

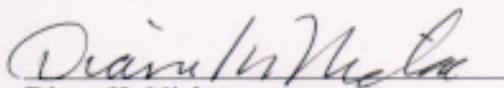


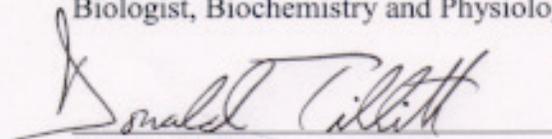
H4IIE bioassay-derived 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalents (TCDD-EQ) in osprey eggs collected along the Willamette River in Oregon.

Biochemistry & Physiology Branch Final Laboratory Report FY 2005 – 30-01

4 April 2005

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*Citation*

Nicks, D. K. and D. E. Tillitt, H4IIE bioassay-derived 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalents (TCDD-EQ) in osprey eggs collected along the Willamette River in Oregon. USGS, Columbia Environmental Research Center, Biochemistry & Physiology Branch, Final laboratory report FY 2005 – 30-01, 2005, 16 p. (1 table, 6 figures)

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## INTRODUCTION

The United States Geological Survey WR Biological Resources Division has initiated research to investigate contaminant levels in the osprey population in the Columbia River Basin in western Oregon. This work includes assessments of osprey egg health including the H4IIE bioassay which is one of the screening measures employed to assess and characterize exposure to planar halogenated hydrocarbons (PHHs), such as chlorinated dioxins, furans and biphenyls (PCBs), as well as polycyclic aromatic hydrocarbons (PAHs). The H4IIE bioassay is a semi-quantitative assay that measures the overall toxic potency of PHH and PAHs in the extracts of animal tissue. The information provided by the H4IIE bioassay complements the biological metrics collected on these ospreys. Thus, the H4IIE bioassay was conducted on osprey egg samples that were collected along the Willamette River.

The dioxin-like toxic potency of chemicals found in osprey eggs collected along the Willamette River is based on the ability of the extracts of those eggs to increase 7-ethoxyresorufin-O-deethylase (EROD) activity in the H4IIE rat hepatoma cell line. The results of the induction caused by the extracts are evaluated relative to that of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). The resultant TCDD equivalents (TCDD-EQs) are a measure of the exposure that the osprey eggs have received to these classes of compounds.

## OBJECTIVE

- 1) To determine H4IIE EROD bioassay-derived 2,3,7,8-tetrachloro-*p*-dioxin equivalents (TCDD-EQ) in extracts of osprey eggs collected at selected sites along the Willamette River in western Oregon.

## MATERIALS AND METHODS

### Sample History:

Osprey egg samples were collected by USGS personnel from selected sites along the Willamette River. These samples were transferred to David Qui under the direction of Dr. Doug Hafner at the University of Windsor, Windsor, Ontario for extraction and preparation for H4IIE bioassay analysis. Sample transmittal and chain of custody forms accompanied samples during transmission. Upon receipt at CERC, extracted samples were unpacked, logged in by Diane Nicks (CERC) and stored at 25° C until H4IIE

analysis. Twenty-seven extracts of osprey eggs collected from the Willamette River in western Oregon were received at CERC in April of 2002.

### **Analytical Sample Preparation Methods Summary:**

No information on extraction procedures was provided with the samples. The extracts were evaporated to near dryness and reconstituted in 500 µl iso-octane, and ampulated for shipment to CERC. Upon arrival at CERC the samples were marked for volume and stored at 25° C.

Quality control (QC) samples (matrix blanks, procedural blanks, and positive control materials) were prepared at CERC. Positive control material was derived from samples of CERC's standard positive control matrix, common carp (*Cyprinus carpio*) tissue, collected from Saginaw Bay, Michigan, December 1988. Matrix blank material was derived from clean bluegill (*Lepomis macrochirus*) raised in CERC's holding pond. Samples were defrosted at room temperature and 10 or 20 g aliquots removed and combined with a, four-fold excess by weight, of anhydrous sodium sulfate. After dehydrating overnight, the mixture was homogenized in a blender, packed into an extraction column, and extracted with methylene chloride (CH<sub>2</sub>Cl<sub>2</sub>). The resultant extract was concentrated via rotary evaporation. The concentrated extract was then subjected to reactive clean up per CERC SOP P.186 (Appendix 1). The QC samples were then prepared for the H4IIE bioassay by concentrating to near dryness followed by transfer to 300 µL dosing vials through a series of three rinses, reconstitution and subsequent concentration cycles. The samples were reconstituted in a final volume of 150 µL in iso-octane and stored in capped conical dosing vials until used for the bioassay.

### **H4IIE Bioassay Method:**

The H4IIE bioassay procedure was a modification of that reported by Tillitt et al. (1991). These modifications miniaturize and expedite the assay procedure, by allowing for sample processing in 96 well microtiter plates, reported in CERC SOP C5.194 (Appendix 1). The H4IIE rat hepatoma cell line (American Type Culture Collection, ATCC) was maintained using standard sterile tissue culture techniques. Cells were cultured in Dulbecco's Modified Eagle's Medium (D-MEM) at 37°C, 5% CO<sub>2</sub> (ambient chamber concentration). Cell cultures and exposures occurred in a humidified, temperature and carbon dioxide level-controlled incubator (Forma Scientific, Marietta, Ohio). Microtiter plates were seeded by pipetting 300 µL of a media/cell suspension (approximately 23,000 cells/mL) into each well. Post seeding, cells were allowed to attach and proliferate for approximately 24 hours. The cell containing plates were then dosed with a serial dilution of the recently sonicated samples or TCDD standard and returned to the incubator for 72 hours to allow for maximal EROD induction. Upon completion of this induction period, the EROD reaction was measured for each well on each plate.

A standardized 2,3,7,8-tetrachlorodibenzo-*p*-dioxin solution was used to generate an analytical dose-response curve to which all other samples would be related. Generally,

six standard dose-response curves were measured on each assay date. Dose-response curves were prepared as a set of 7 serial dilutions along with an iso-octane blank for each sample or standard. The TCDD standard, (10 pg TCDD/ $\mu$ L iso-octane), was diluted in a ratio of 1:2 (v/v) while each experimental sample was diluted 1:3 (v/v). Six basal curves, non-dosed cells, to which were assigned an artificial dose were, also, run on each assay date. These were used to calculate basal induction, limits of detection (LOD), and limits of quantitation (LOQ). The TCDD standard and basal curves were placed in varying plate positions and interspersed among sample curves. All experimental fluorometric data were collected with a Perkin-Elmer BioSystems Cytofluor 4000 instrument.

A resorufin standard curve (range, 0 to 320 pmol) was generated on each assay date using the following procedure. Eight standard solutions were prepared by making a 1:1 (v/v) serial dilution of a prepared 16  $\mu$ M resorufin/phosphate buffered saline (PBS) working stock. This working stock was prepared by making a dilution of a 200  $\mu$ M, resorufin/methanol, super stock. The concentration of the super stock was checked spectrophotometrically at 571 nm on each assay date. Six replicates of each resorufin standard were added to a plate and the average background corrected arbitrary fluorescence units (AFU's) were plotted against the nominal resorufin concentrations to produce the resorufin standard curve and linear regression equation.

The reaction was initiated in experimental plates and fluorescence resulting from resorufin formation in each well was monitored once a minute for 20 minutes. The background corrected AFU's for the experimental plates were compared to the corresponding linear fit of the eight point resorufin standard curve and AFU's were converted into pmol of resorufin formed. The resorufin content in each well was plotted against time to evaluate any deviations from linearity in the progressive formation of resorufin with time. A linear regression analysis was performed on each sample well to obtain the slope and estimate the rate of reaction (pmol/min). The reaction rate observed in each well was normalized according to the measured protein content, generating a value of specific activity in units of pmols resorufin formed/(min\*mg) of protein. Reported results are the average of at least four replicate curves. The linear portions of the slopes derived from each of these curves were normalized to the average initial slope obtained for the TCDD standard curves, resulting in a measure of an equivalent dose of TCDD (TCDD-EQ) for each sample.

The protein content in each well was determined using a fluorescamine-based protein assay (Udenfriend et al. 1972; Bohlen et al. 1973; Lorenzen and Kennedy, 1993). The reaction was allowed to progress for 10 minutes then fluorescence measurements were made. A separate BSA standard curve (range, 0 to 120  $\mu$ g) was generated for each assay day. Eight standard solutions were prepared by making a 1:1 (v/v) serial dilution of a prepared 6 mg/mL BSA stock. Six replicates of each BSA standard were added to a plate and the average background corrected arbitrary fluorescence units (AFU's) were plotted against the nominal BSA concentrations to produce the standard curve and linear regression equation. Fluorescence values were measured for each sample well, the background corrected AFU's were compared to the corresponding linear fit of the eight point BSA standard curve and AFU's were converted into mg of protein.

All EROD assay reagents were incubated for ten minutes at 37° C prior to data collection. The correct sample identification and its associated microtiter plate well were recorded on data log sheets and stored with the laboratory notebook. All electronic files were stored on CD's with names and other pertinent information recorded in the laboratory notebook.

Excess of the tissue extracts was stored at room temperature in sealed conical vials with the volume marked.

### **Quality Assurance and Quality Control**

The objective of the quality assurance plan of this study was to ensure that the biochemical analyses were accurate and representative measures of the TCDD-EQs found in each composite sample generated from those collected in the field portion of this study. The general scheme included replication of assayed samples, comparison of calibration against known standards, proper maintenance and calibration of equipment, accurate sample tracking and chain of custody, proper documentation at all steps of sample processing and other considerations of Good Laboratory Practice (GLP). The specific aspects of the QA plan related to the H4IIE EROD assay are given below.

All experimental information was recorded in bound notebooks and copies maintained in a separate, secured area. Instrument printouts and computer-generated data tables were uniquely labeled and cross-referenced to the project notebook. The accuracy of all such data reductions was independently verified. Hard copies of computerized data files were maintained in a project notebook. Computer files were backed-up and archived on CD's. All equipment used in this study was routinely inspected and preventive maintenance performed. A logbook was kept for each instrument to document its use, performance and maintenance.

Replication and subsequent performance checks were performed at many stages of the H4IIE EROD assay procedure. A composite TCDD dose-response curve was generated from the average of 6 independent determinations for each composite sample. Ten percent (10%) of tissue extract samples were assayed in triplicate, as were positive control and some matrix blank samples. Eight-point resorufin and BSA (bovine serum albumin) standard curves were prepared at 6 replicates for each concentration, and analyzed concurrently with the TCDD standards and samples. Positive control fish tissue extracts were analyzed on each assay date along with the samples. The source and lot number of the BSA and resorufin were recorded in the laboratory notebook. Scatter plots for the resorufin (Figure 1) and BSA (Figure 2) standard curves have been included with this report. The BSA standard curve data is presented in two separate graphs to account for an equipment upgrade that took place while samples for this project were being assayed. A 400 nm excitation filter was added to the Cytofluor 4000 instrument. BSA standard curves assayed with the previous 360 nm filter (CERC SOP C5.194, Appendix 1) are displayed in one graph and those assayed with the 400 nm filter are displayed in the second graph. These scatter plots were prepared to demonstrate the consistency of

the fluorescence response with concentration over the time course of sample evaluation. They were also used to facilitate data analysis. The slope and y-intercept values were recorded electronically.

The concentrations of the resorufin, ethoxyresorufin and NADPH reagents were checked on each assay date using a spectrophotometer and their actual concentrations determined based upon Beer's Law using known extinction coefficients for the different reagents. It was deemed acceptable if the actual concentration was within 10% of the nominal concentration.

Positive control and matrix blank tissue extracts and procedure blank extracts were included along with the samples for H4IIE analysis on each assay date to assure that both the EROD enzyme assay and the reagents were behaving according to specifications. These QA/ QC samples were not provided with the extracted osprey egg samples so the egg samples were assayed in conjunction with the Texas EMAP samples which were extracted and prepared at CERC. The positive control was prepared from CERC's reference material, common carp from Saginaw Bay, Michigan. Five 10g aliquots were separately extracted and carried through reactive absorbent clean-up columns in accordance with CERC's SOP P.186 (Appendix 1). Positive control extracts were designated with their own unique tracking number. Matrix blank extracts were prepared from pond-raised bluegill in the same process as that used for the positive control and were assigned a unique tracking number that was matched with a positive control sample. Procedure blank samples were, also, prepared in the same manner.

**Data Analysis:**

The standard curve data for both the resorufin and BSA standard curves were separately compiled and each plotted as a set, in order to verify the consistency and consequently the reliability of the sample data as a whole. In each case, a linear regression, 95% confidence interval, and prediction interval were plotted (Figures 1 and 2, respectively). The means and error limits (SD or standard deviation, and CV or coefficient of variation) for both the resorufin and BSA standard data are given below.

	slope		y intercept	
	mean	(SD, CV)	mean	(SD, CV)
Resorufin	88.8 AFUs/pmol	(8, 9 %)	-72.3 AFUs	(226, 312 %)
BSA	380 AFUs/mg	(43, 11 %)	1532 AFUs	(411, 27 %)

The time courses for the production of resorufin (i.e. the EROD reaction rates) were evaluated graphically to ensure linearity of the response. Linearity of the reactions verifies that non-saturating substrate levels or enigmatic kinetics did not limit the reactions. In cases where the kinetic reaction did not yield a linear response over the 20-minute sampling time, the linear portion of the curve was used to calculate the rate of reaction.

The linear portion of a plot of EROD specific activity versus gram tissue equivalents per mg cellular protein was used to determine the EROD induction response for the H4IIE cells for a given sample. This measure of EROD induction was translated into an equivalent dose of 2,3,7,8-tetrachloro-*p*-dioxin, TCDD, by dividing the average induction response arising from treatment of the cells with sample by that response arising from treatment of cells with the standard, TCDD (Eq. 1). The resultant metric for potency estimates was TCDD-equivalents (TCDD-EQs) in the extract or tissue sample.

$$\text{TCDD-EQ (pg/g)} = [(\text{EROD/g-equivalent in extract})/(\text{EROD/pg TCDD})] \quad (1)$$

Positive control, matrix blank, and procedure blank samples were included during each data collection and work-up phase of this study. Three dose-response curves were run and evaluated for the positive control sample on each assay date. A composite dose-response curve was developed for the positive control samples used on each assay date. These were generated from combining raw data of individual dose-response curves. The slope and standard error were calculated from these composite curves. The positive control samples assayed in this study were plotted as TCDD-EQ versus assay date. These are illustrated in combination with line plots indicating the lab mean positive control value, high control limit (mean + 2 X SD), and low control limit (mean – 2 X SD) (Figure 3). Data for matrix blank and procedure blank samples were prepared in the same manner and are, also, illustrated (Figures 4 and 5, respectively).

The degree of EROD induction in reagent blanks and basal cells were determined in addition to the measurement of EROD induction for the TCDD standard and positive controls. The limits of detection (LOD) and quantitation (LOQ) for each assay date were calculated, as described by Keith et al. (1983). These parameters were calculated from the observed level of basal EROD activity measured in the H4IIE cells on a given day. The LOD was defined to be equal to the average basal activity plus 3 times the standard deviation of the mean (standard error) associated with that activity. The LOQ was defined to be equal to the average basal activity plus 10 times the standard deviation of the mean (standard error) associated with that activity. These measures were used to evaluate the sample data results and to determine whether they were detectable or measurable above that of the background. Based upon the basal level of EROD activity found in uninduced H4IIE cells, the criteria of LOD and LOQ could be used to judge the significance of the measured results obtained for the samples. Control charts that evaluate the run-to-run variation of LOD and LOQ are presented (Figure 6). In each case, the value for each assay date is plotted in combination with line plots indicating the lab mean value, high confidence limit (mean + 2 X SD), and low confidence limit (mean – 2 X SD).

Replicates of sample data were treated as separate samples until TCDD-EQ's were calculated. These were then averaged for reporting in tables and are indicated. A sample was re-run if the coefficient of variation was over 25%, starting with the concentrated tissue extract in iso-octane. Exceptions were made in the cases of the blanks and basal measurements or when the measured values fell below or very close to the limit of quantitation.

## RESULTS

### *Quality Assurance*

The data generated for all resorufin standard curves were compiled and plotted as background-corrected fluorescence values versus resorufin content per well (Figure 1). As seen in the previous table the y-intercept coefficient of variation (CV), was 312 % while that for the slope was 9 %. While the y-intercept accounts for the day-to-day background fluctuations in the instrument, the slope reveals the correlation between fluorescence and resorufin content. Since the associated error in the slope was low, a high degree of confidence may be placed in the reliability of the mathematical conversion between measured fluorescence and calculated resorufin content throughout the course of the study.

The data generated for all BSA standard protein curves were compiled and plotted in the same manner as the resorufin with background-corrected fluorescence values versus protein content per well (Figure 2). The y-intercept coefficient of variation (CV) was 27 % while that for the slope was 11 %.

Generally, the results for all samples fell within the 25% CV limit as set forth by the QA objectives. Those samples that exceeded the 25% CV maximum fell into the reagent blank or basal classes of sample measurements or were samples that had calculated TCDD-EQs on the order of LOQ or less.

In order to evaluate the reproducibility of the experimental method throughout the time course of the analysis, the TCDD-EQs determined for the positive control, matrix blank, and procedure blank samples were plotted as stated above (Figures 3, 4, and 5). All positive control, matrix blank, and procedure blank samples fell within set confidence intervals of the lab average.

Examination of the LOD and LOQ (Figure 6) control chart illustrates that all values fell within limits.

### *TCDD-EQs in Osprey Eggs*

Many of the sample extracts contained rust colored particles were cloudy or yellow in color or had a combination of these anomalies. Sonication in all but two samples resulted in a homogeneous mixture which was used for dosing. The two samples (OS-Egg-2C & OS-Egg-52) for which sonication did not result in a homogeneous mixture were dosed directly after sonication even though an obvious layer of immiscible substance remained in the vial.

The osprey egg samples analyzed had relatively low dioxin-like potencies. TCDD-EQ's for most of the egg samples were low (Table 1). Fifty-six percent (15/27) egg samples assayed had dioxin-like potencies that were below LOD or LOQ. Thirty-three percent (9/27) of the egg samples had dioxin-like potencies less than ten (TCDD-EQ range: 0.7 – 5.4). Only eleven percent (3/27) of the samples resulted in dioxin-like potencies greater

than ten (TCDD-EQ range: 16.0 – 20.1). These higher samples were os-egg-2A, os-egg-5, and os-egg-165A (Table 1).

## LITERATURE

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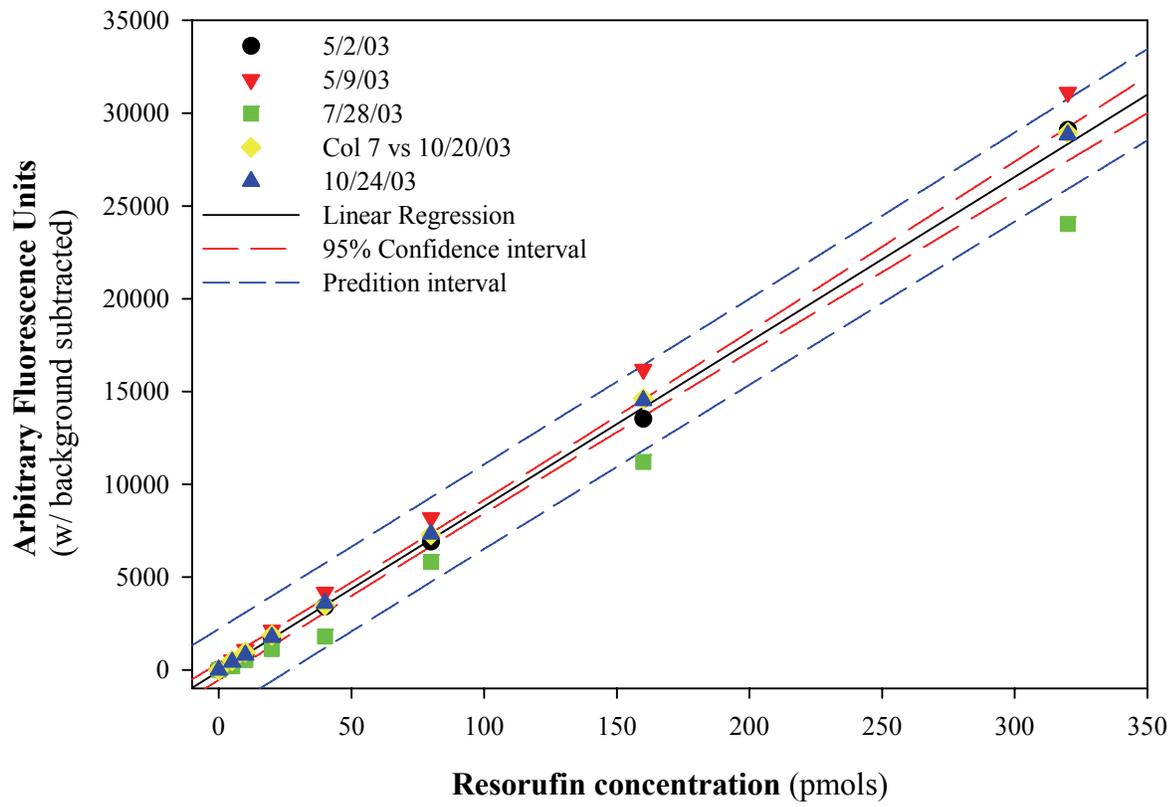
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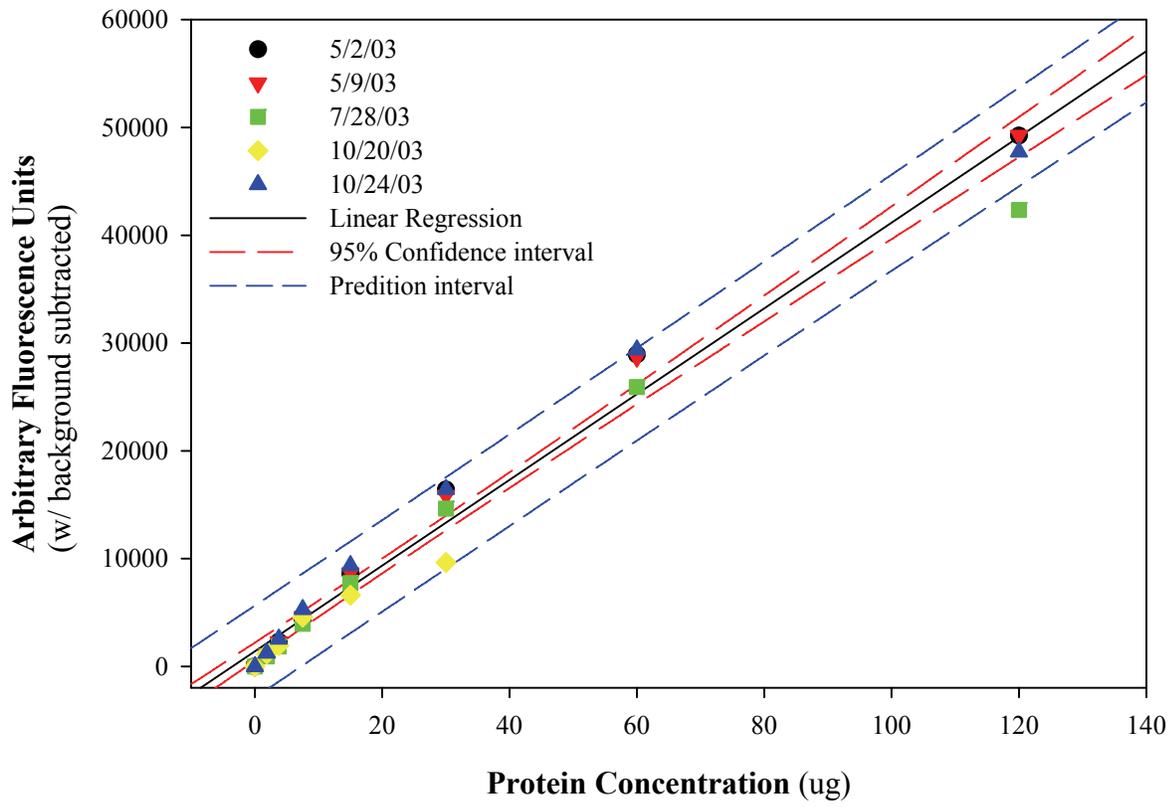
Udenfriend, S., S. Stein, P. Bohlen, W. Dairman, W. Leimgruber and M. Weigele (1972) Fluorescamine: A reagent for the assay of amino acids, peptides, and primary amines in the picomole range. *Science* **178**, 871-872.

**Table 1. TCDD equivalents (pg/g) of osprey egg samples.** Assay triplicate are those samples that were dosed in the assay in triplicate.

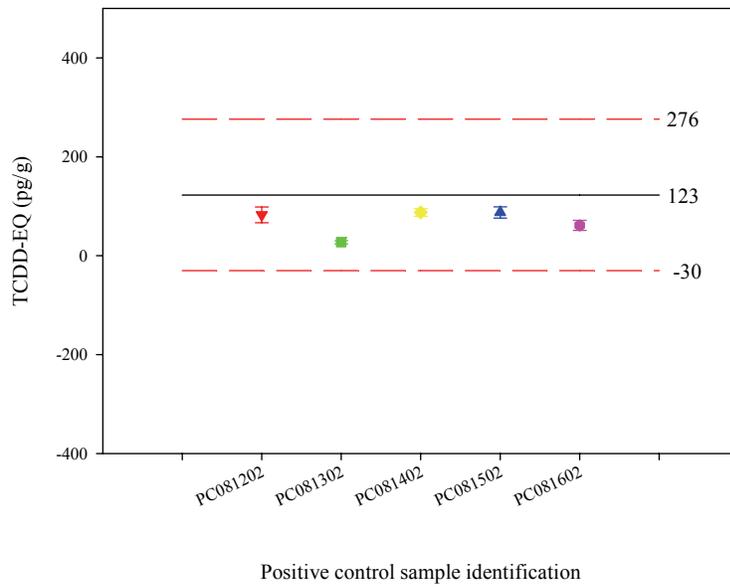
Field ID	Lab Label	Species	TCDD-EQ $\pm$ SD (pg/g)	Assay Notes
Os-Egg-153A	GLIER02-431	Osprey	5.4 $\pm$ 0.5	
Os-Egg-158	GLIER02-432	Osprey	0.7 $\pm$ 0.3	Assay triplicate
Os-Egg-98A	GLIER02-433	Osprey	<LOQ	
Os-Egg-11A	GLIER02-434	Osprey	2.4 $\pm$ 0.6	
Os-Egg-13	GLIER02-435	Osprey	<LOD	
Os-Egg-12	GLIER02-436	Osprey	<LOQ	
Os-Egg-179	GLIER02-437	Osprey	1.1 $\pm$ 0.4	
Os-Egg-110	GLIER02-438	Osprey	<LOQ	
Os-Egg-11	GLIER02-439	Osprey	<LOQ	Assay triplicate
Os-Egg-30B	GLIER02-440	Osprey	<LOQ	
Os-Egg-21	GLIER02-441	Osprey	<LOD	
Os-Egg-2C	GLIER02-442	Osprey	<LOQ	
Os-Egg-80A	GLIER02-443	Osprey	<LOD	
Os-Egg-2A	GLIER02-444	Osprey	16.1 $\pm$ 1.2	
Os-Egg-5	GLIER02-445	Osprey	20.1 $\pm$ 1.3	
Os-Egg-165A	GLIER02-446	Osprey	16.0 $\pm$ 1.3	
Os-Egg-9A	GLIER02-447	Osprey	1.8 $\pm$ 0.4	
Os-Egg-4	GLIER02-448	Osprey	4.8 $\pm$ 0.6	Assay triplicate
Os-Egg-132	GLIER02-449	Osprey	<LOD	
Os-Egg-121A	GLIER02-450	Osprey	<LOD	
Os-Egg-52	GLIER02-451	Osprey	4.9 $\pm$ 0.7	
Os-Egg-194	GLIER02-452	Osprey	2.7 $\pm$ 0.2	
Os-Egg-141C	GLIER02-453	Osprey	5.3 $\pm$ 0.9	
Os-Egg-38B	GLIER02-454	Osprey	<LOD	
Os-Egg-132(2000)	GLIER02-455	Osprey	<LOD	
Os-Egg-27(2000)	GLIER02-456a	Osprey	<LOD	
Os-Egg-194(2000)	GLIER02-457	Osprey	<LOD	



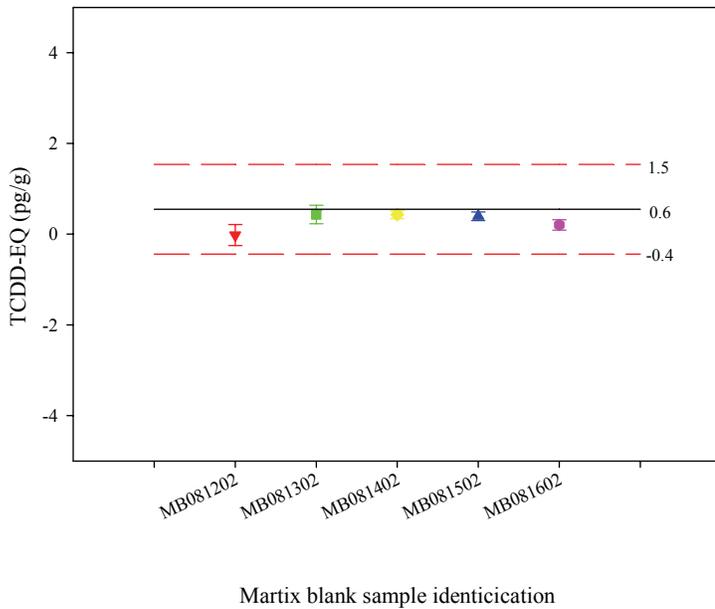
**Figure 1. Resorufin standard chart.** All resorufin standards run with osprey egg samples.



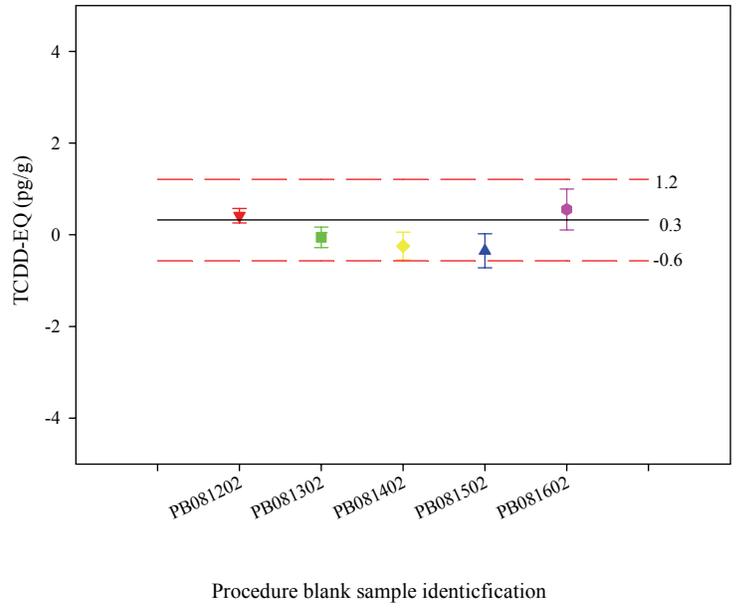
**Figure 2. BSA (protein) standard chart.** All bovine serum albumin (BSA) standard curves run with osprey egg samples.



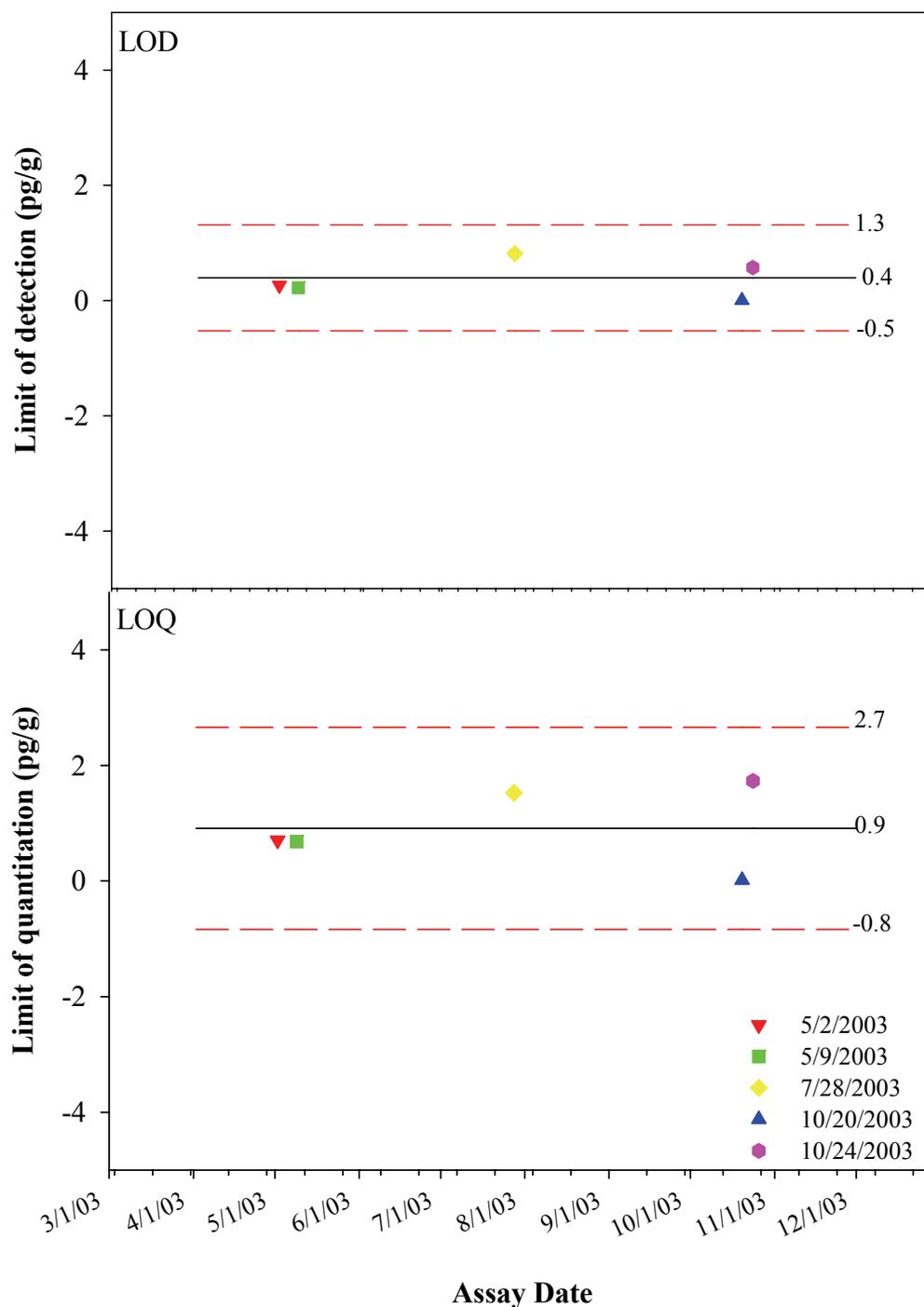
**Figure 3. Positive control, control chart.** Mean positive control (PC) 2,3,7,8-TCDD equivalents (TCDD-EQ)  $\pm$  SD of Saginaw Bay carp PC samples assayed with osprey egg samples. Sample mean values are plotted with a solid line indicating the lab mean PC TCDD-EQ value and dashed lines indicating the upper and lower confidence limits. Confidence limits were calculated using the lab mean TCDD-EQ  $\pm$  two times the standard deviation.



**Figure 4. Matrix blank control chart.** Mean matrix blank (MB) 2,3,7,8-TCDD equivalents (TCDD-EQ)  $\pm$  SD of CERC bluegill matrix blank samples assayed with osprey egg samples. Sample mean values are plotted with a solid line indicating the lab mean MB TCDD-EQ value and dashed lines indicating the upper and lower confidence limits. Confidence limits were calculated using the lab mean TCDD-EQ  $\pm$  two times the standard deviation.



**Figure 5. Procedure blank control chart.** Mean procedure blank (PB) 2,3,7,8-TCDD equivalents (TCDD-EQ)  $\pm$  SD of PB samples assayed with osprey egg samples. Sample mean values are plotted with a solid line indicating the lab mean PB TCDD-EQ value and dashed lines indicating the upper and lower confidence limits. Confidence limits were calculated using the lab mean TCDD-EQ  $\pm$  two times the standard deviation.



**Figure 6. Limit of detection (LOD) and limit of quantitation (LOQ) graph.** Daily assay LODs or LOQs plotted with the lab mean LOD or LOQ (solid black line), upper confidence limit and lower confidence limit (dashed red lines). Confidence limits are the mean plus or minus two times the standard deviation.