g and 19 ng/g, respectively, in the *WYE2002* litter source *(Table 6)*. These levels are somewhat lower then results for the other 6 eastern shore litter samples, but generally in agreement with levels reported previously for broiler litter [Nichols *et al*., 1997; 1998; Shore *et al*., 1995].

FATHEAD MINNOW ASSAY RESULTS

Water Quality

During the three fathead minnow laboratory assays all water quality parameters fell within acceptable ranges except ammonia (NH_3) in the highest poultry litter exposures. Temperature, measured continuously during the three assays, remained stable

Table 5. Partial list of organic contaminants detected in poultry litter *(WYE2000)* from a whole-house scrape-out of a standard broiler operation on the Delmarva Peninsula. Comprehensive analytical methods, detection limits and results are available in McGee *et al*. [2003].

Compound	Sample 1	Sample 2	Sample 3
Organochlorine Pesticides (ng/g)			
α-BHC	0.3	<MQL	MDL
β-BHC	1.3	43	4.2
δ-BHC	0.3	<MDL	<MDL
Lindane	0.6	4.6	0.8
p,p'-DDT	2.9	<MDL	<MDL
p,p'-DDE	<MQL	0.6	0.6
Endrin	<MDL	3.7	0.6
Oxychlordane	<MDL	13	<MDL
trans-Chlordane	<MQL	1.4	0.8
cis-Chlordane	<MDL	0.6	0.6
Current Use Pesticides (ng/g)			
Acetachlor	1.1	11	<MQL
Metolachlor	14	8.3	3.1
Trifluralin	<MQL	5.6	1.6
Diazinon	0.8	18	0.8
cis-Permethrin	6.6	<MDL	<MDL
trans-Permethrin	7.4	4.0	<MDL
λ-Cyhalothrin	3.7	28	12
Atrazine	340	1300	370
Antibiotics (µg/g)			
Chlortetracycline	1.2	1.6	<MDL
PAHs (ng/g)			
Benz[a]anthracene	270	210	250

at 25.0 ±1.0EC. Increases in PLAC concentration had the effect of depressing DO and pH marginally below control levels (≤1.0 mg/L and ≤0.5 units, respectively) while increasing total NH_3 (*PLAC-417* and *PLAC-833* NH_3 levels were ≤3.1 mg/L and ≤6.0 mg/L, respectively). The water quality criterion for chronic ammonia exposure at pH 7.5 and temperature 25EC is 2.2 mg NH_3/L [USEPA, 1999].

Table 6. Sex steroids in poultry litter samples collected from broiler operations on the eastern shore of Maryland, USA, prior to agricultural field application. Water soluble fractions, determined by the methods of Nichols *et al*. [1997], could contain both bound (conjugated) and unbound steroids depending on antibody specificity. Conjugated steroids were removed from aqueous samples via organic extraction to determine un-conjugated (presumably bioactive) compound.

	17 β-Estradiol			Testosterone		
Poultry litter source	Water soluble (ng/g)	Extractable from water soluble (ng/g)	Ratio of extracted to total	Water soluble (ng/g)	Extractable From water soluble (ng/g)	Ratio of extracted to total
PL-1	131	74	0.56	58	58	1.01
PL-2	134	62	0.46	43	40	0.93
PL-3	135	83	0.61	50	60	1.20
PL-4	136	71	0.53	48	38	0.79
PL-5	166	88	0.53	50	65	1.30
PL-6	112	61	0.54	39	38	0.98
WYE2000	108	n/a	n/a	34	n/a	n/a
WYE2002	86	49	0.57	19	18	0.95
Mean	126	70	0.54	42	45	1.03
(±SD)	(23.7)	(13.3)		(11.9)	(16.5)	
range			0.46 - 0.61			0.79 - 1.30

Steroid Chemistry

Analysis of *Control* and *Solvent Control* treatments from the various assays indicated a near complete absence of E2 and T with most measurements below MDLs of 18 ng/L and 6 ng/L, respectively *(Figures 8a, 9a, 10a, & 11a)*. Target E2 concentrations (nominal) for *E2 Control* treatments were 100 ng/L. Measured E2 values in *Assay I* and *Assay II* were 79.2 ng/L (range 27 - 137 ng/L) and 96.0 ng/L (range 59 - 130 ng/L), respectively. As expected, T was not detected in *E2 Control* treatments. *PLAC-417* and *PLAC-833* treatments from *Assay I* had mean E2 levels of 113.6 ng/L and 191.7 ng/L, respectively, and mean T levels of 36.6 ng/L and 31.3 ng/L, respectively – E2 followed the expected pattern of more litter in solution yielding a higher steroid concentration while T did not *(Figure 8a)*. In *Assay II* E2 concentrations in *PLAC-104*, *PLAC-208* and *PLAC-417* treatments remained fairly stable over the 21 d exposure interval with mean levels of 18.3 ng/L, 40.2 ng/L and 67.8 ng/L, respectively, while T levels (measured only during the first 4 days) were 18.8 ng/L, 26.7 ng/L and 51.7 ng/L, respectively *(Figure 9a)*. Despite using the same litter source as *Assay I*, E2 values in *Assay II* were lower than anticipated (*PLAC-417* E2 was <70 ng/L compared to >110 ng/L in the previous assay). In *Assay III* the *PLAC-417* treatment had a mean E2 concentration of 71.1 ng/L but mean T concentration of only 11.2 ng/L *(Figure 11a)*. E2 levels in *PLAC-833* treatments at 4 d, 9 d, and 21 d and *PLAC-833 Flow-Thru* treatments at 21 d were 132.5 ng/L, 138.3 ng/L, 123.0 ng/L, and 105.7 ng/L, respectively. T concentrations for these treatments were 45.9 ng/L, 41.9 ng/L, 36.2 ng/L, and 31.0 ng/L, respectively.

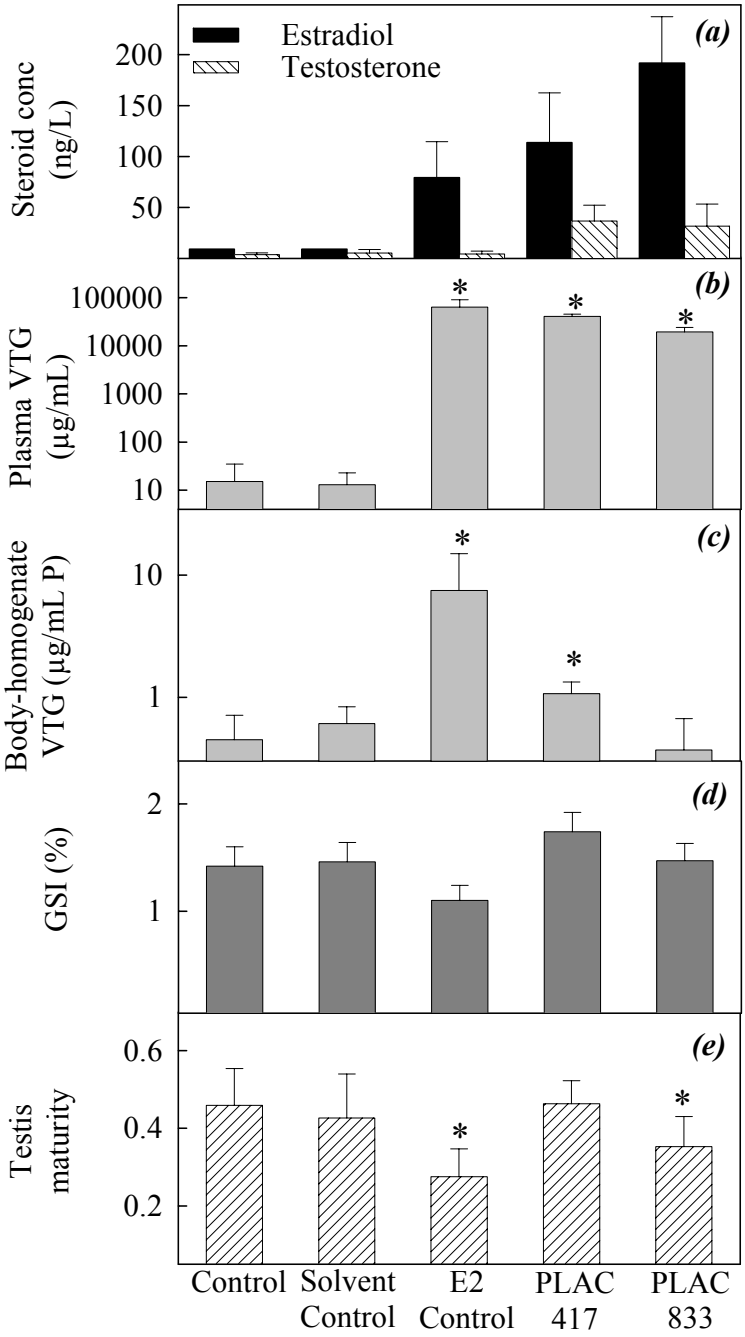


Figure 8. *Fathead Minnow Assay I* mean (±SD) sex steroid (E2 and T) exposure levels ***(a)*** and treatment means (±SD) of various biological indicators: ***(b)*** plasma Vtg levels in adult ♂ fish; ***(c)*** larval body homogenate Vtg levels (normalized to total protein); ***(d)*** gonadosomatic indices in adult ♂ fish; ***(e)*** testis maturity in adult ♂ fish (estimated as the proportion of spermatozoa and spermatids to total germinal epithelium. Asterisks (*) indicate treatment groups that differ significantly ($p < 0.5$) from controls.

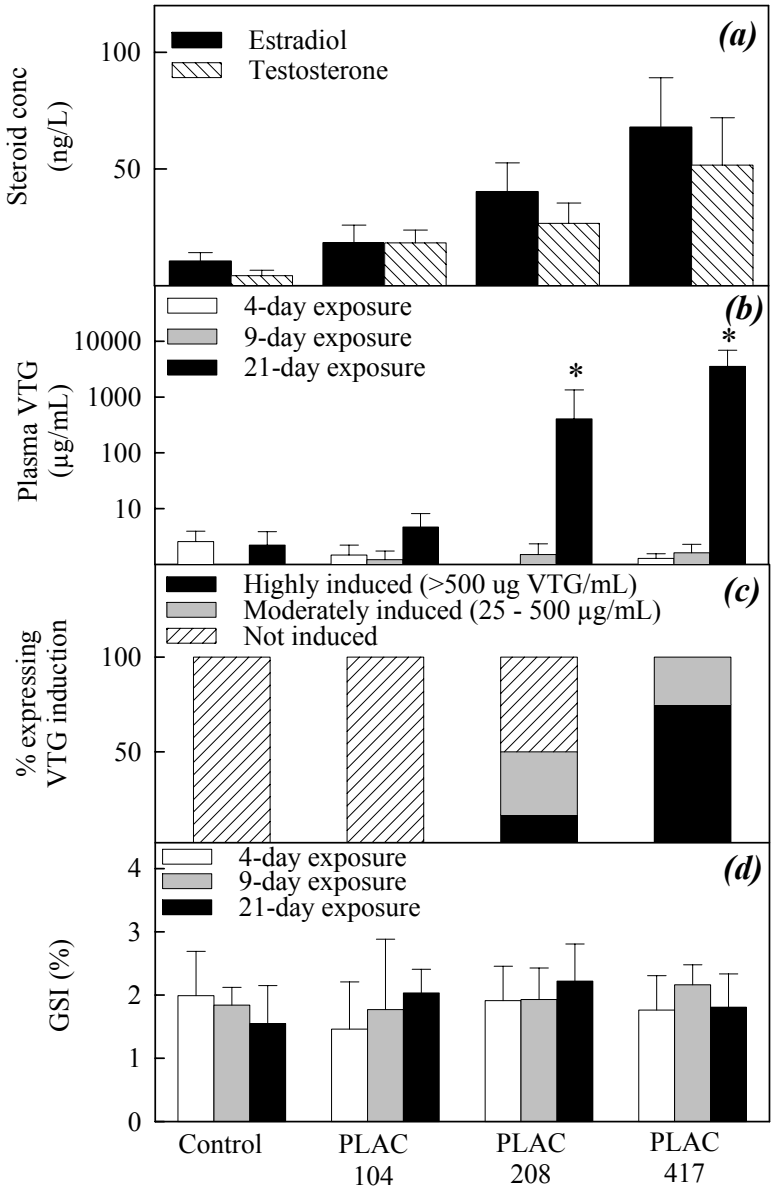


Figure 9. *Fathead Minnow Assay II* mean (±SD) exposure levels of sex steroid (E2 and T) to adult ♂ fish ***(a)*** and resulting treatment means (±SD) of various biological indicators: ***(b)*** plasma Vtg levels; ***(c)*** proportion (%) of fish expressing moderate or high levels of plasma Vtg; ***(d)*** gonadosomatic indices. Asterisks (*) indicate groups that differ significantly ($p < 0.5$) from controls.

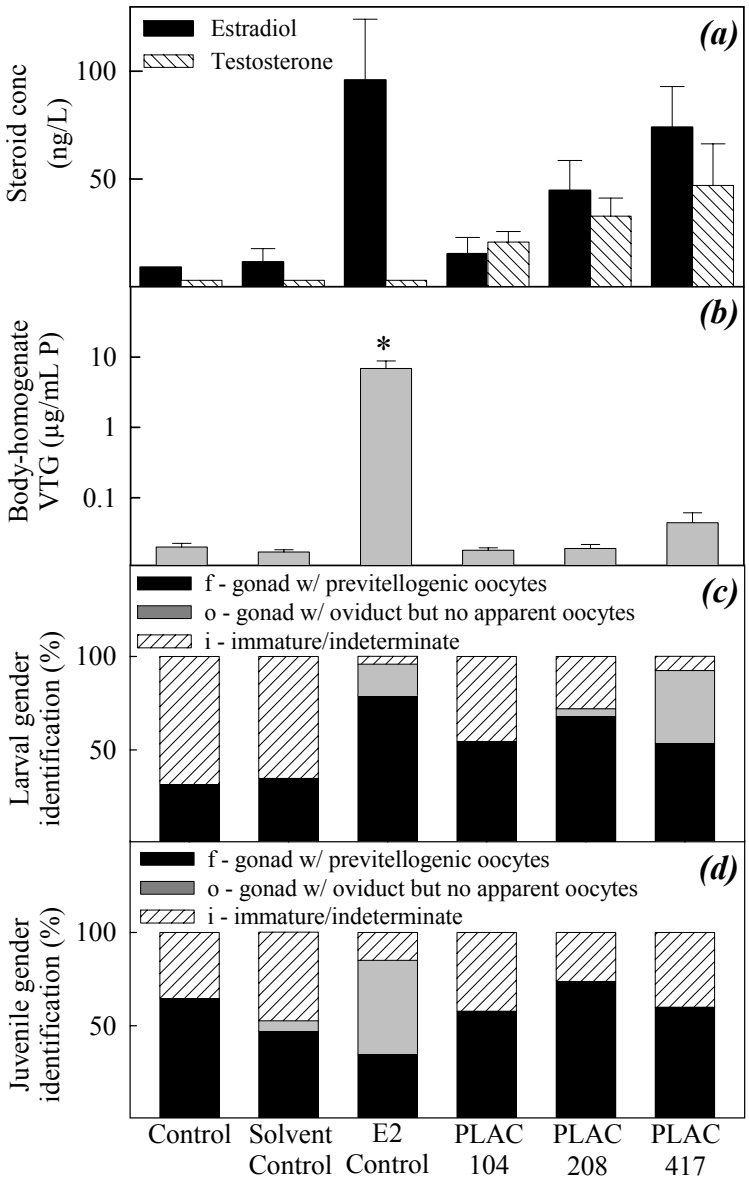


Figure 10. *Fathead Minnow Assay II* mean (±SD) exposure levels of sex steroids (E2 and T) to larval and juvenile fish ***(a)*** and resulting treatment means (±SD) of various biological indicators: ***(b)*** juvenile whole-body homogenate Vtg levels; ***(c)*** larval gender identification (%); ***(d)*** juvenile gender identification (%); (**f** and **o** represent presumptive females, **i** represent fish not sufficiently mature to make a gender determination). Asterisks (*) indicate groups that differ significantly ($p < 0.5$) from controls.

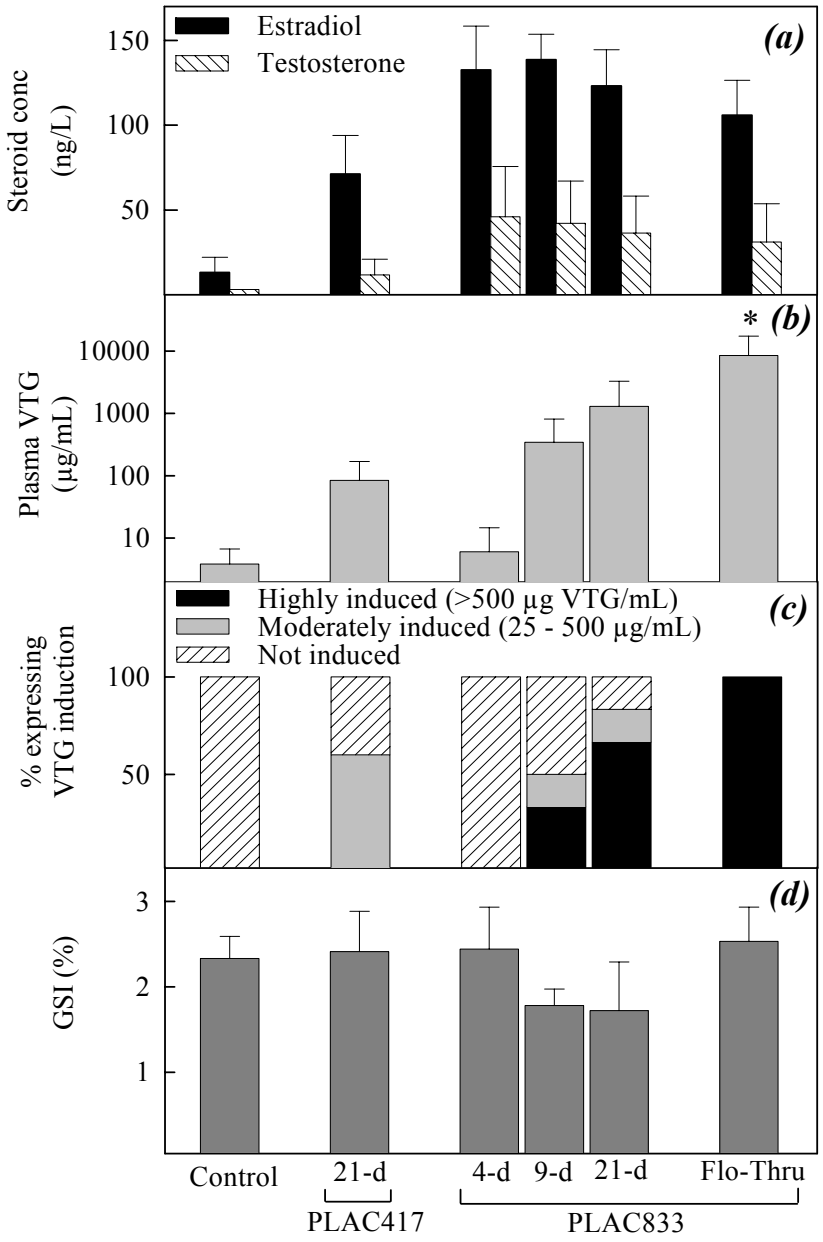


Figure 11. *Fathead Minnow Assay III* mean (±SD) exposure levels of sex steroid (E2 and T) to adult ♂ fish *(a)* and resulting treatment means (±SD) of various biological indicators: *(b)* plasma Vtg levels; *(c)* proportion (%) of fish expressing moderate to high levels of plasma Vtg; *(d)* gonadosomatic indices. Asterisks (*) indicate groups that differ significantly ($p < 0.5$) from controls.

Survival

No adult male fish mortalities occurred in any of the fathead minnow assays. Fish were examined grossly for signs of pathology prior to plasma collection and fixation. Fish appeared healthy and active at the conclusion of each exposure with most individuals exhibiting enlarged dorsal pads and pronounced nuptial tubercles (secondary sex characteristics providing evidence of reproductive preparedness). Exceptions include two *Control* specimens with mild exophthalmia (*Assay I* & *Assay III*) and a *PLAC-104* specimen with moderate caudal fin erosion *(Assay II)*.

Larval mortality was ~50% in *Assay I*, but did not differ between control and exposure treatments. Condition indices of surviving larvae also did not differ between treatments suggesting mortalities were likely the result of physical stress from daily siphoning and water replacement (screen impingement, etc.) rather than actual toxicity. Mortalities in *Assay II* were minimal with larval (3 dph at assay initiation) and juvenile (36 dph at assay initiation) survival of ≥87.5% and ≥97.5%, respectively. Again, condition indices did not differ between treatments. Because *Assay II* was performed flow-through, exposure tanks did not require daily siphoning and water replacement. Reduced mechanical stress may explain improved survival of larval fish.

Plasma Vtg Induction in Adult Male Fish

In *Assay I* Vtg induction occurred in all 10 adult male fish from *E2 Control* and *PLAC-417* and 9 of 10 fish from *PLAC-833 (Figure 8b)*. Mean plasma Vtg levels were 63,600 µg/ml, 40,700 µg/ml, and 19,300 µg/ml, respectively, and differed significantly from the *Control* level of 13 µg/ml (Kruskall-Wallis; $p < 0.05$). There was no evidence

of Vtg in any *Solvent Control* fish indicating that induction in *E2 Control* animals was not the result of the EtOH vehicle.

In *Assay II* no evidence of Vtg induction was seen prior to the 21 d sample interval in any exposure treatment *(Figure 9b)*. At the 21 d test conclusion moderate to high levels of Vtg were detected in 3 of 7 fish from *PLAC-208* and 8 of 8 fish from *PLAC-417*. Plasma Vtg in fish from these treatments differed significantly from the *Control* while levels in *PLAC-104* did not ($p < 0.01$). Note that the statistically significant increase in Vtg in *PLAC-208* was driven largely by a single highly induced individual *(Figure 9c)*.

Mean plasma Vtg concentrations for *Assay III* are presented in *Figure 11b*. No *Control* fish showed signs of Vtg induction. Three of 5 fish from *PLAC-417* had mildly elevated plasma Vtg levels after the 21 d exposure *(Figure 11c)*. In the *PLAC-833* treatments vitellogenesis was not apparent at 4 d, but was apparent in 3 of 6 fish at 9 d, and 5 of 6 fish at 21 d. In the *PLAC-833 Flow-Thru* treatment Vtg was highly induced in all fish and differed significantly from the control (Kruskall-Wallis; $p < 0.05$). Despite the lack of statistical significance (based on mean plasma Vtg levels) the occurrence of several "induced" male fish indicates that estrogenicity in *PLAC-417* were sufficiently high to elicit an effect after a 21 d exposure. Further, higher Vtg levels and a greater proportion of induced fish in *PLAC-833* after 9 d than in *PLAC-417* after 21 d suggests that PLACs exposure concentration may be more relevant than exposure duration.

Whole-Body Homogenate Vtg

Mean measures of larval whole-body homogenate Vtg from *Assay I* are

summarized in *Figure 8c*. Fish were of mixed gender with sex indeterminate at the time of homogenization. While approximately half were presumed to be genotypic females, egg maturation, and therefore Vtg expression, was expected to be minimal at the 28 d age of homogenization. Only fish from the *E2 Control* had appreciable Vtg (7.52 µg/mg P). Mean Vtg in *PLAC-417* was 1.05 µg/mg P, modestly (but significantly) above the *Control* level of 0.45 µg/mg P (Kruskall-Wallis; $p < 0.05$). The *PLAC-833* Vtg level of 0.37 µg/mg P was actually less than that of the *Control*. Juvenile fish from *Assay II* were 58 dph when sacrificed and preserved for analysis of whole-body homogenate Vtg *(Figure 10b)*. At this age precocious females, though still immature, might be expected to have begun early vitellogenesis such that homogenate Vtg levels would be low but discernible. All fish analyzed from the *E2 Control* had substantial amounts of Vtg with a mean level of 7.13 µg/mg P, comparable to that of the *E2 Control* value from *Assay I*. No fish from any of the litter treatments showed signs of Vtg induction. As expected, only the *E2 Control* differed significantly from the *Control* (Kruskall-Wallis; $p < 0.05$).

Gonadosomatic Index

Mean GSI values in adult male fish from *Assay I* ranged from 1.10 in the *E2 Control* to 1.74 in *PLAC-417 (Figure 8d)*. *Control*, *Solvent Control* and *PLAC-833* means were 1.42, 1.46 and 1.47, respectively. While no significant differences were detected between treatments, the reduction in GSI in the *E2 Control* may reflect a subtle effect of exogenous E2 on testis mass in adult male fish. Mean GSI values in adult males from *Assay II* varied from 1.46 (*PLAC-104* at 4 d) to 2.22 (*PLAC-208* at 21 d) *(Figure 9d)*. Despite this variability, significant differences were not detected within or between

treatments at any time interval. In *Assay III* mean GSI values ranged from 1.72 (*PLAC-833* at 21 d) to 2.53 (*PLAC-833 Flow-Thru*) and again did not differ significantly from the control *(Figure 11d)*.

Adult Male Gonadal Histopathology

Multiple sections of testis from each adult male fathead minnow were examined via light microscopy at low (40x) and high (400x) magnification for evidence of pathology. Sections from several specimens from *Assay I* had significant post-mortem artifact resulting either from poor fixation/preservation or inadequate histological processing. Sections were considered tolerable for analysis if representative regions of tissue were free of major artifact. In general, tissues from *Assay I* were healthy and without major pathology. Individual apoptotic (karyorrhexic) cells were encountered infrequently in specimens from all treatments. Most specimens appeared sufficiently mature to indicate reproductive competence (i.e., possessing mature spermatozoa within collecting tubules and/or ductus deferens).

Staging of testis from *Assay I* suggested a reduction in reproductive competence in *E2 Control* and possibly *PLAC-833* compared to the *Control*. Estimates of area within germinal epithelium occupied by mature spermatozoa and spermatids were ≥50% for the *Control*, *Solvent Control* and *PLAC-417* treatments, but only 41% and 32% for the *PLAC-833* and *E2 Control* treatments, respectively. Quantitative assessment of testis maturity via morphometric measurement (described previously) yielded spermatozoa/spermatid proportions of 43% - 46% for *Control*, *Solvent Control* and *PLAC-417* but only 35% and 27.5% for *PLAC-833* and *E2 Control*, respectively *(Figure*

8e). The initial qualitative assessment tended to over-estimate the area of spermatozoa/spermatid by ~10% compared to the quantitative (i.e., morphometric) measurement. Because this over-estimation was consistent across treatments, differences identified in the initial examination were confirmed in the latter. *Control* fish were found to possess a significantly greater proportion of mature gametes than fish from *PLAC-833* and *E2 Control* ($p < 0.05$) (see *Figure 4*).

Tissues from *Assays II* and *III* appeared healthy and without major pathology. Qualitative testis maturity staging in the latter two assays indicated no difference between treatments. As such, morphometric analyses were not undertaken. Single perinucleolar oocytes were found in individual specimens from each of the three assays; *Assay I - Solvent Control* at 21 d (~65 µm diam), *Assay II - PLAC-417* at 21 d (~135 µm diam), and *Assay III - PLAC-833* at 9 d (~200 µm diam) *(Figure 12)*. The latter oocyte occupied nearly the entire lumen of a seminiferous tubule. While notable anomalies, it is difficult to attach statistical (or biological) significance given the limited scope of occurrences.

Larval and Juvenile Histopathology & Sex Ratio

Tissues from larval and juvenile fish *(Assay II)* were examined for pathology and to ascertain phenotypic gender as described previously. Twenty-two to 26 specimens/treatment exposed as larvae (3-24 dph) were examined as were 20 specimens/treatment exposed as juveniles (36-57 dph). At 60 and 58 dph, respectively, males could not be definitively identified. Therefore, gender categories were limited to: ***(f)*** female based on the clear presence of oocytes; ***(o)*** putative female based on the

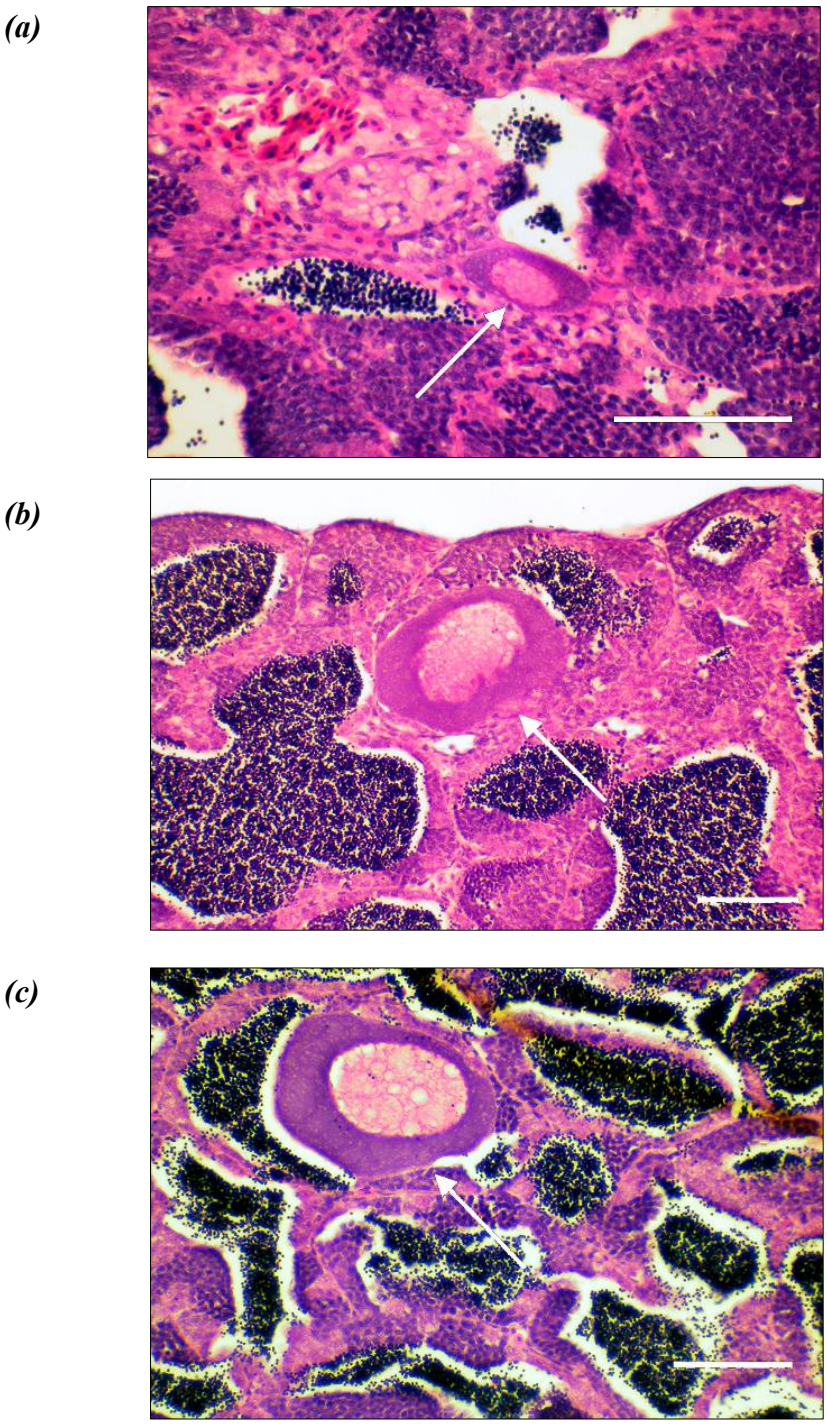


Figure 12. Ovarian follicles (cortical alveolar stage) within testes of mature male fathead minnow *Pimephales promelas* (arrows): ***(a)*** specimen from *Solvent Control* treatment of *Fathead Minnow Assay I*; ***(b)*** specimen from *PLAC417* treatment (21 d exposure) of *Fathead Minnow Assay II;* ***(c)*** specimen from *PLAC833* treatment (9 d exposure) of *Fathead Minnow Assay III;* (H&E; bars ≈ 100 µm).

appearance of an oviduct-like structure in the absence of oocytes; and ***(i)*** immature/indeterminate (*Figure 5*). Of fish exposed as larvae for 21 d (then grown-out to 60 d) a substantial bias in the number of females was apparent (*Figure 10c*). In *Control* and *Solvent Control* treatments 32% and 35% of specimens, respectively, could be identified as females based entirely on the presence of perinucleolar and/or cortical alveolar oocytes. Of the remaining *Control* and *Solvent Control* individuals, none had evidence of an oviduct in the absence of oocytes and none was sufficiently developed to be distinguished as male. Therefore, remaining individuals from these treatments were designated as indeterminate ***(i)***. The most pronounced effect was seen in the *E2 Control* where 19 of 24 individuals were obvious females ***(f)*** and 4 were presumptive females ***(o)*** bringing to 96% the total number of individuals with female characteristics. In litter treatments the number of females (and/or feminized individuals) increased in dose-dependant fashion; 55% in *PLAC-104*, 72% in *PLAC-208*, and 92% in *PLAC-417*. The occurrence of a putative oviduct (often well developed) in the absence of gametogenic oocytes was most conspicuous in *PLAC-417* where 10 of 26 fish demonstrated this characteristic compared to one of 22 in *PLAC-208* and none in *PLAC-104*.

Assessments of gender in fish exposed as juveniles differed considerably from those of the larval exposures *(Figure 10d)*. Again, no males could be definitively identified. Many more females were apparent in the *Control* (65%) then in the larval counterpart. Further, the *E2 Control* was unusual in that only 35% of specimens were females with developing oocytes ***(f)*** while another 50% were presumptive females based on the presence of an oviduct ***(o)***. Litter exposures produced results comparable to the *Control* with gender proportions of 58% female in *PLAC-104*, 74% female in *PLAC-208*,

and 60% female in *PLAC-417*. No juvenile fish from litter treatments had oviducts in the absence of developing oocytes.

SHEEPSHEAD MINNOW AND MUMMICHOG LABORATORY ASSAYS

Water quality

With the exception of NH_3, water quality parameters for all treatments fell within acceptable ranges throughout 21 d exposure intervals. Because substantial amounts of litter were used in creation of exposure treatments in the second assay, unionized NH_3 levels were above suggested exposure criteria for all PLAC treatments. Mean unionized NH_3 levels for *PLAC-417, PLAC-833* and *PLAC-1667* treatments were 0.20 mg/L, 0.37 mg/L and 0.64 mg/L, respectively. While significantly above the 0.035 mg/L EPA *Criteria Continuous Concentration* for estuarine assays [USEPA, 1989], toxicity tests by Poucher [1986] found unionized NH_3 LC_{50} levels for sheepshead minnows in the order of 2.1 - 3.5 mg/L. As fish behavior did not suggest adverse effect and no mortalities were recorded in high litter treatments (except as noted below) we felt it permissible to continue the assays in spite of high NH_3 levels.

Steroid Chemistry

In poultry litter exposure treatments (*PLAC-417, PLAC-833* and *PLAC-1667*) E2 levels were 47 ng/L, 93 ng/L and 148 ng/L, respectively, for sheepshead, and 50 ng/L, 83 ng/L and 143 ng/L, respectively, for mummichog *(Table 7)*. T was consistently less then E2 with levels of 25 ng/L, 38 ng/L and 45 ng/L, respectively, for sheepshead, and 30

Table 7. Summarized results of steroid exposure concentrations and endocrine endpoints for adult male *Fundulus heteroclitus* and *Cyprinodon variegatus* assays. Data expressed as treatment mean ± standard deviation (SD).

Treatment	n	17 β-Estradiol (ng/L)	Testosterone (ng/L)	Plasma Vtg (µg/mL)	GSI (%)	Testis maturity rank	Reproductive competence (%)
Assay I							
Fundulus heteroclitus							
Control	5[b]	---[a]	---[a]	---[a]	2.46 ± 0.24[b]	3.7 ± 0.27[b]	100
Solvent Control	10	---	---	---	1.43 ± 0.26	2.9 ± 0.85	70
E2 Control	10	101.5 ± 14.0	---	8,950 ± 7,040**	1.15 ± 0.34	1.5 ± 1.06	20
Cyprinodon variegatus			---				
Control	10	---	---	---	0.66 ± 0.11	3.1 ± 0.52	80
Solvent Control	10	---	---	---	0.41 ± 0.07	3.3 ± 0.46	100
E2 Control	10	104 ± 15.4		36,832 ± 16,218**	0.44 ± 0.09	2.9 ± 0.39	89
Assay II							
Fundulus heteroclitus							
Control	6	---	6.5 ± 5.8	---	2.13 ± 0.84	4.7 ± 0.19	100
PLAC-417	6	50.1 ± 19.2	30.4 ± 7.8	---	2.19 ± 0.65	4.6 ± 0.13	100
PLAC-833	6	82.7 ± 17.7	41.2 ± 10.7	---	2.10 ± 0.46	4.3 ± 0.22	100
PLAC-1667	6	143.7 ± 26.8	42.5 ± 17.6	213.3 ± 224.6*	2.12 ± 0.36	4.4 ± 0.21	100
Cyprinodon variegatus							
Control	6	---	---	---	0.69 ± 0.11	4.0 ± 0.16	100
PLAC-417	6	46.9 ± 12.4	25.4 ± 12.7	---	1.28 ± 0.58*	3.9 ± 0.13	100
PLAC-833	6	93.2 ± 16.3	37.9 ± 15.6	---	1.05 ± 0.40	3.9 ± 0.31	100
PLAC-1667	5	147.9 ± 26.8	44.9 ± 27.2	---	0.75 ± 0.19	4.0 ± 0.21	100

[a] Below method detection limits: 17 β-estradiol 18 ng/L; testosterone 6 ng/L; vitellogenin 6 µg/mL.
[b] 50% *Control* mortality limits usefulness of results.
* Treatment differs significantly from the control ($p < 0.05$).
** Treatment differs significantly from the control ($p < 0.01$).

ng/L, 41 ng/L and 43 ng/L, respectively, for mummichog. Levels of E2 were below detection in the *Control* and *Solvent Control* treatments and ~100 ng/L in the *E2 Control* treatment. Levels of T were below detection in *Control* and *Solvent Control* and *E2 Control* treatments.

Survival

In *Assay I* there were no adult sheepshead minnow mortalities. All fish were active and appeared healthy at test termination and condition indices were high and consistent across treatments. Survival of larval sheepshead minnow was as follows: *Control* = 85%; *Solvent Control* = 95%; *E2 Control* = 85%. Larval condition indices did not differ across treatments. Numerous *Control* mortalities occurred in the mummichog portion of the assay. Four of 10 fish showed marked wasting and were removed once dead or moribund. Parasites were not apparent under gross or microscopic examinations (e.g., skin scrapes, gill and intestinal squashes). Putative cause of mortality was a viral and/or bacterial pathogen. A fifth *Control* fish with severely distended abdomen, notable inflammation and edema at assay conclusion was excluded from all endpoint calculations. The 5 remaining *Control* fish and mummichog from all other treatments were active and without apparent pathology at the time of sacrifice. Condition indices did not differ between treatments but substantial *Control* mortalities bring into question the utility of this endpoint. A single mortality occurred in the second assay, a sheepshead minnow from *PLAC-1667* that escaped its tank and was found dried on the floor the following morning. All other fish were active and without apparent pathology at the time of sacrifice.

Vitellogenin

In *Assay I* mean plasma Vtg concentrations in *Control* and *Solvent Control* treatments of adult sheepshead minnow and mummichog were below detection with no evidence of induction in any fish *(Table 7)*. Conversely, Vtg levels in *E2 Control* treatments were 36,832 µg/mL for sheepshead minnow, and 8,950 µg/mL for mummichog, with 100% and 75%, respectively, of exposed fish expressing substantial Vtg induction.

Unlike adult males, larval sheepshead minnow were largely unresponsive to E2 exposure. A modest elevation in whole-body homogenate Vtg occurred in the *E2 Control* (20 µg/mL) compared to the *Control* (< 6 µg/mL MDL; samples not adjusted to total protein), but fell well short of the 4 order of magnitude increase that occurred in "induced" adult male fish. This lack of response may suggest that vitellogenesis in this species at this early age is a poorly suited indicator of exogenous estrogenic exposure. Because of this lack of sensitivity to the *E2 Control* larval exposures were eliminated from the second assay.

In *Assay II* moderate Vtg induction occurred in only 3 of 6 mummichog from the highest exposure treatment (*Table 7*) yielding a treatment mean of 213 µg/mL. Plasma Vtg levels in all other mummichog and all sheepshead minnow treatments were below detection. While Vtg in the *PLAC-1667* treatment was found to differ significantly from the *Control* (Kruskall-Wallis; $p < 0.05$), the magnitude of induction was far less then that seen in mummichog from the *E2 Control* treatment of the previous assay, this despite an E2 level of 143 ng/L in *PLAC-1667* vs. 101 ng/L in the *E2 Control*.

Gonadosomatic Index

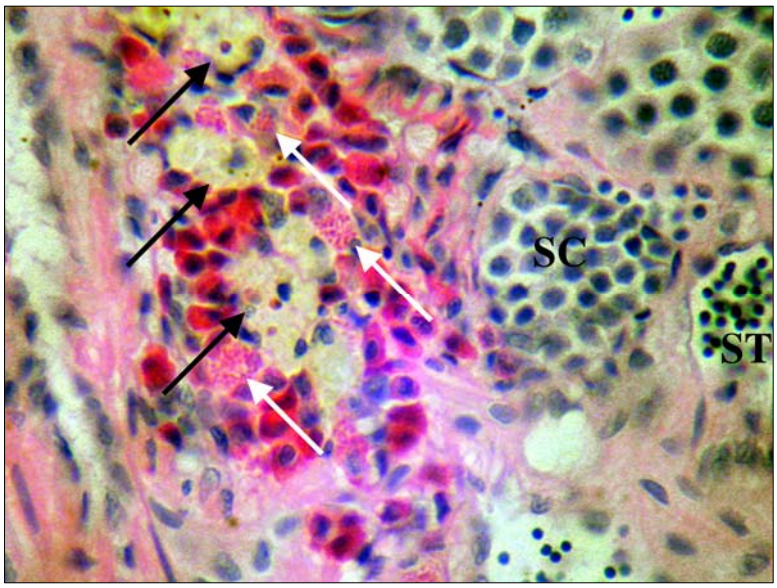
In *Assay I* mummichog GSI results were variable within and across treatments (*Table 9*). GSI was significantly higher in the *Control* then other treatments ($p < 0.05$), however, this result should be interpreted with caution as high *Control* mortalities excluded 5 of 10 fish from statistical calculations. The power of the ANOVA, **$** =0.26, was also well below the desired level of **$** = 0.80. Sheepshead GSI data were also variable, but did not differ between treatments (Kruskall-Wallis; $p = 0.156$).

Mummichog GSI results for *Assay II* were very consistent with no differences between treatments. Results were somewhat more variable for sheepshead minnow where the *PLAC-417* GSI of 1.28 was significantly higher than the *Control* value of 0.69 (Kruskall-Wallis; $p < 0.05$).

Gonadal Histopathology

Sections of sheepshead and mummichog testis were examined for evidence of pathology and to assess maturity levels and concomitant reproductive competence. Minimal to mild infiltration of eosinophilic granulocytes occurred in testis sections from both species *(Figure 13a)*. Approximately half of all individuals were affected with frequency and severity of pathology distributed evenly among groups and not indicative of a treatment effect. Macrophage aggregates were also noted infrequently in testes of several sheepshead *(Figure 13a)*. Again, no treatment effect was indicated. Focal apoptotic germ cell syncytia were encountered infrequently in tissues from both species regardless of treatment *(Figure 13b)*. These pathologies are consistent with field-collected organisms routinely exposed to parasites, pathogens and other natural stressors.

(a)



(b)

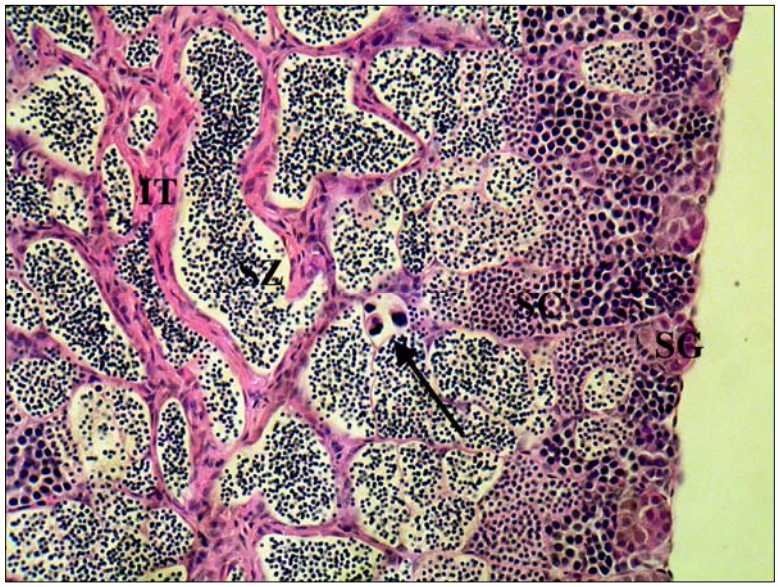


Figure 13. Testes of sheepshead minnow *Cyprinodon variegatus* with ***(a)*** interstitial tissue inflammation consisting of macrophage aggregates (black arrows) and eosinophilic granulocytes (white arrows) (H&E 1000x); and ***(b)*** necrotic regions containing germ cell syncytia (black arrow) (H&E 400x); (IT = interstitial cells; SC = spermatocytes; SG = spermatogonia; ST = spermatid; SZ = spermatozoa).

Testis Maturity Ranking

Mean testis maturity ranks for the various treatments are provided in *Table 7.* Typically, fish (sheepshead and mummichog) with testis ranks greater than 2.5 were judged to be reproductively competent (i.e., spermatozoa were clustered within collecting tubules and individuals appeared capable of spawning) *(Figure 14a & c)*. Fish with ranks below 2.5 were usually judged to not be reproductively competent (i.e., not prepared to spawn at the time of sacrifice) *(Figure 14b & d)*.

Mummichog testis maturity ranks were markedly reduced in the *E2 Control* (1.5) compared to *Control* (3.7) and *Solvent Control* (2.9) ranks. Only 20% of *E2 Control* fish appeared capable of spawning at the time of sacrifice vs. 100% of *Control* fish and 70% of *Solvent Control* fish. The subjective nature of testis maturity ranking and reproductive competence assessment preclude statistical analysis of these endpoints (also note that *Control* mortality of 50% may have left only the most robust fish, skewing the maturity rank for this treatment). Unlike mummichog, sheepshead from the *E2 Control* showed no reduction in testis maturity or reproductive competence. Ranks ranged from 2.9 *(E2 Control)* to 3.3 *(Solvent Control)* with 80% to 100% of fish judged reproductively competent. Despite plasma Vtg levels 4x higher than that of the mummichog (36,832 µg/mL vs. 8,950 µg/mL), sheepshead reproductive competence appeared less affected by exogenous E2 exposure than did mummichog reproductive competence. *Assay II* testis maturity ranks for *Control* and PLAC treatments were very high for both species; mummichog ranks ranging from 4.3 to 4.7 and sheepshead ranks ranging from 3.9 to 4.0. All individuals were deemed to be reproductively competent.

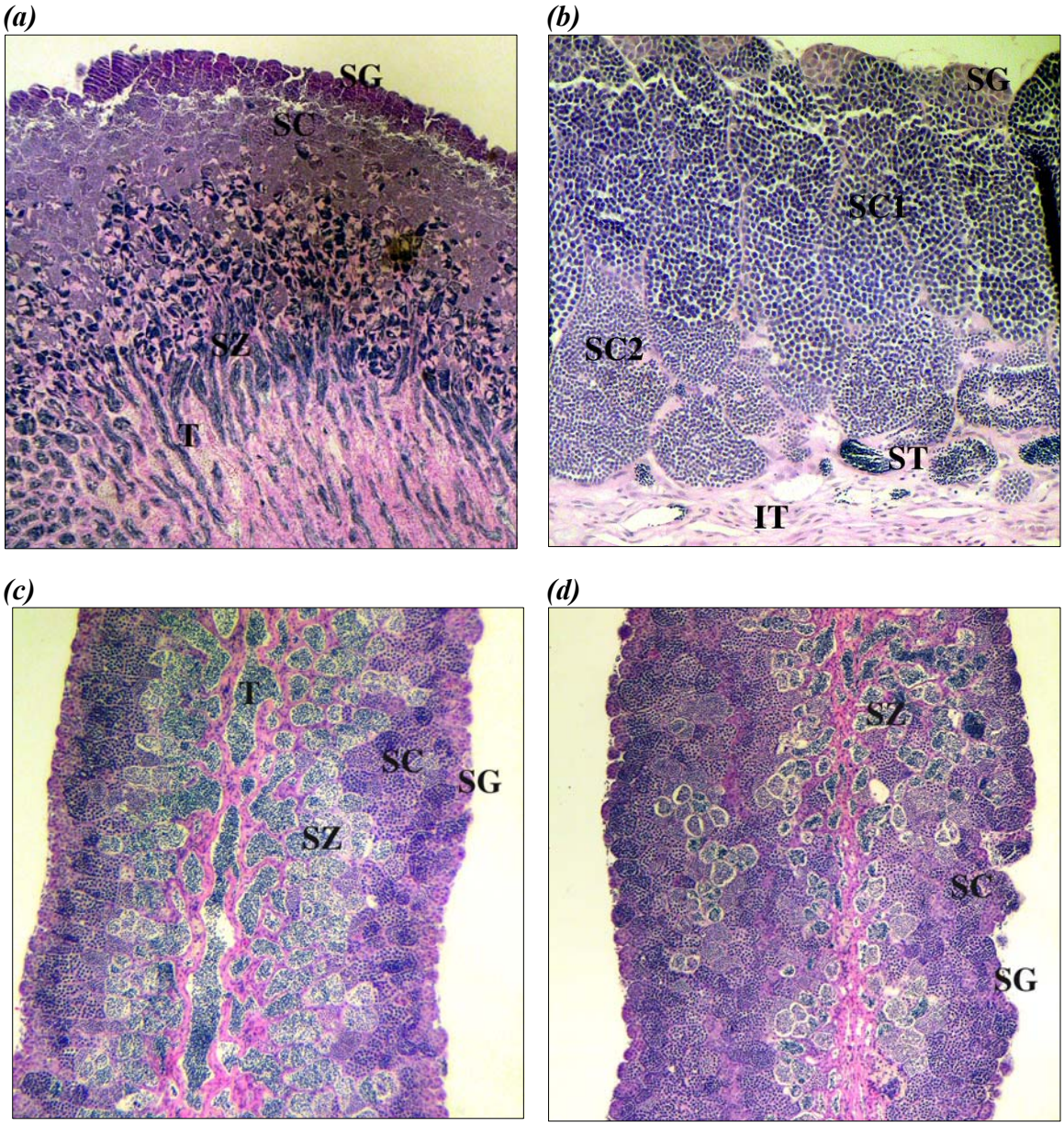


Figure 14. Testis maturity ranking and assessment of reproductive competence in sheepshead minnow *Cyprinodon variegatus* and mummichog *Fundulus heteroclitus* (IT = interstitial cells; SC = spermatocytes; SC1 = primary spermatocytes; SC2 = secondary spermatocytes; SG = spermatogonia; ST = spermatids; SZ = spermatozoa; T = seminiferous collecting tubules): ***(a)*** *Fundulus heteroclitus* with testis maturity rank of 4.0; reproductively competent (H&E 40x); ***(b)*** *Fundulus heteroclitus* with testis maturity rank of 1.5; not reproductively competent (H&E 200x); ***(c)*** *Cyprinodon variegatus* with testis maturity rank of 4.0; reproductively competent (H&E 40x); ***(d)*** *Cyprinodon variegatus* with testis maturity rank of 2.5; reproductively competent (H&E 40x).

CONTROLLED FIELD INVESTIGATIONS

Spring 2000

The first objective of the *Controlled Field Investigation* involved measuring the transport of poultry litter-derived steroids via runoff into receiving waters. The first rain event that produced runoff occurred on 5/22/00 (12 d after litter application) and dropped a total of 5.89 cm of precipitation. Of that total 29% (1.71 cm) came off the *No-Till* field as surface runoff with the remainder staying on the field and infiltrating the soil. In contrast, only 8.4% (0.49 cm) of total precipitation came off the *Conventional-Till* field as surface runoff. *Figure 15* provides measured E2 concentrations from flume discharge samples collected during this and subsequent rain events throughout the 2000 growing season. During the 5/22/00 event levels were considerably higher in *No-Till* runoff (93 – 191 ng/L) than in *Conventional-Till* runoff (42 ng/L). Levels diminished in subsequent runoff events falling as low as 24 ng/L before rebounding to some extent in late summer/early fall.

Surface water samples collected from the *Research Pond* during the 5/22/00 event showed a precipitous increase in E2 from below detection at first runoff to a peak of 82 ng/L after 12 h and finally to 69 ng/L at runoff conclusion *(Figure 16)*. From here the E2 level decreased steadily over the next several months, dropping below the detection limit in early August before rebounding slightly in September. During the 21 d fish exposure interval *Research Pond* E2 levels averaged 50.2 ng/L (range 33 - 63 ng/L excluding the short-lived peak during the actual runoff event. *Reference Pond* E2 levels, measured when fish were loaded (Day 0) and at exposure conclusion (Day 21), were 21 ng/L and 23 ng/L, respectively.

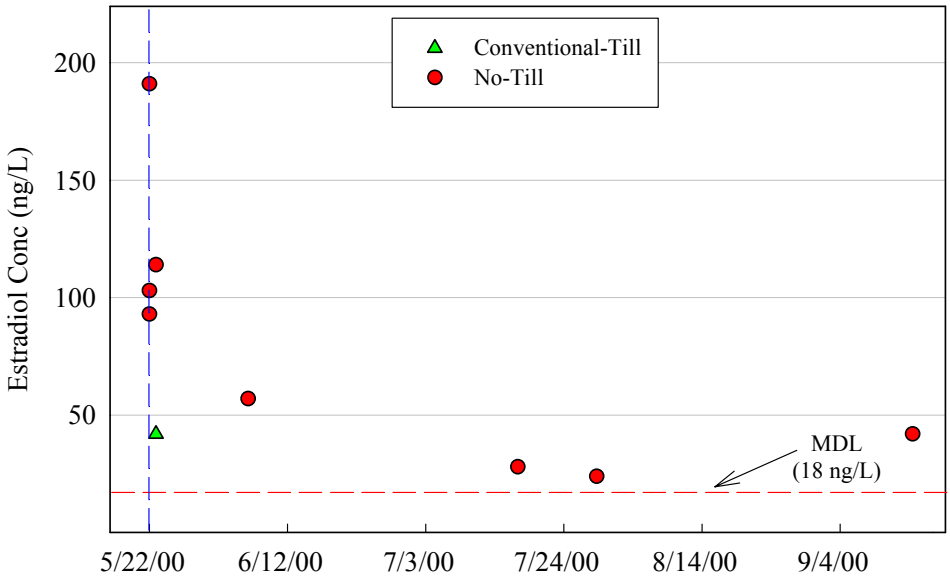


Figure 15. 17 β-estradiol levels in runoff from *No-Till* and *Conventional-till* fields collected throughout the 2000 growing season (blue line indicates the first runoff following litter application; red line indicates method detection limit).

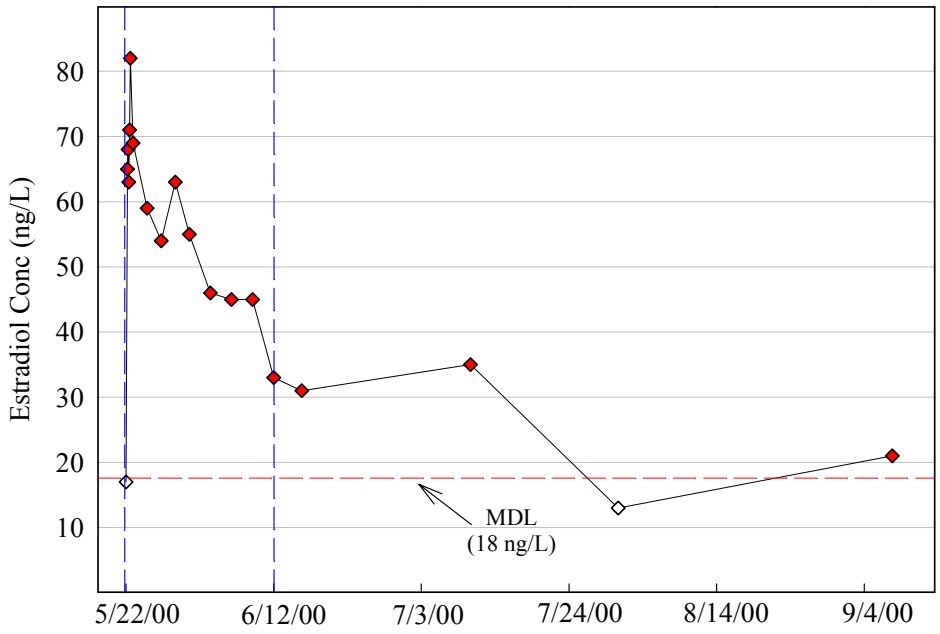


Figure 16. 17 β-estradiol levels in a University of Maryland research pond receiving *No-Till* field runoff following spring 2000 poultry litter application. The 21 d caged fish exposure interval is indicated by the region bounded by the blue lines.

Survival of adult male fish caged in *Research* and *Reference Ponds* was adequate with 2 of 10 fish lost at each location. Water quality parameters within the ponds were altered considerably during the 5/22/00 rain event and over the subsequent 21 d fish exposure interval *(Table 8)*. Water temperature, which had been as high as 25EC prior to the event, dropped to ~17EC as runoff entered the *Research Pond*. Air temperature and sunlight caused daily temperature fluctuations within both shallow ponds with a maximum temperature of 33EC occurring in the *Research Pond* during the fish exposure interval. Dissolved oxygen in the *Research Pond* was severely affected by runoff, dropping from ~9 mg/L to <1.5 mg/L within 2 d of the rain event. Low DO coincided with fish mortalities and was the most likely cause. Aerators were installed at caged fish arrays in both ponds providing gentle bubbling within floating baskets and maintaining adequate DO levels (>3.0 mg/L). *No-Till* runoff also depressed pH within the *Research Pond* to a low of 5.73 which only slowly returned to the pre-event level of ~8.00. Surface water total ammonia levels increased rapidly following runoff introduction to 2.9 mg/L then fluctuated between 0.8 mg/L and 2.1 mg/L for the remainder of the exposure period. Ammonia in the *Reference Pond* never exceeded 0.4 mg/L.

Table 8. Summary of water quality measurements for *Research* and *Reference Ponds* during the *Spring 2000* 21 d controlled fish field exposure.

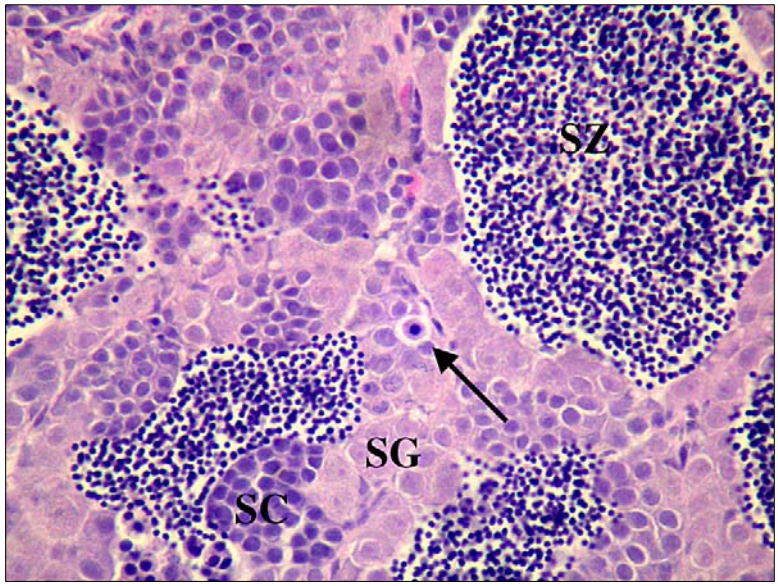
Location		Temperature (EC)	pH	DO (mg/L)	Conductivity (µmhos)	Ammonia (mg/L)
Research Pond	Mean	23	6.7	6.3	230	1.3
	Min	16	5.7	1.4	180	0.1
	Max	34	9.3	15.2	300	2.9
Reference Pond	Mean	22.6	6.4	7.5	127	<0.1
	Min	17	5.9	2.5	105	<0.1
	Max	31	7.8	12.8	190	0.4

After the 21 d exposure period caged fish were held for 3 d in the laboratory before being euthanized and sacrificed for assessment of ED endpoints *(Table 9)*. Plasma Vtg levels from caged fish as well as control animals held in the laboratory were near or below the MDL of 3.0 µg/mL and gave no indication of Vtg induction in any fish. Mean GSI measurements were 1.54 in *Research Pond* animals, 1.30 in *Reference Pond* animals, and 1.32 in laboratory control animals and did not differ significantly between groups. Testis maturity estimates (% spermatids & spermatozoa) were 47% for laboratory *Control* fish, considerably reduced at 25% for *Reference Pond* fish and somewhat increased at 59% for *Research Pond* fish. Pathology within testes of control and pond exposed fish was limited to the occurrence of necrotic/apoptotic spermatogenic cells, usually seen individually *(Figure 17a),* but occasionally appearing in small clusters *(Figure 17b)*.

Table 9. Summary of results of *Spring 2000 Controlled Field Exposure* of mature male fathead minnow *(Pimephales promelas)* to poultry litter-amended agricultural field runoff.

Treatment	n	17 β-Estradiol (ng/L)	Plasma Vtg (µg/mL)	GSI (%)	Testis maturity
Control	8	n/a	3.4 ± 1.58	1.32	0.47
Reference Pond	8	22.0	3.0 ± 2.89	1.30	0.25*
Research Pond	10	37.6	7.3 ± 4.52	1.54	0.59

(a)



(b)

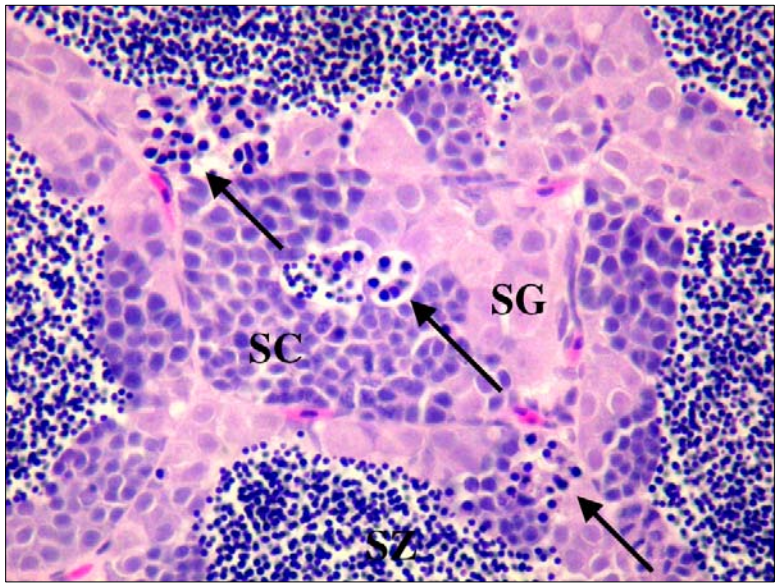


Figure 17. Testes of fathead minnow *Pimephales promelas* with apoptotic cells (arrows) occurring ***(a)*** individually and ***(b)*** in small clusters within luminal walls of seminiferous tubules (SC = spermatocytes; SG = spermatogonia; SZ = spermatozoa; H&E 1000x).

Spring 2002

Water-soluble E2 in *WYE2002* poultry litter was measured at 86 ng/g at the time of field application, 20% less then the 108 ng/g E2 level of *WYE2000* litter. Also, the first rain event following litter application in 2002 (5/18/02) differed significantly from that of 2000. The 2002 event dropped 3.05 cm of precipitation of which only 0.09 cm ran off of the *No-Till* field. This resulted in a concentrating of PLACs within runoff such that samples collected from the *No-Till* discharge flume had an average E2 level of 275 ng/L (range 207 to 350 ng/L) *(Figure 18)*. The 5/18/02 rain event lacked sufficient precipitation to produce runoff from the *Conventional-Till* field. However, the next rain event, starting 17 d later (6/5-7/02), dropped 6.30 cm of rain and produced abundant runoff from both fields (*No-Till* = 0.89 cm, *Conventional-Till* = 0.93 cm) with resulting E2 levels of 38.5 ng/L (range 26 to 65 ng/L) in *No-Till* discharge and 37 ng/L in *Conventional-Till* discharge.

E2 levels in surface water samples collected from the *Research Pond* during and immediately after the 5/18/02 runoff event averaged 39.2 ng/L (range 22 to 70 ng/L) with levels during the subsequent fish exposure interval (17 d) averaging 30 ng/L (range <MDL - 41 ng/L) *(Figure 19)*. High E2 levels in *No-Till* field runoff (275 ng/L) produced only modest increases in surface water E2 in the *Research Pond* because the actual volume of runoff was relatively meager (<0.10 cm) providing for considerable dilution. On 6/4/02, 16 d after fish were caged in the *Reference* and *Research Ponds,* a county road crew resurfaced the road bordering the research fields and ponds by applying tar and chipped stone. Nearly 1 mile of this road drains via ditches on either side into the *Research Pond*. In anticipation of a major thunderstorm, and in order to prevent caged

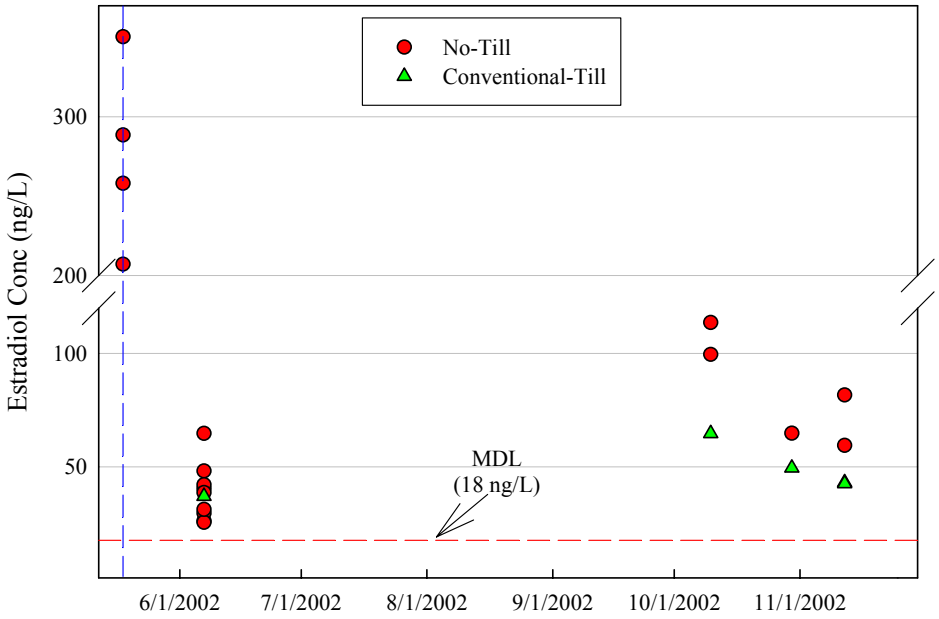


Figure 18 17 β-estradiol levels in runoff from *No-Till* and *Conventional-till* fields collected throughout the 2002 growing season (blue line indicates the first runoff following litter application; red line indicates method detection limit).

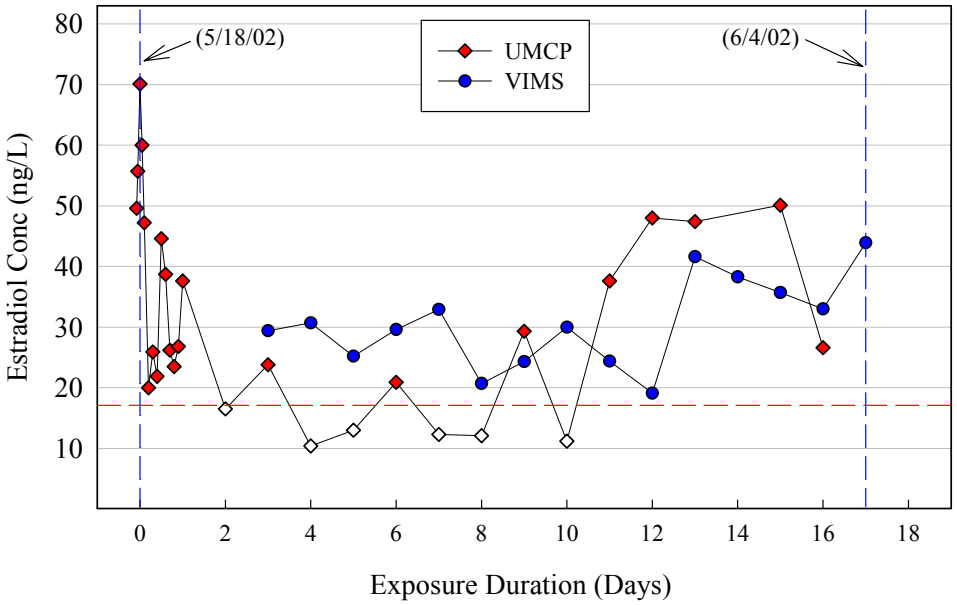


Figure 19. 17 β-estradiol levels in a University of Maryland research pond receiving *No-Till* field runoff following spring 2002 poultry litter application (analyses performed initial by Sara Pollock, University of Maryland, corroborated by Barbara Rutan at Virginia Institute of Marine Sciences). Fish were caged within the pond during the indicated (blue lines) 17 d interval. Values below the MDL (hollow symbols) are presented for illustrative purposes only.

fish from being "oiled" the field exposure was discontinued and animals were returned to the laboratory after only 17 d. Laboratory treatments (*Control*, *Research Pond/Lab*, and *Flume/Lab*) were also discontinued after only 17 d.

After encountering hypoxic conditions in the *Research Pond* in 2000 (consequent to high runoff nutrient loads) aerators were installed on fish cages to avoid low oxygen related stress. Water quality parameters for laboratory and field fish exposures are provided in *Table 10*. Because the 5/18/02 runoff event was less severe then the 5/22/00 event, water quality was not as severely impacted. As before, temperature in the ponds fluctuated daily reflecting changes in air temperature and sunlight intensity. Temperature of laboratory treatments was held constant at 24 ± 1EC. Ammonia remained below 1.0 mg/L in both ponds. However, frozen flume water from the 5/18/02 event, used daily to renew the *Flume/Lab* treatment, had a mean total ammonia level of 3.4 mg/L (range 2.4 – 5.4 mg/L).

Numerous mortalities occurred during the 17 d exposure interval. In the *Research Pond* a single fish died on day 8 and another on day 14 (possibly due to high afternoon water temperatures). Four fish were lost in the *Reference Pond* on day 8 and another just prior to exposure conclusion leaving only 5 of the original 10. After only 1 d a fish with severe ventral inflammation, hemorrhage and edema was removed from the *Research Pond/Lab* treatment. The *Flume/Lab* exposure proved very problematic. On the morning of day 2 all fish were dead and too necrotic to preserve for histology. The treatment was restarted with additional fish from the batch used in the other treatments. On day 6 these fish also died. Hypoxia was not responsible, as aeration maintained DO ≥8.0 mg/L. Total ammonia averaged 3.1 mg/L with a maximum of 5.4 mg/L. However, at 24EC

Table 10. Summary of water quality measurements for *Research* and *Reference Ponds* and for laboratory aquaria during the *Spring 2002* 17 d controlled fish field/lab exposure.

Location		Temperature (EC)	pH	DO (mg/L)	Conductivity (µmhos)	Ammonia (mg/L)
Research Pond	Mean	24.0	7.2	8.3	169	0.3
	Min	18	6.4	5.7	150	<0.1
	Max	30	8.4	10.6	180	0.9
Reference Pond	Mean	24.5	7.3	7.1	218	0.1
	Min	20	6.6	3.4	205	<0.1
	Max	29	8.3	11.4	245	0.4
Control 1	Mean	23.1	7.8	8.2	282	0.3
	Min	23	7.5	7.7	270	<0.1
	Max	24	8.0	8.5	365	0.9
Research Pond/Lab	Mean	23.2	7.2	8.4	188	0.5
	Min	23	6.5	7.6	160	0.1
	Max	25	8.2	10.5	380	2.5
Control 2	Mean	23.5	7.8	8.1	280	0.3
	Min	23	7.3	6.9	270	<0.1
	Max	25	8.1	8.5	295	1.0
Flume/Lab	Mean	23.5	7.2	7.8	640	3.1
	Min	23	6.6	6.7	600	2.4
	Max	25	7.6	9.5	700	5.4

and pH 7.11 unionized NH_3 would be 0.037 mg/L, only slightly above the EPA water quality criterion for chronic ammonia exposure [USEPA, 1999] and similar to levels in previous laboratory studies in which fathead minnow survived 21 d exposures without mortality or apparent pathology. The treatment was restarted a second time. Replacement fish were selected randomly from excess in-house breeders ranging in age from 8 to 12 months. As these animals were distinct from those in other exposure treatments, an additional control treatment *(Control 2)* was also started. These treatments were run 17 d to match the others. Treatment effects were investigated by comparing pond water exposed fish (field and lab) to *Control 1* and the flume water exposed fish to *Control 2*.

Animals retrieved from pond exposures were held in the laboratory under control conditions for 24 h before being euthanized and sacrificed for assessment of ED endpoints *(Table 11)*. No evidence of vitellogenesis was found in control or field caged fish or those exposed to *Research Pond* water in the laboratory. Significantly, male fish exposed to preserved *No-Till* field runoff *(Flume/Lab)* were highly vitellogenic. All were strongly induced to an average plasma Vtg level of 3,146 µg/mL, a clear demonstration of estrogenicity in runoff from a litter-amended field. Mean GSI measurements for all pond water exposures were significantly higher than *Control 1* values ($p < 0.05$). However, testis maturity indices (% spermatids & spermatozoa) for these treatments did not differ. Because *Control 2* and *Flume/Lab* fish were selected from active breeders, GSI and testis maturity indices were very high in all specimens. As in the *Spring 2000* investigation apoptotic spermatogenic cells occurred infrequently within most specimens with no clear treatment related differences.

Table 11. Summary of results of *Spring 2002 Controlled Field Exposure* of mature male fathead minnow *(Pimephales promelas)* to poultry litter-amended agricultural field runoff.

Treatment	n	17 β-Estradiol (ng/L)	Plasma Vtg (µg/mL)	GSI (%)	Testis maturity
Control 1	7	n/a	6.3 ± 4.40	1.27	0.53
Research Pond/Lab	7	30.4 ± 18.95	2.6 ± 1.94	1.89*	0.57
Reference Pond/Field	5	23.9 ± 21.1	3.9 ± 2.01	1.79*	0.43
Research Pond/Field	8	30.4 ± 18.95	6.5 ± 11.08	1.96*	0.47
Control 2	10	n/a	3.2 ± 2.14	1.65	0.65
Flume/Lab	9	146.7	3,146 ± 2,585*	1.95	0.69

CHAPTER V: DISCUSSION

POULTRY LITTER-ASSOCIATED CONTAMINANTS

Contaminants in Poultry Litter

Chemical analysis of poultry litter *(Table 5)* found a variety of contaminants with the potential to cause environmental harm, some at trivial concentrations, but others at levels of concern. Included in this list are persistent organic pollutants (POPs) like DDT and several of its metabolites, which, although banned in the US since 1972, continue to appear in the environment, albeit, usually at miniscule levels. Similarly, regulation has sharply curtailed lindane use, but because it is still approved for corn seed pretreatment (and 18 other US crops) it is still found in environmental samples with great regularity.

Because most POPs are strongly hydrophobic (i.e., high octanol-water partition coefficients), they adsorb readily to available solid (sediments) and/or lipid (biota) phases. Transport to surface waters can occur when contaminated particulates are suspended in field runoff and deposited into receiving waters. Sedimentation of contaminant-bound particulates introduces them to benthic systems where they are able to accumulate in biota with potential toxicological consequences. As described in Chapter I *(Table 1),* numerous POPs possess varying degrees of reproductive toxicity (e.g., estrogenicity and/or androgenicity) in addition to teratogenicity, mutagenicity, and carcinogenicity. While levels of these contaminants in poultry litter are very low, their environmental persistence and potential for bioaccumulation/magnification through food webs makes them an environmental concern.

Water-soluble contaminants found in poultry litter include antibiotics like chlortetracycline, triazine pesticides like atrazine, and sex steroids like E2 and T. These compounds are highly mobile and transport readily from fields to receiving waters. Resulting contaminant concentrations are heavily influenced by application rate, soil type, agronomic practice, and precipitation [Staver *et al.,* 1996]. Runoff during intense rain events can introduce high contaminant concentrations in a slug. Lower background concentrations can result from slow but persistent groundwater seepage [Shore *et al.,* 1995].

Antibiotics, such as chlortetracycline found in our litter sample *(WYE2000),* are widely used in the poultry industry. At sub-therapeutic levels they improve growth-rate and efficiency of feed utilization, reduce mortality and improve reproductive performance. Higher doses are used for prophylactic disease prevention and disease treatment where necessary [Cromwell, 1999]. Birds only metabolize a fraction of administered antibiotics. The majority passes through the digestive system intact, ending up in manure in the active form. Agricultural application of poultry litter introduces these antibiotics to the environment. In a nationwide reconnaissance of pharmaceuticals in US water resources, Kolpin *et al.* [2002] detected tetracycline compounds in 2.4% of the surveyed water bodies. Detection of tetracyclines occurred in samples downstream of urbanized and rural areas suggesting municipal waste water discharge as well as agricultural runoff sources. In a recent news release, researchers from Colorado State University reported identifying a variety of antibiotics in waterways influenced by urban and rural inputs [Carlson, 2004]. Tetracyclines were found at all impacted sites, while

ionophores (e.g., monensin), which are used exclusively in animal applications, occurred only in agriculturally influenced areas.

The biological significance of antibiotics in aquatic systems is unknown. The primary concerns of broad dispersal of antibiotic residues to the environment include widespread development of antimicrobial resistance and potential alterations in bacterial assemblages important to the healthy functioning of aquatic ecosystems [Kolpin et al., 2002; USEPA, 2004]. If measured chlortetracycline levels of approximately 1 μg/g from our poultry litter sample *(WYE2000)* are representative of average levels in the 1.6 billion lbs (7.3×10^8 kg) of litter applied annually on the eastern shore, than nearly 730 kg of chlortetracycline is land applied within Delmarva watersheds.

Atrazine is used abundantly as a grassy and broadleaf herbicide. Once on agricultural fields, it can persist in dry soils for many months (half-life 60 to >100 d). Atrazine has a high potential for surface water contamination via runoff and groundwater seepage because it does not adsorb strongly to soil particles [Howard, 1989]. Hydrolysis is slow at neutral pH and photolysis, evaporation and volatilization do not reduce its presence in water. Atrazine detected in our *WYE2000* litter sample ranged from 0.34 - 1.3 ng/g. At an application rate of 3 ton/ac, this would introduce approximately 1.0 - 3.5 g of atrazine/acre. Normal field application rates of atrazine for corn production are often as high as 2.5 lbs/ac (1.1 kg/ac). This would seem to indicate that poultry litter-associated atrazine is an insignificant source of introduction to aquatic resources [Solomon *et al.,* 1996].

Nutrient contaminants in poultry litter are beyond the scope of this discussion. However, ammonia, which is produced as a by-product of biodegradation and/or

microbial decomposition of organic nitrogen compounds, requires special mention. Ammonia is very highly water-soluble. Once in solution un-ionized ammonia (NH_3) exists in equilibrium with ammonium ion (NH_4^+) with relative proportions influenced by pH, temperature, hardness and salinity. Toxicity to aquatic organisms is attributed primarily to the NH_3 species [USEPA, 1984]. Our *WYE2000* litter source had 7.3 g/kg of ammonia (measured as NH_4^+). Therefore, our 2.5 g/L poultry litter daily stock solutions (from which we made individual exposure treatments) had the potential for total ammonia levels up to 18.3 mg/L, well above USEPA ambient water quality criteria for ammonia exposure [USEPA, 1984; 1989]. Sufficient dilution of this stock was necessary to reduce ammonia of exposure treatments to permissible levels. In this way, the potential for ammonia related toxicity prescribed an upper limit on laboratory PLACs exposure concentrations. Exposures of fish to field runoff, either caged within the *Research Pond* or in the laboratory, presented similar concerns. Obviously endocrine disruption is of little concern if significant mortalities are occurring as a result of ammonia exposure.

Naturally excreted steroid hormones (E2 and T) are the poultry litter contaminants of greatest concern as potential endocrine disruptors. They are excreted by birds either in the free from, or as conjugates that are readily transformed to the free form [Panter *et al.,* 1999]. The steroid of greatest concern is E2, because it exerts physiological effects at lower exposure concentrations then other natural steroids and because it is often reported in the environment at concentrations above known effects thresholds [Shore and Shemesh, 2003]. Although manures from other animals (e.g., cattle, swine) are often used as fertilizer, poultry litter tends to have higher hormone concentrations because

birds exhibit higher fecal and urinary hormone levels then mammals [Shore *et al.,* 1995]. E2 can either be excreted as the parent compound in an unbound form (primarily in feces), or more readily via formation of glucuronic acid or sulfate conjugates (primarily in urine) which are 10 to 50-fold more water soluble then the parent compound [Ingerslev and Halling-Sorensen, 2003]. Several glucuronide conjugates of E2 are known with conjugation occurring at the C_3 position, C_{17} position, or C_3 and C_{17} positions on the molecule *(Figure 20)*. Sulfatation occurs at the same positions and likely occurs together with glucuronidation. Poultry excrete E2 primarily via the urinary route (69%) suggesting that most E2 in litter (at least initially) is in a conjugated form [Hanselman *et al.,* 2003]. Once excreted, de-conjugation of steroids can occur via bacterial and possibly fungal activity, specifically via hydrolyzation by glucuronidase and/or sulfatase enzymes

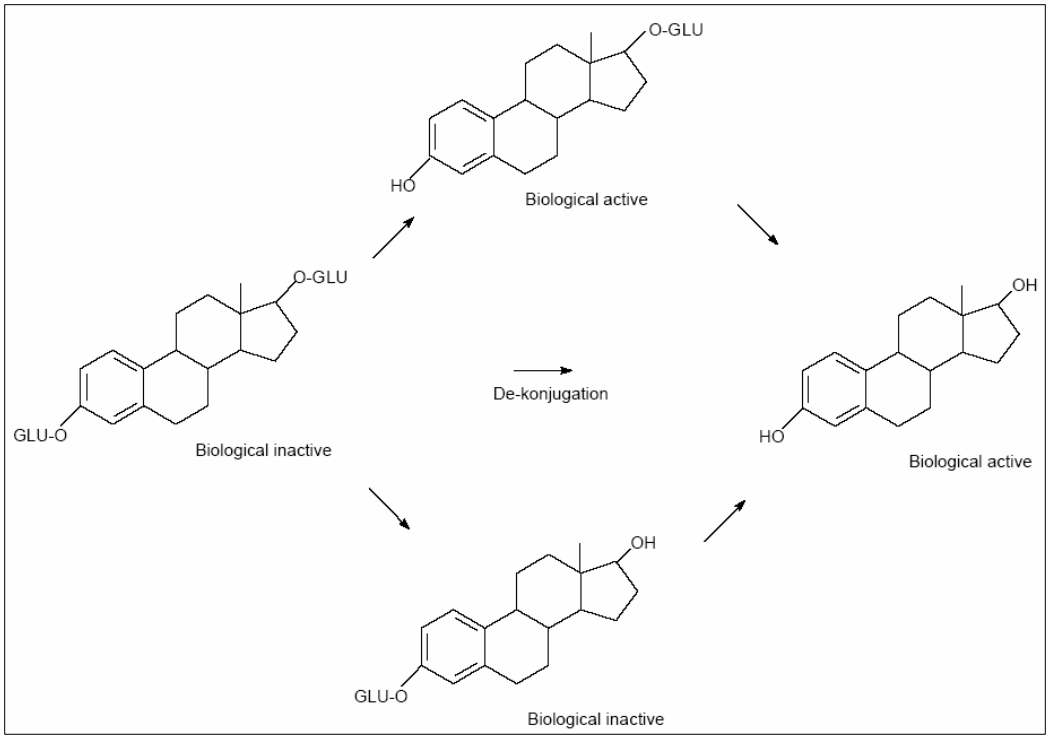


Figure 20. De-conjugation of glucuronidated 17 ß-estradiol into biologically active and inactive compounds [Ingerslev and Halling-Sorensen, 2003].

[Hanselman *et al.,* 2003; Panter *et al.,* 1999]. Ingerslev and Halling-Sorensen [2003] maintain that de-conjugation of E2 need only occur at the C_3 position to allow binding to ER. If so, both unconjugated and at least a portion of conjugated E2 in environmental matrices are of potential toxicological concern.

Numerous researchers have studied the degradation of steroids while still in poultry litter and after entering natural waters [Hanselman *et al.,* 2003]. Litter has been reported to contain up to 904 ng/g E2 and 607 ng/g T on a dry weight basis with concentrations varying according to age, gender and reproductive status [Nichols *et al.,* 1997; 1998; Shore *et al.,* 1995]. Shore *et al.* [2003] found steroid levels in litter remained stable over several months when left in uncovered piles and that thermal processing did not affect steroid concentrations in that time period. E2 levels in the various litter sources measured for this project averaged 126 ± 23.7 ng/g (n = 8). As this material was accumulated within poultry houses over several years prior to transport and storage by end-users, this is a clear indication of steroid persistence with poultry litter.

Once in natural waters steroid degradation can be rapid. In a study of steroid biodegradation in English rivers, Jürgens *et al.* [2002] found E2 was principally transformed to E1 by microorganisms in water. Half-lives were on the order of 0.2 to 8.7 d when incubated at 20EC. E1 was further degraded by microbial cleavage of the steroid ring system (half-lives were 0.1 to 7.2 d). Reductions in estrogenicity, measured by the yeast estrogenicity (YES) assay, paralleled losses of E2 and E1, indicating that degradation did not yield any other persistent estrogenic intermediates.

Laboratory assays performed for this project used poultry litter collected from two eastern shore broiler operations, one in 2000 *(WYE2000)* and one in 2002 *(WYE2002),*

which were subsequently applied as fertilizer on research fields to investigate issues of environmental persistence and transport. Samples of these litter sources were collected for steroid analysis prior to field application. In addition, samples from 6 other poultry litter sources (also eastern shore broiler operations) were analyzed for steroidal constituents *(Table 6)*. The major concern was to identify water-soluble steroids that might move from fields to surface waters in runoff during rain events or by groundwater seepage. Therefore, water-soluble samples were prepared using the methods of Nichols *et al.* [1997], of shaking dry litter in water, centrifuging and analyzing the centrifugate. Resulting E2 and T levels in the eight samples averaged 126 ng/g (SD = 23.7 ng/g) and 42 ng/g (SD = 11.9 ng/g), respectively. Nichols *et al.* [1997] report an average waters-soluble E2 of 133 ng/g (n = 3, SD = 6.0) from broiler litter quantified using an ELIZA kit (Oxford Biomedical Research, Inc. Oxford, MI). The kit claims high specificity to E2, but does not report sensitivity to conjugated E2. Shore *et al.* [1995], on the other hand, report E2 and T in broiler litter of only 28 ng/g and 34 ng/g, respectively, without specifying the method of analysis.

The immunoassay method selected for steroid analysis (RIA) has several advantages over other analytical systems *(Table 12)* [Ingerslev and Halling-Sorensen, 2003]. The most pertinent for this project were high cost effectiveness and rapid throughput of multiple samples. Radioimmunoassay analyses of steroidal contaminants in environmental samples do have a number of disadvantages. First, it is recommended that immunoassay results be independently confirmed, usually with LC-MS-MS or GC-MS-MS methods, which can be very costly. Second, although monoclonal antibodies (MAbs) reported to cross-react minimally (up to 16%) with steroidal metabolites,

Table 12. Advantages and disadvantages of environmental immunoassays (modified from *Evaluation of Analytical Chemical Methods for Detection of Estrogens in the Environment* [Ingerslev and Halling-Sorensen, 2003]).

Advantages	Disadvantages
Sensitivity	*Not 100% specific, vulnerable to cross reactivity*
Rapid	*Requires independent confirmation (e.g., HPLC-MS-MS or GC-MS-MS)*
Cost-Effective	*Not suitable for small sample loads*
Small sample volume	*Synthesis of antibody can be difficult and expensive*
Easy to use	*Only one substance can be analyzed at a time*
Wide applicability	
Reduced sample preparation	
Simultaneous analysis of multiple samples	
Easily automated, ideal for large sample loads	
Suited for field use	

information concerning cross-reactivity of MAbs with other environmental constituents is unavailable. Therefore, whole sample cross-reactivity remains an unknown. A related disadvantage is the problem of false-positive reactions due to interference(s). Specifically, the competitive RIA method relies on unencumbered interaction between a known amount of radiolabeled steroid and latent steroid in the unknown sample. Inhibition of this competition by interfering compounds glomming to antibody attachments sites or binding/sorbing steroid will promote inaccuracies in results (usually overestimations). For example, Huang and Sedlak [2001] found positive E2 ELISA-signals from compounds other than E2 in sewage treatment effluents. They identified the interfering compounds as natural organic matter (NOM) capable of adsorbing onto

antibodies and other surfaces in the immunoassay system. Such NOMs are abundant in aqueous poultry litter solutions and may, therefore, produce similar over-estimations of steroid levels in RIA analyses.

Another potential disadvantage of the selected RIA method involves its sensitivity to conjugated steroid forms. As mentioned previously, conjugated steroids are more water-soluble then free forms, but free steroids are likely more bioactive. It is not entirely clear, at this time, whether the RIA methods employed for this project are sensitive to all or even some of the various conjugated forms of E2 and T. Therefore, a rudimentary attempt was made to determine the levels of free and conjugated steroids within water-soluble poultry litter fractions. This was done by extracting non-polar (i.e. free) steroids in ether, taking ether to dryness, reconstituting residues in RIA buffer, and analyzing. In this way, polar (i.e., conjugated) steroids were removed from samples such that remaining steroids only represented free forms. Of 7 litter samples extracted in this manner, free E2 levels averaged 70 ng/g (SD = 13.3 ng/g) *(Table 6)*. The ratio of free to total E2 was 0.54 (range 0.46 to 0.61) suggesting that somewhat over ½ of measured E2 from aqueous samples is un-conjugated and therefore bioactive. This should not be interpreted to infer that the analytical method employed is detecting all present E2 forms (free and conjugated), only that, of detected E2, ~ ½ is free. Testosterone was a different story. Of the 7 extracted samples, free T levels averaged 45 ng/g (SD = 16.5 ng/g) yielding a ratio of free to total T of 1.0 (range 0.79 to 1.30). This would seem to indicate that all T measured in water-soluble litter samples is un-conjugated. Again, this result does not infer that there are no conjugated forms of T in aqueous litter samples. It is possible (even probable) that the method of analysis employed is insensitive to

conjugated T and only detects free T. While not entirely conclusive, the extraction investigation suggests that RIA measurements of water-soluble poultry litter likely over-estimate free E2 by an approximate factor of 2 while providing reasonably accurate estimates of free T.

17 β-estradiol and Testosterone in Poultry Litter Solutions

In devising the laboratory protocol for exposing fish to PLACs, several assumptions were made. First was the assumption that aqueous mixtures of poultry litter would contain contaminants representative of those found in runoff from litter-amended agricultural fields, thereby yielding meaningful results concerning the potential for ED in resident biota. Second was the assumption that PLACs prepared by proportional dilution would themselves be proportional. As the contaminant of greatest concern E2 was used to track the accuracy of this assumption by measuring steroid levels in daily poultry litter stock mixtures and in exposure treatments derived as dilutions of this stock. These analyses, however, were not performed until weeks or even months after sample collection and, therefore, of no immediate value in assessing trends within assays. Also, because desired steroid levels were often near limits of detection (MDL), and certainly below accurate limits of quantitation (LOQ), strict reliance on results was difficult. More immediate feedback on consistency within and proportionality between exposure treatments was available by measuring ammonia. For example, in the first fathead minnow assay ammonia in the *PLAC-833* treatment ranged from 4.9 mg/L to 5.8 mg/L over the 21 d exposure interval averaging 5.1 mg/L. Ammonia in the *PLAC-417* treatment was approximately ½, averaging 2.5 mg/L (range 2.1 - 2.9 mg/L). As *PLAC-*

417 was a 50% dilution of *PLAC-833*, this is not surprising, but serves as a reasonable indication of linearity in contaminant concentrations. More importantly, narrow ranges of ammonia levels within treatments over the 21 d exposure interval indicate consistency in daily treatment renewals. This trend held in all assays (freshwater and estuarine) where poultry litter treatments were prepared daily as well as in preserved runoff water collected during the spring 2002 rain event which was thawed daily.

Steroid levels within and across assay treatments were somewhat less consistent. For example, in the first fathead minnow assay, average E2 levels in *PLAC-833* and *PLAC-417* treatments were 192 ng/L and 114 ng/L, respectively; reasonable given that one treatment was a 50% dilution of the other. However, E2 levels within treatments (sampled daily at the time of treatment renewal) varied considerably, ranging from 145 - 247 ng/L in *PLAC-833* and 66 - 225 ng/L in *PLAC-417*. Some of this variability may have resulted from analytical interference of organic material as described previously.

Persistence of E2 in exposure treatments was also a concern. Again, actual exposure levels were not known until archived samples had been analyzed. In general, E2 in static positive control treatments dropped by 20% to 70% during 24 h intervals between renewal. The degree of decline tended to increase over the course of 21 d assays such that daily losses were greater at assay conclusion then assay initiation. E2 in poultry litter treatments did not follow this clear trend as levels were found to increase as often as decrease. As mentioned above, interference and/or lack of analytical precision may partially explain discrepancies. In addition, biotransformation of steroids from conjugated to un-conjugated forms may actually increase detectible levels within exposure treatments. For example, if conjugated-E2 degradation to free-E2 is more rapid

then E2 degradation to E1, than detectable E2 will increase in exposure treatments. Conversely, E1 production outpacing E2 de-conjugation will yield a decrease in residual E2. This was the case for Finley-Moore *et al*. [2000] who found levels of unconjugated E2 in poultry waste-impacted runoff increased ≤ 150% in some instances, and decreased ≤ 69% in others.

BIOLOGICAL INDICATORS OF ENDOCRINE DISRUPTION

Vitellogenesis

Induction of Vtg in male fish is an accepted indicator of exogenous estrogenic exposure [Heppell *et al.,* 1995; Folmar *et al.,* 1996]. While not causally linked to a reduction in reproductive competence, Vtg induction in male fish has been found to correlate with reductions in GSI [Panter *et al.,* 1998; Tyler *et al.,* 1999] and pathologic effects within testis [Miles-Richardson *et al.,* 1999]. In our fathead minnow assays the only 21 d litter exposure that did not induce Vtg in any adult male fish was the lowest treatment from *Assay II* with an E2 level of 18 ng/L. Note however that Vtg induction in a 21 d litter exposure with 40 ng E2/L *(Assay II)* was low, averaging only 407 µg/ml, and driven largely by a single individual. Further, a 21 d litter exposure with E2 of ~70 ng/L induced 100% of fish (mean Vtg 3,577 µg/ml) in *Assay II* (flow-through), but only moderately induced 60% of fish (mean Vtg 139 µg/ml) in *Assay III* (static-renewal). Meanwhile, the *E2 Control (Assay I)*, with a concentration of 79 ng/L, produced a mean plasma Vtg level of 63,600 µg/ml, 1 to 3 orders of magnitude higher than levels induced by similar amounts of poultry litter-derived E2. Results from our assays are in close agreement with reports by others of Vtg induction in fathead minnow exposed to

exogenous E2. Panter *et al.* [1998] found an E2 dose of 100 ng/L sufficient to induce Vtg in adult male fathead minnow in a 21 d exposure period. Tyler *et al.* [1999] exposed fathead minnow from 24 h post-fertilization to 30 d post hatch and found as little as 50 ng/L E2 significantly elevated plasma Vtg over control levels.

Responses within and across assays were not always consistent. Twenty-one day exposure to a litter treatment with E2 of 114 ng/L *(Assay I)* induced Vtg in 100% of fish to a mean plasma level of 40,700 µg/ml while a similar 21 d exposure to a treatment with E2 of 123 ng/L *(Assay III)* induced only 5 of 6 fish to a mean Vtg level (in effected fish) of 1,545 µg/ml. Also, in *Assay I* exposure to *PLAC-833* (E2 of 192 ng/L) produced plasma Vtg levels of only 19,300 µg/ml while *PLAC-417* (E2 of 114 ng/L) induced levels of 40,700 µg/ml.

Despite variability in response, plasma Vtg proved a robust biological indicator of poultry litter-derived estrogenicity when exposures lasted 21 days. Responses to shorter exposures were less effective. In *Assay II*, 4 d and 9 d litter exposures with E2 levels up to 70 ng/L did not induce Vtg production in any fish. In *Assay III* a 9 d litter exposure with E2 of 138 ng/L induced Vtg in 50% of fish while a 4 d exposure to E2 of 133 ng/L minimally elevated Vtg in a single fish. Sampling methodology may in part explain the weak Vtg response to short-term exposures. Fish were euthanized and plasma collected immediately after removal from exposure treatments. Therefore, those exposed only 4 d or 9 d had significantly less time for Vtg levels to "ramp-up." Had they been held under control conditions until the conclusion of the 21 d exposure interval, they may have produced Vtg levels equal to those of the longer exposures. On the other hand, were fish held for an extended period after exposure, Vtg may have been lost through elimination

mechanisms. With this in mind, it cannot be concluded that Vtg induction did not occur from short-term exposures, only that it was not expressed at the time of plasma collection.

Hemmer *et al.* [2002], working with mature male sheepshead minnow, report an E2 concentration of 89 ng/L inducing significant vitellogenesis (>40,000 µg/ml) in ≤8 d. They also describe plasma Vtg levels peaking at the conclusion of exposure (16 d) and beginning to drop immediately (Vtg clearance half-life of 14 d), observing that the time-course of Vtg elimination was dependent on the total accumulated amount of Vtg. Schmid *et al.,* [2002] exposed sexually mature male fathead minnow to EE2 (50 ng/L) for 35 days then maintained them another 35 days under control conditions sampling batches of fish at frequent intervals. A modest increase in plasma Vtg was observed beginning at day 3 (first sample), but statistically significant induction was not detected until day 21. The largest increase occurred between days 14 and 28, after which time levels began to plateau. Vtg remained at the plateau level until day 38 (3 days after cessation of exposure) before beginning to decrease, first rapidly, then more gradually. The authors devised a two-compartment model to describe the clearance kinetics with half-lives of 2.2 and 21.3 d for initial and final compartments, respectively.

Measurement of whole-body homogenate Vtg levels proved of minimal use in detecting poultry litter-derived estrogenicity. Exposure of young fathead minnow to *E2 Controls* (96 ng/L) effectively elevated whole-body homogenate Vtg levels in agreement with Tyler *et al.* [1999], who report significant increases in whole-body homogenate Vtg for fathead minnow exposed to E2 (50 ng/L) from 24 h post hatch to 30 d old. In contrast, exposure to poultry litter-derived E2 of 114 ng/L *(PLAC-417)* only slightly

elevated Vtg above control levels, and exposure to poultry litter-derived E2 of 192 ng/L *(PLAC-833)* produced homogenate Vtg somewhat lower than control levels. Given the difficulty of preparing and analyzing body homogenates and poor sensitivity to E2 derived from poultry litter, I believe "whole-bodies" from larval and juvenile fathead minnow exposures would be better used intact for histological examinations of effects on gonadal development then as homogenates.

Twenty-one day aqueous exposure to the *E2 Control* (105 ng/L) readily induced plasma Vtg in adult male sheepshead minnow and mummichog to levels of 36,832 µg/ml and 8,950 µg/ml, respectively, indicating that mature males of both species are capable of expressing potent vitellogenic responses to an exogenous estrogenic stimulus. On the other hand, larval sheepshead minnow had a comparatively weak vitellogenic response (20 µg/ml). Modest elevation of whole-body homogenate Vtg in the *E2 Control* suggests that vitellogenesis in this species at this early age is not a particularly robust indicator of exogenous estrogenic exposure.

Mummichog and particularly sheepshead have been shown previously to be sensitive to exogenous estrogen agonists. Folmar *et al.* [2000] demonstrated in vivo inducability of Vtg in male sheepshead following exposure to natural (E2) and synthetic (17 α-ethynyl estradiol, diethylstilbestrol) estrogens. They report dose dependant induction of Vtg in mature male fish exposed for 16 d to E2 at 231 ng/L, but no effects at 40 ng/L (measured conc.). Hemmer *et al.* [2001] report Vtg induction in adult male sheepshead following aqueous exposures to an alkylphenol (*p*-nonylphenol) and several organic pesticides (methoxychlor, endosulfan). Their E2 positive control (65 ng/L) produced detectable amounts of plasma Vtg in male fish within 5 d of exposure initiation

with levels increasing in linear fashion for the entire 42 d exposure period. Twenty-one d plasma Vtg was approximately 39,000 µg/ml (estimated from graph), comparable to our 21 d sheepshead result of 36,832 µg/ml. Cumulatively, these results suggest a lower E2 response threshold for Vtg induction in male sheepshead somewhere between 40 ng/L and 65 ng/L.

Compared to sheepshead, there is a relative paucity of information on mummichog sensitivity to exogenous E2 exposure. Vitellogenesis has been found in mature male mummichog following intraperoteneal (ip) E2 injection [McArdle *et al.,* 2004; Pait and Nelson, 2003]. Similarly, aqueous E2 exposures of 200 ng/L (nominal conc.) have been used successfully to induce vitellogenesis in male fish for production/purification of Vtg ELISA standards [Denslow *et al.,* 1999; MacLatchy *et al.,* 2003]. However, aqueous E2 exposure levels lower then 200 ng/L are not reported in the literature. To date, the positive control treatment (E2 = 105 ng/L) employed in this project appears to be the lowest E2 concentration tested in aqueous mummichog exposures for Vtg induction, making estimation of a lower effects threshold impossible.

Unlike the positive controls, mature male mummichog were only minimally responsive and sheepshead completely non-responsive to PLAC, even at levels containing poultry litter-derived E2 >140 ng/L. Plasma Vtg was below detection in all sheepshead minnow, and only 3 of 6 mummichog responded modestly to our highest litter treatment (144 ng/L E2). Further, the magnitude of Vtg induction in these fish (213 µg/ml) fell well short of levels encountered in our *E2 Control* (8,950 µg/ml) and in studies with other known EDCs [MacLatchy *et al.,* 2003; Pait and Nelson, 2003]. This finding suggests either that a qualitative difference exists between the available forms of

E2 present in positive control and poultry litter treatments, or that one or several constituents within the complex poultry litter mixture (including testosterone) has an ameliorating effect on E2 exposure.

Pait and Nelson [2003] explored differences in species sensitivity between mummichog and sheepshead to EDCs. Vitellogenic responses in adult males of both species were comparable 21 d after ip E2 injections. However, in similar injections with 4-nonylphenol (NP), the sheepshead vitellogenic response was significantly greater than that of the mummichog. Further, mummichog collected from a reference site free from NP and Bisphenol-A (Bis-A) contamination consistently responded more intensely to NP and Bis-A exposure via ip injection than did fish collected from heavily contaminated sites suggesting intra-species variability in response. The authors hypothesize that prior exposure of fish to these contaminants may have up-regulated Phase I and Phase II enzyme systems, increasing biotransformation and excretion rates of the EDCs, and resulting in less of the compounds being available for endocrine disruption [Pait and Nelson, 2003].

McArdle *et al.* [2000] also explored differences in interspecies responses to EDCs by exposing adult male mummichog and sunshine bass (*Morone saxatilis* x *Morone chrysops*) to municipal sewage treatment plant (MSTP) effluent (75% effluent, 25% seawater) for 21 d. They observed significant elevation of plasma and hepatic Vtg levels in sunshine bass relative to controls, but no such elevation in hepatic Vtg in mummichog (plasma Vtg was not measured). They interpret the findings as indicating ontogenetic and/or species-specific differences in response to estrogenic compounds in MSTP effluent. Hepatic CYP1A protein (140 -145%) and EROD activity (408-598%) were

elevated in both species, indicating exposure to compounds within the effluent capable of altering xenobiotic (and possibly steroid) biotransformation systems in fish [McArdle *et al.,* 2000]. Higher baseline *(Control)* levels of hepatic CYP1A protein and EROD activity in mummichog compared to sunshine bass suggest a greater capacity for bio-transformation and may explain in part why mummichog have a less robust vitellogenic response to complex mixtures of EDCs.

Overall, adult males of the two estuarine species showed similar Vtg responses when exposed to E2 in positive control treatments to that observed in fathead minnow. Fathead minnow, however, were much more sensitive to PLAC, with Vtg induction apparent at exposure levels as low as 40 ng E2/L compared to 144 ng E2/L for the mummichog.

Gonadosomatic Index

Gonadosomatic index was of no use in detecting ED in mature male fathead minnow exposed to PLAC. Treatment effects were not detected in any fathead assay even in instances where plasma Vtg levels exceeded 60,000 µg/ml. This may be explained in part by the selection of sexually mature fish for the assays. As testes were already fully developed, a treatment effect (relative to controls) would require regression of extant tissue rather than inhibition of a developing or recrudescing gonad. Significant effects on GSI have been reported in adult fathead minnow following exposure to various E2 concentrations. Panter *et al.* [1998] observed significant reductions in GSI of mature male fathead minnow when exposed for 21 d to nominal E2 concentrations of 320 and 1000 ng/L, but not 100 ng/L. They also found significant reductions in GSI in fish

exposed 21 d to a nominal estrone (E1) concentration of 318 ng/L. However, a 993 ng/L exposure treatment was not found to differ significantly from controls due to high treatment variability. Their results indicate that extraordinary estrogenic exposure levels are required to induce a significant reduction in GSI in mature male fatheads and even then, results may be tenuous.

Spawning in mummichog (and therefore GSI) is influenced by temperature, photoperiod, lunar cycle, food availability and even hypoxia [Hardy, 1978]. GSI rises steeply in the spring in anticipation of spawning and declines steeply in the autumn at the conclusion of the reproductive season. Levels fluctuate in both sexes throughout the summer with mean levels gradually declining as the season progresses. Several studies describe large differences in spawning condition at the time of animal collection and/or exposure initiation. Dubé and MacLatchy [2000], for example, report male mummichog from New Brunswick Canada with GSI measures of only 0.3% when field collected in July of 1997, of 2.3% in July of 1998, and of 1.2% in August of 1998. Similar differences in spawning condition were encountered in fish used for this project. Male mummichog collected locally in mid August 2001 and examined prior to initiation of estuarine *Assay I* had a GSI of only 1.0. Conversely, animals for estuarine *Assay II* collected in early September 2002 had a mean GSI of 2.1. Although collected later in the spawning season, GSI in these fish was more than twice that of the previous batch.

Jobling *et al.* [1996] noted that testicular inhibition in rainbow trout was strongly influenced by the timing of exposure, and that fish undergoing seasonal gonadal regression were not affected by high exposures to natural estrogens. Pait and Nelson [2003] found a similar result in mummichog given ip injections of E2. Fish tested during

spring/summer had high initial GSI values (~2.5) and showed a significant decline when injected with E2 at level ≥1.0 mg/kg. On the other hand, fish tested during fall/winter began with low GSI values (~0.3) and were unaffected by E2 injections up to 10.0 mg/kg.

While occasionally useful in laboratory exposures [Ankley, et al., 2001], measurement of GSI is of limited use as a field sampling tool. Temporal and spatial variability in GSI among resident fish populations make it difficult to apply as a general marker of endocrine disruption. Similarly, lack of control of factors known to affect GSI (e.g., temperature, tides, food availability, DO, light intensity) limit the utility of GSI as an endpoint in caged field exposures.

Histopathology

While induction of Vtg in male fish has not been causally linked to effects on reproductive competence, it has been found to correlate with histopathological effects in testes [Miles-Richardson et al., 1999]. As such, gonadal histology, in conjunction with hormone and vitellogenin measurements, morphological and fecundity studies, can provide insights into the effects of environmental stressors on reproductive health [Blazer, 2002]. Identification of cellular and tissue level testicular lesions capable of impacting reproductive competence may also shed light on mechanisms of action of potential EDCs. Numerous histological changes have been observed in fish testes following exposure to EDCs. Zillioux *et al.* [2001] report dose-dependent occurrence of testicular fibrosis (LOEC 1.7 ng/L) and testis-ova (LOEC 18 ng/L) in male sheepshead minnow exposed for 59 d (sub-adult to maturity) to 17 α-ethynylestradiol (EE2). Karels *et al.* [2003] describe inflammation comprised of macrophage aggregates and

eosinophilic granules within testis, liver and kidney of 4-*tert*-octylphenol (OP) exposed adult male sheepshead minnow. They also report a dose-dependant increase in interstitial tissue proliferation in testis, coincident with increasing OP concentration. Effects in fathead minnow testes attributed to EDC exposure include: degeneration/necrosis of germ cells and spermatozoa, hypertrophy/hyperplasia of Sertoli cells, frank testicular atrophy and the appearance of testis-ova [Ankley *et al*., 2001; Miles-Richardson *et al*., 1999; Länge *et al*., 2001]. Configurational alterations in reproductive organs have also been reported for fish exposed to suspected estrogens during the period of gonadal differentiation. Prominent among these is development of testis with an oviduct-like structure comprised of dual mesenteric attachments to dorsal peritoneum [Ankley *et al*., 2001; Gimeno *et al*., 1997; 1998].

No significant pathology was evident in male fathead minnow exposed as adults to PLACs. Occasional necrotic/apoptotic cells were seen in most sections but frequency was minimal and not treatment specific. Sperm necrosis and cell syncytia (fusion of cells) have been reported previously in field and laboratory studies after exposure to E2 [Flammarion *et al.,* 2000; Miles-Richardson *et al.,* 1999]. Necrotic cells are characterized by nuclear changes including pyknosis, karyorrhexis and eventually karyolysis. Blazer [2002] suggests necrosis results from arrested gamete maturation during spermatogenesis and subsequent degeneration.

Testes from three fathead minnow were found to contain ovarian follicles within seminiferous tubules, one from each assay *(Figure 12)*. Two were from poultry litter treatments, the third from a *Solvent Control* treatment. In each case only a single perinucleolar oocyte was encountered. Because whole gonads were several mm thick

and only three equally spaced 5 μm sections were examined from each, only a fraction of each organ was actually examined. Thus, there may have been additional oocytes within portions of organs that were not examined. Likewise, testis from other fish may have contained oocytes that did not fall within examined sections. While induction of "intersex" is considered a profound indicator of endocrine disruption, it is unlikely that exogenous E2 exposure could induce a primordial germ cell (PGC) to develop into a 200 μm perinucleolar oocyte in only 9 d. Presumably this condition preceded initiation of the exposures. Intersex was not evident in any sheepshead minnow or mummichog exposed to PLAC (or *E2 Control*) in this project. The only testicular pathologies encountered in estuarine fish were minimal infiltrations of eosinophilic granulocytes (both species) and infrequent macrophage aggregates (sheepshead minnow). These pathologies are consistent with field collected organisms routinely exposed to parasites, pathogens and other natural stressors [Blazer, 2002].

Zillioux *et al.* [2001] observed testis-ova in sub-adult male sheepshead minnow exposed to EE2 concentrations (nominal) of 20 ng/L (rarely after 73 d exposure) and ≥200 ng/L (frequently after 57 and 73 d exposure). Similarly, numerous instances of intersex have been reported in wild fish populations from areas influenced by domestic, industrial and agricultural contaminants [Jobling *et al.,* 1998]. Kavanagh *et al.* [2004] observed a 44 – 85% prevalence of intersex in male white perch collected from several contaminated harbors in western Lake Ontario. Hatchery reared white perch and those collected from a pristine reference site had no incidence of intersex. Similarly, male roach *(Rutilus rutilus)* [Rodgers-Gray *et al.,* 2001] and gudgeon *(Gobio gobio)* [van Aerle

et al., 2001] from English rivers were found to have testis-ova. Prevalence and severity correlated with proximity to municipal and industrial effluents.

Recently testis-ova have been observed in male smallmouth bass *(Micropterus dolomieu)* from upper and middle reaches of the Potomac River, USA [Blazer, personal communication]. Incidences in some regions are as high as 80 - 100%. Contaminant sources vary from mixed urban and rural (middle reaches) to exclusively agricultural (upper reaches). Interestingly, land-use in the upper Potomac has shifted in the past decade to accommodate an intensely growing poultry industry. With limited agriculture in the region, poultry waste is land-applied to pastures as a means of disposal. Steep topography ensures that much of this waste will wash into surrounding surface waters.

Testis maturity indices

According to Ankley *et al.* [2001] testicular staging based on degree of germ cell differentiation is the first step in evaluating histological effects of EDC exposure. The presence and proportion of various spermatogenic stages within germinal epithelium and the abundance of spermatozoa within seminiferous tubules can serve as a measure of testicular maturity [Ankley *et al*., 2001; Gimeno *et al*., 1998]. Several methods are described for estimating testicular maturity. Perhaps the simplest is the USGS method employed as part of the BEST (Biomonitoring of Environmental Status and Trends) Program for identifying contaminant effects in aquatic ecosystems [McDonald *et al.,* 2002; Schmitt and Dethloff, 2000]. This method uses a semi-quantitative approach to staging testis maturity by evaluating the relative proportions of various spermatogenic cell types. Stages are defined as follows:

Stage 0 - Undeveloped: exclusively immature phases (spermatogonia to spermatids) with no spermatozoa

Stage 1 - Early spermatogenic: immature phases predominate, but spermatozoa may also be observed

Stage 2 - Mid-spermatogenic: spermatocytes, spermatids, and spermatozoa are present in roughly equal proportions

Stage 3 - Late spermatogenic: all stages may be observed, however, mature sperm predominate

Alternatives to this method incorporate additional stages (up to *Stage 5*) that more closely define distinction in proportions of gametogenic cell types and include descriptive characteristic concerning thickness of germinal epithelium, relative cyst sizes, etc.

Several quantitative methods also exist for estimating fish testis maturity. They can be manual, partially or fully automated [John Wolfe, Environmental Pathology Laboratory, Sterling VA, personal communication]. Manual methods usually involve counting and measuring individual gametogenic cells either directly from glass slides or using digital images of glass slides. Cells can be manually "tagged" to identify individual types (e.g., spermatozoa, spermatids, spermatocytes (primary and secondary), spermatogonia, and Sertoli cells) as well as pathological conditions (e.g., necrotic/apoptotic germinal cells, vacuolated cells, phagocytic cells, and oocytes). Once tagged, cells types are enumerated using appropriate software (e.g., ImagePro-Plus, Media Cybernetics, Silver Springs, MD; SigmaScan Pro, SPSS Inc, Chicago, IL) to quantify relative proportions. Fully automated systems program image analysis software to identify distinct cell types based on color and size criteria. Immunohistochemical stains can also be employed to preferentially highlight specific cellular characteristics (e.g., nuclei of dividing cells). The software determines total object areas, and

automatically tabulates and transfers data to a designated spreadsheet. Such macro-driven systems can evaluate large numbers of images in a short time, and store information for easy review and revision.

A simplified quantitative method was developed for examining fathead minnow testes in this project (described in *Chapter II: Materials and Methods*). This morphometric method exploits differences in H&E staining characteristics between mature (spermatozoa and spermatids) and immature (spermatocytes, spermatogonia) gametes to assess overall testicular maturity. The method has several advantages over other quantitative systems: (1) it does not require special stains; (2) distinctions in cell types are unambiguous; (3) cost is low compared to automated systems. The method has the disadvantage of being sensitive to histological artifact such as swelling and distortion, a disadvantage shared with most other quantitative methods (i.e., garbage in = garbage out).

In practice, fathead minnow testis maturity was screened using a semi-quantitative staging method with categories ranging from 0 (undeveloped) to 5 (late spermatogenic). Treatments determined to differ from controls were further assessed using the quantitative "morphometric method." Results revealed a significant reduction in proportion of spermatozoa/spermatids within germinal epithelium following exposure to *E2 Control* (E2 = 79 ng/L) and *PLAC-833* (E2 = 192 ng/L). The effect was more pronounced in *E2 Control* (40% reduction) than *PLAC-833* (23% reduction). Furthermore, other PLAC treatments with E2 levels as high as 123 ng/L did not elicit reductions in spermatozoa/spermatids relative to controls. Thus, the endpoint proved capable of detecting an estrogenic exposure, but was not particularly sensitive to steroidal

contaminants derived from poultry litter. Limitations on the utility of GSI likely also apply to testis maturity ranking. Specifically, significant regression of testicular condition in mature male fish over short exposure intervals is not likely to occur. A superior application involves exposing animals as sub-adults and growing them to maturity. As spermatogenesis is steroidally modulated, this allows investigation into contaminant induced inhibition of testis maturation rather than simply regression of extant tissue.

Maturity ranking of testes via the BEST method was able to identify a considerable reduction in reproductive competence in mummichog from the *E2 Control* (105 ng/L) but not from any PLAC treatment. No such reduction in spermatozoa occurred in the sheepshead minnow *E2 Control*. Despite having a plasma Vtg level 3 times higher than that of the mummichog, sheepshead minnow reproductive competence was unaffected by E2 exposure via the *E2 Control*.

Fathead Minnow Gender Assessment

Maintained at 25EC and adequately fed, gametogenesis commences in female fathead minnows at approximately 60 days [van Aerle *et al*., 2004]. In precocious females ovaries may be enlarged with numerous perinucleolar and even cortical alveolar oocytes. However, vitellogenic oocytes with yolk-granules are rare at this age. Oviducts develop prior to the onset of gametogenesis and can be distinguished in cross-section by their two points of attachment with the dorsal peritoneum [van Aerle *et al*., 2002]. Males are difficult to distinguish at 60 days. At this age a testis typically appears in cross-section as a compact "packet" of PGCs and interstitial stroma suspended from the

peritoneum by a single stout point of attachment. Prior to the onset of gametogenesis this packet loosens to reveal cord-like structures surrounding cavities that precurse the seminiferous tubules [van Aerle *et al*., 2004]. Because males could not be positively identified in this study, all specimens of questionable gender were combined into a single group termed *immature/indeterminate*, which precluded calculation of sex ratio within groups. Instead, percent females – *unequivocal* based on the presence of oocytes and *presumed* based on the presence of an oviduct – was used for making comparisons between groups.

It is possible that oocytes were present in animals designated as having an oviduct without oocytes because they were not encountered in the three cross-sections. The converse is highly unlikely. Because cross-sections were taken at anterior, mid and posterior regions of gonad, development of an oviduct, even at its earliest stages, was usually quite obvious in one or several sections.

Fish appear more susceptible to endocrine disruption because of their great plasticity in gonadal development [van Aerle et al., 2002]. Phenotypic gender is not rigidly established in most species. Even in gonochoristic species, despite genetic predilection, actual differentiation and development of reproductive tissues and organs is strongly influenced by endogenous steroid levels [Gimeno et al., 1998]. Exposure to environmental steroids prior to the time of normal sexual differentiation can profoundly affect this process in young fish. Natural estrogens (E2, E1) as well as a variety of estrogen agonists (EE2, DES, NP) have been reported to produce a female gender bias, some at low ng/L concentrations [Colborn *et al*., 1993; Scholz and Gutzeit, 2000]. Androgens (T, 11-KT) and androgen agonist (pulp mill effluents BKMEs, PCBs, dioxin,

DDT) are reported to have the opposite effect, biasing gender ratios toward males [Larsson *et al.,* 2000; Matta *et al.,* 1998].

A clear gender bias appears to have occurred in larval fathead minnow exposures. Fish exposed 3-24 days post-hatch (dph) to the *E2 Control* (no T) were feminized such that nearly 80% were unequivocal females with another 17% possessing oviducts. Litter treatments contained both E2 and T in varying concentrations but typically with an abundance of E2. Exposure of larval fish (3 dph at assay initiation) to these treatments caused a dose-dependant increase in the proportion of identifiable females to levels nearly equaling that of the *E2 Control*. This increase may represent an actual change in phenotypic gender, a precocious onset of oogenesis or some combination of both [van Aerle *et al*., 2004]. There is the possibility that nutrients in litter treatments provided additional food allowing more rapid growth and earlier maturation. This would not explain the increase in females found in the E2 Control which had no such additional food. Also, fish were fed live *Artemia* nauplii in abundance twice daily. Excess material siphoned from aquaria prior to the next feeding indicated continual availability of high quality food. Finally, lengths, weights, and condition indices at the time of sacrifice did not differ between control and poultry litter groups, disputing the idea that nutrient availability encouraged gonadal development in litter treatments.

Perhaps more interesting than the proportion of unequivocal females was the incidence of fish possessing oviducts in the absence of oocytes. None were seen in the *Control* or lowest litter treatment, and only one occurred in the middle treatment. The high treatment, however, had 10 (38%) and the *E2 Control* had 4 (17%). The presence of an oviduct absent oocytes could mean several things: (1) that the specimen was female

with developing oocytes but none were captured in examined sections (recall that only three section totaling ~15 µm were examined for each specimen); (2) that the specimen was an immature female who had not yet begun oogenesis; (3) that the specimen was a male who was sufficiently feminized to develop a distinctly female phenotypic trait, an oviduct. If either of the first two explanations are correct then the fish were actually females placing the sex ratio of the highest litter treatment at ≥12:1 (female:male). On the other hand, if the third case is correct, then the majority of male fish from the high litter treatment were feminized as were several in the middle litter treatment and all but one from the *E2 Control*. In either case, the impact is dramatic. A 21 d exposure of larval fish to contaminants derived from poultry litter had a substantial feminizing effect either by altering phenotypic gender or, at a minimum, inducing the appearance of feminine characteristics within the gonad of immature males. This result was pronounced in a litter exposure with 74 ng E2/L and modest in a litter exposure with 45 ng E2/L. A repeat of these exposures with animals grown-out to maturity would be helpful in determining the nature and permanence of the feminizing effect.

Gender assessments in fish exposed as juveniles (36 - 57 d) differed in several ways from those exposed as larvae. Recall that unequivocal females were more numerous in the juvenile *Control* (65%) and *Solvent Control* (47%) then in their larval counterparts (35% and 32%, respectively). Conversely, the juvenile *E2 Control* had only 35% females with oocytes compared to 79% in the larval *E2 Control*. However, another 50% had presumptive oviducts bringing the total proportion of female and/or feminized fish to 85% in the juvenile *E2 Control* comparable to 96% in the larval *E2 Control*. Proportion of females in low, medium and high poultry litter treatments were 58%, 74%,

and 60%, respectively, with no litter-exposed specimen exhibiting an oviduct in the absence of developing oocytes.

Juveniles exposed to PLACs did not differ from the *Control,* as did their larval counterparts. Age at time of exposure likely explains this difference. van Aerle *et al*. [2002] found sensitivity of larval fathead minnow to EE2, indicated by Vtg induction and appearance in males of an oviduct-like cavity, was effected by age and duration of exposure. Batches of fish were exposed to EE2 (10 ng/L) in discrete 5 day intervals (egg-5 dph, 5-10 dph, 10-15 dph, and 15-20 dph). The authors observed a window of enhanced sensitivity between 10 and 15 dph where 60% of males had feminized ducts.

The importance of observed pathology has yet to be determined. Obviously the consequences of feminizing an entire fish population are dire. However, because assessments were performed on immature fish, the permanence of observed effects is not known. The fathead minnow that Van Aerle *et al.* [2002] exposed as larvae to EE2 (described previously) were grown to 100 dph before being sacrifice for histological analysis. At that age they were approaching maturity and gender could be determined from expression of secondary sex traits. Sex ratio did not differ between groups. However, the average maturity of testes (based on percentage of different spermatogenic cell types) was reduced in several exposed treatments (10-15 dph, 15-20 dph) relative to the control.

The greater number of identifiable females in juvenile control treatments (47 - 65%) compared to larval controls (32 – 35%) is difficult to explain. Both sets of fish were ~60 dph old when fixed for histological preparation. Larval fish were exposed flow-through from age 3 – 24 dph then held static with water replaced 3x/week until 60

dph. Juveniles were grown in static tanks with water replaced 3x/week to an age of 36 dph before initiation of the 21 d flow-through assay. Fish were held in identical aquaria, fed the same diet, and of similar size at the time of sacrifice. If we assume that the fish with oocytes in the juvenile control treatment (65%) were not feminized males, then they must have simply been females that began oogenesis earlier then their larval counterparts. If so, two differences in handling could have contributed to this result. First, assays were performed in aquaria held in a temperature controlled water bath maintained at 25 ± 1EC. Therefore, fish exposed as juveniles were held precisely at 25 ± 1EC for three weeks prior to being sacrificed and assessed for gender. Fish exposed as larvae were moved after the assay conclusion to holding tanks within the culture facility where ambient temperatures can vary from 23 - 25EC. Similarly, light intensity on the exposure table was higher (~200 lux) than ambient levels (~80 lux) in the culture facility. Temperature and light (duration and intensity) are critical cues in the maturation process and could explain differences in rate of development between batches of fish.

SUMMARY OF LABORATORY DERIVED BIOLOGICAL RESULTS

Assays were performed using nominal amounts of poultry litter in solution with actual poultry litter-associated steroids measured afterwards. Without "real-time" information on steroid levels in exposures it was difficult to know with certainty whether anticipated treatment levels were achieved, or whether daily renewal levels were consistent over time. As a result, anticipated E2 levels (based on results of previous assays) were not always achieved. For example *PLAC-417* treatments had an E2 concentration of 114 ng/L in *Assay I* but only 68 ng/L in *Assay II*. Also, not all steroidal

constituents in exposure treatments were measured; E1 for example is excreted by birds, is a known intermediate of E2 degradation, and has high endocrine activity in fish [Panter *et al.,* 1998]. For these reasons, comparisons of treatment effects between assays are based on the amount of litter used to generate treatments rather than resulting measured E2 levels. Results from all three fathead minnow assays (summarized in *Table 13*) are.

1. Vitellogenesis was the most sensitive and consistent indicator of PLAC exposure in adult male fish.
    a. Induced individuals were observed in all 21 d *PLAC-208*, *PLAC-417* and *PLAC-833* treatments as well as the *E2 Control*.
    b. Shorter exposures only produced meaningful vitellogenesis in the 9 d *PLAC-833* treatment.
2. GSI (as applied in this study) was not sensitive to PLAC exposure.
3. Testis maturity was not sensitive to PLAC exposure. Reduction only occurred following 21 d exposure to *PLAC-833,* not at lower exposure levels or shorter exposure durations.
4. Vitellogenesis was not useful in larval fish exposures - results were inconsistent and lacked sensitivity.
5. Exposure of larval fish (3-24 dph) to PLAC produced an increase in proportion of females identifiable at 60 dph above control levels (32% ♀). The effect was dramatic in *PLAC-417* (92% ♀), moderate in *PLAC-208* (74% ♀), and modest in *PLAC-104* (55% ♀).

6. Exposure of juvenile fish (36-57 dph) to PLAC did not produce discernable changes in fish gender identification compared to controls.

Table 13. Summary of reproductive effects occurring in fathead minnow *(Pimephales promelas)* following exposure to PLAC and positive control treatments. Symbols indicate increase (+), decrease (–), or no change (=) in reproductive endpoint. Multiple symbols indicate a more robust response.

Reproductive Endpoint	Assay #	*PLAC-104*			*PLAC-208*			*PLAC-417*			*PLAC-833*			*E2 Control*
		4	9	21	4	9	21	4	9	21	4	9	21	21
Plasma Vtg Induction	I									++			++	++
	II	=	=	=	=	=	+	=	=	+				
	III									+	=	+	++	
Gonadosomatic Index	I									=			=	=
	II	=	=	=	=	=	=	=	=	=				
	III									=	=	=	=	
Testis Maturity	I									=			–	– –
	II	=	=	=	=	=	=	=	=	=				
	III									=	=	=	=	
Whole-body Homogenate Vtg	I									+			=	++
	II			=			=			=				++
Larval sex ratio	II			+?			+?			++				++
Juvenile sex ratio	II			=			=			=				+

Table 14 summarizes results of sheepshead minnow and mummichog assays. Vitellogenesis was robust in males from *E2 Control* treatments of *Assay I*, but only occurred minimally in the highest mummichog PLAC treatment of *Assay II*. Similarly, GSI and testis maturity indicated negative endocrine effects in *E2 Controls*, but not in PLAC exposures. An increase in sheepshead minnow GSI in *PLAC-417* is difficult to explain as higher PLAC treatments produced no similar effect. Limited sensitivity of

these species to environmentally relevant PLAC exposure concentrations makes them poor choices as sentinel species for investigating poultry litter-induced endocrine disruption in natural waters.

Table 14. Reproductive effects occurring in sheepshead minnow (*Cyprinodon variegatus)* and mummichog *(Fundulus heteroclitus)* following exposure to PLAC and positive control treatments. Symbols indicate increase (+), decrease (–), or no change (=) in reproductive endpoint (* high control mortality puts these results in question).

Reproductive Endpoint	Test Species	Assay II			Assay I
		PLAC-417	*PLAC-833*	*PLAC-1667*	*E2 Control*
Plasma Vtg Induction	*C. variegatus*	=	=	=	+++
	F. heteroclitus	=	=	+	+++
Gonadosomatic Index	*C. variegatus*	+	=	=	=
	F. heteroclitus	=	=	=	–*
Testis Maturity	*C. variegatus*	=	=	=	–
	F. heteroclitus	=	=	=	–*

TRANSPORT OF 17 β-ESTRADIOL

Detection of considerable E2 in runoff from litter-amended research fields (up to 350 ng/L) clearly demonstrated the transport of PLACs from agricultural fields to surface waters following rain events. Concentrations in field runoff were dependant on agronomic practices (e.g., *No-Till* vs. *Conventional-Till*) and intensity and duration of rainfall. In 2000, water-soluble E2 in litter applied to the research fields (WYE2000) was measured at 108 µg/kg. Since nearly 100,000 kg of litter were applied to each of the 35 ac research fields (3 ton/ac), approximately 10.3 g of E2 was introduced to each watershed. The first event to produce runoff (5/22/00) dropped 5.89 cm of rain of which 29% (~ 2.3 x 10^6 L) came off the *No-Till* field. Since this material had an average E2 concentration of 125 ng/L, total E2 in runoff from the *No-Till* field for this event was 0.28 g, only 2.7% of the amount originally applied. Runoff from the *Conventional-Till* field represented only 8.4% (6.5 x 10^5 L) of total precipitation with the remainder infiltrating the soil. Because *Conventional-Till* runoff had an averaged E2 concentration of 42 ng/L, total E2 discharged from the field was 0.027 g, only 0.26% of applied E2. This indicates that only 1/10 as much E2 was transported (in runoff) from the *Conventional-Till* field as was transported from the *No-Till* field.

Several characteristics associated with agronomic practices on the two fields explain the discrepancy [Staver, 2004]. Recall that in conventional tillage litter is first applied, then tilled (homogenized) into the top 20 cm of soil. In no-till practices, litter is applied directly to the compacted soil surface. The advantage of no-till lies in minimizing soil loss by leaving cover crop residuals in place and by not disrupting surface material and making it vulnerable to suspension and runoff. However,

disadvantages of no-till include limited water-holding capacity and vulnerability of surface applied materials (e.g., poultry litter, inorganic fertilizer) to rapid suspension and lateral transport. On the other hand, deep furrows on conventionally tilled fields can retain substantial volumes of water allowing a great deal of infiltration (vertical transport) before significant runoff begins. In this way water-soluble contaminants are transported to groundwater rather than surface water. These advantages can diminish with time as the texture of conventionally tilled fields smooth and surfaces "crust-over," inhibiting infiltration and allowing furrows to behave as raceways rapidly transporting precipitation to surrounding surface waters.

Keeping the above characteristics in mind, it is obvious that the *Conventional-Till* field absorbed the vast majority of rainfall from the 5/22/00 event, and that contact with litter-associated E2 (distributed to a depth of 20 cm) was minimal. In contrast, 3 ½ times as much precipitation ran from the *No-Till* field and average E2 concentration in that runoff was 3 times that of the *Conventional-Till* field.

A second event (6/6/00) dropped 1.17 cm of rain of which 4.9% (7.6×10^4 L) ran from the *No-Till* field. Average E2 in the runoff was 58 ng/L so total E2 transported from the field was 0.004 g, (0.04% of field applied E2). Therefore, in the first 30 days (5/8/00 – 6/6/00) after litter application to the research fields less than 3% of available E2 was laterally transported from the *No-Till* field to receiving waters. Only 0.26% of available E2 was transported from *Conventional-Till* in that time period. From that time forward drought conditions prevailed through September so that subsequent E2 in runoff was trivial.

Results in 2002 were somewhat different. Water-soluble E2 in field-applied litter (*WYE2002)* was 86 μg/kg so total E2 applied to each field was 8.2 g. The first runoff event after litter application (5/18/02) dropped 3.05 cm of precipitation of which only 2.9% (1.2×10^5 L) was laterally transported from the *No-Till* field (no runoff occurred in *Conventional-Till*). E2 concentration in *No-Till* runoff was exceptionally high (average 275 ng/L), but given the small volume, only 0.033 g escaped the field via surface transport. This accounted for a mere 0.4% of initial field-applied E2. A second intense rain event (6/5-7/02) dropped 6.30 cm of precipitation of which 14.1% (1.2×10^6 L) ran off the *No-Till* field and 14.8% (1.2×10^6 L) ran off the *Conventional-Till* field. Average E2 levels from both sources were nearly identical (*No-Till* = 38.5 ng/L; *Conv-Till* = 37 ng/L) such that transported E2 from each field was 0.046 g, accounting for only 0.56% of initial field-applied E2. Therefore, slightly less than 1.0% total available E2 applied to the *No-Till* field was transported to surface waters between application and this second rain event (5/8/02 – 6/7/02). Furthermore, only 0.56% of available E2 from the *Conventional-Till* field was transported in this interval.

No-Till vs. Conventional-Till Runoff Characteristics

1. If the initial rain event after litter application is intense and of substantial volume, no-till has the disadvantage of allowing significant transport of E2 to surrounding surface waters. Superior water retention and increased soil infiltration on conventionally tilled fields reduces total runoff volumes and promotes dilution of E2 in water that does run off.

2. If initial precipitation after litter application is moderate, contaminant loads transported from no-till fields are diminished. However, because runoff volume is minimal, contaminants may concentrate into an intense slug. Substantial water holding capacity of conventionally tilled fields minimizes runoff from moderate precipitation
3. Homogenization of poultry litter into soils on conventionally tilled fields limits available surficial E2 contact with precipitation.
4. Differences in runoff characteristics between the two management practices become minimal after multiple rain events have compacted and smoothed conventionally tilled surface soils.

17 $-Estradiol in Receiving Water

Resultant contaminant levels in receiving waters are a consequence of runoff volume, contaminant concentration, and the size and nature of receiving body. Flowing waters (e.g., streams, rivers and tidal estuaries) provide a continual source of dilution such that pulse introductions of water-borne contaminants will be continually diminished. Impounded water bodies (e.g., ponds, lakes) provide dilution of introduced waters proportionate to their volume with excess discharged as overflow. The *Research Pond* used in this project was such an impounded water body. At 75 m x 25 m x 0.67 m the total volume of the pond is 1.3×10^6 L, approximately equal to the volume of water in one centimeter of runoff from the 35 ac *No-Till* field. Therefore, intense rain events can generate runoff volumes well in excess of the ponds holding capacity. In such cases,

exchange and mixing of runoff with existing pond water is rarely optimal so calculation of resulting contaminant concentrations is tenuous.

Even from a limited amount of data, trends emerge. For example, runoff from the 5/22/00 rain event totaled 2.3 x 10^6 L with average E2 concentration of 125 ng/L. Pond E2 before initiation of runoff was below detection. Assuming optimal mixing of runoff and resident water the resulting E2 concentration would be 80 ng/L, a level that agrees very well with our measured pond concentrations which ranged from 63 to 83 ng/L during the period of runoff *(Figure 16)*. The next runoff event (6/6/00) introduced only 76,000 L of runoff with E2 concentration of 58 ng/L. After dilution this would be expected to increase the whole pond E2 concentration < 2 ng/L. Runoff from the 5/18/02 rain event had an E2 concentration of 275 ng/L. However, with a volume of 120,000 L, dilution within the pond would be expected to leave a maximum residual E2 level of only 13 ng/L. *Figure 19* shows E2 at levels up to 70 ng/L during the actual runoff event, but after several days of mixing the pond level had dropped to near the 18 ng/L detection limit.

Estradiol concentrations measured in litter-amended field runoff and within the receiving pond for this project are in general agreement with previous studies on the Delmarva Peninsula [Shore *et al.,* 1995] and elsewhere [Finlay-Moore *et al.,* 2000; Herman and Mills, 2003; Nichols *et al.,* 1997; 1998] that investigated the transport of E2 from poultry litter into surface and groundwater following application to fields and pastures. Shore *et al.* [1995] reported E2 at concentrations of 14 to 20 ng /L in a farm pond receiving runoff from poultry litter-amended agricultural fields. Herman and Mills [2003] found stream E2 concentrations as high as 120 ng/L in an instrumented 1.2-km^2

agricultural watershed in central Virginia. Higher concentrations were observed early in the growing season (shortly after application of poultry litter) with values decreasing over the course of the summer and as a function of hydrological transport distance from the cropped fields. Runoff from small scale (1m x 3m) fescue plots amended with broiler litter was reported to contain E2 at levels of 450 ng/L [Nichols *et al.,* 1998]. Larger (0.8 ha) fescue plots produced E2 levels of 305 to 820 ng/L in runoff following amendment with broiler litter [Finlay-Moore *et al.,* 2000].

CAGED FISH EXPOSURES

Fish were caged within the *Research Pond* to investigate effects of exposure to agriculturally-generated PLACs, rather than basing conclusions exclusively on laboratory-generated PLAC exposure data. Advantages of caging within the *Research Pond* were numerous: (1) Proximity to the research facility simplified daily fish observation, maintenance and feeding; (2) Instrumentation on research fields provided quick and accurate hydrological data; (3) Complete knowledge of cropping history and current agronomic practices ensured ample understanding of all contaminants introduced to the watershed. Despite the degree of control imparted by these various advantages, intangibles are always a part of field work. The study design relied on natural introduction of PLACs to the *Research Pond* via runoff from rain. It was fortunate that rain events sufficient to induce runoff did occur in timely fashion both years in which poultry litter was applied to fields. Runoff events differed significantly between the two years (described above) as did the consequent pond PLAC levels. The average E2 concentration during the 21 d cage exposure in 2000 was 50 ng/L, sufficient to induce

vitellogenesis in male fathead minnow based on laboratory results. Average E2 in 2002 (exposure only 16 d) was a comparatively low 30 ng/L, below levels known to induce Vtg based on laboratory results. However, insight from laboratory assays proved of little use as fish exposures both years failed to induce vitellogenesis in a single specimen. Whether caged within the pond itself or exposed to water transported from the pond to the laboratory, endocrine disruption was not in evidence. On the other hand, water collected at the time of runoff and preserved (i.e., frozen) demonstrated considerable estrogenicity, inducing Vtg in 100% of exposed fish. This runoff was undiluted compared to the pond exposures and was not subject to microbial degradation, but was, nevertheless, the product of standard eastern shore cropping practices; the inference being that poultry litter-associated contaminants maintain the potential for endocrine disruption at the time of their transport from agricultural fields to surrounding water bodies.

Further investigations into the effects of exposure to this "preserved field runoff" are ongoing. Specifically, larval fathead minnow of various ages have been exposed in the laboratory to this material for several time intervals, grown-out to 120 d, and then sacrificed for histological examination *(Figure 21)*. Results of exposures are pending completion of histological preparation.

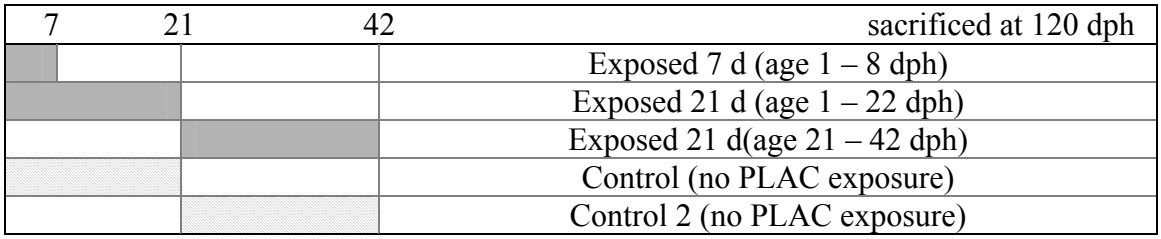


Figure 21. Ages and intervals of exposure of larval fathead minnow *(Pimephales promelas)* to preserved runoff from a poultry-litter amended field. Fish were grown to 120 d then fixed for histological examination.

CONCLUSIONS

1. Exposure to poultry litter-associated contaminants (PLACs) at environmentally relevant concentrations caused endocrine disrupting effect in mature male fathead minnow. The most sensitive indicator of endocrine disruption, detection of plasma Vtg, was observed to levels >40,000 μg/mL in adult male fish exposed in the laboratory to aqueous extracts of poultry litter. Vitellogenesis occurred in >40% of fish exposed for 21 d to a treatment with poultry litter-derived17 β-estradiol (E2) of 40 ng/L.

2. Gender differentiation in larval fathead minnow showed dose-dependant sensitivity to PLAC exposure. The proportion of female/feminized fish exceeded 90% following a 21 d exposure to a treatment with poultry litter-derived E2 of 74 ng /L. Male fish either underwent gender reversal or were feminized to the point of developing an oviduct-like structure. The result occurred to a lesser degree in a treatment with 45 ng E2/L (74% ♀), and less still with E2 at the 18 ng/L detection limit (55% ♀).

3. Sheepshead minnow and mummichog were not sensitive to endocrine disruptive effects of poultry litter. Male sheepshead minnow were completely non-responsive to all tested PLAC treatments and male mummichog were only minimally responsive to the highest tested treatment.

4. Substantial quantities of poultry litter-derived E2 can be transported to surface waters via runoff from agricultural fields. The amount transported is a function of the initial E2 concentration in litter, the frequency, volume and intensity of precipitation and the agronomic practices employed. Fields under “No-Till” management practices can lose up to 10 times more E2 than fields employing conventional tillage. At most, E2

transported to surface waters in runoff amounts to only several percent of total field-applied E2. Maximum measured E2 concentrations in runoff from *No-Till* and *Conventional-Till* fields were 350 ng/L and 42 ng/L, respectively.

5. Poultry litter-derived E2 can enter surface waters via field runoff and persist for weeks to months at environmentally relevant concentrations. For example, E2 in the *Research Pond* was increased to >60 ng/L by introduction of field runoff and required nearly 2 months to return to pre-runoff levels. Average E2 for the 21 d post-runoff interval was 50 ng/L, higher than the 21 d LOEC of 40 ng/L identified in the laboratory.

6. Runoff from poultry litter-amended agricultural fields was capable of causing endocrine disruption in mature male fathead minnow. Preserved field runoff (collected and frozen) was sufficiently estrogenic to induce vitellogenesis in male fish exposed in the laboratory (21 d).

7. However, if allowed to "age" naturally, poultry litter-influenced surface water was not sufficiently estrogenic to promote vitellogenesis in adult male fathead minnow either *in situ* or in the 21 d laboratory exposures.

8. The morphometric method developed to quantify proportions of mature gametes in histological slides of fathead minnow testes proved accurate and reproducible and should be applicable with minimal modification to a variety of small fish species.

9. The caging system developed for this study, using floating baskets within protective barrels, proved sufficiently adaptable for *in situ* exposure of small to medium size fish in a variety of locations. The system works at depths of 0.5 to 4 meters, is robust enough to tolerate strong currents during high flow periods and is tamper resistant to discourage vandalism.

EVIDENCE OF POULTRY LITTER INDUCED ENDOCRINE DISRUPTION

There are several lines of evidence suggesting widespread application of poultry litter may be causing endocrine disruption in resident fish populations. However, most information is anecdotal or preliminary so it is premature to draw conclusions.

1. In 2000 the U.S. Fish and Wildlife Service (FWS) under the supervision of Beth McGee, Ph.D., (Chesapeake Bay Field Office), initiated a three-year project (2000 - 2003) aimed at evaluating potential water quality impacts of animal agriculture on the Delmarva Peninsula [McGee *et al.,* 2003]. Biological indicators of AFO related environmental impacts were investigated by caging mature male fathead minnow in "high risk" areas (based on intensity of agricultural activity within surrounding watersheds) and by collecting resident fish from these same areas. As in my *Research Pond* exposures, there was no evidence of endocrine disruption in the field caged fathead minnow. However, male common carp *(Cyprinus carpio)* collected from a Delmarva location (Slaughter Creek) had average plasma Vtg levels of 53 µg/mL, evidence of exposure to an estrogenic stimulus and significantly higher than Vtg levels of 22 µg/mL in carp collected from a non-agriculturally influenced reference site (Jug Bay Wildlife Refuge, Patuxent River).

2. Members of the USGS, Maryland DNR and DC Fisheries have observed intersex male smallmouth bass *(Micropterus dolomieu)* from upper and middle reaches of the Potomac River, USA. Incidences in some regions are as high as 80 - 100%.

Suspect sources of EDCs include sewage treatment plant effluents and agricultural runoff. In the upper Potomac, where testis-ova were first observed, urban influences are minimal, but a substantial poultry production industry has developed in the past decade. As elsewhere, disposal of poultry litter is accomplished by application to agricultural fields. In regions where agricultural need for fertilizer is insufficient, excess litter is applied directly to pastureland. Steep topography ensures that much of this waste is washed into surrounding surface waters. A collaborative project, initiated by the FWS-CBFO (with participation form USEPA, USGS, MDNR, and others) has been devised to investigate the extent and etiology of intersex within non-tidal portions of the Potomac River. Collection of chemical and biological samples will begin during the summer of 2005. The fish cage system (devised for this project) will be employed in 2006 to house young-of-year smallmouth bass in areas of concern.

ONGOING/FUTURE RESEARCH

The present study addressed the potential of contaminants in poultry litter to induce endocrine disruption in aquatic organisms. Observations of vitellogenesis in mature male fish and biases in gender ratio in sub-adults following laboratory exposure as larvae to aqueous poultry litter clearly demonstrate the estrogenic propensity of poultry litter-associated contaminants. Further, lower effects levels determined in laboratory assays are on the order of contaminant levels encountered in natural waters receiving runoff from litter-amended agricultural fields. However, *in situ* exposure of mature male fish to these natural waters has not demonstrated estrogenic bioactivity. Ambiguities in laboratory results suggest distinctions in sensitivity across fish species and between age categories within species. Likewise, analyses of litter from multiple sources reveal differences in levels of contaminants of concern.

A complimentary project has been funded by the Maryland Center for Agro-Ecology, Inc., with an aim of pursuing several areas of research advanced by the present study. First, additional larval fathead minnow assays will be performed to determine minimum PLAC exposures (concentration vs. duration) required to elicit endocrine disruptive effects. Test design will enable identification of specific fish age related windows of sensitivities. Further, a lengthy post-exposure fish grow-out period will be employed to explore the permanence of abnormal gonadal morphology and confirm gender-biasing effects. African clawed frogs *(Xenopus laevis)* will be used in a similar series of assays to investigate PLAC effects on amphibians. Animals will be exposed at various life-stages (embryo, pre-metamorphic, post-metamorphic, and adult) to establish age related sensitivities.

In 2004 five agriculturally influenced Delmarva watersheds (Choptank River, Nanticoke River, Pocomoke River, Wicomico River, and Marshyhope Creek) were broadly surveyed for surface water steroids. A screening approach was used in which many sites (>90) were visited with limited frequency, twice during spring rain-events and once during a late summer low-flow period. Preliminary results (spring 2004) found more than 60% of sites sampled (55 of 91) had detectable E2 (range 18 – 45 ng/L). E2 was encountered in areas where poultry production and poultry litter field-application is particularly intensive. Sites of greatest concern (~12) will be revisited in 2005 for additional contaminant characterization and to investigate resident fish and amphibian populations for evidence of endocrine disruption. Collection will target species with site fidelity and known sensitivity to exogenous estrogens. Plasma Vtg will be measured in adult male and immature specimens as an indicator of estrogenic exposure. Gonads will be examined histologically for evidence of ED related pathology (e.g., inhibition of maturation, gametogenic regression, intersex). In the event of encountering evidence of ED, sites will be revisited in 2006. Indicator species identified in 2005 will again be collected and screened for effects. In addition, laboratory reared fish will be caged within troubled watersheds to more fully characterize the extent of endocrinological impact.

APPENDIX

APPENDIX 1. Summary of test conditions for laboratory assays.

Fathead Minnow Assay #1: Adult Males

1. Test species:	Fathead minnow *(Pimephales promelas)*
2. Test duration/type:	21 day/static exposure w/ daily renewal
3. Age of test animals:	Sexually mature adults (~ 6 to 8 months old)
4. Gender of test animals:	Male
5. Number of organisms/treatment:	10
6. Dilution water:	Aged aerated unchlorinated deep well water
7. Exposure treatments:	1) Control 2) Positive Control (~100 ng 17 β-Estradiol/L) 3) Solvent Control (~2 µL EtOH/L) 4) Low Poultry Litter (417 mg Dry Litter/L) 5) High Poultry Litter (833 mg Dry Litter/L)
8. Temperature (EC):	25 ± 1
9. Photoperiod:	16 h light/8 h dark
10. Light quality/intensity:	Fluorescent bulbs providing ~ 200 lux at surface of exposure aquaria
11. Test vessel:	37 L all glass aquaria
12. Volume of test solution:	20 L w/ 18 L siphoned and replaced daily
13. Feeding regime:	Tetramin® tropical flake food (~ 2 % body weight) fed once/day prior to water renewal.
14. Aeration:	Gentle aeration via glass pipette
15. Endpoints:	1) Survival 2) Plasma vitellogenin (Vtg) 3) Gonad histopathology 4) Gonadosomatic index (GSI) 5) Condition index (CI)

APPENDIX 1 (continued).

Fathead Minnow Assay #1: Mixed Larvae

1. Test species:	Fathead minnow *(Pimephales promelas)*
2. Test duration/type:	21 day/static exposure w/ daily renewal
3. Age of test animals:	Test initiated w/ 1 day old fish
4. Gender of test animals:	Mixed
5. Number of organisms/treatment:	10
6. Dilution water:	Aged aerated unchlorinated deep well water
7. Exposure treatments:	1) Control 2) Positive Control (~100 ng 17 β-Estradiol/L) 3) Solvent Control (~2 ppm EtOH) 4) Low Poultry Litter (417 mg Dry Litter/L) 5) High Poultry Litter (833 mg Dry Litter/L)
8. Temperature (EC):	25 ± 1
9. Photoperiod:	16 h light/8 h dark
10. Light quality/intensity:	Fluorescent bulbs providing ~ 200 lux at surface of exposure aquaria
11. Test vessel:	10 L all glass aquaria
12. Volume of test solution:	5 L w/ 4.5 L siphoned and replaced daily
13. Feeding Regime:	Fed live < 24 h old Artemia nauplii at a rate of 150 nauplii/fish/day prior to water renewal
14. Aeration:	Gentle aeration via glass pipette
15. Endpoints:	1) Survival 2) Whole-body homogenate vitellogenin (Vtg) 3) Gonad histopathology

APPENDIX 1 (continued).

Fathead Minnow Assay #2: Adult Males

1. Test species:	Fathead minnow *(Pimephales promelas)*
2. Test duration/type:	4, 9, & 21 day/ flow-through w/ 8 volume replacements/day
3. Age of test animals:	Sexually mature adults (~ 5 ½ months old)
4. Gender of test animals:	Male
5. Number of organisms/treatment:	~20 (6 sampled @ day 4, 6 @ day 9, remainder @ day 21)
6. Dilution water:	Aged aerated unchlorinated deep well water
7. Exposure treatments:	1) Control 2) Low Poultry Litter (104 mg Dry Litter/L) 3) Medium Poultry Litter (208 mg Dry Litter/L) 4) High Poultry Litter (417 mg Dry Litter/L)
8. Temperature (EC):	25 ± 1
9. Photoperiod:	16 h light/8 h dark
10. Light quality/intensity:	Fluorescent bulbs providing ~ 200 lux at surface of exposure aquaria
11. Test vessel:	37 L all glass aquaria
12. Volume of test solution:	20 L w/ 8 volume replacements/day
13. Feeding Regime:	Tetramin® tropical flake food (~ 2 % body weight) fed once/day
14. Aeration:	Gentle aeration via glass pipette
15. Endpoints:	1) Survival 2) Plasma vitellogenin (Vtg) 3) Gonad histopathology 4) Gonadosomatic index (GSI) 5) Condition index (CI)

APPENDIX 1 (continued).

Fathead Minnow Assay #2: Mixed Juveniles

1. Test species:	Fathead minnow *(Pimephales promelas)*
2. Test duration/type:	21 day /Flow-through w/ 8 volume replacements/day
3. Age of test animals:	Juvenile - 36 d old @ assay initiation, 60 d old @ assay termination
4. Gender of test animals:	Mixed
5. Number of organisms/treatment:	40 (20 for Vtg analysis; 20 archived for histopathology)
6. Dilution water:	Aged aerated unchlorinated deep well water
7. Exposure treatments:	1) Control 2) Positive Control (~100 ng 17 β-Estradiol/L) 3) Solvent Control (~2 ppm EtOH) 4) Low Poultry Litter (104 mg Dry Litter/L) 5) Medium Poultry Litter (208 mg Dry Litter/L) 6) High Poultry Litter (417 mg Dry Litter/L)
8. Temperature (EC):	25 ± 1
9. Photoperiod:	16 h light/8 h dark
10. Light quality/intensity:	Fluorescent bulbs providing ~ 200 lux at surface of exposure aquaria
11. Test vessel:	20 L all glass aquaria
12. Volume of test solution:	10 L w/ 8 volume replacements/day
13. Feeding Regime:	Fed live < 24 h old Artemia nauplii at a rate of ~ 450 nauplii/fish/day
14. Aeration:	Gentle aeration via glass pipette
15. Endpoints:	1) Survival 2) Whole-body homogenate vitellogenin (Vtg) 3) Condition index (CI) *4) Gonad histopathology*

APPENDIX 1 (continued).

Fathead Minnow Assay #2: Mixed Larvae

1. Test species:	Fathead minnow *(Pimephales promelas)*
2. Test duration/type:	21 day /Flow-through w/ 8 volume replacements/day
3. Age of test animals:	Larval - 3 d old @ assay initiation; 24 d old @ assay termination; 60 d old @ time of preservation
4. Gender of test animals:	Mixed
5. Number of organisms/treatment:	40 individuals in 4 reps of 10
6. Dilution water:	Aged aerated unchlorinated deep well water
7. Exposure treatments:	1) Control 2) Positive Control (~100 ng 17 β-Estradiol/L) 3) Solvent Control (~2 ppm EtOH) 4) Low Poultry Litter (104 mg Dry Litter/L) 5) Medium Poultry Litter (208 mg Dry Litter/L) 6) High Poultry Litter (417 mg Dry Litter/L)
8. Temperature (EC):	25 ± 1
9. Photoperiod:	16 h light/8 h dark
10. Light quality/intensity:	Fluorescent bulbs providing ~ 200 lux at surface of exposure aquaria
11. Test vessel:	20 L all glass aquaria
12. Volume of test solution:	10 L w/ 8 volume replacements/day
13. Feeding Regime:	Fed live < 24 h old Artemia nauplii at a rate of ~ 150 nauplii/fish/day.
14. Aeration:	Gentle aeration via glass pipettes
15. Endpoints:	1) Survival 2) Gonad histopathology 3) Condition index (CI)

APPENDIX 1 (continued).

Fathead Minnow Assay #3: Adult Males

1. Test species:	Fathead minnow *(Pimephales promelas)*
2. Test duration/type:	4, 9, & 21 day/Static exposure w/ daily renewal and flow-through w/ 8 volume replacements/day
3. Age of test animals:	Sexually mature adults (5 - 6 months old)
4. Gender of test animals:	Male
5. Number of organisms/treatment:	5 - 6
6. Dilution water:	Aged aerated unchlorinated deep well water
7. Exposure treatments:	1) Control 2) Low Poultry Litter (417 mg/L) 3) High Poultry Litter (833 mg/L) for 4 days 4) High Poultry Litter (833 mg/L) for 9 days 5) High Poultry Litter (833 mg/L) for 21 days 6) High Poultry Litter (833 mg/L) flow-through for 21 days
8. Temperature (EC):	25 ± 1
9. Photoperiod:	16 h light/8 h dark
10. Light quality/intensity:	Fluorescent bulbs providing ~ 200 lux at surface of exposure aquaria
11. Test vessel:	37 L all glass aquaria
12. Volume of test solution:	Static: 24 L w/ 21 L siphoned and replaced daily Flow-through: 20 L w/ 6 vol. replacements/day
13. Feeding Regime:	Tetramin® tropical flake food (~ 2 % body weight) fed once/day prior to water renewal.
14. Aeration:	Gentle aeration via glass pipette
15. Endpoints:	1) Survival 2) Plasma vitellogenin (Vtg) 3) Gonadosomatic index (GSI) 4) Condition index (CI) *5) Gonad histopathology*

APPENDIX 1 (continued).

Sheepshead Minnow and Mummichog Assay #1: Adult Male Sheepshead Minnows

1. Test species:	Sheepshead minnow *(Cyprinodon variegatus)*
2. Test duration/type:	21 day/Static exposure w/ daily renewal
3. Age of test animals:	Sexually mature adults (~ 6 to 8 months old)
4. Gender of test animals:	Male
5. Number of organisms/treatment:	10
6. Dilution water:	Wye river estuarine water adjusted by dilution with aged aerated unchlorinated deep well water to a salinity of ~ 10 ‰
7. Exposure treatments:	1) Control 2) Positive Control (~100 ng 17 β-Estradiol/L) 3) Solvent Control (~2 µL EtOH/L)
8. Temperature (EC):	25 ± 1
9. Photoperiod:	16 h light/8 h dark
10. Light quality/intensity:	Fluorescent bulbs providing ~ 200 lux at surface of exposure aquaria
11. Test vessel:	37 L all glass aquaria
12. Volume of test solution:	20 L w/ 18 L siphoned and replaced daily
13. Feeding Regime:	Tetramin® tropical flake food (~ 2 % body weight) fed once/day prior to water renewal.
14. Aeration:	Gentle aeration via glass pipette
15. Endpoints:	1) Survival 2) Plasma vitellogenin (Vtg) induction 3) Gonad histopathology 4) Gonadosomatic index (GSI) 5) Condition index (CI)

APPENDIX 1 (continued).

Sheepshead Minnow and Mummichog Assay #1: Mixed Larval Sheepshead Minnow

1. Test species:	Sheepshead minnow *(Cyprinodon variegatus)*
2. Test duration/type:	21 day/Static exposure w/ daily renewal
3. Age of test animals:	Test initiated w/ 1 day old fish
4. Gender of test animals:	Mixed
5. Number of organisms/treatment:	20
6. Dilution water:	Wye river estuarine water adjusted by dilution with aged aerated unchlorinated deep well water to a salinity of ~ 10 ‰
7. Exposure treatments:	1) Control 2) Positive Control (~100 ng 17 β-Estradiol/L) 3) Solvent Control (~2 µL EtOH/L)
8. Temperature (EC):	25 ± 1
9. Photoperiod:	16 h light/8 h dark
10. Light quality/intensity:	Fluorescent bulbs providing ~ 200 lux at surface of exposure aquaria
11. Test vessel:	10 L all glass aquaria
12. Volume of test solution:	5 L w/ 4.5 L siphoned and replaced daily
13. Age of test animals:	Test initiated w/ 1 day old fish
14. Gender of test animals:	Mixed
15. Number of organisms/treatment:	20
16. Feeding Regime:	Fed live < 24 h old Artemia nauplii at a rate of ~ 150 nauplii/fish/day prior to water renewal
17. Aeration:	Gentle aeration via glass pipette
18. Endpoints:	1) Survival 2) Whole-body homogenate Vtg induction 3) Gonadal histopathology

APPENDIX 1 (continued).

Sheepshead Minnow and Mummichog Assay #2: Adult Male Sheepshead Minnow

1. Test species:	Sheepshead minnow *(Cyprinodon variegatus)*
2. Test duration/type:	21 day/Static exposure w/ daily renewal
3. Age of test animals:	Sexually mature adults (~ 6 to 8 months old)
4. Gender of test animals:	Male
5. Number of organisms/treatment:	6
6. Dilution water:	Wye river estuarine water with an ambient salinity of ~ 15 ‰
7. Exposure treatments:	1) Control 2) Low Poultry Litter (417 mg/L) 3) Medium Poultry Litter (833 mg/L) 4) High Poultry Litter (1,667 mg/L)
8. Temperature (EC):	25 ± 1
9. Photoperiod:	16 h light/8 h dark
10. Light quality/intensity:	Fluorescent bulbs providing ~ 200 lux at surface of exposure aquaria
11. Test vessel:	37 L all glass aquaria
12. Volume of test solution:	20 L w/ 18 L siphoned and replaced daily
13. Feeding Regime:	Tetramin® tropical flake food (~ 2 % body weight) fed once/day prior to water renewal
14. Aeration:	Gentle aeration via glass pipette
15. Endpoints:	1) Survival 2) Plasma vitellogenin (Vtg) induction 3) Gonad histopathology 4) Gonadosomatic index (GSI) 5) Condition index (CI)

APPENDIX 1 (continued).

Sheepshead Minnow and Mummichog Assay #1: Adult Male Mummichog

1. Test species:	Mummichog *(Fundulus heteroclitus)*
2. Test duration/type:	21 day/Static exposure w/ daily renewal
3. Age of test animals:	Sexually mature adults (of indeterminate age), field collected and acclimated for ≥14 days prior to test initiation
4. Gender of test animals:	Male
5. Number of organisms/treatment:	10
6. Dilution water:	Wye river estuarine water adjusted by dilution with aged aerated unchlorinated deep well water to a salinity of ~ 10 ‰
7. Exposure treatments:	1) Control 2) Positive Control (~100 ng 17 β-Estradiol/L) 3) Solvent Control (~2 µL EtOH/L)
8. Temperature (EC):	25 ± 1
9. Photoperiod:	16 h light/8 h dark
10. Light quality/intensity:	Fluorescent bulbs providing ~ 200 lux at surface of exposure aquaria
11. Test vessel:	37 L all glass aquaria
12. Volume of test solution:	20 L w/ 18 L siphoned and replaced daily
13. Feeding Regime:	Tetramin® tropical flake food (~ 2 % body weight) fed once/day prior to water renewal
14. Aeration:	Gentle aeration via glass pipette
15. Endpoints:	1) Survival 2) Plasma vitellogenin (Vtg) induction 3) Gonad histopathology 4) Gonadosomatic index (GSI) 5) Condition index (CI)

APPENDIX 1 (continued).

Sheepshead Minnow and Mummichog Assay #2: Adult Male Mummichog

1. Test species:	Mummichog *(Fundulus heteroclitus)*
2. Test duration/type:	21 day/Static exposure w/ daily renewal
3. Age of test animals:	Sexually mature adults (of indeterminate age), field collected and acclimated for ≥14 days prior to test initiation
4. Gender of test animals:	Male
5. Number of organisms/treatment:	6
6. Dilution water:	Wye river estuarine water with an ambient salinity of ~ 15 ‰
7. Exposure treatments:	1) Control 2) Low Poultry Litter (417 mg/L) 3) Medium Poultry Litter (833 mg/L) 4) High Poultry Litter (1,667 mg/L)
8. Temperature (EC):	25 ± 1
9. Photoperiod:	16 h light/8 h dark
10. Light quality/intensity:	Fluorescent bulbs providing ~ 200 lux at surface of exposure aquaria
11. Test vessel:	37 L all glass aquaria
12. Volume of test solution:	20 L w/ 18 L siphoned and replaced daily
13. Feeding Regime:	Tetramin® tropical flake food (~ 2 % body weight) fed once/day prior to water renewal
14. Aeration:	Gentle aeration via glass pipette
15. Endpoints:	1) Survival 2) Plasma vitellogenin (Vtg) induction 3) Gonad histopathology 4) Gonadosomatic index (GSI) 5) Condition index (CI)

APPENDIX 2: FISH DEPLOYMENT CAGES

The second objective of the *Controlled Field Investigation* was addressed by caging fish in the *Research Pond* during and after the first runoff event subsequent to field litter application. The fish caging system consisted of 4 L floating mesh baskets housed within large (approximately 150 L) cylindrical high density polyethylene barrels. The barrels had large windows on the sides and top to facilitate water circulation. Windows were screened with heavy mesh (~1/4") to prevent predators (e.g., largemouth bass, snapping turtles, etc) from gaining access to the caged fish. Barrels were attached via stainless steel cables to wooden poles (2 x 4s) driven into the sediment and locks were installed on the tops to deter tampering (while unlikely at the WREC facility, the caging system was designed for subsequent field deployment in areas where the likelihood of vandalism was much greater). The floating baskets were made from 4 L wide-mouth polyethylene bottles with 2 mm mesh installed over side windows. Floats were installed so baskets could remain at the surface as water levels in the barrels varied according to levels within the pond.

More specifically, exterior protective barrels are made from cylindrical 30 gal heavy-walled polyethylene tanks (US Plastics part # 4152 or equivalent). Tanks are 45 cm (18”) diameter x 76 cm (30”) tall. Four large windows (40 cm x 25 cm) are cut in the sides of each barrel and another is cut in the top. Mesh covers (0.5 – 1.0 cm aperture) are affixed to the windows. In high-energy areas or if security is a concern, perforated stainless steel covers are used. Otherwise, polyethylene mesh covers are sufficient. Stainless covers are attached using stainless bolts, nuts and washers. Plastic mesh can be attached using nylon bolts, nuts and washers. A length of 4” schedule 40 PVC pipe (60

cm) is attached vertically to the outside of the barrel using stainless bolts, nuts, etc. This allows "lacing" of the barrel over the support pole so it can be raised or lowered as necessary. Three eyebolts are secured equidistantly around the perimeter of the barrel 7.5 cm below the top lip (it may be necessary to reinforce the barrel at the points of attachment). Similarly, three eyebolts are attached to the cover such that they align with the bolts on the barrel. Stainless cables are attached to the eyebolts on the barrel and fashioned with loops such that when passed through the eyebolts on the cover they meet in the center thus securing the cover to the barrel. A padlock (brass and stainless steel) can be used to secure the cover minimizing the likelihood of vandalism.

Interior floating baskets selected based on the size and number of fish to be housed. Small (1 L) wide-mouth polyethylene bottles with a 2 mm mesh have been used successfully to contain young sheepshead minnows (10 dph). Similarly, 4 L wide-mouth polyethylene bottles with a 5 mm mesh have been used to contain adult fathead minnows. Windows are cut in the sides of the bottles and mesh is attached with monofilament fishing line and sealed with silicone. Floatation is provided using a flexible closed-foam material (we used swimming pool "noodles" sliced to size).

The support pole can be a sharpened 2" x 4", a length of PVC pipe, or a screw-pile (galvanized pole w/ auger-tip). Eye-bolts are attached to the poles near the top and bottom. Once deployed, a stainless cable (3/16" dia.) is laced through the eyebolts and attached to the barrel so that it can be raised, lowered and locked in place at the desired depth. If lowered to its limit the barrel will rest on the bottom eye-bolt. Screw-piles are significantly more expensive than wood or PVC, but provide several clear advantages: (1) they are easily deployed *and* retrieved (other systems are often difficult to remove);

(2) they can be reused; (3) they are stable in depths up to 15'; (4) they are more secure then other materials.

MATERIALS AND COSTS

Barrel

		Stainless	Plastic	
Barrel 30 gal		$65	$65	
Barrel cover		$14	$14	
Mesh		$30	$5	
Nuts/Bolts		$7	$4	
Eye-bolts		$12	$12	
Stainless Cable		$3	$3	
Lock		$3	$3	
PVC (4")		$2	$2	Construction time
	Total	$136	$108	~3 hrs

Basket

		1 L	4 L	
Wide-mouth bottle		$3	$12	
Mesh		$1	$1	
Monofilament		$0.25	$0.50	
Silicone sealant		$0.50	$0.50	
Floatation		$0.25	$0.25	Construction time
	Total	$5	$14	~1/2 hr

Support Pole

		Wood	PVC	Screw-pile	
Pole		$3	$6	$45	
Eye-bolts		$4	$4	$4	
Stainless cable		$5	$5	$5	
Lock		$3	$3	$3	Construction time
	Total	$15	$18	57	~1 hrs

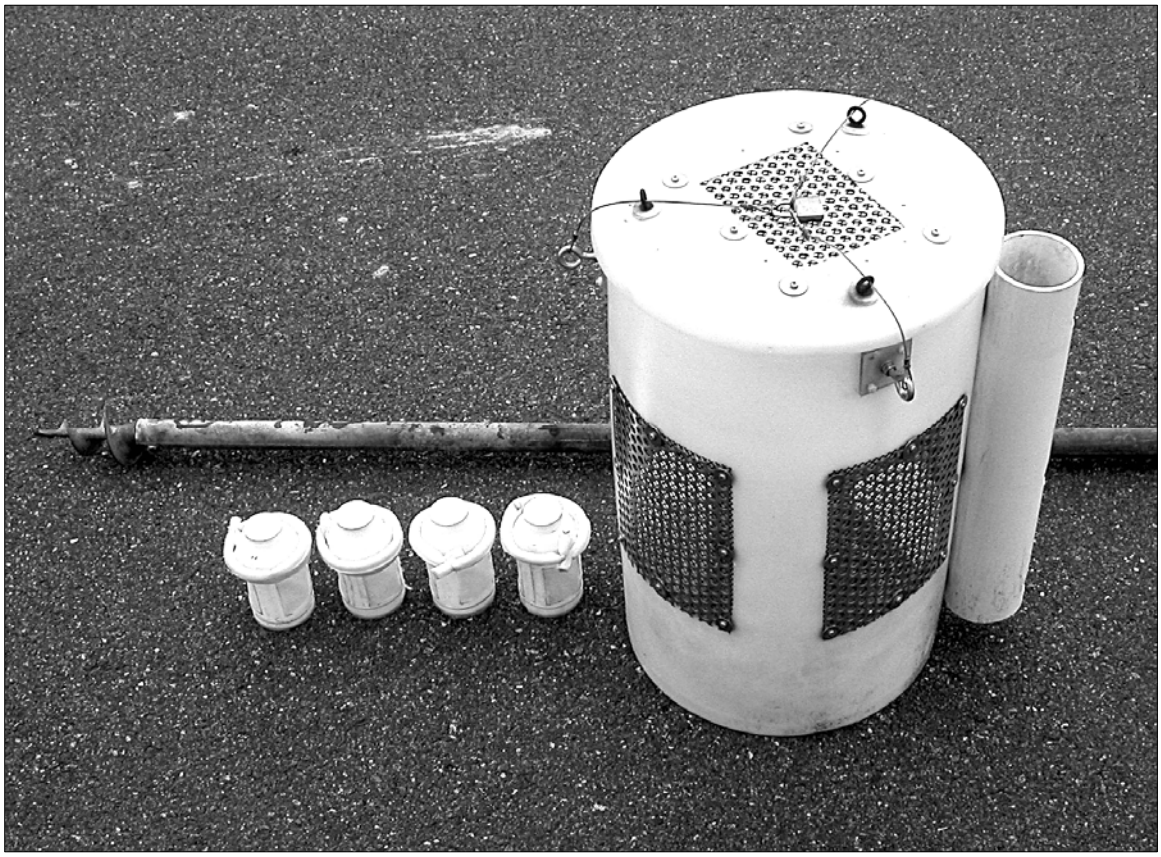


Figure 22. Detail of fish caging apparatus including protective barrel, floating baskets, and galvanized mounting post.

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*TECHNICAL TRAINING:*I have worked continuously at a Maryland State Bioassay Facility, housed variously at the Wye Research and Education Center and the Applied Physics Laboratory, since 1987. One of my primary responsibilities during this time involved conducting acute and short-term chronic bioassay tests on effluents from Maryland industries and municipal wastewater treatment facilities discharging into the Chesapeake Bay and inland tributaries. Test species included the fathead minnow, *Pimephales promelas*, sheepshead minnow, *Cyprinodon variegatus*, water flea, *Ceriodaphnia dubia*, and opossum shrimp, *Americamysis bahia*. In addition, I developed test methods and exposure apparatus for investigating residual toxicity of oxidant biocides (chlorine, chlorine dioxide and degradates) employed in wastewater and cooling water disinfection. My recent research has centered on identification of histopathological lesions and other biomarkers of effects in fish species exposed to endocrine disrupting chemicals. As part of this research I developed a histological atlas illustrating normal tissue conditions of the fathead minnow, *Pimephales promelas* (available online at http://aquaticpath.umd.edu/fhm/) and participant in the *Workshop on the Gonadal Histopathology of Small Laboratory Fish* (February 2004, Hannover, Germany) which provided training was specific to the interpretation of endocrine disruption associated histopathological lesions in fish gonadal tissue.

RESEARCH INTERESTS:

Active Research:

Investigate the environmental persistence and impact to aquatic resources of poultry litter-associated contaminants (including, steroids, antibiotics, pesticides, metals, etc.) in agricultural field runoff. Of principal interest are the sex steroids, 17β-estradiol and testosterone, which occur naturally and abundantly in litter, persist when applied as fertilizer and transport readily into surface and ground waters via runoff during rain events.

Identify and morphometrically quantify histopathological lesions indicative of endocrine disruption in fathead minnow, *Pimephales promelas,* and other small fish.

Investigate poultry litter induced endocrine disruption *in situ* by collecting resident fish and frogs and caging laboratory animals within impacted watersheds.

Modify the *Ceriodaphnia dubia* 7-Day Survival and Reproduction Assay to include production of male neonates as an indicator of endocrine disruption.

Investigate the chronic toxicity of Wye River sediments to the amphipod *Leptocheirus plumulosus* using suborganismal, organismal, population and community level indicators of stress. Develop histological and enzymatic markers of contaminant induced stress in *L. plumulosus.*

Examine the histological and hematological effects of chlorine dioxide and chlorite on the fathead minnow, *Pimephales promelas*.

Previous Research:

Participate in the Maryland Biomonitering Program conducting acute and short-term chronic bioassay tests on effluents from Maryland industries and municipal waste water treatment facilities discharging into the Chesapeake Bay and inland tributaries.

Develop a digital histological atlas illustrating normal tissue conditions of the fathead minnow, *Pimephales promelas* (online at http://medschool.umaryland.edu/AquaticPath/fhm).

Develop Water Quality Criteria for chlorite (ClO_2^-), the primary decay product of chlorine dioxide (ClO_2), a possible substitute for chlorine in certain disinfection and antifouling applications.

Investigate the residual toxicity of chlorine, chlorine dioxide, and chlorite following dechlorination with the sulfur (IV) compound sodium metabisulfide.

Participate in interlaboratory validation of the *Leptocheirus plumulosus* chronic sediment toxicity test method.

Participate in field validation of the *Leptocheirus plumulosus* chronic sediment toxicity test method using contaminated sediments from the Baltimore Harbor/Patapsco River system.

Investigate the acute whole effluent toxicity of storm water from an international airport (BWI) following application of de-icers and anti-icers (ethylene and propylene glycol) during winter storm events.

Determine the acute and chronic effects of chlorine dioxide on freshwater and estuarine organisms under continuous and intermittent (2-h) exposure regimes.

Determine the acute effects of chlorine and bromine on freshwater and estuarine organisms under continuous and intermittent (2-h) exposure regimes.

Determine the acute and chronic effects of chlorine on estuarine organisms under continuous and intermittent (2-, 4-, 6-, 8-h) exposure regimes.

Establish a baseline of coral species diversity and percent coral cover on a threatened reef near La Paguera, Puerto Rico.

Determine the suitability of the sheepshead minnow, *Cyprinodon variegatus,* for use as a test organism in low salinity estuarine waters.

Perform field and laboratory studies investigating the effects of periodic hypoxia/anoxia on benthic organisms in Chesapeake Bay.

Compare the suitability of clam shell, oyster shell, PVC, and rubber as setting substrates for larvae of the eastern oyster, *Crassostrea virginica*.

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