DEPARTMENT OF THE INTERIOR
U.S. FISH AND WILDLIFE SERVICE
REGION 5

FY08 ENVIRONMENTAL CONTAMINANTS PROGRAM
ON-REFUGE INVESTIGATIONS SUB-ACTIVITY

Assessment of Endocrine Disruption in Smallmouth Bass (*Micropterus dolomieu*) and Largemouth Bass (*M. salmoides*) in Region 5 National Wildlife Refuges

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II. INTRODUCTION

II.A. Background and Justification

The Problem

Since the early 1980s, endocrine disruption in humans, fish, and wildlife has been recognized as a global environmental concern (McLachlan 1980; Colburn and Clement 1992). Both man-made chemicals and plant and animal hormones have been shown to have endocrine disrupting effects. In aquatic systems, two major sources are agricultural production, especially intensive activities, such as animal feeding operations (AFOs) (Iwanowicz *et al*. 2004; Orlando *et al*. 2004) and sewage treatment plant effluents (e.g., Harries *et al*. 1996, 1997; Folmar *et al*. 1996, 2001). **Kirk *et al.* (2002) found that sewage treatment plants with secondary treatment transfer up to 30 percent of the androgenic and estrogenic compounds into the effluent. In plants that relied solely on primary treatment, up to 93 percent of the androgenic and estrogenic compounds analyzed in influent were detected in effluent.**

Evidence is mounting to suggest that endocrine disrupting compounds may be affecting reproduction in fish populations. Endocrine-disrupting effects in fish have been characterized through the use of biomarkers and by direct measurement of reproductive success. One key biomarker is the measurement of vitellogenin, a protein precursor of egg yolk (Harries *et al*. 1996). In male fish, vitellogenin concentrations are typically extremely low (non-detected or just above the limit of detection), whereas in male fish exposed to estrogenic compounds Vtg concentrations can be similar to those in females. In addition, ratios of the hormones 17ß-estradiol and testosterone (or 11-ketotestosterone) can be measured and compared (Toft *et al*. 2003). Gonad histopathology is used to detect the presence of oocytes in testes, characterized as intersex (Kirby *et al*. 2004). Finally, laboratory assays can be used to measure sperm quality, fertilization, and hatching success (Cheek *et al*. 2001). Cheek *et al.* (2001) demonstrated the relationship between environmental estrogens, increased vitellogenin protein and decreased gonad development in Japanese medaka (*Oryzias latipes*). This study exposed eight-week post-hatch fish to 0.5, 1.0, 2.5 and 7.5 ppb concentrations of *o,p'* –DDT. The authors concluded that all concentrations induced vitellogenin after eight weeks when compared to the reference (0 ppb). The higher concentrations led to a female-skewed sex ratio in adults and progressive gonadal intersex in male fish. **All doses demonstrated significantly reduced fertility and hatching success in the F2 generation. Recently, Martinovic *et al.* (2007) reported that 9 of 10 male fathead minnows exposed to sewage treatment plant effluent were unable to reproduce in the presence of competing males.** In addition, the steroidal androgen, trenbolone, which is administered to beef cattle, significantly reduced plasma vitellogenin in female fathead minnows (Miller *et al.* 2007). In this study, the authors predicted population-level impacts associated with reduced vitellogenin concentrations.

Numerous studies have indicated the induction of vitellogenin in male fish either captured or caged near sewage treatment plants (e.g., Folmar *et al*. 1996, 2001;

Harries *et al*. 1996, 1997). Folmar *et al.* (1996) reported reduced serum testosterone and vitellogenin induction in male carp (*Cyprinus carpio*) collected near the St. Paul, Minnesota sewage treatment plant. Similar effects were also observed in male walleye (*Stizostedion vitreum*) from the same location. Although intersex was not observed, gonads showed less advanced stages of spermatogenesis relative to fish from a reference location.

Kavanagh *et al.* (2004) studied gonadal intersex in male and female carp, gizzard shad (*Dorosoma cepianum*), brown bullhead (*Ameiurus nebulosus*), pumpkinseed sunfish (*Lepomis gibbosus.*), bluegill (*Lepomis macrochirus*), and white perch (*Morone americana*) in the Lower Great Lakes region. **The authors found that 22 to 83 percent of the young of the year to two year-old male white perch exhibited gonadal intersex compared to 0 percent observed in hatchery-raised fish and at the reference site. Plasma vitellogenin in the male white perch ranged from 49-1,711 μg/ml at the study sites sampled. This study concluded that white perch in the Lower Great Lakes region are likely being exposed to endocrine-disrupting substances from sewage and industrial treatment plant effluents and that this exposure may be responsible for the induction of gonadal intersex.**

Harries *et al.* (1997) placed male trout in cages at varying distances downstream of five sewage treatment plants in the United Kingdom. After exposure to effluent for three weeks, the authors demonstrated that male fish were synthesizing vitellogenin on a decreasing gradient for up to 5 km downstream presumably in response to dilution of endocrine disrupting chemicals in the sewage treatment plant effluent. Generally fish synthesized more vitellogenin the closer they were to the sewage treatment plant.

Orlando *et al.* (2004) found endocrine disrupting effects in populations of fathead minnows (*Pimephales promelas*) collected upstream and downstream of cattle feedlot effluent on the Elkhorn River in Nebraska. The study documented androgenic activity in the water below feedlots. Evidence of lower testicular testosterone syntheses and smaller testes size were observed. The author concluded that feedlot effluent contained a mixture of endocrine active substances that alter the hypothalamic- pituitary-gonadal axis. The authors suggested that these alterations were in response to growth implants, natural hormones and/or endogenous fecal steroids found in the runoff and effluent of the feedlot operations.

In the USGS Biomonitoring of Environmental Status and Trends (BEST) Program, fish health surveys have examined carp as the representative bottom-dweller and largemouth bass (*Micropterus salmoides*) and smallmouth bass (*M. dolomieu*) as the representative predator (Schmitt 2002; Schmitt and Dethloff 2000; Schmitt *et al.* 2005, Hinck *et al.* 2006, 2007). These studies have a reproductive biomarkers component that includes measurement of plasma hormone and vitellogenin concentrations and gonad histopathology. Recently USGS issued a nationwide report on endocrine disruption in carp and bass (Goodbred *et al*. 2007). It is our intention to utilize these studies in the proposed project both as a source of data for comparisons and as a framework to ensure compatibility.

FWS-funded studies in the Chesapeake Bay watershed

In a Division of Environmental Quality (DEQ)-funded on-refuge investigation, McGee *et al.* (2003a) studied vitellogenin in the blood of male carp (*Cyprinus carpio*) exposed to runoff from poultry operations near the Prime Hook National Wildlife Refuge (Milton, DE) and a site at Blackwater National Wildlife Refuge (Cambridge, MD). This study concluded that male carp had vitellogenin concentrations of 0.025 to 0.030 mg/ml plasma at both the Prime Hook and the Blackwater National Wildlife Refuge sites. Further investigation was recommended.

With funding from the National Fish and Wildlife Federation, McGee *et al*. (2003b) conducted a pilot study in the tidal Potomac estuary looking at gonadal histopathology and vitellogenin in five adult male carp near the Blue Plains wastewater treatment plant in Washington, DC. Vitellogenin was detected at low concentrations in all males (0.001to 0.058 mg/ml), including six males from the reference location in the Patuxent River at Jug Bay. This study concluded that there may be widespread low-level exposure of endocrine disrupting compounds to fish populations and recommended further evaluation of endocrine disrupting compounds in the Potomac River.

The Potomac is the second largest tributary to the Chesapeake Bay. It is an important spawning and nursery ground for both migratory and resident fish species including American eel (*Anguilla rostrata*), striped bass (*Morone saxatilis*), white perch, shad and herring (*Alosa sp.*), smallmouth bass (*Micropterus dolomieu*), largemouth bass (*M. salmoides*), sunfish (*Lepomis* sp.), and carp. The upper portions of the Potomac in West Virginia and parts of Virginia are dominated by rural communities and animal agricultural facilities. Closer to the Chesapeake Bay, the Potomac watershed becomes more urban with agricultural discharges being replaced more frequently with municipal sewage treatment plant discharges. According to the Maryland Department of the Environment, there are 747 permitted surface water discharges within the Maryland waters of the Potomac River watershed. One hundred seventeen are municipal wastewater treatment plants (WWTPs; Maryland Department of the Environment 2004).

Studies of endocrine disruption in smallmouth and largemouth bass in the Potomac River watershed have received considerable attention in the media and resulted in Congressional hearings in October 2006 (USGS 2006). According to USGS (2006), the National Fish Health Research Laboratory (NFHRL) initiated fish health assessments of the South Branch of the Potomac River in 2003 and 2004 in response to fish kills and observations of external lesions on smallmouth bass and other species. The studies were a joint effort with the West Virginia Department of Natural Resources. In those studies, 16 of 24 sampling events showed more than 25% of the male bass with intersex characteristics.

In 2005, more extensive sampling to determine the extent of the intersex problem in the Potomac watershed was conducted. A portion of the efforts was funded through an Off-Refuge Investigation (Guy and Pinkney 2007). The 2005 sampling involved collaboration between USFWS-CBFO, USGS-NFHRL, Virginia Department of Game

and Inland Fisheries, Virginia Department of Environmental Quality, West Virginia Department of Natural Resources, Maryland Department of Natural Resources, and Virginia Institute of Marine Sciences.

The Off-Refuge Investigation studied the effects of exposure to smallmouth and largemouth bass at five locations: Upper Conococheague Creek (agricultural), Lower Conococheague Creek (sewage), Upper Monocacy River (agricultural), Lower Monocacy River (sewage), and the main stem of the Potomac River near the Blue Plains wastewater treatment plant in Washington, DC. The investigation is a two-year study with the following testable hypotheses:

- Sewage treatment plants are releasing detectable concentration of endocrine disrupting compounds including natural and synthetic hormones into the Monocacy River, Conococheague Creek and the main stem of the Potomac River near Washington, D.C. as measured by Semi Permeable Membrane Devices (SPMDs) and Polar Organic Compound Integrated Samplers (POCIS).

- Fish exposed to endocrine disrupting compounds will have altered concentrations of vitellogenin and hormones.

- Fish exposed to endocrine disrupting compounds will exhibit histological changes in the gonads such as intersex.

The most dramatic finding from 2005 was that 80-100 percent of male smallmouth bass and 30 percent of the male largemouth bass had intersex gonads (Table 1). Specifically, immature eggs were detected in the testes**.** In addition, fish from all sites (at similar reproductive stage) had relatively low gonadosomatic index (GSI) scores (GSI is the ratio between gonad weight and the total weight of the fish). These results suggest that bass populations in the Potomac River watershed may be experiencing decreased reproductive function. Chemistry data are expected shortly. In 2006, caged studies were performed with smallmouth bass in the Conococheague and Monocacy locations. Results are pending.

Endocrine Disruption and National Wildlife Refuges

A goal of the Clean Water Act (CWA) is to "restore and maintain the chemical, physical and biological integrity of the Nation’s waters." Although the Service and other federal agencies work with the states and USEPA to implement the applicable requirements of the CWA, further progress is needed both to prevent degradation of high quality waters and sensitive aquatic ecosystems and to accelerate the restoration of degraded water resources. The Unified Federal Policy for a Watershed Approach to Federal Land and Resource Management (Federal Register, October 18, 2000; Vol. 65, No.202 pp. 62566-62572) (UFP), to which the Department of the Interior is signatory, provides a foundation to help ensure that federal land and resource management activities meet the CWA's goals and that the federal government serves as a model for water quality stewardship. This investigation is consistent with that policy in that it implements a science-based

approach to watershed assessment, as well as uses a regional landscape approach across multiple states within the Service's Region 5. Agricultural runoff, urban storm water and wastewater effluents, industrial discharges, and waste sites as well as management activities on NWRs, such as historic pesticide use can all negatively influence water quality. Certain NWRs within Region 5 are already protected under a no measurable change anti-degradation classification. The study protocols in this investigation are orchestrated with other federal, state and local monitoring programs to the extent practicable. As such, the study undertaken by the Service and its partners is consistent with the intent of the UFP as well as sound NWR management initiative.

One of the mandates of the Refuge Improvement Act is to ensure adequate water quality on NWRs. In addition, the Biological Integrity, Diversity, and Environmental Health policy (601 FW 3) requires refuge managers to assess, protect, and restore environmental health. Section 3.20 recognizes that events occurring off-refuge can injure or destroy biological integrity, diversity, and environmental health of a refuge and states that refuge managers should address these concerns.

These results should assist in the identification of management opportunities, priorities and development of alternatives to protect or restore watersheds associated with individual NWRs. Since our assessment objectives are focused on fish health indicators, we are also providing a regional snapshot regarding fish health variability across the region that is consistent with the objectives and methods of the U.S. Geological Survey's Biomonitoring of Environmental Status and Trends (BEST) Program, but with a Refuge-specific focus. Results of the fish health assessment can be used to determine which species and NWRs may need further assessment for determination of specific pollutant concentrations at a specific sampling station, while conserving limited financial resources until a biological need is demonstrated. Working with our partners, such as the States, we can develop the needed insight to better understand if fish health has been compromised and what needs to be done to restore species to full health consistent with the Clean Water Act, the National Wildlife Refuge System Improvement Act of 1997, and other mandates. Study results may offer input to future revisions of existing NWR-specific Comprehensive Conservation Plans (CCPs), including acquisition of long-term environmental quality trend data and strengthening partnerships with the States and local watershed partners. Protection of designated and existing uses (fish and wildlife) as well as attainment of anti-degradation classification and policies that are part of State water quality standards cannot be achieved without scientifically-defensible, long-term trend data.

II.B. Scientific Objective(s)

Our goal is to evaluate the extent and magnitude of endocrine disruption (including intersex) in smallmouth and largemouth bass in rivers and impoundments at Region 5 National Wildlife Refuges.

The specific objectives of this project are to:

Years 1 and 2

Identify if fish, from selected locations in rivers on or adjacent to Region 5 NWRs are experiencing endocrine disruption as evidenced by gonad histopathology including intersex; abnormal hormone concentrations; or abnormal plasma vitellogenin concentrations.

To the extent possible, compare the findings in an upstream-downstream design to evaluate the impacts of suspected sources of endocrine-disrupting compounds.

Document whether fish are being exposed to endocrine-disrupting chemicals by performing *in vitro* screening assays on water samples at the collection sites.

Year 3

Identify if fish, from selected locations in ponds within Region 5 NWRs are experiencing endocrine disruption as evidenced by gonad histopathology including intersex;abnormal hormone concentrations; or abnormal plasma vitellogenin concentrations

Document whether fish are being exposed to endocrine-disrupting chemicals by performing *in vitro* screening assays on water samples at the collection sites.

Year 4

Integrate the results of Years 1 through 3 in a report. Prepare manuscripts and submit to journals. Prepare fact sheets and briefing statements for Congress. Work with public affairs to disseminate the results to print and electronic media. Local field offices work with refuges on specific problems and present findings to state agencies with designated Clean Water Act authority.

II.C. Management Action(s)

Our goal is to evaluate the extent and magnitude of endocrine disruption (including intersex) in smallmouth and largemouth bass in rivers and impoundments at Region 5 National Wildlife Refuges. Compromised fish will likely form a gradient across multiple refuges including riverine, wetland, and impoundment habitats. The degree to which these species and habitats differ with respect to displaying abnormal physiological and histopathological characteristics can be useful for both indirect and direct management actions. Largemouth and smallmouth bass are sentinel trust species within the NWR system for assessing the effects of endocrine disrupting chemicals. Given what is already known about these species, they serve as important indicators of ecosystem health.

Indirect Action: In Year 4 (and subsequently), local field offices in concert with the refuges, will communicate the results and concerns to state agencies with delegated water quality authority. Listing of waters as impaired (through the Clean Water Act 303(d) authority) does not require that a putative contaminant linkage be made at the time of listing. Such listings will result in the state agencies conducting investigations to identify a causal factor(s) that can be corrected through load (non-point source pollutants) or waste load (point source pollutants) reductions under the total maximum daily load (TMDL) program. Antidegradation provisions of State water quality standards categorize all waters based on the amount of permissible degradation allowed. Waters within some NWRs receive designations that allow a high quality protection with no measurable decrease in ambient water quality. Field offices have successfully advocated these issues with State agencies to protect trust resources (threatened and endangered species) within specific watersheds via water quality standards and permit changes. Documentation of such cases is available from the New Jersey Field Office (e.g., Day 2003).

Direct Action: **Discretion of NWR managers is quite broad to address direct management action within NWR property lines. Legal authorities such as the Refuge Improvement Act and the Biological Integrity, Diversity, and Environmental Health policy are described in section IIA (*Endocrine Disruption and National Wildlife Refuges*). Impoundments could be managed differently regarding source waters, be drained, used as non-fish management units or altered physically ('staircasing" to increase filtering functions) to minimize unwanted effects. Pesticide use has direct management application through integrated pest management and could be utilized to mitigate compromised habitats.** Waste sites, including Superfund National Priority List (NPL) designated sites, contain opportunities for both remediation and restoration decisions both within the direct purview and discretion of R5 refuge managers. Lastly, the possibility of compelling decisions from outside regulators on refuge management is a form of direct action as well. Working together, local field offices and refuges could apply for funding to conduct more detailed investigations and could interact with industrial facility (such as wastewater treatment plant) managers to reduce loadings.

III. METHODS

III.A. Data Collection and Analysis

Home range of largemouth and smallmouth bass

Studies using fish as indicators of habitat quality should include information on home range as part of the rationale for selecting sampling locations. For ponds, we are collecting single samples and home range information is not needed. For nearfield/farfield comparisons in rivers it is important to gather available information to determine whether the sampling locations are likely to result in non-overlapping groups of fish.

Thus, we have reviewed the literature on home range of these species in flowing waters, both tidal and non-tidal. In the tidal waters of the Northern Chesapeake Bay, Richardson-Heft *et al.* (2000) reported that tagged largemouth moved an average of 2.8 km from one capture site (Susquehanna R.) and 2.1 km from a second capture site (Northeast R.). However, 4% of Susquehanna fish and 6% of Northeast fish had longer movements of 15-21 km. This study showed that while the vast majority of largemouth bass have a restricted home range, a small percentage have greater movements. A similar conclusion was reached by Freund (2003) in a study of largemouth bass in the Ohio River, who refers to "homebodies" and "movers". In the tidal Hudson River, Nack *et al.* (1993) reported movements of 3-16 km as indicative of spawning migration. Paller *et al.* (2005) used radio telemetry to track the movement of largemouth bass in a ^{137}Cs contaminated stream (Steel Creek) and in the Savannah River, near the Savannah River (reactor) Site. Of 30 Savannah River fish, 15 consistently had limited mobility (generally less than 100 meters between relocations and 15 moved longer distances (between 1 and 35 km). Four of the 15 individuals that moved long distances returned to their points of origin. They calculated an average home range of about 500 linear meters of shoreline. There were some individuals that were largely sedentary and others more mobile and some that switched behaviors. Approximately 90% of the relocations of Steel Creek fish in the Savannah River were within 10 km of Steel Creek.

For smallmouth bass in Wisconsin rivers, Langhurst and Schoenike (1990) reported that there was little movement in summer where 89% of electrofishing recaptures were within a 5 km reach of the capture site. In autumn, however, bass traveled from 35 to 109 km of the capture site but most returned to the original capture site in the spring. In contrast, Todd and Rabeni (1989) did not report long range movements and reported longitudinal home ranges of less than 200 m in Jacks Fork River, Missouri. In the Huron River (Michigan), Beam (1990) reported home ranges ranging from 30 to 370 meters with no significant difference between seasons.

Thus, to the extent possible we will attempt to select sampling sites that have isolated populations of fish. This occurs when there are dams or great distances separating the sampling sites. For example, there is a reservoir about 11 km upstream of the Patuxent Research Refuge, Laurel, MD. According to Maryland Department of Natural Resources biologist Mary Groves, there is no fish passage between the reservoir fish and those below the reservoir. At the refuge boundary there is a 7.5 million gallon per day wastewater treatment plant. Thus, we are assured that largemouth bass downstream of the plant (the nearfield fish) are not overlapping with the reservoir (farfield) fish. If isolating dams are not in place, we will attempt to select locations that are at least 10 km apart based on the literature described above.

Site selection

Each Principal Investigator contacted Refuge Biologists, state officials, and local biologists with knowledge of the availability of largemouth and smallmouth bass on or near each refuge. Potential sample sites were placed into a spreadsheet (Tables 2-4) with separate sheets for static and flowing waters**. The spreadsheet summarized available**

information on the expected ease and method of sampling, the land use around the sampling site, any potential sources of endocrine-disrupting chemicals such as wastewater treatment plants or intensive agriculture, and (for rivers) whether a paired impacted/less impacted pairing could be established. Each field office was asked to prioritize separately their river and impoundment sites. The prioritization was based on an evaluation of the likelihood of catching sufficient fish, the potential for observing local impacts of a suspected source, and the potential for management action if a source is identified. A matrix with summary data for all proposed sites is given as Tables 2-4. More detailed rationales for a selection of the refuges are provided in Appendix A.

Fish collection

From each site, ten male and ten female adult fish will be collected from each location using boat or barge electroshocking, or if necessary gill netting. We will collect in the late summer-fall time period to match that of the BEST program (e.g., Hinck *et al.* 2006, 2007). Colleagues with state fisheries agencies and Fisheries Resource Offices will provide expertise and assistance with fish collection, many of which are providing services in-kind. Fish will be euthanized with MS-222 and a thorough necropsy as described by Goede and Barton (1990) and Smith *et al*. (2002a) will be completed. Any grossly visible external or internal abnormalities will be documented. The major organs will be preserved in 10% buffered neutral formalin for histopathology which will be performed outside the scope of this project. Portions of major organs will be frozen and archived for possible genomic workup. Each fish will be weighed, measured, bled, and scales and otoliths taken for aging. In addition, during the necropsy, liver and gonad will be weighed. From these data, condition factor, size at age, hepatosomatic and GSI will be calculated and used in the analysis and interpretation of the results.

Reproductive biomarkers

Methods will be consistent with those of the BEST program (Schmitt *et al.* 2005, Hinck *et al.* 2006, 2007). Gonad histology will be used to confirm sex, determine reproductive stage, and detect the presence of atresia, intersex, neoplasia, ceroid deposits, Sertoli cell proliferation and other abnormalities as described by Blazer (2002). Plasma samples will be analyzed for 17ß-estradiol, 11-ketotestosterone, and testosterone using radio-immunoassay (RIA) procedures (Bevans *et al*. 1995; Goodbred *et al*. 1996; Smith *et al.* 2002b). Vitellogenin, an egg-yolk precursor protein produced in response to estrogen and estrogen agonists, is normally found only in the serum of adult female oviparous vertebrates, but it can be induced in males and immature females by estrogenic compounds or estrogen mimics. Vitellogenin will be quantified using an enzyme-linked immunosorbent assay (ELISA). The assay will use monoclonal antibodies developed for smallmouth and largemouth bass and will follow the methodology described in Smith *et al*. (2002b). Vitellogenin analyses will be conducted according to (Denslow *et al*. 1996, 1997). The ELISA assay used can detect between 10 and 100 ng of VTG per well, resulting in a sensitivity of about 0.001 mg/ml.

In vitro screening assays

Numerous *in vitro* reporter systems have been developed to evaluate the estrogenicity of chemicals. Many of these assay platforms involve the utilization of estrogen sensitive mammalian cell lines that have been genetically modified to produce specific enzymes (which can then be quantified) following exposure to estrogen. While these assay platforms are sensitive, mammalian cells tend to be affected by the inherent toxicity of many chemicals. They are also fairly costly to perform. Recently a BLYES yeast reporter has been developed that is more sensitive than the previous YES strain. Utilization of this yeast reporter is sensitive ($\approx 4 \times 10^{-11}$ M), cost-effective, and less sensitive to toxic chemicals.

***In vitro* screening assays will be utilized to measure estrogenic potential of chemicals present in grab samples of water. If a WWTP or industrial plant is the focus of one of the paired sites, the facility will be contacted so that an effluent sample can be obtained. Effluent and water samples (from both the nearfield and farfield sites) will be collected in clean amber bottles and stored on ice**. Samples will then be filter sterilized and stored in glass bottles at -80°C prior to the assay. Water samples and estradiol standards will then be added to the wells of a 96-well black plate and samples dried using a speed-vac. The yeast reporter (strain BLYES) will be was grown in YMM (leu^-, ura^-) overnight at 30°C and 200 rpm shaking to an approximate optical density at 600 nm (OD_{600}) of 1.0. Cells are then centrifuged and resuspended in fresh YMM (leu^-, ura^-) to an OD_{600} of 1.0 and transferred to the wells of a black 96-well plate containing the dried samples. Bioluminescence is then measured every 60 min for 12 h in a SpectrafluorPlus plate reader (Tecan) with an integration time of 2 s/well. Estrogenicity of the water samples is interpolated from the curve generated with the estradiol standards (Sanseverino *et al.* 2005). Similar methods will be employed to determine androgenicity as yeast reporter strains become available.

Data Analysis:

To the extent possible, USGS statisticians have been and will continue to be consulted on the study design and data analysis procedures to make these results compatible with those in the BEST program. We recognize that this field study cannot prove a cause and effect relationship between a suspected source and biological observation due to factors that cannot be controlled (water quality parameters, stream flow, etc.).

<u>Nearfield/farfield comparisons</u>

For continuous data (e.g., GSI, hormone concentrations, vitellogenin concentrations, intersex severity index, sperm motility parameters), comparisons between the nearfield and farfield locations will be made using traditional statistical procedures treating each sex separately (Sokal and Rohlf 1981). If parametric assumptions are satisfied, means will be compared using t-tests. Log transformation will be used if

necessary. If parametric assumptions are still not satisfied, comparisons of medians will be performed using the Mann-Whitney test. The null hypothesis in these tests is that there is no difference in means (or medians) between the two locations.

For prevalence data (atresia, intersex, and detection of vitellogenin in males, etc.), comparisons will utilize Fisher's Exact test (Stokes et al. 1995), again treating males and females separately. The null hypotheses are that there is no statistical difference in the prevalence data between nearfield and farfield locations. Sigma Stat and SAS software will be used. A *p* value of 0.05 will be the test for significance.

<u>Comparisons with reference locations</u>

Dr. Blazer's group is developing a data base for reproductive health parameters for "reference" areas for both largemouth and smallmouth bass. To the extent possible, this data base will be used for comparisons with site data. Data will also be compared with values obtained through the BEST program national reconnaissance studies. Comparisons will be performed on a refuge basis keeping the flowing waters separate from the ponds:

a) Null hypothesis: There is no statistically significant difference in the prevalence of intersex at the sampling site(s) compared with Dr. Blazer's pooled reference locations (and/or BEST program recon data) [critical value: $p<0.05$, Fisher's Exact test]

b) Null hypothesis: Mean (median if parametric assumptions are not met) plasma vitellogenin concentrations in each sex at the sampling site(s) are not significantly different from those at Dr. Blazer's pooled reference locations (and/or BEST program recon data) [critical value: $p<0.05$, two tailed t-test or Mann-Whitney U-test if one site, ANOVA followed by Tukey's and Kruskal-Wallis followed by Dunn's method if more than one site}

c) Null hypothesis: Mean (median if parametric assumptions are not met) hormone concentrations and ratios in each sex at the sampling site(s) are not significantly different from the average hormone concentrations and ratios measured at Dr. Blazer's pooled reference locations (and/or BEST program recon data) [critical value: $p<0.05$, two tailed t-test or Mann-Whitney U-test if one site, ANOVA followed by Tukey's and Kruskal-Wallis followed by Dunn's method if more than one site]

<u>Histopathological data</u>

We will evaluate the patterns of ovarian follicular development and maturation of the testes by determining the proportion of each developmental stage. We will explore the approach of Snyder *et al.* (2004) in which multivariate profile analysis was performed based on arc-sine transformed proportions.

III.B. Proposed Schedule of Milestones

This is a four-year proposal. In Year 1, we will collect fish from 17 sites from 8 prioritized refuges. One refuge will have three sites and the other seven will be paired. In Year 2, we will collect fish from an additional 14 paired sites from seven refuges, which will complete the matrix for river sites. In Year 3, we will collect from 13 impoundments at ten refuges. Yearly interim reports will be prepared. A final report fact sheets, and briefing statements will be prepared in Year 4. Separate journal articles will be prepared for the river and pond surveys.

In Year 4, outreach to refuges and advocacy for resolving refuge-specific problems will be conducted by the local field office in concert with the refuge. State agencies with delegated water quality authority will be contacted by the local field offices and approaches for proposing waterways for TMDLs and other actions will be discussed.

IV. INTERIM REPORT

IV.A. Results to Date

None. This is a new proposal.

IV.B. Significant Changes to Previous Proposal

None. This is a new proposal

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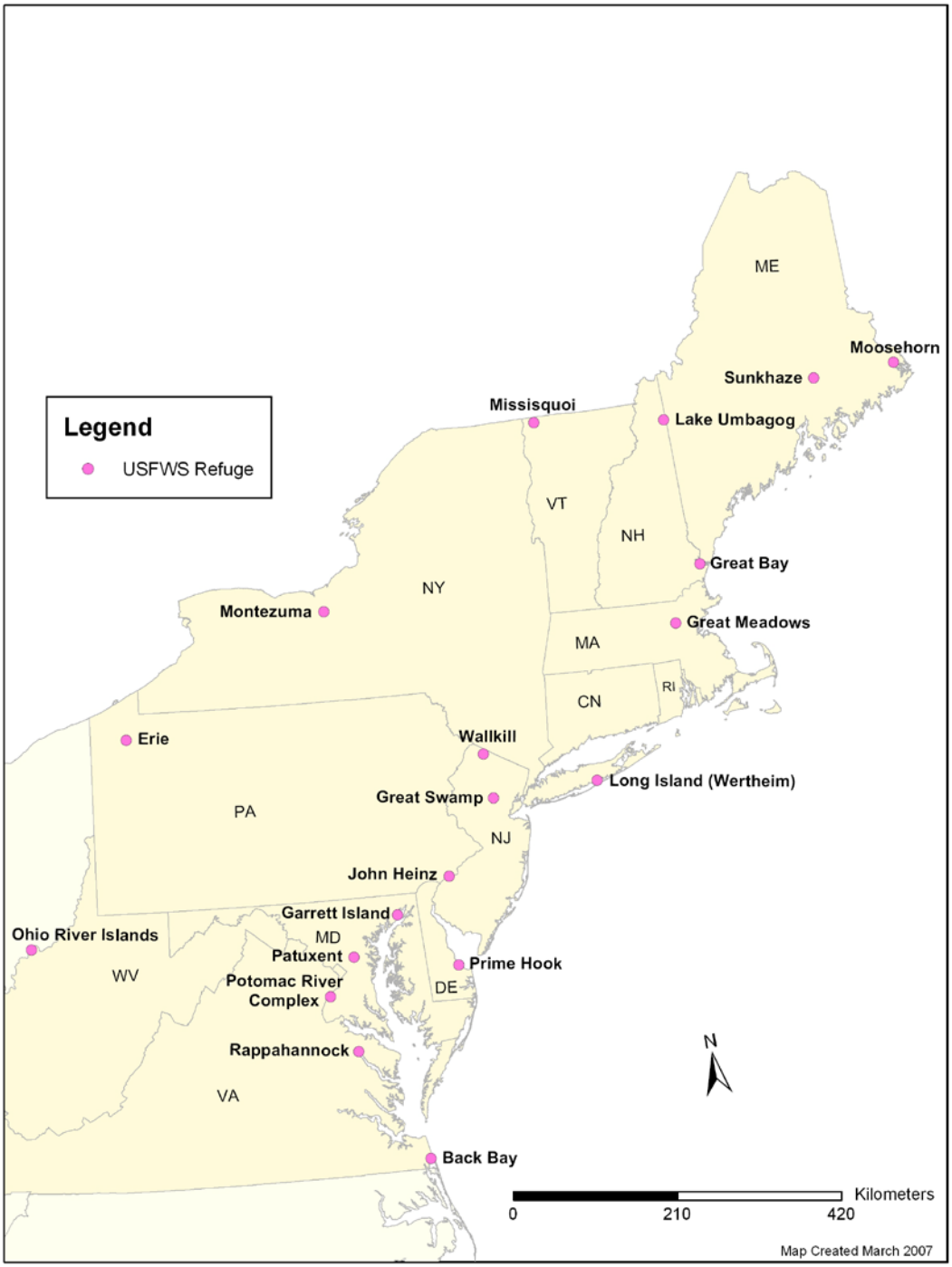

Figure 1. Map of Region 5 National Wildlife Refuges proposed for sampling.

Table 1**.** Intersex (males with primary oocytes in the testes) and Gonadosomatic Indices (GSI) in smallmouth bass collected in the Potomac River watershed and reference areas in fall 2005; (USGS data from V.S. Blazer, pers. comm., summarized in Guy and Pinkney 2006).

Site	**Mean Male GSI***	**Prevalence of Intersex**	**Mean Severity of Intersex****	**Mean Female GSI***
Shenandoah				
Cowpasture	0.62	100%	2.0	1.83
South Fork	0.64	80%	1.2	1.32
North Fork	0.83	100%	1.2	1.02
Mainstem	0.55	100%	1.7	0.89
Potomac – MD				
Upper Conococheague	0.39	100%	2.1	1.24
Lower Conocheague	0.13	90%	1.8	0.62
Upper Monocacy	0.32	80%	1.3	0.85
Lower Monocacy	0.30	100%	1.9	0.81
Washington, D.C. (largemouth bass)	0.58	23%	0.2	0.43
Out of Basin				
Gauley River	0.55	16.7%	0.2	2.17
Back Fork Elk River	0.59	36.4%	0.2	2.66
West Fork Greenbrier	0.55	27.8%	0.1	2.77

*****GSI = gonadosomatic index (gonad weight in grams/total fish weight in grams) X 100
** severity scale 0-3 with 3 being the highest.
Note: The Lower Conococheague and Lower Monocacy sites were just downstream of waste water treatment plants. There were statistically significant decreases (t-test, $p<0.05$) in mean male and mean female GSI in the Lower vs. Upper Conococheague.

Table 2. Proposed Year One Sites							
Site ID	**Refuge**	**Site Name**	**Tidal**	**Small-**	**Large-**	**Collection**	**Rationale**
			(Y/N)	**mouth**	**mouth**	**method**	
NEFO1	Great Meadows	Sudbury River upstream	N	N	Y	shocking boat	Hg contamination, NPL Site, POTW, mixed urban/ag watershed
NEFO2	Great Meadows	Sudbury River downstream	N	N	Y	shocking boat	On refuge below source
PAFO1	John Heinz	Darby Creek upstream	Y	Y	Y	shocking boat	Upstream of refuge
PAFO2	John Heinz	Darby Creek downstream	Y	Y	Y	shocking boat	On refuge. Last stop before the Delaware River. Receives multiple urban sources including landfills, industries, storm water runoff
NYFO1	Montezuma	Seneca R. upstream	N	Y	N	shocking boat/gill net	Seneca R. ~10 km upstream of the Refuge where it exits Cayuga lLke
NYFO2	Montezuma	Seneca River and Erie Canal downstream	N	Y	N	shocking boat/ gill net	Seneca R. adjacent to refuge: Historical pesticides (OCs) detected in converted wetlands used for agriculture; current use farming includes pesticides such as atrazine
MEFO1	Moosehorn	St. Croix River upstream	N	Y	N	shocking boat	Above pulp and paper mill upstream, WWTP
MEFO2	Moosehorn	St. Croix River downstream	N	Y	N	shocking boat	On refuge, receives influences from pulp and paper mill , WWTP.
WVFO1	Ohio River Islands	Ohio River upstream	N	Y	Y	shocking boat/trapnets	Above the major discharges and urban areas on the Ohio River.
WVFO2	Ohio River Islands	Ohio River downstream	N	Y	Y	shocking boat/trapnets	There is an abundance of discharges along the refuge.
CBFO1	Patuxent Research Refuge	Patuxent River upstream	N	N	Y	shocking boat	Rocky Gorge reservoir-drinking water supply above dam (11 km upstream of Refuge boundary)
CBFO2	Patuxent Research Refuge	Patuxent River upstream	N	N	Y	barge shocker	Patuxent R. on-refuge about 0.8 km downstream of 7.5 mgd Parkway Plant. This is about 10.2 km from Rocky Gorge which has an isolated fish population due to a high dam.
VAFO1	Rappahannock	Rappahannock	Y	N	Y	shocking boat	Above Little Falls WWTP and below Fredericksburg WWTP that directly discharges into Rappahannock
VAFO2	Rappahannock	Rappahannock	Y	N	Y	shocking boat	On refuge below the Falls Run WWTP on the Rappahannock River
VAFO3	Rappahannock	Little Falls tributary	Y	N	Y	shocking boat	Reference tributary for Falls Run WWTP
NJFO1	Wallkill	Upstream	N	?	Y	shocking boat/trapnets	Above the WWTP on the Wallkill River
NJFO2	Wallkill	Downstream	N	?	Y	shocking boat/trapnets	On refuge below the WWTP, agricultural runoff, and turf farms

Table 3. Proposed Year Two Sites							
Site ID	**Refuge**	**Site Name**	**Tidal**	**Small-**	**Large-**	**Collection method**	**Rationale**
			(Y/N)	**mouth**	**mouth**		
CBFO3	Chesapeake Island Complex	Garrett Island upstream	Y	Y	Y	shocking boat/trapnets	Above Harrisburg, and the major dams/ agricultural influences of the Susquehanna River.
CBFO4	Chesapeake Island Complex	Garrett Island downstream	Y	Y	Y	shocking boat/trapnets	The Susquahanna River is a targeted river of concern for both species. Substantial agricultural and industrial influences. Refuge, at the mouth of the river, receives all influences
VAFO5	Potomac R. Complex	Farm Creek/MarumscoGreat Marsh	Y	Y	Y	shocking boat	Urban Influences from D.C. metropolitan area.
VAFO6	Potomac R. Complex	Farm Creek/MarumscoGreat Marsh	Y	Y	Y	shocking boat	On refuge below and possibly outside the influences of the Urban anrea.
CBFO5	Prime Hook	Prime Hook Creek	N	N	Y	small shocking boat	High mercury in bass fillets, sewage spills upstream into Sowbridge Creek which flows into Prime Hook Creek
CBFO6	Prime Hook	Broad Kill Creek	N	N	Y	small shocking boat	Adjacent reference watershed to Prime Hook Creek
NJFO3	Great Swamp	Great Brook	N	?	Y	shocking boat/trapnets	POTW upstream, urban watershed, landfill upstream on refuge
NJFO4	Great Swamp	Lamington River	N	?	Y	Shocking boat/trapnets	Adjacent but similar watershed to serve as a reference for Great Brook on refuge
NYFO3	Long Island Complex Wertham	Carmans River upstream	?	N	Y	shocking boat/trapnets	This river passes through developed and undeveloped land on Long Island; potential for PAHs, pesticides and other residential/urban/industrial runoff. Groundwater input to river is significant.
NYFO4	Long Island NWR Complex Wertham	Carmans River downstream	?	N	Y	shocking boat/trapnets	On refuge; receives influences from upstream
NEFO3	Missisquoi	Missisquoi River upstream	N	Y	N	electroshocker	POTW upstream plus agricultural watershed,
NEFO4	Missisquoi	Missisquoi River downstream	N	Y	N	electroshocker	On refuge receives influences from upstream
MEFO3	Sunkhaze Meadows	Penobscot River upstream	N	Y	N	shocking boat	Pulp and paper mill upstream
MEFO4	Sunkhaze Meadows	Penobscot River downstream	N	Y	N	shocking boat	On refuge receives influences from upstream

Table 4. Proposed Year Three Sites						
Site ID	**Refuge**	**Site Name**	**Small-mouth**	**Large-mouth**	**Collection method**	**Rationale**
PAFO3	John Heinz	Hoy Pond	Y	N	shocking boat	Regional groundwater and air deposition from Philadelphia international airport
PAFO4	Erie	Pool K	N	Y	shocking boat	Cooperative agricultural sources
PAFO5	Erie	Pool 9 (138 acre impoundment)	N	Y	shocking boat	No known sources
NJFO5	Great Swamp	Nursery pond	N	Y	hook and line	Old nursery pond-pesticides
NEFO5	Missisquoi	Big Marsh Slough	Y	Y	jon boat shocker, gillnet, trapnet	Agricultural watershed, connected to lake Champlain
NEFO6	Great Bay	Upper Peverly Pond	N	Y	shocking boat	Former military base, urban watershed
NEFO7	Great Meadows	Heard Pond	N	Y	shocking boat	Hg contamination and agricultural inputs.
NEFO8	Lake Umbagog	Lake Umbagog	Y	N	hook and line, gillnet	Possible control site
CBFO5	Patuxent Research Refuge	Cash Lake	N	Y	shocking boat	Public fishing; good water quality
CBFO6	Patuxent Research Refuge	Snowden Pond	N	Y	shocking boat	Some nutrient inputs from failing stormwater pond off-refuge (Lake Allen, where there is public fishing and historical nutrient problems form horse stables is an alternative)
VAFO4	Rappahannock	Wilna Pond	N	Y	shocking boat	Agriculture along private side of pond; grasslands on Refuge side of pond
VAFO5	Rappahannock	Chandler's Mill Pond	N	Y	shocking boat	Agriculture
VAFO6	Back Bay	to be named	N	Y	shocking boat	No known sources; good reference site

VI. ROLES, RESPONSIBILITIES, AND PARTNERSHIPS

VI.A. Roles and Responsibilities

Ten Region 5 Environmental Contaminants Biologists will serve as co-Principal Investigators. They will assess, select, and prioritize the sampling sites, coordinate with Refuge biologists and state collaborators on collection and sampling, and assist USGS NFHRL with sampling and logistics. The primary USFWS role in data analysis and report preparation will be performed by Fred Pinkney and Chris Guy of CBFO who are conducting a similar Off-Refuge Investigation.

Dr. Vicki Blazer and (soon to be) Dr. Luke Iwanowicz of USGS- Leetown Science Center, NFHRL will also serve as Co-Principal Investigators. Through this proposal, USGS will be funded for travel expenses and to conduct necropsies, obtain plasma and tissue samples, prepare and conduct histopathology, and conduct the vitellogenin, hormone, and *in vitro* assays. They will be assisted in these efforts by the fisheries agencies, the EC Biologists, and the Lamar Fish Health Unit. Dr. Blazer and Mr. Iwanowicz are providing in-kind services as partners on the project. They will donate the time needed for their portions of the report writing, data analysis, presentation, and publication efforts. This salary time has been estimated at $50,000 per year for Years 1 through 3.

We have received in-kind offers of assistance in the collection of the fish from the following agencies: Maryland Fisheries Resources Office, Maryland Department of Natural Resources, Virginia Department of Environmental Quality, Delaware Department of Natural Resources and Environmental Control, West Virginia Department of Natural Resources, New Jersey Department of Environmental Protection, and New York State Department of Environmental Conservation. The value of these contributions is provided in Appendix B.

A final report to the Division of Environmental Quality will be a collaborative effort of the partners. The results will be presented at regional and national meetings and publication in a peer-reviewed journal will be pursued. Fact sheets and briefing statements will be prepared by EC biologists. Field office EC staff will work together with refuge managers on specific problems identified through this proposal. Examples of joint efforts may include interactions with state agencies, contacts with plant managers of upstream facilities, and outreach to local watershed groups.

VII. Budget

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VI. BUDGET					
EXPENDITURES	Year 1 FY 2008	Year 2 FY 2009	Year 3 FY 2010	Year 4 FY 2011	All Years
Field Operations - Collection Costs	$8,000	$7,800	$10,000	$0	$25,800
Personnel - Field (Bioday = $700)	$39,900	$31,500	$50,400	$0	$121,800
Personnel - Data Analysis	$0	$0	$0	$7,000	$7,000
Personnel - Report Writing	$0	$0	$0	$3,500	$3,500
Travel/Per Diem	$1,000	$1,400	$2,000	$0	$4,400
Supplies	$0	$0	$0	$0	$0
Equipment	$0	$0	$0	$0	$0
Non-ACF Analytical (see below)					
USGS - Histopathology and hormone tests ($138/fish)	$46,920	$44,160	$35,880	$0	$126,960
Lamar Fish Health Unit	$10,800	$9,600	$7,800	$0	$28,200
Other (USGS Per Diem)	$1,500	$1,500	$1,500	$0	$4,500
Other (USGS Yeast Estrogen Screen)	$1,380	$1,380	$1,380	$0	$4,140
Regional Overhead (10%)	$12,045	$10,707	$11,986	$1,155	$35,893
					$0
Operational Subtotal	$109,500	$97,340	$108,960	$10,500	$326,300
					$0
Total Funding	$120,450	$107,074	$119,856	$11,550	$358,930

* The Lamar Fish Health Unit will assist USGS with sample processing for histopathology, hormone, and vitellogenin analysis. They will utilize the proposed sampling to collect additional data (outside the scope of this project) for the National Wild Fish Health Survey, J. Coll, pers. comm.

In-Kind Services		
Description	**Amount**	**Notes**
CBFO Off-Refuge Study (2005 – present)	$197,000	
Staff time for Dr Blazer and staff	$150,000	50k/year for 3 years
Fish Collection, FWS Fisheries and state agencies	$22,250	fisheries and various state agencies; see year by year rationale for details
CBFO report writing (Federal Activities Branch (C.Guy) 20 days@$700/day)	$14,000	
Total	$383,250	

Rationale

A year by year rationale for the budget and a detailed tally of in-kind services are given in Appendix B

Scientific Peer Review Form

1. Is the experimental design well thought out and scientifically valid? Please comment:

The proposed study is scientifically valid in that it will further describe the incidence of intersex in black bass from multiple Region 5 NWRs and uses established reproductive biomarkers. The design will allow for comparisons within a site (one NWRs) but will be more difficult to make comparisons among sites (temporal issues, collections time, etc). Largemouth and smallmouth bass data from the Biomonitoring of Environmental Status and Trends (BEST) Program was named as "a source of data for comparisons and as a framework to ensure compatibility" (page 3). While this program has a large reproductive biomarker dataset for these species, the majority of fish were collected post-spawn (typically in the late summer and fall). The proposed study describes that fish will be collected as they "approach spawning condition" (page 10). Comparability of these data may therefore be inappropriate as steroid hormones, vitellogenin, GSI, and gonadal stage vary throughout the reproductive cycle of black basses.

2. Is there a good probability of achieving the objectives of the investigation? Please comment:

Fish collections and field necropsies in Years 1-3 are achievable. The authors should not underestimate the time required to perform the comprehensive histopathology, steroid hormone analysis, and vtg analysis as well as the interpretation of these data. It is unclear how their findings will be compared to the impacts of suspected sources (general trends or some sort of modeling?). Appendix B outlines approximately 15 days for data analysis and report writing – this seems inadequate considering the amount of data this project will produce (perhaps some in-kind time will be allocated to this as well but was not included in the table?).

3. Does the investigation integrate current information with accepted methodologies to close data gaps, and establish a cause and effect relationship?

Much is still unknown about intersex in bass including background prevalence, cause, etc. even though it has received much interest in the past few months (including in Washington D.C.). This study would provide useful information on the occurrence of this condition in the northeastern United States. However, the reproductive biomarker responses proposed will not establish cause-effect relationships between these responses and endocrine disrupting chemicals, but rather trends or patterns may be revealed. Other environmental factors that can influence these responses cannot be controlled in field studies like the one proposed. Nevertheless, the study will provide important and useful information to refuge managers on fish health and possible exposure to endocrine disrupting compounds.

4. Are the costs well researched, clearly spelled out and defensible? Please comment:

A few clarifications are needed on the budget.

What is the rationale behind the "Travel/Per Diem" line? Footnote "a" of Appendix B indicates that cost of travel and per diem are included in the "Field Operations-Collection Costs". Footnote "a" also states that it includes the cost of personnel – isn't this included in the $700 bioday charge?

The Proposed Milestones (page 12) state that fish will be collected from 17 sites in Year 1, 14 sites in Year 2, and 13 sites in Year 3. At first glance, one would expect the "Personnel-Field" expenditures would be greatest for the year with the most sites samples. Instead, the cost is greatest for Year 3 (fewest sites). Is this because Years 1 and 2 have paired sites and impoundments will be sampled in Year 3? It appears there are eight locations to be sampled in Year 2 (not seven as indicated on page 12).

Are you assuming 2 field personnel in your bioday charges? For example, 6 bioday would mean 2 field biologists at the site for 3 days? This cost seems somewhat high, especially considering that the Lamar Fish Health Unit will be receiving 1,200 per site for assistance in collection and fish health assessment. What will these field folks be doing if many of the state agencies will be collecting the fish? And, if my 3 day assumption is correct, why will it take 3 days to collect 40 fish from a site?

The cost for histopathology and hormone analysis is quite reasonable. Does this cost also include vitellogenin analysis?

5. Commensurate with investigation objectives, does the proposal describe or cite scientifically acceptable operating procedures that include QA/QC sufficient to ensure the integrity of the data? Please comment:

The field and lab methods described in the proposed study are well described in the scientific literature. The data analysis needs to be more clearly outlined. The citation provided (Schmitt and Dethloff, 2000) does not describe any data analysis but rather describes the rationale of including various fish health indicators and reproductive biomarkers in the BEST Program (with some reference of "normal" and "abnormal" values for certain fish health indicators but few reproductive biomarkers). This document is somewhat dated and other more recent publications from peer-reviewed journals are available. As previously described, it may be erroneous to compare the proposed study results with BEST Program studies due to differences in collection times (pre-spawning vs. post-spawning).

Please check one of the following:

__ Proposal is acceptable *as is* required (no changes required) _X_ Minor revisions required (rectify the costs and some minor edits) __ Major revisions

PROPOSAL TITLE: Assessment of Endocrine Disruption in Smallmouth Bass (*Micropterus dolomieu*) and Largemouth Bass (*M. salmoides*) in Region 5 National Wildlife Refuges

REVIEWER* Jo Ellen Hinck TITLE Biologist DATE 5-8-07

*If peer reviewer is anonymous, EC coordinator should indicate such and initial the signature line.

Response to Reviewer's Comments:

1. Text altered on P. 10 (Fish collection): The timing of the collections has been moved to late-summer/fall to match BEST.

2. In-kind has been added to Appendix B. Findings on suspected sources will be compared as described in the data analysis section. Language added to P. 11: We recognize that this field study cannot prove a cause and effect relationship between a suspected source and biological observation due to factors that cannot be controlled (water quality parameters, stream flow, etc.).

3. P. 11: We recognize that this field study cannot prove a cause and effect relationship between a suspected source and biological observation due to factors that cannot be controlled (water quality parameters, stream flow, etc.).

4. Cost justifications have been added to Appendix B. Bio day rates do not include travel and per diem. The number of days for FWS field office personnel includes time needed for site reconnaissance, preparation for the field work, shipping of samples, and maintenance of data.

5. P. 10: Text added: Methods will be consistent with those of the BEST program (Schmitt *et al.* 2005, Hinck *et al.* 2006, 2007).

2008 National Criteria Score Sheet

TITLE: **Assessment of Endocrine Disruption in Smallmouth Bass (*Micropterus dolomieu*) and Largemouth Bass (*M. salmoides*) in Region 5 National Wildlife Refuges**

PROJECT I.D.: _____ REGION: 5 RO RANK: _____ TARGET STATES:

Pass/Fail Criteria

The investigation proposal ***DOES X DOES NOT*** ___ pass the minimum required standards of the Environmental Contaminants Program.

Yes/No Proposal clearly identifies (1) an environmental problem related to anthropogenic contaminants and (2) site-specific management actions designed to resolve that problem. If not, explain:

Yes/No The proposal clearly identifies a level of biological impacts that must be investigated. Abiotic only sampling is clearly linked to an established threshold level of concern. If not, explain:

Yes/No At least one substantive peer review has been conducted and is attached. The proposal has been revised as appropriate. The study design is sufficient to meet the objectives of the proposal. If not, explain:

Yes/No The required surnames have been obtained. If not, explain:

Ranking Criteria

For the above referenced proposal, determine a score for each of the following criteria in accordance with the criteria definitions described in Chapter 5 of the investigations manual. Identify the location of the text that supports the score. If you disagree with a score previously provided, explain why.

A. Threats to resources are ***DOCUMENTED (20 pts) or SUSPECTED (15 pts)****.*

Field Office Supporting Text (**in bold**): Section IIA, ¶1-4, Table 1 Score: 20

Regional Office Supporting Text: Section _____, ¶ _____ Score:
Explanation (if scores differ):

Reviewer Supporting Text: Section IIA, Score:
Explanation (if scores differ):

B. Management actions are ***DIRECT (15 pts)*** *or* ***INDIRECT (10 pts)****.*

Field Office Supporting Text (**in bold**): Section IIC , ¶ 3 Score: 15
Regional Office Supporting Text: Section _____, ¶ _____ Score:
Explanation (if scores differ):

Reviewer Supporting Text: Section IIC, Score:
Explanation (if scores differ):

C.1. The study question(s) or hypotheses being addressed by the investigation ***ARE (4 pts) or ARE NOT (0 pts)*** *clearly stated.*

Field Office Supporting Text (**in bold**): Section IIIA , Data analysis section and IIB. Objectives ¶ Score: 4

Regional Office Supporting Text: Section _____, ¶ _____ Score:
Explanation (if scores differ):

Reviewer Supporting Text: Section IIIA, ¶ Score:
Explanation (if scores differ):

C.2. The study design as described in the proposal ***WILL (4) or WILL NOT (0 PTS) answer the study question(s)/hypotheses.***

Field Office Supporting Text (**in bold**): Section IIIA, Data analysis section and IIB. Objectives ¶ Score: 4

Regional Office Supporting Text: Section _____, ¶ _____ Score:
Explanation (if scores differ):

Reviewer Supporting Text: Section IIIA, ¶ Score:
Explanation (if scores differ):

C.3. The scope or complexity of impacts being addressed by the investigation ***IS (4 pts)*** *or* ***IS NOT (0 pts)*** *appropriate.*

Field Office Supporting Text (**in bold**): Section IIB, Specific objectives Score: 4

Regional Office Supporting Text: Section _____, ¶ _____ Score:
Explanation (if scores differ):

Reviewer Supporting Text: Section IIB, ¶ Score:
Explanation (if scores differ):

C4. The most severe type of biological impact addressed by the investigation is an ***INDICATOR OF ADVERSE EFFECTS (4 pts) or ACTUAL ADVERSE EFFECTS (7 pts)****.*

Field Office Supporting Text (**in bold**): Section IIA, ¶ 2 , 4, Table 1
Section IIIA, Score: 7

Regional Office Supporting Text: Section _____, ¶ _____ Score:
Explanation (if scores differ):

Reviewer Supporting Text: Section IIA,
Explanation (if scores differ): Score:

*C.5. Source of the contaminant **IS (3 pts) or IS NOT (0 pts)** sufficiently addressed.*

Field Office Supporting Text (**in bold**): Section IIIA, ¶ 5 , Tables 2-4
Score: 3

Regional Office Supporting Text: Section _____, ¶ _____ Score:
Explanation (if scores differ):

Reviewer Supporting Text: Section IIIA, ¶ _ Score:
Explanation (if scores differ):

*C.6. Pathway of the contaminant **IS (3 pts) or IS NOT (0 pts)** sufficiently addressed.*

Field Office Supporting Text (**in bold**): Section IIA, ¶1, 2
Section IIIA, 5, Tables 2-4 Score: 3

Regional Office Supporting Text: Section _____, ¶ _____ Score:
Explanation (if scores differ):

Reviewer Supporting Text: Section IIA , ¶
IIIA, Score:
Explanation (if scores differ):

D. Final regional rank order is ___ of ___ proposals submitted. Score:

E1. Regional Performance Score Score:

E2. Total Partnership Effort
Field Office Supporting Text: Section VIB, ¶ _
Section VII, ¶ _ Score:

Regional Office Supporting Text: Section _____, ¶ _____ Score:
Explanation (if scores differ):

Reviewer Supporting Text: Section VI A+B, ¶ _____ Score:
Explanation (if scores differ):

General Reviewer Comments or Major Concerns:

APPENDIX A: Examples of reasoning supporting specific sampling sites

River sites

Great Meadows NWR (MA)

Great Meadows National Wildlife Refuge is one of eight refuges of the Eastern Massachusetts National Wildlife Refuge Complex. Established in 1944, the current extent of the refuge includes 1563 hectares and extends into eight towns. In addition to numerous impoundments, the Sudbury River is an integral part of the refuge. The Sudbury River is 66 km long, and drains 438 square kilometers. It has three distinct sections: the first section is a narrow, rapidly flowing stream; the second section consists of two large impoundments; and the third section which has been compared to an elongated lake (in this 19-km section of the river, the elevation changes by only one foot). It is this third section which runs through the Great Meadows NWR.

Multiple pollution point sources impact water quality in the Sudbury River. In addition to two wastewater treatment plants, the Nyanza Superfund site in Ashland and the Raytheon brook wetlands in Sudbury are two major sources of pollution near the refuge. Both sites have introduced mercury into the Sudbury River. Other heavy metals detected in the river system include lead, arsenic, cadmium and chromium. Concentrations of polychlorinated biphenyls (PCBs) and polynuclear aromatic hydrocarbons (PAHs) in sediments in the vicinity of the Raytheon site are high.

Sampling of largemouth bass would occur in the third section of the Sudbury River, within the refuge boundary. The first section of the Sudbury will serve as the upstream or reference site. It is located approximately 32 km upstream of the refuge boundary.

Great Swamp NWR

The Great Swamp National Wildlife Refuge is located approximately 42 kilometers west of Manhattan, New York, in Morris County, New Jersey. The Great Swamp watershed covers approximately 142 square kilometers, ultimately draining to the Passaic River through Millington Gorge. Fish sampling would be conducted at and around the confluence of the Great Brook and the Passaic River, along the refuge's western boundary. Bisecting the refuge, Great Brook receives POTW municipal effluent and landfill run off via Loantaka Brook. The Lamington River at the Black River Wildlife Management Area northwest of the refuge will serve as the paired reference site with Great Brook. The distance between the reference and refuge sampling sites is approximately 25 km. There is no hydrologic connection between Lamington River and the Passaic/Great Brook system.

John Heinz NWR (PA)

The John Heinz National Wildlife Refuge is located along Darby Creek in Philadelphia and Delaware Counties, Pennsylvania, about one mile north of the Philadelphia

International Airport. The watershed drains a largely urbanized region containing numerous stormwater discharges, large areas of imperviousness, combined sewer outfalls, and eight permitted industrial and municipal wastewater discharges, including one 1.4 mgd municipal sewage treatment plant discharge located within the Refuge boundaries. In addition, an old landfill is located on the Refuge; this site, another landfill just upstream of the Refuge, and a two-mile reach of Darby Creek (including portions within the Refuge) are listed on EPA's National Priority List (Lower Darby Creek Area NPL Site) and are currently undergoing remedial investigations. Hazardous substances of concern include heavy metals, polycyclic aromatic hydrocarbons, volatile organic compounds, PCBs, and dioxins. Further information on ambient contaminant levels in Darby Creek will be available from EPA in the near future. The Refuge is located upstream from the tidal Delaware River, and water from the river reaches the Refuge during high tide.

Largemouth and smallmouth bass will be collected near the sewage treatment plant outfall in Darby Creek, and at an upstream location, approximately 5 km from the outfall (fish are not believed to be available further upstream). The upstream locations are away from the immediate influence of the STP effluent, but within the influence of the host of contaminant sources described above. The situation provides an opportunity to compare the extent of endocrine disruption resulting from two very different types of contaminant scenarios.

Montezuma NWR (NY)

The Montezuma National Wildlife Refuge was established in upstate New York in 1938. It was developed in an area that supported thousands of acres of wetlands that had been converted to agricultural "mucklands." The same properties of moisture, warmth, and high organic matter that made these muckland soils productive for growing crops also made them conducive to disease, unwanted plant growth, and a proliferation of insects. Consequently, agricultural practices in converted wetlands around the Refuge included and continue to include the use of a variety of pesticides. Information on historic pesticide use is incomplete, however, organochlorine insecticides, such as DDT, dieldrin, chlordane, endrin, toxaphene, endosulfan and hexachlorocyclohexane (HCH) were believed used there and have been detected in soil and sediment samples. A variety of inorganic insecticides, herbicides, and fungicides were also likely used in the Montezuma mucklands. These include arsenic and lead-based products, herbicides including ammonium salts, iron sulfate, copper sulfate, borate compounds, and fungicides such as the Bordeaux mixture, a copper-based compound. More recently, carbamate and organophosphate insecticides and herbicides, including atrazine, linuron, oxyfluorfen, and paraquat, have also been used in agricultural practices.

There may be implications for endocrine disruption in fish within the Seneca River and at the Refuge as a result of exposure to pesticides such as HCH, DDE, dieldrin, linuron, and atrazine. Dieldrin and DDE were reported to alter circulating hormones and steady state mRNA expression levels of a set of genes chosen to represent three possible mechanisms of endocrine disruption in largemouth bass (Garcia-Reyero *et al.* 2006). Freshwater catfish exposed to the pesticide gamma-HCH exhibited changes in the gonadosomatic

index, plasma gonadotropin, and sex steroids (Singh and Canario 2004). Linuron has been identified as a weak androgen receptor antagonist (Cook *et al.* 1993) and the triazines, including atrazine, are suspected of having endocrine disrupting properties (Moore and Waring 1998).

The Seneca River flows through these intensively farmed areas and also adjacent to Montezuma NWR. There is periodic water exchange between the Refuge and the Seneca River during high flow events. Sampling of largemouth bass would occur on the Seneca River adjacent to agricultural areas. The Cayuga-Seneca canal section of the Seneca River upstream of the Refuge as it exits Cayuga Lake will serve as a reference site. It is approximately 10 km upstream from the agriculturally influenced site. There is a dam/lock combo (Mud Lock) in between, although the lock is not a complete barrier.

Moosehorn NWR (ME)

Moosehorn NWR was established in 1937 as a refuge and breeding ground for migratory birds and other wildlife. The refuge has two units - Baring and Edmunds. The 6960-hectare Baring Unit, the unit associated with this proposal, has approximately one mile of frontage along the St. Croix River. Magurrewock Stream flows into the St. Croix at the northeast corner of the unit. The river floods into the stream during high flows. A bald eagle pair and up to four osprey pair nest on platforms located less than one-half mile from the river. The expansive Magurrewock Marsh associated with the stream provides important breeding and feeding habitat for waterfowl, wading birds, and other wildlife.

The St. Croix River is an international boundary between Canada and the United States. The river is approximately 100 km in length with a watershed encompassing 3885 square km. Although much of the upper St. Croix has very good water quality, lower reaches of the river suffer from reduced water quality. A pulp and paper mill, municipal wastewater treatment facilities, and runoff associated with residential and commercial activities from three large communities likely contribute to lower water quality in the lower reaches. Spills of paper-processing liquor from the mill have occurred in the past, with the most recent spill of 583,000 liters of weak black liquor occurring in September 2002. Thermal discharges from the mill, residential septic discharges, and non-point sources have also affected water quality in the lower St. Croix. Moosehorn NWR is 3.1 km downriver from the mill in the lower reach of the St. Croix near Calais, Maine, and Milltown, New Brunswick. Sampling would be conducted at the refuge and about 10 km upstream (above the mill dam which serves as a fish barrier).

Patuxent Research Refuge (MD)

Patuxent Research Refuge was established in 1936. It is the only National Wildlife Refuge established to support wildlife research. With land surrounding the Patuxent and Little Patuxent Rivers between Washington, D.C. and Baltimore, MD, the Refuge has grown from the original 2,670 acres to its present size of 12,750 acres and encompasses land formerly managed by the Departments of Agriculture and Defense.

A 7.5 mgd wastewater treatment plant (Parkway Plant operated by Washington Suburban Sanitation Commission) discharges to the Patuxent River approximately 0.8 km upstream of the refuge boundary. From that point, the Patuxent River flows through the refuge. About 11 km upstream from the refuge boundary is Rocky Gorge, a drinking water reservoir for suburban Washington, DC. A high dam controls the outflow from the reservoir and there is no fish passage. Largemouth bass are available in both the reservoir and stream.

Rappahannock NWR (VA)

Rappahannock River Valley is one of three National Wildlife Refuges that comprise the Eastern Virginia Rivers National Wildlife Refuge Complex. Rappahannock River Valley was established in 1996 to conserve fish and wildlife habitat along this vital tributary of the Chesapeake Bay. The focus is primarily on protecting and managing tidal and inland wetlands, and adjacent uplands, to benefit bald eagles, other migratory birds, and resident wildlife. Agriculture is the predominant land use in the watershed. Forested and agricultural lands make up 69 percent of the land in this watershed.

There are several wastewater treatment facilities on the Rappahannock River whose effluent contributes to the nutrient loading of the Chesapeake Bay and may or may not affect fish species at the Rappahannock River Valley Refuge. The river stations and impoundments chosen for the Virginia component of this proposal are part of the Virginia Department of Environmental Quality's (VADEQ) fish tissue monitoring program and are in the Rappahannock River watershed with relevance to our Rappahannock River Valley National Wildlife Refuge.

A large wastewater treatment plant that discharges directly to the Rappahannock River is located in the City of Fredericksburg. It has a discharge rate of 3.5 million gallons per day (mgd). In December 2006, a Consent Special Order was issued by the VADEQ for the Fredericksburg Facility for violations of total Kjeldahl nitrogen, ammonia, and phosphorous and failing to meet the minimum limit for total residual chlorine. Little Falls Run is another wastewater treatment facility with discharge to the Rappahannock River. It is designed for a daily flow average of 6 mgd with a peak flow of 12 mgd. Little Falls Run received a grant from the Chesapeake Bay Program in 2001 for the installation of biological nutrient removal facilities to reduce nitrogen loads to the river.

Our proposed sampling strategy is to sample three locations: one on a tributary as a reference for Falls Run, a second below the Falls Run WWTP on refuge property, and a third below the Fredericksburg WWTP that discharges into the Rappahannock River. All locations are separated by about 10 km. Our preliminary data suggest that largemouth bass should be available at all three sites.

Wallkill River NWR (NJ, NY)

The Wallkill River National Wildlife Refuge (WRNWR) located in Sussex County, New Jersey, and Orange County, New York, is a riverine floodplain refuge. Its bottomlands provide one of the few large areas of high quality waterfowl habitat remaining in northwest New Jersey. The effluent of the Sussex County Municipal Utilities Authority's wastewater treatment plant, located in Hamburg, New Jersey, discharges into the Wallkill River just upstream of the WRNWR. During periods of low river flow and high withdraw demands (*e.g.*, irrigation) the effluent may be a principal contributor of river water. The potential threats to the WRNWR include treatment plant overflow or failure, introducing elevated levels of nutrients or quasi-treated sewage to the WRNWR. Additionally, the chronic input of effluent into the Wallkill River presents the potential for elevated levels of endocrine disrupting substances to enter the WRNWR, including, but not limited to, natural and synthetic hormones, pharmaceuticals/breakdown products, and other effluent-related compounds with possible endocrine activity. As a reference, an upstream location above the WWTP will be identified. The two sites will be approximately 20 km apart.

Pond Sites

Back Bay NWR (VA)

The 32 km² fresh water Back Bay NWR borders the Atlantic Ocean on the east and Back Bay on the west. The northern edge of North Carolina's Outer Banks is located immediately to the south. The refuge's barrier islands feature large sand dunes, maritime forests, fresh water marshes, ponds, ocean beach, and large impoundments for wintering wildfowl. It is considered by conservationists to be an important link along the Atlantic Flyway for migratory birds such snow geese. The majority of refuge marshlands are on islands contained within the waters of Back Bay. There are 10 freshwater impoundments on site covering over 880 acres (356 hectares). There are no known sources of contaminants to these impoundments. In particular, agriculture and wastewater treatment plant discharges are not a concern, making these impoundments an important area for consideration as a reference site for largemouth bass. One pond (name to be determined) will be targeted for Year 3.

Erie NWR (PA)

The Erie National Wildlife Refuge is located about 35 miles (56 km) south of Lake Erie in Crawford County, Pennsylvania. The Refuge’s Sugar Lake Division contains over 2023 hectares of creeks, beaver ponds, pools and wetlands, bounded by forested slopes, farmland, grasslands, and meadows. Two ponds support bass: Pool K (4 hectares) and Pool 9 (56 hectares). Pool K’s watershed has a small amount of farming about ¼ mile upstream; otherwise the watershed is entirely Refuge lands. Pool 9, an impoundment on Lake Creek, flows through the small town of Guys Mills. There is one other impoundment between the refuge and Guys Mills. There are no major point source discharges in the watershed. The Refuge allows cooperative farming in the Pool 9

watershed. Pesticides approved for use on the Refuge (dimethylamine salt of 2-methyl-4 chlorophenoxyacetic acid and primisulfuron methyl) are not currently known to be endocrine disruptors (www.pesticideinfo.org). These impoundments appear to be ideal reference locations.

Great Swamp NWR (NJ)

The Hidden Valley Nursery parcel gently slopes from about 260 to 240 feet (79 to 73 meters) elevation. The parcel's topography promotes surface run-off to drain into two small ponds, and several depositional areas. There are several dilapidated outbuildings on the parcel that may have been used for equipment or pesticide storage. A review of historical aerial photographs revealed the presence several greenhouses on the parcel. No previous contaminant data regarding the site was found. Given the combined topography and historical land use, sediment and biotic contamination is highly probable.

John Heinz NWR (PA)

Hoy Pond, about 2.3 hectares in size, is periodically stocked by the Pennsylvania Fish and Boat Commission, supports both largemouth and smallmouth bass. There are no point source discharges to this pond, but it is exposed to regional air pollution.

Patuxent Research Refuge (MD)

Cash Lake is a 21-hectacre impoundment that is used for recreational fishing. There are no major local sources of contaminants, although the dam is adjacent to a state highway. In the past, there were concerns about releases from a nearby landfill but sediments were not found to contain concentrations of organic contaminants that raised concern (Pinkney 2000). A second impoundment, Lake Allen (9.2 hectares), is part of the North Tract. In the past, water quality was poor due to nutrient loading from adjacent horse stables. Several years ago, these stables were closed and water quality has improved (H. Obrecht, pers. comm.). Both sites are viewed as possible reference sites and of interest to refuge managers because of public use. A third site, Snowden Pond (3 hectares) is an alternative to Lake Allen. It receives nutrient inputs from an off-refuge stormwater pond that is not fully functioning.

Rappahannock NWR (VA)

Rappahannock River Valley was established in 1996 to conserve fish and wildlife habitat along this vital tributary of the Chesapeake Bay. The focus is primarily on protecting and managing tidal and inland wetlands, and adjacent uplands, to benefit bald eagles, other migratory birds, and resident wildlife. Wilna Pond, a 14.2-hectare impoundment, is open to public fishing. The pond supports largemouth bass, bluegill sunfish, fliers, yellow bullhead, and American eel. The Refuge requires catch and release fishing only for largemouth bass. Other finfish species may be taken in accordance with State regulations. During the Level I Preacquisition survey for Wilna Pond and surrounding habitat, past farming activities were noted as the cause of environmental contamination by Law

Engineering and Environmental Services, Inc. Farming continues along portions of the pond that are not part of the Refuge system.

Chandler's Millpond is a 30-hectare impoundment located in Westmoreland County in the "Northern Neck" of Virginia. The dam was breached in September 1993 following a large storm event during which up to 16" (40 cm) of rain fell in some parts of the watershed. During the winter of 1994-1995, the dam was rebuilt and a Denil fish ladder was added to accommodate potential spawning runs of river herring that historically ascended the stream. The pond was stocked with bluegill, largemouth bass, redear sunfish and channel catfish and was closed to fishing to allow these fish to establish a self-sustaining population. On July 1, 1998 the pond was re-opened to public fishing. The pond has an average depth of about 6 feet (1.8 meters) supports good stands of aquatic vegetation and the shoreline is largely wooded. In addition to bluegill, largemouth bass, redear sunfish and channel catfish, other species include black crappie, redbreast sunfish, flier, redfin pickerel, and gizzard shad. The Virginia Department of Game and Inland Fisheries report that the largemouth bass fishery appears to be in great shape with numerous bass in the 4- to 6-pound (1.8 to 2.7 kg) range, but that no bass between 12 and 15 inches (30.5 to 38.1 cm) may be kept. Both Wilna and Chandler ponds are part of the DEQ's statewide fish monitoring and are VAFO's highest priority for impoundment sampling during Year Three.

Appendix B

Budget rationale and In-kind Services Summary

				Year1								
Field Office	Refuge	River	Collection By	Cost[a]	Biodays	Cost	Per Diem[b]	Analysis-1[c]	Analysis-2[d]	Totals	In-Kind $$	Comments
MEFO	Moosehorn	St. Croix River	Nashua FRO	2,000	6	4,200	200	5,520	1,200	13,120		
NEFO	Great Meadows	Sudbury	Nashua FRO	2,000	6	4,200	200	5,520	1,200	13,120		
NYFO	Montezuma	Seneca	NYFO	2,000	6	4,200	0	5,520	1,200	12,920	1,000	NYDEC
PAFO	John Heinz	Darby Creek	PAFO	2,000	6	4,200	200	5,520	1,200	13,120		
NJFO	Wallkill	Wallkill	NJDEP	0	6	4,200	200	5,520	1,200	11,120	2,500	NJDEP
WVFO	Ohio River Islands	Ohio River	WVDNR	0	6	4,200	200	5,520	1,200	11,120	2,000	WVDNR
CBFO	Patuxent	Patuxent	MD DNR	0	6	4,200	0	5,520	1,200	10,920	1,500	MD DNR
VAFO	Rappahannock	stream 1 and stream 2	VA DEQ	0	9	6,300	0	5,520	1,200	13,020	2,000	VA DEQ
VAFO	Rappahannock	Rappahannock	VA DEQ	0	6	4,200	0	2,760	1,200	7,560	1,000	VA DEQ
USGS Per Diem										1,500		
USGS Yeast Estrogen Screen										1,380		
Totals				**8,000**		**39,900**	**1,000**	**46,920**	**10,800**	**108,900**	**10,000**	

[a]Includes cost of personnel, travel, and per diem for one night (if necessary)

[b]Assumes one overnight stay ($100) per biologist

[c]USGS - Histopathology and hormone tests ($138/fish) x 40 fish

[d] Lamar Fish Health Unit for assistance in collection and fish health assessment

				Year 2								
Field Office	Refuge	River	Collection By	Cost[a]	Biodays	Cost	Per Diem[b]	Analysis-1[c]	Analysis-2[d]	Totals	In-Kind $$	Comments
MEFO	Sunkhaze Meadows	Penobscot	Nashua FRO	2,000	3	2,100	0	5,520	1,200	10,820		
NEFO	Missisquoi	Missisquoi	Lake Champlain FRO	1,000	6	4,200	200	5,520	1,200	12,120		
NYFO	Long Island	Carmens	NYFO	2,000	6	4,200	200	5,520	1,200	13,120		
NJFO	Great Swamp	Great Brook	NJDEP	0	6	4,200	200	5,520	1,200	11,120	2,500	NJDEP
WVFO	Ohio River Islands	Ohio River	WVDNR	0	6	4,200	200	5,520	1,200	11,120	2,000	WVDNR
CBFO	Chesapeake Island	Garrett Island	MFRO	2,800	6	4,200	200	5,520	1,200	13,920	2,800	MFRO
CBFO	Prime Hook	Prime Hook	DNREC	0	6	4,200	200	5,520	1,200	11,120	1,000	DNREC
VAFO	Potomac River	Farm Creek	VA DEQ	0	6	4,200	200	5,520	1,200	11,120	2,000	VA DEQ
USGS Per Diem										1,500		
USGS Yeast Estrogen Screen										1,380		
Totals				**7,800**		**31,500**	**1,400**	**44,160**	**9,600**	**97,340**	**10,300**	

[a]Includes cost of personnel, travel, and per diem for one night (if necessary)

[b]Assumes one overnight stay ($100) per biologist

[c]USGS - Histopathology and hormone tests ($138/fish) x 40 fish

[d]Lamar Fish Health Unit for assistance in collection and fish health assessment

Year 3

Field Office	Refuge	River	Collection By	Cost[a]	Biodays	Cost	Per Diem[b]	Analysis-1[c]	Analysis-2[d]	Totals	In-Kind $$	Comments
MEFO	Lake Umbagog	Lake Umbagog	Nashua FRO	2,000	6	4,200	200	2,760	600	9,760		
NEFO	Great Meadows	Heard Pond	Nashua FRO	1,000	6	4,200	0	2,760	600	8,560		
NEFO	Great Bay	Upper Peverly	Nashua FRO	1,000	6	4,200	0	2,760	600	8,560		
NEFO	Missisquoi	Big Marsh Slough	Lake Champlain FRO	1,000	6	4,200	200	2,760	600	8,760		
NJFO	Great Swamp	HVN Pond	NJFO	1,000	6	4,200	0	2,760	600	8,560		
PAFO	John Heinz	Hoy Pond	PAFO	2,000	6	4,200	200	2,760	600	9,760		
PAFO	Erie	Pool K	PAFO	2,000	3	2,100	200	2,760	600	7,660		
PAFO	Erie	Pool 9	PAFO	0	3	2,100	200	2,760	600	5,660		
CBFO	Patuxent	Cash Lake	MD DNR	0	6	4,200	200	2,760	600	7,760	2,250	MD DNR
CBFO	Patuxent	Snowden Pond	MD DNR	0	6	4,200	200	2,760	600	7,760		MD DNR
VAFO	Rappahannock	Wilna Pond	VA DEQ	0	6	4,200	200	2,760	600	7,760	1,000	VA DEQ
VAFO	Rappahannock	Chandlers Mill Pd	VA DEQ	0	6	4,200	200	2,760	600	7,760	1,000	VA DEQ
VAFO	Back Bay	Unnamed Impoundment	VA DEQ	0	6	4,200	200	2,760	600	7,760	1,000	VA DEQ
USGS Per Diem										1,500		
USGS Yeast Estrogen Screen										1,380		

[a]Includes cost of personnel, travel, and per diem for one night (if necessary)

[b]Assumes one overnight stay ($100) per biologist

[c]USGS - Histopathology and hormone tests ($138/fish) x 20 fish

[d]Lamar Fish Health Unit for assistance in collection and fish health assessment

Totals				**10,000**		**50,400**	**2,000**	**35,880**	**7,800**	**108,960**	**5,250**

Year 4

Field Office	Activity	Biodays	Cost	Activity	Biodays	Cost	In-Kind Activity	By	Program Fed. Activities	Bio days	Cost
CBFO	Data analysis	10	$7,000	Writing	5	$3500	Writing/ analysis	C. Guy		20	$14,000

Notes on costs:
Bio day rate of $700 is average for R5 field offices.
FWS field office number of field days includes reconnaissance, prep, assistance with sampling personnel, sample shipping, data record maintenance

In-Kind Services		
Description	**Amount**	**Notes**
CBFO Off-Refuge Study (2005 – present)	$197,000	
Staff time for Dr Blazer/ Mr. Iwanowicz (writing and analysis)	$150,000	50k/year for 3 years
Fish Collection, FWS Fisheries and state agencies	$22,250	fisheries and various state agencies; see year by year rationale for details
CBFO report writing and data analysis (Federal Activities Program, C. Guy, 20 d @$700/day)	$14, 000	
Total	$383,250	