



BASE YEAR STUDY REPORT

**INVESTIGATION OF THE IMPACTS OF  
COMMON ENDOCRINE DISRUPTING COMPOUNDS  
ON MULTIPLE EARLY LIFE STAGES OF ENDANGERED  
ATLANTIC SALMON AND SHORTRNOSE STURGEON AND  
THREATENED ATLANTIC STURGEON**

PREPARED FOR

NATIONAL MARINE FISHERIES SERVICE

NORTHEAST REGIONAL OFFICE

BY

KIER ASSOCIATES

TURNERS FALLS, MASSACHUSETTS

AND

BLUE LAKE, CALIFORNIA

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SEPTEMBER 2011



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NATIONAL MARINE FISHERIES SERVICE  
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BY

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## EXECUTIVE SUMMARY

This study investigates whether ‘whole effluent testing’, or ‘WET’, a standard wastewater discharge regulatory tool recognized by the U.S Environmental Protection Agency, is effective in predicting negative impacts of regulated discharges on federal Endangered Species Act- (ESA) listed endangered Atlantic salmon (*Salmo salar*), endangered shortnose sturgeon (*Acipenser brevirostrum*), and threatened Atlantic sturgeon (*Acipenser oxyrinchus*). For those receiving waters that are the federally-designated habitat for these ESA-listed species, the WET test uses fathead minnow (*Pimephales promelas*), brook trout (*Salvelinus fontinalis*) and rainbow trout (*Oncorhynchus mykiss*) as test species.

Concern has been raised among the responsible fisheries conservation agencies whether the WET test is effective in determining the impacts of endocrine disrupting compounds (EDCs) on the ESA-listed species. The question becomes, therefore, whether the species commonly used in the WET test are suitable surrogates for, and sufficiently protective of the ESA-listed salmon and sturgeon species.

The investigation has three principal objectives. The first is to determine if EDCs that are commonly discharged into U.S. waters containing Atlantic salmon, shortnose sturgeon and Atlantic sturgeon can negatively affect the species (and, if such negative effects appear, at what EDC concentrations and exposure durations do they occur?). The second is to examine differences in sensitivity to EDCs at different life stages of shortnose sturgeon, Atlantic salmon and Atlantic sturgeon. The final objective is to provide information as to whether test species commonly used for WET testing are, in fact, suitable surrogates for shortnose sturgeon, Atlantic sturgeon, and Atlantic salmon.

In its first year the project team completed short-term (four-day) exposures of three common EDCs (nonylphenol, 17  $\beta$ -estradiol and  $\alpha$ -ethinylestradiol - three concentrations of each compound plus a control/vehicle) in Atlantic salmon and shortnose sturgeon. The team exposed three life-stages of each species - embryos, yolk-sac larvae, and feeding fry. An additional older life-stage (smolt) of Atlantic salmon was exposed.

Where none were available, the team developed three biological assays to serve as indicators of exposure to EDCs. Two of the assays were developed to measure the expression of vitellogenin (‘Vtg’, a precursor egg protein) in Atlantic salmon, an Enzyme-Linked Immunosorbant Assay (ELISA) for measuring plasma Vtg and a quantitative real-time PCR for Vtg mRNA.

Eighty percent of the data concerning salmon has been completed and analyzed. A qPCR assay for shortnose sturgeon Vtg mRNA has been completed and the team is developing an ELISA to measure plasma Vtg in this species. Shortnose sturgeon samples are in storage awaiting analysis.

Initial results from Atlantic salmon indicate that both plasma Vtg and Vtg mRNA are appropriate biomarkers of EDC exposure in this species, with differential responses based on the particular compound and dosage rate. To date we have seen no statistically significant dose-dependent or contaminant-related mortality in any of the four-day exposures of Atlantic salmon, nor in any of the shortnose sturgeon life-stage tests that we have conducted.

Since our short-term ‘median lethal dose’, or ‘LD<sub>50</sub>’, trial elicited neither chronic nor acute responses, it suggests that the standard LD<sub>50</sub> may not be an appropriate measure of single-contaminant impact on the long-term fitness of Atlantic salmon or shortnose sturgeon.

## 1 INTRODUCTION

### ***1.1 Origins of this Investigation***

Under Section 7 of the federal Endangered Species Act, (ESA, 7 U.S.C. § 136, 16 U.S.C. § 1531 et seq.), the National Marine Fisheries Service (NOAA Fisheries) conducts consultations, as needed, with agencies and parties engaged in actions within the designated habitat of ESA-listed species to identify and negotiate the reduction of activities capable of further jeopardizing the existence of the listed species. Such consultations extend to agencies and parties permitted to discharge wastewater into public waterways under the National Pollution Discharge Elimination System (NPDES).

Questions have arisen among the responsible agencies concerning the efficacy of the standard ‘whole effluent test’ (WET), which is a biological assessment required of NPDES permittees to detect harm to ESA-listed Atlantic salmon (*Salmo salar*), shortnose sturgeon (*Acipenser brevirostrum*), and Atlantic sturgeon (*Acipenser oxyrinchus*). The questions concern both the fish species normally used in the WET testing (whether they are true biological surrogates for the ESA-listed species) and the efficacy of the testing to identify wastewater components like endocrine disrupting compounds (EDCs) which may be contributing further to the jeopardy of the ESA-listed species.

#### ***1.1.2 EDCs have been shown to impair the fitness of fish***

The compounds referred to as EDCs include a large number of organic compounds that may act as estrogen mimics or act to block normal hormonal control, thus causing imbalanced hormonal regulation in fish through a number of pathways. EDCs have been shown to impair the fitness of freshwater, estuarine, and marine fish by causing reproductive failure, skewed sex ratios, and developmental abnormalities (Sumpter 2005).

These compounds are most often associated with municipal and industrial wastewater effluent and can feminize male fish in environments that receive large volumes of effluent (Sumpter 2005, Desbrow et al. 1998, Blazer et al. 2007) and result in local extinction (Kidd et al. 2007). Two of the most prevalent estrogenic compounds in rivers and estuaries include the natural steroid 17  $\beta$ -estradiol (E2) and a synthetic estrogen, 17  $\alpha$ -ethinylestradiol (EE2) (Desbrow et al. 1998). Additionally, less potent but often more concentrated estrogenic compounds, such as nonylphenol (NP), are also present in effluent (Servos et al. 2003). Routine wastewater treatment does not completely remove these compounds and in some cases increases their efficacy (Johnson & Sumpter 2001), and any fish in the receiving waters of wastewater effluent may be subject to endocrine disruption (Oberdorster & Cheek 2001, Matthiessen 2003).

Under certain conditions, exposure to endocrine disruptors can elicit severe and long-lasting impacts, primarily when fish are exposed early in their development (Strussmann & Nakamura 2002, Mills & Chichester 2005). Several studies have indicated that particular developmental stages may be more susceptible to EDCs than others. For example, Liney et al. (2005) exposed the common roach, *Rutilus rutilus*, to a gradient of wastewater effluent and found little response in adults but 100% feminization in fish exposed during the embryonic through juvenile period. Similar life-stage-dependent results have been demonstrated in zebrafish (Brion et al. 2004), medaka (Koger et al. 2000), and fathead minnow (Van Aerle et al. 2002).

One factor that may severely undermine current efforts to conserve Atlantic and shortnose sturgeon and Atlantic salmon is degraded water quality (Collins et al. 2000, Thorstad et al. 2008). Over-wintering and spawning of these species within the Gulf of Maine and other Northeast watersheds often occur near large urban centers (Kynard et al. 2000, Fernandes et al. 2010). For example, shortnose sturgeon were found to be present year-round in the Penobscot River estuary downstream from urban areas and several pulp and paper processing plants (Fernandes et al. 2010). The same study found that Atlantic sturgeon also spent several months in the Penobscot River estuary and that both sturgeon species migrated between the Penobscot River estuary and the Kennebec River, another urbanized estuary. Therefore, both spawning adults and developing offspring may be exposed to effluent with high concentrations of EDCs, potentially leading to recruitment failure (Kidd et al. 2007).

Relatively little is known of the impacts of EDCs on Atlantic salmon or any species of sturgeon, including the life-stage sensitivity to EDCs of these species. Such knowledge, were it available, could prove critical to the conservation of these species.

### ***1.2 Purpose and Scope of this Investigation<sup>1</sup>***

The purpose of this investigation is to gather data on the impacts of impaired water quality on Atlantic sturgeon, shortnose sturgeon, and Atlantic salmon and to determine whether the surrogate species commonly used in WET tests provide results that are applicable to Atlantic salmon and Atlantic and shortnose sturgeon sensitivity. The investigation will:

1. provide information as to whether EDCs commonly discharged into U.S. rivers that contain Atlantic sturgeon, shortnose sturgeon and Atlantic salmon, and identified by NMFS as biologically relevant, can negatively affect the species and, if possible, at what concentrations and exposure durations such negative effects occur;
2. investigate the most vulnerable life stages of Atlantic and shortnose sturgeon; and
3. provide information as to whether test species commonly used for WET testing [e.g., fathead minnow (*Pimephales promelas*), brook trout (*Salvelinus fontinalis*), rainbow trout (*Oncorhynchus mykiss*)] are suitable surrogates for Atlantic sturgeon, shortnose sturgeon, and Atlantic salmon.

It is anticipated that the investigation will take three years to accomplish these goals and requirements.

#### ***1.2.1 Year One (base year)***

The goals of the base year project are to 1) determine baseline responses to three common endocrine disrupting compounds when exposed for four days (96 hours); 2) further determine differential responsiveness to these compounds across different life-stages, and 3) develop and validate assays that will be used as biomarkers for endocrine disruption in these species. This will determine which contaminants have the greatest influence on Atlantic salmon and shortnose sturgeon, which life-stages are most sensitive to these contaminants, and how these species respond

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<sup>1</sup> From NMFS' 17 August 2010 Request for Quotes # 495852 'Sturgeon and Salmon Water Quality Study'

(i.e. reduced growth, altered gonad development, etc.) to the contaminants. Atlantic sturgeon were unavailable during year one and, pending availability, will be studied in year two.

### ***1.2.2. Year Two (option year one)***

The goals of project year two are to 1) determine short-term responses to endocrine disruptors in Atlantic sturgeon life stages (thereby completing all short-term response testing), 2) expose shortnose sturgeon and Atlantic salmon to environmentally relevant levels of EDCs and determine long-term effects of exposure, and 3) determine the impacts of EDCs on a commonly used WET test species (fathead minnow or brook trout) for comparison.

### ***1.2.3 Year Three (option year two)***

Year three will focus on additional contaminants that NMFS has identified as potential hazards for both salmon and sturgeon in Gulf of Maine watersheds. We will incorporate whole-effluent testing if we have access to representative wastewater effluent and determine responses of salmon and/or sturgeon to paper- and pulp-processing effluent. Additionally, we will analyze impacts to normal animal development and growth. The data will be synthesized from the three years of the investigation to determine the best strategy for the protection of Atlantic salmon, shortnose sturgeon, and Atlantic sturgeon.

## **2 METHODS AND ASSAY DEVELOPMENT**

### ***2.1 Year One Experimental Design<sup>2</sup>***

The researchers carried out short-term, 4-day (96 hour) exposures on both Atlantic salmon and shortnose sturgeon in year one. In these exposures we utilized three common EDCs with known estrogenic impacts on wild and lab-reared fish populations (Desbrow et al. 1998, Servos et al. 2003). The EDCs used in the investigation include:

- 1. 17 $\beta$ -Estradiol (E2)**

Four concentrations: one solvent control (0 nM), one high dose/positive control (4.0 nM), one dose mimicking an urbanized river (0.04 nM), and a concentration of E2 seen in wastewater effluent (0.4 nM).

- 2. Ethinylestradiol (EE2)**

Four concentrations: 0.4 nM, 0.04 nM, and 0.004 nM, along with a solvent control. EE2 is often 10-50 times more potent than E2, and smaller concentrations may elicit a larger response. Therefore, 0.4 nM was thought to be a concentration that would elicit a positive response to EE2.

- 3. Nonylphenol (NP)**

Four concentrations: one solvent control (0 nM), one high dose/positive control (400 nM), and two environmentally relevant doses, characteristic of urbanized portions of U.S. rivers or undiluted wastewater, respectively (4 and 40 nM). Concentrations of NP were chosen

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<sup>2</sup> See 'Revised Atlantic salmon and sturgeon wastewater impacts investigation plan, including timeline and description of the contaminant concentrations to be used in the study', memo from Kier Associates to Ms. Kimberly Damon-Randall, NOAA Fisheries, Northeast Region, 29 October 2010.

based on previous exposures of Atlantic salmon to NP in previous Conte Lab investigations (Lerner et al. 2007).

In the original study plan, the team suggested using additional doses for each compound across the range of what would be found within a moderately impacted river system. However, due to the limited number of fish available at each life stage, the team chose to focus on three doses of each compound, in addition to a control. The doses examined represent the range of doses initially proposed, and for each compound there is a representative dose of contaminant that would elicit a positive response, a dose similar to what would be found in sewage effluent, and a dose that would be found in an urbanized estuary. The lower range of doses originally proposed were eliminated from the study since four-day exposures may not be sufficient to elicit a response by the organism (Dwyer et al. 2005).

## ***2.2 Preliminary exposures***

Preliminary exposures to adult salmon and shortnose sturgeon were carried out between November and December 2010. Ten one-year old salmon parr were injected intraperitoneally with 5 mg/kg E2 dissolved in peanut oil to elicit Vtg induction (Arukwe et al. 1997). Five fish were sampled by anesthetizing in MS-222 and immediately drawing blood with heparinized syringes. An additional five parr were sampled on the same day from a tank of non-injected fish to serve as controls. Blood was centrifuged at 4 °C for five minutes, and plasma was snap-frozen and stored at -80 °C. Additionally, liver tissue was dissected and snap-frozen at -80 °C. The additional five fish injected with E2 were sampled in the same manner after ten days. These plasma and liver tissue samples were produced to use in validation of the salmon Vtg ELISA and mRNA assays.

Two lab-reared juvenile shortnose sturgeon were also injected intraperitoneally with 5 mg/kg E2. Plasma and livers were sampled from both fish 11 days after the injection. Plasma and liver tissue were stored as above. Control liver tissue was collected from non-injected juvenile shortnose sturgeon.

### ***2.2.1 Salmon experiments***

In all, six experiments were carried out using Atlantic salmon (*Salmo salar*) encompassing four life-stages: late-stage embryos, newly hatched larvae, feeding fry, and smolts. Salmon of three early life-stages were acquired from White River National Fish Hatchery in Vermont between February and May 2011. A total of 1,200 salmon were donated to this project by White River's hatchery manager Ken Gillette. Late-stage embryos (approximately two-three weeks prior to hatch) were transported from White River hatchery in February 2011 to the Conte Lab where they were randomly assigned to treatments carried out in 1.1 L aerated flow-through containers. Twenty embryos were allocated to each treatment in duplicate. Embryos were acclimated from 5 °C to 7.5 °C over 72 hours before exposures were carried out. Exposures were carried out in duplicate 1.1 L containers with a single 19 L head tank for each treatment. Head tanks were refilled daily with dechlorinated and aerated city water and the compound of interest, ensuring a turnover time of 7 times d<sup>-1</sup> and an average flow rate of 0.25 L h<sup>-1</sup>. All exposures were carried out in a walk-in cooler/cold room to control temperature. Head tanks were aerated and covered to minimize photo-degradation of compounds. All compounds used were solubilized in methanol (used as a vehicle for each compound) at a final concentration of <0.0001% to minimize solvent toxicity (Lerner et al. 2007). Control treatments

received solvent only. At the end of 96 hours, embryos were briefly rinsed in clean dechlorinated city water, snap-frozen on dry ice, and stored at -80 °C prior to analysis.

Yolk-sac larvae and feeding fry were exposed as above with the following changes. Both stages were exposed at 15 °C. A lower temperature exposure of salmon embryos was necessary to prevent developmental abnormalities associated with higher temperatures. However, both larvae and fry can be easily reared at 15 °C. Yolk-sac larvae exposures were carried out in mid-March at 18 fish per replicate and feeding fry exposures in early May 2011 at 15 fish per replicate. Both life-stages were acclimated in the exposure chambers for 72 hours prior to beginning of the experiment. Feeding fry were acclimated to pelleted salmon feed in the hatchery and fish were fed daily during acclimation and during the experiment. In addition, feces and uneaten food were siphoned from experimental tanks daily to minimize bacterial degradation of contaminants.

Atlantic salmon smolts were also exposed to these contaminants in three, four-day experiments in mid-April 2011 encompassing the preparation for transition to saltwater. Smolts were placed in approximately 30 L of water in circular tanks at six fish per tank on the morning the exposures were initiated. Fish were not fed during the four-day period. Large 560 L circular tanks served as head tanks, which were filled prior to the beginning of the experiment and allowed to fill tanks prior to introduction of fish. Head tanks were replenished daily with the appropriate volume of stock contaminant in methanol and aerated, dechlorinated city water, yielding a turnover time of at least 2 times d<sup>-1</sup> and flow rate of approximately 10.5 L hr<sup>-1</sup>.

Following the 96-hour exposures, fish were removed from experimental tanks, anesthetized with MS-222, and immediately bled with heparinized syringes. Blood was centrifuged at 5000 x g for 5 minutes at 4 °C and plasma was aliquoted into tubes and stored at -80°C. Fish were immediately placed on ice, and gill and liver tissue were removed and snap-frozen and stored at -80 °C until analysis.

### ***2.2.2 Shortnose sturgeon experiments***

Three experiments were carried out with early life-stage shortnose sturgeon: embryos, yolk-sac larvae, and feeding fry. Two female sturgeon in spawning condition were collected from the Connecticut River using gill nets (methods outlined in Damon-Randall 2010) in April 2011 and brought back to the Conte Lab where they were held in a 5-foot flow-through tank and fed daily. In early May, four male shortnose sturgeon were collected from the Connecticut River and brought to Conte where they were held in a separate tank and fed daily. Females were injected with carp pituitary hormone at 36 and 24 hours prior to strip-spawning to induce egg maturation.

After 36 hours, milt was harvested by abdominal stripping, thus leaving the fish unharmed. Immediately following, mature eggs were released from females in a similar fashion. Eggs from both fish were mixed with milt from both male sturgeon to allow fertilization, resulting in four half-sib families. Embryos were then mixed with dechlorinated city water and Fullers earth powder (Parker 2007) to prevent eggs from clumping together. Embryos were de-adhesed in this solution for 20 minutes, then placed into McDonald hatching jars at 15 °C (see Parker 2007 for sturgeon spawning methods).

Embryos were kept in aerated McDonald jars with moderate flow until hatched. Upon hatching, fish were directed into a 10 L container with circular flow. Temperatures were maintained at 15-17 °C

throughout this period, with large rocks in the bottom of the container to act as substrate for that period during which the fish are photonegative (as yolk-sac larvae). Upon absorption of the yolk-sac, the fish were fed live brine shrimp nauplii, dry starter feed, and frozen copepods. The adults used in spawning were returned unharmed to the Connecticut River.

A subset of fertilized embryos were immediately placed into clean 1.1L exposure chambers at 15°C at four hours post fertilization (hpf) at a density of 32 embryos per treatment, across two replicates. Treatments were initiated 24 hpf. Exposures on embryos were carried out as previously described with salmon embryos. Due to the short developmental periods of each life stage (approximately 10 days as embryos and another 10 days as yolk-sac larvae at 15 °C), exposures were initiated soon after fertilization or hatch. Three day old yolk-sac larvae were randomly assigned to treatments at 12 fish per treatment (6 per replicate) and acclimated to the exposure chambers for 48 hours before exposures.

Feeding fry exposures were carried out in a similar manner, with 8 fish per treatment. Sturgeon fry exposures were initiated two weeks after feeding first began to ensure that no first-feeding mortality was seen during exposures. During the experiment, fish were fed dry starter feed daily and live brine shrimp every other day. Tanks were siphoned daily. Photographs (for length) and weight measurements were taken on all fish at the end of the 96-hour exposures at which point they were snap-frozen and stored at -80°C.

The fourth stage of sturgeon (1-year old juveniles) will be exposed in fall, 2001. Because of high temperatures of both river and city water during the summer and the limitations to chilling the large volume of water needed for this experiment, the team has decided to delay exposures of these fish. Cooler temperatures in the fall will allow Dr. Duffy to keep experimental temperatures consistent among experiments (15 °C).

## ***2.3 Development of the assays***

### ***2.3.1 Salmon Vtg mRNA assay development***

The salmon mRNA assay was developed using the methods for qRT-PCR as described in Arukwe and Roe (2007). The primers for the housekeeping gene (EF-1a) were taken directly from Ingerselv et al. (2006). Both primer sets were validated using the Roche 480 LightCycler in Dr. McCormick's lab. Each set yielded optimal melting curves indicating the production of one amplicon (~200 base pairs) each. Additionally, a small set of samples run to date indicate that EF-1a is not differentially regulated in response to exposure of contaminants, validating its use as a housekeeping gene. Extraction of mRNA and subsequent production of cDNA for use in this assay is underway.

### ***2.3.2 Salmon Vtg ELISA assay development***

Development of the salmon protein Vtg ELISA has proven to be difficult. Initial efforts to develop this assay were based on a mouse anti-salmon *monoclonal* antibody that was developed to identify salmon Vtg protein. The company that produces this antibody, Biosense (Norway) sells a commercially available assay for detection of Vtg using this antibody. Because the costs of a single kit are prohibitive for direct use and because the kit is not quantitative (only samples on the same 96-well plate can be compared), the team chose to develop a more quantitative assay. After multiple attempts to develop this assay with the Biosense antibody, Dr. McCormick and Dr. Duffy decided it would not allow for proper quantification of Vtg among experimental samples.

In February 2011, a new paper was published detailing methods of Vtg quantification in Pacific salmon (Peck et al. 2011). The team contacted the lead author (Karen Peck, NMFS Northwest) for detailed methods. This method, using a new *polyclonal*, rabbit anti-salmon Vtg antibody available from Biosense, was more successful. Troubleshooting for this assay was needed to determine the proper sample dilution, additional assay components, incubation times, and to validate for use with Atlantic salmon plasma. The assay development and validation is now complete and this assay is currently in use by the team.

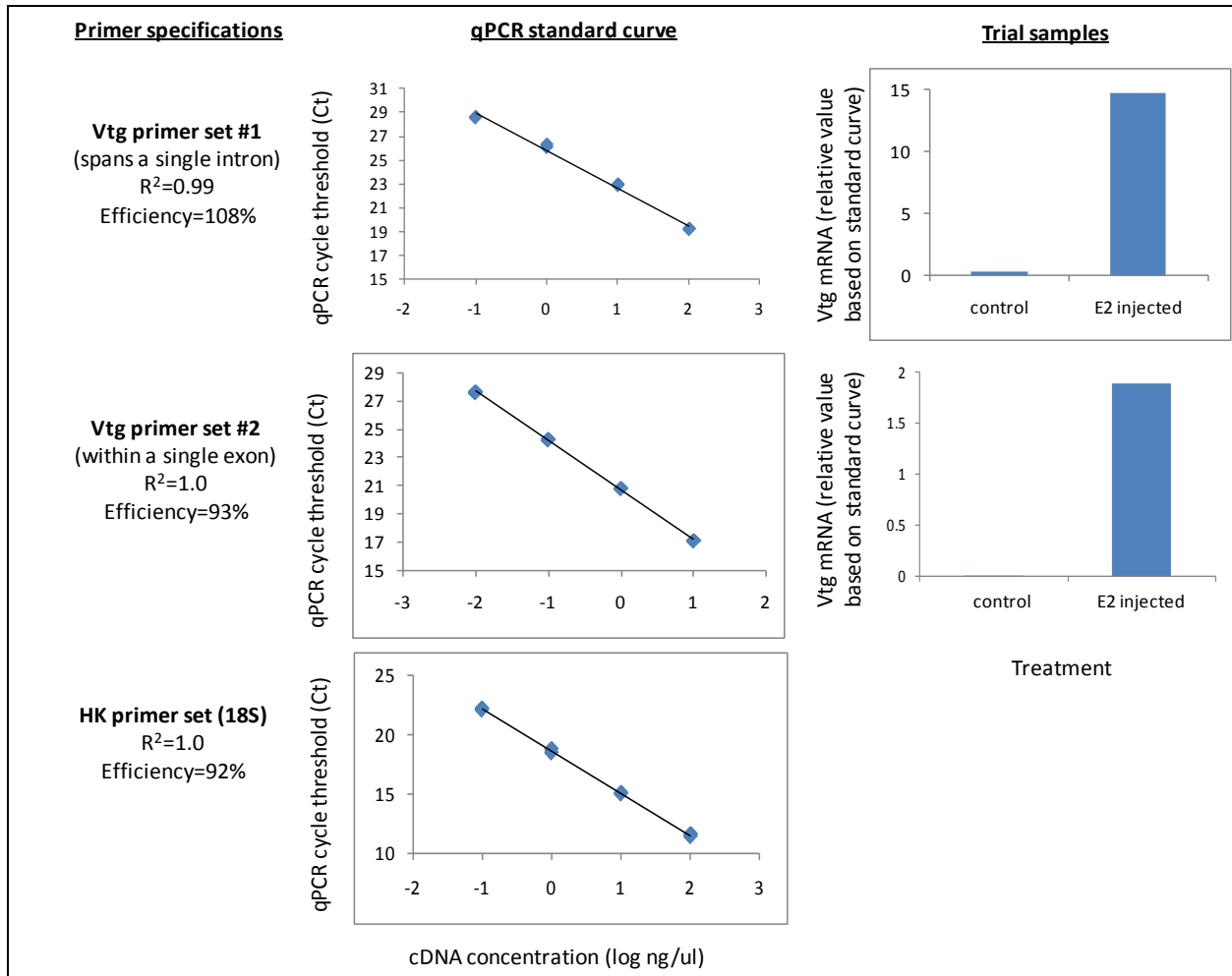
To our knowledge, there is no published study that has validated the use of whole-body homogenates for the measurement of Vtg protein in small animals. Therefore, our analysis of Vtg protein is necessarily limited to animals that are large enough to collect plasma. Because feeding fry and smaller life-stages were not large enough to collect plasma from, we are unable to quantify plasma Vtg from these animals. If future investigations use larger juvenile salmon, then we may be able to collect enough plasma to use this assay for Vtg quantification in smaller animals.

### ***2.3.3 Sturgeon Vtg RNA assay development***

The assay for shortnose sturgeon Vtg mRNA was developed by partially cloning a portion of the sturgeon vitellogenin gene. To accomplish this, sturgeon Vtg sequences in GenBank were aligned with Vtg sequences from other fish species. Mixed base primers were designed based on regions of the Vtg gene that are conserved across lineages. These primers then amplified putative Vtg sequences which were sequenced at the University of Massachusetts-Amherst genomic sequencing facility. One primer set yielded two partial sequences (amplicons) which closely matched the sequences the primers were intended to amplify. Next, these amplicons were cloned into vectors (circular rings of DNA) and inserted into *E. coli* bacteria. The bacteria were grown on plates containing X-gal, allowing for blue/white screening of bacterial colonies with the insert of choice. Colonies containing the putative insert (the vector containing the partial Vtg gene) were grown overnight, cleaned, and sequenced.

These sequences yielded matches to other Vtg genes, including ~98% homology to other sturgeon Vtg genes. The product from the cloning reactions is a 627 base pair partial sequence that contains two exons and a putative intron. This partial sequence will be published in GenBank immediately prior to publication of this work, as is customary with cloned molecular sequences. Sequence data is available upon request.

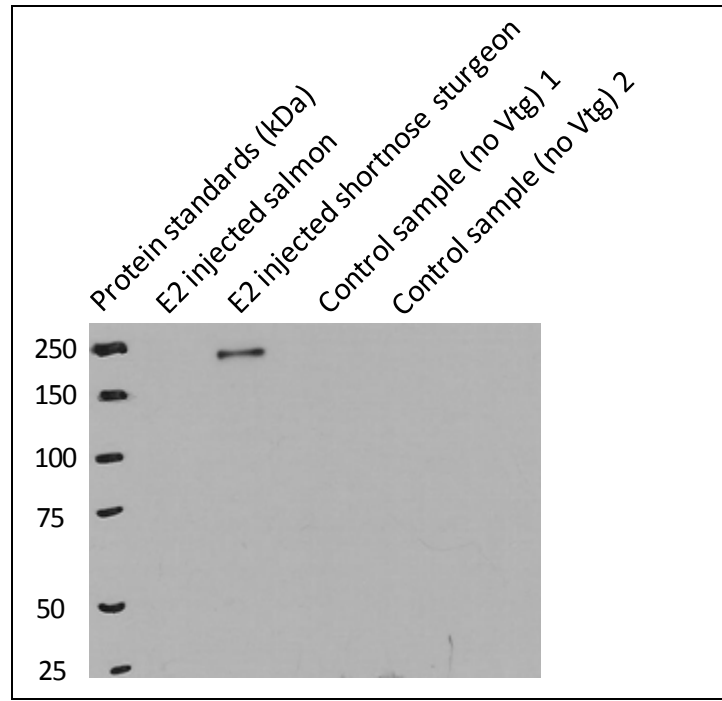
Primers for qRT-PCR assays were designed to amplify small fragments of the cloned gene (<200bp). Dr. Duffy designed three primer sets to amplify Vtg cDNA (amplified from mRNA) and two primer sets to amplify housekeeping genes. Two primer sets for Vtg yielded excellent results and will be further optimized (Figure 1). However, the team is confident this assay is ready to proceed upon preparation of the samples. In addition, the housekeeping gene 18S yielded similar results, and can be immediately used in this assay. Primer sequences will be published in GenBank along with the Vtg sequence data.



**Figure 1.** Validation data for design of the qRT-PCR assay to measure shortnose sturgeon Vtg mRNA. Column 1 contains primer specifications for the three primer sets discussed, including data on the standard curves created with shortnose sturgeon cDNA (column 2). Column 3 contains sample Vtg mRNA transcription levels quantified with the two Vtg primers. These are based on one control and one E2 injected shortnose sturgeon analyzed in duplicate.

### 2.3.4 Sturgeon Vtg ELISA assay development

One Vtg antibody for Gulf sturgeon (*Acipenser oxyrinchus desotoi*), a subspecies of Atlantic sturgeon, is commercially available. Dr. Duffy has purchased this antibody, which the team confirmed to recognize Vtg protein in shortnose sturgeon (Figure 2), identifying it as a candidate for use in Western Blot and/or ELISA analysis of shortnose sturgeon Vtg. Dr. Duffy has reviewed the literature available to determine the best method to extract and purify Vtg from sturgeon plasma. Extraction and purification of Vtg from shortnose sturgeon is currently in progress, and the team will next proceed to develop the ELISA assay for shortnose sturgeon. Additionally, we have recently received a second antibody from a colleague of Dr. McCormick's, Dr. Dianna Papoulias (USGS), which can be used if the commercially available antibody proves to be difficult in assay development. Because the salmon Vtg assay is completed, we are confident that we will be able to develop an assay for shortnose sturgeon within two months.



**Figure 2.** Western blot analysis of a commercially available Gulf sturgeon antibody for cross-reactivity with shortnose sturgeon Vtg. Samples analyzed include E2-injected salmon plasma (for cross-reactivity), E2 injected shortnose sturgeon plasma, and two control samples which do not contain sturgeon Vtg. Plasma from E2 injected shortnose sturgeon demonstrated cross-reactivity with the antibody in a band of Vtg approximately 220 kDa which is the characteristic of vitellogenin in other sturgeon species. Protein standards have been highlighted within the figure.

### 3 RESULTS

#### *3.1 Assay Development Results*

The team validated an assay for quantifying Atlantic salmon Vtg mRNA and three-quarters of the analyses are complete at this time. Additionally, the team now has a fully validated protocol for quantifying Vtg protein in plasma using a competitive ELISA. Eighty percent of the samples collected from Atlantic salmon smolts have been quantified and are presented in this report. The shortnose sturgeon Vtg mRNA assay is complete and is ready to be used to quantify samples collected to date. Additionally, the design of an assay to measure shortnose sturgeon Vtg protein is currently underway and is anticipated to be completed by the end of 2011.

#### *3.2 Growth/Weight Loss Data Results*

Dr. Duffy has analyzed a portion of the growth/weight loss data for a subsample of the salmon and sturgeon experiments. Smolting salmon do not show any statistically significant contaminant-dependent weight loss during the four day exposures ( $p > 0.111$ , 3 df). Additionally, neither sturgeon embryos nor yolk-sac larvae demonstrate statistically significant dose-dependent weight loss over the four days of exposure. Fish from a single treatment, 0.04 nM E2, showed significantly different weights at the end of the experiment, but this was not consistent with higher doses of E2, so dose-

dependent effects may be ruled out. Preliminary analysis of these data indicate that sublethal impacts to growth or weight loss are unlikely to be detectable over short periods of exposure.

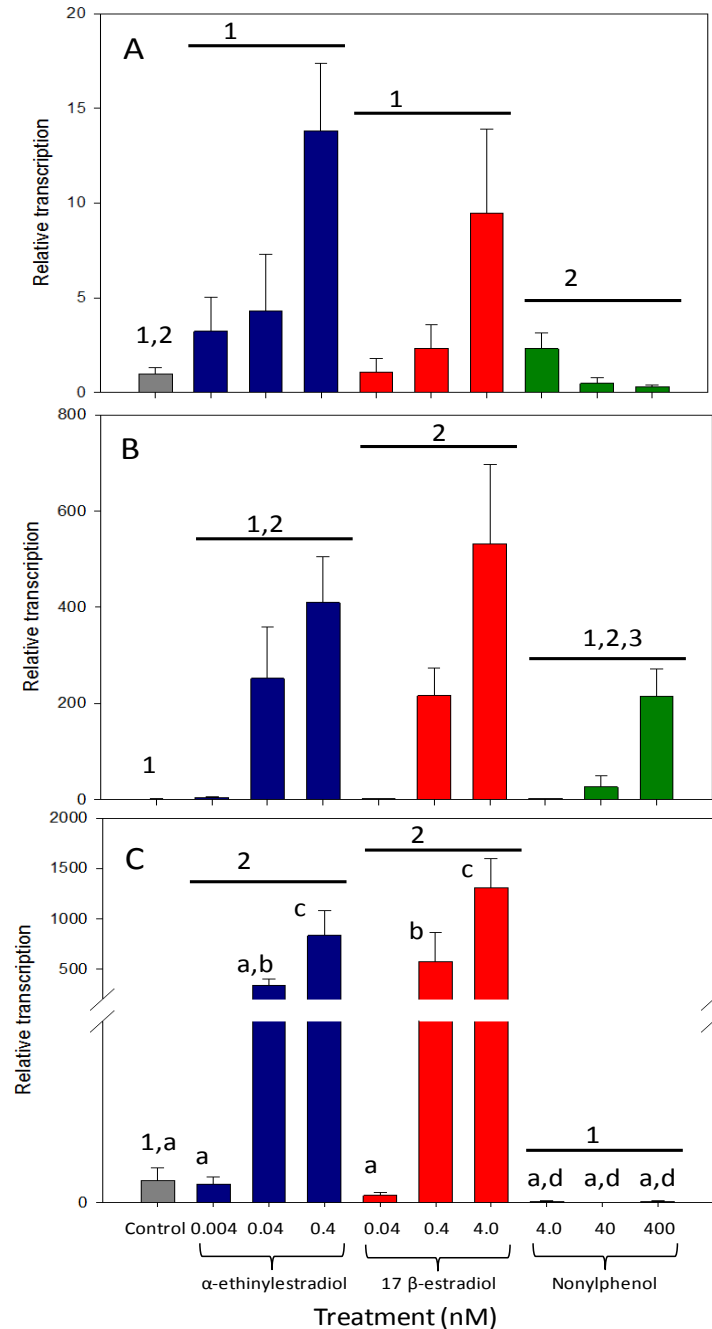
### ***3.3 Atlantic salmon Vtg mRNA and protein quantification***

Initial analyses of the three life-stages of Atlantic salmon analyzed for Vtg mRNA transcription indicates that Vtg mRNA is a useful biomarker of four-day exposure to two of the compounds studied here. The data demonstrate overall significant effects of treatment with E2 and EE2, and in some cases demonstrate clear dose-dependent responses (primarily in smolts) (Figure 3). The magnitude of response clearly differs among the three life-stages tested to date. Embryos show limited and variable response with few statistically significant differences among treatments (Figure 3A). Maximal Vtg transcription was limited to one order of magnitude response greater than the control, and this was limited to the highest doses of E2 and EE2.

Feeding fry demonstrated significant effects of all three treatments, but the magnitude of response among compounds varied significantly (Figure 3B), without evidence of statistically significant dose-dependent response, owing largely to high variance among samples. However, maximal average transcription exceeded two orders of magnitude difference from controls. Additionally, NP treatments showed some evidence of upregulation, although the treatment effects were not statistically different from controls.

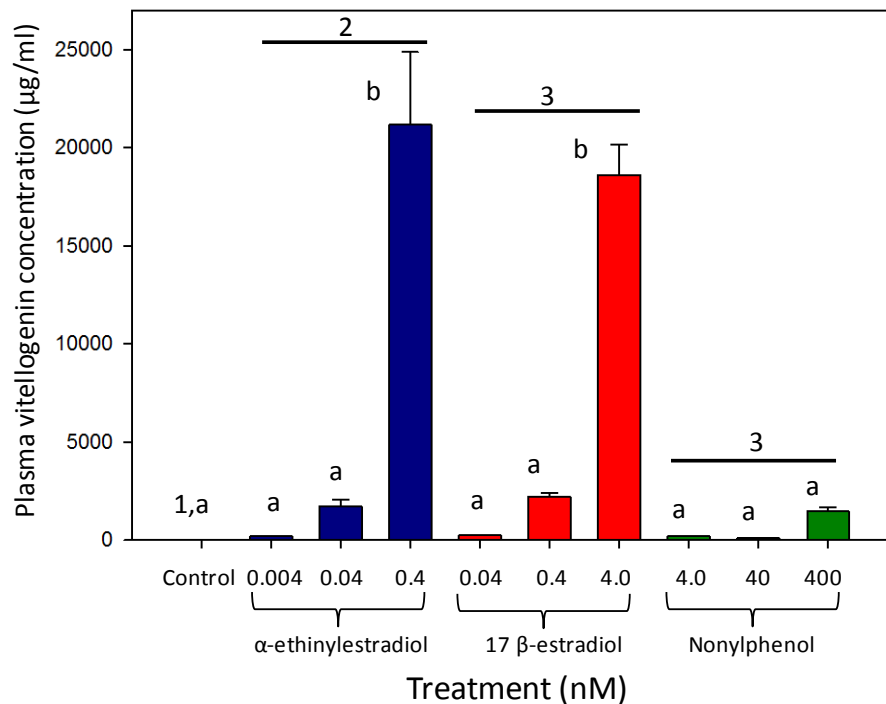
Relative transcription in salmon smolts showed the highest maximal expression levels (~three orders of magnitude greater than controls) of any life-stage, but this may be owing to expression quantified from liver (site of Vtg production) rather than whole body samples. Significant responses were limited to the medium and high concentrations of E2 and EE2, with non-significant response in any NP treatments or low doses of E2 or EE2.

Plasma Vtg quantification in salmon smolts showed elevated production of Vtg in both high, positive control doses of E2 and EE2 in the partial data set included in this report (Figure 4). Overall, the data suggest significantly different treatment effects. Additionally, there is some indication of a dose-dependent response for these compounds, but these were not statistically significant after analyzed with a two-way ANOVA. The pattern seen here is similar to that seen in salmon smolt Vtg mRNA, but protein Vtg concentration appears to be a less sensitive measure of response to E2 and EE2 than Vtg mRNA since there was no statistically significant increase in protein Vtg at intermediate doses of E2 and EE2.



**Figure 3.** Relative Vtg transcription in three life-stages of Atlantic salmon. The three panels represent A) embryos, B) feeding fry, and C) salmon smolts for controls (grey) and each of three doses of single compound exposure. Data from exposure to  $\alpha$ -ethinylestradiol is depicted in blue, 17  $\beta$ -estradiol in red, and Nonylphenol in green. Each bar represents eight individuals from each treatment pooled from two replicates. Data are shown in values of Vtg transcription normalized to a HK gene (EF-1 $\alpha$ ) and then normalized to the control (=1). Significant differences among treatments (two-way ANOVA,  $p \leq 0.05$ ) are indicated by different group numbers and significant differences among doses within and among treatments are indicated by different letters. Note the axes differences in relative transcription among the life stages.

Atlantic salmon analyses and conclusions drawn here are preliminary and subject to change when additional data are analyzed. Therefore, we stress that the overall patterns are unlikely to change, but analyses may be altered when additional data are included.



**Figure 4.** Plasma Vtg in Atlantic salmon smolts exposed to estrogenic compounds for 4 days. Treatments are displayed as in Figure 3; each bar represents 3-8 samples. Two-way ANOVA revealed significant differences across treatments, concentrations of compounds, and a significant interaction term (all  $p < 0.001$ ). Treatment differences are indicated by asterisk, and differences among doses are indicated by different letters.

## 4 SUMMARY OF YEAR ONE PROJECT ACCOMPLISHMENTS

### 4.1 Laboratory Assays Developed and Experiments Performed

The primary objectives of project year one were to obtain information regarding the response of these animals to short-term (96 hour) exposure to common wastewater contaminants, develop the assays necessary to compare sublethal responses to these contaminants, and compare life-stage sensitivity across the initial species examined. To date, the principal investigators, Drs. Stephen McCormick and Tara Duffy, have achieved the majority of these goals and additional activities planned for the next four months will complete the project goals. By the end of year one, we will be able to provide information as to how Atlantic salmon and shortnose sturgeon respond to common endocrine disrupting contaminants. The responses to short-term exposure will guide us to a better understanding of how we will expose vulnerable life-stages to contaminants in a more rigorous, long-term exposure that more closely mimics what the organism experiences in the wild. Long-term exposures will be carried out in years one and two.

**Table 1. Summary of assays developed and experiments performed in project year one**

	<b>Assay status</b>	<b>Experiments completed</b>
<b>Atlantic salmon</b>	Vtg mRNA qRT-PCR (completed)	Embryos (completed), yolk-sac larvae (completed) feeding fry (completed)
	Vtg protein ELISA (completed)	smolts (completed)
<b>Shortnose sturgeon</b>	Vtg mRNA qRT-PCR (completed with minimal optimization)	Embryos (completed) yolk-sac larvae (completed)
	Vtg protein ELISA (currently in progress)	feeding fry (completed) older juveniles (to be completed this fall)

Our year-one efforts have been focused on shortnose sturgeon and Atlantic salmon. In year two, we will determine responses to endocrine disruptors in Atlantic sturgeon and a common WET test species (fathead minnow or brook trout) for comparison. To this end, we have provided information for a Conte Lab permit application for the acquisition of Atlantic sturgeon should they be placed on the ESA List. This will allow us to possess Atlantic sturgeon embryos in the future for comparative exposures. Additionally, we anticipate that our development of vitellogenin mRNA and protein assays will be transferrable to Atlantic sturgeon, requiring minimal assay development once the shortnose sturgeon assays are validated.

The development of EDC biomarker assays has been an essential yet complicated step in year one efforts. Because most of these assays are not available for sturgeon, and are available in only a limited capacity for Atlantic salmon, we have been and will continue to develop these tools for use in years two and three of the project. Since previous EDC work has demonstrated impairments to growth, sex determination and differentiation, and normal physiological processes critical to anadromy, we will continue to develop additional assays that will measure the extent to which these processes are impacted. For example, in year two we will be able to accurately measure the extent to which growth may be delayed in our longer-term assays, while providing a biomarker to measure growth impairment in future studies of these species.

#### **4.2 Outreach Activities Accomplished**

An additional, yet vital, dimension of the project is to maintain communications and outreach among Gulf of Maine agencies, researchers, and parties permitted to discharge wastewater into public waterways under the terms of the National Pollution Discharge Elimination System (NPDES). This outreach is pivotal to the investigation’s larger purpose which is to determine whether federally recognized water quality monitoring procedures required of wastewater managers are providing adequate protection for salmon and sturgeon species as required by the Endangered Species Act. As such, Field Coordinator, Barbara Arter, conferred with numerous parties to identify relevant literature, brief fellow colleagues and researchers, and attend meetings of the Maine Department of Environmental Protection Penobscot River NPDES Stakeholder Group. The Kier Associates team outreach activities during the reporting period included:

- Dr. Duffy and Ms. Arter worked with Ms. Vaccaro (NMFS) to identify and upload project-relevant documents to NMFS' shared-access FTP site. The posted scientific literature focuses on developing immunochemical assays with which to measure vitellogenin, experimental designs for exposure to several contaminants, and assessing the endpoints of exposure in anadromous species.
- Information was gathered concerning other endocrine disruptors as well as studies of sturgeon habitat and migration underway in the principal area of interest, Maine's Penobscot and Kennebec watersheds.
- The Maine Department of Environmental Protection (DEP) provided information identifying WET test contractors for the three major cities in the target region (Bangor, Brewer, and Augusta), Maine surface water ambient toxins (SWAT) reporting, Maine surface water criteria, and EPA Publicly Owned Treatment Works (POTW) studies.
- Ms. Arter reviewed the project with Barry Mower of Maine's DEP, Steve Mierzykowski of the U.S Fish & Wildlife Service, Adria Elskus of the U.S Geological Survey/ Orono, and Gayle Zydlewski of the University of Maine.
- Ms. Arter presented a briefing about the project to the Penobscot River Science Exchange at the University of Maine in Orono on 4 March 2011. The presentation generated interest regarding possible testing for other contaminants (the presentation can be viewed at: [http://www.umaine.edu/searunfish/penobscotexchange/PSE\\_Spring2011mtg.html](http://www.umaine.edu/searunfish/penobscotexchange/PSE_Spring2011mtg.html)).
- Ms. Arter also attended Maine Department of Environmental Protection Penobscot River MEPDES Stakeholder Group Meetings in November 2010 and March 2011 in Bangor to learn more about the MEPDES process and river discharge issues in general. She also participated in a statewide MEPDES Stakeholder group focused on marine (bay and estuary) discharge criteria in June 2011 in Augusta, Maine.
- Volunteer project co-investigator Dr. Stephen McCormick attended the NOAA sturgeon conservation workshop in February 2011 in Virginia where he met with sturgeon experts to discuss conservation strategies and current sturgeon research. Dr. McCormick presented a brief overview of the project to US Geological Survey staff at the Conte Lab on April 2011. The presentation was well received, no significant concerns were raised.
- Dr. Duffy submitted an abstract for a presentation to the annual meeting for the Society for Environmental Toxicology and Chemistry. The abstract has been accepted and results from the salmon and sturgeon work will be presented at the conference in November 2011.
- Dr. Duffy discussed the goals of this project with a group of high school students at the Women in Science summer camp at Smith College.
- Ms Arter discussed the goals of the project at the Diadromous Species Restoration Research Network Natural Variability Workshop at the University of Maine, Orono in May 2011.

## 5 FINDINGS AND RECOMMENDATIONS TO DATE

### *5.1 Mortality data*

The U.S EPA suggests that WET tests use LD<sub>50</sub> as the endpoint for short-term, four-day exposures to wastewater effluent. To date we have seen no statistically significant dose-dependent or contaminant-related mortality in any of the four-day exposures of Atlantic salmon, nor in any of the shortnose sturgeon life-stage tests that we have conducted.

The maximum concentration of each contaminant used was chosen to elicit a strong, positive response. These concentrations represent contaminant levels that are ten to fifteen times higher than what fish would be exposed to in the wild with regard to the concentration of a single contaminant.

While the LD<sub>50</sub> test may be an adequate indicator of a substance's acute toxicity, it does not, however, provide any information regarding population-level responses or information concerning long-term impacts on growth, saltwater challenge, or reproduction. Our results indicate that the high doses of each of the compounds tested elicited a vitellogenic response, an indicator of long term reproductive abnormality. Since our LD<sub>50</sub> investigation found no significant mortality at these same doses, the results suggest that LD<sub>50</sub> may not be an adequate measure of single-contaminant impact on long-term fitness of Atlantic salmon or shortnose sturgeon.

### 5.2 Atlantic salmon Vtg mRNA and protein

Both Vtg mRNA and protein have proven to be adequate biomarkers of four-day exposure to both 17  $\beta$ -estradiol and  $\alpha$ -ethinylestradiol, but this is less clear for exposure to nonylphenol. Owing to partial completion of analyses, we cannot make any concrete recommendations at this time pertaining to the validity of Vtg as a biomarker across life-stages. There is some evidence of a dose-dependent response by Vtg transcription in Atlantic salmon, but this is not consistent across life-stages. However, initial data collected from Atlantic salmon suggests that exposure to these compounds during the embryo stage is the least responsive of the life-stages tested to date in terms of Vtg gene expression. Therefore, long-term exposure in the next stages of this project will likely focus on older life-stages. A full recommendation will be presented when the data analysis for Atlantic salmon data is complete.

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