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Final Report

Poultry Litter-Associated Contaminants: Environmental Fate and Effects on Fish

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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 U.S. 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* (PLACs). 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 PLACs dynamics in natural systems and our incomplete understanding of endocrine disruption (ED) as a consequence of PLACs exposure make any assessment of risk premature. This study was conducted to provide information on some of these areas of uncertainty by quantifying steroid levels in poultry litter, agricultural field runoff and receiving waters, and by exposing fish to PLACs concentrations in laboratory and controlled field settings.

Major objectives of the study were to:

(1) survey steroid concentrations in poultry litter/manure from regional poultry operations.
(2) determine steroid levels in groundwater from research fields at the University of Maryland Wye Research and Education Center (UMD – WREC) which have previously received poultry litter application.
(3) determine the surface transport of steroids and metals via runoff from conventional and no-till fields at UMD – WREC immediately following application of poultry litter and throughout the year.
(4) determine the capacity of actual runoff from a poultry litter amended field to induce endocrine disruption in the laboratory and the field. This objective addresses the discrepancy [Fisher *et al.*, 2003] between PLACs effects from aqueous poultry litter extracts prepared in the laboratory and those from natural field runoff.
(5) determine the effects of PLACs exposure on the reproductive capacity of adult fathead minnows (*Pimephales promelas*) in the laboratory.

These objectives were investigated by: (1) applying poultry litter to large scale research fields under standard agronomic practices; (2) measuring resultant steroid and metal levels in groundwater runoff and receiving waters; and (3) exposing test animals to runoff material in laboratory and controlled field settings.

Application of poultry litter on research fields at the University of Maryland Wye Research and Education Center (UMD-WREC) in 2002 allowed specific monitoring of runoff over the entire planting season. Likewise, measurement of steroids in litter prior to application and subsequently in field runoff and a receiving pond facilitated determination of PLACs persistence and transport from fields into receiving bodies. Fish *(P. promelas)* were caged within the pond receiving litter-influenced agricultural runoff to discover "real world" effects of PLACs exposure on laboratory-reared animals. Additionally, exposure of *P. promelas* in the laboratory to litter-amended field runoff (frozen at the time of collection) was used to investigate PLACs effects under controlled conditions. Finally, a *Fathead Minnow Reproduction Assay* with breeding groups of *P. promelas* (USEPA approved protocol) was employed to investigate the effects of exposure to poultry litter-associated contaminant on fish reproductive capacity. Endocrine endpoints developed previously in the laboratory were used to assess effects in field and laboratory exposed animals.

RESULTS

Detection of considerable E2 in runoff from litter-amended research fields and in the receiving pond clearly demonstrated the transport of PLACs from fields to surface waters following rain events. Groundwater E2 concentrations were below detection, suggesting surface runoff was the primary mechanism of steroid transport. Steroid concentrations in field runoff were dependent on agronomic practices (e.g., No-Till vs. Conventional-Till) as well as rainfall intensity and duration. Resultant surface water E2 levels (measured in the No-Till receiving pond) were a direct function of runoff volume and contaminant concentration. Mature male fish caged in the receiving pond did not demonstrate evidence of ED. Similarly pond water returned to the laboratory did not induce ED in adult fish. However, 17-d exposure of mature male *P. promelas* to rations of litter-influenced field runoff (thawed and renewed daily) induced plasma vitellogenin (Vtg) levels >3,000 µg/mL, confirming that environmental contaminants resulting from standard agricultural practices are capable of causing ED in aquatic animals. Previous laboratory assays w ith larval *P. promelas* demonstrated endocrine disruption in adult and larval fish from PLACs samples generated in the laboratory. Results from the *Fathead Minnow Reproduction Assay* indicated that exposure to PLACs did not reduce reproductive output of sexually mature *P. promelas*, despite the induction of Vtg in breeding males, a known marker for endocrine disruption.

CONCLUSIONS

As of 2003, two separate experiments had been conducted by Dr. Fisher's research group at the WREC concerning the fate and effects of poultry litter runoff on the aquatic environment. Following is a review of the pertinent findings from both studies:

1. Substantial quantities of poultry litter-derived E2 can be transported to surface waters via runoff from agricultural fields [Current study and Fisher *et al.* [2003]]. 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. Thus, agricultural practices

intended to reduce nutrient runoff from fields (i.e., conventional tillage) can also help to reduce runoff of other contaminants, in this case steroids.

2. Poultry litter-derived E2 can enter surface waters via field runoff and persist for weeks to months at environmentally relevant concentrations [Current study and Fisher *et al.*, 2003]. 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 during the first study, higher than the 21 d Lowest Observable Effects Concentration (LOEC) of 40 ng/L identified in the laboratory [Fisher *et al.*, 2003]. The current field runoff study indicated levels in the Research Pond of only 30 ng/L, less than the laboratory-identified 21-d LOEC.

3. Runoff from poultry litter-amended agricultural fields in the current study was capable of causing endocrine disruption in mature male fathead minnows. Preserved field runoff (collected and frozen) was sufficiently estrogenic to induce vitellogenesis in male fish exposed in the laboratory (21-d). However, if allowed to "age" naturally, poultry litter-influenced surface water was not sufficiently estrogenic to promote vitellogenesis in adult male fathead minnows either *in situ* or in the 21-d laboratory exposures.

4. The current research shows that sexually mature male fathead minnows were induced to produce plasma Vtg to levels as high as 14,500 µg/ml after a 21-d laboratory exposure to PLACs. The reproductive competence of these fish was not reduced, nor were there measurable changes in gonad histopathology.

ONGOING RESEARCH

This study, in conjunction with previous laboratory research, clearly demonstrates the capacity of PLACs to induce endocrine-active effects on fish in laboratory settings. Further, transport of estrogenic PLACs has been demonstrated in runoff from research fields at concentrations sufficiently high to be of environmental concern. With continuing support from the MD Center for Agro-Ecology, Inc., several steps have been taken to assess PLACs-related risks to fish and other wildlife.

1. Surface waters in agricultural regions engaged intensively in poultry production have been broadly surveyed for evidence of poultry litter-derived estrogens. Nearly 60% of tested surface waters from the Choptank, Nanticoke, Wicomico, and Pocomoke watersheds had detectable E2 in spring 2004 samples.
2. Fish and amphibians have been collected from sites where E2 levels were highest and/or persistent. Plasma and gonadal tissues from representative specimens have been preserved and await final processing and analysis for Vtg induction and reproductive effects, respectively.
3. Confirmation of PLACs effects has been undertaken by repeating fathead minnow laboratory assays with additional exposure regimes and greater treatment replication. An assay to investigate the interplay between exposure concentration and exposure duration on Vtg induction and larval gender modification has recently been completed. Tissues await analysis upon completion of histological processing.
4. Development of a model for investigating PLACs effects on amphibian gender differentiation, Vtg induction, and time to completion of metamorphosis has also begun. Initial exposures of the African clawed frog *(Xenopus laevis)* are underway with tabulation of results expected early in 2006.

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INTRODUCTION

ENVIRONMENTAL ESTROGENS AND REPRODUCTIVE EFFECTS

The issue of endocrine disrupting chemicals (EDCs) came to public attention most recently after researchers connected environmental contaminants and reproductive and developmental abnormalities in various animal models and humans [Colborn and Clement, 1992]. These scientists concluded that anthropogenic chemicals may interfere with normal hormone function in ways that are not apparent in short term toxicity tests. Subsequently, Colborn *et al.* [1993] identified a large number of chemicals in use today (pesticides and industrial chemicals) that may act at low doses to disrupt endocrine function. The result was the endocrine disruptor hypothesis, which has since been studied by the National Academy of Sciences [NRC, 1999], professional organizations [Kendall *et al.*, 1996; Rolland *et al.*, 1997; deFur *et al.*, 1999] and federal agencies [Kavlock *et al.*, 1996]. This hypothesis states that some exogenous chemicals can interact with hormonal systems to alter normal function of those systems and the processes they control. Often this action occurs at low dose exposures, in subtle ways, over long periods and on sensitive life stages.

The premise of the endocrine disruptor hypothesis is based on observations that some chemicals are known to mimic natural hormones (agonists), while other chemicals are known to interfere with hormones (antagonists). In this way, synthetic chemicals interfere by initiating or suppressing hormonal activities at the wrong times and/or in the wrong tissues [see Colborn *et al.*, 1993; Kavlock *et al.*, 1996]. Exposure of vertebrates to EDCs has been reported to produce a variety of deleterious effects associated with developmental, reproductive and metabolic function [Colborn *et al.*, 1993; Guillette *et al.*, 1994; Kavlock *et al.*, 1996; Ankley *et al.*, 1998]. Fish and amphibians are particularly sensitive to EDCs during early development [Tyler *et al.*, 1999; Bevan *et al.*, 2003; Mackenzie *et al.*, 2003] as are amphibians during metamorphosis [Crump *et al.*, 2002]. Exposure to EDCs, including exogenous steroid hormones, can reduce fecundity, hatch, and larval survival, compromise growth, modify sex hormone concentrations, alter gonadal differentiation and morphology and produce developmental abnormalities [Jobling *et al.*, 1998; Ankley *et al.*, 2001; Mackenzie *et al.*, 2003]. Detection of the egg-yolk protein precursor, vitellogenin (Vtg) in males is a robust indicator of exposure to an estrogenic stimulus [Colborn *et al.*, 1993; Sumpter and Jobling, 1995; Panter *et al.*, 1998] and has been shown in some instances to be predictive of subsequent reproductive and histopathological effects [Tyler *et al.*, 1999].

Most of the better understood biological effects of EDCs involve compounds that interfere with the normal action of estrogen. Estrogens, especially 17 β-estradiol (E2), play a critical role in the development of the reproductive tract and sexual differentiation in vertebrates. Estrogens regulate growth, differentiation and function of diverse tissues within and outside of the reproductive system. Some of these pathways have been relatively well characterized while others are poorly understood or remain to be discovered. Most activities of estrogen(s) are dependant upon binding to the nuclear estrogen receptor (ER) [O'Malley *et al.*, 1991]. Environmental agents with estrogenic activity including organochlorine pesticides, PCBs and alkylphenol polyethoxylates have been shown to disrupt reproductive development in humans and wildlife [Ankley *et al.*, 1998; Jobling *et al.*, 1996]. However, the actual estrogenic potency of these environmental contaminants correlates to their affinity for the ER and is typically very

low when compared to E2 and other natural estrogens (e.g. estrone). For this reason, environmental exposure to natural sex steroids is of particular concern.

ENVIRONMENTAL DEGRADATION ASSOCIATED WITH AGRICULTURE/ANIMAL FEEDING OPERATIONS

The 2002 US Census of Agriculture reports 400,000 animal feeding operations (AFOs) in the US 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 industry has experienced the greatest 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 application of manure in excess of crop requirements and ultimately ecosystem enrichment [Lander and Moffitt, 1996]. The past quarter-century has seen a dramatic increase in water quality problems associated with the disposal of animal waste. Of US streams and rivers classified as impaired approximately 70% are degraded as a result of agricultural runoff [USEPA/USDA, 1998].

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 produces 600 million birds and 1.6 billion lbs. of manure/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]. 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, National Oceanic and Atmospheric Administration, 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, 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].

Finally, concerns have arisen over release to the environment of natural and synthetic endocrine disrupting chemicals (EDCs) as a result of agricultural activity. 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) [Shore *et al.*, 1995; Nichols *et al.*, 1997; 1998]. 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 fields amended with litter. Increases in poultry production and consolidation into dense AFOs have dramatically increased the volume of poultry litter on the Delmarva Peninsula. Field application of this material, often in excess of crop requirements, introduces copious poultry litter-associated steroids to regional watersheds. 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 a 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.

IMPACTS TO WILDLIFE - GENERAL

Exposure of vertebrates to EDCs has been reported to produce a variety of deleterious effects associated with reproduction and metabolic function [Colborn *et al.*, 1993; Kavlock *et al.*, 1996]. 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 the 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, the result being a reduction in egg viability and subsequent decline in the number of juvenile alligators [Woodward *et al.*, 1993]. In birds, EDCs are particularly potent in the embryo and impact the action of endogenous hormones at critical stages of development. Normally, endogenous steroids are maternally deposited in eggs or are produced by embryos later in development [Adkins-Regan *et al.*, 1995; Ottinger *et al.*, 2001a,b]. Laboratory embryonic exposures to exogenous steroids or EDCs have caused permanent reproductive axis abnormalities resulting in reduced fertility, decreased production, and behavioral impairment [Ottinger and Abdelnabi, 1997]. In the field, decreased growth and reproduction have occurred following EDC exposure, ultimately resulting in population decline [Fry *et al.*, 1987; Rattner *et al.*, 1993; McGary *et al.*, 2001; Ottinger *et al.*, 2002; Quinn *et al.,* 2002]. Fish are also most sensitive to the effects of EDCs during reproduction and early development. Exposure to exogenous estrogens can reduce fecundity, hatch, and larval viability, compromise growth, and produce developmental anomalies especially in reproductive tissues [Purdom *et al.*, 1994; Panter *et al.*, 1998; Tyler *et al.*, 1999]. In these ways, EDC exposure jeopardizes future reproduction and compromises population stability.

ENDOCRINE DISRUPTION IN FISH RESULTING FROM POULTRY LITTER APPLICATION

Because E2 is present in high concentrations in poultry litter and has been measured in field runoff and receiving waters at concentrations known to elicit estrogenicity in birds and fish, there is the potential for adverse impacts on resident species. Historic and current-use application of persistent pesticides on the Delmarva Peninsula has resulted in contaminant accumulations in sediment [Miller *et al.*, 1999]. Many such contaminants are known or suspected EDCs. Of potential EDCs identified in poultry litter, naturally occurring E2 demonstrates the highest bioactivity. Given the intensity of agricultural land use and the enormous quantities of poultry litter generated and applied to fields on the Delmarva Peninsula (1.6 billion lbs. annually) the magnitude of adverse impacts could be substantial.

In 2000, a NOAA National Sea Grant College study was initiated by Fisher *et al.* [2003] to develop and apply biomarkers to evaluate endocrine disruption in fish as a result of poultry

litter application. The work was conducted at the University of Maryland Wye Research and Education Center aquatic toxicology laboratory and at associated experimental fields. The project was divided into laboratory and field components. Application of poultry litter on research fields allowed specific monitoring of runoff over an entire planting season. Measurement of steroids, especially E2, in poultry litter prior to application and subsequently in field runoff and a receiving pond demonstrated persistence and movement of poultry litter-derived contaminants from fields into receiving bodies. A series of laboratory assays were conducted in which the freshwater fathead minnow, *Pimephales promelas*, and the estuarine sheepshead minnow, *Cyprinodon variegatus*, and mummichog, *Fundulus heteroclitus*, were exposed to aqueous solutions of poultry litter. Tissue effects, especially on gonads, were assessed histologically and plasma vitellogenin (Vtg) levels were measured as a gauge of estrogenic exposure. Vtg, a phospholipoglycoprotein precursor to egg yolk protein, is synthesized in the liver of sexually mature females of all oviparous species. Production of Vtg is controlled by interactions of estrogens, predominantly E2, with the estrogen receptor [Sumpter and Jobling, 1995]. Males of many species maintain the capacity to produce Vtg in response to stimulation from estrogens and/or estrogen agonists. Detection of Vtg in males can, therefore, be exploited as a biomarker of exposure to estrogenic compounds of exogenous origin. Similarly, detection of Vtg in immature fish of either gender can be indicative of exogenous estrogenic exposure.

The principal aims of the laboratory assays were to identify sensitive reproductive endpoints and determine lower effects levels of poultry litter-derived contaminants. In a controlled field exposure, fish were caged in a research pond receiving runoff from the research fields following the application of poultry litter mentioned above. Fish were also caged in several streams on the Delmarva Peninsula in watersheds believed to be at high risk of impact from intensive animal agriculture. Resident fish were also taken from these streams. Sediment and water column samples were collected from the sites to identify contaminants of likely poultry litter origin.

Results from this research provide direct evidence that endocrine disrupting effects in fathead minnows can result from laboratory exposure to poultry litter-derived contaminants at environmentally relevant concentrations. Plasma Vtg was induced to levels of >40,000 µg/mL in adult male fathead minnows exposed in the laboratory to aqueous extracts of poultry litter. Induction of Vtg occurred in >40% of fish exposed for 21 days to a litter treatments containing 40 ng/L E2. Histologic examination of testes revealed reductions in the proportion of mature gametes but only in a high litter treatment and in an E2 positive control.

Larval fathead minnows showed the most consequential effects of exposure to litter-derived contaminants. The proportion of fish identifiable as females at 60 days post hatch (dph), based on the presence of oocytes within the ovary and/or a presumptive oviduct, exceeded 90% in a 21 day exposure to a poultry litter treatment containing 74 ng/L E2. Presumably genotypic male fish either underwent a gender reversal, becoming phenotypic females, or were sufficiently feminized to express a distinctly female oviduct. This result also occurred to a lesser degree (75% identifiable females) in a litter treatment containing E2 at 40 ng/L. Identifiable females at 60 dph in control treatments numbered 30% to 35%.

Detection of E2 in runoff and in the retention pond clearly demonstrated PLACs transport from research fields to receiving waters following rain events. Mean E2 levels measured in the research pond during *in situ* exposures were actually above effects concentrations identified in laboratory assays. Despite this, similar effects to those seen in laboratory exposures of fathead

minnows did not occur in caged *in situ* exposures of adult male fish. One unanswered question from this study was: *Why can we induce Vtg in the laboratory with aqueous PLACs samples but do not get Vtg induction in field exposed fish?* Reported environmental concentrations of poultry litter-derived contaminants, especially E2, were sufficiently high to warrant additional *in situ* investigations.

Sheepshead minnows and mummichogs were not particularly sensitive to the endocrine disruptive effects of poultry litter. Positive controls (100 ng E2/L) readily induced Vtg in adult males of both species. Despite this finding, male sheepshead minnows were completely non-responsive to poultry litter-derived E2 at all test concentrations while male mummichogs were only slightly responsive. Male fathead minnows were much more sensitive to poultry litter-associated contaminants with Vtg induction apparent at exposure levels as low as 40 ng E2/L compared to 144 ng E2/L for the mummichog. Unlike adult males, larval sheepshead minnows were not particularly responsive to E2 even in the *E2 Control* exposure. A very modest increase in whole-body homogenate Vtg suggests that this species at this early age is not well suited as an indicator of exogenous estrogenic exposure. Histologic assessment of mummichog testes was used to identify a reduction in reproductive competence based on abundance and distribution of spermatozoa within germinal epithelium, but only in the E2 positive control treatment. No such reduction occurred in the sheepshead minnow positive control.

Results from a companion US Fish and Wildlife Service (FWS) study (McGee *et al.*, 2003), performed coincident with the Sea Grant study, provides evidence for transport of tetracycline compounds, and possibly E2, from poultry litter applied fields to adjacent water bodies on the Delmarva Peninsula. Furthermore, Vtg analyses suggest that fish (i.e., carp) on the Delmarva Peninsula are being exposed to estrogenic compounds. While field data are limited, the potential for system-wide estrogen and antibiotic contamination affecting fish and other aquatic resources of the Delmarva Peninsula merits further investigation.

OBJECTIVES

The primary intent of this project is to assess the fate and consequence of PLACs, primarily the natural hormone E2, following introduction to the environment via application of poultry manure/litter to agricultural fields as fertilizer. Components of the project will address the persistence, bioavailability, transport and ecotoxicology of E2 associated with poultry litter.

Research conducted at the Wye Research and Education Center (WREC) has addressed the flow of nutrients from poultry litter amended fields into streams and receiving waters [Staver and Brinsfield, 1995]. However, no systematic survey of steroid hormones, endocrine disrupting chemicals (EDCs), or other contaminants in litter has been conducted in our region, despite routine use of poultry litter as a fertilizer on agricultural fields. Additionally, little research exists on the consequences of long-term environmental exposure of vertebrate populations to contaminants originating from poultry litter. Building on the findings from the earlier NOAA Sea Grant study [Fisher *et al.,* 2003], the specific objectives for the current project are as follows:

(1) to survey steroid levels in poultry litter/manure from regional poultry operations.
(2) to determine steroid levels in groundwater from research fields at WREC which have previously received poultry litter application.

(3) to determine the surface transport of steroids and metals via runoff from conventional and no-till fields at WREC immediately following application of poultry litter and throughout the year.

(4) to determine the capacity of actual runoff from a poultry litter amended field to induce endocrine disruption in the laboratory and the field. This objective addresses the discrepancy [Fisher *et al.*, 2003] between PLACs effects from aqueous poultry litter extracts prepared in the laboratory and those from natural field runoff.

(5) to determine the effects of PLACs on the reproductive capacity of adult fathead minnows (*P. promelas*) in the laboratory.

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. 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 seed 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 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 this study 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 2002. 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 -20°C until required. Samples for contaminant analysis were collected either directly from the pile or as material was transported for field application. Samples from six additional poultry litter sources, all originating from Eastern Shore broiler operations, were also analyzed for E2 and T for this project. Water-extractable E2 and T samples were prepared from poultry litter material according to the methods of Nichols *et al.* [1997; 1998]. Resulting aqueous samples were analyzed via radioimmunoassay (RIA) by personnel at the Virginia Institute of Marine Science (VIMS), Gloucester Point, VA. Sample preparation and analysis are described in the *Chemical Analysis* beginning on page 12.

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. Monthly reference toxicity tests (KCl) were employed to assess sensitivity of the cultures. Advantages of this 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 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 1)*. Typically 40 to 70 µl of whole blood was obtained from each adult male specimen. 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 calculation of gonadosomatic index (GSI).

Brain Aromatase

Cytochrome P450 aromatase is responsible for conversion of C19 androgens to C18 estrogens in brain and gonadal tissues of vertebrates [Callard *et al.,* 1978]. As such, aromatase activity is an important modulator of E2 levels and is critical in the regulation of processes under E2 control. Evidence that a variety of chemicals are capable of inhibiting aromatase activity has led to concern that environmental contaminants might elicit adverse reproductive and developmental effects via this mechanism of action [Ankley *et al.,* 2002].

Aromatase activity measurement. Following plasma collection, brains from male and female fathead minnows were dissected whole, placed in potassium phosphate buffer (100 µL) and stored at -80°C for future processing. Samples were homogenized for 15 sec. using a Fisher Powergen 125 homogenizer (Fisher Scientific, Pittsburgh, PA, USA). After addition of 30 µL

Figure 1. Collection of whole blood from an adult male fathead minnow *(Pimephales promelas)* via incision into the caudal sinus.

NADPH, and 12 µL ^{3}H-androstenedione (the precursor metabolite produced during aromatization from androgen to estrogen [Griffin and Ojeda, 2000]), samples were incubated at 25˚C in a shaking water bath for three hours. The reaction was stopped with addition of 1.7 mL deionized H_2O and 4 mL methylene chloride. After centrifuging for 15 min. at 3000 rpm, the supernatant was removed and incubated for 20 min. in a 150 mL microcentrifuge tube containing dextran coated charcoal (made up in a 5% solution and air-dried), at 4˚C for 20 minutes. After 20 min., tubes were centrifuged at 2000 rpm for 10 min. The supernatant was again removed (200 µL) and added to a scintillation vial containing 300 µL H_20 and 5 mL of scintillation cocktail. Vials were then counted in a beta counter for 5 min. per vial. Counts were measured as disintegrations per minute (DPM) with aromatase activity, reported as the rate (femtamoles/hour*organ), determined by the formula:

$$\frac{((\text{DPM}) - 0 \text{ for Blanks (DPM)}) * (\text{Total vol. in sample } (\mu\text{L})/\text{Aliquot amount } (\mu\text{L}))}{(\text{Specific activity of } ^{3}\text{H-androstenedione } (\mu\text{Ci})) * (\text{Time for incubation of the assay (h)})}$$

Condition Index

General health and somatic vigor of adult fish was estimated by calculation of a condition index (CI): body wt expressed as a proportion of the length cubed: [CI = (body wt/lgth^{3}) x 100] where body weight and length were recorded in mg and mm, respectively. Typical mature male fathead minnow CIs are ~1.0 (unitless measure).

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 = (gonad wt/body wt) x 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.

Histology

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 exposures 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, phenotypic 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, hematoxylin and eosin (H&E) stained and covered with glass cover-slips. Testes 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.

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 2)*.

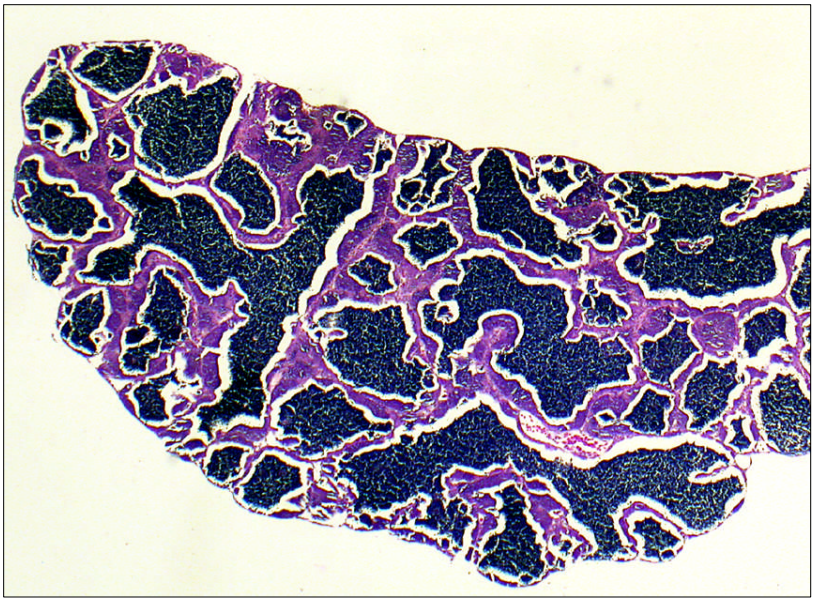
Several steps were required to produce this ratio. Initially, multiple sections of tissue from each specimen were digitally photographed and images stored to disk. Digital traces were made of entire segments of interest and of basophilic regions within these segments (i.e., regions occupied by spermatozoa 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.

CHEMICAL ANALYSIS

17β-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

(a)



(b)

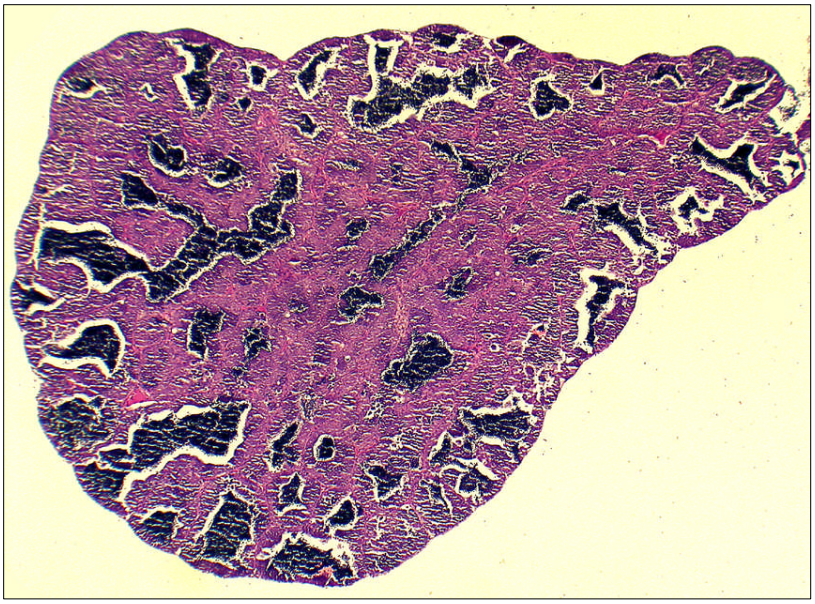


Figure 2. Examples of fathead minnow *(Pimephales promelas)* testis sections characterized by high ***(a)*** and low ***(b)*** proportions of spermatozoa and spermatids (dark blue regions) relative to total area. Morphometric analysis determined areas of sections ***(a)*** and ***(b)*** occupied by spermatozoa/spermatids to be 55.2% and 18.8%, respectively (H&E; 40x).

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 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 poultry litter from several sources; material used in the field exposure and material from the 6 other whole-house scrape-outs of Eastern Shore broiler operations. Aqueous PLACs 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 -20°C 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 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 50°C water bath and stored (-20°C) 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 plasma Vtg was measured 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 the 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) received 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.).

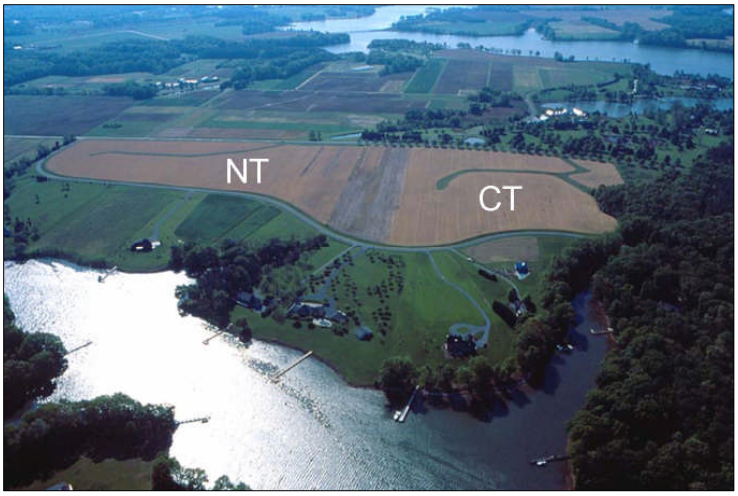
FIELD INVESTIGATIONS

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 growing season using two adjacent ~35 acre agricultural research fields at the UMD - WREC *(Figure 3a)*. 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. An earlier study using poultry litter application was conducted on these fields in 2000 [Fisher *et al.*, 2003].

During the 2002 planting season one field was cultivated according to conventional-tillage practices of turning surface soil (~20 cm depth) into furrows before planting while the other was cultivated using no-till practices. 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 3b)*. 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 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 2c)*. 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 investigation 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

(a)



(b)

(c)

Figure 3. 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 (NT = No-Till; CT = Conventional-Till).

events that produced runoff from No-Till and/or Conventional-Till fields for the remainder of the growing season 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 (*Figure 3c*). 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 PLACs 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 4)*. 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.

On 3/19/02 poultry litter from an Eastern Shore broiler operation was delivered to UMD - WREC. 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. Litter was applied to the research fields at 3 ton/ac on 5/8/02. The first rain event after application occurred on 5/18/02 with rain falling from 0330 – 1030 hrs. 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 -20°C 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 the first rain event (05/18/02) 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 -20°C in anticipation of subsequent steroid analysis.

Fish exposures in 2002 were similar to those described in Fisher *et al.* [2003]. Barrels in Research and Reference Ponds each received 10 mature male fathead minnows (5/replicate basket). To avoid undue stress during the actual rain event, fish were not deployed until 1930 hr, near the conclusion of runoff into the Research Pond. In addition, 3 groups of fish were also

(a)

(b)

(c)

Figure 4. 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.

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 this event by thawing batches of 6 cubitainers (24 L) for daily water renewal. Laboratory exposures were conducted at 23±1°C 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® Tropical 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* in the Research Pond for 21 d after the 5/18/02 rain event 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 field investigation was only 17 d.

SAMPLING OF GROUNDWATER FOR STEROIDS

Researchers have reported that T, but not E2, may reach receiving waters via groundwater transport following field litter application [Shore *et al*., 1995]. Therefore, groundwater within the Conventional-Till and No-Till watersheds was monitored for evidence of residual steroids from previous poultry litter application before initiation of the 2002 field study. The two research fields had received litter applications twice previously, in 1998 and 2000. The groundwater data collection system, installed during a previous study at this site, consisted of a stratified network of wells within the groundwater discharge zone, and was designed to monitor water chemistry and determine rates of hydrologic discharge. Eighteen wells on or adjacent to the conventionally tilled (n=11) and No-Till (n=7) fields were sampled on 4/22/02, 16 days prior to spring litter application. Polyethylene bottles (250 mL) received 200 mL of sample which was promptly frozen (-20°C) in anticipation of subsequent steroid analysis.

REPRODUCTIVE ASSAY WITH FATHEAD MINNOWS

Previous assays performed in our laboratory have shown that contaminants in poultry litter are capable of inducing vitellogenin (Vtg) in adult male fathead minnows *(Pimephales promelas)* at environmentally relevant exposure levels [Fisher et al, 2003]. We performed a *Fathead Minnow Reproduction Assay* to investigate the effects of PLACs on fecundity and hatching success using the method of Ankley *et al.*, [2001]. Four replicate breeding groups of 6 month old reproductively competent adult fathead minnows (2 male and 4 female/ replicate) were exposed to each of two aqueous poultry litter solutions (E2 levels of 33 and 70 ng/L) and to positive (85 ng E2/L) and aged aerated well water controls for 21 days in a flow-through system. Three breeding substrates, made from 15 cm sections of PVC pipe cut in half lengthwise, were added to each aquarium at the beginning of the test. Females lay eggs on the ceiling of the substrates where they are fertilized and guarded by attendant males until hatch *(Figure 5)*.

A 14-d pre-exposure breeding period was conducted prior to the initiation of contaminant exposures to allow for selection of actively breeding fish for the actual exposures. Twenty

breeding groups were maintained during the pre-exposure period. The 16 groups with the greatest reproductive output during this time were randomly assigned to the various exposure treatments. Breeding substrates were collected daily and eggs that had been laid in the previous 24 h period were counted. New breeding substrates were added after the removal of the substrates. *Figure 6* shows a breeding substrate with fertilized and eyed egg. The substrates were then maintained in control water until hatch. Egg fertilization/viability was recorded daily until all eggs had hatched. The assay endpoints were plasma Vtg expression in male fish, fecundity, egg production, egg fertilization and hatching success. Fish were fed Tetramin® flake food daily and observed for stress/mortality.

A schematic of the test system is shown in *Figure 7*. New poultry litter stock solution was made daily by introducing 475 g of litter to 190 L of well water (i.e., 2.5 g/L). This mixture was stirred/aerated for 24 h before being filtered (1 μm) and transferred to the poultry litter stock chamber. Litter used in this experiment was from the same batch used in field exposures discussed previously. An E2 positive control stock was prepared daily by spiking 60 L of well water with a concentrated E2 solution to achieve a final concentration of 250 ng/L. Poultry litter and E2 solutions were pumped from stock chambers to mixing funnels suspended above individual 40 L test aquaria using peristaltic pumps. Diluent water (aged aerated well water) was delivered from the diluent water delivery pipe to the mixing funnels using adjustable "dippy bird" glass siphons. The flows from the siphons and the peristaltic pumps were calibrated to maintain the appropriate PLACs or pure E2 concentrations. Temperature in exposure aquaria was maintained via thermostatically controlled water bath. Similarly, temperature of diluent water was maintained using heaters in an elevated head tank and a large capacity holding/recirculation tank. Standard water quality parameters (temperature, dissolved oxygen, pH, conductivity and NH_3) were monitored daily in all exposure aquaria.

DATA ANALYSIS

Differences in reproductive output, plasma chemistry, and GSI between exposure groups were tested using one way analysis of variance (ANOVA, SigmaStat3.0). Where assumption of normality and homogeneity were met, ANOVA was followed by Dunnett's multiple comparison versus the control. When assumptions were not satisfied, data were analyzed using Kruskal-Wallis one way ANOVA on ranks followed by Student Newman-Keuls multiple comparison procedure. Statistical significance throughout the experiment was assumed at $p < 0.05$.

Figure 5. Fathead minnow *(Pimephales promelas)* male guarding breeding substrate.

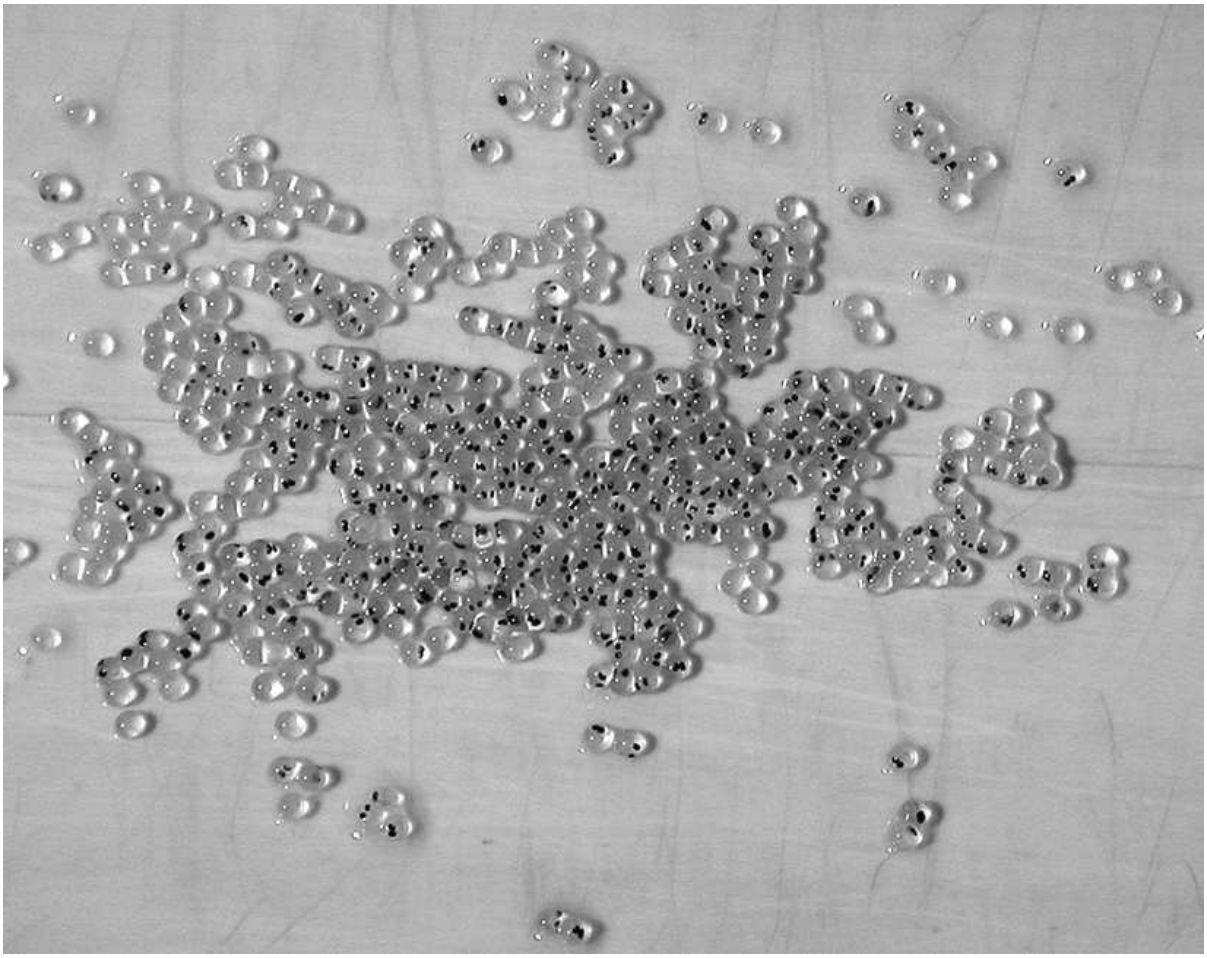


Figure 6. Fathead minnow breeding substrate with embryos at various developmental stages.

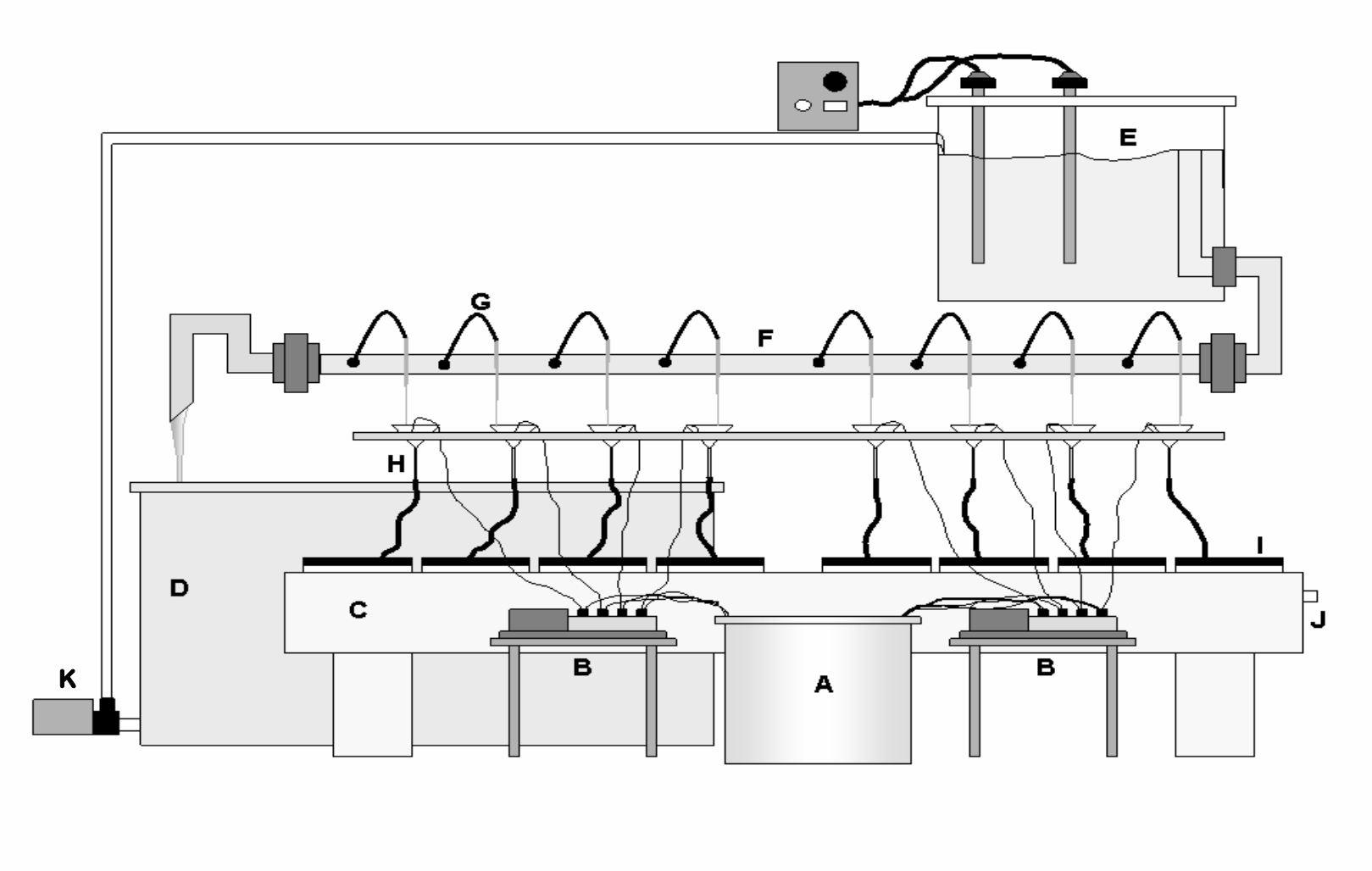


Figure 7. Exposure apparatus for Fathead Minnow Reproduction Assay. A) Poultry litter stock chamber; B) Peristaltic pumps and delivery lines to mixing funnels; C) Water bath (heated); D) Diluent storage tank; E) Diluent head tank with heaters; F) Diluent delivery pipe; G) Diluent delivery "dippy birds"; H) Mixing funnels and delivery tubes to aquaria; I) Exposure aquaria; J) Waste to septic system, K) Pump.

RESULTS

STEROIDS IN POULTRY LITTER

Water-soluble E2 and T levels in litter used in field applications and for the *Fathead Minnow Reproduction Assay (WYE2002)* were 86 ng/g and 19 ng/g, respectively *(Table 1)*. 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]. Also included in *Table 1* for comparative purposes are E2 (108 ng/g) and T (34 ng/g) values for a broiler litter used at WREC in an earlier study [Fisher *et al.*, 2003].

STEROIDS IN GROUNDWATER, FIELD RUNOFF, AND RECEIVING WATERS

Estradiol was not detected in groundwater collected from any of the 18 wells located on or adjacent to the research fields. An 18 ng/L method detection limit (MDL) precluded measurement of E2 below this level. Testosterone was only detected in groundwater from a single well (NT-2) at a level of 6.3 ng/L, only slightly above the 6.0 ng/L MDL for T.

Water-soluble E2 in *WYE2002* poultry litter was measured at 86 ng/g at the time of field application. The first rain event after litter application occurred on 5/18/02. This 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 8)*. The 5/18/02 rain event lacked sufficient precipitation to produce runoff from the Conventional-Till field. However, the next rain event, starting 17 days later, 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 9)*. 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.

FIELD EXPOSURE OF FISH

On 6/4/02, 16 days after fish were caged in the *Reference* and Research Pond*s,* 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 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.

Table 1. 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 (±SD)	126 (23.7)	70 (13.3)	0.54	42 (11.9)	45 (16.5)	1.03
range			0.46 - 0.61			0.79 - 1.30

† Wet weight basis

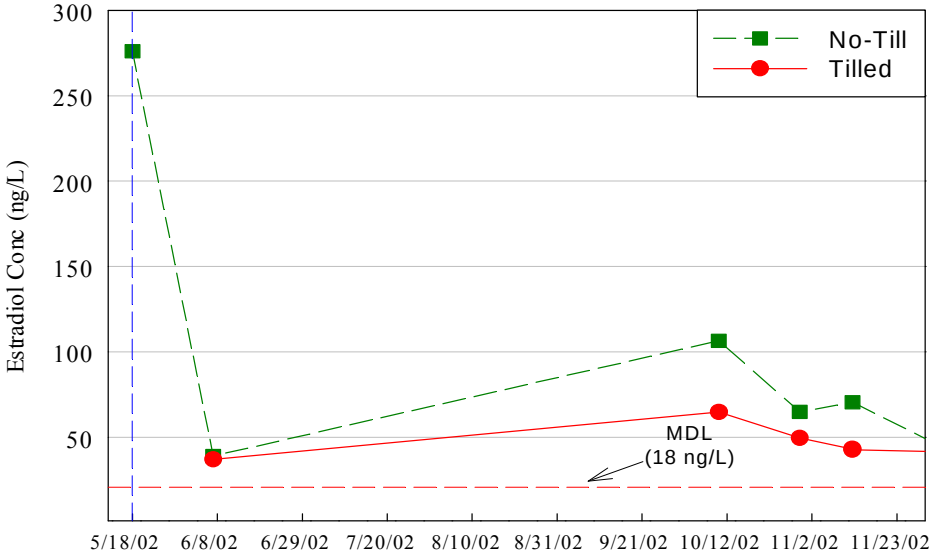


Figure 8 17 β-estradiol levels in runoff from No-Till and Conventional-Till fields collected throughout the 2002 growing season (blue line indicates the first runoff following litter application; red line indicates method detection limit).

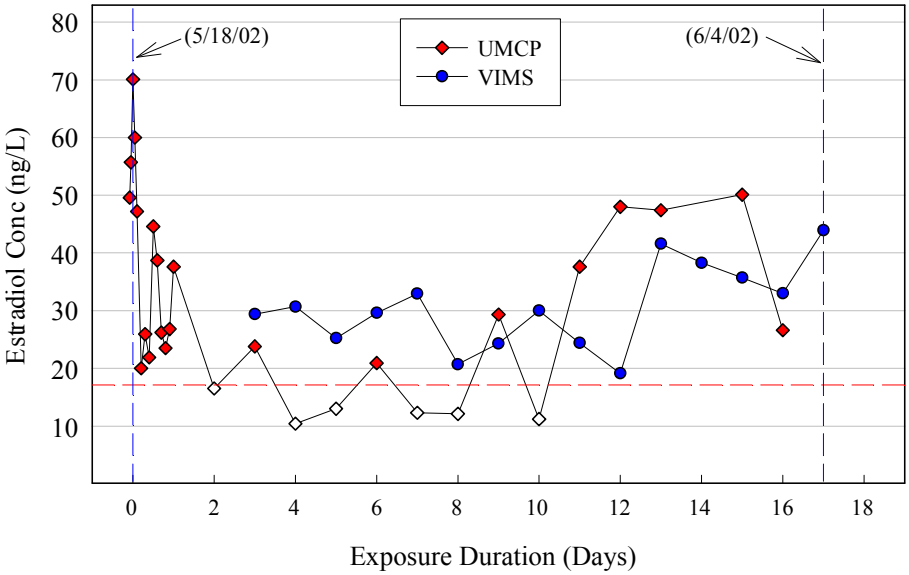


Figure 9. 17 β-estradiol levels in a University of Maryland research pond receiving No-Till field runoff following spring 2002 poultry litter application (analyses performed initially by Sara Pollock, University of Maryland, corroborated by Barbara Rutan, Virginia Institute of Marine Sciences). Fish 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.

After encountering hypoxic conditions in the Research Pond in an earlier 2000 field study (consequent to high runoff nutrient loads), aerators were installed in fish cages to avoid low oxygen related stress. Water quality parameters for laboratory and field fish exposures are provided in *Table 2*. Because the 5/18/02 runoff event was less severe then the 2000 event, water quality was not as severely impacted. Temperature in the ponds fluctuated daily reflecting changes in air temperature and sunlight intensity. Temperature of laboratory treatments was held constant at 24 ± 1°C. 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 24°C 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 minnows 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 for assessment of ED endpoints *(Table 3)*. 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. Aromatase activity in adult males was variable within and across groups with no indication of a treatment effect. 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 investigations of poultry litter effects in 2000 (Fisher *et al.*, 2003), apoptotic spermatogenic cells occurred infrequently within most specimens with no clear treatment related differences.

Table 2. 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 (°C)	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

Table 3. Summary of results of *Spring 2002 Controlled Field Exposure* of mature male fathead minnows *(Pimephales promelas)* to poultry litter-amended agricultural field runoff.

Treatment	n	17 β-Estradiol (ng/L)	Plasma Vtg (µg/mL)	Aromatase activity (fmol/hour)†	GSI (%)	Testis maturity	
						Rank‡	Proportion‡
Control 1	7	n/a	6.3 ± 4.40	1,813 ± 663	1.27	4.1	0.53
Research Pond/Lab	7	30.4 ± 18.95	2.6 ± 1.94	2,078 ± 1,838	1.89*	4.2	0.47
Reference Pond/Field	5	23.9 ± 21.1	3.9 ± 2.01	1,216 ± 621	1.79*	3.7	0.57
Research Pond/Field	8	30.4 ± 18.95	6.5 ± 11.08	1,792 ± 954	1.96*	3.9	0.47
Control 2	10	n/a	3.2 ± 2.14	1,201 ± 448	1.65	4.5	0.65
Flume/Lab	9	146.7	3,146 ± 2,585*	1,465 ± 896	1.95	4.5	0.69

† Aromatase activity measured in whole brains of individual fish

‡ See methods section titled *Testis maturity ranking*.

* Treatment differs significantly form corresponding *Control* level ($p < 0.05$)

METALS IN POULTRY LITTER AND FIELD RUNOFF

Copper (Cu) and zinc (Zn) were measured in *WYE2002* litter samples collected from discrete manure spreader loads (see *Figure 3b*) immediately prior to field application. Mean (±SD) levels for Cu and Zn were 364.8 ± 45.5 µg/g and 411.0 ± 126.4 µg/g, respectively. Arsenic (As), Cu, and Zn were measured in all runoff events from conventionally tilled and No-Till fields resulting from precipitation between May 2002 (immediately after litter application) and March 2003. Mean metals levels for each runoff event are presented in *Figure 10.* In general, concentrations are greatest in the first event following litter application and decrease in subsequent events. Further, metals levels tend to be higher in runoff from the No-till field then the conventionally tilled field. This holds particularly for As and Cu, but not so much so for Zn.

REPRODUCTIVE ASSAY WITH FATHEAD MINNOWS

Water quality data for the reproductive assay are presented in *Table 4*. During the 21 d exposure interval all water quality parameters fell within acceptable ranges except ammonia (NH_3) in the *High PLAC* treatment. Temperature, measured continuously during the assay, remained stable at 25.0 ±1.0°C. Increases in PLACs concentration had the effect of depressing DO and pH marginally below control levels (#0.5 mg/L and #1.0 units, respectively) while increasing total NH_3 (*Low PLAC* and *High PLAC* NH_3 levels averaged 2.2 mg/L and 5.0 mg/L, respectively). The water quality criterion for chronic ammonia exposure at pH 7.5 and temperature 25°C is 2.2 mg NH_3/L [USEPA, 1989]. *High PLAC* and *Low PLAC* treatments had average E2 concentrations of 70 and 33 ng /L, respectively, while the *E2 Control* treatment had an average E2 level of 85 ng/L.

Vtg was expressed in 100% of males (n = 8) from *E2 Control* and *High PLAC* treatments and 37% of males from the *Low PLAC* treatment (*Figure 11*). Vtg expression in male *Control* fish was near or below detection limits and not indicative of exposure to an estrogenic stimulus. Aromatase activity was again variable within and across treatments and between genders. Mean activities appeared reduced in *E2 Control* animals (both male and female), but high variability precluded detection of statistical differences. There was a statistically significant 30% reduction in GSI in male fish from the *E2 Control* compared to the *Control* ($p < 0.05$). Egg production was very good in poultry litter treatments but poor in *Control* and *E2 Control* treatments (*Figure 12*). By the end of the exposure period breeding pairs in the *Low* and *High PLAC* treatments had cumulatively produced 5,323 and 4,580 eggs, respectively, compared to cumulative production in *Control* and *E2 Control* treatments of 1,416 and 890 eggs, respectively. Egg production in both litter treatments differed significantly from *Control* levels (one-way ANOVA with Dunnett's multiple comparison versus control; $p < 0.05$). Percent egg fertilization was fair in *Control* (70%) and *E2 Control* (64%) treatments but poor in *Low PLAC* (52%) and *High PLAC* (53%) treatments. However, treatments did not differ significantly (one-way ANOVA; $p = 0.455$). Hatching success of fertilized eggs was good (80 – 90%) in all treatments.

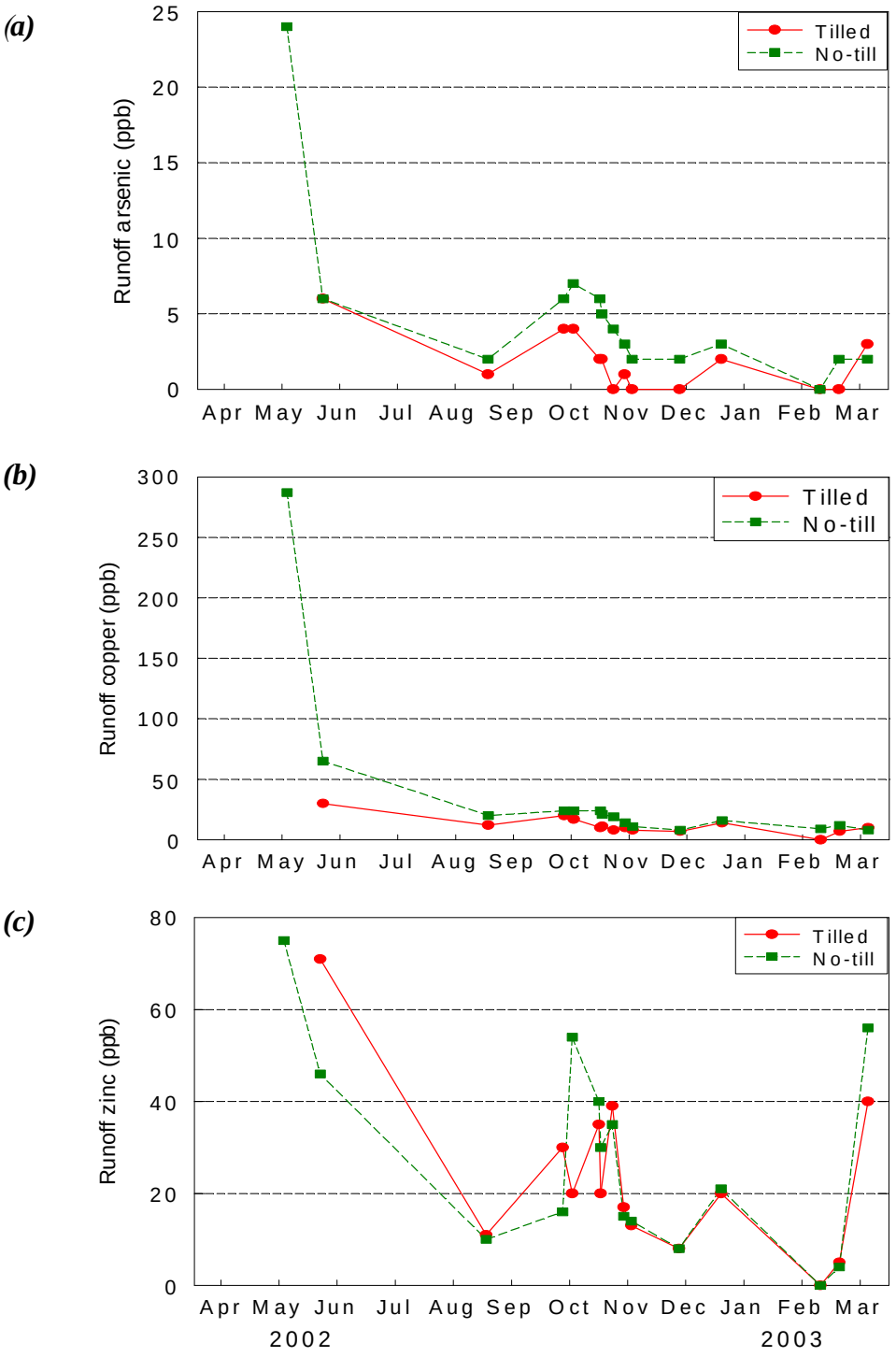


Figure 10. Mean concentrations of ***(a)*** arsenic, ***(b)*** copper and ***(c)*** zinc in runoff from "Tilled" and "No-Till" research fields at the University of Maryland Wye Research and Education Center from May 2002 (immediately post litter application) to March 2003.

Table 4. Summary of water quality measurements for the *Fathead Minnow Reproduction Assay.*

Treatment		pH	DO (mg/L)	Conductivity (µmhos)	Ammonia (mg/L)
Control	Mean	7.62	7.8	276	0.2
	Min	7.36	7.4	250	<0.1
	Max	7.90	8.4	300	0.4
Low PLAC	Mean	7.35	7.2	332	2.2
	Min	7.04	6.3	320	1.0
	Max	7.59	8.1	350	2.7
High PLAC	Mean	7.40	7.0	389	5.0
	Min	7.17	3.6	330	2.8
	Max	7.68	8.0	410	5.8
E2 Control	Mean	7.64	7.8	277	0.2
	Min	7.20	7.3	260	<0.1
	Max	7.88	8.4	290	0.4

Table 5. 17 β-Estradiol exposure concentrations and results of *Fathead Minnow Reproduction Assay* (mean ± SD).

Treatment	Sex	n	17 β-Estradiol Exposure Conc. (ng/L)	Plasma Vtg (µg/mL)	Aromatase Activity (fmol/hour) †	GSI (%)
Control	♂	8	n/a	1.9 ± 1.3	2,230 ± 976	1.58
	♀	16			1,988 ± 1,096	
Low PLAC	♂	8	32.5 ± 12.1	441 ± 621*	2,245 ± 2,001	1.68
	♀	16			2,876 ± 1,264	
High PLAC	♂	8	70.2 ± 16.8	14,517 ± 18,989*	2,927 ± 1,969	1.67
	♀	16			2,197 ± 1,718	
E2 Control	♂	8	85.8 ± 35.6	125,738 ± 50,124*	1,645 ± 815	1.11**
	♀	16			1,341 ± 595	

† Aromatase activity measured in whole brains of individual fish

* Treatment differs significantly form corresponding *Control* (Kruskal-Wallis one way ANOVA on ranks; Student-Newman-Keuls multiple comparison procedure; $p < 0.05$)

**Treatment differs significantly form corresponding *Control* (One way ANOVA with Dunnett's multiple comparison versus the control; $p < 0.05$)

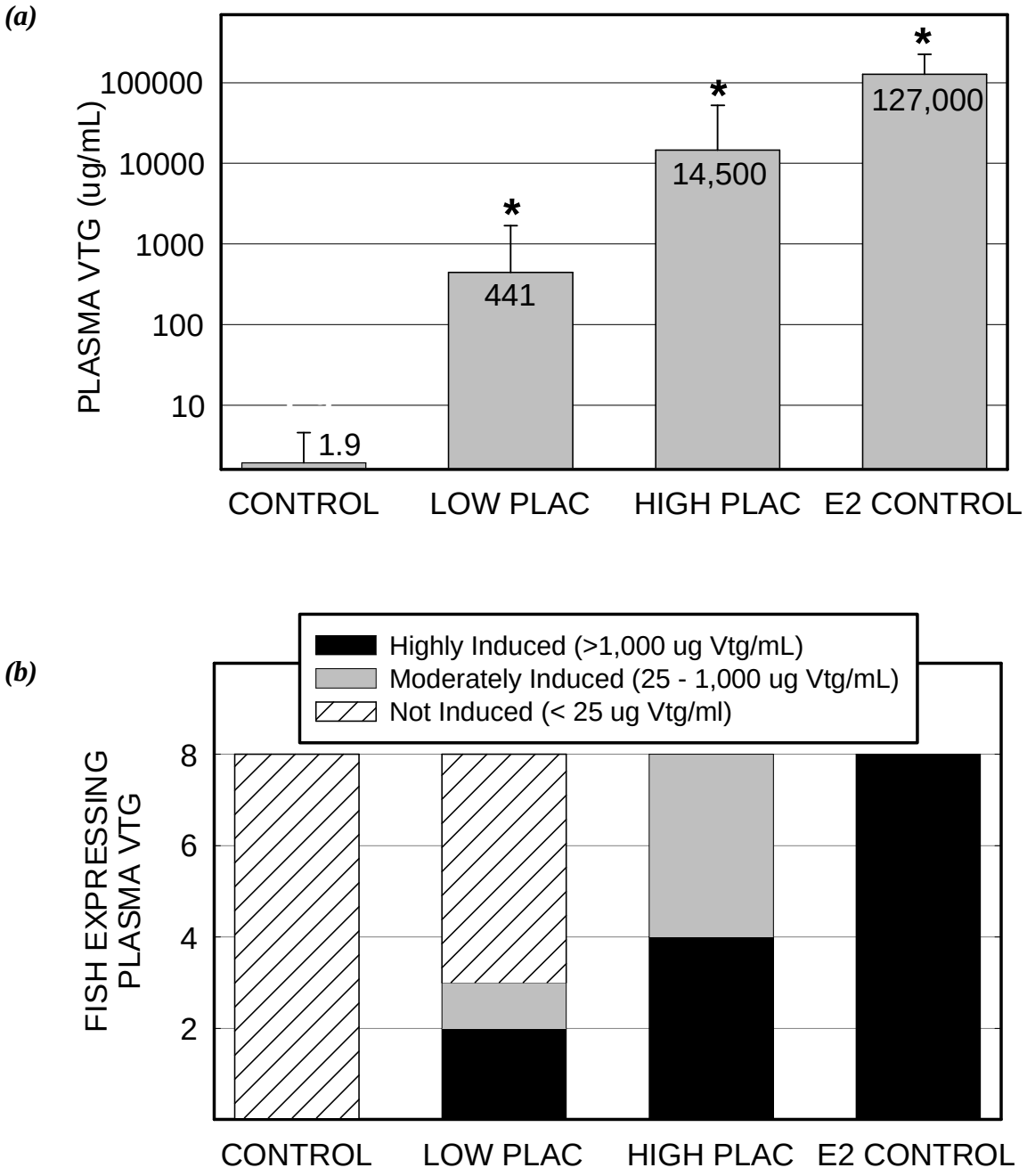


Figure 11. ***(a)*** Mean plasma vitellogenin (Vtg) levels in male fish from the *Fathead Minnow Reproduction Assay* and ***(b)*** proportion of male fish expressing moderate and high Vtg levels; (n = 8 fish/treatment). Asterisks (*) indicate treatments which differ significantly from the Control (Kruskal-Wallis one way ANOVA on ranks with Student-Newman-Keuls multiple comparison procedure; $p < 0.05$).

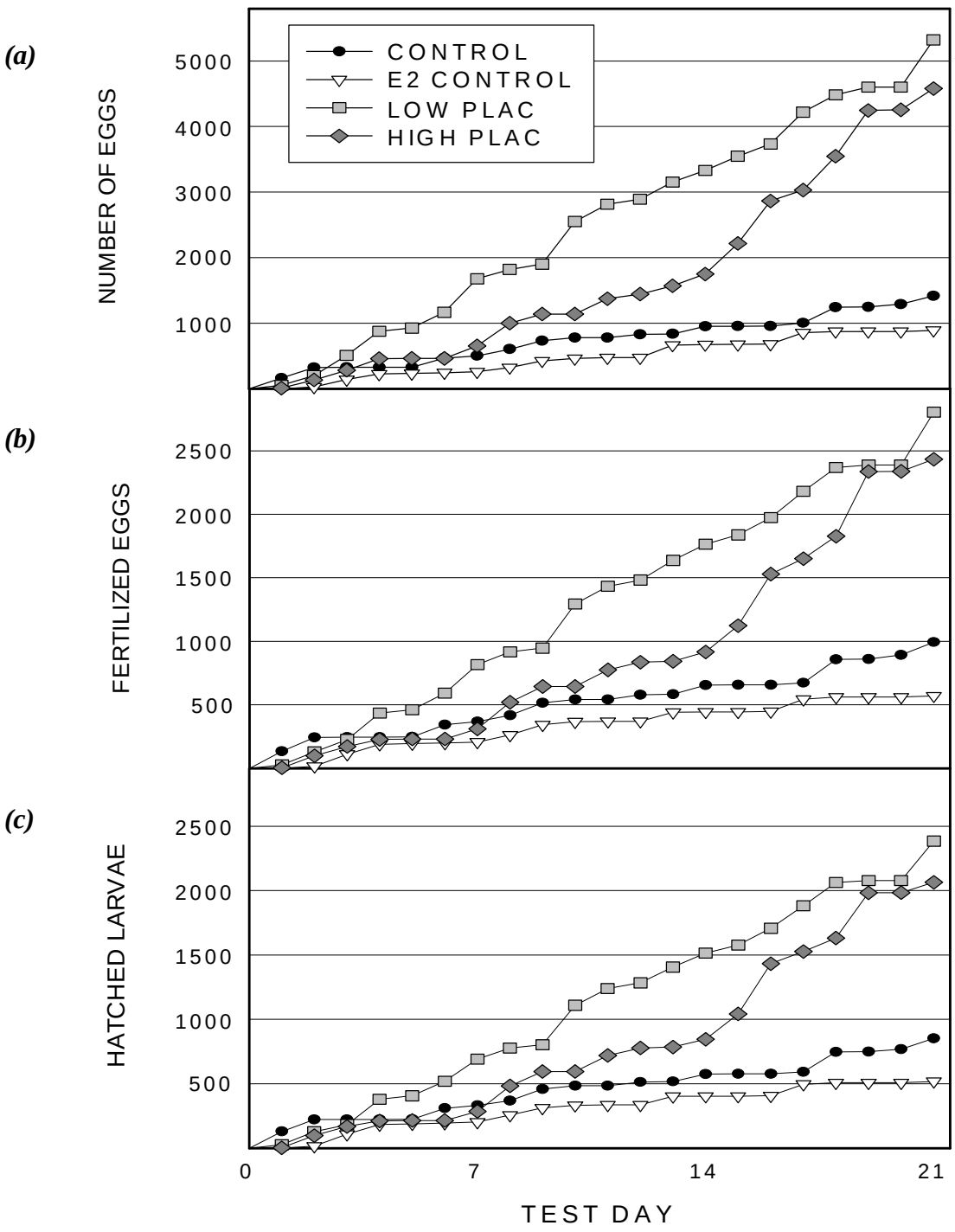


Figure 12. Fecundity endpoints for fathead minnow breeding groups exposed to poultry litter and 17 β-estradiol (E2) in the *Fathead Minnow Reproduction Assay:* cumulative number of ***(a)*** eggs produced; ***(b)*** eggs fertilized; and ***(c)*** hatched larvae.

DISCUSSION

CONTAMINANTS IN POULTRY LITTER

Chemical analysis of poultry litter *(Table 5)* conducted in an earlier project [Fisher *et al.*, 2003] 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 field corn seed pretreatment (as well as small grains and sorghum) it is still found in environmental samples with great regularity [USEPA, 2002]. 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 this litter sample, 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 1.0 µg/g from this 2000 poultry litter sample 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.

Table 6. Partial list of contaminants detected in poultry litter from a whole-house scrape-out of a standard broiler operation on the Delmarva Peninsula. Comprehensive analytical methods and results from [McGee *et al.*, 2003]. This litter was applied to the UMD - WREC fields in 2000 [Fisher *et al.*, 2003]

Compound	**Conc. Range**
Steroids (ng/g)	
17 β-Estradiol (E2)	245 - 449
Estrone (E1)	200
Testosterone (T)	74 - 114
Organochlorine Pesticides (ng/g)	
α-BHC	<MQL – 0.3
β-BHC	1.3 - 43
δ-BHC	<MDL – 0.3
Lindane	0.6 - 4.6
p,p'-DDT	<MDL – 2.9
p,p'-DDE	<MQL - 0.6
Endrin	<MDL - 3.7
Oxychlordane	<MDL - 13
trans-Chlordane	<MDL - 1.4
cis-Chlordane	<MDL - 0.6
Current Use Pesticides (ng/g)	
Acetachlor	<MQL - 11
Metolachlor	3.1 - 14
Trifluralin	<MQL - 5.6
Diazinon	0.8- 18
cis-Permethrin	<MDL – 6.6
trans-Permethrin	<MDL – 7.4
λ-Cyhalothrin	3.7 - 28
Atrazine	340 - 1300
Antibiotics (µg/g)	
Chlortetracycline	<MDL - 1.6
PAHs (ng/g)	
Benz[a]anthracene	210 - 270
Metals(µg/g)	
Copper	428 - 500
Zinc	450 - 539
Arsenic	30 – 45

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) [Briggs, 1992]. 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 2000 litter sample ranged from 0.34 - 1.3 ng/g. At a litter 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) [Solomon *et al.,* 1996]. This would seem to indicate that poultry litter-associated atrazine is an insignificant source of introduction to aquatic resources.

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]. The 2000 litter source, described above, 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.

17 β-ESTRADIOL (E2) AND TESTOSTERONE (T) LEVELS IN POULTRY LITTER/MANURE FROM REGIONAL POULTRY OPERATIONS

The advent of factory type farms for raising poultry has led to a dramatic increase in production and a consequent increase in poultry waste. This waste is high in steroid hormones (E2 and T) which are excreted naturally via normal metabolic processes. Agricultural field application of this material on the Delmarva Peninsula introduces copious poultry litter-associated steroids to regional watersheds. 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]. 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].

E2 and T were found in high concentrations in all eight litter sources that we have examined (*Table 1*). The steroid of greatest concern is E2, because it exerts physiological effects at lower exposure concentrations then other natural steroids and anthropogenic compounds and because it is often reported in the environment at concentrations above known effects thresholds [Shore and Shemesh, 2003]. 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. 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 [Hanselman *et al.,* 2003; Panter *et al.,* 1999]. Ingerslev and Halling-Sørensen [2003] maintain that de-conjugation of E2 need only occur at the C_3 position to allow binding to the estrogen receptor. 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 and Shemesh [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) (*Table 1*). 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 in 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 an Eastern Shore broiler operation which was 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, as discussed above, samples from 6 other poultry litter sources (also Eastern Shore broiler operations) were analyzed for steroidal constituents along with an earlier poultry litter that had been applied to the WREC fields in 2000 *(Table 1) (*Fisher *et al.*, 2003*)*. 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 water-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) at WREC has several advantages over other analytical systems [Ingerslev and Halling-Sørensen, 2003]. The most pertinent for this project were high cost effectiveness and rapid throughput of multiple samples. Radioimmunoassay analysis of steroidal contaminants in environmental samples does have

several 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) are reported to cross-react minimally (up to 16%) with steroidal metabolites, 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 the 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 1)*. 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, approximately one-half is free. Testosterone gave different results. 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)*(Table 1)*. 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, 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, 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 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, total ammonia in the *High PLAC* treatment of the reproduction assay averaged 5.0 ± 0.79 mg/L over the 21 d exposure interval. Ammonia in the *Low PLAC* treatment was approximately one-half, averaging 2.2 ± 0.45 mg/L. As this treatment was a 50% dilution of the higher treatment, 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 renewal. This trend, seen in the reproduction assays where poultry litter treatments were prepared daily, also held in preserved runoff water field collected during the 2002 rain event and thawed daily.

Steroid levels within and across assay treatments were somewhat less consistent. For example, in the reproduction assay, average E2 levels in *High PLAC* and *Low PLAC* treatments were 70 ng/L and 33 ng/L, respectively; reasonable given that one treatment was a 50% dilution of the other. However, E2 levels within treatments varied considerably over the 21 d exposure interval, ranging from 46 - 115 ng/L in the *E2 Control,* 52 – 85 ng/L in the *High PLAC* treatment and 22 - 46 ng/L in the *Low PLAC* treatment. Some of this variability may have resulted from analytical interference of organic material as described previously. However, 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, the measured concentration of E2 in the positive control treatment tended to decrease over the exposure period (despite identical daily augmentation of the stock chamber) such that exposure levels were ~ 60% lower at assay conclusion then assay initiation. E2 in poultry litter treatments followed a similar but less dramatic trend. 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 as much as 150% in some instances, but decreased as much as 69% in others.

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 an earlier 2000 study by Fisher *et al.* (2003), water-soluble E2 in litter applied to the research fields at UMD - WREC 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% (~ 2.3×10^6 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% (6.5×10^5 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 in this earlier study, and that contact with litter-associated E2 (distributed to a depth of 20 cm) was minimal. In contrast, 3½ 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 in this 2000 study (6/6/00) dropped 1.17 cm of rain of which 4.9% (7.6×10^4 L) ran from the No-Till field [Fisher *et al.*, 2003]. 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 from the current 2002 project were somewhat different. Water-soluble E2 in field-applied litter (*WYE2002)* was 86 µg/kg so total E2 applied to each field was approximately 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 runoff 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; Conventional-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% of 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.

Results from the two poultry litter runoff studies conducted at WREC illustrate some basic differences in No-Till and 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 β-ESTRADIOL IN RECEIVING WATER

Resultant contaminant levels in receiving waters are a consequence of runoff volume, contaminant concentration, and the size and nature of the 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 and earlier poultry litter studies at WREC 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. Despite limited data, trends emerge. For example, Fisher *et al.* [2003] found runoff from their first runoff event totaled 2.3×10^6 L with an average E2 concentration of 125 ng/L. Pond E2 before initiation of runoff was below detection. Assuming optimal mixing of runoff and pond water, the resulting E2 concentration would be 80 ng/L, a level that agrees very well with their measured pond concentrations which ranged from 63 to 83 ng/L during the period of runoff. The second 2000 event introduced only 7.6×10^4 L of runoff with an average E2 concentration of 58 ng/L. After dilution, this would be expected to increase the whole pond E2 level < 2 ng/L.

Runoff from the first rain event in the current study (5/18/02) had an average E2 concentration of 275 ng/L (*Figure 8*). 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 9* 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 and the earlier Fisher *et al.* [2003] 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 PLACs 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 in this project and an earlier poultry litter runoff project [Fisher *et al.*, 2003] after application of the litter. Runoff events differed significantly between the two years (described above) as did the consequent pond PLACs levels. The average E2 concentration during the 21 d cage exposure in 2000 was 50 ng/L, sufficient to induce vitellogenesis in male fathead minnows based on laboratory results [Fisher *et al.*, 2003]. Average E2 in 2002 (exposure only 17 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 (both years) or exposed to water transported from the pond to the laboratory (current study), endocrine disruption was not in evidence. On the other hand, water collected at the time of runoff in the current study 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. 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. Even male fish with elevated Vtg from the preserved flume exposures did not show reduced GSI or testis maturity indices compared to the control fish.

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

FATHEAD MINNOW REPRODUCTION ASSAY

The induction of Vtg in male fish has been accepted as a robust indicator of exogenous estrogenic exposure [Sumpter & Jobling, 1995]. Researchers have suggested that Vtg expression in male fish "may be interpreted as a warning of reproductive consequences, but that a lack of expression cannot be interpreted as absence of consequences" [Cheek *et al.*, 2001].

While reproductive consequences may exist, our research suggests that significant expression of Vtg in male fish does not preclude reproductive success nor does it cause histological changes in gonads as examined by GSI or testis maturity indices. This may be a result of starting these exposures with sexually mature adults which are producing sperm and eggs at test initiation. This test method did not examine larval exposures of young fish (e.g., one day post hatch) and possible changes in gonad differentiation that could lead to reproductive consequences as the fish grow. Fisher *et al.* [2003] found that these early life stage exposures to poultry litter can have dramatic effects on reproductive tissue development. The current research shows that sexually mature male fathead minnows attained a mean plasma Vtg levels of 14,000 µg/ml after a 21-day exposure to poultry litter-associated contaminants with no apparent reduction in reproductive competence.

CONCLUSIONS

As of 2003, two separate experiments have been conducted by Dr. Fisher's research group at the WREC concerning the fate and effects of poultry litter runoff on the aquatic environment. Following is a review of the pertinent findings from both studies:

1. Exposure to PLACs at environmentally relevant concentrations caused endocrine disrupting effect in mature male fathead minnows [Fisher *et al.*, 2003]. 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-derived 17 β-estradiol (E2) of 40 ng/L.

2. Gender differentiation in larval fathead minnows showed dose-dependant sensitivity to PLACs exposure [Fisher *et al.*, 2003]. 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 [Fisher *et al.*, 2003]. Male sheepshead minnows were completely non-responsive to all tested PLACs treatments and male mummichogs 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 [Current study and Fisher *et al.,* 2003]]. 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. Thus, agricultural practices intended to reduce nutrient runoff from fields (i.e., conventional tillage) can also help to reduce runoff of other contaminants, in this case steroids.

5. Poultry litter-derived E2 can enter surface waters via field runoff and persist for weeks to months at environmentally relevant concentrations [Current study and Fisher *et al.*, 2003]. 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 during the first study, higher than the 21-d Lowest Observable Effect Concentration (LOEC) of 40 ng/L identified in the laboratory [Fisher *et al.*, 2003]. The current field runoff study indicated levels in the Research Pond less than the 21-d LOEC of 40 ng/L identified in the laboratory by Fisher *et al.* [2003].

6. Runoff from poultry litter-amended agricultural fields in the current study was capable of causing endocrine disruption in mature male fathead minnows. Preserved field runoff (collected and frozen) was sufficiently estrogenic to induce vitellogenesis in male fish exposed in the laboratory (21 d). However, if allowed to "age" naturally, poultry litter-influenced surface

water was not sufficiently estrogenic to promote vitellogenesis in adult male fathead minnows either *in situ* or in the 21 d laboratory exposures.

7. 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 [Current study and Fisher *et al.*, 2003].

8. The current research shows that sexually mature male fathead minnows were induced to produce plasma Vtg to levels as high as 14,000 µg/ml after a 21-day exposure to poultry litter-associated contaminants. The reproductive competence of these fish was not reduced, nor were there measurable changes in gonad histopathology.

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.

ONGOING RESEARCH

This study, in conjunction with previous laboratory research, clearly demonstrates the capacity of PLACs to induce endocrine-active effects on fish in laboratory settings. Further, transport of estrogenic PLACs has been demonstrated in runoff from research fields at concentrations sufficiently high to be of environmental concern. With continuing supported from the MD Center for Agro-Ecology, Inc., several steps have been taken to assess PLACs-related risks to fish and other wildlife.

Beginning in 2004, surface waters in agricultural regions engaged intensively in poultry production were broadly surveying for evidence of poultry litter-derived estrogens. Nearly 100 sample sites on four Delmarva watersheds (Choptank, Nanticoke, Wicomico, and Pocomoke) were visited twice during spring rain events (shortly after litter application) and again during a fall low-flow period (shortly after crop harvesting). Nearly 60% of tested surface waters had detectable E2 in spring samples. Only a handful of sites had detectable E2 levels during fall sampling. In most instances E2 concentrations were below the 21-d LOEC of 40 ng/L established in laboratory exposures of fathead minnows. In the fall of 2005 fish and amphibians were collected from a variety of sites where E2 levels were highest and/or persistent. Plasma and gonadal tissues from representative specimens have been preserved and await final processing and analysis for Vtg induction and reproductive effects, respectively.

Confirmation of PLACs effects has been undertaken by repeating several previous fathead minnow laboratory assays with additional exposure regimes and greater treatment replication to allow more robust statistical analysis of test endpoints. A definitive assay to investigate the interplay between exposure concentration and exposure duration on Vtg induction and larval gender modification has recently been completed. Tissues await analysis upon completion of histological processing.

Development of a model for investigating PLACs effects on amphibian gender differentiation, Vtg induction, and time to completion of metamorphosis has also begun. Initial exposures of the African clawed frog *(Xenopus laevis)* are underway with tabulation of results expected early in 2006.

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