# *EVOSTC ANNUAL PROJECT REPORT*

Recipients of funds from the *Exxon Valdez* Oil Spill Trustee Council must submit an annual project report in the following format by **Sept. 1 of each fiscal year** for which project funding is received (with the exception of the final funding year in which a final report must be submitted). **Please help ensure that continued support for your project will not be delayed by submitting your report by Sept. 1. Timely receipt of your report allows more time for court notice and transfer, report review and timely release of the following year's funds.**

Satisfactory review of the annual report is necessary for continuation of multi-year projects. Failure to submit an annual report by **Sept. 1** of each year, or unsatisfactory review of an annual report, will result in withholding of additional project funds and may result in cancellation of the project or denial of funding for future projects. **PLEASE NOTE:** Significant changes in a project's objectives, methods, schedule, or budget require submittal of a new proposal that will be subject to the standard process of proposal submittal, technical review, and Trustee Council approval.

*Project Number: 090839*

*Project Title: Evaluating injury to harlequin ducks (***Histrionicus histrionicus***) caused by sublethal hydrocarbon exposure in Prince William Sound using species-specific cell lines*

*PI Name: Hollmen, T. and Springman, K.*

*Time period covered: October 1, 2008 – August 31, 2009*

*Date of Report: September 1, 2009*

*Report prepared by: Hollmen, T., Springman, K., and Riddle, A.*

**Work Performed:**

**Objective 1:** *Develop harlequin duck and surrogate (mallard) cell lines to evaluate injury from site-specific hydrocarbons in harlequin ducks*

**FY09 Tasks: Year 2 cell isolation, cell line characterization**

**Progress:**

Isolation of cells and development of cell lines for bioassays continued. In Year 2, cells were harvested from eggs of harlequin ducks (*Histrionicus histrionicus*, HADU; n=9) and mallards (*Anas platyrhynchus*, MALL; n=60). Additionally, rainbow trout (RT) hepatocytes were acquired from American Type Culture Collection (ATCC) for use as reference material for bioassay development.

Development of protocols to isolate fibroblast and hepatocyte cells from mallard and harlequin duck eggs continued. Protocols were established for harvesting cells, disaggregation of cell extracts, routine maintenance and monitoring, subculture of fibroblast cell lines, and cryopreservation of fibroblast cell lines. Protocols were tested for freeze-thaw of hepatocyte cells and results indicate that conducting hepatocyte-based assays using cultures without a freezing step is preferable to maximize cellular yields. Therefore, it will be necessary to plan hepatocyte-based assays to occur during the annual breeding season when eggs and fresh hepatocyte cultures are available. Availability of harlequin duck eggs will determine the total hepatocyte-based assay capacity on an annual basis.

For quality control and preparation of bioassay development, cell cultures were tested for non-specific toxic effects from reagents and solvents required for bioassays, including S9 fraction cofactors and the carrier solvent used, dimethylsulfoxide (DMSO).

A total of 19 hepatocyte cell extractions from harlequin duck or mallard eggs were performed, with a total yield of approximately 500,000 cells from each harlequin duck egg and 590,000 cells from each mallard egg. The harvested hepatocytes were utilized for bioassay development without a freezing step.

A total of 13 fibroblast cell extractions from harlequin duck or mallard eggs were performed. Subsamples of harvested fibroblasts were utilized for bioassay development without a freeze-thaw cycle. The remaining fibroblasts were frozen and stocks are stored in liquid nitrogen to be used for further bioassay development and in bioassay panels. At this time, we have cryopreserved and archived a total of up to 116 mallard fibroblast stock vials and up to 118 harlequin duck stock vials (estimated cell count per vial approximately 1 x $10^6$).

**Objective 2:** *Develop bioassays using harlequin duck and surrogate (mallard) cell lines to assess and quantify injury due to lingering oil in PWS*

**FY09 Tasks: Endpoint bioassay development**

**Progress:**

Endpoint bioassay development is in progress, including bioassays for determining cytopathic effects, dye exclusion cell viability assay, lactate dehydrogenase (LDH) release assay, ethoxyresorufin-*o*-deethylase (EROD) assay, PAH/DNA adduct formation assay, and comet assay.

Bioassay development was performed in duck hepatocyte, duck fibroblast, and/or rainbow trout hepatocyte cell cultures. Cells were cultured in microwell plates and monitored for consistency (cell morphology, cell layer development) to prepare for assay validations and determine optimum time lines for assays. Bioassay development was initiated by dosing with test materials with known composition, including chrysene, β-naphthoflavone (BNF), diesel, and intact Alaska North Slope (ANS) crude oil. The dosing using known reference materials involved range finding studies and comparisons among mallard cell lines, harlequin duck cell lines, and rainbow trout reference cell lines. The fibroblast cell lines were supplemented with standardized external liver homogenate (S9) fraction to facilitate enzymatic processes.

<u>Summary of endpoint bioassay panel development:</u>

*Cytopathic effects (CPE)*

Cytopathic effects were monitored in cell cultures dosed with known composition test materials, including chrysene, BNF, diesel, and ANS oil reference material. Types of CPE observed were characterized and described for each cell line and test material used, and a time series experiment was conducted to monitor CPE at 24, 48, and 72 hours post challenge The range of CPE observed using different test doses with known composition and concentration will be used to develop a scoring protocol for testing semipermeable membrane device (SPMD) extracts from study sites in Prince William Sound (PWS), and for comparisons with other bioassay results.

*Dye exclusion cell viability assay*

Laboratory operating protocol has been tested in fibroblast cell cultures for estimation of cellular viability using a trypan blue dye exclusion method.

*LDH release assay*

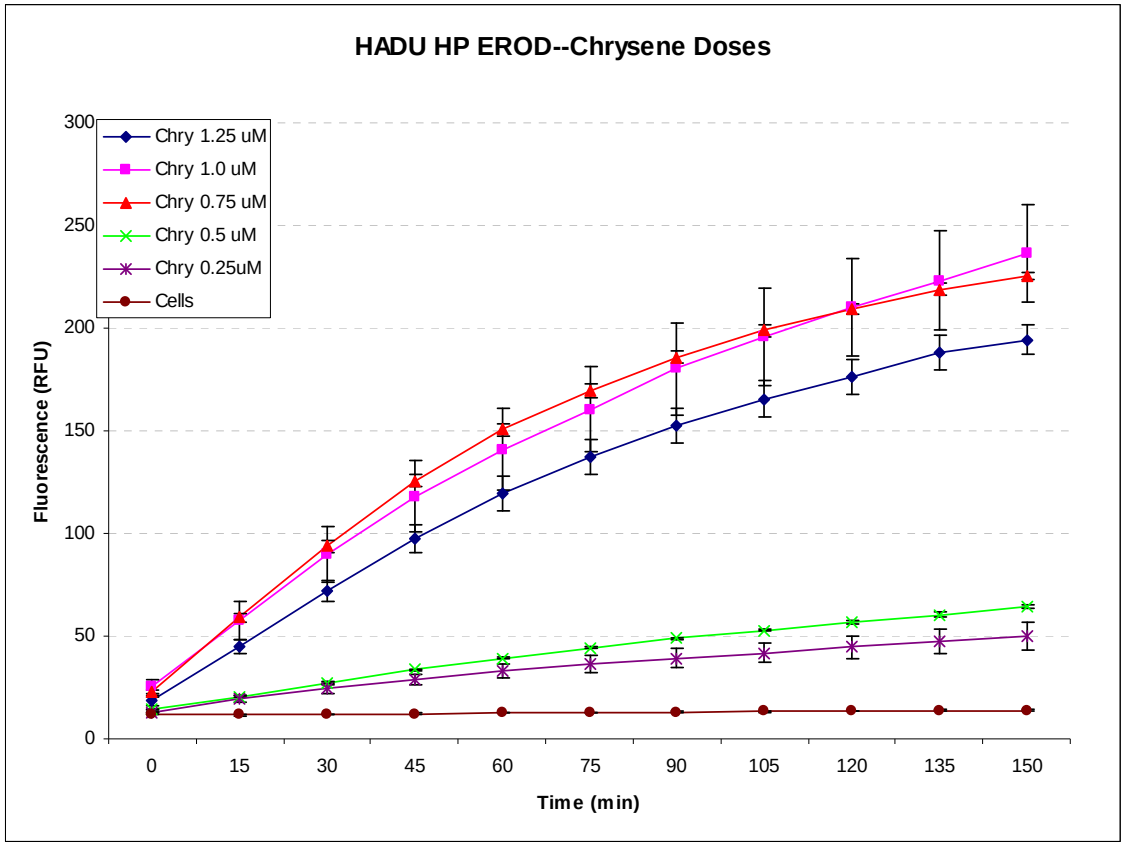
Assay development and validation is in progress using a Cytotoxicity Detection Kit (LDH) (Roche Applied Science, Indianapolis, IN), which measures cell death and cell lysis based on the LDH activity released from damaged cells. Protocols have been tested in hepatocytes from mallards and in fibroblasts without S9 fraction. Repeats of

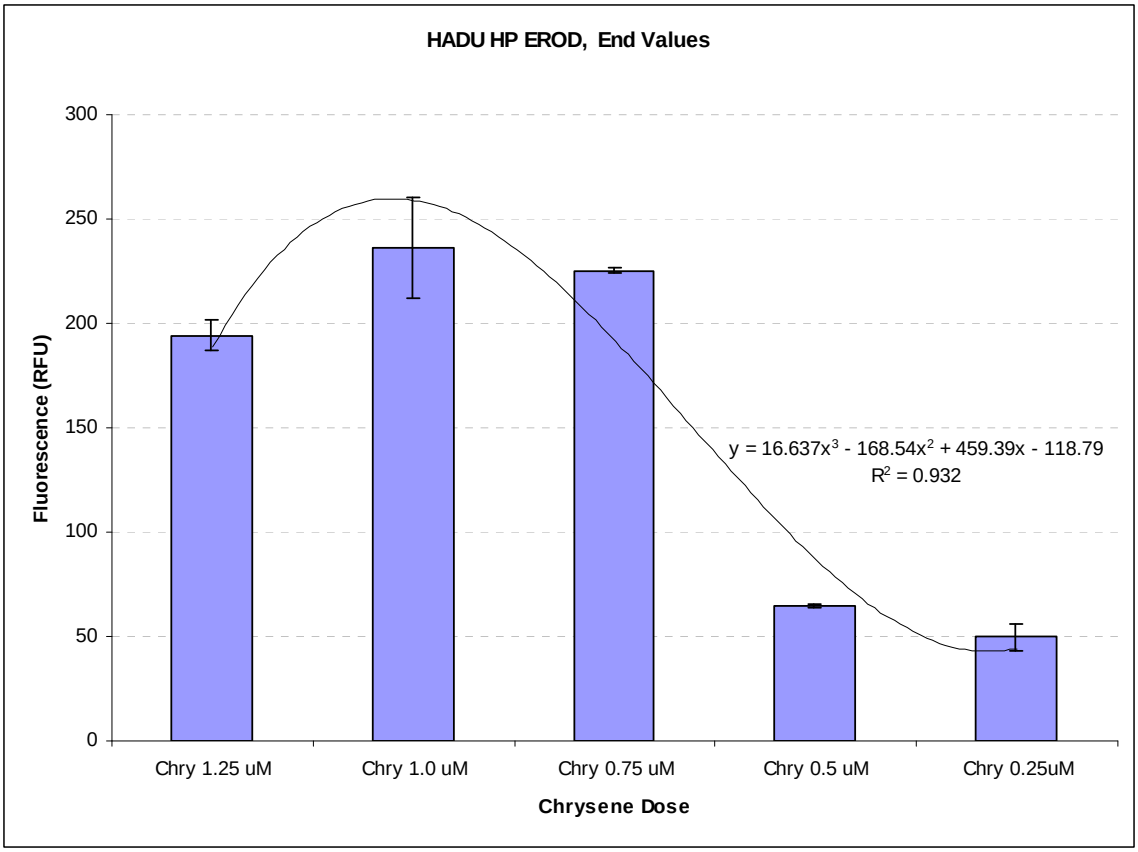
experiments using cell cultures supplemented with S9 fraction and comparison between mallard and harlequin duck cell lines are scheduled for FY10.

*EROD assay*

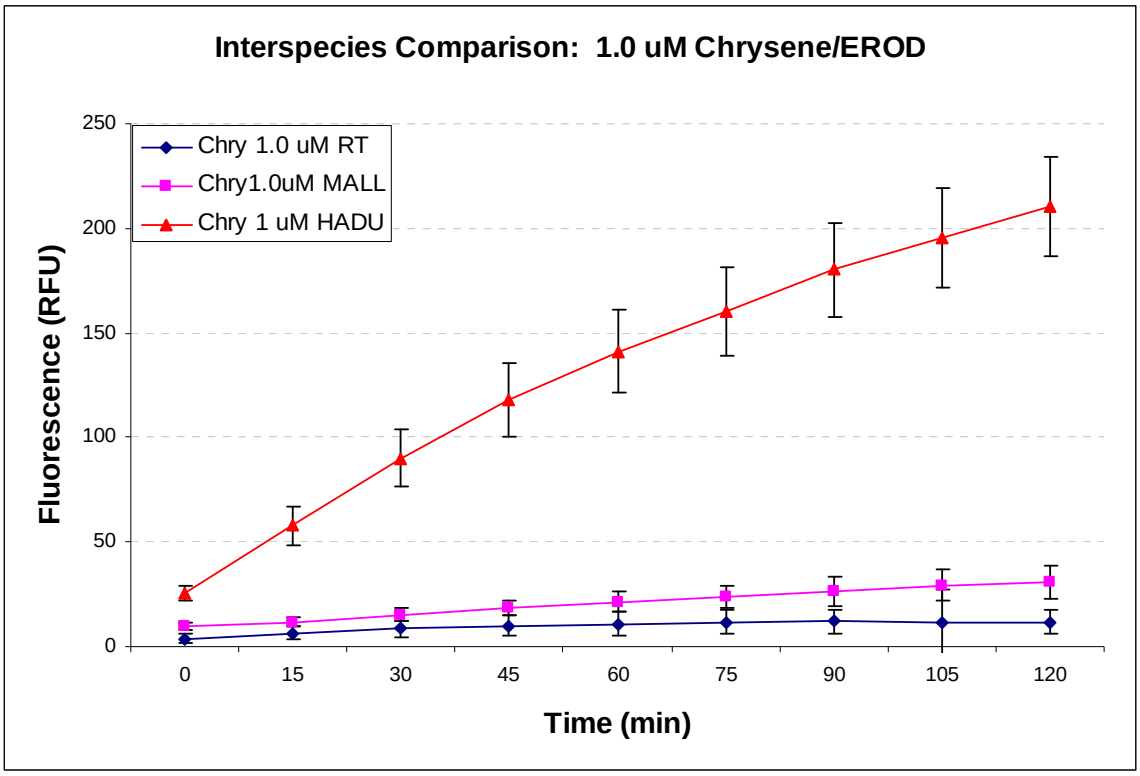
Equipment (Gemini EM Dual-Scanning Microplate Spectrofluorometer, Molecular Devices, Sunnyvale, CA) has been set up in our laboratory for validation of the EROD assay. Assay validations have been conducted with hepatocyte cultures from mallards, harlequin ducks, and rainbow trout. Range finding and species comparisons are in progress.

Surrogate (mallard) cell lines have been used in the EROD assay to determine dose ranges for chrysene and a model CYP1A inducer (BNF), as well as the doses for SPMDs made from ANS-intact. Other protocol specifics and quality controls for method development were performed with MALL cell lines. These have been compared to results obtained in EROD assays analyses with HADU hepatocytes with chrysene. These results are shown below, both kinetically and the endpoint results:

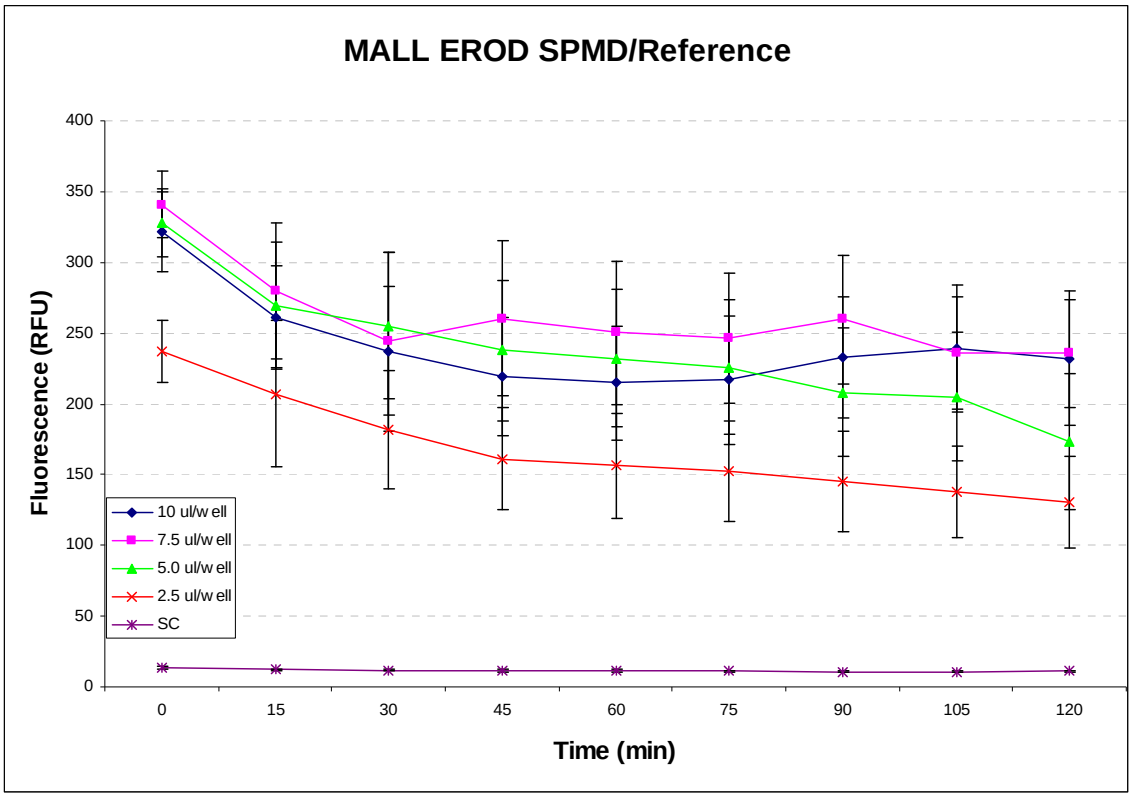



HADU HP EROD, End Values
300
250
200
150
100
50
0
Fluorescence (RFU)
y = 16.637x3 - 168.54x2 + 459.39x - 118.79
R2 = 0.932
Chry 1.25 uM
Chry 1.0 uM
Chry 0.75 uM
Chry 0.5 uM
Chry 0.25uM
Chrysene Dose

The doses selected are not cytotoxic, but the highest dose appears to be overwhelming the HADU cells' capacity to synthesize the dosing material. For this reason, we have chosen to use 1.0 µM chrysene as the testing standard. When HADU, MALL and RT (rainbow trout) hepatocytes are dosed with this dose of chrysene, the responses are markedly different, as shown below:



The EROD assay was repeated with SPMD reference material and MALL hepatocytes to estimate appropriate dosing. These SPMDs were composed of ANS-intact in lieu of triolein and processed normally. These results have provided upper and lower bounds to dosing with SPMD extracts, and will be repeated with HADU hepatocytes and SPMD site material upon availability of HADU eggs. The MALL/SPMD/EROD results are shown below:




This will be repeated with this extract as well as SPMD extract from weathered ANS in HADU cell lines with consideration to the greater sensitivity of the HADU cell lines when compared to their MALL counterparts.

As the chrysene range-finding for chrysene in HADU and MALL is complete, we can now test the responses with priority pollutant PAH as well as the full array of chrysenes.

*PAH/DNA adduct formation*

Analysis of PAH/DNA adduct formation in duck hepatocytes is underway. Hepatocyte cell cultures from mallards were dosed and prepared, and shipped to collaborator's laboratory for testing of protocols developed for adduct analysis in mammalian species. Additionally, six liver biopsy samples available from free ranging harlequin ducks through collaboration were prepared and shipped for testing of adduct analysis protocols. Preliminary results show positive staining reactions using previously developed antibodies and protocols when applied to ducks. Method validation will continue in FY10. See section on coordination/collaboration for more specifics about collaborative efforts regarding this assay.

*Comet assay*

Protocol validations have been initiated for this assay, providing a potential alternative for testing of genotoxicity. Validations will be continued in FY10 using fibroblast cell lines supplemented with S9 fraction.

*Summary*

A suite of validations have been conducted to provide a panel of endpoint assays in cell cultures to evaluate cytotoxic and genotoxic effects of SPMD extracts collected from PWS. Development of endpoint assays for this project is focusing on the following suite of assays: microscopy for cytopathic effects, LDH release assay, EROD assay, and PAH/DNA adduct formation assay. Dye exclusion cell viability assay and comet assays are also being evaluated. Assay validations will continue during FY10, as described in the FY10-FY11 study plan. Due to more limited availability of source materials for harlequin duck eggs, compared to surrogate species, some assay validations need to be conducted during the 2010 laying season of harlequin ducks (anticipated to occur in the 3rd quarter of FY10)..

## Future Work:

Project plan for FY10-11 is described in a proposal submitted July 15, 2009.

## Coordination/Collaboration:

This study includes collaborative efforts from Dr. Dan Esler at Simon Fraser University and Dr. Miriam Poirier at the National Cancer Institute (NCI).

In order to assess the potential of lingering oil to affect HADU by the formation of PAH/DNA adducts, we are working with Dr. Miriam Poirier, Head of the Carcinogen-DNA Interactions Section of the National Cancer Institute. Prior to sending HADU cells dosed with SPMD site extracts, pilot studies were conducted with chrysene and SPMD reference material. This involved dosing HADU hepatocytes in the lab with SPMD extract from ANS-intact, and sending these samples for immunohistochemical (IHC) analyses and scoring at NCI. For comparative evaluation of the HADU response from birds at both impacted and clean sites, Dr. Dan Esler has shared liver biopsies from HADU and Barrow's goldeneye (*Bucephala islandica*) taken in PWS. Dr. Poirier's section has processed some of these biopsies in conjunction with the laboratory samples using the same IHC analyses to determine the presence of adducts. Some changes in the protocol for the laboratory component of this study are planned, and the remainder of the biopsies will be stained and scored at NCI.

## Information Transfer:

The Principal Investigators attended the Alaska Marine Science Symposium in Anchorage, Alaska on January 19-22, 2009. Principal Investigator Hollmen participated in a Metadata workshop organized in association with the Marine Science Symposium on January 23, 2009.

## Budget:

During Year 2, we requested and received approval to allocate funds from remaining Year 1 funds towards a purchase of laboratory equipment for fluorescence based enzymatic measurements. This equipment has supported on-site validation and analysis of enzymatic EROD assays, and established a capacity for these assays at the Alaska SeaLife Center.