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thesis entitled

2,3,7,8 - TETRACHLORODIBENZO-P-DIOXIN EQUIVALENTS IN TISSUE SAMPLES FROM THREE SPECIES IN THE DENVER METROPOLITAN AREA

presented by

Katherine Kemler

has been accepted towards fulfillment
of the requirements for
Master's degree in Zoology

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Date **April 17, 2000**

2,3,7,8 – TETRACHLORODIBENZO-*P*-DIOXIN EQUIVALENTS IN TISSUE SAMPLES FROM THREE SPECIES IN THE DENVER METROPOLITAN AREA

By

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A THESIS

Submitted to
Michigan State University
In partial fulfillment of the requirements
for the degree of

MASTERS OF SCIENCE

Department of Zoology

2000

ABSTRACT

2,3,7,8 – TETRACHLORODIBENZO-*P*-DIOXIN EQUIVALENTS IN TISSUE SAMPLES FROM THREE SPECIES IN THE DENVER METROPOLITAN AREA

By

Katherine K. Kemler

Polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and polychlorinated biphenyls (PCBs) are possible contaminants of concern in areas with a history of organochlorine pesticide production and various other industrial chlorination processes. The Rocky Mountain Arsenal (RMA) is a Superfund site near Denver, CO. This site has a history of various industrial processes that may have led to the release of these compounds and subsequent exposure risk to surrounding biota. Non- and mono-ortho-substituted PCBs, PCDDs, and PCDFs cause a common set of toxic effects that are mediated through the aryl-hydrocarbon receptor (AhR). The total AhR-mediated activity of complex mixtures was determined by both instrumental and bioanalytical techniques in a range of biota samples from the RMA. Concentrations of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) equivalents (TCDD-EQ and TEQ) were measured in carp eggs, great horned owl livers, and American kestrel eggs collected from the RMA and from off-post reference sites. The tissue samples were collected from 1995 through 1998. Two carp egg samples were collected from reference areas and 16 samples were collected from the RMA. Eleven great horned owl livers were collected from reference areas and 16 samples were collected from the RMA. Sixteen American kestrel eggs were collected from reference areas and 30 samples were collected from the RMA. Detected concentrations of dioxin equivalents were used to assess risk to exposed wildlife through the use of Hazard Quotients (HQs). Mean concentrations of TCDD-EQ and TEQ in extracts of carp and kestrel eggs were not significantly different between groups of samples collected on and off the RMA. Concentrations of TCDD-EQ in several owl livers collected on the RMA were significantly greater than concentrations in owl livers from off of the RMA. Significant differences in TCDD-EQ concentrations were also observed between different age classes of great horned owls.

ACKNOWLEDGEMENTS

Thanks to Dr. Paul Jones, Dr. John Giesy, and Dr. Alan Blankenship for their help and encouragement while working on this project.

Thanks also to my committee members, Dr. Steve Bursian, Dr. Karen Chou, and Dr. Patrick Muzzall, for their guidance.

I would also like to thank the Biological Advisory Subcommittee (BAS) at RMA for funding the project.

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LIST OF SYMBOLS AND ABBREVIATIONS

AhR	Aryl hydrocarbon Receptor
ANOVA	Analysis of Variance
BAS	Biological Advisory Subcommittee, Rocky Mountain Arsenal
CERCLA	Comprehensive Environmental Response, Compensation and Liability Act
DRE	Dioxin Responsive Enhancer
EPA	Environmental Protection Agency
GC/MS	Gas Chromatography / Mass Spectrometry
H4IIE-luc	Rat hepatoma cell line stably transfected with the luciferase gene under control of AhR
HQ	Hazard Quotient
LOAEL	Lowest Observable Adverse Effect Level
MDL	Method Detection Limit
MRI	Midwest Research Institute
MSU	Michigan State University
NOAEC	No Observable Adverse Effect Concentration
NOAEL	No Observable Adverse Effect Level
PCB	Polychlorinated biphenyl
PCDD	Polychlorinated dibenzo-*p*-dioxin
PCDF	Polychlorinated dibenzofurans
QA/QC	Quality Assurance/ Quality Control
REP	Relative Effects Potency
RMA	Rocky Mountain Arsenal

RLU	Relative Luminescence Unit
TCDD	Tetrachlorodibenzo-*p*-dioxin (usually reserved for 2,3,7,8-TCDD)
TCDD-EQ	Tetrachlorodibenzo-*p*-dioxin equivalent (bioassay)
TEF	Toxic Equivalency Factor
TEQ	2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalent (instrumental analysis)
TEQ_{WHO}	2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalent based on WHO TEF values
TEQ_{REP}	2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalent based on relative potency factors
TRV	Toxicity Reference Value
TU	Toxic Unit, dimensionless unit used in Hazard Quotient calculations
UF	Uncertainty Factor
WHO	World Health Organization

Introduction

This study was conducted to determine the concentrations of polychlorinated diaromatic hydrocarbons (PCDHs), such as polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and non- and mono-ortho- substituted polychlorinated biphenyls (PCBs) in tissues from three animal species from the Rocky Mountain Arsenal (RMA) and adjacent areas. The overall objective of the investigation was to determine whether concentrations of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) equivalents in samples present on the RMA were significantly greater than those from reference sites in adjacent off-site locations. Concentrations of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalents were determined by instrumental and bioanalytical techniques (*in vitro* bioassays) to measure TEQs and TCDD-EQs, respectively. Concentrations of TEQs were compared to TCDD-EQs to determine if all of the AhR-mediated activity determined in the H4IIE-luc assay could be accounted for by the identified PCDH congeners. Possible risks that concentrations of TCDD-EQs or TEQs pose to wildlife on the RMA were also assessed. The U.S. Environmental Protection Agency's "Framework for ecological risk assessment" was used as an outline for reporting the formulation and results of this study (U.S. EPA 1992).

Problem Formulation:

In 1996, the Colorado Department of Public Health released results from a survey of trace organic compounds on the grounds of the Rocky Mountain Arsenal. Anomalous concentrations of PCBs, PCDDs, and PCDFs were detected in the Basin F wastepile, a

waste area adjacent to the North Plant facility in the core region of the RMA, and in some biota samples. This suggested that dioxin-like compounds could be potential contaminants of concern (COCs) on the RMA. Consequently, the Biological Advisory Subcommittee (BAS) began an investigation of the prevalence and availability of dioxin-like compounds to wildlife on the RMA (U.S. Army 1999).

PCDHs, including PCBs, PCDDs, and PCDFs, are widely distributed in the environment. A subset of the PCB congeners, including non-ortho and some mono-ortho-substituted congeners, are structurally similar to PCDDs and PCDFs and are referred to as co-planar PCBs. PCDDs, PCDFs, and co-planar PCBs are persistent, subject to bioaccumulation, and some classes are toxic to biota at small concentrations (Giesy *et al.* 1994a, Giesy *et al.* 1994b, Peterson *et al.* 1993). A subset of these chemicals is characterized by a common mechanism of action via binding to the cytoplasmic aryl-hydrocarbon receptor (AhR) (Giesy and Kannan 1998, Sanderson *et al.* 1996). PCDDs, PCDFs, and some PCB congeners that can attain a planar configuration bind to the AhR. Upon binding of the ligand, the AhR-ligand complex translocates to the nucleus where it dimerizes and binds to dioxin responsive enhancers (DREs) on the DNA (Sanderson *et al.* 1996). Hence, specific changes in gene expression can then result from receptor binding (Giesy and Kannan 1998). Toxic effects of PCDDs, PCDFs, and co-planar PCBs include impaired reproduction of several avian and fish species (Jones *et al.* 1993, Peterson *et al.* 1993, Williams *et al.* 1995, Xu *et al.* 1997), severe weight loss, fatty deposition in the liver, edema, fetotoxicity, and teratogenicity in laboratory animals and wildlife (Couture *et al.* 1990, Giesy *et al.* 1994a, Giesy *et al.* 1994b, Giesy and Kannan 1998).

The RMA is a 27-square-mile Superfund site located in Commerce City, CO. The vegetation of the site is primarily composed of grassland flora with some riparian woodlands. The wildlife composition of RMA is diverse, including 26 species of mammals, 17 species of reptiles and amphibians, aquatic communities, and 127 species of birds have been observed on-post (U.S. Army 1999). The avian community on the RMA consists of raptors (eagles, hawks, great horned owls, burrowing owls), ground-nesting songbirds, as well as bird species preferring open, grassy habitats. Due to the historical presence of multiple agencies and multiple chemical production facilities on this site, the RMA was placed on the National Priorities List in accordance with the Comprehensive Environmental Response, Compensation and Liability Act (CERCLA) in 1987. The RMA is an army facility composed of two main chemical-producing plants, the South Plants and the North Plants, and numerous support buildings and other infrastructures **(Figure 1)**. These plants were used to produce a diversity of chemicals throughout the Arsenal's history (U.S. Army 1999). The plants are located in the central area of the RMA, which is designated as the core area. The core area is 6 square miles and is surrounded by 21 square miles of periphery area. It is expected that the periphery is relatively uncontaminated, since no major chemical producing activities took place in the peripheral area. From 1942 to 1945, the RMA was used to produce chemical warfare agents, such as Levinstein mustard and Lewisite by the U.S. Army. During this time, incendiary bombs, organophosphate nerve agents, such as Sarin, and Hydrazine rocket fuel were also manufactured at the RMA. From 1946 to 1982, some of the RMA facilities were leased to various companies that used its plants to produce pesticides, including dieldrin, aldrin, chlordane, carbamate insecticides, soil fumigants, and various other chlorinated hydrocarbon pesticides (U.S. Army 1999).

The source of dioxin-like compounds on the RMA is unknown. However, PCDDs, PCDFs, and PCBs have been detected in wastes from the North Plants. This waste consisted of chemical byproducts like hydrogen chloride from nerve agent production processes and was deposited into Basins A and F (U.S Army 1999). Dioxin-like chemicals can be formed as byproducts from the production of pesticides, nerve agents, and other processes involving chlorination (Kannan *et al.* 1998). PCDDs and PCDFs have never been manufactured for any beneficial purposes, but rather were released as byproducts from many human activities (Giesy and Kannan 1998). PCDDs and PCDFs were investigated on the RMA to determine if they were released as byproducts in the production of other chlorinated compounds. PCDDs and PCDFs are known to be released from chloralkali plants, the manufacture of some types of pesticides, incineration of chlorine-containing wastes, and fossil fuel combustion (Kannan *et al.* 1998). Routine application of various pesticides such as pentachlorophenol (PCP) on the RMA may also have been a source for surface soil contamination with these compounds (U.S. Army 1999).

Endpoints

Assessment Endpoints:

Assessment endpoints are the ultimate focus when characterizing risk in a given environment. These endpoints are selected to link measured endpoints to an evaluation of risk (U.S. EPA 1992). To assess the risk to wildlife on RMA, concentrations of TCDD-equivalents in the tissues of sentinel species were selected as the assessment endpoints and were used as an integrative measure of the bioavailability and toxicity of dioxin-like compounds in the RMA environment. A simplified risk assessment based on hazard quotients (HQs) was utilized. HQs are ratios based on a reference dose of toxicant determined to have some adverse effect on the organism of interest. Toxicity Reference Values (TRVs) were used as the accepted reference doses in this study, and Hazard Quotients were calculated based on the amount of toxicant detected in the sample extract relative to the TRV derived for that organism. The HQ is measured in toxic units (TU), and a HQ value greater than 1.0 is indicative of potential adverse effects on the organisms of interest. In this study, HQs were calculated based on concentrations of both TCDD-EQs, which were calculated from chemical concentrations determined by an *in vitro* bioassay and TEQs, which were measured by instrumental analyses.

Measurement Endpoints:

Measurement endpoints are directly measured components of an ecosystem that can be related to assessment endpoints (U.S. EPA 1992). The specific measurement endpoints used in this study were concentrations of individual PCDDs, PCDFs, and PCB

congeners (Van de Berg *et al.* 1998), patterns of the congeners, and measures of total dioxin-like activity in tissue samples. Eggs from the American kestrel (*Falco sparverius*) and common carp (*Cyprinus carpio*) and livers from adult and juvenille great horned owls (*Bubo virginianus*) were selected to compare the dioxin-like activity between the on- and off post populations. The working hypothesis for the use of these tissue samples was that if PCDDs, PCDFs, and PCBs were released into the environment, they could persist and possibly biomagnify to hazardous concentrations in some portions of the local foodchain. PCDDs and PCDFs are lipophilic compounds; they tend to accumulate in fats or fatty stores within organisms (Peterson *et al.* 1993). Dioxin-like toxicants can also be transferred maternally to offspring via the egg yolk (Williams *et al.* 1995, Jones *et al.* 1993). The liver often accumulates dioxin-like chemicals. For these reasons, kestrel and carp eggs, as well as owl livers, were used as indicators of the availability of PCDDs, PCDFs, and PCBs present in the RMA environment. Kestrels and owls were selected as sentinel species because they are near the top of the foodchain, and thus are useful for making integrative measures of the terrestrial environment. These birds prey upon insects, small mammals and, at times, other birds. Carp eggs were used as bioindicators of dioxin-like activity in the limited aquatic systems of the RMA. The chemical composition of all tissue samples were considered to be representative of the surrounding environment. Great horned owl adults are the only organisms collected that may not be indicative of the local environment in which they were sampled, since the adult bird has relatively high dispersion ability.

In order to determine possible risks to RMA wildlife, two methods of estimating TCDD-equivalents were applied. In the first method, concentrations of TCDD-equivalents

were calculated as the sum product of Toxic Equivalency Factors (TEFs) derived by the World Health Organization (WHO), which are multiplied by the concentration of each congener detected in a mixture (**Equation 1**) (Van den Berg *et al.* 1998). This measure of TCDD-equivalency is designated as $TEQs_{WHO}$. In addition, TCDD-equivalents were determined by use of the H4IIE-luc *in vitro* cell bioassay, and these equivalent concentrations are designated as TCDD-EQs.

$$\mathbf{TEQ_{WHO} = \Sigma C_i * TEF_i} \qquad \mathbf{(1)}$$

where C_i indicates the concentration of the congener and TEF_i is the TEF for that congener

To assess the potential risk of TCDD-equivalents to wildlife, consensus TEFs, derived from a variety of species and endpoints, were applied (Van den Berg *et al.* 1998). TEFs are relative values assigned to dioxin-like compounds based on the most potent AhR binding dioxin known, which is 2,3,7,8-TCDD. As it is the most potent PCDH, TCDD is assigned the TEF value of 1.0, and TEFs for other dioxins, furans, and PCBs are calculated relative to TCDD. Determination of TEF values vary depending on the organism and endpoint used. TEF values relevant to birds and fish were applied for samples of avian tissues and fish tissues respectively. This was done because it is known that fish, birds, and mammals respond differently to dioxin-like compounds (Van den Berg *et al.* 1998).

Concentrations of TEQ_{WHO} are not directly comparable to bioassay derived TCDD-EQ concentrations. Derivation of a mass balance relationship between concentrations of TEQs and TCDD-EQs was accomplished by applying a relative potency factor (REP) to

detected congener concentrations **(Equation 2)**. The REPs used are derived from the same endpoint in the same bioassay system (H4IIE-luc) (Sanderson *et al.* 1996, Giesy *et al.* 1997). These REPs allowed calculation of concentrations of TEQ_{REP} that could be compared directly to concentrations of TCDD-EQ measured in the same bioassay.

$$\mathbf{TEQ_{REP} = \Sigma C_i * REP_i,} \qquad \mathbf{(2)}$$

where C_i indicates the concentration of the congener and REP_i is the REP for that congener

Total AhR-mediated activity in complex mixtures (TCDD-EQ) was determined by use of an integrating bioassay, the H4IIE-luc (CALUX) assay (Sanderson *et al.* 1996). TCDD-EQs calculated from the H4IIE-luc bioassay were used in conjunction with TEQs calculated from concentrations of individual congeners (Midwest Research Institute; MRI) on the same set of tissues. This *in vitro* bioassay is an integrative measure of all the dioxin-like activity of PCB, PCDD, and PCDF mixtures in a given sample. Concentrations of TCDD-EQ are a single aggregate measure of toxicity to examine the AhR-mediated activities of complex mixtures (Giesy and Kannan 1998, Sanderson *et al.* 1996, Williams *et al.* 1995). In the H4IIE-luc cell line, the luciferase gene has been stably transfected into rat hepatoma cells so that AhR binding causes promoter activation and upregulation of the luciferase gene. The luciferase gene increases production of the enzyme product, luciferase. Luciferase cleaves luciferin (a substance dosed onto the cells on the day of analysis) and luminescence is produced in a dose-dependent manner. Therefore, mixtures of dioxin-like chemicals are measured by the upregulation of the luciferase gene and consequent increase of luminescence intensity. *In vitro* bioassays are advantageous due to their rapidity and decreased cost as compared to instrumental analyses. Bioassays also integrate various

interactions among the toxicants of interest and are therefore more biologically relevant (Giesy and Kannan 1998).

Analysis Plan

Sampling Design and Methods:

Kestrel eggs were collected from nestboxes located on the RMA and at selected reference areas off the RMA. At the RMA, kestrel nest boxes were located at the corners of the sqaure mile demarcations (28 nestboxes in all) **(Figure 2)**. Sixteen kestrel eggs were collected from nestboxes in the reference areas off of the RMA. The reference areas for kestrel egg collection included a fairground, a cemetery, a wastewater facility, a lakeside area, a reservoir, and a former airport. Twenty-nine kestrel eggs were collected on-site; ten eggs were collected from the core region of the RMA and 19 samples originated from the periphery **(Figure 2)**.

Carp eggs were collected from lakes on and off the RMA. Two samples of carp eggs were collected from reference sites, while 16 samples of carp eggs were collected from the RMA. Eggs were collected from Banner Lake and Lower Derby Lake on the RMA. Carp were collected by use of electroshocking procedures and gill nets. Eggs were collected from the adult carp.

Livers were collected from juvenile and adult great horned owls. Samples were collected when owls were found dead or moribund as part of the US Fish and Wildlife Services' opportunistic sampling program. Eleven great horned owl livers were collected from reference areas **(Figure 3)** and 16 livers were collected on-site. Reference areas from which owl specimens were collected were within a 130-mile radius of the RMA.

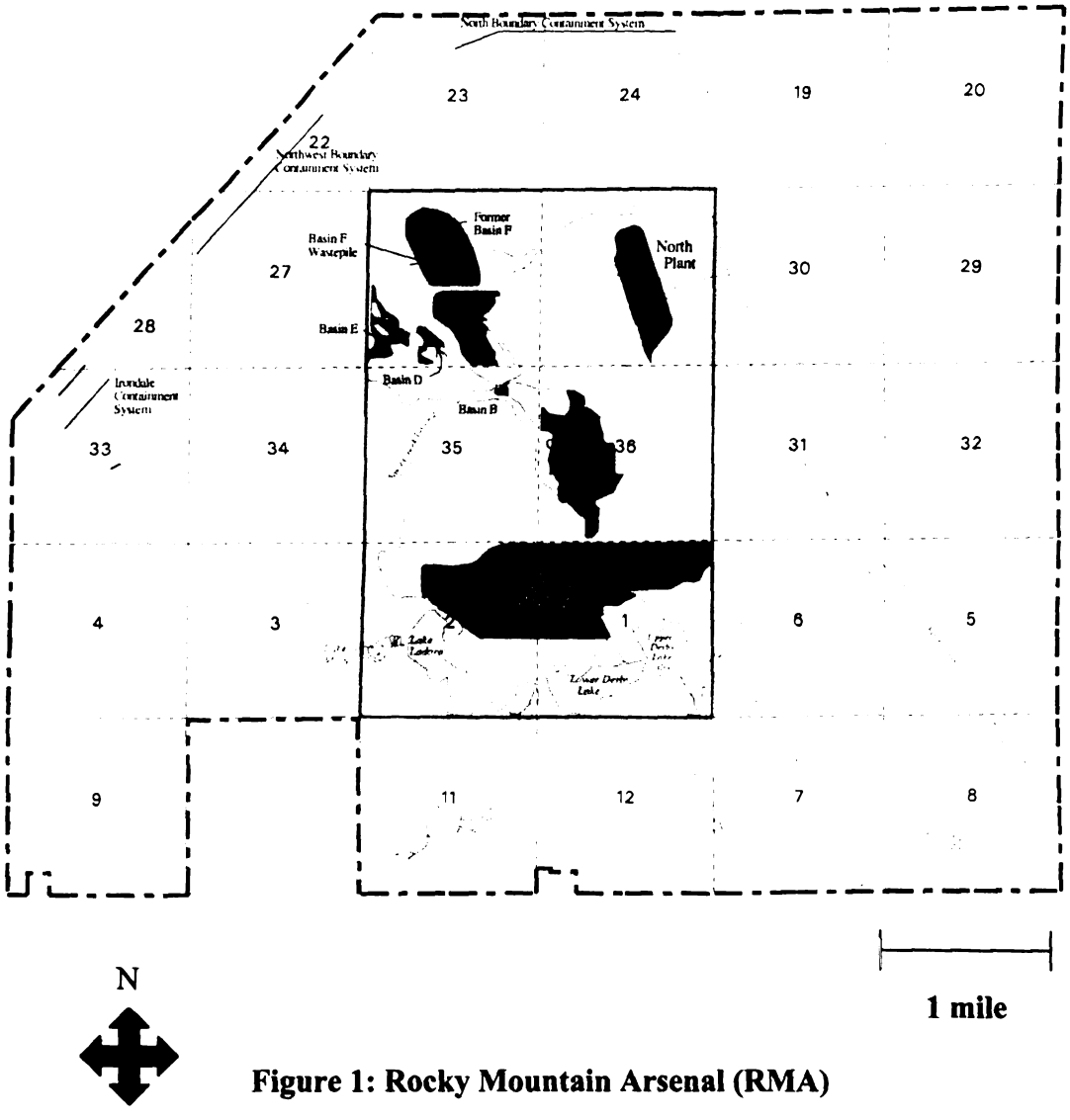

Figure 1: Rocky Mountain Arsenal (RMA)

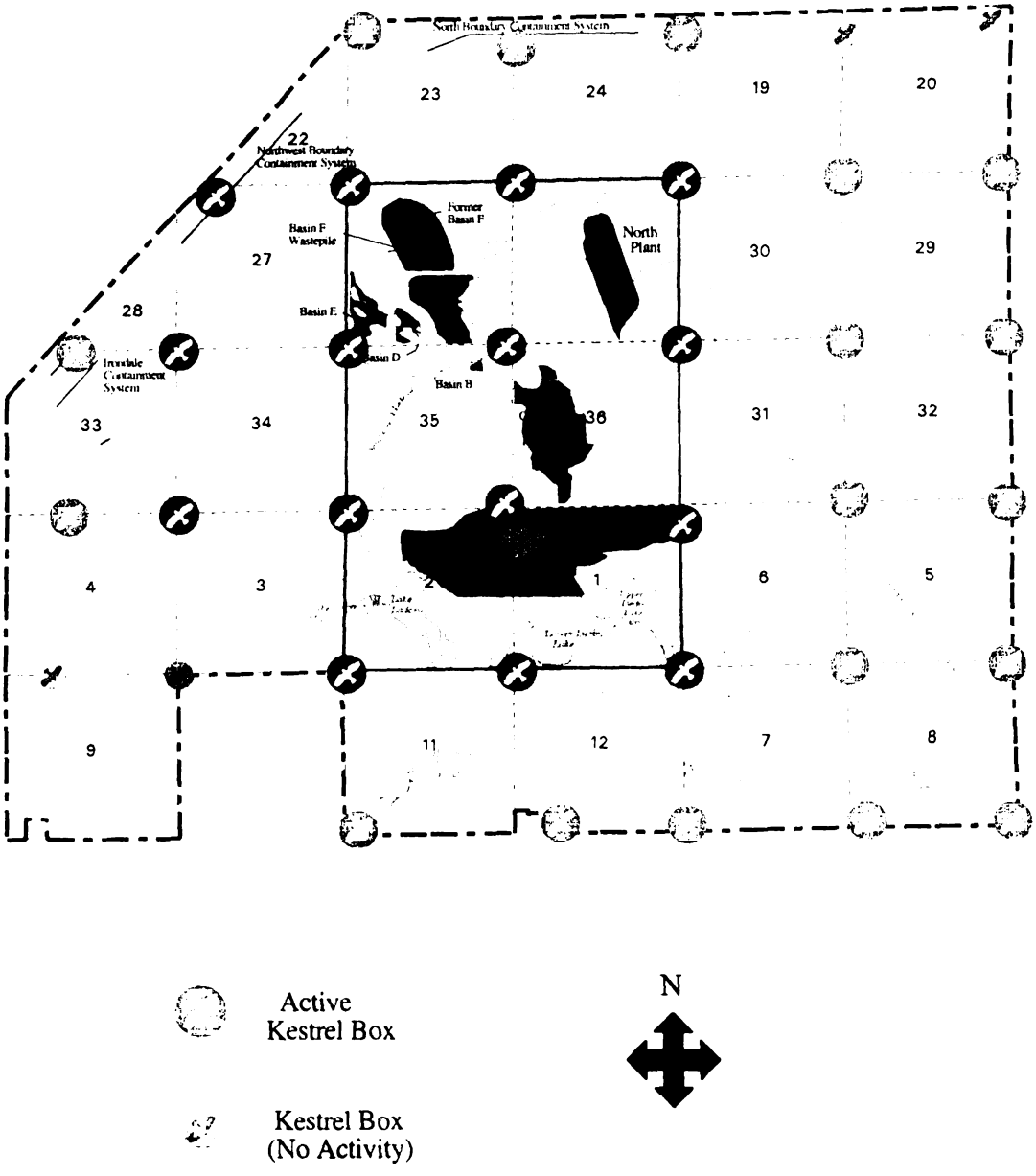

Figure 2: American kestrel nestbox locations on the RMA

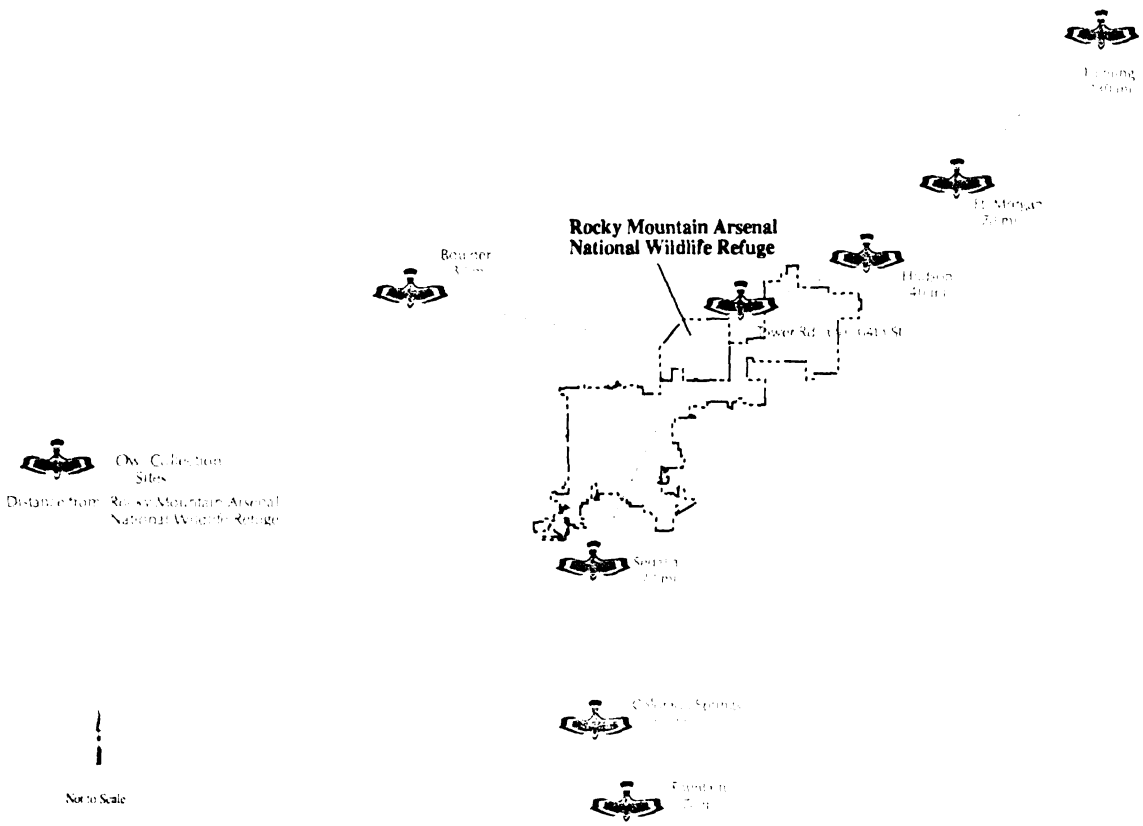

Figure 3: Reference locations of great horned owl specimen collection

Laboratory Methods:

Carp and kestrel egg samples, as well as owl livers, were delivered to Michigan State University (MSU) for chemical extraction, clean-up, and determination of TCDD-EQ concentrations using the H4IIE-luc *in vitro* bioassay. Coded tissue samples were supplied "blind" to MSU so that analyses were performed without knowledge of sample origin. Homogenization of tissue samples was accomplished by adding a sufficient amount of Na_2SO_4 to dry the sample (usually in a 10:1 ratio to the tissue weight). The tissue-Na_2SO_4 mixture was mixed until the tissues were dry. The mixture was then blended at high speed in a homogenizer to produce a fine powder.

With each batch of approximately twenty samples, method blank and method spike samples were extracted to determine the background and method recovery for dioxin-like chemicals. The method blanks and spikes consisted of 50 grams of Na_2SO_4, approximately equivalent to that used for each sample. For the method spikes, twenty-five μL of method spike solution, containing a mix of PCB congeners, was added to Na_2SO_4. When extracting kestrel egg tissue, matrix spikes and matrix blank quality control samples were analyzed with each block of 20 samples. These samples consisted of approximately five grams of chicken egg homogenate to which a known amount of PCB congeners had been added. Twelve quality control quail egg samples were also included in the kestrel egg sample group. Eight of these samples were spiked with known amounts of PCB 126, while four un-spiked quail eggs served as blanks. With each batch of approximately twenty samples, method blank and method spike samples were extracted to determine the background and method recovery for dioxin-like chemicals.

After homogenization, samples were extracted in a soxhlet apparatus with 300 ml of 1:1 acetone/hexane for 18 hr. After extraction, sample extracts were concentrated to near dryness by rotary evaporation at 35° C. Five ml of concentrated H_2SO_4 was added to each sample to remove lipids by oxidation. Each sample was then left for approximately 3 hr to allow separation of acidic (lipid) and hexane layers. When the layers had separated, the hexane portion, containing the analytes of interest, was removed from the acidic lipid layer and placed in a round bottom flask. The acid phase was washed with an additional aliquot of hexane, which was removed and combined with the first hexane fraction. The volume of the hexane extract was again reduced to near dryness by rotary evaporation, and five ml of nanopure water was used to rinse the extracts. After rinsing, hexane extracts were evaporated under a stream of nitrogen to 0.1 ml and diluted to 1.0 ml with isooctane for use in the H4IIE-luc bioassay (Sanderson *et al.* 1996).

Pigments and other compounds that interfere with the H4IIE-luc bioassay were removed from extracts of kestrel eggs by use of column chromatography with a silica, acidic silica, and KOH-silica gel column. This column removes almost all compounds except for dioxins, furans, and co-planer PCBs (dioxin-like chemicals) (Kannan *et al.* 1998).

Concentrations of TCDD-EQs were determined by the response of rat hepatoma cells (H4IIE) transfected with the luciferase gene under the control of the dioxin-responsive enhancer (DRE) (Sanderson *et al.* 1996). The H4IIE-luc bioassay was conducted over a period of 5 d. Cells were plated on day 1, dosed on day 2 with 1.25 μL of the sample extract, and dioxin-like activity and cell viability were measured on day 5. Analyses of individual extracts from tissue samples were conducted in separate 96-well

cell culture plates concurrently with a range of concentrations of 2,3,7,8-TCDD as the standard. The TCDD standard was made from a 10 ppm stock solution. TCDD standard dilutions were made to deliver doses of 30, 10, 3, 1, 0.3, and 0.1 pg TCDD/well. Extracts were applied in serial dilution, which delivered 1x, 0.3x, 0.1x, 0.03x, 0.01x, and 0.003x diluted extracts to the wells. A series of wells that contained cells but were not used in the analyses were interspersed between the active wells to minimize cross-talk between wells. Cell viability was measured at the time of the luminescence assay to ensure that the tissue extracts were not causing cell death. Viability was determined by both detailed visual inspection of cell growth and a cell viability test kit (Molecular Probes Inc.). A standard operating procedure for the H4IIE-luc bioassay is included **(Appendix I)**.

Concentrations of individual congeners were measured by gas chromatography and mass spectrometry (GC/MS) using standard analytical techniques. Midwest Research Institute (MRI) chemically analyzed all sample types. These chemical analyses were conducted under a separate contract between the BAS and MRI. All data generated by MRI was subject to US-EPA quality assurance and quality control assessment and validation before use in this report.

Data Analysis - TEQs:

Consensus TEFs were used in the calculation of TEQ_{WHO} values for carp egg, owl liver, and kestrel egg extracts to assess risks to exposed species (Van den Berg *et al.* 1998). Avian TEF values were applied to the owl and kestrel sample extracts, while fish TEF values were applied to the carp sample extracts. TEFs were multiplied by the congener concentrations of the dioxin-like analytes that were chosen for measurement

(Van den Berg *et al.*, 1998). This product was then summed to yield a total TEQ_{WHO} for the sample. Several concentrations of TEQ_{WHO} were calculated based on varying manipulations of the congener data below the method detection limit (MDL). $TEQ_{WHO-MAX}$ was calculated by substituting the MDL for those congeners that were below the MDL. $TEQ_{WHO-MIN}$ was calculated when congeners below the MDL were set to 0.0. Therefore, the $TEQ_{WHO-MAX}$ values are the most conservative and greatest possible overestimates of the dioxin-like activity present in the samples, while the $TEQ_{WHO-MIN}$ values are the least conservative and lowest possible underestimates reported.

Data Analysis – TCDD-EQs:

Bioassay derived TCDD-EQs were determined for each sample by comparing mean luminescence readings of sample extracts to luminescence readings from the TCDD standard curve analyzed on the same plate. Data were plotted and evaluated using Dunnett's test to identify samples where the extract response was greater than background and greater than the method detection limit (MDL) (Kuehl 1994). Samples for which the response of the undiluted extract was less than the least standard (0.1 pg TCDD/well) were determined to exhibit no detectable response ("non-detect") because extrapolation to concentrations less than those included in the standard curve was not acceptable (Villeneuve *et al.* in press). "Non-detect" samples were evaluated using the detection limit method to determine the sample-specific TCDD-EQ based on the MDL. Concentrations of TCDD-EQ were determined for samples for which a significant response was achieved by use of the slope-ratio method.

The detection limit method involves the calculation of sample-specific MDLs. MDLs for the analyses were based on the response of the sample in undiluted extract (1x) and the response of the minimum standard (0.1 pg TCDD/well). These responses varied among assays and among plates because of differences in samples sizes and responsiveness of the H4IIE-luc cells. To determine the MDL, the relative luminescence units (RLUs) measured in the solvent blank wells were subtracted from the sample response, and from the response of the lowest standard, usually 0.1 pg TCDD. The sample response was then divided by the standard response to determine the maximum possible TCDD-equivalent amount present in the sample well. This TCDD amount was then converted to a tissue concentration by determining the gram equivalents of sample in the well. The measured dioxin-like activity calculated using this method for "non-detect" samples was reported as the TCDD-EQ_{MAX} value. The TCDD-EQ_{MIN} reported "non-detect" samples with a value of zero. Sample calculations for this method are provided **(Appendix II)**.

In the slope-ratio method, the un-transformed TCDD standard and sample dose response curves were evaluated to determine the linear portion of the curve (Villenueve in press). The sample dilution series was first converted to gram equivalents/well using initial tissue weight, final extract volume and dilution factor. It was sometimes necessary to remove 'saturated' response values and the blank values from the standard curve. This was due both to the non-linear effect of plateau values and an initial non-linear threshold effect in the dose response curve due to the undue influence of the six blank values (3 solvent blanks and 3 no-treatment blanks) on the curve containing at most 12 other data points. Statistical rigor in the analysis was achieved by determining the correlation

coefficient for the line, after each data elimination. When the maximum r^2 value was achieved, the slope of the standard curve was calculated and used in subsequent calculations. The same assessment was performed for the sample to determine the slope of the linear portion of the dose-response relationship for each sample. The ratio of the slopes (sample/standard) was then used to determine the relative potency of the sample. Sample calculations for the slope-ratio method are provided (**Appendix II**). These calculations determined the mass of sample required to cause the same response as a specific mass of TCDD in a well. The result of the analysis was an equivalent concentration of 2,3,7,8-TCDD (TCDD-EQ) expressed as pg/g wet weight of tissue.

Since most undiluted extracts of carp eggs contained concentrations of TCDD-EQ less than the minimum TCDD standard concentration, the greater proportion of the carp egg extracts were analyzed by use of the detection limit method. Three extracts of carp eggs were analyzed using the slope-ratio method, since their luciferase induction was within the range of the TCDD standard curve. Concentrations of TCDD-EQs in owl livers and kestrel eggs were calculated in the same manner as the carp egg samples, where concentrations of TCDD-EQs were calculated by both the detection limit and slope-ratio methods, depending on the level of AhR-induced luciferase activity of the samples in comparison to the TCDD standard curve.

Statistical Methods to Detect for Differences in TCDD-Equivalent Concentrations Among Locations:

In order to establish whether or not tissue samples collected from the RMA had greater concentrations of TCDD-equivalents than tissue samples from reference areas, concentrations of $TEQ_{WHO\text{-}MAX}$ and TCDD-EQ_{MAX} were compared between on-site and reference areas using either the Mann-Whitney U test or the two group t-test. The two group t-test was utilized if the data set was normally distributed and had variances that were not statistically different. The Mann-Whitney U test was used in cases where the assumptions of a normal distribution were not met (Rand 1995). The one-way ANOVA and Dunnett's multiple comparison were used in cases where more than one population of RMA samples (originating from the core or periphery areas) was compared to reference populations. The level of statistical significance for all tests was set at α= 0.05. Power, the probability of avoiding a type II error (Kuehl 1994), was set at 0.80 (β = 0.20). For any analyses for which sufficient power (0.80) was not achieved, the results were deemed inconclusive. SYSTAT software (SPSS Inc. 1998) was used for statistical analyses, and Pass 60 computer software was used to calculate the power of each analysis.

Exposure Assessment

Quality Control/ Quality Assurance Results:

MRI quality control samples were analyzed in accordance with US-EPA regulations and will not be reported here. Quality control/quality assurance samples performed at MSU are examined in this document. The BAS supervised and authorized all quality control and quality assurance measures taken during the course of this study. All quality control and quality assurance criteria established by the MSU aquatic toxicology lab were met. Method spikes for carp eggs and great horned owl livers were evaluated together to determine their acceptability. Likewise, method and matrix spikes for American kestrel samples were evaluated as a group. Mean method and matrix spikes for each group were determined, and spike responses were expected to be within 20% of the mean value. All method and matrix spikes were within the expected range. Quail eggs, included in the kestrel egg sample blocks, were spiked with varying concentrations of PCB 126 and did not show significant dioxin-like activity when measured by the H4IIE-luc bioassay. These results reflect the low dioxin-like induction potential of PCB-126 in the H4IIE-luc cell line (Giesy *et al.* 1997). Method blank samples were less than the least TCDD standard concentration as expected, and did not display any dose-response relationships. A summarization of quality control/quality assurance samples is listed in **Appendix III.**

Residue Concentrations of TEQ_{WHO} in Carp Eggs:

The average total dioxin-like activities (reported as TEQ_{WHO}) for the carp egg samples were not greater than 1.6 pg/g. The inclusion of MDL values for congeners with values less than the MDL influenced the resulting TEQ_{WHO}. The average concentration of TEQ_{WHO} in carp eggs from the RMA ranged from **0.38** to **1.6** pg TEQ/g egg, while the average concentration of TEQ_{WHO} in samples from reference locations ranged from **0.34** to **0.84** pg TEQ/g egg depending on what surrogate proxy values were applied. The average contribution of PCBs to the total dioxin-like activity ranged from 1.5 to 20% among samples and methods of calculation **(Table 1)**. Only three non-ortho substituted PCB congeners were measured in carp egg samples. The contribution of mono-ortho-substituted PCBs to the TEQ_{WHO} was not determined as these congeners were not measured in the carp egg samples. Detailed results of concentrations of TEQ in individual samples of carp eggs are summarized in **Appendix IV**.

Table 1: Mean TEQ concentrations for carp egg samples (± 95% confidence intervals)

Sample Origin	N	Total $TEQ_{WHO\text{-}MIN}$ pg/g	%PCBs (min)	Total $TEQ_{WHO\text{-}MAX}$ pg/g	%PCBs (max)
RMA	16	0.38±0.07	20±6.3	1.6±0.17	4.5±0.10
Reference	2	0.34±1.3	4.6± 43	0.84± 0.97	1.5±12

Total TEQ_{WHO} = Total concentration of TEQ (PCBs, PCDDs, and PCDFs wet weight)
$_{MIN}$= 0 used for "non-detect" congeners
$_{MAX}$ = MDL used for "non-detect" congeners

Concentrations of TCDD-EQ in Carp Eggs:

Mean concentrations of bioassay derived TCDD-EQs in carp eggs from the RMA ranged from **4.3** to **8.5** pg TCDD-EQ/g egg, while mean concentrations in carp eggs from reference areas ranged from **0** to **2.5** pg TCDD-EQ/g egg depending on the choice of surrogate values chosen for "non-detect" values (**Table 2**). Concentrations of TEQ_{REP} were calculated by multiplying concentrations of individual congeners by their H4IIE-luc REP values. Concentrations of TEQ_{REP} were compared with concentrations of TCDD-EQs. Differences between TCDD-EQ and TEQ_{REP} were a function of surrogate values applied. When "non-detect" congeners were assigned MDL values (denoted $_{MAX}$), there was better agreement between concentrations of TCDD-EQ and TEQ_{REP}. This indicates that congeners that occurred at concentrations less than the MDL had the potential to contribute significantly to the total dioxin-like activity detected in the H4IIE-luc bioassay. The concentrations of $TEQ_{REP\text{-}MAX}$ were found to be significantly different (α=0.05) from concentrations of $TCDD\text{-}EQ_{MAX}$ when all data points were considered in the chi-square analysis ($X^2 = 94.3$, df = 17) (**Figure 4**). When three outlying points were removed from the analysis, the remaining concentrations of $TEQ_{REP\ MAX}$ and $TCDD\text{-}EQ_{MAX}$ were not significantly different from each other ($X^2 = 21.2$, df = 14). The three outlying carp egg samples were collected on the RMA and had greater TCDD-EQ concentrations than predicted by the TEQ_{REP} concentrations (**Appendix IV**). This is to be expected since the bioassay measures the total activity of complex mixtures and accounts for interactions among congeners (Sanderson *et al.* 1996). Instrumental analyses measured only target analytes. The H4IIE-luc bioassay can also detect additional dioxin-like activity from compounds not measured in analytical chemistry

procedures. Complete information on concentrations of TCDD-EQ and TEQ_{REP} in samples of carp egg are summarized **(Appendix IV)**.

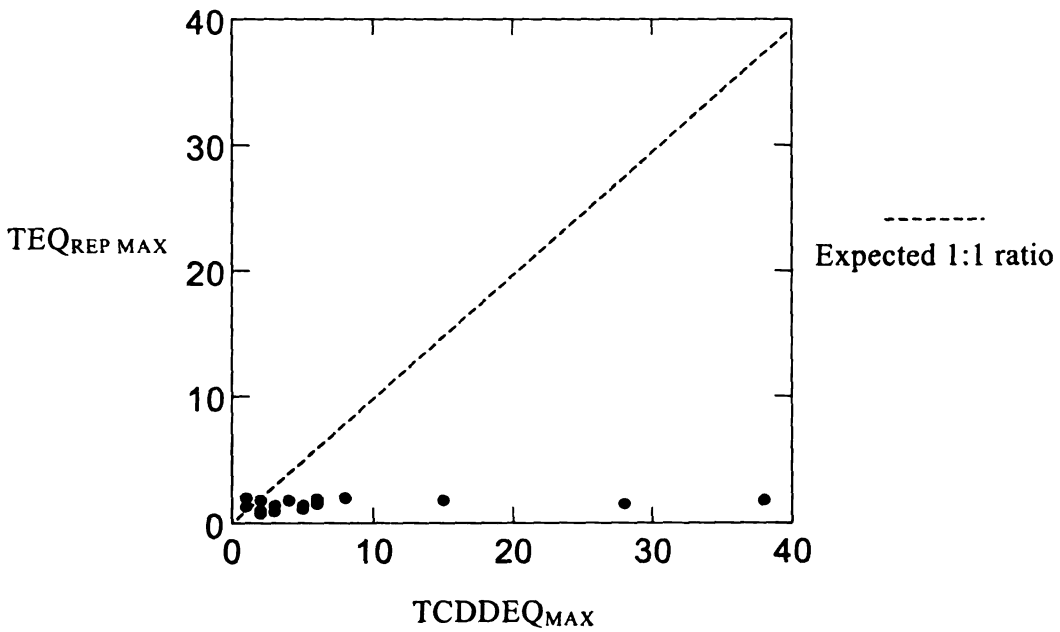

Figure 4: Comparison of carp egg $TEQ_{REP-MAX}$ and $TCDD\text{-}EQ_{MAX}$ values

Table 2: Comparison of mean TCDD-EQ and TEQ_{REP} concentrations in carp egg samples (± 95% confidence intervals)

Sample Origin	N	TCDD-EQ_{MIN} (pg/g)	$TEQ_{REP-MIN}$ (pg/g)	$TCDD\text{-}EQ_{MAX}$ (pg/g)	$TEQ_{REP-MAX}$ (pg/g)
RMA	**16**	4.3 ±6.1	0.64±0.10	8.5±5.5	3.4±0.59
Reference	**2**	0±0.0	0.33±0.95	2.5±6.4	1.5±1.3

TEQ_{REP} = sum of concentrations of individual congeners multiplied by their respective H4IIE relative potencies
$_{MIN}$ = 0 used for "non-detect" congeners
$_{MAX}$ = MDL used for "non-detect" congeners

Since the concentrations of TEQ_{WHO} in carp were normally distributed and the variances between the two groups were not significantly different, the 2-group Student's t-test was used to compare the mean values of on-site and reference concentrations. At $\alpha = 0.05$, TEQ_{MAX} concentrations from carp egg samples on the RMA were significantly lower from reference samples using both separate and pooled variances. TCDD-EQ_{MAX} concentrations were also significantly different between locations when separate variances were used, but there were no significant differences when the variances were pooled **(Table 3)**. The small sample size of carp eggs collected from reference areas greatly influenced the difference in variance measurements. Therefore, the pooled estimate may therefore be a more accurate test statistic. Power ($1-\beta$) was designated as 0.80 in this study. The power of this analysis is low due to small sample sizes.

Table 3: Significance of differences (p values) between locations as determined by the Student's t-test ($\alpha = 0.05$)

N		TEQ_{MAX}			TCDD-EQ_{MAX}		
On-site	Reference	Sep	Pool	$1-\beta$	Sep	Pool	$1-\beta$
16	2	**0.004**	**0.007**	1.0	**0.045**	0.457	0.67

Sep = tests using separate variances Pool = tests using pooled variances
Significant differences are in bold.

Residue Concentrations of TEQ_{WHO} in Owl Livers:

Average concentrations of TEQ_{WHO} in samples of owl livers from the RMA ranged from $\mathbf{3.3x10^2}$ to $\mathbf{3.4x10^2}$ pg TEQ_{WHO} /g liver, while the average concentration of TEQ_{WHO} in owl livers from reference areas ranged from **35** to **42** pg TEQ_{WHO}/g liver

depending on the proxy values used for congeners below the MDL (**Table 4**). The average contribution of PCBs to total TEQ_{WHO} ranged from 24 to 39%, again depending on the proxy value chosen **(Table 4).** The relative contribution of PCB congeners to the total TEQ_{WHO} was greater in owl livers than in carp egg samples. Overall, there was greater variation in TEQ_{WHO} concentrations among individual owl liver samples collected on and off the RMA than was evident in carp egg TEQ_{WHO} concentrations. Complete information on concentrations of TCDD-EQs and TEQs in samples of owl livers are summarized **(Appendix V)**.

Table 4: TEQ_{WHO} concentrations in owl liver samples (± 95% confidence intervals)

Sample Origin	N	Total $TEQ_{WHO\text{-}MIN}$ (pg/g)	% PCB_{MIN}	Total $TEQ_{WHO\text{-}MAX}$ (pg/g)	% PCB_{MAX}
RMA	16	$3.3x10^2 \pm 3.7x10^2$	39±9.0	$3.4 \times 10^2 \pm 3.7x10^2$	34±6.0
Reference	11	35±18	29±13	42±29	24±9.0

Total TEQ_{WHO} = Total concentration of TEQ_{WHO} (PCBs, PCDDs, and PCDFs)
$_{MIN}$ = 0 used for "non-detect" congeners
$_{MAX}$= MDL used for "non-detect" congeners

Concentrations of TCDD-EQ in Owl Livers:

Average concentrations of TCDD-EQ in owl livers collected in reference areas ranged from **17** to **23** pg TCDD-EQ/g liver and the average concentration in samples from the RMA was **$1.3x10^2$** pg TCDD-EQ/g liver **(Table 5)**. Again, concentrations of TCDD-EQs were variable among samples of individual owl livers collected on and off of

the RMA **(Appendix V)**. Concentrations of TEQ_{REP} were compared with TCDD-EQ. In this case, concentrations of TEQ_{REP} were, on average, greater than concentrations of TCDD-EQ **(Figure 5)**. This outcome was possibly observed due to the fact that congeners, when tested as a mixture in the bioassay, can antagonize one another causing interference and reduced measures of dioxin-like activity that are not predictable in the additive TEQ_{REP} model (Sanderson *et al.* 1996). Using the Chi-square analysis, it was found that concentrations of TCDD-EQ_{MAX} and $TEQ_{REP\text{-}MAX}$ differ significantly at α= 0.05 (X^2 = 86, df = 26) when all data points are considered. By removing a single outlying point from the analysis **(Appendix V)**, TCDD-EQ_{MAX} and $TEQ_{REP\text{-}MAX}$ concentrations were found to not be statistically different (X^2 = 14.2, df = 25). Removal of the outlying point illustrates that most of the great horned owl samples examined had similar results when analyzed by instrumental and bioanalytical techniques.

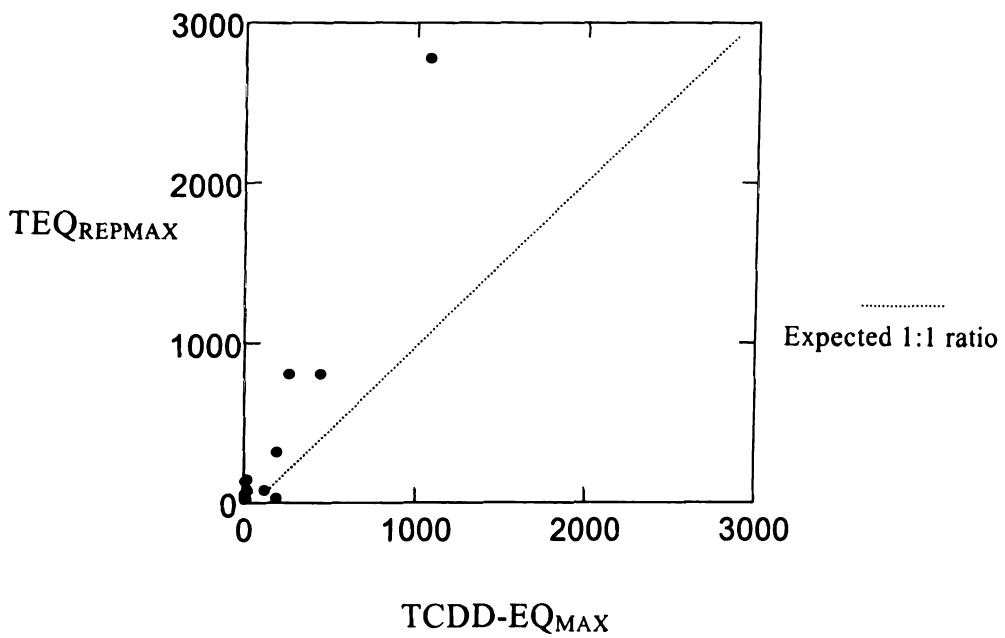

Figure 5: Comparison of great horned owl $TEQ_{REP\text{-}MAX}$ and TCDD-EQ_{MAX} values

Table 5: Comparison of TCDD-EQ and TEQ_{REP} concentrations in great horned owl liver samples (± 95 % confidence intervals)

Sample Origin	N	TCDD-EQ_{MIN} (pg/g)	$TEQ_{REP-MIN}$ (pg/g)	TCDD-EQ_{MAX} (pg/g)	$TEQ_{REP-MAX}$ (pg/g)
RMA	16	$1.3x\ 10^2$ $\pm 1.5x10^2$	$2.3x\ 10^2$ $\pm 2.8x10^2$	$1.3x\ 10^2$ $\pm 1.5x10^2$	$2.6x\ 10^2$ $\pm 3.1x10^2$
Reference	11	17±38	18±18	23±37	30±25

TEQ_{REP} = sum of concentrations of individual congeners multiplied by their respective H4IIE relative potencies
$_{MIN}$ = 0 used for "non-detect" congeners
$_{MAX}$ = MDL used for "non-detect" congeners

Since the great horned owl liver TCDD-equivalent concentrations were not normally distributed, the Mann-Whitney U test was used to test for significant differences between adult and juvenile owl populations (Rand 1995). In order to investigate the possible outcomes of the analysis between age classes, owls of unknown age were treated as adults for the first analysis, treated as juveniles for the following analysis, and eliminated in the final analysis **(Table 6)**. There were no significant differences among age classes in concentrations of $TEQ_{WHO-MAX}$. Therefore, comparisons between concentrations of $TEQ_{WHO-MAX}$ from on-site and reference areas were performed on pooled age classes. Significant differences in concentrations of TCDD-EQ_{MAX} were detected among age classes. Therefore, comparisons of concentrations of TCDD-EQ_{MAX} between on-site and reference locations were performed separately. Concentrations of

$TEQ_{WHO\text{-}MAX}$ between locations were not significantly different. However, concentrations of TCDD-EQ_{MAX} in livers of adult owls were significantly different between locations **(Table 7)**. The power of this analysis was found to be less than 0.8, therefore the results are inconclusive, and it could not be determined if there were any differences among locations.

Table 6: Significance of differences between age classes as determined by the Mann-Whitney U test ($\alpha = 0.05$).

Manipulation of owl specimens of unknown age	N		TEQ_{MAX}	TCDD-EQ_{MAX}
	Adults	Juveniles		
Unknowns treated as adults	13 (14)	13	0.144	**0.046**
Unknowns treated as juveniles	9	17 (18)	0.146	**0.020**
Unknown eliminated	9	13	0.117	**0.021**

Values in parentheses indicate sample sizes for the TCDD-EQ_{MAX} analysis.
Significant differences are in bold.

Table 7: Significance of differences between locations as determined by the Mann-Whitney U test ($\alpha = 0.05$).

	N		TEQ_{MAX}	TCDD-EQ_{MAX}
	On-site	Reference		
Analysis across age classes	16	10	0.077	.
Adults	4	5	.	**0.014**
Juveniles	8	5	.	0.604
Power (1-β)			0.50	0.44

Significant differences are in bold.

Residue Concentrations of TEQ_{WHO} in American Kestrel Eggs:

Mean concentrations of TEQ_{WHO} in American kestrel eggs collected from the RMA were in the range of **21** to **24** pg TEQ/g egg. Concentrations of TEQ_{WHO} in American kestrel eggs collected from reference areas were in the range of **55** to **57** pg TEQ/g egg **(Table 8).** Concentrations of TEQ_{WHO} in American kestrel eggs collected from reference areas were variable (**Appendix VI**), possibly reflecting the diversity of reference locations utilized in this study. The average contribution of PCBs to the total dioxin-like activity ranged from 21 to 57% among samples and methods of calculation, which is similar to the results reported for the great horned owl livers. Complete information on concentrations of TCDD-EQs and TEQs in samples of kestrel eggs is summarized **(Appendix VI)**.

Table 8: Mean TEQ_{WHO} concentrations in American kestrel egg samples (± 95 % confidence intervals)

Sample Origin	N	Total $TEQ_{WHO\text{-}MIN}$ (pg/g)	% PCB_{MIN}	Total $TEQ_{WHO\text{-}MAX}$ (pg/g)	% PCB_{MAX}
RMA	30	21±6.6	55±8.6	24±6.6	46±6.8
Reference	16	55±58	60±14	57±57	54±13

Total TEQ_{WHO} = Total concentration of TEQ (PCBs, PCDDs, and PCDFs wet weight)
$_{MIN}$ = 0 used for "non-detect" congeners
$_{MAX}$ = MDL used for "non-detect" congeners

Concentrations of TCDD-EQ in American kestrel eggs:

Average concentrations of TCDD-EQ in samples of American kestrel eggs from the RMA contained **2.4** to **6.7** pg TCDD-EQ/g egg. Average concentrations of TCDD-EQ in kestrel eggs collected in reference areas ranged from **14** to **18** pg TCDD-EQ/g egg **(Table 9)**. The variability in concentrations of TCDD-EQ were among individual kestrel egg samples collected from reference areas as is apparent in the large 95% confidence intervals for these samples **(Table 9)**. This phenomenon may reflect the diversity of reference sites. Concentrations of TEQ_{REP} were compared with TCDD-EQ. As with the owl livers, concentrations of TEQ_{REP} in the kestrel eggs were, on average, greater than concentrations of TCDD-EQ **(Figure 6)**. Concentrations of $TEQ_{REP\text{-}MAX}$ values were statistically different from $TCDD\text{-}EQ_{MAX}$ when all data points were analyzed by the Chi-square test ($X^2 = 668$, df = 56, $\alpha = 0.05$). By removing a single outlier from the analysis, $TCDD\text{-}EQ_{MAX}$ and $TEQ_{REP\text{-}MAX}$ concentrations were not significantly different ($X^2 = 40$, df = 55, $\alpha = 0.05$). Complete information on TCDD-EQ and TEQ_{REP} concentrations is provided **(Appendix VI)**.

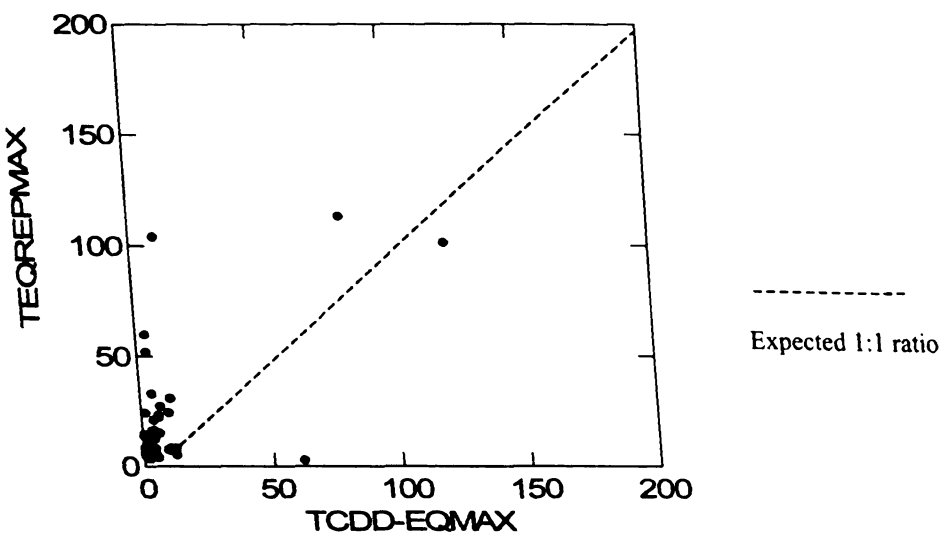

Figure 6: Comparison of concentrations of $TEQ_{REP\text{-}MAX}$ with concentrations of $TCDD\text{-}EQ_{MAX}$ in American kestrel eggs

Table 9: Comparison of TCDD-EQ and TEQ_{REP} concentrations in American kestrel egg samples

(± 95 % confidence intervals)

Sample Origin	N	TCDD-EQ_{MIN} (pg/g)	$TEQ_{REP-MIN}$ (pg/g)	TCDD-EQ_{MAX} (pg/g)	$TEQ_{REP-MAX}$ (pg/g)
RMA	31	2.4±4.3	15±7.6	6.9±4.1	19±7.9
Reference	16	14±19	19±18	17±18	21±18

TEQ_{REP} = sum of concentrations of individual congeners multiplied by their respective H4IIE relative potencies

$_{MIN}$ = 0 used for "non-detect" congeners

$_{MAX}$ = MDL used for "non-detect" congeners

A one-way ANOVA followed by a Dunnett's multiple comparison were used to test kestrel eggs collected from the core and periphery areas of the RMA as well as the reference areas. No significant differences in concentrations of $TEQ_{WHO-MAX}$ or TCDD-EQ_{MAX} were found among locations (α=0.05) **(Table 10)**. Concentrations of both TEQ and TCDD-EQ in eggs from core and peripheral area were grouped together in order to compare all on-site and reference areas by use of a Student's two group t-test. No significant differences in concentrations of either TEQ or TCDD-EQ were observed (α = 0.05). However, the power level was not sufficient in the t-test to be conclusive **(Table 11)**.

Table 10: Analysis of variance (ANOVA) results for comparison of TEQ_{MAX} concentrations in American kestrel egg samples among location

Source	Sum of Squares	df	Mean Square	F-ratio	p
Site	11470	2	5735	1.345	0.271
Error	183282	43	4262		

Table 11: Student's t-test results for the comparison of on-site and reference TEQ_{MAX} and TCDD-EQ_{MAX} concentrations in American kestrel egg samples

N		TEQ_{MAX}			TCDD-EQ_{MAX}		
On-site	Reference	Sep	Pool	1-β	Sep	Pool	1-β
30	16	0.241	0.105	0.219	0.246	0.126	0.214

Sep = tests using separate variances Pool = tests using pooled variances

Effects Characterization

Possible adverse effects of the TEQ and TCDD-EQ concentrations measured in carp eggs, owl livers, and kestrel eggs are determined by calculation of hazard quotients (HQs). Observed concentrations were compared to toxicant-specific Toxicity Reference Values (TRVs). TRVs are protective values that are calculated based on either a No Observable Adverse Effect Level (NOAEL) or a Lowest Observable Adverse Effect Level (LOAEL) of the toxicant on the species of interest (**Equation 3**). A NOAEL is defined as the greatest dose of toxicant tested at which no significant deleterious effect has been observed, and the LOAEL is defined as the lowest dose of toxicant tested at which a deleterious effect has been observed (Rand *et al.* 1995). The TRVs used in this study were derived from well-crafted preexisting studies providing NOAEL and LOAEL data. Uncertainty factors (UFs) were applied in TRV calculations if the NOAELs and LOAELs used were in some way non-representative of the species or toxicant of interest.

$$\textbf{TRV = NOAEL or LOAEL/ UFs} \qquad \textbf{(3)}$$

Derivation of the Carp Egg TRV:

Fish embryos are often the most sensitive life stage to dioxins and dioxin-like compounds (EPA 1993). The TRV used for carp egg samples was based on a combination of studies determining sensitivities of various freshwater fish species to dioxin-like compounds (Elonen *et al.* 1998, Henry *et al.* 1997, Walker and Peterson 1994). The No Observable Adverse Effect Concentration (NOAEC) from these studies was found to be between 170 and 500 pg TCDD/g egg wet weight. The UF for this TRV

was based on the extrapolation of laboratory studies to field situations, but only amounted to 1.0. Thus, a TRV for the carp eggs was calculated and found to be in the range of **170** to **500 pg/g** egg TCDD-equivalent (**Equation 4**). The calculation of UFs is explained in **Appendix VII.**

$$TRV = [NOAEL / UF]$$

$$\mathbf{TRV = [170\text{-}500pg/g /1.0] = 170\text{-}500\ pg/g} \qquad \mathbf{(4)}$$

Derivation of the American Kestrel TRV:

The TRV for American kestrel egg samples was derived from a study in which PCB 126 was injected into kestrel eggs (Hoffman *et al.* 1998). Eggs were examined for embryotoxic effects at the close of the study. The route of exposure, egg injection, is assumed to closely mimic natural, maternal contribution to the developing embryo. Kestrel embryos were used as the test subject, since it has been observed that they are more sensitive to dioxin-like compounds than are hatchlings (Hoffman *et al.* 1996). Of all the studies examined for TRV derivation, the Hoffman *et al.* 1998 study presented the fewest uncertainties. The test organism was the species of interest, the American kestrel, and even though PCB 126 was used as the test compound instead of dioxin, a TEF value of 0.1 was used in order to make the conversion to dioxin equivalents. The uncertainty factor was determined to be 3.5 based on uncertainties in the extrapolation of a lab study to field conditions and the uncertain NOAEL endpoint. The NOAEL for effects of PCB 126 on kestrel embryos was found to be 2300 pg PCB 126/g egg wet weight. This

NOAEL value was used to calculate a TRV value of **70 pg/g** TCDD-equivalent (**Equation 5**).

$$TRV = [NOAEL * TEF/ UF]$$

$$\textbf{TRV = [2300 pg/g * 0.1/ 3.5] = 70 pg TCDD/g egg} \quad \textbf{(5)}$$

Derivation of the Great Horned Owl Liver TRV:

No studies of the effects of TCDD on owls were found in the literature. For this reason, the derivation of the great horned owl TRV was based on the American kestrel TRV (Hoffman *et al.* 1998). More uncertainties were associated when determining the TRV for the great horned owl. Since the NOAEL value had to be extrapolated from kestrels to owls, an uncertainty factor of 5.0 was applied. Other uncertainties included extrapolation from the lab to the field (UF = 2), an unclear NOAEL endpoint (UF= 1.5), and an uncertain organ ratio (UF = 1). The organ ratio for converting from egg tissue to liver tissue was determined to be 1.5 (on a lipid basis) in herring gulls (Braune and Norstrom 1989). In total the uncertainty factor amounted to 22.5 ((2+1+1.5)*5). Based on these parameters, the TRV for great horned owls was found to be **15 pg/g** wet weight TCDD-equivalent (**Equation 6**).

$$TRV = [(NOAEL * (organ\ ratio) *TEF)/ UF]$$

$$\textbf{TRV = [(2300 pg/g * (1.5) *0.1)/ 22.5] = 15 pg TCDD/g liver} \quad \textbf{(6)}$$

Risk Characterization

Risk Estimation Using Hazard Quotients:

The potential for adverse effects of TEQs and TCDD-EQs concentrations on wildlife was assessed by calculating a hazard Quotient (HQ). The HQ was determined as the ratio of the concentration of TCDD-equivalent in the tissue of interest to the reference dose of toxicant (TRV) determined to have some meaning within a given organism. The values of HQs are reported as dimensionless Toxic Units (TU) **(Equation 7)**.

$$\textbf{HQ (TU) = concentration detected in the tissue/ TRV} \qquad (7)$$

Carp Eggs:

Values of HQs for samples of carp egg from neither on nor off of the RMA exceeded 1.0. The upper 95% confidence intervals for carp HQs were an order of magnitude less than 1.0 indicating quite conservatively that there are no significant adverse effects to carp as a result of exposure to dioxin-like chemicals **(Table 12 and Appendix IV)**.

Table 12: Mean Hazard Quotients (HQs) for carp egg samples

(± 95 % confidence intervals)

Sample Origin	N	HQ_{MIN} (TEQ based)	HQ_{MAX} (TEQ based)	HQ_{MIN} (TCDD-EQ based)	HQ_{MAX} (TCDD-EQ based)
RMA	16	0.002±0.001	0.01±0.001	0.02±0.035	0.05±0.032
Reference	2	0.002±0.008	0.005±0.006	0.000±0.000	0.015±0.064

Owl Livers:

Mean HQ values for owl livers collected from reference areas were less than mean HQ values for owl livers collected on-post for all methods of calculation employed **(Table 13)**. HQ values for several liver samples collected from on or off of the RMA exceeded 1.0 TU. The HQ_{MAX} (TEQ based) exceeded 1.0 TU for 8 samples from reference areas and 14 samples from the RMA. The HQ_{MAX} (TCDD-EQ based) exceeded 1.0 TU for 3 owl liver samples from reference sites and 5 samples from on the RMA **(Appendix V)**. HQ values differed considerably among individual owl liver samples collected on and off the RMA **(Appendix V).** This variation could be a result of the diversity in age, location, and cause of death of the owl specimens.

Table 13: Mean Hazard Quotients (HQs) for great horned owl liver samples (± 95 % confidence intervals)

Sample Origin	N	HQ_{MIN} (TEQ based)	HQ_{MAX} (TEQ based)	HQ_{MIN} (TCDD-EQ based)	HQ_{MAX} (TCDD-EQ based)
RMA	16	22±25	22±25	8.7±10	8.9±10
Reference	11	2.3±1.7	2.8±1.9	1.1±2.5	1.6±2.5

American Kestrel Eggs:

Mean HQ values for samples of kestrel egg from neither on nor off of the RMA exceeded 1.0. The results of the risk assessment indicate that there is no significant adverse effects toward American kestrels as a result of exposure to dioxin-like chemicals **(Table 14 and Appendix VI)**.

Table 14: Mean Hazard Quotients (HQs) for American kestrel egg samples (± 95 % confidence intervals)

Sample Origin	N	HQ_{MIN} (TEQ based)	HQ_{MAX} (TEQ based)	HQ_{MIN} (TCDD-EQ based)	HQ_{MAX} (TCDD-EQ based)
RMA	30 (31)	0.30±0.10	0.34±0.09	0.04±0.06	0.10±0.06
Reference	16	0.78±0.82	0.81±0.82	0.19±0.27	0.24±0.26

The value in parenthesis indicates sample size for TCDD-EQ analyses

Risk Description: Ecological Significance of the Results

If the HQ is greater than 1.0 TU, then the detected concentrations in tissue samples are greater than the desired threshold concentration. Due to the inclusion of uncertainty factors, TRVs are conservative values calculated to be protective of wildlife. Therefore, adverse population effects may not occur at levels of contamination resulting in HQ values slightly exceeding 1 TU, but TU values of 10-20 can be indicatative of population-level effects, depending on the slope of the dose-response curve (Giesy and Kannan 1998).

The results of the risk assessment, based on HQs, for the carp eggs indicate that aquatic systems on the RMA are not at risk as a result of exposure to dioxin-like compounds. Carp eggs collected from reference areas contained statistically less concentrations of $TEQ_{WHO\text{-}MAX}$ and $TCDD\text{-}EQ_{MAX}$ than did carp eggs collected on the RMA. However, the small sample sizes utilized in this analysis (n =2 from reference areas) may not be representative of the locations from which they were collected. Therefore, it is inconclusive whether concentrations of TCDD-equivalents are greater in samples of carp eggs from the RMA. A larger sample size in the reference population would allow for more representative results from this location.

In this study, livers of great horned owls contained the greatest concentrations of TEQs and TCDD-EQs, and exhibited the greatest HQ values of the three species studied. The terrestrial RMA environment had varying results in the risk assessment analysis. Some individual great horned owls on the RMA contained sufficient concentrations of TCDD-equivalents to result in HQ values greater than 1.0. No owl livers collected from reference areas had HQs greater than 20 TUs. However, depending on the use of proxy

values, HQ values of 2 to 4 samples of owl livers from the RMA were greater than 20 TUs (**Appendix V**). This result indicates that great horned owls on the RMA may be at risk due to exposure to dioxin-like chemicals. Adult great horned owl livers collected from the RMA had statistically greater concentrations of TCDD-EQ_{MAX} than the adult owl livers collected from surrounding reference areas. This may indicate that there is a localized source of dioxin-like compounds in the terrestrial environment at the RMA. However, the results are inconclusive due to the low power associated with the comparison of concentrations of TEQ and concentrations of TCDD-EQ among locations. An increased sample size would be required to increase the power to 0.80.

HQ values for samples of American kestrel eggs were not greater than 1.0 for any locations. Concentrations of TEQ and TCDD-EQ in kestrel eggs also did not show significant differences between on-site and reference locations, but the power in these analyses was low so that the results were inconclusive. The differing results in overall TCDD-EQ concentrations and TEQ concentrations between kestrel egg and owl liver samples may indicate that there are separate terrestrial pathways for exposure to dioxin-like chemicals.

Study Uncertainties

Quantification of concentrations of TCDD-EQs by use of the H4IIE-luc *in vitro* bioassay is based on luminescence readings and is thus an indirect measure of the relative dioxin potency of the sample extract. Activation of the luminescence gene only indicates that translocation and binding of the AhR has occurred in the nucleus. The bioassay was not used as a direct measure of the potential toxic effect of dioxin-like activities, but rather used as an integrative measure of the total concentration of TCDD-EQ. The responses to the effects of TCDD-EQs vary among species and endpoints. Thus, there is some uncertainty in interpreting the toxicological relevance of the TCDD-EQs measured by the induction of luciferase under control of a dioxin-responsive enhancer (DRE). Another uncertainty associated with the application of the H4IIE-luc bioassay is that the responses detected can not be solely attributed to PCDD, PCDFs, and PCBs since this bioassay is an integrative measure of all of the individual AhR-active compounds and all of the potential interactions among both AhR-active and inactive congeners (Giesy and Kannan 1998, Williams *et al* 1995). Uncertainties also exist when results are extrapolated from an *in vitro* situation to an *in vivo* situation. Complex interactions, like metabolism by endogenous enzymes, may occur *in vivo* to cause activation or inactivation of dioxin-like chemicals, and this phenomenon may not be measured in an *in vitro* test. In the risk assessment, concentrations of TCDD-EQs estimated in the bioassay were compared to TRV values.

TEQ values calculated by the application of TEFs or REPs are uncertain because there is an inability to account for chemical interactions that occur in complex mixtures.

TEFs and REPs assume that interactions between chemicals are additive, and these two factors can not account for synergistic or antagonistic interactions that may increase or decrease toxic activity.

There are also inherent uncertainties in the derivation of TRV values for the three species utilized in this experiment. These uncertainties, including lab to field extrapolation, species extrapolation, inadequate study designs, and uncertain endpoints are taken into account when calculating a final TRV, but do not lead to accurate values. TRVs are formulated in order to be protective of organisms, therefore they are conservative estimates. In cases such as the great horned owl, there is not sufficient experimental evidence on the test species for the estimation of an accurate TRV.

The three species selected in this study were chosen to act as sentinels for the entire RMA wildlife population. These sentinel species yielded general information on the distribution of TCDD-EQ and TEQ concentrations in tissue samples from the RMA environment, but could not accurately represent every species present on the RMA. Therefore, some uncertainty in the results is associated with the inability to measure concentrations of TCDD-EQs and TEQs in every species present on and off the RMA. In addition, great horned owl specimens were collected opportunistically, which meant that some owls were found dead in response to an environmental stressor independent from PDH exposure. The type of death of some owl specimens may have affected the measurement of TCDD-equivalents in these samples. For example, loss of lipid content occurs in the liver tissue of organisms that expire due to dieldrin poisoning (Stickel *et al.* 1969). This loss of lipid content in dieldrin-poisoned owls may have led to an increased

partitioning of TCDD-equivalents in liver samples, resulting in higher observed TCDD-equivalent concentrations.

These uncertainties should be taken into account when interpreting the results of this study. However, the dioxin-equivalent concentrations determined by instrumental analyses and the H4IIE-luc bioassay were similar for most of the collected tissue samples, giving strength the accuracy of the results. Overall, some significant differences in dioxin-equivalent concentrations were found between RMA and reference locations depending on which dioxin-equivalent methodology was used. TEQ concentrations in carp eggs and TCDD-EQ concentrations in adult great horned owl livers differed significantly among RMA and Reference locations. In contrast, dioxin-equivalent concentrations in juvenile owl livers and in kestrel eggs did not vary significantly among locations. These conflicting results, the small sample sizes, and the low power associated with most of the analyses, render the study inconclusive as to whether or not dioxin-like chemicals are contaminants of concern on the RMA. It is apparent from the Hazard Quotient results that carp and American kestrel populations are not likely at risk due to dioxin-like exposure. However, some great horned owls from both RMA and Reference locations do appear to be at risk if the calculated TRV is reliable. It appears that adult great horned owls from the RMA may be at risk due to a combination of both dieldrin poisoning and dioxin-like contamination.

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Appendix I: Standard Operating Procedure for the H4IIE-luc Bioassay for the Detection of AhR Agonists (MSU)

Michigan State University
National Food Safety and Toxicology Center
Aquatic Toxicology Laboratory

STANDARD OPERATING PROCEDURE

H4IIE-luc Bioassay For The Detection Of Ah Receptor Agonists

Version 1 September 14, 1998

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DEFINITIONS AND ACRONYMS

AhR	Aryl hydrocarbon receptor
ATL	Aquatic Toxicology Laboratory (Michigan Sate University)
DCCFBS	Fetal bovine serum that has been charcoal-stripped
EC50	Conentration of test agent that causes 50% of maximal response
FBS	Fetal bovine serum
H4IIE-luc	Rat hepatoma cells stably transfected with an AhR-controlled luciferase reporter gene construct
PBS	Phosphate-buffered saline
RLU	Relative luminescent units
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin

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1.0. PURPOSE

The H4IIE-luciferase induction assay is an *in vitro* technique for the identification of aryl hydrocarbon (Ah) receptor-active compounds. The technique uses rat hepatoma cells (H4IIE-luc) stably transfected with an AhR-controlled luciferase reporter gene construct were developed at Michigan State University by Dr. Jac Aarts (Univ. of Wageningen, The Netherlands; Sanderson et al., 1996). The assay is also referred to as the chemical activated luciferase gene expression (CALUx) system (Murk et al., 1996). These cells express firefly luciferase in response to Ah receptor agonists. Luciferase activity is measured conveniently and with high sensitivity as light emission using a plate-scanning luminometer. Luciferase induction potential is assessed by comparison of the response to that of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the most potent agonist for the mammalian Ah receptor.

2.0 SCOPE AND APPLICATION

Ah receptor agonists include polyhalogenated aromatic hydrocarbons (PHAHs), such as polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) which are persistent environmental contaminants found in all parts of the world. A number of these compounds cause a variety of adverse effects in laboratory studies on rats and mice (reviewed by Poland and Knutson 1982). These include hepatotoxicity, certain types of cancer, thymic atrophy and other immunotoxicities, a wasting syndrome, reproductive toxicities, terata and the induction of enzymes and porphyrins. A number of these toxicities have also been observed in wildlife in areas with elevated levels of PHAHs, particularly in fish-eating birds in the Great Lakes (reviewed by Gilbertson *et al.* 1991; Giesy *et al.* 1994a; 1994b).

Interest exists in assessing the risk posed by these PHAHs to fish and wildlife, which may also reflect the risk to humans. One aspect of risk assessment is the use of bioanalytical assays to detect and determine the toxicity of complex mixtures of these chemicals in extracts of environmental compartments such as soil, water and biota. Quantitative instrumental analysis of complex mixtures of these compounds is a difficult and expensive task. Furthermore, demonstrating the presence of one or many of these compounds in samples provides only limited information on their biological potency, particularly when present in a complex mixture with many potential interactions.

In order to develop a suitable bioassay, an understanding of the mechanism of action for the compounds is required. In the case of a number of PHAHs, considerable knowledge of the mechanism by which they cause their toxicities has been acquired. As previously mentioned, PHAHs are persistent agonists for the Ah receptor (Poland and Knutson 1982). Binding of agonist to receptor results in an activated receptor-ligand complex that translocates to the nucleus. Here it interacts with specific sequences on the DNA, termed dioxin-responsive elements (DREs;

also called xREs or AhREs), in order to alter gene transcription (reviewed by Whitlock 1990; Okey *et al.* 1994). A rapid and sensitive response that is under direct regulation of the Ah receptor is the induction of cytochrome P-450 1A isoenzymes (CYP1A1 and 1A2) and their associated ethoxyresorufin O-deethylase (EROD) activity (Nebert and Gonzalez 1987). Good correlations exist between the Ah receptor-binding affinity of persistent PHAHs and their EROD-inducing potency *in vitro*, and, dependent on the endpoint, their toxic potential *in vivo* (Safe 1986; 1990). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), in particular, binds tightly to the Ah receptor, and is a potent inducer of EROD activity and highly toxic. For the above reasons, the capacity of single compounds or complex environmental mixtures to induce EROD activity is considered to be a reasonable measure of their toxic potential. This mechanistic knowledge has been applied *in vivo* in biological monitoring of fish and birds, and *in vitro* in the use of bioassays for the screening of environmental extracts for Ah-active components. The H4IIE rat hepatoma cell bioassay (Tillitt *et al.* 1991) is widely used for this purpose. In this assay, EROD-inducing potencies (ED_{50} values) of single compounds and environmental samples are determined from complete dose-response curves and compared to that of TCDD in order to express the biological potency of the tested samples in TCDD-equivalents (TCDD-EQs). The bioassay integrates potential non-additive interactions among Ah receptor agonist and between Ah receptor agonists and other compounds by measuring a final receptor-mediated response (Giesy *et al.* 1994a).

For the same purpose as the currently used wild-type H4IIE bioassay (H4IIE-wt), a recombinant H4IIE cell line (H4IIE-luc) has been developed that exhibits Ah receptor-mediated luciferase expression (Aarts *et al.* 1995). This cell line has been stably transfected with a luciferase reporter gene under transcriptional control of several DREs from mouse (Aarts *et al.* 1993; Denison *et al.* 1993). These DRE sequences are highly conserved among species, unlike the Ah receptor which can exhibit greatly different ligand-binding properties among species. A preliminary report using luciferase-transfected mouse Hepa1c1c7 hepatoma cells indicated that these cells are more sensitive to Ah receptor agonists than the wild-type cells (Aarts *et al.* 1993; Sanderson *et al.*, 1996). It has been suggested that luciferase-transfected cell lines would have more favorable properties than their respective wild-types, such as greater selectivity, sensitivity and dynamic range. This has been postulated because the Ah receptor-mediated expression of luciferase, being foreign to the cell, is probably not affected by post-transcriptional and -translational events which influence CYP1A1 expression. Furthermore, the recombinant cells would not be dependent on a functional CYP1A1 gene or protein for responsiveness, although the Ah receptor-mediated pathway would still need to be present. Another theoretical consideration is that luciferase is assayed on the basis of light production for which extremely sensitive detectors exist; also, the turnover number or molecular activity of luciferase is so high that it allows the detection of very few molecules of the enzyme, relative to CYP1A1. Further, recombinant cells are readily

amenable to further improvements in responsiveness by genetic engineering of the reporter gene construct.

The threshold dose (*i.e.*, detection limit) and ED_{50} (*i.e.*, effective dose to elicit 50% of the maximal response) for luciferase induction in H4IIE-luc cells were approximately 0.1 and 1.2 pg/well, respectively, as determined from 41 separate standard TCDD curves analyzed in 1997 (Figure 1). Coefficients of variation (standard deviation/mean x 100) for the assay were under 10% at all concentrations tested. For a sample size of 20 g tissue and a final extract volume of 0.25 ml, the H4IIE-luc assay will detect 1 part per trillion (ppt; pg/g, wet weight) TCDD-equivalents. With a sample size of 5 g tissue, 4 pg/g (wet weight) TCDD-equivalents will be detected.

3.0. SAFETY CONSIDERATIONS

TCDD and many related compounds have been found to be carcinogenic. In addition, the ethidium homodimer used in the cell viability assay is a powerful mutagen. Care should be taken to minimize exposure. According to institutional guidelines (refer to the Safety Manual for the Aquatic Toxicology Laboratory at Michigan State University), medium should be collected in a liquid trap for disposal as hazardous waste.

4.0. EQUIPMENT, MATERIALS, AND REAGENTS

4.1. Maintenance, preparation and use of H4IIE-luc cells

The assay uses a stably transfected cell line developed by Dr. Jac Aarts at Michigan State University (Sanderson et al., 1996). Briefly, rat hepatoma cells [American Type Culture Collection (ATCC) catalog #CRL 1548)] were stably-transfected with an inducible reporter plasmid, pGudLuc1.1. This plasmid contains the firefly luciferase gene under PHAH-inducible control of four DREs. Exposure of these cells to Ah receptor-active chemicals results in induction of luciferase activity in a time-, dose-, and AhR-dependent manner.

4.1.1. Maintain adherant cells in continuous culture in 100 mm tissue culture plates (Corning #25020-100, Cambridge, MA; 1-800-492-1110), 75 cm^2 flasks (Corning #25113-75) or any appropriate vessel at a maximum density of 80-90% confluence in 10% Full Medium (See **Media Preparation**). Incubator conditions are 37 C, 5% CO_2 humidified atmosphere.

4.1.2. Subculture cells 1:6 every week (depending upon density) maintaining a minimum cell density of 15-25%.

4.2. Instruments

4.2.1. Dynatech ML3000 Luminometer
(Dynatech Technical Support: (800) 336-4543; Chantilly, VA)

4.2.2. Cytofluor 2300/2350 Fluorescence Measurement System
(Millipore Technical Support: (800) 645-5476; Bedford, MA)

***NOTE**: All users of the Dynatech ML3000 Luminometer and the Cytofluor 2300 must read and be familiar with the Operators Manual before using the instrument. A working knowledge of Microsoft Windows is also necessary. For both the luminometer and fluorescence measurement system, instrument use is recorded in a log book (located next to the instrument). For each use, the person, date, number of samples, type of samples, and results of proficiency standards are recorded. In addition, any abnormal operation of the instrument is recorded. Calibration is performed by analyzing a positive control or sample with known activity each day that the assay is run. If the positive control exceeds ± 20% of the on-going average (as determined by a comparison to a proficiency curve maintained in the instrument log book), the positive control will be rerun. If exceedance is confirmed with the second analysis, the manufacturer will be contacted as a corrective action so that the instrument can be recalibrated.*

4.2.3. Pipets

a. Eppendorf Repeat Pipetter (Brinkmann Instruments #22 26 000-6; Westbury, NY) with sterile, Biopur 12.5 ml combitips (Brinkmann Instruments #22 49 520-8) for dispensing cells into the 96-well plate and for changing media. Calibration checked weekly by weight of water check and the results are entered in calibration log book. Adjustments made by MSU Biochemistry Instrument Shop when accuracy exceeds manufacturer's specifications of ± 0.3%.

b. Rainin Pipetman Pipets (0.5 - 10 µl capacity, Rainin #P-10; 20 - 200 µl, Rainin #P-200 ; 100 - 1000 µl, Rainin #P-1000; Woburn, MA) for making sample dilutions, dosing cells, etc. Calibration checked weekly by weight of water check and the results are entered in calibration log book. Adjustments made by MSU Biochemistry Instrument Shop when accuracy exceeds manufacturer's specifications of ± 2.5% for P-10, 1.0 % for P-200, and 3.0% for P-1000.

c. Brinkmann Eppendorf Multichannel Pipetter 30-300 µl (Brinkmann Instruments #22 45 120-1) for washing cells and adding reagents for viability and Luclite reagents. Calibration checked weekly by weight of water check and the results are entered in calibration log book. Adjustments made by MSU

Biochemistry Instrument Shop when accuracy exceeds manufacturer's specifications of ± 1.5%.

d. Drummond Pipet-Aid Filler/Dispenser (Drummond #4-000-111-TC, available from Fisher #13-681-15D) for dispensing volumes greater than 1 ml, such as for changing media on cells.

NOTE: *For all pipettors, except the Drummond Pipet-Aid, calibration test results are recorded weekly, along with the name of the person and any abnormal operation of the instrument.*

4.3. Supplies and Biochemicals

4.3.1. 96-Well ViewPlates™ (Packard Instruments #6005181; Meriden, CT); white 96-well plates, sterile, tissue culture treated, with lids and self-adhesive sticker for bottom of plates

Cost: $297/50 plates;
Ordering: (800) 856-0734
Technical support: (800) 323-1891

4.3.2. Cell viability assay reagents; sold either as a kit from Molecular Probes (#L-3224; Eugene, OR) or as individual components:

Calcein AM (Molecular Probes #C-3100);
MW = 994.87; made up as 4000x (2 mM) stock (50 μg/12.56 μl DMSO)

Ethidium homodimer I (Molecular Probes #E 1169)
MW = 857; made up as 2000x (1 mM) stock in DMSO

Ordering: (800) 438-2209
Technical support: (541) 465-8353

4.3.3. LucLite™ Kit (Packard Instruments # 6016911 - 1000 assay kit; Meriden, CT). Make fresh on same day as assay. Dissolve one bottle of lyophilized reagent with 10ml buffer (supplied) for every 133 assays (individual wells) to be analyzed.

Cost: $420/1333 assays using 75 μl/assay (or 1000 assays if using the manufacturer's suggested volume of 100 μl/assay. Preliminary studies showed equivalent responses at both 75 μl and 100 μl).
Ordering: (800) 856-0734
Technical support: (800) 323-1891

4.3.4. 10x Trypsin-EDTA solution (Sigma #T-4174, St. Louis, MO) for dissociation of cells from plates. From a 10x concentrated solution, a 1x working solution is prepared using sterile PBS as the diluent.

4.3.5. Dulbecco's phosphate-buffered saline (PBS) with Ca^{2+} and Mg^{2+}

First, make up 10 L of Ca^{2+}-free and Mg^{2+}- free Dulbecco's PBS:

2.0 g	KCl
2.0 g	KH_2PO_4
80.0 g	NaCl
11.5 g	Na_2HPO_4
10.0 L	H_2O[a]

[a]distilled/deionized or Nanopure biological grade

To each 1 L of PBS, add 0.1 g anhydrous $CaCl_2$ and 0.1 g $MgCl_2 \bullet 6H_20$ Keep at room temperature (good for at least 2 - 4 weeks).

4.4. Media Preparation

4.4.1. 10% Full Medium

Dulbecco's Modified Eagle's Medium (Sigma #D-2902) **without phenol red** (known to be estrogenic and with unknown influence on H4IIE cells), and sodium bicarbonate. Prepare as instructed by the manufacturer, adjust the pH to ~7.3, and then add :

10% fetal bovine serum (Hyclone defined FBS # SH30070.03; Logan, UT; 1-800-492-5663)

4.4.2. 10% DCCFBS Medium

Prepared as for the full medium, except the fetal bovine serum is replaced with dextran/charcoal-stripped fetal bovine serum (available from Hyclone #SH30068.03).

5.0 METHOD, PROCEDURES, AND REQUIREMENTS

5.1. Sample Preparation

Two types of samples may be assayed: pure compounds and mixtures derived from environmental or tissue samples. For pure compounds, sample preparation consists of dissolving the material in an appropriate solvent. The solvent of choice is isooctane (because of its low toxicity and for direct comparison to TCDD, which is dissolved in isooctane). However, if the material is insoluble in isooctane, the following solvents can be tried:, ethanol, acetone, *p*-dioxane, acetonitrile, and methanol. A stock solution should be prepared at 5 mM for compounds of unknown activity and stored in amber glass vials at -20^{0}C. However, it may be necessary to test concentrations near the limit of solubility of the compound. Ideally, a volume of 1-2 ml at the highest possible concentration should be obtained for standards and samples. This volume is sufficient for splitting samples for instrumental analysis and for preparation of serial dilutions.

Samples derived from environmental matrices such as tissue, water, or sediment should be extracted and concentrated according to appropriate protocols (in accordance with SOP #211 - Extraction and Analysis of PCBs and Non-ortho Coplanar PCBs in Biological Matrices). The volume of sample should be recorded before re-dissolution in the assay solvent so that a dilution or concentration factor can be calculated. To prepare dilutions, 0.5 ml of isooctane (assuming that this is the solvent of choice) is added to five appropriately labeled 2 ml amber GC glass vials with teflon lined caps. Then 0.5 ml of the 1x extract (original extract that is undiluted) is added to the first vial (labeled 0.5x), the lid enclosure tightened, and then the sample is vortexed well. Then 0.5 ml of the 0.5x stock is added to the next vial (labeled 0.25x), the lid enclosure tightened, and then the sample is vortexed well. These steps are repeated until all dilutions are prepared. The sample dilutions will be 100, 50, 25, 12.5, 6, and 3% of the extract. If less than 1 ml of original 1x extract is available, the above mentioned volumes should be proportionately reduced. After dissolution in the assay solvent, the samples should be stored at -20^{0}C.

5.2. Standards Preparation

A large range of standards should be prepared to deliver a final concentration of **TCDD** between 0.03 - 100 pg/well in a volume of 1.25 µl (*i.e.*, make 200x stock solutions). Generally, 6 concentrations will achieve a full dose response curve (final = 0.1, 0.3, 1, 3, 10, 30 pg/well) for TCDD. Ideally, standards should be dissolved in the same solvent as the samples, but this is not always possible. In this case, be sure to conduct assays with both solvent controls and compare them to blanks (see Dosing Cells). A stock of TCDD in isooctane is maintained in Room 181, URCF, in a 1 liter volumetric flask. The concentration is written on the flask and is tested for purity and accuracy by GC/MS and GC/ECD by

comparison to commercially available certified standards. To make dilutions, prepare the following stock concentrations (assuming a stock concentration of 10000 ng/ml):

final mass. in wells (pg/1.25 ul)	stock concentration (ng/ml)
12500	10000
10000	8000
1000	800
100	80
The following six concentrations are used for the bioassay:	
30	24
10	8
3	2.4
1	0.8
0.3	0.24
0.1	0.08

***NOTE**: Adherence to these procedures will insure consistent response of cells. Timing and cell density are critical. An assay may be completed in 5 days.*

5.3. Plating Cells (Day 1)

Prior to confluence, write down the passage number of the cells, aspirate media from cell culture dishes, wash the cells with sterile PBS without Ca^{2+} and Mg^{2+}, and trypsinize the cells with 3 ml of 1x sterile trysin-EDTA solution for 5 minutes at 37^0C. Add trysinized cell suspension to 27 ml of "10% Full Medium" and determine the number of cells/ml with a hemacytometer (for more information on cell counting, refer to "Cell Culture: A Manual of Basic Techniques", by Dr. Ian Freshney, 1996) . Dilute the cell solution to a concentration of 60,000 cells/ml with media. Add 0.25 ml of cell suspension to each well (15,000 cells) of a 96-well ViewPlate™ using an Eppendorf™ repeat pipettor. Care must be taken that the cell suspension is uniform each time that the pipettor is refilled. This is done by gently inverting the tube or bottle of cell suspension end-over-end several times immediately prior to refilling the pipettor. If the outer 36 wells are not being used for the experiment (recommended), fill them with either sterile media or PBS to maintain humidity consistently across the plate. Use of the outer wells is not recommended because of an edge effect caused by inconsistent growth of cells in these wells.

***NOTE**: cell number per well is one of the largest contributors to variation in the data - so take plenty of time to do this step properly. Cell passage number should be noted to monitor long-term changes in the responsiveness and growth characteristics of the cells.*

5.4. Dosing Cells (Day 2)

Dose cells 24 hours after plating and continue exposures for 3 days. First, examine cells to ensure consistent plating. Aspirate media (attach p-10 tips to the suction line to minimize cell scraping), and replace with 0.25 ml of DCCFBS media that is prewarmed to 37^0C.

***NOTE**: the use of cold media should be avoided because it may "shock" or stress the cells and it may cause precipitation of test agents that have poor water solubility).*

A typical plate design is shown in Figure 2. Use at least three replicates per treatment. Note that with TCDD, only two replicates are used for a typical plate design. Each control and TCDD concentration are averaged for all plates within a given experiment. Use a negative control with no treatment (blank) and a solvent control treated with pure solvent only. Use at least five concentrations of each compound or extracts tested. Prepare 200x chemical stocks in the appropriate solvent (see Sample Preparation). In general, it is best to dose cells directly in the well; however, for water miscible solvents, the test agent can be added to a sterile glass vial containing 2 ml of media, mixed, and then added to each well. Each well shall receive 1.25 µl of sample or standard or solvent control as noted in the plate design below.

Earlier versions of this assay determined that cross-talk can occur when high activity samples are directly adjacent to low activity samples. Therefore, as a corrective action, the plate design shown in Figure 2 was developed. Note that two blank columns border samples, so that there is no cross-talking between samples and TCDD and between samples and controls.

Row/ Col	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		0.1 pg TCDD	0.1 pg TCDD			C1 Conc. 1	C1 Conc. 1	C1 Conc. 1			solvent control	
C		0.3 pg TCDD	0.3 pg TCDD			C1 Conc. 2	C1 Conc. 2	C1 Conc. 2			solvent control	
D		1 pg TCDD	1 pg TCDD			C1 Conc. 3	C1 Conc. 3	C1 Conc. 3			solvent control	
E		3 pg TCDD	3 pg TCDD			C1 Conc. 4	C1 Conc. 4	C1 Conc. 4			blank	
F		10 pg TCDD	10 pg TCDD			C1 Conc.5	C1 Conc.5	C1 Conc.5			blank	
G		30 pg TCDD	30 pg TCDD			C1 Conc. 6	C1 Conc. 6	C1 Conc. 6			blank	
H												

Figure 2. A typical plate design for determination of Ah receptor agonist activity in H4IIE-luc cells. Note that blanks, solvent controls, and a standard curve for TCDD can be analyzed on the same plate with one test sample (labeled C1, at six different concentrations). Two blank columns are recommended between samples and either TCDD or controls to prevent any possible cross-contamination.

5.5. Conducting the Bioassay (Day 5)

5.5.1. Preparation steps (prior to assay)

a. Inspect plates visually with and without microscope - check degree of confluence, homogeneity from well-to-well, and any signs of cytotoxicity or altered morphology

b. Set up cytofluor for viability assay:

set 1: excitation = B emission = B sensitivity = 3
set 2: excitation = C emission = E sensitivity = 4

***NOTE**: sensitivity can be adjusted if values are too high (9999) or low (not different from blanks)*

c. Preparation of viability assay reagent (refer to Supplies and Biochemicals Section):

Each plate will need 1.8 ml or 3 ml, depending on whether 36 or 60 wells are used per plate, respectively plus a little extra (2 ml). Dilute the appropriate amounts of calcein and ethidium with the appropriate volume of media without FBS as shown below:

Number of plates	Volume of viability assay reagent needed [total volume (ml); calcein stock (µl); Ethidium stock (µl)]	
	using 36 wells	Using 60 wells
1	3.8 ml; 0.95 µl; 1.9 µl	5 ml; 1.25 µl; 2.5 µl
2	5.6 ml; 1.4 µl; 2.8 µl	8 ml; 2 µl; 4 µl
3	7.4 ml; 1.85 µl; 3.7 µl	11 ml; 2.75 µl; 5.5 µl
4	9.2 ml; 2.3 µl; 4.6 µl	14 ml; 3.5 µl; 7 µl
5	11 ml; 2.75 µl; 5.5 µl	17 ml; 4.25 µl; 8.5 µl
6	12.8 ml; 3.2 µl; 6.4 µl	20 ml; 5 µl; 10 µl
7	14.6 ml; 3.65 µl; 7.3 µl	23 ml; 5.75 µl; 11.5 µl
8	16.4 ml; 4.1 µl; 8.2 µl	26 ml; 6.5 µl; 13 µl

***CAUTION**: ethidium homodimer is a powerful mutagen - handle with care and throw contaminated tips, etc., into biohazard bags*

d. Set up luminometer:

Mode = Cycle; **Pause** = 2 sec;
Data = All; **Mix** = Off;
Gain = High; **Temp.** = 30^0C;
Cycle = 1-3; **A/D reads** = 20 (number of times that each well is analyzed per cycle)

e. Set up vacuum aspirator (attach p-10 tips to each suction line to minimize scraping surface area and only use the plate washer to aspirate and **not** to dispense PBS)

f. Prepare LucLite substrate solution and luciferase positive control (must be used on the same day as prepared)

1) Reconstitute lyophilized substrate solution by adding 10 ml of Assay buffer solution A (provided with kit) to one vial of lyophilized substrate (each vial is enough for 133 assays if 75 µl is used). Agitate gently until a homogeneous solution is formed (a slight turbidity is acceptable). Equilibrate to room temperature before use.

***NOTE**: if more than one vial is reconstituted, combine and mix to prevent any variation from the substrate between plates.*

2) Reconstitute the lyophilized luciferase positive control with 200 µl of distilled (or nanopure water). Each vial contains sufficient luciferase for 20 controls.

5.5.2. Cell Viability Assay Procedure (process one plate at a time)

a. remove plate from incubator and aspirate media, then rinse 1 time with PBS

b. Add 50 µl of PBS with Ca^{2+} and Mg^{2+} to all wells using a 8-channel pipet

c. Add 50 µl of viability assay reagent to all wells using a 8-channel pipet

d. Incubate at room temperature for 10 minutes and then scan plate in the Cytofluor instrument

e. Export/print data (check that values are appropriate, otherwise adjust sensitivity and rescan). Password protect data files with a project-specific password. Data analysis are discussed in Section E along with an example raw data file and a Microsoft Excel spreadsheet version of a sample data analysis.

f. Aspirate viability reagent/PBS, and rinse 1 time with PBS using vacuum aspirator

g. Seal the bottom of the ViewPlates with self-adhesive TopSeal (or tape very well). This increases the signal detection in the luminometer.

h. Add 75 µl of PBS with Ca^{2+} and Mg^{2+} (or media without serum or phenol red) to all wells using an 8-channel pipet

i. Working under SUBDUED light conditions, add 75 µl/well of reconstituted LucLite substrate solution, cover with foil, and agitate gently.

j. Wait twenty minutes before counting the plate to allow full signal generation.

k. Measure luminescence on the Dynatech ML3000 luminometer

l. Save, print, and copy data to disk. Password protect data files with a project-specific password. Data analysis are discussed in Section E along with an example raw data file and a Microsoft Excel spreadsheet version of a sample data analysis.

5.6. Protein Determination

Protein determination is readily accomplished in the same plates with same cell lysates after measuring luminescence. A fluorescamine-based protein assay (Sanderson *et al.*, 1996) or Micro-BCA Assay (available from Pierce (800) 874-3723) can be used. Degree of confluency of wells was verified microscopically, and in most cases this made normalization to protein unnecessary; therefore, luciferase activity is reported as either relative light units (RLU) or percent of solvent control.

5.7. Data Analysis

5.7.1. Viability Data

Average the three measurements for calcein AM and ethidium. Divide the average calcein AM fluorescence for each sample by its ethidium homodimer fluorescence to obtain a live to dead ratio. Graph the average calcein AM fluorescence and standard deviation for the negative control, solvent control, and each concentration tested. Examine the calcein AM data visually. If the blank has greater viability than the other treatments, the solvent may be toxic to the cells. If viability decreases with increasing concentration of the test substance, the test substance may be toxic to the cells. In either of these cases, the luciferase data must be regarded with great suspicion. If the solvent is toxic, try a different solvent or a lower concentration of solvent. If the test substance is toxic, try extracting the toxic component (*e.g.*, removing sulfur compounds from sediment extracts), or conclude that cytotoxicity is likely to preclude any dioxin-like effects.

5.7.2. Luciferase Data

Calculate averages and standard deviations for each treatment. Graph the change in response and its standard deviation against concentration. Relative potencies should be calculated through conversion of the data to probit values or any other appropriate transformation that linearizes the sigmoidal dose-response curve. For a complete description and decision tree for data analysis methods, refer to the Standard Operating Procedures entitled, "Estimation of Relative Potencies Based on In Vitro Bioassay Results". To convert data to probit values, the concentration producing maximal induction is set at 100% and the responses of all other concentrations are converted to a percentage of this maximal level. A lookup table function can be used in a spreadsheet program such as Excel or Lotus 1,2,3 to convert percentages to probit values (contact the Aquatic Toxicology Laboratory for a copy of this table on disk).

6.0. RECORDS, DOCUMENTATION, AND QC REQUIREMENTS

6.1 Records and Documentation

The primary analyst shall document any anomalies and/or deviation from the specified method in a bound, serially numbered, laboratory notebook with tear-out carbon copies. All electronic files and hardcopies will be kept at the Aquatic Toxicology Laboratory at Michigan State University and a duplicate

copy will be kept in the Archive Room of Dr. John Giesy (Dept. of Zoology, Michigan State University). This information will also be recorded in the data package and listed in the Case Narrative Form (in accordance with SOP 802). The primary analyst will sign and date any forms as the analyst.

The technical reviewer will record any problems noted during the technical review. The technical reviewer will return the items to the analyst for corrections prior to inclusion into the data package. The technical reviewer will sign and date all forms as the reviewer.

6.2 QC Requirements and Data Quality Objectives

The threshold dose and EC_{50} for luciferase induction in H4IIE-luc cells were approximately 0.1 and 1.2 pg/well, respectively, as determined from 41 separate standard TCDD curves analyzed in 1997 (Figure 1). Coefficients of variation (standard deviation/mean x 100) for the assay were under 10% at all concentrations tested for any single day of experiments. A proficiency curve is maintained for the EC_{50} and threshold doses for TCDD standard curves (every plate has a standard curve). If for a particular plate, the EC_{50} or threshold for the TCDD standard exceeds $\pm$ 20 % of the proficiency curve, then the sample on the plate in question will be reanalyzed. For a sample size of 20 g tissue and a final extract volume of 0.25 ml, the H4IIE-luc assay will detect 1 part per trillion (ppt; pg/g, wet weight) TCDD-equivalents. With a sample size of 5 g tissue, 4 pg/g (wet weight) TCDD-equivalents will be detected.

7.0. RESPONSIBILITIES

The primary analyst will complete the analysis as specified in this SOP and provide documentation of raw data and any anomalies and provide data to the data analyst who will perform data calculations in accordance with SOP 202.

The technical reviewer will determine if data quality objectives were met, notify the analyst if any problems were found.

8.0. REFERENCES

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ATTACHMENTS

Attachment 201-1: Figure 1 - TCDD Dose-response Curve with H4IIE-luc Cells
Attachment 201-2: Figure 3 - Viability Assay Sample Data Set (Raw Data)
Attachment 201-3: Figure 4 - Viability Assay Sample Data Set (Calculated Data)
Attachment 201-4: Figure 5 - Luciferase Assay Sample Data Set (Raw Data)
Attachment 201-5: Figure 6 - Luciferase Assay Sample Data Set (Calculated Data)

Appendix II: Sample Calculation of the Detection Limit Method and the Slope-Ratio Method

Appendix II - Sample Calculation: Detection Limit Method

Note: Luminescence was measured in relative units. The term 'units' was used to describe this in the calculations below.

To determine sample-specific detection limits the following calculations re performed:

Lot:	BBC
Sample:	96RFGH04
Tissue:	Owl liver
Tissue weight:	0.912 g
Extract volume:	1000 µL
µl extract added per well:	1.25
Mean blank value:	0.005128
Mean standard response at 0.1 pg/well:	0.018183 (least standard concentration)
Mean sample response undiluted:	0.005711 (greatest sample concentration)

Gram equivalents/µL extract $\frac{\text{tissue wt}}{\text{Final volume}} = \frac{0.192\text{g}}{1000\mu\text{L}} = 0.000912\ \text{g}/\mu\text{L}$

Therefore g-eq added to well in 1.25 µL = 0.000912*1.25 = 0.00114 **(A)**

Relative response sample/standard

$$= \frac{\text{(mean sample – mean blank)}}{\text{(mean std at 0.1 pg/well – mean blank)}}$$

$$= \frac{(0.005711-0.005128)}{(0.018183-0.005128)}$$

$$= 0.04466$$

Standard was 0.1 pg therefore the well contains 0.04466*0.1 pg TCDD

$$= 0.004466 \text{ pg TCDD}$$

Tissue equivalents in the well $= 0.00114$ g **(A)**

Equivalents concentration $= \frac{0.004466 \text{ pg}}{0.00114 \text{ g}}$

$$= 3.918 \text{ pg/g TCDD-EQ}$$

$TCDD\text{-}EQ_{MAx}$ Reported detection Limit = 4 pg/g TCDD-EQ w/w

$TCDD\text{-}EQ_{MIN}$ Reported detection Limit = 0 pg/g TCDD-EQ w/w

Appendix II- Sample Calculation: Slope Ratio Method.

This method requires the determination of the slope of the linear portion of the dose response curve for the standard and the sample run on the same plate. This evaluation is best performed in SYSTAT where interactive data point selection, graphing and regression analysis are available. The determination of the linear portion of the curve is a judgement and experience based assessment not amenable to strict standardization. To attain statistical rigor in the analysis, two criteria are applied to each sample analyzed by this method.

1. Minimize data removal to obtain slope
2. Maximize r^2 for the regression line.

Once slopes for the two lines have been determined the simple ratio of these slopes is the TCDD-EQ concentration of the sample, since the dependent for the standard is pg/well and the dependent for the sample is g-eq/well.

Lot:	**BBB**
Sample:	**96FGH007**
Tissue:	**owl liver**
Tissue Weight:	**3.9553 g**
Extract Volume:	**1000 µL**
µL extract added per well:	**1.25**
Slope Standard dose response:	**0.073**
Slope sample dose response:	**78.268**

Gram equivalents/µL extract = $\frac{\text{tissue wt}}{\text{Final volume}} = \frac{3.9553\ \text{g}}{1000\ \mu\text{L}}$

= 0.0039553 geq/µL

Sample concentration = $\frac{\text{Sample Slope}}{\text{Standard Slope}}$

= $\frac{78.268}{0.073}$

= 1072.16 pg/g TCDD-EQ w/w

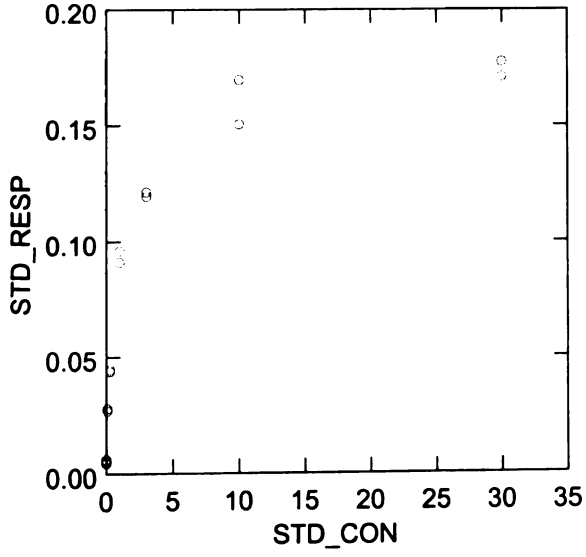
Reported value = 1072 pg/g TCDD-EQ w/w

All anotations added after SYSTAT output are identified in bold underlined italic

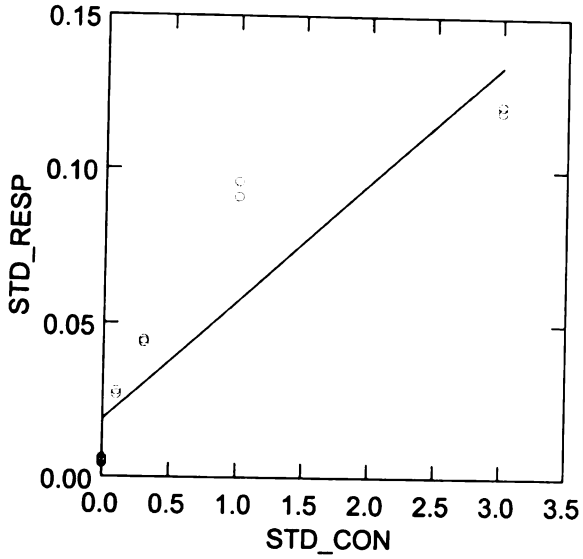
SYSTAT Rectangular file R:\lot BBB\96fgh007 positive.SYD,
created Tue Oct 05, 1999 at 15:47:18, contains variables:

SAMP$	TYPE$	SAM_DIL	SAM_RESP	STD_CON	STD_RESP
POS	TISSUE	GEQ			

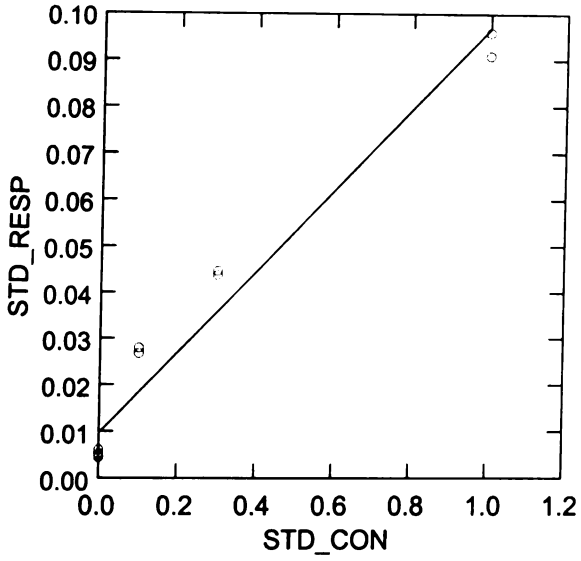
This is the frst plot of the standard response (Y) against pg TCDD added to each well (x). Values used are the mean luminesence for each replicate. Clearly the points at 10 and 30 pg are not part of the linear portion of the curve.



This is the same plot with the data for 10 and 30 pg removed and a line added to provide an estimator for linearity. Clearly the points at 3 pg/well are not part of the linear portion of the curve.



Below is the curve with the 3 pg/well data removed providing a much better linear fit.



Below is the linear regression and correlation data for the above plot. The only concern would be that the 6 blank values are pulling the end of the line down unduely - try removing these points.

Dep Var: STD_RESP N: 12 Multiple R: 0.980 Squared multiple R: 0.961

Adjusted squared multiple R: 0.958 Standard error of estimate: 0.007

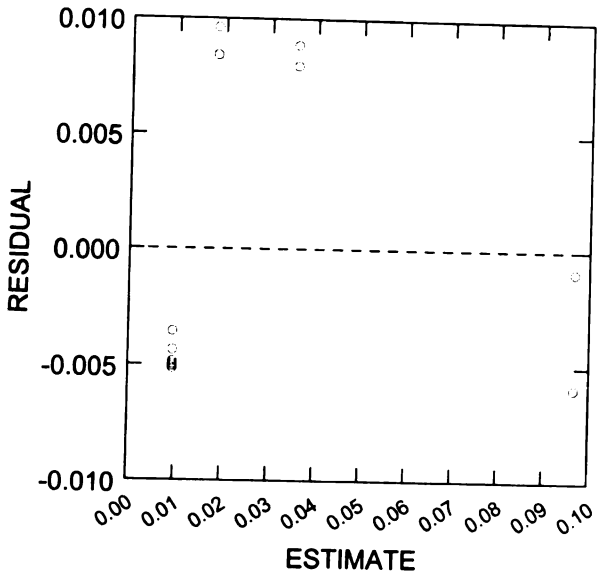
Effect	Coefficient	Std Error	Std Coef	Tolerance	t	P(2 Tail)
CONSTANT	0.010	0.002	0.000	.	4.039	0.002
STD_CON	0.087	0.006	0.980	1.000	15.776	0.000

Analysis of Variance

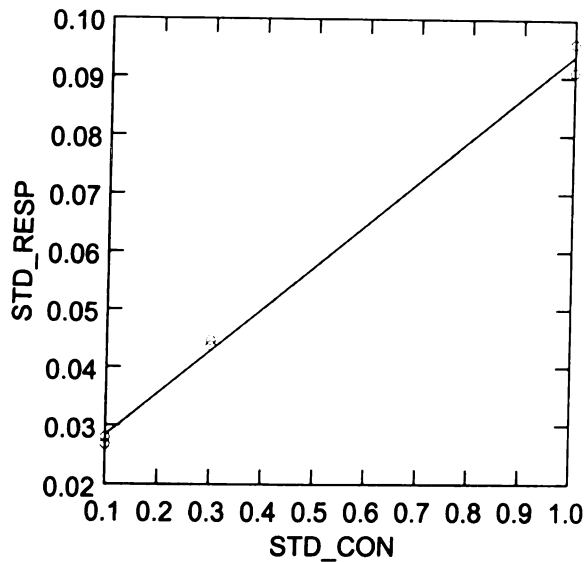
Source	Sum-of-Squares	df	Mean-Square	F-ratio	P
Regression	0.012	1	0.012	248.879	0.000
Residual	0.000	10	0.000		

Durbin-Watson D Statistic 0.233
First Order Autocorrelation 0.76

Plot of Residuals against Predicted Values



Here is the plot with the blanks removed, clearly a very good linear fit.



Dep Var: STD_RESP N: 6 Multiple R: 0.998 Squared multiple R: 0.996

Adjusted squared multiple R: 0.995 Standard error of estimate: 0.002

Effect	Coefficient	Std Error	Std Coef	Tolerance	t	P(2 Tail)
CONSTANT	0.021	0.001	0.000	.	14.971	0.000
STD_CON	0.073	0.002	0.998	1.000	31.278	0.000

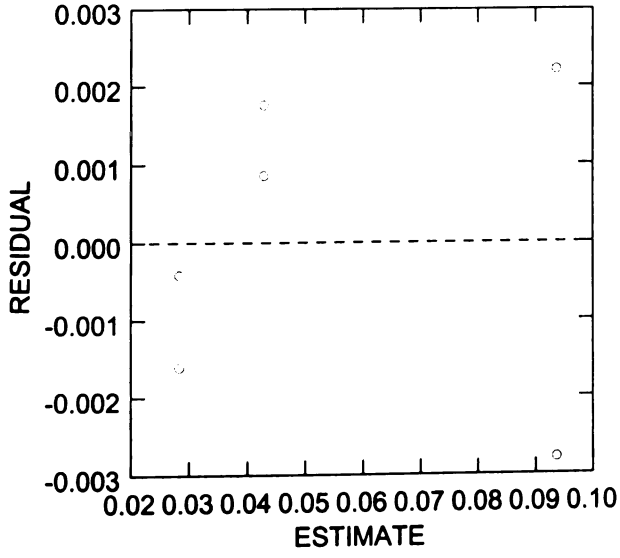
Analysis of Variance

Source	Sum-of-Squares	df	Mean-Square	F-ratio	P
Regression	0.005	1	0.005	978.342	0.000
Residual	0.000	4	0.000		

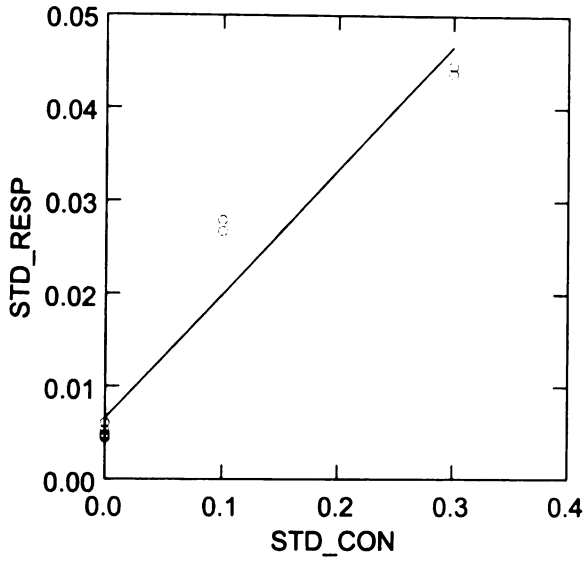
*** WARNING ***
Case 31 is an outlier (Studentized Residual = -3.306)

Durbin-Watson D Statistic 2.099
First Order
Autocorrelation.....0.256

Plot of Residuals against Predicted Values



But we could also have removed the two higher points and kept the blank values in - what would this have done ?



Dep Var: STD_RESP N: 10 Multiple R: 0.972 Squared multiple R: 0.945

Adjusted squared multiple R: 0.938 Standard error of estimate: 0.004

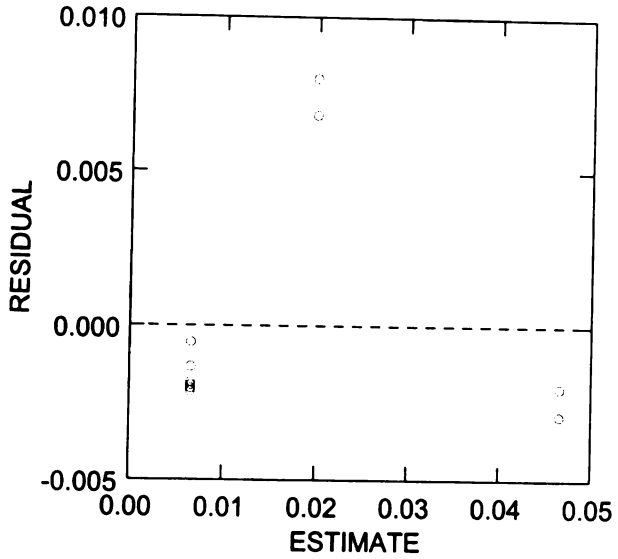
Effect	Coefficient	Std Error	Std Coef	Tolerance	t	P(2 Tail)
CONSTANT	0.007	0.002	0.000	.	4.067	0.004
STD_CON	0.134	0.011	0.972	1.000	11.741	0.000

Analysis of Variance

Source	Sum-of-Squares	df	Mean-Square	F-ratio	P
Regression	0.002	1	0.002	137.845	0.000
Residual	0.000	8	0.000		

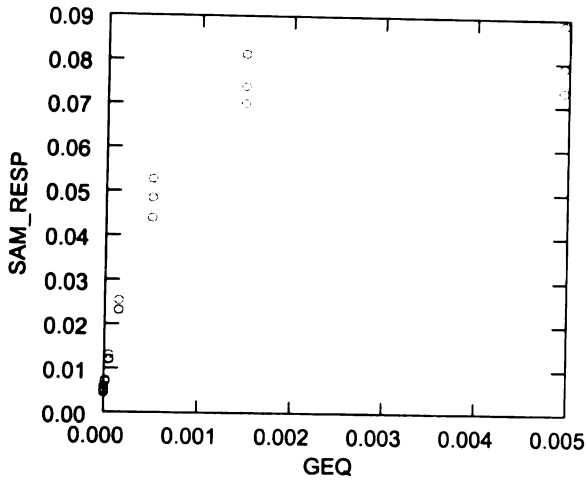
*** WARNING ***
Case 36 is an outlier (Studentized Residual = 2.680)
Durbin-Watson D Statistic 0.595
First Order Autocorrelation 0.469

Plot of Residuals against Predicted Values

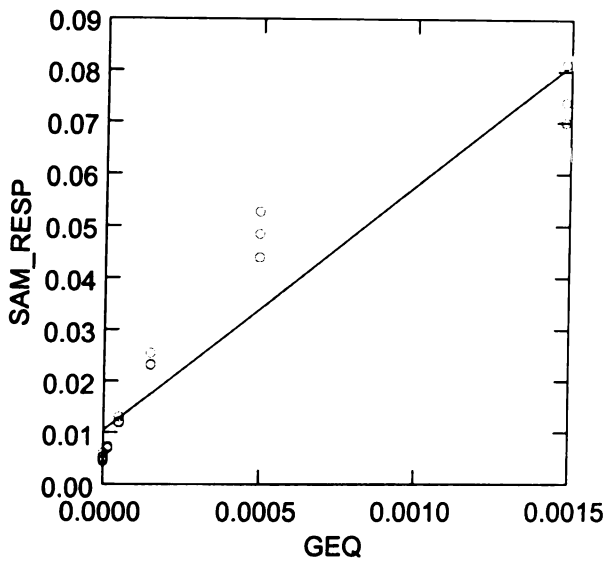


Not a bad line but the correlation data is worse than the previous scenario. For this standard slope is 0.072 with r2 = 0.995

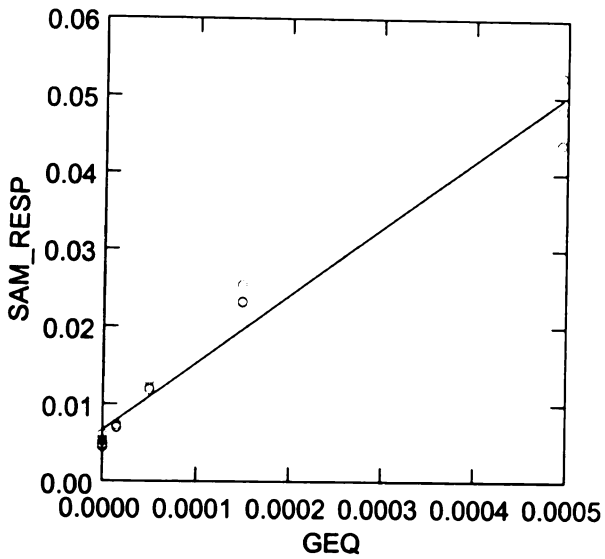
Now here is the first plot for the sample extract. Again the upper most points hace clearly plateaued. This sample could be analysed by the probit transform method but to ensure comparability with other samples it will be analysed with the slope ratio method.



Remove the top two data points and fit a line - new top points still not linear - remove two more



This is a better fit but as with the standard the blanks are pulling the line down - remove



Dep Var: SAM_RESP N: 18 Multiple R: 0.985 Squared multiple R: 0.970

Adjusted squared multiple R: 0.968 Standard error of estimate: 0.003

Effect	Coefficient	Std Error	Std Coef	Tolerance	t	P(2 Tail)
CONSTANT	0.007	0.001	0.000	.	8.101	0.000
GEQ	87.224	3.860	0.985	1.000	22.597	0.000

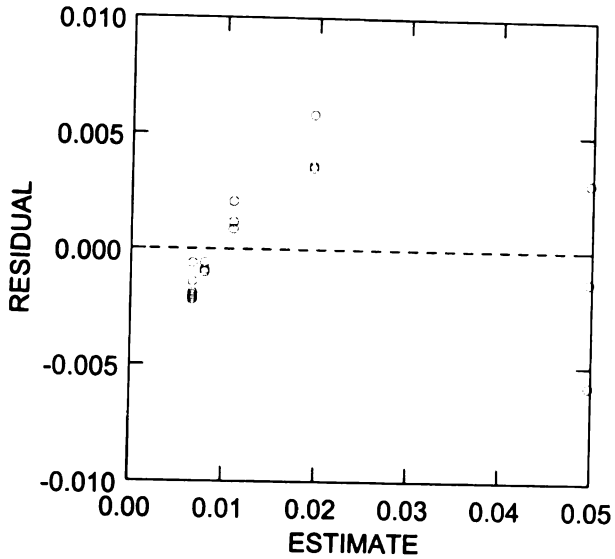
Analysis of Variance

Source	Sum-of-Squares	df	Mean-Square	F-ratio	P
Regression	0.004	1	0.004	510.628	0.000
Residual	0.000	16	0.000		

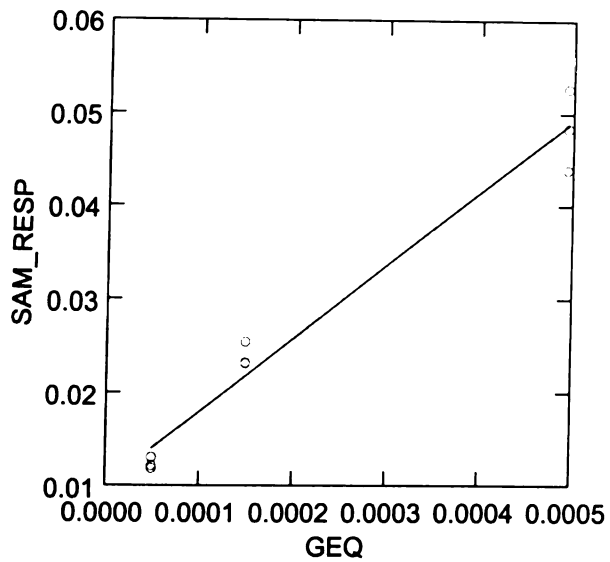
*** WARNING ***
Case 9 is an outlier (Studentized Residual = -2.986)

Durbin-Watson D Statistic 1.572
First Order Autocorrelation 0.193

Plot of Residuals against Predicted Values



This is a better fit and a better correlation - for this sample slope is 78.268 with r2 = 0.9



Dep Var: SAM_RESP N: 9 Multiple R: 0.984 Squared multiple R: 0.968

Adjusted squared multiple R: 0.963 Standard error of estimate: 0.003

Effect	Coefficient	Std Error	Std Coef	Tolerance	t	P(2 Tail)
CONSTANT	0.010	0.002	0.000	.	6.286	0.000
GEQ	78.268	5.377	0.984	1.000	14.556	0.000

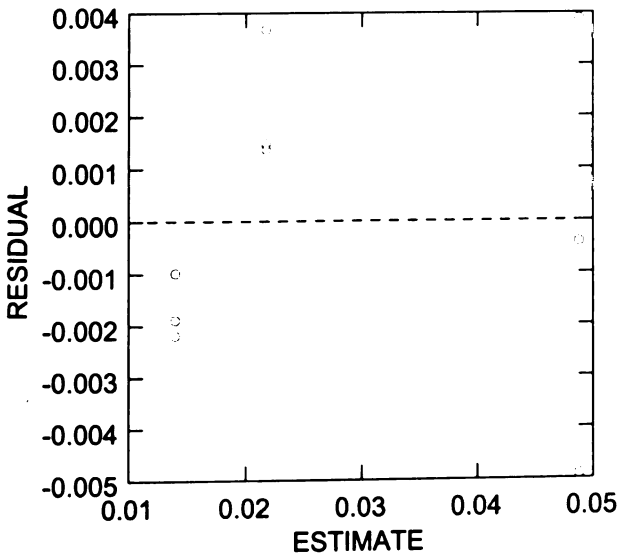
Analysis of Variance

Source	Sum-of-Squares	df	Mean-Square	F-ratio	P
Regression	0.002	1	0.002	211.877	0.000
Residual	0.000	7	0.000		

*** WARNING ***
Case 9 is an outlier (Studentized Residual = -2.647)

Durbin-Watson D Statistic 2.401
First Order Autocorrelation -0.238

Plot of Residuals against Predicted Values



Appendix III: Quality Control/ Quality Assurance Samples

Table III-a: MSU Quality Control / Quality Assurance Samples

Sample number	Tissue	TCDD-EQ_{MAX}	TCDD-EQ_{MIN}
Method Blank (lot BBA)	carp eggs	1	0
Method Blank (lot BBB)	Carp eggs/ owl livers	1	0
Method Blank (lot BBC)	owl livers	1	0
Method Blank (KKA)	kestrel eggs	1	0
Method Blank (lot KKB)	kestrel eggs	1	0
Method Blank (lot KKC)	kestrel eggs	2	0
Matrix Blank (KKA)	kestrel eggs	5	0
Matrix Blank (KKB)	kestrel eggs	1	0
Matrix Blank (KKC)	kestrel eggs	2	0
Mean		**1.67**	**0**
Method Spike (lot BBA)	carp eggs	1818	1818
Method Spike (lot BBB)	Carp eggs/ owl livers	2169	2169
Method Spike (lot BBC)	owl livers	1856	1856
Mean		**1943**	**1943**
Min		**1555**	**1555**
Max		**2332**	**2332**
Method Spike (KKA)	kestrel eggs	908	908
Method Spike (lot KKB)	kestrel eggs	866	866
Method Spike (lot KKC)	kestrel eggs	986	986
Matrix Spike (KKA)	kestrel eggs	1120	1120
Matrix Spike (KKB)	kestrel eggs	1065	1065
Matrix Spike (KKC)	kestrel eggs	888	888
Mean		**972**	**972**
Min		**778**	**778**
Max		**1167**	**1167**

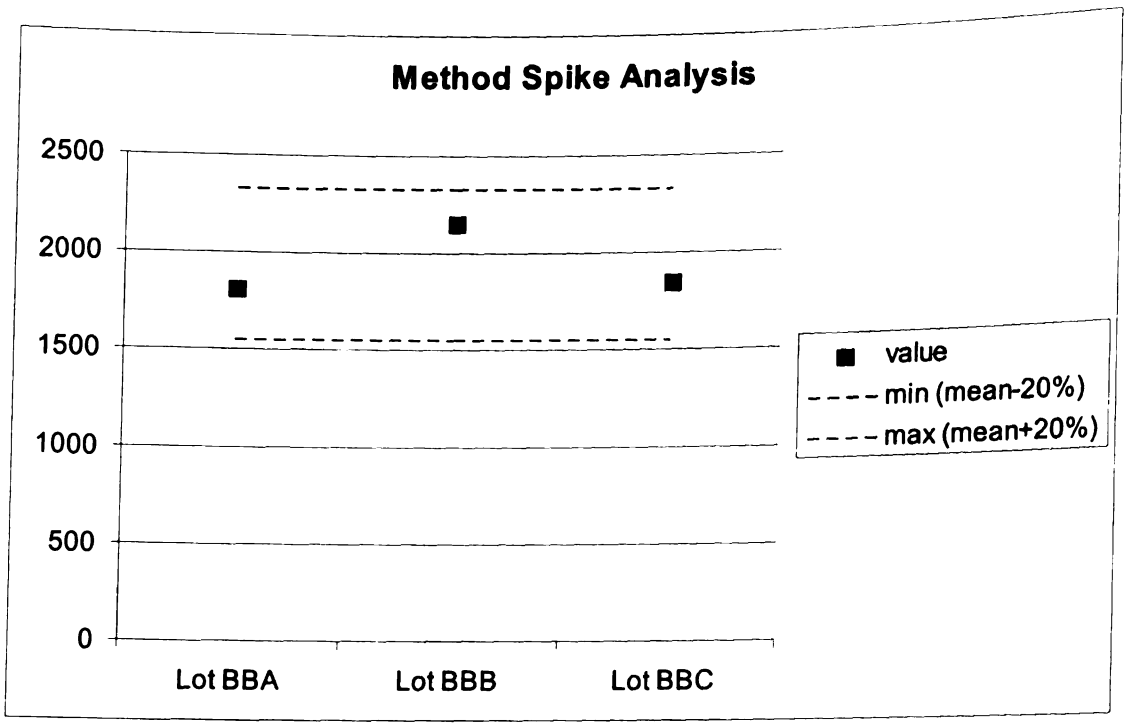


Figure III-a: MSU method spike quality control for lots BBA, BBB, and BBC

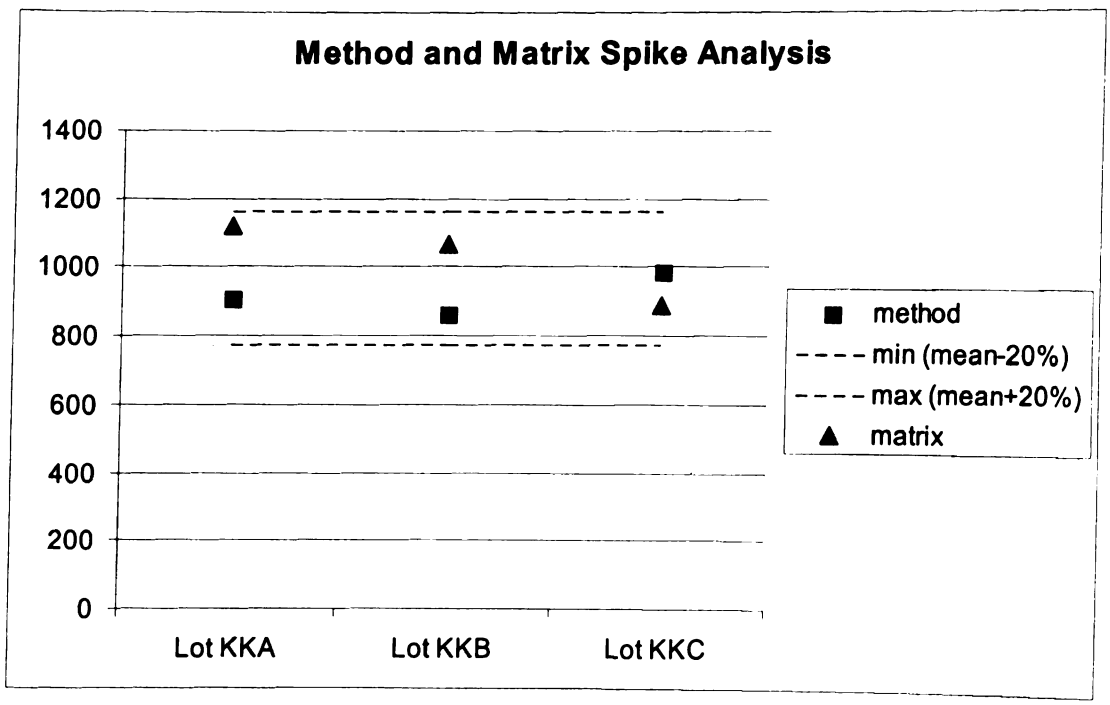


Figure III-b: MSU method and matrix spike quality control for lots KKA, KKB, and KKC

Appendix IV – Complete Results for Carp Egg Samples

Table IV-a: Complete listing of concentrations of TEQ and HQ values for carp egg extracts (pg/g w/w)

SAMPLE On-post	TOT TEQ_{MIN} (pg/g w/w)	% PCBs MIN	TOT TEQ_{MAX} (pg/g w/w)	% PCBs MAX	† HQ_{MIN} (TEQ based)	† HQ_{MAX} (TEQ based)	† HQ_{MIN} (TCDD-EQ based)	† HQ_{MAX} (TCDD-EQ based)
H96DCP01	$5.6x10^{-1}$	$1.0x10^{1}$	$1.9x10^{0}$	$3.0x10^{0}$	$0.0x10^{0}$	$1.0x10^{-2}$	$0.0x10^{0}$	$4.0x10^{-2}$
H96DCP02	$3.9x10^{-1}$	$3.3x10^{1}$	$1.5x10^{0}$	$8.9x10^{0}$	$0.0x10^{0}$	$1.0x10^{-2}$	$0.0x10^{0}$	$4.0x10^{-2}$
H96DCP06	$2.6x10^{-1}$	$1.7x10^{1}$	$1.4x10^{0}$	$3.2x10^{0}$	$0.0x10^{0}$	$1.0x10^{-2}$	$0.0x10^{0}$	$2.0x10^{-2}$
H96DCP07	$2.0x10^{-1}$	$5.4x10^{1}$	$2.0x10^{0}$	$5.4x10^{0}$	$0.0x10^{0}$	$1.0x10^{-2}$	$0.0x10^{0}$	$5.0x10^{-2}$
H96DCP08	$4.3x10^{-1}$	$2.3x10^{1}$	$1.8x10^{0}$	$5.7x10^{0}$	$0.0x10^{0}$	$1.0x10^{-2}$	$0.0x10^{0}$	$9.0x10^{-2}$
H96DCP11	$3.2x10^{-1}$	$1.9x10^{1}$	$1.4x10^{0}$	$4.2x10^{0}$	$0.0x10^{0}$	$1.0x10^{-2}$	$0.0x10^{0}$	$2.0x10^{-2}$
H96DCP12	$2.6x10^{-1}$	$1.1x10^{1}$	$1.1x10^{0}$	$2.5x10^{0}$	$0.0x10^{0}$	$1.0x10^{-2}$	$0.0x10^{0}$	$3.0x10^{-2}$
H96DCP14	$4.5x10^{-1}$	$1.6x10^{1}$	$1.8x10^{0}$	$3.8x10^{0}$	$0.0x10^{0}$	$1.0x10^{-2}$	$1.0x10^{-2}$	$1.0x10^{-2}$
H96DCP15	$2.9x10^{-1}$	$2.3x10^{1}$	$1.8x10^{0}$	$3.6x10^{0}$	$0.0x10^{0}$	$1.0x10^{-2}$	$0.0x10^{0}$	$2.0x10^{-2}$
H96DCP16	$4.4x10^{-1}$	$2.4x10^{1}$	$2.0x10^{0}$	$5.1x10^{0}$	$0.0x10^{0}$	$1.0x10^{-2}$	$0.0x10^{0}$	$1.0x10^{-2}$
H96DCP17	$5.6x10^{-1}$	$7.2x10^{0}$	$1.4x10^{0}$	$2.9x10^{0}$	$0.0x10^{0}$	$1.0x10^{-2}$	$0.0x10^{0}$	$3.0x10^{-2}$
H96DCP18	$5.5x10^{-1}$	$2.2x10^{1}$	$1.5x10^{0}$	$8.1x10^{0}$	$0.0x10^{0}$	$1.0x10^{-2}$	$1.6x10^{-1}$	$1.6x10^{-1}$
H96DCP19	$4.1x10^{-1}$	$8.5x10^{0}$	$1.0x10^{0}$	$3.6x10^{0}$	$0.0x10^{0}$	$1.0x10^{-2}$	$0.0x10^{0}$	$1.0x10^{-2}$
H96DCP20	$3.1x10^{-1}$	$1.1x10^{1}$	$1.3x10^{0}$	$2.8x10^{0}$	$0.0x10^{0}$	$1.0x10^{-2}$	$0.0x10^{0}$	$1.0x10^{-2}$
H96DCP10	$4.3x10^{-1}$	$1.7x10^{1}$	$1.4x10^{0}$	$5.0x10^{0}$	$0.0x10^{0}$	$1.0x10^{-2}$	$0.0x10^{0}$	$3.0x10^{-2}$
H96DCP13	$2.0x10^{-1}$	$3.2x10^{1}$	$1.8x10^{0}$	$3.5x10^{0}$	$0.0x10^{0}$	$1.0x10^{-2}$	$2.2x10^{-1}$	$2.2x10^{-1}$
MEAN (On-Post)	**$3.8x10^{-1}$**	**$2.0x10^{1}$**	**$1.6x10^{0}$**	**$4.5x10^{0}$**	**$0.0x10^{0}$**	**$1.0x10^{-2}$**	**$3.0x10^{-2}$**	**$5.0x10^{-2}$**
Reference								
H97BCP10	4.4x10-1	$1.2x10^{0}$	$9.2x10^{-1}$	$5.5x10^{-1}$	$0.0x10^{0}$	$1.0x10^{-2}$	$0.0x10^{0}$	$2.0x10^{-2}$
H97CP13	$2.4x10^{-1}$	$8.0x10^{0}$	$7.6x10^{-1}$	$2.5x10^{0}$	$0.0x10^{0}$	$0.0x10^{0}$	$0.0x10^{0}$	$1.0x10^{-2}$
MEAN (Reference)	**$3.4x10^{-1}$**	**$4.6x10^{0}$**	**$8.4x10^{-1}$**	**$1.5x10^{0}$**	**$0.0x10^{0}$**	**$0.0x10^{0}$**	**$0.0x10^{0}$**	**$1.0x10^{-2}$**

TOT = Contribution of PCBs, PCDDs, and PCDFs

$_{MIN}$ = 0 used for "non-detect" congeners; $_{MAx}$ = MDL used for "non-detect" congeners

† 170 pg/g TCDD was used as the TRV for these calculations

Table IV-b: Complete listing of concentrations of TCDD-EQ and TEQ_{REP} values for carp egg extracts

SAMPLE On-Post	TCDD-EQ $_{MIN}$ (pg/g w/w)	$TEQ_{REP\ MIN}$ (pg/g w/w)	$TCDD\text{-}EQ_{MAX}$ (pg/g w/w)	$TEQ_{REP\ MAX}$ (pg/g w/w)
H96DCP01	$0.0x10^{0}$	$6.7x10^{-1}$	$6.0x10^{0}$	$4.0x10^{0}$
H96DCP02	$0.0x10^{0}$	$8.5x10^{-1}$	$6.0x10^{0}$	$3.1x10^{0}$
H96DCP06	$0.0x10^{0}$	$4.3x10^{-1}$	$3.0x10^{0}$	$2.7x10^{0}$
H96DCP07	$0.0x10^{0}$	$7.9x10^{-1}$	$8.0x10^{0}$	$3.6x10^{0}$
H96DCP08	$0.0x10^{0}$	$7.9x10^{-1}$	* $1.5x10^{1}$	$4.1x10^{0}$
H96DCP11	$0.0x10^{0}$	$5.4x10^{-1}$	$3.0x10^{0}$	$3.1x10^{0}$
H96DCP12	$0.0x10^{0}$	$3.1x10^{-1}$	$5.0x10^{0}$	$2.1x10^{0}$
H96DCP14	$2.0x10^{0}$	$6.5x10^{-1}$	$2.0x10^{0}$	$3.4x10^{0}$
H96DCP15	$0.0x10^{0}$	$6.4x10^{-1}$	$4.0x10^{0}$	$5.6x10^{0}$
H96DCP16	$0.0x10^{0}$	$8.2x10^{-1}$	$1.0x10^{0}$	$5.5x10^{0}$
H96DCP17	$0.0x10^{0}$	$6.4x10^{-1}$	$5.0x10^{0}$	$3.2x10^{0}$
H96DCP18	$2.8x10^{1}$	$8.7x10^{-1}$	* $2.8x10^{1}$	$3.0x10^{0}$
H96DCP19	$0.0x10^{0}$	$3.9x10^{-1}$	$2.0x10^{0}$	$1.3x10^{0}$
H96DCP20	$0.0x10^{0}$	$4.8x10^{-1}$	$1.0x10^{0}$	$2.7x10^{0}$
H96DCP10	$0.0x10^{0}$	$6.6x10^{-1}$	$5.0x10^{0}$	$3.0x10^{0}$
H96DCP13	*$3.8x10^{1}$	$6.7x10^{-1}$	* $3.8x10^{1}$	$4.1x10^{0}$
MEAN (On-Post)	**$4.3x10^{0}$**	**$6.4x10^{-1}$**	**$8.5x10^{0}$**	**$3.4x10^{0}$**
Reference				
H97BCP10	$0.0x10^{0}$	$4.0x10^{-1}$	$3.0x10^{0}$	$1.4x10^{0}$
H97CP13	$0.0x10^{0}$	$2.5x10^{-1}$	$2.0x10^{0}$	$1.6x10^{0}$
MEAN (Reference)	**$0.0x10^{0}$**	**$3.3x10^{-1}$**	**$2.5x10^{0}$**	**$1.5x10^{0}$**

TCDD-EQ = bioassay results

TEQ_{REP} = analytical chemistry results multiplied by H4IIE relative potencies

$_{MIN}$ = 0 used for "non-detect" congeners; $_{MAX}$ = MDL used for "non-detect" congener

*** indicates outlying carp egg samples in the Chi-square analysis**

Appendix V: Complete Results for Owl Liver Samples

Table V-a: Complete listing of concentrations of TEQ and HQ values for owl liver extracts

SAMPLE	AGE	TOT TEQ_{MIN} (pg/g w/w)	% PCB_{MIN}	TOT TEQ_{MAX} (pg/g w/w)	% PCB_{MAX}	HQ_{MIN} (TEQ based)	HQ_{MAX} (TEQ based)	HQ_{MIN} (TCDD-EQ based)	HQ_{MAX} (TCDD-EQ based)
On-post									
96FGH002	A	$8.0x10^{2}$	$5.1x10^{1}$	$8.1x10^{2}$	$5.0x10^{1}$	$5.3x10^{1}$	$5.4x10^{1}$	$3.0x10^{1}$	$3.0x10^{1}$
96FGH007	A	$2.8x10^{3}$	$1.5x10^{1}$	$2.8x10^{3}$	$1.5x10^{1}$	$1.8x10^{2}$	$1.9x10^{2}$	$7.2x10^{1}$	$7.2x10^{1}$
96FGH017	A	$8.0x10^{2}$	$2.6x10^{1}$	$8.1x10^{2}$	$2.6x10^{1}$	$5.4x10^{1}$	$5.4x10^{1}$	$1.7x10^{1}$	$1.7x10^{1}$
96FGH026	J	$7.3x10^{1}$	$4.8x10^{1}$	$7.9x10^{1}$	$4.4x10^{1}$	$4.8x10^{0}$	$5.3x10^{0}$	$7.9x10^{0}$	$7.9x10^{0}$
96FGH027	J	$8.3x10^{1}$	$3.0x10^{1}$	$8.3x10^{1}$	$3.0x10^{1}$	$5.5x10^{0}$	$5.5x10^{0}$	$0.0x10^{0}$	$1.0x10^{0}$
96FGH044	J	$1.6x10^{2}$	$3.9x10^{1}$	$1.6x10^{2}$	$3.9x10^{1}$	$1.1x10^{1}$	$1.1x10^{1}$	$0.0x10^{0}$	$6.7x10^{-2}$
96FGH144	A	$3.2x10^{2}$	$3.5x10^{1}$	$3.2x10^{2}$	$3.5x10^{1}$	$2.1x10^{1}$	$2.1x10^{1}$	$1.3x10^{1}$	$1.3x10^{1}$
95FGH162	U	$2.8x10^{1}$	$2.5x10^{1}$	$3.0x10^{1}$	$2.3x10^{1}$	$1.9x10^{0}$	$2.0x10^{0}$	$0.0x10^{0}$	$3.3x10^{-1}$
96FGH190	J	$1.1x10^{1}$	$5.9x10^{1}$	$1.3x10^{1}$	$4.6x10^{1}$	$7.0x10^{-1}$	$8.9x10^{-1}$	$0.0x10^{0}$	$6.7x10^{-2}$
96FGH231	U	$2.0x10^{1}$	$3.7x10^{1}$	$2.3x10^{1}$	$3.3x10^{1}$	$1.3x10^{0}$	$1.5x10^{0}$	$0.0x10^{0}$	$6.7x10^{-1}$
96FGH232	U	$4.2x10^{1}$	$2.5x10^{1}$	$4.3x10^{1}$	$2.4x10^{1}$	$2.8x10^{0}$	$2.9x10^{0}$	$0.0x10^{0}$	$6.7x10^{-2}$
96FGH250	U	$1.3x10^{2}$	$2.7x10^{1}$	$1.3x10^{2}$	$2.7x10^{1}$	$8.7x10^{0}$	$8.8x10^{0}$	$0.0x10^{0}$	$4.0x10^{-1}$
96FGHL217	J	$5.0x10^{1}$	$3.9x10^{1}$	$5.3x10^{1}$	$3.8x10^{1}$	$3.3x10^{0}$	$3.5x10^{0}$	$0.0x10^{0}$	$2.7x10^{-1}$
96FGHL219	J	$2.0x10^{1}$	$6.9x10^{1}$	$2.2x10^{1}$	$6.1x10^{1}$	$1.3x10^{0}$	$1.5x10^{0}$	$0.0x10^{0}$	$3.3x10^{-1}$
GHL31SE96	J	$2.6x10^{0}$	$7.2x10^{1}$	$5.0x10^{0}$	$3.7x10^{1}$	$1.7x10^{-1}$	$3.3x10^{-1}$	$0.0x10^{0}$	$1.3x10^{-1}$
GHL34SE96	J	$2.2x10^{1}$	$2.2x10^{1}$	$2.4x10^{1}$	$2.1x10^{1}$	$1.5x10^{0}$	$1.6x10^{0}$	$0.0x10^{0}$	$5.3x10^{-1}$
MEAN (On-Post)		$3.3x10^{2}$	$3.9x10^{1}$	$3.4x10^{2}$	$3.4x10^{1}$	$2.2x10^{1}$	$2.2x10^{1}$	$8.7x10^{0}$	$8.9x10^{0}$
Reference									
96RFGH01	A	$1.2x10^{1}$	$4.9x10^{1}$	$2.1x10^{1}$	$3.0x10^{1}$	$8.1x10^{-1}$	$1.4x10^{0}$	$0.0x10^{0}$	$6.7x10^{-2}$
96RFGH02	J	$1.2x10^{1}$	$2.2x10^{1}$	$1.9x10^{1}$	$2.1x10^{1}$	$8.1x10^{-1}$	$1.3x10^{0}$	$0.0x10^{0}$	$1.3x10^{-1}$
96RFGH03	A	$1.3x10^{2}$	$1.1x10^{1}$	$1.4x10^{2}$	$1.0x10^{1}$	$8.6x10^{0}$	$9.5x10^{0}$	$0.0x10^{0}$	$1.1x10^{0}$
96RFGH04	J	$3.8x10^{1}$	$1.3x10^{1}$	$4.0x10^{1}$	$1.3x10^{1}$	$2.6x10^{0}$	$2.7x10^{0}$	$0.0x10^{0}$	$2.7x10^{-1}$
96RFGH05	A	$2.2x10^{1}$	$4.5x10^{1}$	$2.3x10^{1}$	$4.4x10^{1}$	$1.5x10^{0}$	$1.5x10^{0}$	$0.0x10^{0}$	$2.7x10^{-1}$
96RFGH07	A	$1.4x10^{1}$	$2.2x10^{1}$	$1.5x10^{1}$	$2.0x10^{1}$	$9.5x10^{-1}$	$1.0x10^{0}$	$0.0x10^{0}$	$3.3x10^{-1}$
96RFGH08	U	.	.	.	.	.	.	$0.0x10^{0}$	$8.7x10^{-1}$
96RFGH09	J	$5.6x10^{1}$	$6.1x10^{1}$	$7.7x10^{1}$	$4.5x10^{1}$	$3.7x10^{0}$	$5.1x10^{0}$	$0.0x10^{0}$	$1.3x10^{0}$
96RFGH10	J	$3.3x10^{1}$	$4.1x10^{1}$	$4.3x10^{1}$	$3.3x10^{1}$	$2.2x10^{0}$	$2.8x10^{0}$	$0.0x10^{0}$	$1.3x10^{-1}$
96RFGH11	J	$7.0x10^{0}$	$1.6x10^{1}$	$1.2x10^{1}$	$1.1x10^{1}$	$4.7x10^{-1}$	$7.9x10^{-1}$	$0.0x10^{0}$	$6.7x10^{-2}$
96RFGH12	A	$2.7x10^{1}$	$1.4x10^{1}$	$2.8x10^{1}$	$1.3x10^{1}$	$1.8x10^{0}$	$1.9x10^{0}$	$1.3x10^{1}$	$1.3x10^{1}$
MEAN (Reference)		$3.5x10^{1}$	$2.9x10^{1}$	$4.2x10^{1}$	$2.4x10^{1}$	$2.3x10^{0}$	$2.8x10^{0}$	$1.1x10^{0}$	$1.6x10^{0}$

A = Adult: J = Juvenile; U= unknown age

TOT = Contribution of PCBs, PCDDs, and PCDFs

$_{MIN}$ = 0 used for "non-detect" congeners; $_{MAX}$ = MDL used for "non-detect" congeners

Table V-b: Complete listing of concentrations of TCDD-EQ and TEQ_{REP} values for owl liver extracts

SAMPLE On-post	AGE	TCDD-EQ MIN (pg/g w/w)	TCDD-EQ MAX (pg/g w/w)	TEQ (REP) MIN (pg/g w/w)	TEQ (REP) MAX (pg/g w/w)
96FGH002	A	$4.4x10^{2}$	$4.4x10^{2}$	$3.4x10^{2}$	$5.3x10^{2}$
96FGH007	A	$1.1x10^{3}$	$1.1x10^{3}$	$2.1x10^{3}$	* $2.3x10^{3}$
96FGH017	A	$2.6x10^{2}$	$2.6x10^{2}$	$5.1x10^{2}$	$5.8x10^{2}$
96FGH026	J	$1.2x10^{2}$	$1.2x10^{2}$	$6.8x10^{1}$	$7.5x10^{1}$
96FGH027	J	$0.0x10^{0}$	$1.5x10^{1}$	$9.1x10^{1}$	$9.1x10^{1}$
96FGH044	J	$0.0x10^{0}$	$1.0x10^{0}$	$1.8x10^{2}$	$1.8x10^{2}$
96FGH144	A	$1.9x10^{2}$	$1.9x10^{2}$	$1.2x10^{2}$	$1.3x10^{2}$
95FGH162	U	$0.0x10^{0}$	$5.0x10^{0}$	$2.2x10^{1}$	$2.6x10^{1}$
96FGH190	J	$0.0x10^{0}$	$1.0x10^{0}$	$4.1x10^{0}$	1.5x101
96FGH231	U	$0.0x10^{0}$	$1.0x10^{1}$	$1.7x10^{1}$	$2.3x10^{1}$
96FGH232	U	$0.0x10^{0}$	$1.0x10^{0}$	$3.6x10^{1}$	$4.1x10^{1}$
96FGH250	U	$0.0x10^{0}$	$6.0x10^{0}$	$1.2x10^{2}$	$1.2x10^{2}$
96FGHL217	J	$0.0x10^{0}$	$4.0x10^{0}$	$2.5x10^{1}$	$3.3x10^{1}$
96FGHL219	J	$0.0x10^{0}$	$5.0x10^{0}$	$8.7x10^{0}$	$1.1x10^{1}$
GHL31SE96	J	$0.0x10^{0}$	$2.0x10^{0}$	$1.8x10^{0}$	$3.5x10^{0}$
GHL34SE96	J	$0.0x10^{0}$	$8.0x10^{0}$	$4.3x10^{1}$	$4.7x10^{1}$
MEAN (On-Post)		**$1.3x10^{2}$**	**$1.3x10^{2}$**	**$2.3x10^{2}$**	**$2.6x10^{2}$**
Reference					
96RFGH01	A	$0.0x10^{0}$	$1.0x10^{0}$	$3.1x10^{0}$	$1.2x10^{1}$
96RFGH02	J	$0.0x10^{0}$	$2.0x10^{0}$	$3.7x10^{0}$	$1.0x10^{1}$
96RFGH03	A	$0.0x10^{0}$	$1.6x10^{1}$	$8.6x10^{1}$	$1.2x10^{2}$
96RFGH04	J	$0.0x10^{0}$	$4.0x10^{0}$	$2.4x10^{1}$	$3.6x10^{1}$
96RFGH05	A	$0.0x10^{0}$	$4.0x10^{0}$	$8.3x10^{0}$	$1.2x10^{1}$
96RFGH07	A	$0.0x10^{0}$	$5.0x10^{0}$	$1.1x10^{1}$	$1.3x10^{1}$
96RFGH08	U	$0.0x10^{0}$	$1.3x10^{1}$	.	.
96RFGH09	J	$0.0x10^{0}$	$1.9x10^{1}$	$1.7x10^{1}$	$4.3x10^{1}$
96RFGH10	J	$0.0x10^{0}$	$2.0x10^{0}$	$1.1x10^{1}$	$2.8x10^{1}$
96RFGH11	J	$0.0x10^{0}$	$1.0x10^{0}$	$1.9x10^{0}$	$5.8x10^{0}$
96RFGH12	A	$1.9x10^{2}$	$1.9x10^{2}$	$1.7x10^{1}$	$2.0x10^{1}$
MEAN (Reference)		**$1.7x10^{1}$**	**$2.3x10^{1}$**	**$1.8x10^{1}$**	**$3.0x10^{1}$**

A = Adult; J = Juvenille; U = Unknown age; TCDD-EQ = bioassay results; TEQ_{REP}= analytical chemistry results multiplied by H4IIE relative potencies
$_{MIN}$ = 0 used for "non-detect" congeners; $_{MAX}$ = MDL used for "non-detect" congeners; * indicates the outlying sample in the Chi-square analysis

Appendix VI: Complete Results for Kestrel Egg Samples

Table VI-a: Complete listing of concentrations of TEQ and HQ values for kestrel egg samples

SAMPLE	SITE	TOT TEQ_{MIN} (pg/g)	% PCB_{MIN}	TOT TEQ_{MAX} (pg/g)	% PCB_{MAX}	HQ_{MIN} (TEQ based)	HQ_{MAX} (TEQ based)	HQ_{MIN} (TCDD-EQ based)	HQ_{MAX} (TCDD-EQ based)
AKEG003	P	$6.0x10^{0}$	$6.4 x10^{1}$	$8.3 x10^{0}$	$4.4 x10^{0}$	$8.5x10^{-2}$	$1.2x10^{-1}$	$0.0 x10^{0}$	$2.9x10^{-2}$
AKEG008	P	$1.2x10^{1}$	$5.2 x10^{1}$	$1.4 x10^{1}$	$6.4 x10^{0}$	$1.8x10^{-1}$	$2.0x10^{-1}$	$1.6x10^{-1}$	$1.6x10^{-1}$
AKEG014	P	$1.2x10^{1}$	$6.4 x10^{1}$	$1.3 x10^{1}$	$7.5 x10^{0}$	$1.7x10^{-1}$	$1.9x10^{-1}$	$0.0 x10^{0}$	$1.4x10^{-2}$
AKEG015	P	$3.9x10^{1}$	$9.3 x10^{1}$	$4.3 x10^{1}$	$3.6 x10^{1}$	$5.6x10^{-1}$	$6.2x10^{-1}$	$0.0 x10^{0}$	$1.4x10^{-2}$
AKEG018	P	$9.1x10^{0}$	$6.0 x10^{1}$	$1.0 x10^{1}$	$5.4 x10^{0}$	$1.3x10^{-1}$	$1.4x10^{-1}$	$0.0 x10^{0}$	$1.4x10^{-2}$
AKEG020	P	$1.4x10^{1}$	$4.7 x10^{1}$	$1.5 x10^{1}$	$6.8 x10^{0}$	$2.0x10^{-1}$	$2.1x10^{-1}$	$0.0 x10^{0}$	$2.9x10^{-2}$
AKEG022	P	$1.5x10^{1}$	$3.7 x10^{1}$	$1.5 x10^{1}$	$5.5 x10^{0}$	$2.1x10^{-1}$	$2.2x10^{-1}$	$0.0 x10^{0}$	$1.4x10^{-2}$
AKEG024	P	3.7x101	$3.2 x10^{1}$	$3.7 x10^{1}$	$1.2 x10^{1}$	$5.2x10^{-1}$	$5.3x10^{-1}$	$0.0 x10^{0}$	$7.1x10^{-2}$
AKEG025	P	$5.9x10^{0}$	$6.4 x10^{1}$	$7.6 x10^{0}$	$3.8 x10^{0}$	$8.5x10^{-2}$	$1.1x10^{-1}$	$0.0 x10^{0}$	$7.1x10^{-2}$
AKEG029	P	$6.5x10^{0}$	$6.1 x10^{1}$	$7.8 x10^{0}$	$3.9 x10^{0}$	$9.2x10^{-2}$	$1.1x10^{-1}$	$0.0 x10^{0}$	$1.4x10^{-1}$
AKEG030	P	$3.5x10^{1}$	$1.3 x10^{1}$	$3.6 x10^{1}$	$4.8 x10^{0}$	$5.1x10^{-1}$	$5.1x10^{-1}$	$0.0 x10^{0}$	$5.7x10^{-2}$
AKEG036	P	$8.6x10^{0}$	$9.5 x10^{1}$	$1.2 x10^{1}$	$8.2 x10^{0}$	$1.2x10^{-1}$	$1.8x10^{-1}$	$0.0 x10^{0}$	$1.7x10^{-1}$
AKEG037	P	$1.2x10^{1}$	$3.5 x10^{1}$	$1.2 x10^{1}$	$4.2 x10^{0}$	$1.7x10^{-1}$	$1.8x10^{-1}$	$0.0 x10^{0}$	$7.1x10^{-2}$
AKEG039	P	$3.1x10^{1}$	$2.9 x10^{1}$	$3.1 x10^{1}$	$9.0 x10^{0}$	$4.4x10^{-1}$	$4.5x10^{-1}$	$0.0 x10^{0}$	$1.7x10^{-1}$
AKEG045	P	$4.8x10^{0}$	$7.9 x10^{1}$	$7.0 x10^{0}$	$3.8 x10^{0}$	$6.8x10^{-2}$	$1.0x10^{-1}$	$0.0 x10^{0}$	$4.3x10^{-2}$
AKEG048	P	$4.1x10^{1}$	$6.9 x10^{1}$	$4.3 x10^{1}$	$2.8 x10^{1}$	$5.9x10^{-1}$	$6.1x10^{-1}$	$0.0 x10^{0}$	$7.1x10^{-2}$
AKEG052	P	$4.3x10^{1}$	$6.6 x10^{1}$	$4.6 x10^{1}$	$2.8 x10^{1}$	$6.2x10^{-1}$	$6.6x10^{-1}$	$0.0 x10^{0}$	$7.1x10^{-2}$
AKEG054	P	$3.0x10^{1}$	$7.7 x10^{1}$	$4.0 x10^{1}$	$2.4 x10^{1}$	$4.4x10^{-1}$	$5.7x10^{-1}$	$0.0 x10^{0}$	$5.7x10^{-2}$
AKEG058	P	$3.1 x10^{1}$	$2.3 x10^{1}$	$3.3 x10^{1}$	$7.1 x10^{0}$	$4.4x10^{-1}$	$4.8x10^{-1}$	$0.0 x10^{0}$	$1.6x10^{-1}$
AKEG005	C	$6.5 x10^{0}$	$8.1 x10^{1}$	$1.1 x10^{1}$	$5.3 x10^{0}$	$9.3x10^{-2}$	$1.6x10^{-1}$	$0.0 x10^{0}$	$1.4x10^{-2}$
AKEG007	C	$4.4 x10^{0}$	$9.6 x10^{1}$	$9.8 x10^{0}$	$7.7 x10^{0}$	$6.3x10^{-2}$	$1.4x10^{-1}$	$8.9x10^{-1}$	$8.9x10^{-1}$
AKEG011	C	$6.6 x10^{0}$	$6.0 x10^{1}$	$7.1 x10^{0}$	$3.9 x10^{0}$	$9.4x10^{-2}$	$1.0x10^{-1}$	$0.0 x10^{0}$	$2.9x10^{-2}$
AKEG017	C	$1.4 x10^{1}$	$4.7 x10^{1}$	$1.5 x10^{1}$	$6.4 x10^{0}$	$2.0x10^{-1}$	$2.2x10^{-1}$	$0.0 x10^{0}$	$2.9x10^{-2}$
AKEG027	C	$3.8 x10^{1}$	$2.3 x10^{1}$	$3.8 x10^{1}$	$8.8 x10^{0}$	$5.4x10^{-1}$	$5.4x10^{-1}$	$0.0 x10^{0}$	$1.4x10^{-1}$
AKEG038	C	$1.4 x10^{1}$	$4.5 x10^{1}$	$1.5 x10^{1}$	$6.2 x10^{0}$	$2.0x10^{-1}$	$2.1x10^{-1}$	$0.0 x10^{0}$	$1.0x10^{-1}$
AKEG040	C	$1.1 x10^{1}$	$5.1 x10^{1}$	$1.2 x10^{1}$	$5.5 x10^{0}$	$1.6x10^{-1}$	$1.7x10^{-1}$	$0.0 x10^{0}$	$7.1x10^{-2}$
AKEG049	C	$2.0 x10^{1}$	$3.4 x10^{1}$	$2.5 x10^{1}$	$6.9 x10^{0}$	$2.9x10^{-1}$	$3.5x10^{-1}$	$0.0 x10^{0}$	$2.9x10^{-2}$
AKEG050	C	$2.2 x10^{1}$	$2.0 x10^{1}$	$3.2 x10^{1}$	$4.4 x10^{0}$	$3.2x10^{-1}$	$4.6x10^{-1}$	$0.0 x10^{0}$	$5.7x10^{-2}$
AKEG051	C	$1.2 x10^{1}$	$5.6 x10^{1}$	$2.3 x10^{1}$	$6.8 x10^{0}$	$1.7x10^{-1}$	$3.2x10^{-1}$	$0.0 x10^{0}$	$5.7x10^{-2}$
AKEG057	C	$8.6x10^{1}$	$7.6 x10^{1}$	$8.8 x10^{1}$	$6.5 x10^{1}$	$1.2 x10^{0}$	$1.3 x10^{0}$	$0.0 x10^{0}$	$1.0x10^{-1}$
Mean	**Onsite**	**$2.1x10^{1}$**	**$5.5x10^{1}$**	**$2.4x10^{1}$**	**$1.1x10^{1}$**	**$3.0x10^{-1}$**	**$3.4x10^{-1}$**	**$3.5x10^{-2}$**	**$9.8x10^{-2}$**
AKEG001	R	$2.8 x10^{1}$	$7.8 x10^{1}$	$3.0 x10^{1}$	$2.2 x10^{1}$	$4.1x10^{-1}$	$4.2x10^{-1}$	$0.0 x10^{0}$	$2.9x10^{-2}$
AKEG002	R	$1.1 x10^{1}$	$4.7 x10^{1}$	$1.3 x10^{1}$	$7.0 x10^{0}$	$1.6x10^{-1}$	$1.9x10^{-1}$	$0.0 x10^{0}$	$1.9x10^{-1}$
AKEG004	R	$7.0 x10^{0}$	$8.9 x10^{1}$	$1.4 x10^{1}$	$1.0 x10^{1}$	$1.0x10^{-1}$	$2.0x10^{-1}$	0.0x100	$4.3x10^{-2}$
AKEG010	R	$6.2 x10^{0}$	$6.8 x10^{1}$	$7.9 x10^{0}$	$4.7 x10^{0}$	$8.9x10^{-2}$	$1.1x10^{-1}$	$0.0 x10^{0}$	$4.3x10^{-2}$
AKEG012	R	$2.3x10^{2}$	$1.3 x10^{1}$	$2.3 x10^{2}$	$3.1 x10^{1}$	$3.3 x10^{0}$	$3.3 x10^{0}$	$1.2 x10^{0}$	$1.2 x10^{0}$
AKEG016	R	$2.0 x10^{1}$	$8.7 x10^{1}$	$2.2 x10^{1}$	$1.7 x10^{1}$	$2.8x10^{-1}$	$3.2x10^{-1}$	$0.0 x10^{0}$	$7.1x10^{-2}$
AKEG021	R	$1.7 x10^{1}$	$7.1 x10^{1}$	$2.0 x10^{1}$	$1.2 x10^{1}$	$2.4x10^{-1}$	$2.9x10^{-1}$	$0.0 x10^{0}$	$1.4x10^{-2}$
AKEG028	R	$3.2 x10^{1}$	$7.5 x10^{1}$	$3.3 x10^{1}$	$2.4 x10^{1}$	$4.5x10^{-1}$	$4.7x10^{-1}$	$0.0 x10^{0}$	$1.0x10^{-1}$
AKEG031	R	$4.1 x10^{1}$	$2.4 x10^{1}$	$4.2 x10^{1}$	$9.9 x10^{0}$	$5.8x10^{-1}$	$6.0x10^{-1}$	$0.0 x10^{0}$	$1.1x10^{-1}$
AKEG032	R	$9.2 x10^{0}$	$5.4 x10^{1}$	$1.1 x10^{1}$	$4.9 x10^{0}$	$1.3x10^{-1}$	$1.5x10^{-1}$	$0.0 x10^{0}$	$4.3x10^{-2}$
AKEG033	R	$1.3 x10^{1}$	$8.3 x10^{1}$	$1.7 x10^{1}$	$1.1 x10^{1}$	$1.8x10^{-1}$	$2.5x10^{-1}$	$1.9x10^{-1}$	$1.9x10^{-1}$
AKEG035	R	$3.2 x10^{0}$	$5.1 x10^{0}$	$3.7 x10^{0}$	$1.6x10^{-1}$	$4.5x10^{-2}$	$5.3x10^{-2}$	$0.0 x10^{0}$	$7.1x10^{-2}$
AKEG041	R	$4.0x10^{2}$	$8.7 x10^{1}$	$4.1 x10^{2}$	$3.5 x10^{2}$	$5.8 x10^{0}$	$5.8 x10^{0}$	$1.7 x10^{0}$	$1.7 x10^{0}$
AKEG043	R	$8.4 x10^{0}$	$5.5 x10^{1}$	$8.6 x10^{0}$	$4.6 x10^{0}$	$1.2x10^{-1}$	$1.2x10^{-1}$	$0.0 x10^{0}$	$8.6x10^{-2}$
AKEG044	R	$1.8 x10^{1}$	$8.0 x10^{1}$	$2.2 x10^{1}$	$1.4 x10^{1}$	$2.5x10^{-1}$	$3.1x10^{-1}$	$0.0 x10^{0}$	$4.3x10^{-2}$
AKEG047	R	$2.4 x10^{1}$	$4.1 x10^{1}$	$2.5 x10^{1}$	$9.9 x10^{0}$	$3.5x10^{-1}$	$3.5x10^{-1}$	$0.0 x10^{0}$	$2.9x10^{-2}$
Mean	**Ref**	**$5.8x10^{1}$**	**$6.3x10^{1}$**	**$6.0x10^{1}$**	**$3.6x10^{1}$**	**$7.8x10^{-1}$**	**$8.1x10^{-1}$**	**$1.9x10^{-1}$**	**$2.4x10^{-1}$**

R= reference sites; P = periphery of the RMA; C = core of the RMA

TOT = Contribution of PCBs, PCDDs, and PCDFs

$_{MIN}$ = 0 used for "non-detect" congeners

$_{MAx}$ = MDL used for "non-detect" congeners

Table VI-b: Complete listing of concentrations of TCDD-EQ and TEQ_{REP} values for kestrel egg samples

SAMPLE	SITE	TCDD-EQ_{MIN} (pg/g)	TCDD-EQ_{MAX} (pg/g)	$TEQ_{REP\ MIN}$ (pg/g)	$TEQ_{REP\ MAX}$ (pg/g)
AKEG003	P	$0.0x10^{0}$	$2.0\ x10^{0}$	$1.6\ x10^{0}$	$3.2\ x10^{0}$
AKEG008	P	$1.1\ x10^{1}$	$1.1\ x10^{1}$	$5.7\ x10^{0}$	$7.8\ x10^{0}$
AKEG014	P	$0.0\ x10^{0}$	$1.0\ x10^{0}$	$5.4\ x10^{0}$	$6.2\ x10^{0}$
AKEG015	P	$0.0\ x10^{0}$	$1.0\ x10^{0}$	$6.3\ x10^{0}$	$8.0\ x10^{0}$
AKEG018	P	$0.0\ x10^{0}$	$1.0\ x10^{0}$	$3.9\ x10^{0}$	$4.4\ x10^{0}$
AKEG020	P	$0.0\ x10^{0}$	$2.0\ x10^{0}$	$5.2\ x10^{0}$	$6.3\ x10^{0}$
AKEG022	P	$0.0\ x10^{0}$	$1.0\ x10^{0}$	$1.4\ x10^{1}$	$1.4\ x10^{1}$
AKEG024	P	$0.0\ x10^{0}$	$5.0\ x10^{0}$	$3.1\ x10^{1}$	$3.3\ x10^{1}$
AKEG025	P	$0.0\ x10^{0}$	$5.0\ x10^{0}$	$6.8\ x10^{0}$	$7.8\ x10^{0}$
AKEG029	P	$0.0\ x10^{0}$	$1.0\ x10^{1}$	$6.0\ x10^{0}$	$7.0\ x10^{0}$
AKEG030	P	$0.0\ x10^{0}$	$4.0\ x10^{0}$	$5.9\ x10^{1}$	$5.9\ x10^{1}$
AKEG036	P	$0.0\ x10^{0}$	$1.2\ x10^{1}$	$5.6\ x10^{0}$	$7.7\ x10^{0}$
AKEG037	P	$0.0\ x10^{0}$	$5.0\ x10^{0}$	$1.5\ x10^{1}$	$1.6\ x10^{1}$
AKEG039	P	$0.0\ x10^{0}$	$1.2\ x10^{1}$	$2.4\ x10^{1}$	$3.1\ x10^{1}$
AKEG045	P	$0.0\ x10^{0}$	$3.0\ x10^{0}$	$1.2\ x10^{0}$	$3.3\ x10^{0}$
AKEG048	P	$0.0\ x10^{0}$	$5.0\ x10^{0}$	$9.9\ x10^{0}$	$1.2\ x10^{1}$
AKEG052	P	$0.0\ x10^{0}$	$5.0\ x10^{0}$	$1.4\ x10^{1}$	$2.1\ x10^{1}$
AKEG054	P	$0.0\ x10^{0}$	$4.0\ x10^{0}$	$6.5\ x10^{0}$	$1.4\ x10^{1}$
AKEG058	P	$0.0\ x10^{0}$	$1.1\ x10^{1}$	$2.0\ x10^{1}$	$2.4\ x10^{1}$
AKEG005	C	$0.0\ x10^{0}$	$1.0\ x10^{0}$	$3.2\ x10^{0}$	$5.4\ x10^{0}$
AKEG007	C	$6.2\ x10^{1}$	$6.2\ x10^{1}$	$1.1\ x10^{0}$	$2.4\ x10^{0}$
AKEG011	C	$0.0\ x10^{0}$	$2.0\ x10^{0}$	$4.0\ x10^{0}$	$4.5\ x10^{0}$
AKEG017	C	$0.0\ x10^{0}$	$2.0\ x10^{0}$	$6.7\ x10^{0}$	$7.9\ x10^{0}$
AKEG027	C	$0.0\ x10^{0}$	$1.0\ x10^{1}$	$1.0\ x10^{2}$	$1.0\ x10^{2}$
AKEG038	C	$0.0\ x10^{0}$	$7.0\ x10^{0}$	$2.1\ x10^{1}$	$2.2\ x10^{1}$
AKEG040	C	$0.0\ x10^{0}$	$5.0\ x10^{0}$	$2.0\ x10^{1}$	$2.1\ x10^{1}$
AKEG049	C	$0.0\ x10^{0}$	$2.0\ x10^{0}$	$1.5\ x10^{1}$	$2.4\ x10^{1}$
AKEG050	C	$0.0\ x10^{0}$	$4.0\ x10^{0}$	$2.3\ x10^{1}$	$5.2\ x10^{1}$
AKEG051	C	$0.0\ x10^{0}$	$4.0\ x10^{0}$	$7.2\ x10^{0}$	$1.6\ x10^{1}$
AKEG057	C	$0.0\ x10^{0}$	$7.0\ x10^{0}$	$1.8\ x10^{1}$	$2.3\ x10^{1}$
Mean (On-Post)		$\mathbf{2.4\ x10^{0}}$	$\mathbf{6.9\ x10^{0}}$	$\mathbf{1.5\ x10^{1}}$	$\mathbf{1.9\ x10^{1}}$
AKEG001	R	$0.0\ x10^{0}$	$2.0\ x10^{0}$	$6.6\ x10^{0}$	$8.3\ x10^{0}$
AKEG002	R	$0.0\ x10^{0}$	$1.3\ x10^{1}$	$3.6\ x10^{0}$	$4.8\ x10^{0}$
AKEG004	R	$0.0\ x10^{0}$	$3.0\ x10^{0}$	$1.3\ x10^{0}$	$3.5\ x10^{0}$
AKEG010	R	$0.0\ x10^{0}$	$3.0\ x10^{0}$	$1.5\ x10^{0}$	$3.2\ x10^{0}$
AKEG012	R	$8.2\ x10^{1}$	$8.2\ x10^{1}$	$1.1\ x10^{2}$	$1.1\ x10^{2}$
AKEG016	R	$0.0\ x10^{0}$	$5.0\ x10^{0}$	$4.0\ x10^{0}$	$5.9\ x10^{0}$
AKEG021	R	$0.0\ x10^{0}$	$1.0\ x10^{0}$	$1.0\ x10^{1}$	$1.3\ x10^{1}$
AKEG028	R	$0.0\ x10^{0}$	$7.0\ x10^{0}$	$1.4\ x10^{1}$	$1.5\ x10^{1}$
AKEG031	R	$0.0\ x10^{0}$	$8.0\ x10^{0}$	$2.6\ x10^{1}$	$2.7\ x10^{1}$
AKEG032	R	$0.0\ x10^{0}$	$3.0\ x10^{0}$	$3.2\ x10^{0}$	$6.0\ x10^{0}$
AKEG033	R	$1.3\ x10^{1}$	$1.3\ x10^{1}$	$5.2\ x10^{0}$	$7.8\ x10^{0}$
AKEG035	R	$0.0\ x10^{0}$	$5.0\ x10^{0}$	$4.0\ x10^{0}$	$4.5\ x10^{0}$
AKEG041	R	$1.2\ x10^{2}$	$1.2\ x10^{2}$	$9.7\ x10^{1}$	$1.0\ x10^{2}$
AKEG043	R	$0.0\ x10^{0}$	$6.0\ x10^{0}$	$2.5\ x10^{0}$	$3.5\ x10^{0}$
AKEG044	R	$0.0\ x10^{0}$	$3.0\ x10^{0}$	$4.4\ x10^{0}$	$7.4\ x10^{0}$
AKEG047	R	$0.0\ x10^{0}$	$2.0\ x10^{0}$	$9.3\ x10^{0}$	$1.0\ x10^{1}$
Mean (Reference)		$\mathbf{1.4\ x10^{1}}$	$\mathbf{1.8\ x10^{1}}$	$\mathbf{2.0\ x10^{1}}$	$\mathbf{2.2\ x10^{1}}$

R= reference sites; P = periphery of the RMA; C= core area of RMA

TCDD-EQ = bioassay results

TEQ_{REP}= analytical chemistry results multiplied by H4IIE relative potencies

$_{MIN}$ = 0 used for "non-detect" congeners

$_{MAX}$ = MDL used for "non-detect" congeners

Appendix VII- Calculation of Uncertainty Factors for TRV Determination

Overall Uncertainty Factors (UFs) are calculated and applied to existing NOAEL or LOAEL data in order to determine conservative Toxicity Reference Values (TRVs) for a given species and toxicant of interest. UFs are based on several parameters, which are assigned a certain uncertainty value within a specified range. UFs are based on the following parameters:

1. Intertaxon extrapolation
2. Study duration
3. Study endpoint

Uncertainty factors are also based on the following modifying factors:

1. Threatened species
2. Relevance of endpoint
3. Extrapolating lab to field
4. Co-contaminants
5. Unclear endpoint
6. Study species sensitivity
7. Organ ratios
8. Intraspecies variability

Values are assigned to the first three parameters listed depending on the amount of uncertainty associated with that parameter. These values are multiplied together and the product is multiplied by the sum of all values assigned to the modifying parameters. Modifying parameters are assigned values pertinent to TRV calculation (EPA 1993). The following is an example of an Overall Uncertainty Factor calculation:

Calculation of the great horned owl TRV from the Hoffmann *et al.* 1998 study using American kestrel egg and PCB126

Uncertainty Factors	NOAEL =2,300 pg/g
Intertaxon extrapolation	5
Study Duration	1
Study endpoint	1
Modifying Factors	
Threatened species	0
Relevance of endpoint	0
Extrapolating lab to field	2
Co-contaminants	0
Endpoint Unclear	1.5
Study species sensitivity	0
Organ ratios	1
Intraspecies variability	0
Overall UF for TRV	(1*1*5)*(2+1.5+1)= **22.5**
Proposed TRV	TRV= NOAEL * organ ratio*TEF/UF =(2300pg/g*(1.5)*0.1)/22.5 =15 pg TCDD/ g liver