Evaluating the Potential Water Quality Impacts of Animal Feeding Operations on National Wildlife Refuges on the Delmarva Peninsula

CBFO-C03-03

Beth L. McGee[1]
Lance T. Yonkos [2]
Jim D. Petty [3]
Daniel J. Fisher [2]
David A. Alvarez [3]
Tom May [3]
Walter Cranor [3]
Jim Huckins [3]

Under supervision of:
John P. Wolflin, Field Supervisor[1]
Thomas McCabe, Deputy Supervisor[1]

[1] USFWS Chesapeake Bay Field Office
177 Admiral Cochrane Drive
Annapolis, MD 21401

[2] University of Maryland
Wye Research and Education Center
P.O. Box 169
Queenstown, MD 21658

[3] USGS Columbia Environmental Research Center
4200 New Haven Road
Columbia, MO 65201

July 2003

EXECUTIVE SUMMARY

The Delmarva peninsula is one of the largest poultry areas in the U.S., producing some 600 million chickens and 1.6 billion pounds of manure annually. In addition to nutrients, poultry litter contains feed additives such as trace metals and antibiotics, as well as naturally produced hormones. In spring 2000, a two-year field study was initiated to evaluate potential water quality impacts on National Wildlife Refuges (NWRs) associated with animal feeding operations (AFOs). Specifically, our objectives were to: 1) map locations of AFOs in the vicinity of refuge lands and identify watersheds at high and low risk for AFO-related impact; 2) conduct studies to evaluate chemical and biological impacts to these watersheds, with an emphasis on assessing the potential impact of animal feed additives and other "nontraditional" contaminants. The study included the deployment of polar organic compound integrative water samplers (POCIS), organic analysis of poultry litter and sediments, and the analysis of trace elements in litter, water, sediments and fish. Biological endpoints included analysis of vitellogenin (Vtg) in the blood of male fish as an indicator of exposure to endocrine disrupting compounds and benthic macroinvertebrate surveys.

In Year 1, a preliminary investigation was conducted at Prime Hook NWR, Sussex County, DE and Blackwater NWR, Dorchester County, MD because refuge staff expressed the concern that AFO activities located upstream may be impacting the refuge waters. Three sites were sampled at each refuge, two potentially impacted sites, upstream of the refuge, and one downstream, "reference" site.

Poultry litter contained measurable concentrations of several organic analytes, most notably the naturally occurring estrogenic hormone,17β-estradiol, at an average concentration of 210 ng/g and the antibiotic chlortetracycline, found in two samples at levels of 1.2 and 1.6 µg/g. In addition, concentrations of arsenic, copper, and zinc, known poultry feed additives, were elevated in poultry litter samples. However, results from Year 1 indicated no differences between the "impacted" and "reference" sites on the Delmarva peninsula in terms of measured chemical and biological parameters. For example, results of Vtg analysis indicated that all male carp showed some level of Vtg induction, with Vtg concentrations averaging 30 and 25 µg/ml at the Prime Hook and Blackwater reference sites, respectively. Similarly, concentrations of arsenic, copper, and zinc in sediments and surface waters at the study sites were not found to be enriched relative to the reference sites, despite the high concentrations of these elements measured in poultry litter samples. Results of fish fillet analyses indicated no evidence of food chain bioaccumulation of either arsenic or selenium, but mercury in largemouth bass collected from around Prime Hook NWR did exceed EPA's fish tissue residue criterion (0.3 µg/g wet weight) for the protection of human consumers. The likely source of mercury in this area is atmospheric deposition due to air emissions from coal fired power plants and waste incinerators. At present, there is no fish consumption advisory in these waterbodies; therefore, we recommend follow-up sampling to confirm and extend our findings.

These results led us to question our initial experimental design and the ability to detect chemical or biological differences between "upstream" and "downstream" stations. Consequently, we modified the experimental design in Year 2 to include a reference station that we were certain would not be impacted by poultry AFOs (Jug Bay Wetlands Sanctuary on the Patuxent River on Maryland's western shore) and a "positive control" to maximize our chances of detecting water quality impacts related to AFOs on the Delmarva (Cod Creek, a tributary of the Nanticoke in DE), in addition to two sites located upstream of Prime Hook NWR, Beaverdam Creek and Slaughter Creek.

Several organic compounds were detected in the POCIS in Year 2; however, tetracycline and 17β-estradiol were only detected at the Delmarva sites, not at the Jug Bay site. The finding of detectable concentrations of tetracycline in the water at all three Delmarva sites indicates that there is transport of this antibiotic from poultry litter amended fields to adjacent waterbodies, as there are no other likely sources (e.g., wastewater treatment plants) within these tributaries. This family of compounds is widely used in poultry feed. At low doses, chlortetracycline and oxytetracycline are used to promote growth. At higher doses, these compounds and tetracycline are used to control various types of bacterial infections. Because of their widespread use, these compounds may be a useful tracer of poultry litter contaminants.

Interestingly, although there are numerous natural sources of 17β-estradiol (e.g., fish and waterfowl), this hormone was found only in the POCIS deployed at the Delmarva sites, suggesting a poultry litter-related source. We found no plasma Vtg in adult male fathead minnows that were caged for three weeks at each site; however, Vtg was detected in all resident male carp collected, at levels indicative of exposure to an estrogenic stimulus. Furthermore, concentrations were significantly higher in the fish from Slaughter Creek than at Jug Bay, potentially indicating that fish on the Delmarva are being exposed to higher levels of estrogenic compounds.

There was some indication that sediment concentrations of copper, zinc and selenium at the Delmarva sites were enriched relative to Jug Bay. In addition, water column concentrations of zinc at Delmarva sites appeared to be higher than at Jug Bay. It is not known, however, if this enrichment is natural or due to AFO run-off. Fish tissue data do not indicate bioaccumulation of arsenic and selenium at Delmarva sites, but as in Year 1, mercury concentrations in largemouth bass from Slaughter Creek were above the EPA fish tissue residue criterion.

Our study provides direct evidence for transport of tetracycline compounds, and possibly 17β-estradiol, from poultry litter-applied fields to adjacent waterbodies on the Delmarva peninsula. Our results also suggest that fish on the Delmarva are being exposed to estrogenic compounds. We recognize that our data are limited, both in terms of the number of samples as well as the geographic coverage. Nonetheless, the potential for system-wide estrogen and antibiotic contamination effects on the fish and wildlife resources of the Delmarva peninsula merits further investigation.

ACKNOWLEDGMENTS

Keisha Johnson, Devin Ray and Chris Dobony (FWS, Chesapeake Bay Field Office) assisted with the field work. We appreciate the technical and logistical support of refuge staff at Prime Hook and Blackwater NWRs, particularly biologists George O'Shea and John Morton. Greg Breese of the Service's Delaware Bay Estuary Project acquired and assembled Geographic Information System data layers used to target watersheds. We thank Patty McCawley for assistance in report preparation. Thanks also to Chris Swarth, Jug Bay Wetlands Sanctuary, for giving us access to their facilities during Year 2. This project was funded through the U.S. Fish and Wildlife Service Division of Environmental Quality, project number 12615N31.

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INTRODUCTION

Background

According to the Clean Water Action plan, there are approximately 450,000 animal feeding operations (AFOs) throughout the United States. The marked intensification of animal production, particularly in the last 25 years, has resulted in increased water quality problems associated with the production and disposal of animal waste generated by these operations. The Delmarva peninsula, which consists of eastern Maryland, most of Delaware and the part of Virginia east of Chesapeake Bay (Figure 1), is one of the largest poultry areas in the United States, producing some 600 million chickens and 1.6 billion pounds of manure annually (USDA 1997). Excessive land application of poultry litter has resulted in severe water quality problems in surface and groundwater in this geographic area. The primary pollutants of concern are nutrients; however, recent studies have indicated that AFO-related pollutants may not be limited to nutrients. Animal feed additives include trace metals and metalloids such as arsenic, selenium, zinc, and copper, as well as antibiotics. The majority of these additives are excreted and can be found at elevated concentrations in manure (Sims and Wolf 1994). In addition, natural hormones produced by livestock, specifically 17β-estradiol and testosterone, are also present in high concentrations in animal manure. Chicken manure has been reported to contain 14 to 533 mg/g 17β-estradiol and 133 to 670 ng/g testosterone on a dry weight basis with concentrations varying according to gender, maturity and reproductive status (e.g., broilers versus laying hens) (Shore et al. 1995).

Studies have also indicated that these contaminants may be migrating to nearby surface waters. Miller et al. (2000) analyzed trace metals, organic compounds and pesticides in surface waters and sediments in tributaries of the Pocomoke River, located on the Delmarva peninsula. Results indicated elevated water column concentrations of trace elements, such as arsenic and zinc, which are contained in poultry-feed additives. Sediment samples contained organic compounds in the class of hormones related to estradiol (Miller et al. 2000). Similarly Shore et al. (1995), Nichols et al. (1997, 1998) and Finlay-Moore et al. (2000) found that concentrations of 17β-estradiol in run-off from fields to which chicken litter had been applied, were at levels known to elicit estrogenic effects in fish.

The Delmarva peninsula supports significant ecological resources, including six National Wildlife Refuges (NWRs), a National Seashore, breeding areas for waterfowl, wading birds, and bald eagles, spawning habitat for anadromous fish including striped bass, American shad and river herring and is a prime stop-over point for migratory birds. Due to the high density and intensity of agricultural operations and the documented water quality problems throughout the peninsula, it seems likely that contaminants associated with AFOs may represent a threat to these ecological resources.

Objectives

In spring 2000, a two-year field study was initiated to evaluate water quality impacts on NWRs associated with AFOs. Specifically, our objectives were to: 1) map locations of AFOs in the vicinity of refuge lands and identify watersheds at high and low risk for AFO-related impact; 2) conduct studies to evaluate chemical and biological impacts to these watersheds, with an emphasis on assessing the potential impact of animal feed additives and other "nontraditional" contaminants.

METHODS

Targeting Watersheds

The U.S. Fish and Wildlife Service's Delaware Bay Estuary Project was responsible for data compilation and mapping. The specific location of AFOs and the number of animals they contain is confidential; therefore, a variety of sources were used to acquire and/or derive the information. Location of AFOs on the Delmarva peninsula in the vicinity of NWRs was compiled from USGS data (Young and Morton, in prep) and the Delaware Department of Natural Resources and Environmental Control. Other data layers used to target sites in Year 2 included hydrogeomorphic regions identified by USGS (Hamilton et al. 1993), land use, watershed basins, roads, and county delineations.

Study Sites

Year 1

A preliminary investigation was conducted at Prime Hook NWR, Sussex County, DE and Blackwater NWR, Dorchester County, MD from April - June 2000 because refuge staff expressed the concern that AFO activities located upstream may be impacting the refuge waters. Three sites were sampled at each refuge, two potentially impacted sites, upstream of the refuge but downstream of several AFOs and one downstream "reference" site (labeled PH-R and BW-R) located on the refuge (Figure 2). Prime Hook sites were located in Prime Hook Creek (PH-1 and PH-R) and Slaughter Creek (PH-2). Blackwater sites were located in the Little Blackwater River (BW-1 and BW-2) and the Blackwater River (BW-R).

Year 2

Based on Year 1 results, we modified the experimental design to include a "reference" station in the Patuxent River on Maryland's western shore (Jug Bay) and a "positive control" in a watershed with high probability of AFO-related impact (Cod Creek, Nanticoke watershed, Sussex County, DE). Based on the available information on the location of AFOs, Prime Hook NWR appeared to be the refuge with the highest probability of water quality problems associated with AFOs. Therefore, two sites were located in tributaries upstream of Prime Hook NWR (Figure 3): Beaverdam Creek and Slaughter Creek. The Slaughter Creek site was also sampled in Year 1 (PH-2).

Sample Collection

Poultry Litter and Sediments

Three poultry litter samples were collected over two days from a ≥200 ton pile at the University of Maryland - Wye Research and Education Center (UM-WREC), Queenstown, MD prior to field application. The source of the litter was a whole-house "scrape-out" containing manure, bedding material (i.e., sawdust, wood chips) and feathers accumulated over two years from a standard broiler operation on Maryland's Eastern Shore. One sample (#1) was taken directly from the pile on May 8, 2000 and two samples (#2 & #3) on May 9, 2000 as litter was removed from the pile to be spread on the fields. The first sample consisted predominantly of fine particles while the other two samples contained both fine particles and chunks. Two pre-cleaned and labeled amber sample jars, one 500 ml and one 250 ml, were filled at each sampling time. Samples were immediately placed in a -20°C freezer for storage, then shipped on ice to the USGS Columbia Environmental Research Center (CERC) for analysis.

Sediment collection methods followed those described in USEPA/USACE (1995) and briefly highlighted here. Sediments were collected with a stainless steel 0.023 m^2 petite Ponar grab sampler. At each station, the top 2 to 3 cm of several grabs were placed into a pre-cleaned stainless steel bowl, homogenized with a stainless steel spoon until uniform in color and texture and then placed into pre-cleaned containers for sediment chemistry analyses. Duplicate samples were collected at each station. Between stations, the grab sampler, stainless steel bowl and mixing utensils were rinsed sequentially in 10% nitric acid, distilled water, acetone and distilled water to remove residual contaminants. In addition, the first grab taken at each station was discarded and considered an *in situ* rinse. Collected sediments were kept on ice, subsequently refrigerated, and shipped on ice to CERC where they were frozen (-15°C) until initiation of the analytical process. Samples were collected during the deployment period for the integrated water samplers described below.

POCIS Deployment

We deployed a prototype integrated water sampler, similar in concept to the semi-permeable membrane device (SMPD), to detect the presence and relative amounts of polar organic compounds in the water column at the study sites. These Polar Organic Compound Integrative Samplers (POCIS) consist of a hydrophilic polyethersulfone membrane containing an admixture of a hyper-crosslinked polystyrene-divinylbenzene solid-phase extraction resin and S-X3 BioBead-dispersed Ambersorb®1500. The membrane-sorbent "sandwich" is secured between two stainless steel washers and four of these devices were mounted in a stainless steel canister (Figure 4). The POCIS were analyzed for agrichemicals, estrogens and tetracycline antibiotics. In Year 1, the POCIS were deployed for six weeks, from mid-April until the end of May. In Year 2, POCIS were deployed from the beginning of May to mid-June. POCIS were deployed by attaching the cages containing the sampling devices to wooden posts driven into the sediments. The cages were suspended vertically, approximately 10 cm (4 inches) off the bottom. Once a week, the devices were brushed to prevent excessive fouling and water quality measurements (temperature, dissolved oxygen, pH, conductivity, and salinity) were taken with a Hydrolab Surveyor II (Hydrolab Corp., Austin, TX). After six weeks, the POCIS were removed from the cages and stored frozen in closed one-gallon pre-cleaned, airtight "paint cans" and then shipped to CERC for extraction and analyses. Field blanks were used during deployment, retrieval and shipping to account for any interferences introduced to the POCIS during these processes. The field blank was resealed once the POCIS were placed into the water. The field blanks consisted of a POCIS kept in closed paint cans at -20 °C, which were taken to the sampling sites during deployment and retrieval and opened to the atmosphere while the devices were exposed to air. These devices are sensitive enough to potentially absorb hydrophilic organic compounds from the air.

Water Column Trace Elements

Two water samples were collected at each site, one during low flow (no rain in preceding 48 hours) and one during high flow (more than 1.5 cm rain in preceding 24 hours) following procedures outlined in the USGS National Field Manual for the Collection of Water-Quality Data (http://water.usgs.gov/owq/FieldManual). In brief, samples for analysis of dissolved metals were collected using a pumping system consisting of a Masterflex® peristaltic pump using acid-washed C-Flex® tubing, with an in-line Gelman® 0.45-µm cartridge filter. Filters were rinsed with 1 L of distilled water before collecting samples. Samples for dissolved iron, manganese, arsenic, copper, selenium, and zinc were collected in 500-ml glass I-Chem® bottles. At least one blank and one duplicate were collected during each sampling event. Trace metal samples were kept on ice and shipped within 24 hours to CERC where they were acidified upon receipt.

Benthic Macroinvertebrate Community

No standardized methods are currently available for sampling benthic macroinvertebrate communities in shallow, tidal freshwater or oligohaline systems. Consequently, we employed different techniques in order to sample the available benthic habitat. In Year 1, at Blackwater NWR, two petite Ponar grab samples (0.023 m^2 per grab) were collected, sieved through a 500 µm mesh and composited. At Prime Hook, benthic samples consisted of a petite Ponar grab, sieved through a 500 µm mesh and composited with the contents of a 600 µm mesh D-net which was used to trap organisms dislodged from approximately 20 square feet of vegetation. In Year 2, a single benthic grab and a D-net "jab" sample were composited at each station. Samples were preserved in 95% ethanol with rose bengal, sorted in the laboratory and identified to the lowest practical taxon. In Year 1, sorting and identification was conducted by Dr. Leonard Ferrington of the University of Minnesota. In Year 2, sorting and identification was performed by Versar, Inc., an environmental consulting firm in Maryland. Benthic measures included number of organisms, species richness, and number of taxa in the generally intolerant insect orders of Ephemeroptera (mayflies), Plecoptera (stoneflies) and Trichoptera (caddisflies), known as the "EPT taxa". The number of EPT taxa can indicate stream conditions, since these organisms tend to become more scarce with increasing levels of disturbance. In addition, this index is one of the metrics used in the Maryland Benthic Index of Biotic Integrity (Stribling et al. 1998).

Fish

To evaluate the potential for endocrine disrupting effects on resident fish populations in Year 1, our initial plan was to measure vitellogenin (Vtg) concentrations in adult male fathead minnows, *Pimephales promelas*, that were caged for three weeks at each site. Vitellogenin, an estrogen-inducible phospholipid found in fish and other oviparous vertebrates, is normally synthesized in the liver of females during oogenesis and is a precursor of egg yolk. The presence of Vtg in the plasma of male fish has been widely used as an indication of exposure to an estrogenic stimulus and high Vtg concentrations have been associated with a decrease in testes weight and changes in the development of sperm (Jobling et al. 1996, Goodbred et al. 1997, McDonald et al. 2002). Unfortunately, due to unfavorable water quality conditions (i.e., high temperature and low dissolved oxygen), there was high fish mortality at all but one station in Year 1; so instead we measured Vtg in indigenous male carp, *Cyprinus carpio* or mummichogs, *Fundulus heteroclitus.* These species were chosen for study because of their widespread distribution. In addition, carp appear to be relatively sensitive to endocrine disrupting effects (Folmar et al. 1996, Goodbred et al. 1997). Fish were collected in June by either electrofishing or with gill nets. Male fish were initially, tentatively identified by external characteristics i.e., as those that "milked" or produced sperm when the ventral portion of the fish was squeezed (carp) or those with broader heads (mummichogs). Sex was confirmed upon necropsy and internal examination of the gonads.

Fish were also collected analyzed for tissue concentrations of trace metals including mercury, selenium, and arsenic because of the concern that some elements (e.g., selenium) in poultry litter may be accumulating in aquatic food chains. Fish tissue analyzed included the carp collected for Vtg assays as well as brown bullhead catfish (*Ictalurus nebulosus*) and largemouth bass (*Micropterus salmoides*).

In Year 2, we employed a substantially different fish caging design than that used in Year 1 (Figure 5). The *in situ* cages consisted of floating mesh baskets housed within a large (approximately 100 L) cylindrical high density polyethylene container (Figure 5). Three replicate baskets containing four adult male fathead minnows were deployed at each station. Fish were fed TetraMin, three times per week, and retrieved after 21 days. Water quality measurements were taken at each visit. As in Year 1, we also attempted to collect resident male carp at each station for Vtg analysis as well as histopathological evaluation of the gonads. Carp and largemouth bass were collected for tissue analysis.

In addition, larval fathead minnows were exposed to water collected from the three eastern shore sites during the same 21-day interval that the adult fish were caged. Test methods for the exposures were modified from the EPA "Short-Term Methods for Estimating Chronic Toxicity of Effluents and Receiving Water to Freshwater Organisms" (USEPA 1994). Briefly, sufficient water was retrieved from each sample site three times/week and returned to the UM-WREC facility to make daily water renewals on four replicate beakers, each housing ten fish. A control treatment receiving only aged well water was also run. The exposures were initiated with ≤ 24 h old larvae and discontinued after 21 days. Water quality parameters (pH, DO, conductivity, hardness, alkalinity) were monitored and mortality recorded daily. Larval fish were allowed to grow for 14 to 16 days post exposure before being sacrificed at an age of 34 to 36 days. Assay endpoints included: survival; growth, as indicated by wet weight at the time of sacrifice; and *Condition Index (CI)* calculated as the body weight expressed as a percent of the length cubed:

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

where body weight and length are recorded in mg and mm, respectively. Additionally, induction of Vtg was investigated using a whole-body homogenization method. Briefly, whole bodies of individual larval fish were homogenized in phosphate buffer at a ratio of 1:3 wet body weight:buffer volume, using a chilled, hand-held, glass tissue-grinder. Wet weights varied from ~ 30 to 160 mg. Homogenates were centrifuged at 3000 g for 10 minutes (Eppendorf centrifuge, Model # 5415) with supernatant withdrawn and discharged into heparinized and aprotinated cryovials as described further in the *Sampling Processing and Analysis* section. Samples were stored in liquid nitrogen until required for analysis.

Sample Processing and Analysis

Fish

Large fish (i.e., adult carp) were processed for tissue and blood analysis in the field while smaller captured (i.,e., mummichogs) and caged (i.e., fathead minnows) fish were transported live to laboratory facilities at the University of Maryland Wye Research and Education Center for blood plasma collection. Fish were maintained in live wells after capture, usually for not more than 3 hours before processing. Methods for plasma collection followed those of Goodbred et al. (1997) and are briefly described here. All fish were anaesthetized in MS 222 or subdued by direct blunt trauma, weighed and measured. Blood samples were collected from carp using a 3 or 5 ml syringe to draw approximately 1.5 ml of blood from the posterior caudal artery. Blood was ejected into heparinized centrifuge tubes and spun for 10 minutes at 4000 rpm to separate plasma from whole cells. The resulting plasma was aspirated with a transfer pipette into heparinized (35 USP/vial) and aprotinated (0.132 TIU/vial) 1.5 ml conical bottom cryovials (Sigma Chemical Inc., cat. # H-6279 and A-6279, respectively) and placed on dry ice during transport, before storage in liquid nitrogen until required for analysis. Blood samples were collected from adult fathead minnows and mummichogs in 70 µl heparinized microhematocrit tubes via incision into the caudal sinus. Typically 40 to 70 µl of whole blood was obtained from each specimen with additional tubes collected from prolific “bleeders” as a means of investigating the reproducibility of analytical results. Whole blood samples were centrifuged at 3000 g for 10 minutes (International Micro-Capillary centrifuge, Model *MB*) with the resulting plasma discharged into heparinized and aprotinated 1.5 ml conical bottom cryovials and stored in liquid nitrogen until required for analysis. Carp and mummichog plasma samples were shipped on dry ice to Dr. Nancy Denslow at the University of Florida - Protein Chemistry and Molecular Biomarkers Laboratory, Gainesville, FL for analysis of Vtg using Enzyme-Linked Immunosorbent Assay (ELISA) (Folmar et al. 1996). Plasma from several female field-collected fish was also submitted to serve as “positive controls” for the Vtg analyses. The detection limit for plasma Vtg was 1.0 µg/ml. Fathead minnow plasma and whole-body homogenate samples were shipped on dry ice to the College of William and Mary, Virginia Institute of Marine Sciences, Gloucester Point, VA for determination of Vtg using a competitive ELISA method modified from Parks et al. (1999).

Skin-off fillets were taken from a subsample of fish. Filleting was conducted on a board covered with heavy duty aluminum foil. The foil covering was changed between fish. A stainless steel knife was cleaned prior to use by washing sequentially in laboratory detergent, tap water, deionized water, 10% nitric acid, deionized water, pesticide grade acetone and deionized water. In between use, the knife was rinsed sequentially in tap water, nitric acid, deionized water, acetone, and deionized water. At least 20 grams of tissue were wrapped in heavy duty aluminum foil, labeled with the sample identification number and date of collection. The foil containers were placed into small whirl-pak bags which were also labeled. Samples were stored in the freezer until they were shipped on dry ice to CERC for analysis of mercury, selenium, and arsenic.

In Year 2, histopathological analyses of gonads were also conducted on resident carp. Gonads were removed and weighed to calculate the gonadosomatic index (GSI), expressed as the gonad weight as a percentage of total body weight. After weighing, five to six 1-cm pieces were cut from the bottom end of the testes, fixed with 10% neutral buffered formalin then paraffin embedded using standard histological procedures for thick section light microscopy. Five µm thick sections were mounted on glass slides and stained with hematoxylin and eosin, according to Luna (1968).

Chemical Analysis

The poultry samples and replicate sediment samples from the study sites were analyzed for trace metals and metalloids, current use pesticides, natural and synthetic estrogens, tetracycline compounds, as well as select polynuclear aromatic hydrocarbons (PAHs), organochlorine pesticides and total polychlorinated biphenyls (PCBs) (Table 1). In addition, POCIS were used to estimate the relative concentrations of dissolved agrichemicals, natural and synthetic estrogens, and tetracycline compounds. Trace metals and metalloids were measured in the water column and resident fish. Details of chemical preparation of the samples as well as instrumental analyses, including detection limits for all media, spike recoveries and other Quality Assurance/Quality Control (QA/QC) measures, can be found in Appendices 1 through 4.

Data Analysis

Sediment and Water Chemistry

Water chemistry data were compared to EPA's water quality criteria (WQC). All Prime Hook NWR sites and BW-1 at Blackwater NWR were compared to freshwater criteria. Hardness-based criteria were calculated for copper and zinc, using the equations provided in EPA (2002) and a hardness value of 40 mg/L $CaCO_3$. This value is based on average hardness concentrations measured at the Delmarva sites during larval fish exposures in Year 2 (L. Yonkos, unpublished data) and are within the range reported for Prime Hook Creek during another study conducted by the U.S. FWS Chesapeake Bay Field Office (Guy et al. 2002). There was measurable salinity at BW-2 and BW-R (ranging from 2 to 6 ppt); hence, these samples were compared to salt water WQC. Comparisons were made to both the criteria maximum concentration (CMC), a measure of acute toxicity for short term exposures and the criteria continuous concentration (CCC), a measure of the potential for chronic toxicity due to longer term exposures.

Sediment concentrations were compared to sediment quality guidelines (SQGs) in the literature. The guidelines used for comparison in this study are the freshwater consensus-based SQGs proposed by MacDonald et al. (2000, Table 2). In brief, MacDonald et al. (2000) compiled SQGs derived using several different approaches including: sediment quality advisory levels based on equilibrium partitioning theory (EPA 2002), Long and Morgan's ERM and ERLs (1991), and the TEL and PELs developed by Smith et al. (1996). The consensus-based guidelines were then calculated from the geometric mean of these different SQGs. Similar to other empirical approaches, the consensus-based SQGs were derived for threshold effect concentrations (TEC), the concentration below which adverse effects are not expected to occur and probable effects concentration (PEC), the level above which adverse effects are expected to occur more often than not. In this study, sediment concentrations were compared to PECs.

A useful tool to compare trace element concentrations across sites, and to estimate the degree to which concentrations may be elevated due to anthropogenic input, is the enrichment factor (EF) (Sinex and Helz 1981, 1982; Velinsky et al. 1994). This approach also minimizes trace element concentration differences related to variability in grain size. The enrichment factor is defined as:

$$EF = \frac{(X/Fe)\ \text{sediment}}{(X/Fe)\ \text{earth's crust}}$$

where X/Fe is the ratio of element X to Fe. Iron was chosen as the element for normalization because it is naturally very abundant, in most cases the anthropogenic sources are small compared to the amount of natural Fe present, and the ratio of elements to Fe is fairly constant in the crust of the earth. An EF value of one or less implies there is no enrichment relative to the average earth's crust. Turekian and Wedepohl's (1961) average shale data were used in the denominator, representing the reference level (Sinex and Helz 1982).

Fish

Data on total length, weight, GSI, Vtg and tissue concentrations in fish from each site were first tested to determine if they met the assumptions for parametric statistics, equal variance and a normal distribution. For data meeting these assumptions, mean concentrations were compared with one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test. Data not meeting assumptions for parametric statistics were analyzed via the Kruskal-Walllis (K-W) test, followed by Dunn's method for multiple comparisons. A p value of 0.05 was used for all tests of significance.

Fish tissue data were compared to EPA's Screening Values for the protection of human health (USEPA 2000), 1.2 and 20 µg/g wet weight (ww), for arsenic and selenium, respectively, and to the water quality criterion for mercury based on fish tissue residue concentrations, 0.3 µg/g ww (USEPA 2001). Data were also compared to the 85th percentile concentrations of the 1986 wholebody fish collection from the National Contaminant Biomonitoring Program (NCBP), as an additional benchmark. These values are 0.24, 0.66, and 0.18 µg/g ww for arsenic, selenium and mercury, respectively (Schmitt et al. 1999).
In addition to specimens being examined for pathology, a semi-quantitative method was used to assess the level of maturity of testicular tissues. Briefly, a scale from 0 to 5 was devised to rank

specimens based on the proportion of spermatogenic cells at various stages of differentiation (e.g., spermatogonia, spermatocytes, spermatids and mature spermatozoa). A greater proportion of mature spermatozoa relative to less mature cells was assumed to indicate a more advanced level of maturity. Multiple sections from different regions of gonad were assessed to determine ranks for individual fish with higher ranks indicating more advanced maturity. A maturity index for each location was calculated as the mean of all individual ranks from that location.

RESULTS

Chemical Analyses

Details of results of Quality Assurance/Quality Control measures for the analyses, can be found in Appendices 1 through 4.

Poultry Litter

Poultry litter was found to contain trace concentrations of a variety of organic compounds including lindane, chlordane, DDT compounds, atrazine, metolachlor, diazinon, λ cyhalothrin, and benz[a]anthracene (Table 3). The antibiotic chlortetracycline was also found in two of the samples at concentrations of 1.2 μg/g and 1.6 μg/g dry weight, respectively. Initial analyses did not detect any concentrations of estrogens in the litter samples (Appendix 1). This result was surprising and not consistent with the literature (Shore et al. 1995); consequently, an additional litter sample was analyzed in Year 2 using different HPLC conditions (Appendix 3). Results indicated that under the original HPLC system, an unidentified interference peak was obscuring the 17β-estradiol. Using the new system, the 17β-estradiol peak was resolved from the interference, and quantification of triplicate samples indicated 17β-estradiol at concentrations of 210, 200, and 200 ng/g dry weight.

High concentrations of several trace metals including copper, zinc, and arsenic were found in poultry litter samples from the Delmarva peninsula (Table 4, Figure 6). A “reference manure” sample was also analyzed. This sample came from poultry not exposed to feed additives. Concentrations of arsenic and copper were substantially lower in this samples than those from the Delmarva peninsula (Table 4).

Sediments

In Year 1, duplicate sediment samples were collected from three sites in tributaries of Blackwater NWR (BW-1a and b, BW-2a and b, BW-Ra and b) and 3 sites at Prime Hook NWR (PH-1a and b, PH-2a and b, PH-Ra and b). Trace levels of DDTs, and the herbicide, trifluralin and the insecticides, tefluthrin, trans-permethrin, and λ- cyhalothrin were found at all sites (Table 5). Low concentrations of several PAHs were also found at several Blackwater and Prime Hook sites. The highest PAH concentrations were found at BW-R, with several values exceeding their respective PECs. Measurable concentrations of total PCBs were detected only at the Blackwater sites. The highest concentrations of DDT compounds were found at PH-2, Slaughter Creek, at levels PEC. Concentrations of other organic compounds at all stations were well below PECs. No tetracycline compounds were detected in any of the sediments, but samples from PH-R and BW-1 and 2, contained quantifiable concentrations of 17β-estradiol (Table 5). There were no discernable differences between upstream and reference sites in sediment concentrations of organic compounds.

Concentrations of trace metals in sediments from all sites were well below PECs (Table 6). There was fairly good agreement between sediment concentrations in field duplicates, with the exception of iron at some of the sites (e.g., PH-R and BW-2). Trace element concentrations were lowest at PH-1, probably reflected the coarse-grained nature of the sediments. Enrichment factors were calculated as a way to evaluate the potential for anthropogenic enrichment of trace elements and minimize concentration differences related to grain size. The majority of EFs were less than one, except for zinc, which was enriched at all stations. Comparison of EFs from the reference sites (PH-R and BW-R) to the study sites at each refuge, did not suggest concentrations of trace elements in upstream sediments were enriched relative to the reference sites. Comparison of whole sediment concentrations also did not suggest differences between “reference” and “impacted” sites at either Prime Hook or Blackwater NWRs (Figure 6).

In Year 2, trace levels of several organic compounds, including lindane, chlordane, diazinon, dieldrin, trifluralin and several PAHs, were detected at all sites (Table 7); however, there were no exceedances of PECs. Concentrations of several compounds including DDTs, diazinon, chlorpyrifos, chlordane, total PCBs, and dieldrin were highest at Slaughter Creek. No tetracyclines or hormones were found in sediments from any sites in Year 2.

Whole sediment concentrations of copper, zinc and selenium were highest in the replicates from Slaughter Creek; however, all trace element concentrations were well below PECs (Table 8, Figure 7). In addition, whole sediment concentrations of trace elements in sediments from Jug Bay were within the range of concentrations from the Delmarva sites (Table 8, Figure 7). With the exception of zinc, most EFs were less than one; however, a comparison of EFs among sites suggests that concentrations of copper, zinc, and selenium are enriched at that Delmarva sites relative to the Jug Bay reference (Figure 7).

Polar Organic Compound Integrative Samplers (POCIS)
In Year 1, duplicate POCIS were deployed at the study sites from mid-April to the end of May (six weeks). Unfortunately, two of the POCIS were lost during shipping and could not be replaced. Consequently, we were unable to deploy the POCIS at the Blackwater NWR reference site (BW-R). Water temperatures ranged from 10.1 ^{o}C at the beginning of the deployment period to 31.6 ^{o}C, at the end (Table 9).

Several current use pesticides were detected in the POCIS (Table 10). In particular, the herbicides, atrazine and alachlor were detected in at least one POCIS at all stations, whereas the insecticide diazinon was detected only at BW-2 and chlorpyrifos only at the Prime Hook sites. The Prime Hook downstream site, PH-R, had similar concentrations of pesticides as potentially impacted sites, PH-1 and PH-2. No tetracycline compounds were detected in the POCIS at any sites. Estrogens were not detected at Blackwater NWR, but 17β-estradiol was detected in the POCIS deployed at all three stations at Prime Hook NWR.

In Year 2, duplicate POCIS were deployed from the beginning of May until mid-June. Water temperatures ranged from 13.5 ^{o}C at the beginning of the deployment to 24.4 ^{o}C at the end (Table 9). Due to a malfunction with the analytical equipment, only concentrations of tetracycline antibiotics were measured in the POCIS samples from Slaughter Creek. As in Year 1, atrazine was detected at all stations, with the highest concentrations found at Cod Creek. The highest concentrations of metolachor were also found at Cod Creek Tetracycline was detected in POCIS at all three stations on the Delmarva, but not at the reference site in Jug Bay (Table 11). Similarly, 17β-estradiol was also detected only in POCIS deployed on the Delmarva and not at the reference site, with the highest concentrations detected in the POCIS from Cod Creek.

Water Column Trace Elements

In Year 1, concentrations of trace elements in the field blanks were below detection for all analytes except iron. Reagent blanks contained detectable concentrations of all elements except manganese; however, results were within acceptable quality control limits of CERC. Concentrations of arsenic, selenium and zinc in most surface water samples were below the detection limits and in all samples, concentrations of these elements were below acute and chronic water quality criteria (Table 12) . There are no water quality criteria for iron and manganese. Copper was detected in only one sampling event at Prime Hook NWR, but at least once at all stations around Blackwater NWR. The Prime Hook sample, PH-R (high flow), was a field duplicate and one replicate may have been contaminated with copper as concentrations were substantially higher in one replicate (249 ng/ml) compared to the other (18 ng/ml). Both these concentrations were above the CMC and CCC freshwater values for copper. High and low flow samples collected at BW-2 and BW-R were above the salt water CMC and CCC for copper (Table 12).

In Year 2, detection limits were lower than in Year 1 for all elements except for selenium (Table 13). Field blanks and reagent blanks contained low, but detectable levels of manganese and copper. Field duplicate concentrations of copper and zinc were quite different, perhaps reflecting contamination from filtration or other field handling processes, or perhaps field variability in elemental concentrations. Duplicate concentrations of other elements were similar between replicates. Concentrations of trace elements from all sites were below acute water quality criteria, although one sample, Cod Creek-low flow, exceeded the chronic value for copper (Table 13).

Fish Tissue Analysis for Trace Elements
Average tissue concentrations of selenium, mercury and arsenic in fillets from carp, largemouth bass and brown bullhead catfish from Year 1 are presented in Table 14. Despite devoting much effort to fish collection at both refuges, we were only able to collect enough carp to enable statistical comparisons of tissue concentrations among Prime Hook sites (Table 14). Results of ANOVA indicated no difference in mean length or wet weight of carp collected from the three Prime Hook sites; however, there were significant differences in concentrations of selenium and mercury. PH-2 had significantly lower concentrations of selenium than PH-1 and PH-R, while tissue concentrations of mercury were highest at PH-R and significantly different than the mean concentration in carp from PH-2. In addition, concentrations of mercury in largemouth bass collected at PH-2 and PH-3 were above the 85^{th} percentile in the NCBP database, as well as the EPA fish tissue residue criterion.

In Year 2, fish were only successfully collected at Slaughter Creek and Jug Bay (Table 15). Carp collected at Jug Bay were significantly larger than those from Slaughter Creek (Table 15). In addition, they contained significantly higher tissue concentrations of arsenic and selenium. Mercury concentrations of largemouth bass collected from Jug Bay and Slaughter Creek were higher than the NCBP 85^{th} percentile. In addition, bass from Slaughter Creek also exceeded the EPA fish tissue residue criterion for mercury.

Biological Effects

Benthic Macroinvertebrate Community Analysis
A variety of benthic macroinvertebrates were found at the Prime Hook sites, dominated by midges (chironomidae), amphipods, and oligochaetes, but also including sensitive taxa such as mayflies and caddisflies. The number of taxa, EPT taxa, and abundance was lowest at PH-2; however, these parameters were similar between PH-1 and PH-R. Diversity and abundance was substantially lower at Blackwater sites compared to Prime Hook sites. Benthic macroinvertebrate taxa included midges, oligochaetes, amphipods and mussels. Number of taxa ranged from 3 to 5 and no EPT taxa were collected. In general, at both refuges there did not appear to be differences between upstream and downstream sites.

In Year 2, midges, oligochaetes, and amphipods were abundant at all sites. Other dominant taxa included snails (*Littorinopsis sp*) at Jug Bay, dragonfly nymphs (*Ischnura spp*) at Slaughter Creek and caddisfly larvae (*Hydroptila spp*) at Beaverdam Creek. Taxa richness ranged from 61 at Cod Creek to 39 at Jug Bay and abundance from 455 at Cod Creek to 1,516 at Jug Bay. Number of EPT taxa was highest at Beaverdam Creek and lowest at Jug Bay. As in Year 1, there were no evidence that study sites on the Delmarva were impaired relative to the Jug Bay reference.

Fish Vitellogenin and Histopathology
In Year 1, indigenous carp were collected at five of the six sites. We were unable to capture carp at BW-1, in the Little Blackwater River. Results of Vtg analysis indicated that 100% of male carp showed some level of Vtg induction, with the highest value, 76 µg/ml, found in the

single male carp collected in Slaughter Creek (PH-2; Table 8). Average Vtg concentrations in male carp from PH-R were significantly higher than in carp from PH-1 (t-test, $p=0.022$). Plasma Vtg concentration in an immature female carp from PH-1 was 96 µg/ml. We were able to collect indigenous mummichogs at stations BW-2 (n=8) and BW-R (n=11) at Blackwater NWR. Vtg was detected in one male from each station, but at concentrations just above the detection limit, 2 and 4 µg/ml, respectively. Plasma Vtg concentration in a female mummichog collected from BW-2 was 3,595 µg/ml.

In Year 2, we were able to collect carp at Slaughter Creek and the Jug Bay reference site (Table 8). As in Year 1, Vtg was found at detectable levels in all male carp; however, mean Vtg concentrations in male carp collected from Slaughter Creek (53 µg/ml Vtg) were significantly higher than mean concentrations in male carp from Jug Bay (22 µg/ml Vtg)(Table 6, t-test, $p < 0.05$; t-test). Plasma Vtg concentration in a female carp collected from Slaughter Creek was 56,306 µg/ml.

Testicular tissue was processed for histological examination from all adult male carp collected at the Slaughter Creek (n=8) and Jug Bay (n=5) sample sites. Testes from all specimens appeared healthy and without pathology at the time of collection. While some autolysis was apparent in the Jug Bay specimens, this likely occurred post-mortem as a result of incomplete fixation. The mean maturity index for the Slaughter Creek carp was 4.25 with a range of 3.5 5.0. As 5.0 is maximum, this indicates a very high level of maturity. The mean maturity index for the Jug Bay fish was 3.0 with a range of 2.5 to 3.5. Because of the limited number of specimens and semi-quantitative morphometric methods employed, no statistical analyses were employed to interpret histological results. There were no statistical differences between sites in the gonadosomatic index, GSI (t-test, $p>0.05$).

Survival of male fathead minnows caged for three weeks at each site was very good: 100 % at Cod Creek and Jug Bay, 92 % at Slaughter Creek and Beaverdam Creek. No Vtg was detected in blood plasma from the caged fish.

Larval Fish Exposures

Water collected from all three Delmarva sites caused mortality in larval fathead minnows during the 21-day laboratory assay. Control survival at the conclusion of the exposure was 97.5 % while survival in Slaughter Creek, Beaverdam Creek and Cod Creek treatments were 42.5 %, 65.0 % and 67.5 %, respectively. Analysis of variance indicated a statistically significant difference ($p = 0.030$) existed between mean treatment survival values. Post hoc means testing indicated that only mortality in the Slaughter Creek treatment differed significantly from the control ($p < 0.05$, Dunnett's test). Growth, measured as wet wt 14 days post-exposure, was not affected in the surviving fish. Also, there was no effect on *Condition Indices* suggesting that the health of surviving fish was not compromised. Vitellogenin concentration in whole-body homogenates, after normalization to total protein content, typically ranged from below the detection limit of ~ 0.06 µg Vtg/mg P to 0.40 µg Vtg/mg P with one outlier at 1.22 µg Vtg/mg P. Mean Vtg concentrations for the Control (0.25 µg Vtg/mg P), Slaughter Creek (0.29 µg Vtg/mg P), Beaverdam Creek (0.17 µg Vtg/mg P) and Cod Creek (0.18 µg Vtg/mg P) treatments did not differ significantly (Kruskal-Wallis ANOVA on ranks; $p = 0.181$). Details of these results will be published elsewhere (Yonkos, in prep).

DISCUSSION

Year 1

Poultry litter contained measurable concentrations of several organic analytes, most notably 17β-estradiol at an average concentration of 210 ng/g and the antibiotic chlortetracycline, found in two chicken litter samples at levels of 1.2 and 1.6 _g/g. Similarly, concentrations of arsenic, copper and zinc, known poultry feed additives, were elevated in poultry litter samples. However, results from Year 1 indicated no differences between the "impacted" and "reference" sites on the Delmarva peninsula in terms of measured chemical and biological parameters. For example, 17β-estradiol was detected in sediments and surface waters at several of the sites, including the downstream reference site at Prime Hook NWR. Concentrations of arsenic, copper, iron, zinc, selenium and manganese in sediments and surface waters at the study sites were not found to be enriched relative to the reference sites. Sediment concentrations of these elements were below published PECs and, with the exception of copper, elemental concentrations in water were below water quality criteria. Copper concentrations in some water samples from Prime Hook Creek, Little Blackwater River, and Blackwater River were above acute and chronic criteria. There was no evidence of bioaccumulation of poultry-litter associated arsenic or selenium in these aquatic systems, as fish concentrations of these compounds were below EPA screening values and typically below the NCBP 85th percentile. Our data do indicate that mercury concentrations in fish are at levels warranting concern. Mercury concentrations in largemouth bass, a piscivorous fish, collected at PH-1 in Prime Hook Creek and PH-2 in Slaughter Creek were above the EPA fish tissue residue criterion, 0.3 µg/g, for the protection of human consumers. Similarly, largemouth bass collected from Slaughter Creek in Year 2, also had mercury tissue concentrations exceeding the EPA criterion. The likely source of mercury in this area is atmospheric deposition due to air emissions from coal fired power plants and waste incinerators.

Biological endpoints also did not indicate differences between "impacted" sites and the downstream "reference" sites in Year 1. Benthic macroinvertebrate data indicated that taxonomic richness, abundance and number of sensitive (i.e., EPT) taxa were similar across the three sites at each refuge; however, diversity and abundance were substantially lower at Blackwater than at Prime Hook sites. Several factors may account for the observed differences in the benthic macroinvertebrate communities. First, two of the Blackwater sites (BW-2 and BW-R) were oligohaline and benthic macroinvertebrate diversity is typically lowest in this salinity range. In addition, the acute water quality criteria for copper was exceeded at these stations during high and base flow conditions. BW-1 was a tidal freshwater station, but dissolved oxygen dropped to as low as 2.2 mg/L, during our study period. Finally, the Blackwater samples were a composite of two benthic grabs, whereas, the Prime Hook samples included multiple habitats, a grab sample and a D-net jab sample in the vegetation, which would increase the diversity of fauna collected.

Results of Vtg analysis indicated that all male carp showed some level of Vtg induction, with concentrations ranging from 13 μg/ml at PH-1 to 76 μg/ml in the single fish collected from PH-2. Carp Vtg concentrations averaged 30 and 25 μg/ml at the Prime Hook and Blackwater reference sites, respectively (Table 8). This level of Vtg appears to be above what is considered normal based on the results of McDonald et al. (2002) and indicates exposure of these fish to some type of estrogenic stimulus (Steve Smith, USGS BRD, personal communication).

Male mummichogs collected at two Blackwater stations did not have appreciable plasma Vtg concentrations. Current research at the UM-WREC, involving laboratory exposures of fathead minnows and mummichogs to poultry litter-associated contaminants, indicates that mummichogs may be less sensitive to the vitellogenic response than some of the cyprinids. Vitellogenin induction has been found to occur in fathead minnows when exposed to 17 β-estradiol derived from poultry litter for three weeks at a concentration of <60 ng/L. Mummichogs required a similarly derived 17 β-estradiol concentration in excess of 120 ng/L before Vtg induction was seen.

The results of Year 1 led us to question our initial experimental design and the ability to detect chemical or biological differences between "upstream" and "downstream" stations. These watersheds have received decades of inputs from agricultural run-off and nutrient problems are systemic; therefore, in hindsight, it seemed likely that the presence of "non-traditional" contaminants may also be widespread. Consequently, we modified the experimental design in Year 2 to include a reference station that we were certain would not be impacted by poultry AFOs (Jug Bay Wetland Sanctuary on the Patuxent River on Maryland's western shore) and a "positive control" to maximize our chances of detecting water quality impacts related to AFOs on the Delmarva, Cod Creek, a tributary of the Nanticoke in DE, in addition to the two sites located upstream of Prime Hook NWR, Beaverdam Creek and Slaughter Creek.

Year 2 - Antibiotics

The finding of detectable concentrations of tetracycline in the POCIS at all three Delmarva sites in Year 2, but not at the reference site at Jug Bay, indicates that there is transport of this antibiotic from litter-applied fields to adjacent waterbodies, as there are no other likely sources (e.g., wastewater treatment plants) within these tributaries. This family of compounds is widely used in poultry feed. At low doses, chlortetracycline and oxytetracycline are used to promote growth. At higher doses, these compounds and tetracycline are used to control various types of bacterial infections. Because of their widespread use, these compounds may be a useful tracer of poultry litter contaminants, in systems with no human wastewater inputs.

In a nationwide survey of streams, Kolpin et al. (2002) detected tetracycline compounds in up to 2.4% of the waterbodies surveyed. The biological significance of the presence of these antibiotics in aquatic systems is unknown; however, the increasing evidence for antibiotic resistance in microbial communities, particularly arising from transfer of resistance among microbes, point toward an area of significant concern. Based on our determined levels of approximately 1 _g/g (1 ppm) chlortetracycline in poultry litter and a total of 1.6

billion pounds (7.3 x 10^8 kg) of litter, a minimum of 730 kg of chlorotetracycline is potentially land applied on the Delmarva peninsula. While this does not seem to be a particularly large amount, this is a minimum value based on a limited sample set. Perhaps more importantly, it must be remembered that poultry production involves the use of a variety drugs, including but not limited to, tetracyclines, sulfadrugs, and organoarsenicals. As such, the massive amount of chicken litter applied each year to the soil represents a significant source of potential antibiotic input into the ecosystems of the Delmarva Peninsula.

Year 2 - Trace Elements

There is some indication that sediment concentrations of copper, zinc and selenium at the Delmarva sites are enriched relative to Jug Bay, as EFs for these sites were up to 6 times greater than those at Jug Bay. However, it should be noted that, with the exception of zinc, EFs at most sites were less than one, suggesting that relative to concentrations in the earth's crust, they are not enriched. Normalization to concentrations in the earth's crust may not account for natural variations due to regional differences in geology (Velinsky et al. 1994, Schropp et al. 1990); hence, no definitive conclusions can be drawn from the sediment data. Future research directed at determining enrichment factors for metals not associated with AFO run-off may provide additional insight. Water column concentrations of zinc at Delmarva sites appeared to be higher than at Jug Bay; however, concentrations of other elements were similar among sites. With such limited sampling, it is difficult to discern whether higher zinc concentrations are due to natural or anthropogenic enrichment or sampling variability. Fish tissue data were also limited, but indicate that average concentrations of selenium and arsenic in Jug Bay carp were significantly higher than at Slaughter Creek. This comparison may have been influenced by fish size, as fish from Jug Bay were also significantly larger than those from Slaughter Creek and larger/older fish tend to have higher contaminant concentrations.

Year 2 - Estrogens

Interestingly, 17β-estradiol was found only in the POCIS deployed at the Delmarva sites. Although there are numerous natural sources of this hormone in water bodies (e.g., fish, waterfowl), the fact that it was not detected in the water at the reference site is suggestive of a poultry litter- related source at the Delmarva sites. Several studies have indicated that estrogen is readily leached from soils containing chicken litter into aquatic systems via surface run-off (Shore et al. 1995, Nichols et al. 1997). In addition, follow up analyses by one of our laboratories has indicated that 17β-estradiol and estrone are also present in aqueous extracts of poultry litter (Appendix 3). Concentrations ranged from 85 to 140 ng/L for 17β-estradiol and 260 to 600 ng/L of estrone. It is unclear why estrone was not detected in the POCIS. One possibility is that the estrone may be conjugated and the conjugated forms would not be detected by our analytical procedure. Atkinson et al. (2003) have demonstrated that one-half to two-thirds of total estrone in coastal samples occur as polar conjugates.

We used the POCIS data to estimate ambient water column concentrations of 17β-estradiol, using the sampling rate data obtained during laboratory exposures (Alvarez 1999) and the following equation:

$$C_W = M_{\text{Analyte in POCIS}} / (R_s)\,(t)$$

where C_W is the estimated water concentration, $M_{Analyte\ in\ POCIS}$ is the total mass of the analyte of interest in the POCIS sample extract, R_s is the sampling rate in L/d, and t is the time in days. Estimated water concentrations of 17_-estradiol ranged from about 100 to 280 ng/L in Year 1 and 3.3 to 44 ng/L, in Year 2. Higher average concentrations in Year 1 may be related to the timing of litter application and deployment of the POCIS and/or differences in rainfall during the deployment period. Finlay-Moore et al. (2000) reported flow weighted run-off concentrations for estradiol from litter applied fields ranging from a high of 305 to 820 ng/L, 4 days after litter application to a range of 60 to 125 ng/L, 88 days after application. Similarly, Shore et al. (1995) reported stream concentrations on Maryland's eastern shore averaging 5.4 ng estrogen/L after poultry litter application. In the nationwide survey, Kolpin et al. (2002) found 17β-estradiol in approximately 10% of the samples, at average concentrations ranging from 9 to 160 ng/L, depending on the analytical method. Estrone was found in 7% of their samples, at an average concentration of 27 ng/L. Exogenous 17β-estradiol at concentrations as low as 1 ng/L have been found to induce Vtg production in male fish (Purdum et al. 1994). A two month exposure of immature male carp to a 100 ng/L nominal concentration of 17β-estradiol induced plasma Vtg levels in the order of 10^5 μg/ml compared to control levels of 10 μg/ml (Gimeno et al. 1998).

Year 2 - Biological Effects

As in Year 1, we found no evidence that the benthic macroinvertebrate communities at the study sites were impaired relative to the Jug Bay reference. In fact, Jug Bay had the lowest number of taxa and EPT taxa of the four sites sampled. We did not detect Vtg in the plasma of fathead minnows that were either caged *in situ* or exposed in the laboratory to surface water samples from the Delmarva sites. As indicated above, estimated concentrations of 17_-estradiol in Year 2 were 3.3 to 44 ng/L, approaching the lower limit of levels that induce Vtg production in fathead minnows. Tyler et al. (1999) exposed fathead minnows from 24 hours post-fertilization to 30 days post hatch and found fish exposed to 50 ng/L and 100 ng/L 17-β estradiol had significantly higher concentrations of plasma Vtg than control fish. Panter et al. (1998) reported that a dose of 100 ng/L was required to induce Vtg in adult males in a three week exposure period. Hence, estrogen concentrations during our exposure appear to be below that threshold. We did, however, observe effects on larval fish survival in three week exposure to surface waters from the Delmarva sites. Average survival was low in all exposures and significantly reduced in the water from Slaughter Creek. The cause of the toxicity is unknown. Water quality parameters measured in the laboratory indicated dissolved oxygen, pH, and ammonia were all within acceptable limits. Trace element concentrations in surface water samples were below acute and chronic water quality criteria. Unfortunately, no data on surface water concentrations of dissolved agrichemicals are available for Year 2, as there was an analytical malfunction on the sample from Slaughter Creek. However, sediments from Slaughter Creek had the highest concentrations of several pesticides, including chlorpyrifos, diazinon and dieldrin, suggesting that agrichemicals may have played a role. Interestingly, Slaughter Creek had the lowest number of EPT taxa of the Delmarva sites, 3 versus 8 and 13 at Cod Creek and Beaverdam Creek, respectively.

As in Year 1, plasma Vtg was detected in all resident male carp collected in Year 2. We found significantly higher average concentrations in the fish from Slaughter Creek than at Jug Bay, 53 versus 20 μg/ml Vtg, respectively, suggesting that fish on the Delmarva are being exposed to higher levels of estrogenic compounds. Vitellogenin concentrations in all fish, including those from the Jug Bay reference, appear to be above normal concentrations based on the results of McDonald et al. (2002). McDonald et al. (2002) reported detecting Vtg in only 28 of 384 male carp collected from the Mississippi River Basin. They detected trace amounts of Vtg (1 to 10 μg/ml) in at least one male carp from 15 stations and greater concentrations (10 to 30 μg/ml) in only one carp from each of two stations. Vtg concentrations equivalent to that commonly found in females (958 and 2,645 μg/ml) were detected in only two fish in their study. By comparison, Folmar et al. (1996) collected carp from a sewage effluent canal near St. Paul, Minnesota and found 6 of 10 male carp had plasma Vtg concentrations ranging from 30 to 10,000 μg/ml, within the range measured in females (35 to 1357 μg/ml) that were also collected from the site. We should note that our samples may have been inadvertently biased by collecting males that we could identify externally, by "milking". That is, we may have selected for functioning males, rather than randomly sampling all males in the population. Future efforts should focus on a more random sample of the population and include assessment of males and females as well as histopathological analyses.

The finding that concentrations of Vtg in male carp from the reference site, Jug Bay on the Patuxent River, also suggest exposure to estrogenic compounds, is somewhat surprising. The surrounding land use is a combination of agriculture (~ 40%), forest and wetlands (~ 50%), with very little urbanization (~ 6%). However, there are some agricultural chemicals, most notably the herbicide atrazine, that are thought to have endocrine modulating effects (Goodbred et al. 1997). We found fairly high levels of atrazine in the POCIS deployed at this station. Therefore, it is possible that this herbicide, possibly in conjunction with other estrogen mimics, is inducing Vtg production in indigenous carp.

Conclusions

Our study provides direct evidence for transport of tetracycline compounds, and possibly 17β-estradiol, from poultry litter applied fields to adjacent waterbodies on the Delmarva peninsula. Furthermore, Vtg analyses suggest that fish on the Delmarva are being exposed to estrogenic compounds. We recognize that our data are very limited, both in terms of the number of samples as well as the geographic coverage. Nonetheless, the potential for system-wide estrogen and antibiotic contamination on the fish and wildlife resources of the Delmarva peninsula merits further investigation. In future studies, we recommend random sampling and analysis of carp for Vtg, histopathology as well as an evaluation of potential population-level effects of endocrine disrupting effects. This study represents one of the first attempts to deploy POCIS in the field. We are very encouraged by the ability of the POCIS to detect AFO-related compounds and recommend their use in future assessments of AFOs and other relevant applications. Finally, an unexpected finding was that mercury concentrations in piscivorous fish around Prime Hook NWR were at levels of human health concern. At present, there is no fish consumption advisory in these waterbodies; therefore, we recommend follow-up sampling to confirm our initial findings.

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