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CHARACTERIZATION STUDIES OF THE H4IIE BIOASSAY FOR ASSESSMENT OF PLANAR HALOGENATED HYDROCARBONS IN FISH AND WILDLIFE

By

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ABSTRACT

CHARACTERIZATION STUDIES OF THE H4IIE BIOASSAY FOR ASSESSMENT OF PLANAR HALOGENATED HYDROCARBONS IN FISH AND WILDLIFE

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Planar halogenated hydrocarbons (PHHs) are a group of environmental contaminants which include polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs). PHH contamination in the environment is of particular concern because these compounds are extremely toxic, bioaccumalate in aquatic food chains, and are relatively persistent. Even though there are hundreds of PHHs, they are approximate isostereomers, produce the same characteristic toxic symptoms in a variety of phyla, and are believed to act through the same receptor-based mode of action. These similarities of PHH have lead to the development of quantitative structure-activity relationships (SAR) for PHHs in both *in vivo* and *in vitro* systems. One particularly interesting and potentially useful SAR has been observed between the induction potency of individual PHH congeners in H4IIE rat hepatoma cells toward ethoxyresorufin-O-deethylase activity and their *in vivo* toxicity in rats. This relationship between *in vitro* induction potency and *in vivo* toxic potency to the whole organism, along with the

ability of the H4IIE cells to act as integrators at the cellular level, has led to their use in assessment of complex mixtures of PHH in the environment.

The studies reported here focused on characterization of the H4IIE bioassay for use as a bioanalytical monitoring tool for the assessment of toxicity of PHH mixtures in fish and wildlife. Initial studies were directed toward analytical characterization of this bioassay system for use with extracts of environmental samples. Results of these studies indicate that the H4IIE bioassay is precise, has good reproducibility, is reasonably accurate when tested with fortified samples, and has no discernable interferences caused by common extraction protocols or sample matrices.

The H4IIE bioassay was used to screen PHH mixtures in eggs of colonial fish-eating waterbirds from around the Great Lakes. The results of the bioassay concurred with residue analysis and environmental effect data on these waterbird colonies. Comparison of the H4IIE bioassay with PHH residue analysis indicated that the bioassay may also be useful in understanding and interpreting chemical residue data. When used in combination, the H4IIE bioassay and chemical residue analysis complement each other and the information gained can be much more than provided by either type of analysis alone. The H4IIE bioassay results from double-crested cormorant eggs were also compared with reproductive success of these waterbirds. There was a significant correlation between the

H4IIE bioassay results on these eggs and egg mortality rates from colonies around the Great Lakes. Therefore, this *in vitro* bioassay system may be useful for assessing complex mixtures of PHHs in environmental samples.

Dedicated to my best friend and wife, Robin.

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Thanks also goes to my parents for their endless faith in me and love, and to my wife, Robin, for being my best friend. Additionaly, I would like to thank all the people in the laboratory for the work that they have done toward the completion of this project and for the friendship that they have given me over the years. All of you have enriched my life.

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GENERAL INTRODUCTION

Our society has become increasingly dependent on chemicals in most aspects of our lives. Very little in our lives has not been affected by some sort of chemical technology. The benefits, and comfort which have come from agricultural, pharmaceutical, and industrial chemicals are enormous. However, as with most things in life, the use of chemicals has had severe consequences in some cases. In particular, many of the industrial chemicals have found their way into the environment and many of the agricultural chemicals used in the past continue to stay with us. There was little concern over the release of chemicals into the environment from the early part of this century and up until the mid-1960's. Toxicological assessments were grossly incomplete in many cases and the prevailing thought was that such a small amount of chemical diluted into such a vast environment couldn't hurt. Twenty years later, many of the persistent chlorohydrocarbon pesticides and industrial chemicals have been banned, their residues in the environment have decreased, and many appear to have reached an asymptote at the bottom of a degradation curve. However, we are still

left with measurable amounts of hundreds of compounds, particularly in the Great Lakes environment. The questions raised from this are: Which of these compounds are important? Are these chemicals causing adverse effects in fish, wildlife, and/or humans? How do we assess the potential effects of these complex mixtures of chemicals? My dissertation centers around an important group of environmental contaminants and tries to address some of the questions posed above. The group of compounds I studied are the planar halogenated hydrocarbons (PHHs) which include, among others, polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs). PHHs are a group of environmental contaminants that have proven to be very toxic in laboratory studies with fish, wildlife, and laboratory mammals and they can have fairly long half-lifes in most compartments of the environment. These attributes make PHHs important pollutants given the extent of PHH contamination of the Great Lakes. The proper assessment of potential toxicity of PHHs has been skewed, however, because of the large number of compounds in this group and the complex interactions which occur among the individual PHH congeners. The problem of assessing PHHs has been further compounded by a lack of proper analytical techniques for many of the PHHs. Only recently have there been methods developed for the individual PCB congeners, for example. Even after the analytical methods have been developed, quite often only a few laboratories have

these analytical capabilities because the methods can be costly, time-consuming, or require specialized equipment and personnel.

Two characteristics of PHHs can be utilized in the assessment of their potential hazards to exposed organisms in the environment. First, all PHHs are believed to elicit their effects through a common receptor-mediated mode of action. All PHHs have the same suite of toxicological effects when given to an organism, however the individual PHHs vary greatly in their potency. The prototypic and most potent compound in this group is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). The putative receptor for PHHs has been identified in a variety of species and appears to be conserved phylogenetically. This is exemplified by the fact that PHHs can cause the same effects across many phylogenetic lines. In other words, PHHs cause the same type of effects in fish and in avians as well as mammals. Of course, there are grave differences in PHH sensitivity among species. However, limited data indicate that the rank order of potency is consistent among species. The second characteristic of PHHs which can be utilized for the assessment of their potential effects is the intercorrelation of the suite of toxic symptoms caused by these compounds. Some of the biochemical responses of organisms to PHHs are correlated to the toxic effects of these chemicals. In particular, cytochrome P450IA1 induction potency is correlated to the potency of individual PHHs to cause

thymic atrophy and weight loss or wasting syndrome in rats. This relationship has been seen not only *in vivo*, but also *in vitro* with the H4IIE rat hepatoma cell line. Because of this correlation between *in vitro* induction and *in vivo* effects of PHHs seen in rats, the use of these cells as a screening assay for PHHs has been suggested. My dissertation focuses on the characterization of the H4IIE rat hepatoma cell bioassay as a bioanalytical tool for the assessment of complex PHH mixtures from the environment.

The first chapter of this dissertation deals with the analytical characterization of the H4IIE bioassay. The objectives of this chapter were to determine the reproducibility, accuracy and precision of the H4IIE bioassay under conditions similar to those expected for environmental extracts. Chapter Two provides an example of an environmental monitoring program with this bioassay which involved a survey of waterbird eggs from around the Great Lakes. The objectives of this chapter were to determine the potency of waterbird egg extracts with the H4IIE bioassay compare the differences based on locations. The third chapter compares the H4IIE bioassay results with residue analysis in an effort to understand the relationships, if any, between these two methods of analysis. The fourth and last chapter compares the bioassay results with environmental effects data. The H4IIE bioassay results from PHH extracts of double-crested cormorant eggs were compared with the reproductive success in these colonies to determine

if this bioassay system could be useful to integrate and/or predict environmental effects of these complex mixtures of PHH in other species.

CHAPTER 1

Characterization Studies of the H4IIE Rat Hepatoma Cell Bioassay for Use in Analysis of Planar Halogenated Hydrocarbons (PHHs) in Environmental Samples

Introduction

Planar halogenated hydrocarbons (PHH) are a group of chemicals with isosteric configurations or structures and include, among other environmental contaminants, polychlorinated biphenyls (PCB), polychlorinated dibenzo-p-dioxins (PCDD), and polychlorinated dibenzofurans (PCDF). PHHs were used industrially for decades or were contaminants of chemical synthesis and entered the environment by both intentional and inadvertent release. The recalcitrant nature of PHHs, along with their inherent toxic properties and a propensity to bioaccumulate, has caused concern that these environmental contaminants may reach concentrations in organisms at the top of the food chain great enough to elicit toxic effects (1-4). The problem that scientists face in this respect is the evaluation of PHH residues that occur in the environment. Currently, there are analytical techniques to extract, concentrate, isolate, separate, and quantitate PHHs from environmental samples (5-9). However, concentrations of PHHs only provide part of the information necessary to evaluate their potential for adverse effects on fish, wildlife, and humans. This is because PHH congeners each have different toxic potencies (10-13) and the complex interactions of synergism, antagonism, and additivity which are known to occur within mixtures of PHHs (14-24), are not understood completely at this time. These interactions are not considered when attempts are made to predict biological effects from

concentrations of PHHs alone.

PHHs are proximate isostereomers which exert their toxic effects through the same biological receptor (10-12). Although differing in potency, PHHs elicit the same suite of toxicological effects across many phylogenetic lines (12). The characteristic symptoms of PHH poisoning include: weight loss (wasting syndrome), thymic atrophy, subcutaneous edema, immune suppression, hormonal alterations, P450IA1 associated enzyme induction, and the reproductive effects of fetotoxicity and teratogenesis (see reviews 25-26). Additionally, there are strong correlations between the enzyme induction potency of individual congeners and their potency for causing effects such as weight loss and thymic atrophy (27-30). These correlations are significant ($r > 0.90$) for both *in vivo* enzyme induction potency versus the toxic potency *in vivo* with rats, and for *in vitro* enzyme induction potency in H4IIE rat hepatoma cells versus the toxic potency *in vivo* in rats (31). In other words, the response of the H4IIE cells to the individual congeners was predictive of the toxic responses of whole organisms to these PHH congeners. Therein lies the potential utility of this *in vitro* bioassay as an integrative bioanalytical tool for screening PHH extracts of environmental samples.

The H4IIE cells were derived from the Reuber Hepatoma H-35 (32) by Pitot and coworkers (33). It is a continuous cell line and was characterized with regard to aryl hydrocarbon

hydroxylase (AHH) activity by Nebert and coworkers (34). Besides excellent growth characteristics and low basal cytochrome P450IA1 activity, the H4IIE cells have inducible AHH enzymes. These researchers went on to characterize the AHH induction response of the H4IIE cells to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the prototypic PHH, and suggested that the H4IIE rat hepatoma cell culture bioassay might be useful in detecting TCDD and other such compounds, for example, in foodstuffs (35). They found that the H4IIE cells are exquisite in their response to TCDD with a detection limit of 10 fmoles.

Simultaneous to the developments of the H4IIE bioassay structure-activity relationships of PHHs indicated that halogen substitution in the lateral positions of the dioxin, furan, or biphenyl molecules imparted a greater receptor affinity, AHH induction potency, and toxicity to these compounds (10-13). The relationship between AHH induction potency and the toxic potency of individual PHH congeners started to take form. In particular, a strong correlation between AHH or ethoxyresorufin-O-deethylase (EROD) induction potency *in vitro* in the H4IIE cells and the toxic potency *in vivo* of individual dioxin (36), biphenyl (29,37), and furan (28,30) congeners was observed. These reports were summarized by Safe (31). The correlations of -log ED50 for weight loss in rats versus -log EC50 for AHH induction in H4IIE cells and -log ED 50 for thymic atrophy in rats versus -log EC50 for AHH

induction in H4IIE cells had correlation coefficients of 0.93 and 0.92, respectively (31). These strong correlations between *in vitro* induction potency and *in vivo* toxic potency were critical validations for the use of this bioassay for prediction of potential toxicity of PHHs.

The first use of the H4IIE bioassay as a tool for assessing complex mixtures of PHHs in extracts was by the U.S. Food and Drug Administration. They performed the initial characterization of the H4IIE bioassay as an environmental extract assay (36,38-40). These authors reported various aspects of the analytical characterization of the H4IIE bioassay (38). Isooctane as the solvent carrier for extracts or pure compounds optimally enhanced bioassay sensitivity (38). A detection limit of 10 pg TCDD was reported with the isooctane solvent carrier system and the ED50 was 45 pg/plate (0.14 pmol/plate, 28 pM). A quantitation limit for this solvent system was not reported, however, in subsequent publications the limit of quantitation was 25 pg TCDD and the linear response range was 25-500 pg TCDD/plate (39-40). Thus, the sensitivity of this bioassay system for detection of PHHs had been established.

The H4IIE bioassay has been shown to be a sensitive tool for detection of PHHs in extracts of environmental samples (38-40) and much of the initial analytical characterization was performed by these scientists. However, due to improvements in PHH extractions, clean-up, and quantitation

techniques in the past 10 years, studies to confirm and expand on the work already done are necessary if this bioassay technique is to be adapted as a bioanalytical tool. In this study we reexamine the isooctane carrier solvent system, detection and quantitation limits, and some reference toxicants. Additionally, we investigate potential endogenous and exogenous interferences of matrices or extraction protocols. We also investigate the quantitative ability of the H4IIE bioassay with spike/bioanalysis experiments.

Experimental Section

Extractions, Spike/Bioanalysis Protocols

Three extraction and clean-up protocols were investigated: the method used by FDA scientists in the original bioassay reports, Pesticide Analytical Manual (PAM) Sec. 212 (41); an improved method used for PCB analysis which utilizes column extractions with dichloromethane (26); and a modification of this latter method which results in extracts that contain PCBs, PCDDs, and PCDFs in one fraction (42). Briefly, the PAM procedure includes acetonitrile extraction/homogenization, acetonitrile to petroleum ether transfer, aqueous acetonitrile/petroleum ether partitioning, solvent reduction, Florisil column clean-up, another solvent reduction, and final transfer to isooctane (41). This extraction method was used in PCB spike/recovery bioanalysis experiments with chicken eggs and with environmental waterbird

egg samples. Samples extracted using the PAM procedure were 30 g. Extraction efficiencies of the PAM procedure for [14C]-2,4,5,2',4',5'-hexachlorobiphenyl (PCB 153) were 57-61% from fortified chicken eggs. A second, more contemporary extraction method with dichloromethane (DCM) was used in PCB spike/recovery bioanalysis experiments with chicken eggs and various environmental samples. This method is routinely used to extract environmental samples for PCB congener-specific analysis. We felt that it would be important to compare the older PAM method with that of the more contemporary procedure. The DCM method of Ribick et al. (6) consists of grinding the sample with anhydrous sodium sulfate, column extraction with dichloromethane, gel permeation chromatography (GPC), Florisil and silica gel clean-up, and solvent transfer to isooctane. The resultant fraction contains PCBs (90-98% extraction efficiency) without dioxins, furans, polar pesticides, or most organochlorine pesticides (6). Last, we assessed a modified version of the Ribick et al. procedure in which an acidic silica gel (AS) column clean-up was used after GPC. This AS procedure was previously described (42) and briefly consists of grinding the sample with anhydrous sodium sulfate, column extraction with dichloromethane, gel permeation chromatography, acidified silica gel/silica gel column clean-up, and solvent transfer to isooctane. The resultant fraction contains PCBs with extraction efficiencies of 90-100% (42). External standardization of the AS procedure for PCDD

extraction efficiency had not been described previously. Therefore, duplicate 10 g portions of chicken egg homogenates were spiked with 7, 21, 70, 210, or 700 K DPM of ^{3}H-TCDD (specific activity approximately 45 Ci/mmol). The resultant extraction efficiencies (± SD) were 95.4 ± 4.0, 93.2 ± 1.0, 96.3 ± 0.7, 97.7 ± 3.3, and 103.5 ± 3.5%, respectively, with an average of 97.2 ± 4.2%. It should be noted that none of the bioassay results were corrected based on external standard recovery efficiencies.

A series of experiments were performed in which "clean" samples were spiked with a PCB (3,4,3',4'-tetrachlorobiphenyl, congener 77) or TCDD, extracted, and then analyzed with the H4IIE bioassay. The sample matrix used in these studies consisted of chicken eggs from a retail store because many of the samples we are currently analyzing are bird eggs. The first spike/bioanalysis experiment was PCB 77 spiked into chicken eggs at 0.1, 0.5, 1.0, 5.0, 10, 50, and 100 μg/g, extraction with either the PAM (41) or DCM (6) procedure, and then bioanalysis of the extracts. A second spike/bioanalysis experiment consisted of a TCDD spike of 0.01, 0.1, 0.5, 1.0, or 10 ng/g into chicken eggs. The eggs were then extracted with the acidic silica gel (AS) protocol (42), and extracts were subjected to bioanalysis.

Cell Culture and Bioassay Procedure

The H4IIE rat hepatoma cells were obtained from the

American Type Culture collection (ATCC No. CRL 1548). Cells were cultured in Dulbecco's Modified Eagle's Medium (D-MEM) base (Sigma, D5030) supplemented with 1X glutamine, 1.5X vitamins (Sigma, M6895), 2X non-essential amino acids, 1.5X essential amino acids, 1uM pyruvate, 1000 mg/l d-glucose, 2200 mg/l sodium bicarbonate, 15% fetal bovine serum (Gibco, 200-6140AJ), and 50 mg/l gentamicin. These conditions provided optimal growth and EROD induction potential of the H4IIE cells. Stock cultures were grown in 75 cm^2 flasks at 37°C in a humidified 95:5 air:CO_2 atmosphere. New cultures were started from frozen cells after 9 or less passages. The bioassay conditions were slight modifications of previous reports (36-40). Cells, trypsinized from stock flasks at confluency, were seeded in petri dishes (15 x 100 mm) at 0.8 x 10^6/plate in 10 ml D-MEM media. After 24 h incubation, the cells were dosed with extract, an appropriate control, or reference compound, in 100 ul of isooctane. There was no effect of dosing volume between 10 and 150 ul of isooctane when either an extract or standard (TCDD) was tested. Dosed cells were incubated 72 h, rinsed with phosphate-buffered saline (PBS), and then harvested with cell scrapers (Gibco) into Tris-sucrose (0.05 - 0.2M) buffer, pH 8.0 (40). Cells were then centrifuged for 10 min. at 5000X g, resuspended in Tris-sucrose buffer, and protein determined in duplicate (43). Duplicate EROD determinations, by the method of Pohl and Fouts (44), were made with 100 ul aliquots of the standardized (1

mg protein/ml) cell suspensions. Briefly, this method has a final reaction volume of 1.25 ml consisting of 1.0 ml NADPH generator system (5mM glucose-6-phosphate, 5 mM $MgSO_4$, 3.5 mM NADP, and 1.6 mg bovine serum albumin/ml in 0.1 M HEPES buffer, pH 7.8), 0.1 ml of 25 units/ml glucose-6-phosphate dehydrogenase (G6PDH), 0.1 ml of cell suspension, and 0.05 ml of 15 μM ethoxyresorufin (ER) in methanol. The reaction mixtures (less the ER) were preincubated 10 min at 37 °C, after which reactions were initiated by the addition of the ER at 10-s intervals. After 10 min the reactions were stopped by the addition of 2.5 ml of cold methanol, again at 10-s intervals. Proteins were allowed to flocculate for 5 min at 37 °C and then the samples were centrifuged at 5000 g, 4 °C, for 10 min. Resorufin in the supernatant was determined spectrofluorometrically (550 nm excitation, 585 nm emission) against a standard curve which was calibrated with a resorufin standard each bioassay. EROD specific activity was calculated as pmol resorufin formed per mg protein per min (pmol/mg/min) at 37 °C.

Along with each set of extracts, appropriate standards were analyzed on the same day. All environmental extracts were calibrated against a TCDD standard curve for calculation of "TCDD-equivalents" (TCDD-EQ) in the extract. The effective doses for half-maximal EROD induction (ED50) were calculated by probit analysis (45). Calculations of extract potency for each sample were made according to equation 1 as reported by

Sawyer et al. (46).

$$\text{Extract potency} = \text{TCDD } ED_{50}/\text{Extract } ED_{50} \qquad (1)$$

where; TCDD ED_{50} = pg/plate

Extract ED_{50} = ul/plate

Exrtact potency = pg TCDD-EQ/ul

The calculations to TCDD-EQ in an environmental sample were not corrected for extraction efficiencies of the various extraction methods. Variance estimates were calculated according to equation 2 and an additive model of variance (47).

$$CV_T = [(CV_E^2) + (CV_S)^2]^{1/2} \qquad (2)$$

where;

CV_T = coefficient of variation for TCDD-EQ

CV_E = coefficient of variation for extract ED_{50}

CV_S = coefficient of variation for standard ED_{50}

Standard deviations (SD) were obtained by multiplying the fractional CV_T by the estimated TCDD-EQ of the sample or extract. Goodness of fit test of a normal distribution for TCDD ED_{50} values was according to Kolmogorov-Smirnov (47).

Results and Discussion

The H4IIE bioassay has traits which make it a particularly useful technique for the determination of PHHs in environmental extracts. The basal EROD activity of the H4IIE cells ranges from 0.5 - 5.0 pmol/mg/min. Isooctane, which gave optimal response and sensitivity in previous reports (36,38,40), was also an ideal carrier in our studies. Solvent controls had no induction over basal EROD in the H4IIE cells. The dosing volume of extracts or standards was constant at 100 ul (1% media volume). However, there was no effect of dosing volume on the inductive response of the H4IIE cell cultures when volumes between 10 and 100 ul of extracts or standards were tested. Additionally, there was no effect of this concentration of isooctane on the H4IIE cells as measured by cell viability or cellular protein. The use of isooctane as a carrier is also compatible with most chemical residue analysis techniques for PHHs.

Sensitivity of the H4IIE bioassay is quite exquisite for PHHs and TCDD in particular. The limit of detection was 10 pg TCDD (31 fmol) per plate in our studies, which is the same as that reported by others (35-36, 38,40). The coefficient of variation for within bioassay variance of TCDD ED_{50} estimates were generally small with an average of 3.70%. The coefficients of variation associated with extract ED_{50} estimates are generally in the range of 5-15%. Precision of

this bioassay, therefore, is fairly high with the final estimates of TCDD-EQ in environmental samples having coefficients of variation between 10-20%. However, this type of precision is only when a TCDD standard curve is run with each set of environmental samples. Our average ED_{50} for TCDD over a two year period and 54 standard curves was 55.9 pg/plate (0.17 pmol/plate), also very similar to the 45 pg/plate (0.14 pmol/plate) ED_{50} reported previously with a similar solvent system (38). This demonstrates the reproducibility of the H4IIE bioassay, even among different laboratories. The ED_{50} values for TCDD in this system followed a normal distribution ($p < .001$) with a range of 30-115 pg/plate and standard deviation of 18.9 pg/plate (Fig. 1). This corresponds to a coefficient of variation among bioassays of 33.8%. If the average TCDD ED_{50} value with its 34% CV were used, resultant estimates of TCDD-EQ would have CV = 40-50%. Therefore, in bioanalytical applications of the H4IIE bioassay it is important to run a TCDD standard with each set of environmental extracts.

Another test of reproducibility with this bioassay is inter-laboratory comparisons of other PHH standards. For this purpose we tested 2,3,7,8-tetrachlorodibenzofuran (TCDF), and three PCB congeners (3,4,3',4'-tetrachloro- biphenyl, PCB 77; 3,4,5,3',4'-pentachlorobiphenyl, PCB 126; and 2,3,4,3',4',5'-hexachlorobiphenyl, PCB 156), in addition to TCDD (Table 1). In most cases there is fairly good agreement among ED_{50} values

Figure 1. Frequency Distribution of TCDD ED_{50} Values in the H4IIE Bioassay.

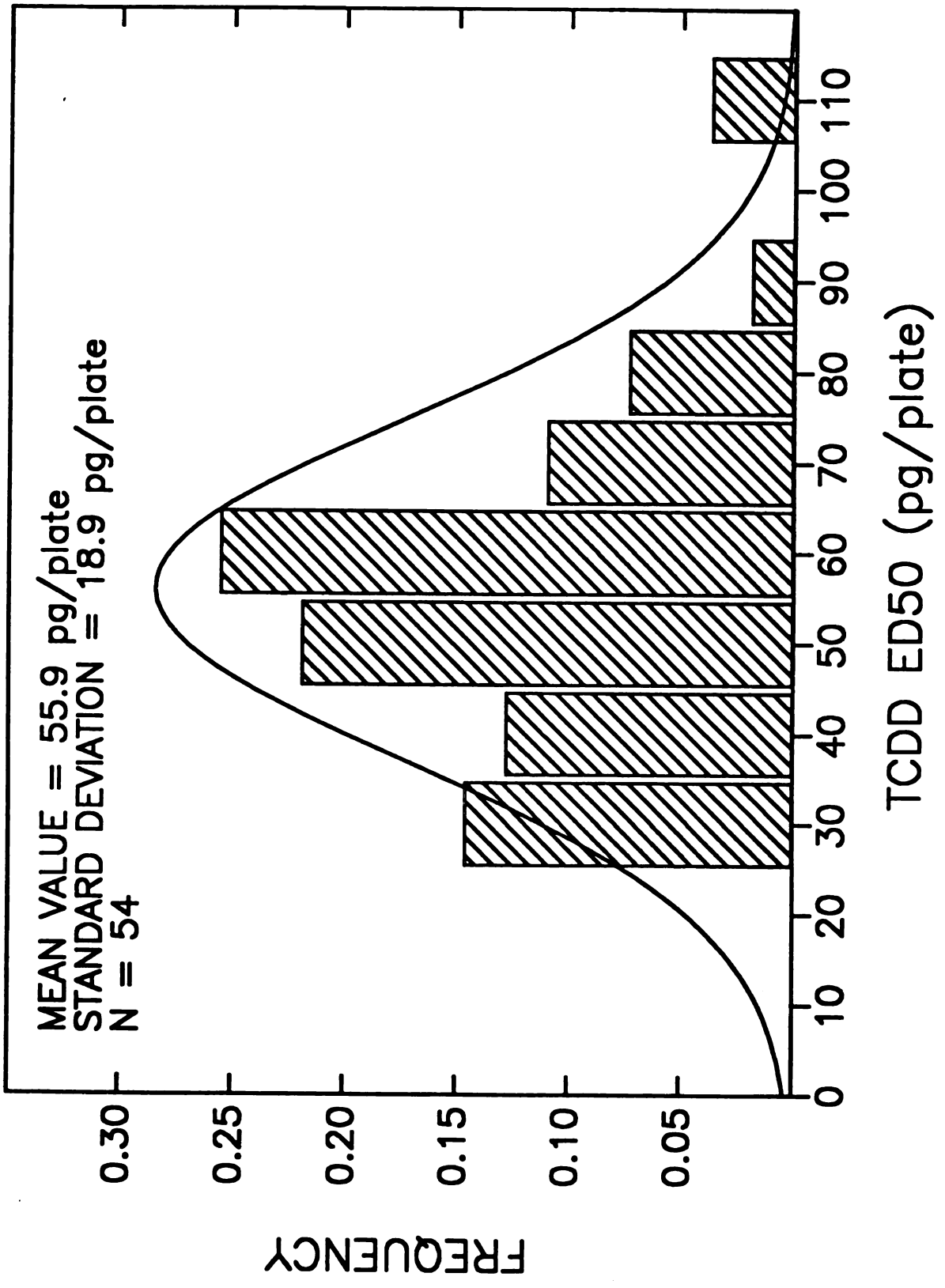

MEAN VALUE = 55.9 pg/plate
STANDARD DEVIATION = 18.9 pg/plate
N = 54
FREQUENCY
0.30
0.25
0.20
0.15
0.10
0.05
0
10
20
30
40
50
60
70
80
90
100
110
TCDD ED50 (pg/plate)

Table 1. ED_{50} Values and Relative Potencies of Selected PHHs for EROD Induction in the H4IIE Bioassay[a]

Compound	ED_{50} (pg/plate ± SD)	ED_{50} (pmol/plate ± SD)	Relative Potency
TCDD	$5.59 \pm 1.89 \times 10^{1}$	0.17 ± 0.06	1.0
TCDF	$8.08 \pm 0.16 \times 10^{3}$	26.4 ± 0.52	6.4×10^{-3}
PCB126	$2.48 \pm 0.02 \times 10^{3}$	7.59 ± 0.07	2.2×10^{-2}
PCB156	$1.13 \pm 0.03 \times 10^{6}$	3120 ± 89.9	5.5×10^{-5}
PCB77	$2.74 \pm 0.10 \times 10^{6}$	9370 ± 341	1.8×10^{-5}

a) All bioassays carried out in duplicate except TCDD where n = 54, r = 2-3. ED_{50} = Effective Dose for half-maximal EROD induction, and potencies are calculated relative to TCDD as (TCDD ED_{50}, pmol/plate)/ (compound ED_{50}, pmol/plate)

of the various PHHs estimated in different laboratories where the same solvent carrier is used. A comparison of PHH ED_{50} values from different laboratories is given (Table 2). ED_{50} values from this study were very close to those reported by others (37-38,48-49) when isooctane was used as a carrier solvent (Table 2), even when different substrates were used to monitor P450IA1 catalytic activity of the cells. Discrepancies begin to appear among ED_{50} values when comparisons are made among solvent carrier systems. Isooctane increases the sensitivity of the H4IIE bioassay towards TCDD as compared to dimethylsulfoxide (DMSO) (38). ED_{50} values for TCDD were 4 to 10 times less when isooctane was the carrier as compared to when DMSO was used (Table 2). However, greater sensitivity was not seen with the isooctane carrier solvent system for all PHHs. There appears to be little effect of carrier solvent on TCDF or PCB 156 potency and DMSO seems to result in greater bioassay sensitivity for PCB 126 and PCB 77 as compared to isooctane (Table 2). This phenomenon of apparent differential sensitivity caused by the carrier solvent system may be due to PHH solubility differences. It should also be mentioned at this point that the similarities in potency noted above are based on ED_{50} values. If effective concentration values (EC_{50}) are compared, there is no good agreement between values from different laboratories. The size of petri dish and volume of media used varied among all laboratories, but the cell densities were

Table 2. Comparison of Reported ED_{50} Values (pmol/plate) in the H4IIE Bioassay for Selected PHHs

Reference	Solvent System	Enzyme Assay	PHH				
			TCDD	TCDF	PCB 77	PCB 126	PCB156
Bradlaw & Casterline (38)	ISO	AHH	0.14	13	10,250	6	--
	DMSO	AHH	1.54	--	--	--	--
Sawyer & Safe (40)	DMSO	AHH	0.77	--	281	1.92	16,600
	DMSO	EROD	0.64	--	708	1.98	7,170
Sawyer & Safe (48)	DMSO	AHH	--	15.6	--	--	3,540
	DMSO	EROD	--	8.1	--	--	4,000
Zacharewski et al. (49)	DMSO	AHH	0.73	--	--	--	--
	DMSO	EROD	0.51	--	--	--	--
This study	ISO	EROD	0.17	26.4	9,370	7.6	3,120

fairly constant between 0.8 - 1.0 x 10^6 cell/plate. The fact that ED_{50}s and not EC_{50}s are similar among laboratories, along with the similarity in cell seeding rates, suggests that most of the PHH dose is effectively reaching the cells. However, radiotracer studies are required to understand if differential solubilities can explain this phenomenon. This also has implications on calculations of relative potency factors of PHHs based on their H4IIE cell induction potency. If ED_{50} values are more reliable and consistent estimates of PHH induction potency, as they appear to be, perhaps ED_{50} values instead of EC_{50} values should be used in calculation of H4IIE-derived potency factors of individual PHH congeners relative to TCDD. These potency factors are being used with increasing frequency (50), in particular to calculate TCDD-EQ from chemical residue analysis (51-52).

Use of the H4IIE bioassay for the determination of TCDD-EQ in environmental samples requires a knowledge of potential endogenous and exogenous interferences caused by the matrix or extraction protocols. To address these issues we examined matrix and procedural blanks, and performed spike/bioanalysis studies. Characterization of extraction protocols was done to insure that the fractions known to contain PHHs induced EROD in the H4IIE cells and fractions containing pesticides did not contain measurable amounts of inducible materials. The three extraction procedures tested, PAM (41), DCM (6), and AS (42) showed no induction with procedural blanks or

pesticide fractions and significant induction with PHH fractions from these methods (Table 3). The PAM characterization was similar to results reported by previous authors of this method (37). Matrix blanks (unfertilized chicken eggs, fertilized 10 day old chicken eggs, salmon eggs, and rainbow trout flesh), with ≤ 0.01 ug total PCBs/g, caused no EROD induction in the H4IIE cells at 1 - 3 g-equivalents of sample/plate. This indicated that endogenous substances in these matrices did not cause false positive responses in the H4IIE bioassay. Because p,p'-DDE is a major co-contaminant of PCBs in these extraction procedures, we exposed the H4IIE cells to 10, 100, 1000, or 10,000 ng p,p'-DDE/plate. There was no EROD induction or cytotoxicity, as measured by cell growth, at any dose of p,p'-DDE.

To assess the quantitative ability of the H4IIE bioassay, PHH spike/bioanalysis studies were conducted. The information to be gained by these experiments is three-fold. First, the actual induction magnitude and dose-response of the extract may be compared with that of the pure congener. Second, the slopes of the extracted and pure congener dose-response curves may be compared in a situation where only a single compound is present. Third, a threshold for detection inclusive of both extraction and bioassay efficiency may be estimated. PAM (41) and DCM (6) extraction methods were used in combination with the H4IIE bioassay to assess quantitation of PCB 77 and AS (42) extraction methods were used to assess quantitation

Table 3. EROD Induction Response of H4IIE Cells to Various Extraction Eluates.

Method	Procedural Blank	Pesticides Fraction	PHH Fraction
PAM	-	-	+
DCM	-	- / -	+
AS	-	NA	+

(a) Inductive (+) and non-inductive (-) fractions from PAM (41), DCM (6), and AS (42) extraction/clean-up protocols. No pesticide fraction is obtained with the AS protocol (NA = not applicable).

of TCDD.

Extracts of PCB 77 spiked chicken eggs produced a dose-response curve which was in good concordance with PCB 77 added directly to the cell cultures (Fig. 2). There were similar slopes in all three cases indicating no extraction or matrix effects on the dose-response curves. ED_{50} values calculated for the extracts varied less than 25% compared to the standard. Correction for the extraction efficiency of each method could reduce these differences among ED_{50} values.

Spike/bioanalysis experiments with TCDD and the AS extraction procedure indicated that the H4IIE bioassay could accurately predict extract potency. Extracted TCDD produced a similar dose-response curve in the H4IIE cells compared to the standard (Fig. 3). Slopes of the curves were not significantly different indicating no extraction or matrix effect on the response of the bioassay. Extract potency was calculated from observed ED_{50} values for each spike concentration, as would be done with environmental extracts, and these were compared with the known concentration of TCDD in the extract (Fig. 4). The nominal concentrations of the extracts were 0.1, 1.0, 5.0, 10 and 100 pg TCDD/ul. The extract at 0.1 pg TCDD/ul was below the limit of quantitation, however, the bioassay predicted extract potency within a factor of two for the other concentrations. Predictions of extract potency by the ED_{50} method were linear between 1.0 and 100 pg TCDD/ul and the regression slope of observed versus

Figure 2. PCB 77 Spike/Bioanalysis with PAM (41) and DCM (6) Extraction Protocols. Dose to cells was calculated based on 50 ul (5%) of 1 ml extract, spike concentration in chicken egg, and 100 % extraction efficiency.

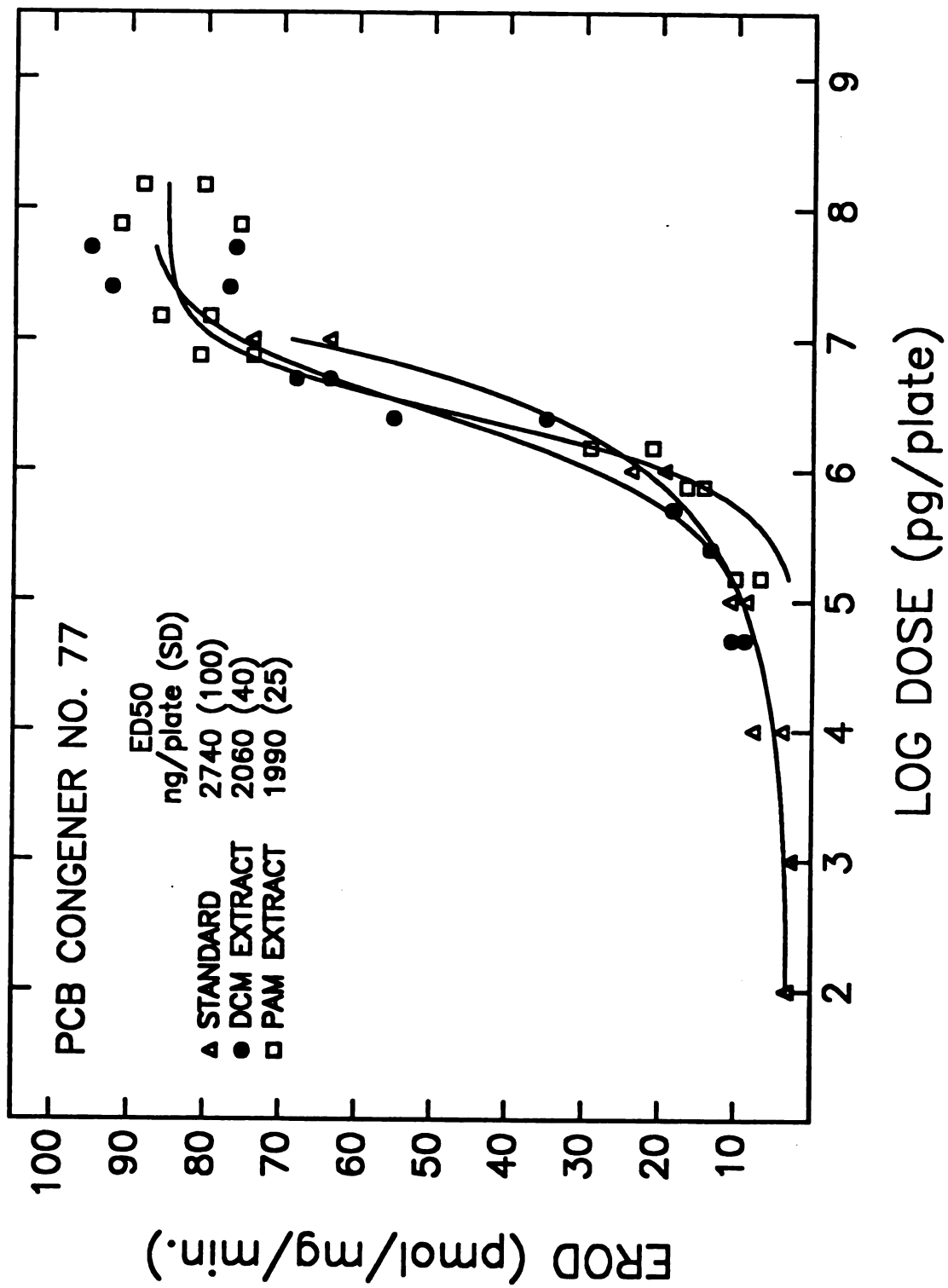

PCB CONGENER NO. 77
ED50
ng/plate (SD)
▲ STANDARD 2740 (100)
● DCM EXTRACT 2060 (40)
□ PAM EXTRACT 1990 (25)
EROD (pmol/mg/min.)
LOG DOSE (pg/plate)
100
90
80
70
60
50
40
30
20
10
2
3
4
5
6
7
8
9

Figure 3. TCDD spike bioanalysis with the AS extraction protocol (42). Dose to cells was calculated based on dosing volume (1, 10, 25, or 100 ul) of a 1 ml extract, spike concentration in chicken egg, and 100% extraction efficiency.

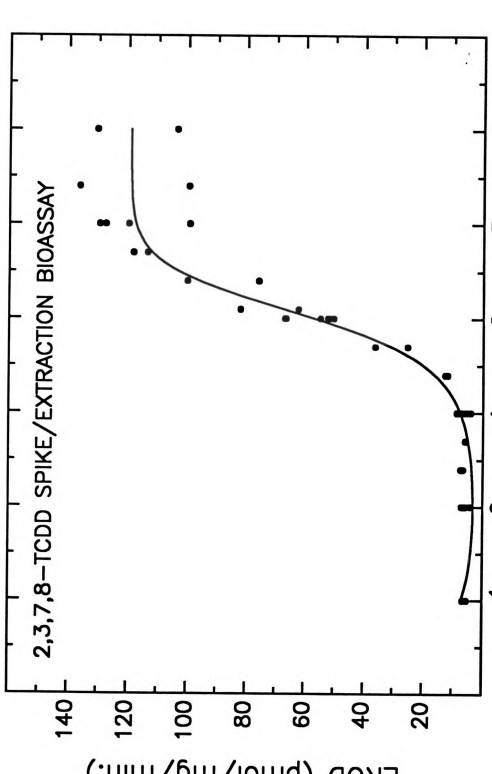
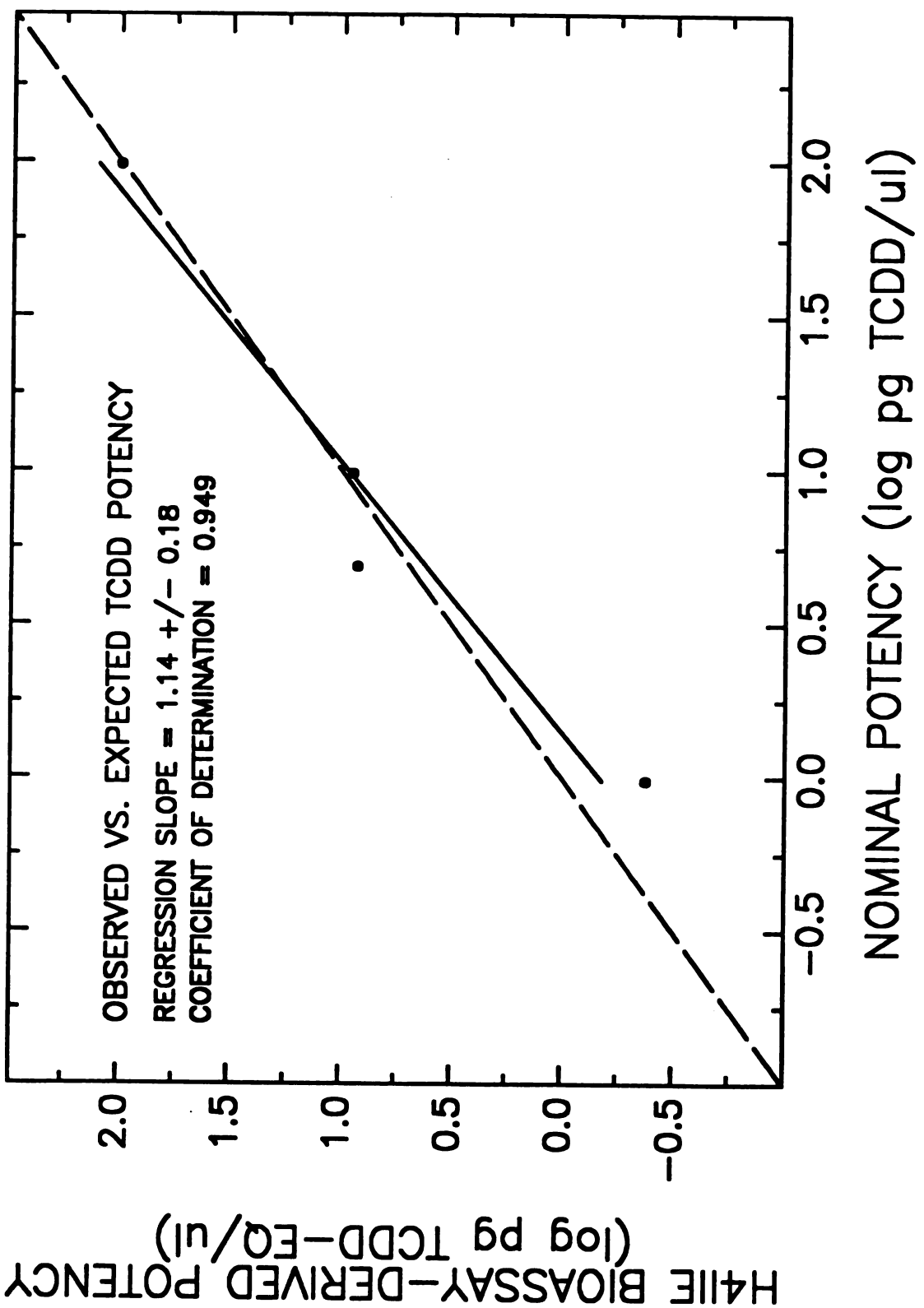

2,3,7,8–TCDD SPIKE/EXTRACTION BIOASSAY
EROD (pmol/mg/min.)
140
120
100
80
60
40
20
–1
0
1
2
3
4
LOG DOSE (pg/plate)

Figure 4. Observed versus Expected Extract Potency of TCDD in Spike/Bioanalysis Experiment.


OBSERVED VS. EXPECTED TCDD POTENCY
REGRESSION SLOPE = 1.14 +/- 0.18
COEFFICIENT OF DETERMINATION = 0.949
H4IIE BIOASSAY-DERIVED POTENCY (log pg TCDD-EQ/ul)
NOMINAL POTENCY (log pg TCDD/ul)
2.0
1.5
1.0
0.5
0.0
-0.5
-0.5
0.0
0.5
1.0
1.5
2.0

expected was not different from 1.0, the ideal. It is clear from this set of experiments that the H4IIE bioassay can accurately and precisely determine the potency of PHH extracts. Comparison of extract and standard ED_{50}s from the H4IIE bioassay is a simple and accurate method of calculating potencies and associated error estimates for PHH extracts. We found over the course of these experiments that single point determinations from the standard curve can lead to grossly inaccurate estimates of extract potency.

Previous studies have used the H4IIE bioassay to estimate the potency of individual PHHs (36-37), assess environmental extracts of PHHs (46,48-49), and address the complex interactions of synergism, antagonism and additivity (18-21,24) impossible to do by chemical residue analysis alone. The H4IIE bioassay has been shown to be a sensitive bioanalytical tool (35-40,48-49) with potential for predicting the toxic effects of PHHs in whole organisms (31). In this study we demonstrate the reproducibility of the H4IIE bioassay among laboratories and its repeatability over time within a laboratory. We also provide experimental data of its ability to quantitatively predict known concentrations of PHHs in biological extracts. The potential utility of this bioassay is as an integrative tool which can complement chemical residue analysis and biological effects data from environmental studies. The H4IIE bioassay can also be used to screen or prioritize chemical residue analysis and thereby

save valuable time and funds.

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Registry No. TCDD, 1746-01-6; PCB 153, 35065-27-1; PCB 77, 32598-13-3; PCB 126, 57465-28-8; PCB 156, 69782-90-7; TCDF, 51207-31-9; p,p'-DDE, 72-55-9; AHH, 9037-52-9; EROD, 59793-97-4.

Literature Cited

(1) Hutzinger, O.; van den Berg, M; Olie, K; Opperhuizen, A., Safe, S. In Dioxins in the Environment; Kamrin, M.A., Rodgers, P.W., Eds.; Hemisphere; New York, 1984, pp. 9-32.

(2) Esposito, M.P., Tiernan, T.O., Dryden, F.E. Dioxins: U.S. Environmental Protection Agency EPA-600/2-80-197, Cincinnati, OH, 1980, 351 pp.

(3) Evans, M.S. Toxic Contaminants and Ecosystem Health: A Great Lakes Focus, John Wiley and Sons, New York, 1988, 602 pp.

(4) Travis, C.C., Hattemer-Frey, H.A., Silbergeld, E.

Environ. Sci. Technol. 1989, 23, 1061-1063.

(5) Smith, L.M, Stalling, D.L., Johnson, J.L. Anal. Chem., 1984, 56, 1830-1842.

(6) Ribick, M.A., Smith, L.M, Dubay, G.R., Stalling, D.L. In Aquatic Toxicology and Hazard Assessment, Branson, D.R., Dickson, K.L., Eds. ASTM STP 737, Philadelphia, PA, 1981, pp 249-269.

(7) Smith, L.M. Anal. Chem. 1981, 53, 2152-2154.

(8) Tiernan, T.O. In Chlorinated Dioxins in the Total Environment, Choudhary, G., Keith, L.H., Rappe, C., Eds, Butterworth, Boston, MA, 1983, pp. 211-237.

(9) Rappe, C. Environ. Sci. Technol., 1984, 18, 78A-90A.

(10) Poland, A., Greenlee, W.F., Kende, A.S. Annals N.Y. Acad. Sci., 1979, 320, 214-230.

(11) Poland, A., Knutson, J.C. Ann. Rev. Pharmacol. Toxicol., 1982, 22, 517-554.

(12) Goldstein, J. In Halogenated biphenyls, terphenyls, naphthalenes, dibenzodioxins and related products; Kimbrough, R., Ed., Elsevier/North-Holland, New York, NY. 1980, pp. 151-190.

(13) Yoshimura, H. Yoshihara, S., Ozawa, N., Miki, M. Annals N.Y. Acad. Sci., 1979, 320, 179-192.

(14) Birnbaum, L.S., Weber, H., Harris, M.W., Lamb, J.C., McKinney, J.D. Toxicol. Appl. Pharmacol., 1985, 77, 292-302.

(15) Bannister, R., Safe, S. Toxicology, 1987, 44, 159-169.

(16) Keys, B., Piskorska-Pliszczynska, J., Safe, S. Toxicol. Lett., 1986, 31, 151-158.

(17) Weber, H., Harris, M.W., Haseman, J.K., Birnbaum, L.S. Toxicol. Lett., 1985, 26, 159-167.

(18) Leece, B., Denomme, M.A., Towner, R., Li, A., Landers, J., Safe, S. Can. J. Physiol. Pharmacol., 1987, 1908-1912.

(19) Bannister, R., Davis, D., Zacharewski, T., Tizard, I., Safe, S. Toxicol., 1987, 46, 29-42.

(20) Haake, J.M., Safe, S., Mayura, K., Phillips, T.D. Toxicol. Lett., 1987, 38, 299-306.

(21) Davis, D., Safe, S. Toxicol. Appl. Pharmacol., 1988, 94, 141-149.

(22) Pluss, N., Poiger, H., Hohbach, C., Schlatter, C. Chemosphere, 1988, 17, 973-984.

(23) Pluss, N., Poiger, H., Hohbach, C., Suter, M., Schlatter, C. Chemosphere, 1988, 17, 1099-1110.

(24) Biegel, L., Harris, M., Davis, D., Rosengren, R., Safe, L., Safe, S. Toxicol. Appl. Pharamacol., 1989, 97, 561-571.

(25) Safe, S. Ann. Rev. Pharmacol. Toxicol., 1986, 26, 371-399.

(26) Whitlock, J.P. Pharmacological Rev., 1987, 39, 147-161.

(27) Bandiera, S., Sawyer, T., Campbell, M.A., Robertson, L., Safe, S. Life Sci., 1982, 31, 517-525.

(28) Bandiera, S., Sawyer, T., Romkes, M., Zmudzka, B., Safe,

L., Mason, G., Keys, B., Safe, S. Toxicol., 1984, 32, 131-144.

(29) Leece, B., Denomme, M.A., Towner, R., Li, S.M.A., Safe, S. J. Toxicol. Environ. Health, 1985, 16, 379-388.

(30) Mason, G., Farrell, K., Keys, B., Piskorska-Pliszczynska, J., Safe, L., Safe, S. Toxicol. 1986, 41, 21-31.

(31) Safe, S. Chemosphere 1987, 16, 791-802.

(32) Ruber, M.A. J. Nat. Cancer Inst., 1961, 26, 891-897.

(33) Pitot, H.C., Peraino, C., Morse, P.A. Jr., Potter, V.R. Nat. Cancer Inst. Monogr. 1964, 13, 229-245.

(34) Benedict, W.F., Gielen, J.E., Owens, I.S., Niwa, A., Nebert, D.W. Biochem. Pharmacol. 1973, 22, 2766-2769.

(35) Niwa, A., Kumaki, K., Nebert, D.W. Mol. Pharmacol. 1975, 11, 399-408.

(36) Bradlaw, J.A., Garthoff, L.H., Hurley, N.E., Firestone, D. Fd. Cosmet. Toxicol. 1980 18, 627-635.

(37) Sawyer, T., Safe, S. Toxicol. Lett. 1982 , 13, 87-84.

(38) Bradlaw, J.A., Casterline, J.L. J. Assoc. Off. Anal. Chem. 1979, 62, 904-918.

(39) Trotter, W.J., Young, S.J.V., Casterline, J.L., Bradlaw, J.A., Kamps, L.R. J. Assoc. Off. Anal. Chem. 1982, 65, 838-841.

(40) Casterline, J.L., Bradlaw, J.A. Puma, P.J. Ku, Y. J. Assoc. Off. Anal. Chem. 1983, 66, 1136-1139.

(41) Pesticide Analytical Manual, Food and Drug Administration, Washington, D. C., 1979, Volume 1, Section 212.13a.

(42) Schwartz, T.R., Lehman, R.G. Bull. Environ. Contam. Toxicol. 1982, 28, 723-727.

(43) Lowry, O.H., Rosenbrough N.J., Farr A.L., Randall, R.J. J. Biol. Chem. 1951, 193,265.

(44) Pohl, R.J., Fouts, J.R. Analyt. Biochem., 1980, 107, 150-158.

(45) Finney, D.J. Statistical Methods in Biological Assay, Charles Griffin, London, U.K., 1980, 333 pp.

(46) Sawyer, T.W., Vatcher, A.D., Safe, S. Chemosphere 1984, 13, 695-701.

(47) Zar, J.H. Biostatistical Analysis, Prentice Hall, Englewood Cliffs, N.J. 1974, 620 pp.

(48) Sawyer, T.W., Safe, S. Chemosphere 1985, 14, 79-84.

(49) Zacharewski, T., Safe, L., Safe, S., Chittim, B., DeVault, D., Wiberg, K., Bergquist, P., Rappe, C. Environ. Sci. Technol. 1989, 23, 730-735.

(50) Olson, J.R., Bellin, J.S., Barnes, D.G. Chemosphere 1989, 18, 371-381.

(51) Niimi, A.J., Oliver, B.G. Chemosphere 1989, 18, 1413-1423.

(52) Kubiak, T.J., Harris, H.J., Smith, L.M., Schwartz, T.R., Stalling, D.L., Trick, J.A., Sileo, L., Docherty, D., Erdman, T.C. Arch. Environ. Contam. Toxicol. 1989, 18, 706-727.

CHAPTER 2

H4IIE Rat Hepatoma Cell Bioassay-Derived 2,3,7,8-Tetrachlorodibenzo-p-dioxin Equivalents (TCDD-EQ) in Colonial Fish-Eating Waterbird Eggs from the Great Lakes.

Introduction

Over the past two decades, populations of fish-eating birds in the Great Lakes of North America have had incidences of elevated mortality rates in adults, chicks, and embryos, altered reproductive behavior, and increased incidence of tetratogenesis. Within populations individual birds have had gross physical and histopathological anomalies, immune suppression, and altered biochemical homeostasis. These problems with fish-eating birds have occurred in areas of environmental pollution (see Peakall 1988 for review). Recently, field studies of various colonies of double-crested cormorants (Phalacrocorax auritis) and Caspian terns (Hydroprogne caspia) in the Great Lakes have noted differences in reproductive success and embryological defects based upon regional distribution of the waterbird colonies (Kurita et al. 1987). One geographic area which is known to be contaminated, the Saginaw Bay Confined Disposal Facility (CDF), had greatly reduced hatching success (28%) and no survival past fledging in Caspian terns (Kurita et al. 1987). The gross physical and histopathological anomalies that have occurred in Caspian terns at the Saginaw Bay CDF, in addition to reproductive impairment seen in waterbirds from around the Great Lakes, are similar to the effects which have been observed in birds exposed to planar halogenated hydrocarbons (PHHs) in controlled laboratory studies (McCune et al. 1962; Dahlgren and Linden 1971; Carlson and Duby 1973; Cecil et al 1973;

Peakall and Peakall 1973; Platonow and Reinhart 1973; Tusamonis et al 1973; Cecil et al 1974; Ax and Hansen 1975; McKinney et al 1976). It has been suggested that PHHs are, at least in part, responsible for some of the reproductive problems and lethality in fish-eating waterbirds at contaminated locations of the Great Lakes (Gochfeld 1975; Gilbertson and Fox 1977; Gilbertson 1983; Mineau et al. 1984; Kubiak et al. 1989).

Planar halogenated hydrocarbons (PHHs) are a group of chemicals that include, among others, polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs). These compounds, although no longer intentionally manufactured in the United States, continue to be of environmental concern because of their toxicity, persistence, and bioaccumulation potential. PHHs were used industrially or were contaminants from chemical synthesis processes for decades and are the focus of continuing public concern because of their widespread distribution and potential to bioaccumulate through the food chain (Tanabe et al. 1987). PHHs are found in all compartments of the ecosystem, however, aquatic systems are the primary areas of environmental contamination, this is particularly true for the Great Lakes (Baumann and Whittle 1988; Colburn 1988; Evans 1988). Therefore, it is not surprising that organisms at the top of the food chain such as fish-eating birds and mammals, including humans, receive

the greatest doses of PHHs.

PHHs are proximate isostereomers which exert their toxic effects through the same biological receptor (Poland and Knutson 1982). Although differing in potency, PHHs elicit the same suite of toxicological effects across many phylogenetic lines (Goldstein 1980). The characteristic symptoms of PHH poisoning include: weight loss (wasting syndrome), thymic atrophy, cutaneous edema, immune suppression, hormonal alterations, liver enzyme induction, and the reproductive effects of fetotoxicity and teratogenesis (see reviews by Safe 1986; Whitlock 1987). It is the presence of PHHs in these fish-eating waterbirds from contaminated areas around the Great Lakes (Gilbertson 1983; Niemi et al. 1986; Fox et al. 1988; Peakall 1988; Kubiak et al. 1989) along with the ability of PHHs to impair reproduction of avian species laboratory in studies, (McCune et al. 1962; Dahlgren and Linder 1971; Carlson and Dudy 1973; Cecil et al. 1973; Peakall and Peakall 1973; Platonow and Reinhart 1973; Tusamonis et al. 1973; Cecil et al. 1974; Ax and Hansen 1975; McKinney et al. 1976) that has lead to questions about their role as potential causal agents of the adverse effects observed in colonial waterbirds (Gilbertson 1983; Harris 1988; Kubiak et al. 1989).

Although analytical techniques exist for the detection of minute quantities of PHHs, these procedures can be extremely costly and time-consuming, particularly when samples may theoretically contain up to 209 different PCB, 75 PCDD,

135 PCDF congeners (Safe 1987). Moreover, even if reliable determination of PHH concentrations can be achieved in a timely, cost-effective manner, it is nearly impossible to predict biological effects of this mixture of compounds, because the toxicity of PCBs, PCDFs and PCDDs varies tremendously among congeners, and because toxic interactions among the PHHs have been variously shown to exhibit synergism, additivity or antagonism (Birnbaum et al. 1985; Weber et al. 1985; Eadon et al. 1986; Keys et al. 1986; Bannister and Safe 1987; Bannister et al. 1987; Leece et al. 1987, Haake et al. 1987; Davis and Safe 1988; Pluss et al. 1988a; Pluss et al. 1988b; Biegel et al. 1989; Bannister et al. 1989).

It has been well established that the most toxic PCB congeners are those which are planar, with lateral halogen substitution (Greenlee and Neal 1985). Although various planar congeners of PCBs differ greatly in their biological potencies, they are approximate isostereomers along with PCDDs and PCDFs, and all produce similar and characteristic patterns of toxic responses in mammals (Poland and Knutson 1982; Safe 1987). It is generally accepted that the toxic properties of different PHHs are expressed via a common mode of action, and therefore, it has been possible to calculate the biological potencies of complex mixtures of PHHs by expressing their potency relative to the most toxic PHH known, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Bradlaw and Casterline 1979; Trotter et al. 1982; Casterline et al. 1983; Eadon et

al. 1986; Safe 1987; Kubiak et al. 1989; Niimi and Oliver 1989).

One approach for the calculation of TCDD-equivalents of PHH mixtures involves congener specific conversion factors developed from the ability of the PCH mixture to induce cytochrome P-450-dependent aryl hydrocarbon hydroxylase (AHH) or ethoxyresorufin-O-deethylase (EROD) activity in H4IIE rat hepatoma cell cultures (Kubiak et al. 1989; Niimi and Oliver 1989). The potency of each PHH congener as determined in the H4IIE bioassay is expressed as a fraction relative to TCDD. TCDD-equivalents are calculated by mutiplying the concentration of each congener by its relative potency factor. Total toxic potency of the mixture can be determined by summing the equivalents attributableto each PHH congener under the assumption of an additive model of toxicity. Various researchers have used this approach of assigning TCDD-equivalents to complex mixtures of PHHs. This technique is limited, however, by the fact that it is strictly additive and does not allow for interactions among active congeners or account for the actions of inactive congeners that are known to modulate the toxicity of active congeners.

Use of the H4IIE rat hepatoma cell line to detect minute (pg) quantities of PHHs in a fast, inexpensive manner was first suggested by Nebert and coworkers (Benedict et al 1973; Niwa et al. 1975). The principle of the H4IIE bioassay is that the potency of complex mixtures of PHHs to induce AHH

or EROD activity is correlated with toxic potency of the mixtures. H4IIE rat hepatoma cells have low basal AHH and EROD enzyme activities, yet are highly inducible by PHHs. The advantage of this bioassay is that it can integrate the complex interactions of PHHs which are kniwn to occur at the cellular level. Investigators at the FDA used this bioassay system to evaluate complex mixtures of PHHs from environmental samples and in foodstuffs (Bradlaw and Casterline 1979; Trotter et al. 1982; Casterline et al. 1983). Studies into the validation of this bioassay system were continued by Safe and his coworkers (Bandiera et al. 1982; Sawyer and Safe 1982; Bandiera et al. 1984; Sawyer et al. 1984; Leece et al. 1985; Sawyer and Safe 1985; Denomme et al. 1986; Keys et al. 1986; Safe 1987). Their work has shown that the potency of individual PHHs to induce cytochrome P450IA1 activities in the H4IIE cells *in vitro*, is positively correlated with the toxicity of the isomers *in vivo* to rats (Sawyer et al. 1984; Mason et al. 1985; Safe 1987). Thus, this relatively simple and sensitive *in vitro* technique has been proposed as a screening assay to evaluate the relative toxicity of complex mixtures of PHHs (Bradlaw and Casterline, 1979; Sawyer and Safe 1982; Casterline et al. 1983; Sawyer et al 1984; Sawyer and Safe 1985; Safe 1987). It was felt that this bioassay could be used to screen waterbird eggs for bioactive PHHs.

It was the purpose of this study to use the H4IIE bioassay system to evaluate the relative potency of extracts

from fish-eating waterbird eggs. In particular, the objectives were: (1) To determine TCDD-equivalents (TCDD-EQ) in PCB extracts of waterbird egg composites from various colonies of double-crested cormorants and Caspian terns based on relative induction of cytochrome P450-dependent EROD activity in H4IIE rat hepatoma cells; (2) Compare H4IIE bioassay-derived TCDD-EQ in composites of waterbird eggs with the location of and biological effects within the colonies.

Materials and Methods

Samples

Individual waterbird eggs (217), from seven regional areas in the Great Lakes (Figure 5), were collected in 1986 and 1987 by Dr. James Ludwig, Ecological Research Services, Bay City, MI, USA. The eggs were from 14 double-crested cormorant (DCC), 10 Caspian Tern (CPT), 2 common tern (CMT,*Sterna airundo*), 1 ring-billed gull (RBG, *Larus delawarensis*), and 1 black-crowned night heron (BCH, *Nycticorax*) colonies, representing 41 different collection sites, dates, and/or species (Table 4). Because the bioassays were conducted on a "blind" basis, they were given arbitrary colony numbers.

The eggs were thawed at room temperature and homogenized in an Omni mixer (Ivan Sorvall, Inc., Norwalk, Conn.). Equal portions (generally 5-6 ml) of each egg within a colony (5-6 eggs/colony) were combined to make a single 30g

Figure 5. Collection Locations of Waterbird Eggs from the Great Lakes.

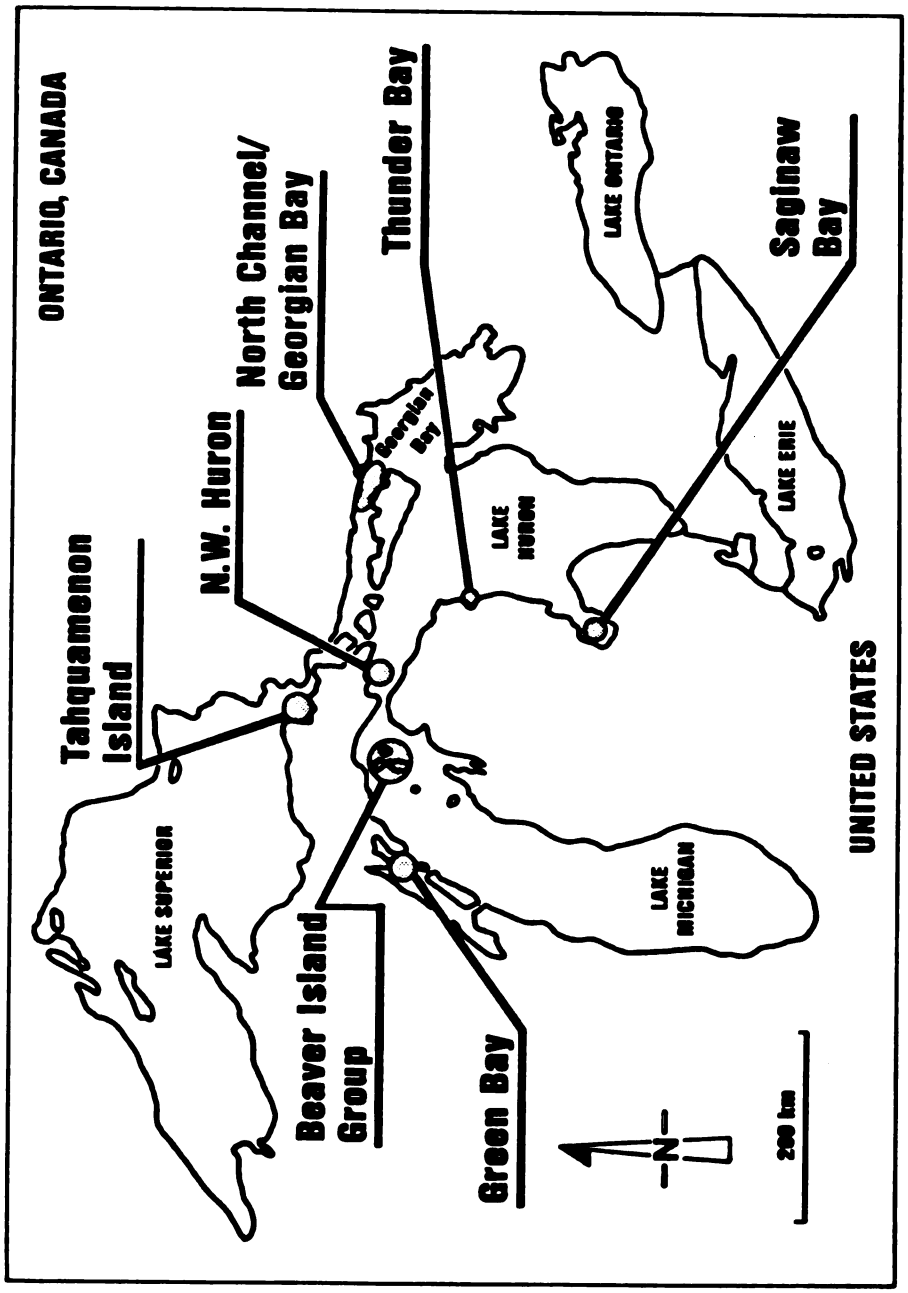

ONTARIO, CANADA
Tahquamenon Island
N.W. Huron
North Channel/ Georgian Bay
Thunder Bay
Saginaw Bay
Beaver Island Group
Green Bay
LAKE SUPERIOR
LAKE MICHIGAN
LAKE HURON
Georgian Bay
LAKE ONTARIO
LAKE ERIE
UNITED STATES
N
200 km

Table 4. Collection Sites and Date, Species and Numeric Designation of Egg Composites

Colony ID.	Collection Site (Region, Date)	Species
1	Gravelly Is. (Green Bay, 5-4-86)	DCC
2	Gravelly Is. (Green Bay, 5-4-86)	CPT
3	Big Gull Is. (Beaver Is., 5-2-86)	DCC
4	Gull Is. (Green Bay, 5-21-86)	CPT
5	Pismire Is. (Beaver Is., 5-23-86)	DCC
6	High Is. (Beaver Is., 5-23-86)	CPT
7	St. Martin's Shoal (N.W. Huron, 5-18-86)	DCC
8	Hat Is. (Beaver Is., 5-20-86)	CPT
9	Gull Is. (Thunder Bay, 5-25-86)	DCC
10	Snake Is. (Green Bay, 5-29-88)	DCC
11	Little Gull Is. (Green Bay, 5-21-86)	DCC
12	Hat Is. (Beaver Is., 5-20-86)	DCC
13	Black River IS. (Thunder Bay, 5-25-86)	DCC
14	Scarecrow Is. (Thunder Bay, 5-25-86)	DCC
15	Tahquamenon Is. (5-29-86)	DCC
16	Ile Aux Galets (Beaver Is., 5-24-86)	CPT
17	Ile Aux Galets (Beaver Is., 5-24-86)	DCC
18	West Grape Is. (Beaver Is., 5-23-86)	DCC
19	Sand Products (N. Beaver Is., 5-29-86)	CMT
20	Thunder Bay Is. (Thunder Bay, 5-30-86)	CMT
21	CDF Saginaw Bay (single egg) (6-11-86)	CPT
22	CDF Saginaw Bay (5-3-86)	RBG
23	CDF Saginaw Bay (5-10-86)	CPT
24	CDF Saginaw Bay (5-3-86)	BCH
25	Gull Is. (Georgian Bay, Ont., 6-12-86)	DCC
26	Gull Is. (Georgian Bay, Ont., 6-12-86)	CPT
27	Elm Is. (North Channel, Ont., 6-12-86)	CPT
28	Papoose Is. (Ontario, 6-12-86)	CPT
29	Cousins Is. (N. Channel, Ont., 6-5-86)	CPT
30	Cousins Is. (N. Channel, Ont., 6-5-86)	DCC
31	CDF Saginaw Bay (6-28-86)	CPT
32	CDF Saginaw Bay (5-25-87)	CPT
33	Ile Aux Galets (Beaver Is., 5-15-87)	CPT
34	High Is. (Beaver Is., 5-15-87)	CPT
35	Gravelly Is. (Green Bay, 5-15-87)	CPT
36	Gull Is. (Green Bay, 5-15-87)	CPT
37	Gravelly and Gull Is. (Green Bay, 5-18,87)	DCC
38	St. Martin's Shoal (N.W. Huron, 5-15-87)	DCC
39	Big Gull Is. (Beaver Is., 5-15-87)	DCC
40	Big Gull Is. (Beaver Is., 5-15-87)	DCC
41	Tahquamenon Is. (5-25-87)	DCC

composite sample from each collection site/date (Table 4). The composite samples and the individual eggs were stored in a -20°C walk-in freezer until they were extracted.

Extractions

Extraction of the composited eggs was done according to the Pesticide Analytical Manual (PAM) Sec. 212 (1979) designed for the extraction and clean-up of nonionic organochlorine residues from foodstuffs. These methods were used and characterized by the original authors of the H4IIE bioassay (Trotter et al. 1982; Casterline et al. 1983). Briefly, the procedures include acetonitrile extraction/homogenization, transfer from acetonitrile to petroleum ether, aqueous acetonitrile/petroleum ether partitioning, solvent reduction, and Florisil column clean-up. The Florisil column was eluted with 6% ethyl ether/petroleum ether (v/v, 6% fraction), the eluate collected, and solvent transferred to isooctane. The 6% fraction contains the PCBs and not dioxins or furans (Trotter et al. 1982). Therefore, since the cells were dosed with the 6% fraction, the TCDD-EQ reported in this study reflect only the contribution of PCBs to the total PHH burden in these waterbird eggs.

Extraction efficiency was assessed for PCBs with [14C]-2,4,5,2',4',5'-hexachlorobiphenyl (PCB 153, 5.3 mCi/mmol, New England Nuclear) by external standardization. Mean extraction efficiency was 49.4 ± 3.9% for triplicate extractions.

Calibration of extractions by internal standards addition was not possible because of the unknown effect an internal standard may have on the bioassay response. Therefore, the PCB 153 external standard was run for comparative purposes, however, the results of the bioassay were not corrected for extraction efficiencies from these or any other external standards.

Bioassay procedures

Bioassay procedures were slightly modified from previous reports (Bradlaw and Casterline 1979; Sawyer and Safe 1982). The H4IIE rat hepatoma cells were obtained from the American Type Culture Collection (ATCC No. CRL 1548). Cells were cultured in Dulbecco's Modified Eagle's Medium (D-MEM) base (Sigma, D5030) that was supplemented with 1X glutamine, 1.5X vitamins (Sigma, M6895), 2X non-essential amino acids, (Sigma, M7145), 1.5X essential amino acids (Sigma M7020), 1uM pyruvate, 1000 mg/l d-glucose, 2200 mg/l sodium bicarbonate, 15% fetal bovine serum (Gibco, 200-6140 AJ), and 50 mg/l gentamicin. The substituents were optimized for growth and induction potential of the cells. H4IIE cells were kept in continuous culture in our laboratory under typical mammalian cell conditions (37°C, 95:5 air:CO_2, humidified) and were regularly restarted from frozen cells to insure the cultures were not altered. H4IIE cells were cultured in 75 sq. cm. tissue culture flasks in 25 ml of the modified D-MEM. When

the cells obtained confluence (15-20 x 10^6 cells) they were washed twice with cold 0.1M phosphate buffered saline (PBS) at pH 7.4 and trypsinized with a 0.53 mM Trypsin-EDTA solution. Cells were counted on a hemocytometer and then diluted with medium to 10^6 cells/ml. Nine milliliters of D-MEM were placed in petri dishes (100 mm x 20 mm), along with 1 ml of the H4IIE cell suspension to give a seeding rate of 10^6 cells/plate in 10 ml of medium. The cells were incubated for 24 h and allowed to attach and begin to grow on the petri dishes. At the end of this 24 h period the cells were dosed with extracts or standards. The carrier solvent for dosing was isooctane at a volume of 100 ul/plate (1.0%). This amount of isooctane had no effect on survival or the basal EROD activity of the H4IIE cells under standard culture conditions. Triplicate plates were used at each dose and the dose range for extracts was across 3-orders of magnitude (0.01 - 1.0% of the total extract). A TCDD standard curve (4-5 doses in triplicate) was conducted with each set of samples for calculation of TCDD-EQ. Therefore, bioassay-to-bioassay variations in cell response were taken into account. The petri dishes of H4IIE cells, which were dosed with the appropriate extract, control, or standard, were incubated for 72 hours. After the incubation period, the cells were rinsed with PBS and then scraped from the plates while in a Tris-sucrose (0.05 - 0.2M) buffer. The harvested cells were centrifuged for 7 minutes at 10,000X g and resuspended in

Tris-sucrose buffer. Protein determinations (Lowry et al. 1951) were made in duplicate for each sample. Cell suspensions were then diluted to 1 mg protein/ml with appropriate volumes of Tris-sucrose buffer.

EROD assays

The monitor for extract potency in this bioassay system is cytochrome P-450-dependent monooxygenase activity associated with the H4IIE cells. In particular, we chose to used the indirect EROD assay described by Pohl and Fouts (1980). This assay procedure is sensitive, specific for the planar-type induction of P-450 enzyme activity, and is correlated with AHH activity in this cell line (Bandiera et al. 1984). Duplicate EROD assays were performed on each sample in polycarbonate tubes in a shaking water bath incubator at 37°C. A 1.25 ml final reaction volume was made up of 1.0 ml NADPH generator system (5 mM glucose-6-phosphate, 5 mM $MgSO_4$, 3.5 mM NADP, and 1.6 mg BSA/ml in 0.1 M HEPES buffer, pH 7.8), 0.1 ml of 25 unit/ml glucose-6-phosphate dehydrogenase (G6PDH), and 0.1 ml of the 1 mg protein/ml cell suspension. This mixture was preincubated 10 min and the reactions were started at 10s intervals by addition of 0.05 ml of 15 uM ethoxyresorufin (Molecular Probes) in methanol. Reactions were terminated after 10 minutes by addition of 2.5 ml cold methanol and allowed to flocculate an additional 5 minutes at 37°C. Samples were then centrifuged for 8 minutes

at 10,000X g and the supernatant was collected for fluorometric analysis of resorufin.

Fluorescence of resorufin in the samples was determined with an SLM 4800 spectrofluorometer (Urbana, IL) at an emission wavelength of 585 nm and excitation wavelength of 550 nm. The machine was first calibrated with a standard rhodamine B solution. Consequently, the fluorescence in samples was measured relative to this internal standard. Each reading was an average of 20 automatic scans. A resorufin standard was also used with each set of samples, for calibration to a resorufin standard curve and calculation of specific enzyme activities. EROD activity was then calculated and reported as pmoles resorufin/mg protein/min for each sample.

Data analysis

The relationship between EROD activity and dose to the cell was described by probit analysis. All probit analyses were performed with PLOT-IT graphical and statistical software (Scientific Programming Enterprises, Haslett, Michigan). The effective dose to elicit half maximal response (ED_{50}) was calculated for all samples according to Finney (1978). The maximal response of the extract was used to normalize submaximal responses to obtain fractional values for probit transformation. Calculation of the potency for each sample extract was according equation 1 as reported by Sawyer et al.

(1984).

$$\text{Extract potency} = \text{TCDD } ED_{50}/\text{Extract } ED_{50} \quad (1)$$

The probit derived TCDD ED_{50} (pg/plate) was compared with the sample extract ED_{50} (ul/plate) with the resultant extract potency, expressed in units of pg TCDD-EQ/ul of extract. TCDD-EQ were then calculated by equation 2.

$$\text{TCDD-EQ(pg/g)} = \frac{\text{(Extract potency) (Extract vol., ul)}}{\text{(Sample weight, g)}} \quad (2)$$

Variance estimates were calculated according to Finney (1978) by an additive model for variance (equation 3).

$$CV_T = [(CV_E^2) + (CV_S^2)]^{1/2} \quad (3)$$

where:

CV_T = coefficient of variation for TCDD-EQ

CV_E = coefficient of variation for extract ED_{50}

CV_S = coefficient of variation for standard ED_{50}

The standard deviation (SD) of TCDD-EQs in the samples was obtained by multiplying the fractional CV_T by the estimated TCDD-EQ for that sample.

Differences in TCDD-EQs from composites of eggs among regional areas was compared by a non-parametric Kruskal-Wallis

one way analysis of variance of the ranks within each region (Zar 1974) and the parametric General Linear Models procedure (GLM) of SAS (SAS 1982; 1987). Comparison of regional average TCDD-EQs were made by Tukey's and Scheffe's tests for comparison of means (SAS 1985). The value for the single egg collected at the Saginaw Bay CDF (June 11, 1986) was not included in these calculations.

Results

There was a significant amount of EROD induction in H4IIE cells caused by the extracts of all waterbird eggs and a complete dose-response curve was generated by the extracts from all sites tested. Representative extract (Fig. 6) and TCDD standard (Fig. 7) dose-response curves run the same day are presented. All extracts had a complete range of responses within the dosing scheme of 1-100 ul of extract/plate. This volume of extract represents 0.015 - 3.0 g-equivalents of waterbird egg. The range of extract ED_{50} values was 10.14 - 34.34 ul extract/plate while the TCDD ED_{50} values had a range of 40.14 to 82.59 pg TCDD/plate. Coefficients of variation for ED_{50} estimates were in the range of 3.83 to 11.42% and 1.40 to 2.81% for extract ED_{50} and TCDD ED_{50} values, respectively. The resultant estimates of TCDD-EQ in the waterbird eggs generally had coefficients of variation less than 15%. Thus, the analytical error associated with the cell culture, dosing, and enzyme assay of this bioassay system is relatively small.

Figure 6. Representative Dose-Response Curve for a Great Lakes Waterbird Egg Extract in H4IIE Rat Hepatoma Cells.

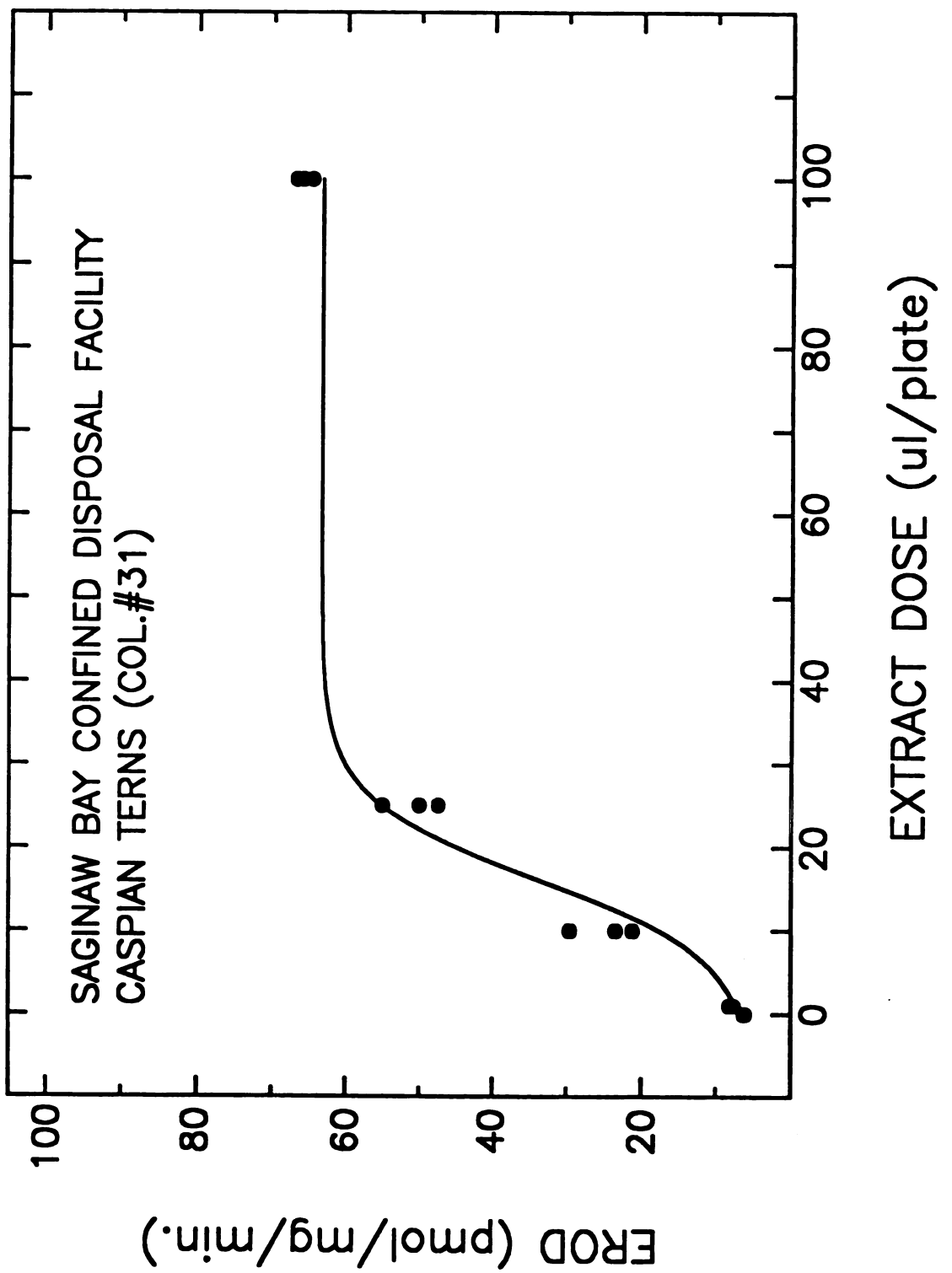
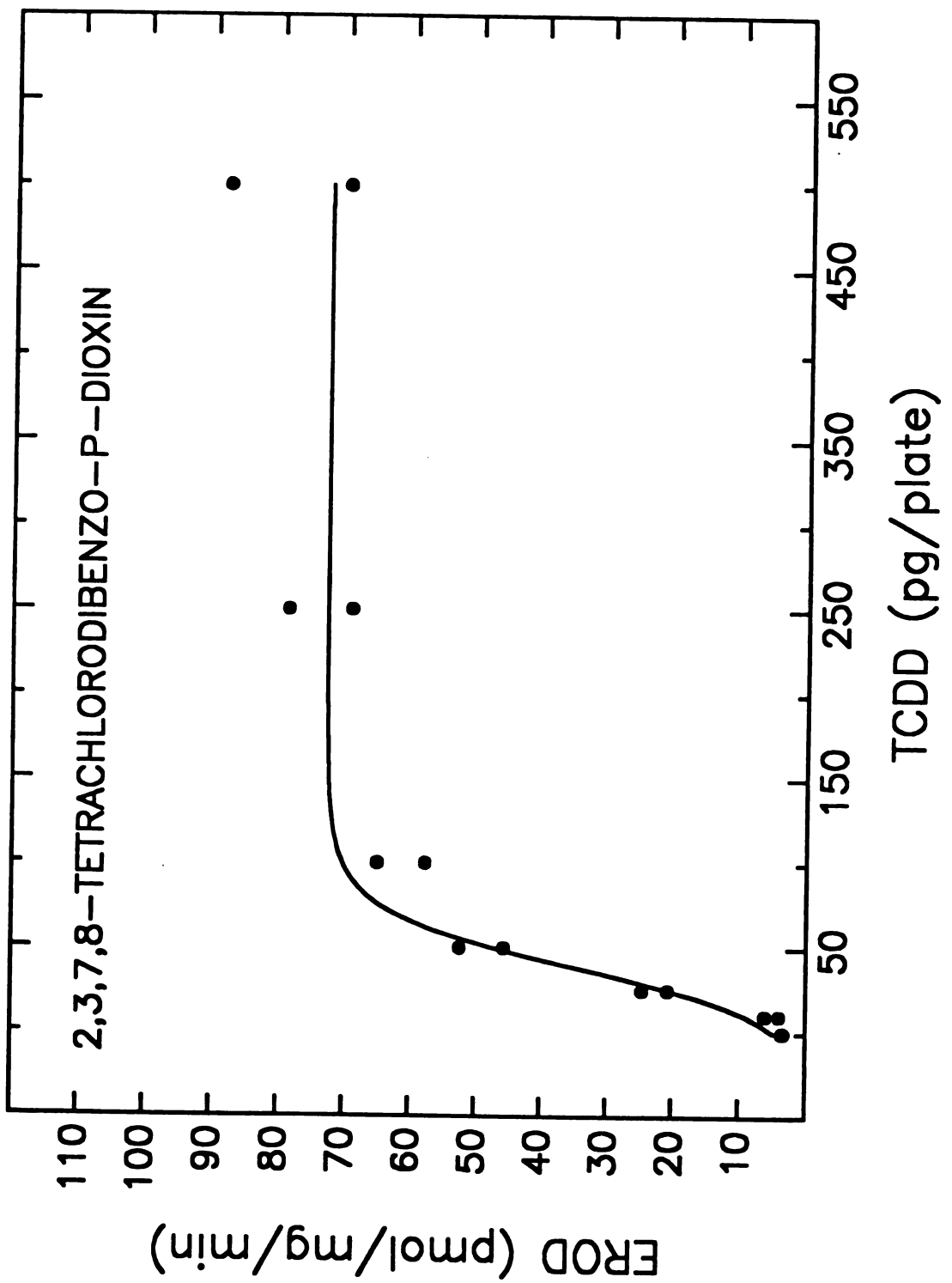

SAGINAW BAY CONFINED DISPOSAL FACILITY
CASPIAN TERNS (COL.#31)
EROD (pmol/mg/min.)
EXTRACT DOSE (ul/plate)
100
80
60
40
20
0
20
40
60
80
100

Figure 7. **TCDD Dose-Response Curve in H4IIE Rat Hepatoma Cells.**


2,3,7,8–TETRACHLORODIBENZO–P–DIOXIN
EROD (pmol/mg/min)
TCDD (pg/plate)
10
20
30
40
50
60
70
80
90
100
110
50
150
250
350
450
550

Significant concentrations of TCDD-EQs were found in waterbird eggs from all areas of the Great Lakes examined. TCDD-EQs (pg/g) in double-crested cormorant egg composites (Fig. 8) and Caspian tern egg composites (Fig. 9) are presented for regional and species comparisons. The bioassay-derived TCDD-EQs in the composite waterbird egg samples varied from a low value of 49.7 pg TCDD-EQ/g for the 1986 High Is. Caspian tern colony (No. 6) to a high of 415.7 pg TCDD-EQ/g for the 1986 Caspian tern eggs from the Saginaw Bay Confined Disposal Facility (Colony No. 31). There were no significant differences among the TCDD-EQs found in cormorant and tern eggs based on a comparison of their ranks (Mann-Whitney U test, $p > 0.20$) or actual TCDD-EQs (GLM SAS, $p > 0.98$) when compared across regions. Within regions which had both species, cormorant eggs had slightly greater TCDD-EQ values (12%), but they were not statistically significant (GLM, $p = 0.57 - 0.81$). Additionally, there was no difference between TCDD-EQ in waterbird eggs collected in 1986 and 1987 (GLM SAS, $p > 0.80$).

TCDD-EQs in waterbird eggs from the various regions were not the same. When these two species were ranked in descending order of TCDD-EQ and the colony sites were classified according to regional distribution in the Great Lakes, a significant effect of regional distribution was observed. The average rank of the cormorant and Caspian tern

Figure 8. H4IIE Bioassy-Derived TCDD-EQ (pg/g) in Double-Crested Cormorant Eggs. The numbers under each bar refer to colony ID numbers (see Table 4).

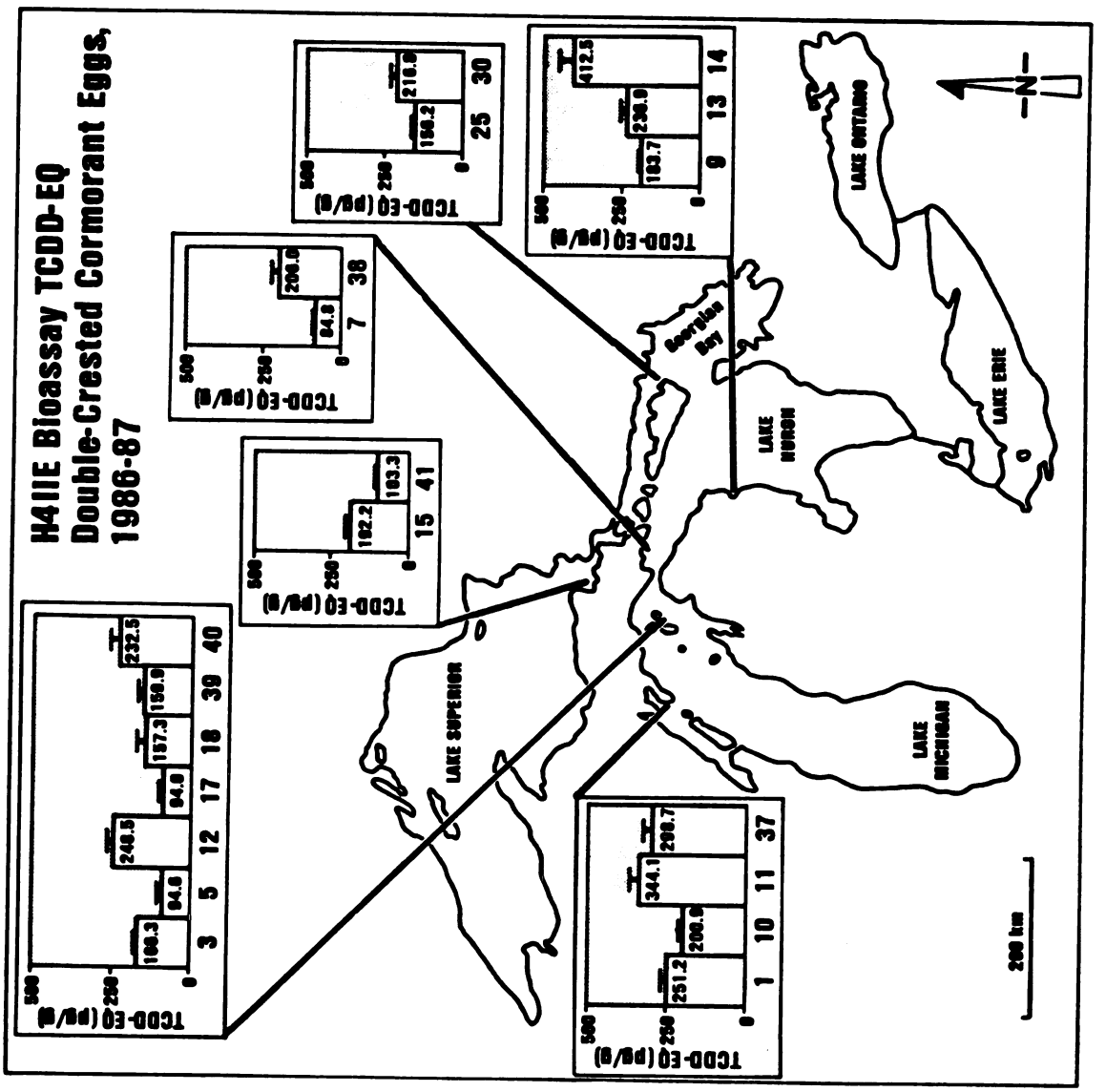
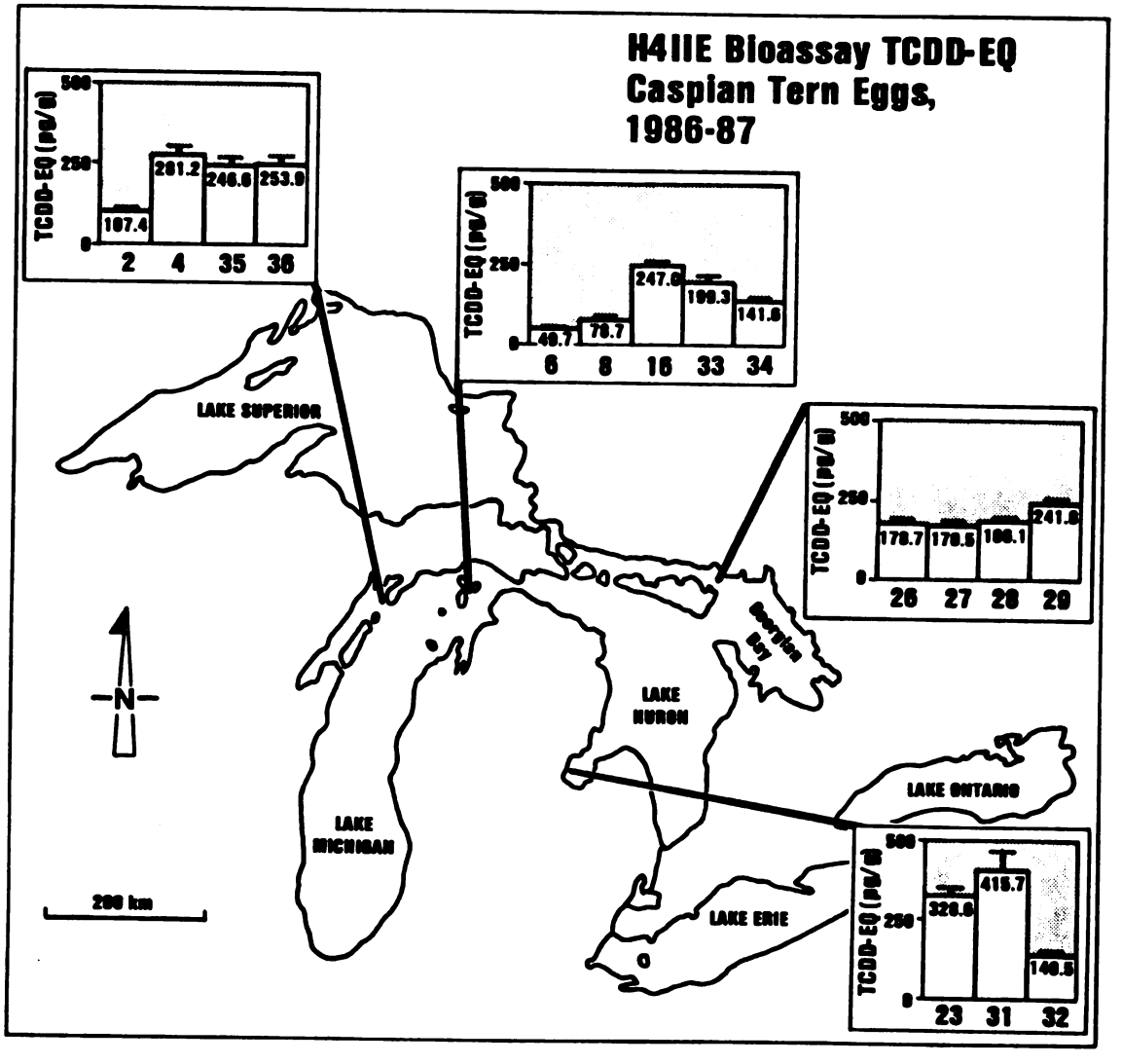

H4IIE Bioassay TCDD-EQ
Double-Crested Cormorant Eggs,
1986-87
TCDD-EQ (pg/g)
500
250
0
3 5 12 17 18 30 40
100.3
84.8
246.5
84.0
157.3
159.0
232.5
15 41
182.2
193.3
7 38
84.8
200.0
25 30
190.2
210.0
0 13 14
183.7
238.0
412.5
1 10 11 37
251.2
200.0
344.1
298.7
LAKE SUPERIOR
LAKE MICHIGAN
LAKE HURON
Georgian Bay
LAKE ERIE
LAKE ONTARIO
-N-
200 km

Figure 9. H4IIE Bioassay-Derived TCDD-EQ (pg/g) in Caspian Tern Eggs. The numbers under each bar refer to colony ID numbers (see Table 4).


H4IIE Bioassay TCDD-EQ
Caspian Tern Eggs,
1986-87
TCDD-EQ (pg/g)
500
250
0
187.4
281.2
246.8
253.9
2
4
35
36
49.7
78.7
247.8
199.3
141.8
6
8
16
33
34
178.7
178.5
188.1
241.8
26
27
28
29
328.8
415.7
146.5
23
31
32
LAKE SUPERIOR
LAKE MICHIGAN
LAKE HURON
Georgian Bay
LAKE ONTARIO
LAKE ERIE
N
200 km

egg samples for the regions was calculated (Table 5). Comparison of ranks showed a regional effect (Kruskal-Wallis, $0.10 > p > 0.05$) as did comparison of TCDD-EQ in the waterbird eggs (GLM SAS, $p = 0.0058$). Regional averages of TCDD-EQ for cormorant and tern egg composites are presented in Table 6. The variation of TCDD-EQ in eggs within regions was relatively large. Coefficients of variation for among colony averages within regions were between 16 - 59% with a mean value of 38%. Therefore, there is a significant variation of PHH contaminant burden in double-crested cormorant and Caspian tern eggs among colonies. Individual eggs, within colonies, were not analyzed to address within colony-bird, or clutch variations of PHH burdens in these eggs.

Three other species of waterbird eggs were collected and analyzed with the H4IIE bioassay for comparative purposes (Table 7). TCDD-EQ in these species' eggs were within the range of values found for cormorants and terns. Because of the limited number of samples from these species it is impossible to make within-regional comparisons. However, when among species differences are ignored, the Saginaw Bay waterbird eggs fall in the upper half of the overall ranks and the common tern egg composites from Beaver Island and Thunder Bay are in the lower half of the overall ranks.

Table 5 Average Rank of H4IIE TCDD-EQ in Double Crested Cormorant and Caspian Tern Egg Composites by Region.

Region	N	Average Rank
Green Bay, Lake Michigan	8	10.9
Saginaw Bay, Lake Huron	3	11.3
Thunder Bay, Lake Huron	3	12.0
Georgian Bay/N. Channel, Lake Huron	6	19.8
Beaver Island, Lake Michigan	12	24.2
N.W. Lake Huron	2	25.0
Tahquamenon Island, Lake Superior	2	25.0

Egg composites ranked from highest to lowest TCDD-EQ and the average ranks within regions were determined.

N = number of egg composites in region.

Table 6 Regional Averages of TCDD-EQ in Egg Composites from Double Crested Cormorants and Caspian Terns

Region/Species	Species	Average TCDD-EQ (SD)
Green Bay, Lake Michigan	DCC	273.7 (62)
	CPT	222.3 (78)
Saginaw Bay, Lake Huron	CPT	294.9 (141)
Thunder Bay, Lake Huron	DCC	277.7 (120)
Georgian Bay/North Channel Lake Huron	DCC	186.5 (43)
	CPT	194.3 (32)
Beaver Island, Lake Michigan	DCC	164.7 (60)
	CPT	143.3 (82)
N.W. Lake Huron	DCC	145.4 (86)
Tahquamenon Island, Lake Superior	DCC	147.8 (63)

TCDD-EQ (pg/g) and standard deviation (SD)

Table 7. TCDD-EQ in Egg Composites of Black Crowned Night Heron, Ring-Billed Gull and Common Tern

Species	Colony ID	TCDD-EQ (SD) (pg/g)
Black-crowned night heron	24	221.8 (20)
Ring-billed gull	22	208.1 (17)
Common tern	19	187.4 (17)
Common tern	20	104.3 (5.0)

TCDD-EQ(pg/g) and standard deviation (SD); Colony ID number from Table A1.

Discussion

Significant concentrations of TCDD-EQ were detected in PCB extracts of eggs from fish-eating waterbirds from all areas of the Great Lakes. The concentrations of PHHs were not only significant enough for induction in the H4IIE bioassay, but significant enough to cause a maximal induction at the highest dose of each extract (100 ul/plate) in all cases. This relates to maximal induction by 1-3 g-equivalents of the egg composites. This has not always the case when we have examined other samples (fish and bird flesh) from around the Great Lakes with the H4IIE bioassay. Procedural blanks from the PAM procedure, solvent blanks, extracts of chicken eggs from a retail store, and extracts of fertilized chicken eggs did not cause induction of EROD in the H4IIE cells. Therefore, induction by the waterbird egg extracts can not be accounted for by any of these factors. The fact that fairly high TCDD-EQ were detected even in remote, non-urban, non-industrialized areas is not completely unexpected based on the recalcitrant nature of these environmental contaminants and the estimates that the majority of PCBs enter the Great Lakes through atmospheric transport and deposition (Eisenreich et al., 1981).

H4IIE bioassay-derived TCDD-EQ in eggs of double-crested cormorants and Caspian terns ranged from 47.9 to 415.7 pg/g. The biological significance of this is not completely

understand at this time. However, what is known is the fact that the inductive response in the H4IIE bioassay is quantitative with respect to PHH dose (Bradlaw and Casterline 1979; Sawyer and Safe 1982) The potential utility of the H4IIE bioassay results lies in two distinct applications for assessment of complex mixtures of PHHs in environmental samples. One is strictly as a bioanalytical tool for relative comparisons of overall PHH potency and the other is as a predictive tool for comparison with environmental effects. Applications for the H4IIE bioassay as a bioanalytical tool are numerous and many of the initial characterization studies to this end suggest that it has good potential (Niwa et al. 1975; Bradlaw and Casterline 1979; Trotter et al. 1982; Casterline et al 1983). The H4IIE bioassay can act as a data reduction tool by integrating the overall interactions of complex PHH mixtures at the cellular level. Even if the values obtained are not predictive of toxicological effects, the H4IIE bioassay can serve as an integrative standard for relative comparisons within and among environmental matrices. As a predictive tool the H4IIE bioassay has been validated for use in rats by the strong correlations between its <u>in vitro</u> response and the toxic responses of rats <u>in vivo</u> to individual PHH congeners (Safe 1987). If the H4IIE bioassay is to become a tool for predicting environmental effects in various species, an understanding will be required of the relative structure-activity relationships within those species as well

as an understanding of the acute versus chronic potency within the same species. In other words, it must be demonstrated that the rank order of PHH potencies is similar among rats and the species of interest. There are no such data for the waterbirds tested in this study, however, the limited data available in chickens indicates that the relative rank order of potency for PCDDs (Poland and Glover 1973; Bradlaw and Casterline 1979) and PCBs (Brunstrom and Anderson 1988) are similar to that of rats.

The relative ranks of the TCDD-EQs in egg composites from this study correlates well with those areas known to have PHH contamination and known to have increased rates of deformities in fish-eating waterbirds. Forster's terns (Sterna forsteri) from Green Bay have had impaired reproduction including an increased incidence of embryo toxicity, increased incidence of deformities, and altered parental behavior (Trick 1982; Heinz et al. 1985; Harris et al. 1985; Hoffman et al. 1987). A comprehensive study of Forster's terns from Green Bay and a reference site in Wisconsin compared several biological endpoints and chemical residues from these two colonies (Kubiak et al. 1989). Concentrations of TCDD and several bioactive PCBs were found to be significantly greater in eggs from the Green Bay colony as compared to the reference colony on Lake Poygan, WI. These authors found decreased hatching success (both in the field and in laboratory-reared eggs), decreased body weights of the

hatchlings, and increased liver to body weight ratios in Green Bay terns as compared to those from the reference site, Lake Poygan (Kubiak et al. 1989) A similar etiology of chick edema disease was described by Gilbertson (1983) for the severe reproductive failure of herring gulls (Larus agentatus) in PHH contaminated areas of Lake Ontario. Elevated TCDD-EQ in Caspian tern eggs from Saginaw Bay (up to 415 pg TCDD-EQ/g) in this study concur with the field data from this site which has had depressed hatching success and no survival past fledge (Kurita et al. 1987) in some years. Additionally, Weseloch et al. (1985) found the incidence of congenital anomolies in double-crested cormorants from Green Bay (1983 and 1984) to be 0.95% (31 of 3249) as compared to zero (0 to 2265) from the other study areas of Lake Erie, Lake Superior, and Lake-of-the-Woods. Recently, Fox et al. (1988) have monitored various PHHs in herring gull livers from around the Great Lakes and compared these residues to the concentrations of carboxylated porphyrins in the livers of birds from the same area. Altered heme biosynthesis resulting in an accumulation of carboxylated porphyrins is also a symptom of PHH-induced toxicity (Elder 1978). Fox et al. (1988) found that herring gulls from Green Bay and Saginaw Bay had the greatest concentrations of carboxylated porphyrins in their livers compared to all other Great Lake sites.

Laboratory studies clearly indicate that both PCBs and PCDDs can cause the same suite of effects, including

reproductive impairment and altered biochemical homeostasis, that have been reported in field studies of fish-eating waterbirds (McCune et al. 1962; Higginbotham et al. 1968; Dahlgren and Linder 1971; Carlson and Duby 1973; Cecil et al. 1973; 1974; Peakall and Peakall 1973; Platnow and Reinhart 1973; Poland and Glover 1973; Tusamonis et al. 1973; Ax and Hansen 1975; McKinney et al. 1976; Cheung et al. 1981). It has also been established, both by residue analysis (see Baumann and Whittle 1988, for recent review) and now by H4IIE extract bioassay described in this study, that PHH residues exist within the aquatic food chain in the Great Lakes and that they are elevated in areas of industrial pollution such as Green Bay and Saginaw Bay. Evidence for a causal role of PHHs in the reproductive impairment of these waterbirds is circumstantial. The studies making the direct link between these compounds and an adverse effect in the field have not been undertaken. The evidence, however circumstantial, for such a relationship appears to be mounting.

Whether the TCDD-EQ obtained for these complex mixtures reflect absolutely what effect that amount of TCDD in the organism would have was beyond the scope of work in this study and has not been addressed by other investigators. However, comparisons that can be made between the TCDD-EQ observed in waterbird eggs in this study and laboratory data on the effects of TCDD in birds, are found within the literature on chicken embryos exposed to TCDD. To make such comparisons,

one must make the assumption that these values are absolute predictions of the potential toxic effects of TCDD at the equivalent concentrations. This assumption has not been validated by this study. However, with this in mind, the lowest observed adverse effect concentration (LOAEC) in chicken embryos is 6.4 pg TCDD/g (Cheung et al. 1981). These investigators found a 21% increase in cardiovascular malformations in white leghorn chicken embryos at this concentration (estimated based on 50 g egg weight) and a 200% increase at 65 pg TCDD/g. The LD_{50} for hatchability in this same species was estimated at 140 pg TCDD/g (Goldstein 1980) and 1000 pg TCDD/g caused 100% embryonic death (Higginbotham 1968). The LD_{50} for adult white leghorn chickens is between 25,000 and 50,000 pg/g (Sawyer et al. 1986), thus showing the sensitivity difference between adult and embryonic stages. The other issue that must be considered when making these comparisons is that of differences in species sensitivity. Clearly, chickens appear to be one of the most, if not the most sensitive avian species to the toxic effects of TCDD (Goldstein 1980; Brunstrom 1988). However, we are uncertain where Caspian terns and double-crested cormorant embryos lay along the scale from sensitive to relatively insensitive species.

Recently, in a study of Forster's terns on Green Bay (Kubiak et al. 1989), relative potency factors developed from the H4IIE bioassay were used to convert chemical residue

analysis data into dioxin-equivalents. The authors used "conversion factors" (similar to "toxic equivalency factors," TEFs) which were derived by comparison of an individual congener's potency in the H4IIE bioassay to that of TCDD (Sawyer et al. 1984). Dioxin-equivalents for individual congeners were then calculated by multiplying the congener's "conversion factor" by its concentration in the sample. These individual values of dioxin-equivalents were then summed, assuming an additive model of toxicity, to estimate total dioxin-equivalents in the sample. Using this method, these authors calculated dioxin-equivalents in Forster's tern eggs from Green Bay, 1983 to have a median value of 2175 pg/g. This value is approximately an order of magnitude greater than the concentration of H4IIE-derived TCDD-EQ in Green Bay waterbird eggs from this study. While there are temporal and species differences to consider, these discrepancies may be due to less than additive effects or antagonism which is only assessed by the H4IIE bioassay. It is also interesting to note that 2,3,7,8-TCDD accounted for less than 2% of the total dioxin-equivalents in their calculations (Kubiak et al. 1989), the remainder being made up of only a few of the bioactive PCB congeners.

In conclusion, the H4IIE bioassay may be a useful tool for assessment of PHH mixtures from environmental matrices. It provides a determination of the potency of the mixture which incorporates the synergistic and antagonistic

interactions which can occur at the cellular level. Additionally, because the extracts are taken from the target organ, differences in pharmacokinetics among congeners and species are incorporated into this bioassay system. Further studies are required to determine whether the relative potency of PHHs in rat cells are similar to their relative potencies in other species. Until such work is complete, caution should be taken when using this bioassay to assess toxic risks to other species. However, the potential utility of this simple, fast, and integrative bioassay remains.

Summary

The H4IIE bioassay was used to assess the potency of PCB extracts from colonial fish-eating waterbirds from around the Great Lakes. Significant concentrations of TCDD-EQ were found in all of the waterbird eggs. The range of values was 49-415 pg TCDD-EQ/g. There was a significant effect of regional distribution which corresponds with the existing data on PHH residues and adverse effects seen in DCC and CPTs. Waterbird eggs from urban and industrialized areas had the greatest amount of TCDD-EQ while the more remote areas had less contaminated eggs. At this time it is impossible to understand the exact significance of H4IIE-derived TCDD-EQ in this range for these species. However, the bioassay data appear to corroborate residue analysis and the adverse environmental effects seen in the colonial fish-eating waterbirds of the Great Lakes.

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References

Ax EL, Hansen LG (1975) Effects of purified polychlorinated biphenyl analogs on chicken reproduction. Poult Sci 54:895-900.

Bandiera S, Sawyer T, Romkes M, Zmudka B, Safe L, Mason G, Keys B, Safe S (1984) Polychlorinated dibenzofurans (PCDFs): effects of structure on binding to the 2,3,7,8-TCDD cytosolic receptor protein, AHH induction and toxicity. Toxicol 32:131-144.

Bandiera S, Sawyer T, Campbell MA, Robertson L, Safe S (1982) Halogenated biphenyls as AHH inducers: effects of

different halogen substituents. Life Sci 31:517-525.

Bannister R, Biegel L, Davis D, Astroff B, Safe S (1989) 6-Methyl-1,3,8-trichlorodibenzofuran (MCDF) as a 2,3,7,8-tetrachlorodibenzo-p-dioxin antagonist in C57BL/6 mice. Toxicol 54:139-150.

Bannister R, Davis D, Zacharewski T, Tizard I, Safe S (1987) Aroclor 1254 as a 2,3,7,8-tetrachlorodibenzo-p-dioxin antagonist: effects on enzyme induction and immunotoxicity. Toxicol 46:29-42.

Bannister R, Safe S (1987) Synergistic interactions of 2,3,7,8-TCDD and 2,2',4,4',5,5'-hexachlorobiphenyl in C57BL/GJ and DBA/2J mice: role of the Ah receptor. Toxicol 44:159-169.

Baumann PC, Whittle DM (1988) The status of selected organics in the Laurentian Great Lakes: an overview of DDT, PCBs, dioxins, furans, and aromatic hydrocarbons. Aquatic Toxicol 11:241-257.

Benedict WF, Gielen JE, Owens IS, Niwa A, Nebert DW (1973) Aryl hydrocarbon hydroxylase induction in mammalian liver cell culture - IV. Stimulation of the enzyme activity in established cell livers derived from rat or

mouse hepatoma and from normal rat liver. Biochem. Pharmacol. 22:2766-2769.

Biegel L, Harris M, Davis D, Rosengren R, Safe L, Safe S (1989) 2,2',4,4',5,5'-Hexachlorobiphenyl as a 2,3,7,8-tetra-chlorodibenzo-p-dioxin antagonist in C57BL/6J mice. Toxicol Appl Pharmacol 97:561-571.

Birnbaum LS, Weber H, Harris MW, Lamb JC, McKinney JD (1985) Toxic interaction of specific polychlorinated biphenyls and 2,3,7,8-tetrachlorodibenzo-p-dioxin: Increased incidence of cleft palate in mice. Toxicol Appl Pharmacol 77:292-302.

Bradlaw JA, Casterline JL (1979) Induction of enzyme activity in cell culture: A rapid screen for detection of planar polychlorinated organic compounds. J Assoc Off Anal Chem 62:904-916.

Brunstrom B (1988) Sensitivity of embryos from duck, goose, Herring gull, and various chicken breeds to 3,3',4,4'-tetrachlorobiphenyl. Poultry Sci 67:52-57.

Brunstrom B, Andersson L (1988) Toxicity and 7-ethoxyresorufin O-deethylase-inducing potency of coplanar polychlorinated biphenyls (PCBs) in chicken embryos.

Arch Toxicol 62:263-266.

Carlson RW, Duby RT (1973 Embryo toxic effects of three PCBs in the chicken. Bull Environ Contam Toxicol 9:261-266.

Casterline JL, Bradlaw JA, Puma BJ, Ku Y (1983) Screening of fresh water fish extracts for enzyme-inducing substances by an aryl hydrocarbon hydroxylase induction bioassay technique. J Assoc Off Anal Chem 66:1136-1139.

Cecil HC, Bitman J, Lillie RJ, Fries GF, Verret J (1974) Embryotoxic and teratogenic effects in unhatched fertile eggs from hen fed polychlorinated biphenyls (PCBs). Bull Environ Contam Toxicol 11:489-495.

Cecil HC, Harris SJ, Bitman J, Fries GF (1973) Polychlorinated biphenyl-induced decrease in liver Vitamin A in Japanese quail and rats. Bull Environ Contam Toxicol 9:179-185.

Cheung MO, Gilbert EF, Peterson RE (1981) Cardiovascular teratogenicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin in the chick embryo. Toxicol Appl Pharmacol 61:197-204.

Colborn T (1988) Great Lakes Toxics Working Paper. Report to Toxics Data Base Project-Steering Committee, Environment Canada, Contract KE 144-7-6336.

Dahlgren RB, Linder RL (1971) Effects of polychlorinated biphenyls on pheasant reproduction, behavior, and survival. J Wildl Manage 35:315-319.

Davis D, Safe S (1988) Immunosuppressive activities of polychlorinated dibenzofuran congeners: quantitative structure - activity relationships and interactive effects. Toxicol Appl Pharmacol 94:141-149.

Denomme, MA, Homonko K, Fujita T, Sawyer T, Safe S (1986) Substituted polychlorinated dibenzofuran receptor binding affinities and aryl hydrocarbon hydroxylase induction potencies - a QSAR analysis. Chem Biol Interaction 57:175-187.

Eadon G, Kaminsky L, Silkworth J, Aldous K, Hilker D, O'Keefe P, Smith R, Gierthy J, Hawley J, Kim N, DeCaprio A (1986) Calculation of 2,3,7,8-TCDD equivalent concentrations of complex environmental contaminant mixtures. Environ Health Perspect 70:221-227.

Elder GH (1978) Porphyria caused by hexachlorobenezene and

other polyhalogenated aromatic hydrocarbons. In: F DeMatteis and WD Aldridge (eds) Heme and hemoproteins. Springer-Verlag, New York, NY, pg 157-200.

Eisenreich SJ, Looney BB, Thorton JD (1981) Airborne organic contaminants in the Great Lakes ecosystem. Environ Sci Technol 15:30.

Evans MS (1988) Toxic contaminants and ecosystem health: A A Great Lakes focus. John Wiley and Sons, p. 602.

Finney DJ (1978) Statistical Methods in Biological Assay, 3rd ed. Charles Griffin and Company, London. 333 pgs.

Fox GA, Kennedy SW, Norstrom RJ (1988) Porphyria in herring gulls: A biochemical response to chemical contamination of Great Lakes food chains. Environ Toxicol Chem 7:831-839.

Gilbertson M (1983) Etiology of chick edema disease in herring gulls in the Lower Great Lakes. Chemosphere 12:357-370.

Gilbertson M, Fox GA (1977) Pollutant-associated embryonic mortality of Great Lakes herring gulls. Environ Pollut 12:211-216.

Gochfeld, M (1975) Development defects in common terns of western Long Island, New York. The AUK 92:58-65.

Goldstein JA (1980) Structure-activity relationships for the biochemical effects and relationship to toxicity. In Rd Kimbrough (ed) Topics in Environmental Health: Vol. 4. Halogenated biphenyls, terphenyls naphthalenes, dibenzodioxins and related products. Elsevier North-Holland Inc. New York, NY, pp. 151-190.

Greenlee, WF, Neal RA (1985) The Ah receptor: a biochemical and biologic perspective. In The Receptors Vol. II pp. 89-129.

Haake JM, Safe S, Mayura K, Phillips TD (1987) Aroclor 1254 as an antagonist of the teratogenicity of 2,3,7,8-tetrachloro-dibenzo-p-dioxin. Toxicol Lett 38:299-306.

Harris JH (1988) Persistent toxic substances and birds and mammals in the Great Lakes. In: M.S. Evans (ed) Toxic contaminants and ecosystem health: a Great Lakes focus. John Wiley and Sons Inc. pg. 557-569.

Harris HJ, Kubiak TJ, Trick J (1985) Micro-contaminants and reproductive impairment of the Forster's Tern on Green

Bay. Final Rept, USFWS, U-W Sea Grant Institute, WI DNR, and Green Bay Metro, Sewage Dist, 39 pp. U.W. Sea-Grant Institute, ES-105, UW-Green Bay, Green Bay, WI.

Heinz GH, Erdman TC, Haseltine SD, Stafford C (1985) Contaminant levels in fish-eating birds from Green Bay and Lake Michigan, 1975-1980. Environ Monitor Assessm 5:223-236.

Higginbotham GR, Huang A, Firestone D, Verrett J, Ress J, Campbell AD (1968) Chemical and toxicological evaluations of isolated and synthetic chloroderivatives of dibenzo-p-dioxins. Nature 220:702-3.

Hoffman DJ, Rattner BA, Sileo L, Docherty D, Kubiak T (1987) Embryotoxicity, teratogenicity, and aryl hydrocarbon hydroxylase activity in Forster's terns in Green Bay, Lake Michigan. Environ Res 42:176-184.

Keys B, Piskorska-Pliszczynska J, Safe S (1986) Polychlorinated dibenzofurans as 2,3,7,8-TCDD antagonists: in vitro inhibition of monooxygenase induction. Toxicol Lett 31:151.

Kubiak TJ, Harris HJ, Smith LM, Schwartz T, Stalling DL, Trick

JA, Sileo L, Docherty D, Erdman TC (1989). Microcontaminants and reproductive impairment of the Forster's tern in Green Bay, Lake Michigan - 1983. Arch Environ Contam Toxicol 18:706-727.

Kurita H, Ludwig JP, Ludwig ME (1987) Results of the 1987 Michigan colonial waterbird monitoring project on Caspian terns and Double-crested cormorants: Egg incubation and field studies of colony productivity, embryologic mortality and deformities. Ecological Research Services. Report to Mich Dept Nat Res.

Leece B, Denomme MA, Towner R, Li A, Landers J, Safe S (1987) Nonadditive interactive effects of polychlorinated biphenyl congeners in rats: role of the 2,3,7,8-tetrachlorodibenzo-p-dioxin receptor. Can J Physiol Pharmacol 65:1908-1912.

Leece B, Denomme MA, Towner R, Li SMA, Safe S (1985) Polychlorinated biphenyls: correlation between in vivo and in vitro quantitative structure-activity relations (QSAR). J Toxicol Environ Health 16:379-388.

Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ, (1951) Protein measurement with Folin phenol reagent. J Biol Chem 193:265.

Mason G, Sawyer T, Keys B, Bandiera S, Romkes M, Piskorska-Pliszczynska J, Zmudzka B, Safe S (1985) Polychlorinated dibenzofurans (PCDFs): Correlation between in vivo and in vitro structure-activity relationships. Toxicol 37:1-12.

McCune EL, Savage JE, O'Dell BL (1962) Hydropericardium and ascites in chicks fed a chlorinated hydrocarbon. Poult Sci 41:295-299.

McKinney JD, Chae K, Gupta BN, Moore JA, Goldstein JA (1976) Toxicological assessment of hexachlorobiphenyl isomers and 2,3,7,8-tetrachlorodibenzofuran in chicks. 1. Relationship of chemical parameters. Toxicol Appl Pharmacol 36:65-80.

Mineau P, Fox GA, Norstrom RJ, Weseloh DV, Hallett DJ, Ellenton, JA (1984) Using the Herring gull to monitor levels and effects of organochlorine contamination in the Canadian Great Lakes. In: JO Nriagu and MS Simmons (eds) Toxic contaminants in the Great Lakes. John Wiley and Sons Inc. New York, pp 425-452.

Niemi GJ, Davis TE, Vieth GD, Vieux B, (1986) Organochlorine chemical residues in Herring gulls, Ring-billed gulls,

and common terms of Western Lake Superior. Arch Environ Contam Toxicol 15:313-320.

Niwa A, Kumaki K, Nebert DW (1975) Induction of aryl hydrocarbon hydroxylase activity in various cell cultures by 2,3,7,8-tetrachlorodibenzo-p-dioxin. Mol Pharmacol 11:399-408.

Peakall DB, (1988) Known effects of pollutants on fish-eating birds in the Great Lakes of North America. In: Proceedings of the World Conference on Large Lakes. May 18-21, 1986, Mackinac Island, MI. Vol 1. Chronic effects of toxic contaminants in large lakes. Chapt 4.

Peakall DB, Peakall ML (1973) Effect of a polychlorinated biphenyl on the reproduction of artificially and naturally incubated dove eggs. J Appl Ecol 10:863-868.

Pesticide Analytical Manual (1979) Volume I. Food and Drug Administration, Washington, DC, Sec. 212.13a.

Platonow NS, Reinhart BS (1973) The effects of polychlorinated biphenyls (Arclor 1254) on chicken egg production ,fertility and hatchability. Can J Compar Med 37:341-346.

Pluss N, Poiger H, Hohbach C, Schlatter C (1988a) Subchronic toxicity of some chlorinated dibenzofurans (PCDFs) and a mixture of PCDFs and chlorinated dibenzodioxins (PCDDs) in rats. Chemosphere 17:973-984.

Pluss N, Poiger H, Hohbach C, Suter M, Schlatter C (1988b) Subchronic toxicity of 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) in rats. Chemosphere 17:1099-1110.

Pohl RJ, Fouts Jr (1980) A rapid method for assaying the metabolism of 7-ethoxyresorufin by microsomal subcellular fractions. Analyt Biochem 107:150-158.

Poland A, Glover E (1973) Studies on the mechanism of toxicity of the chlorinated dibenzo-p-dioxins. Environ Health Perspec 5:245-251.

Poland A, Knutson JC (1982) 2,3,7,8-tetrachlorodibenzo-p-dioxin and related halogenated aromatic hydrocarbons: examination of the mechanism of toxicity. Ann Rev Pharmacol Toxicol 22:517-554.

Safe S (1986) Comparative toxicology and mechanism of action of polychlorinated dibenzo-p-dioxins and dibenzofurans. Ann Rev Pharmacol Toxicol 26:371-399.

Safe S (1987) Determination of 2,3,7,8-TCDD toxic equivalent factors (TEFs): support for the use of the in vitro AHH induction assay. Chemosphere 16:791-802.

SAS Institute Inc. (1982) SAS Users Guide: Statistics. Box 8000 Cary, NC.

SAS Institute Inc. (1987) SAS/STAT Guide for Personal Computers, Version 6 Edition. Box 8000 Cary, NC.

Sawyer T, Safe S (1982) PCB isomers and congeners: induction of aryl hydrocarbon hydroxylase and ethoxyresorufin O-deethylase enzyme activities in rat hepatoma cells. Toxicol Lett 13:87-94.

Sawyer TW, Vatcher AD, Safe S (1984) Comparative aryl hydrocarbon hydroxylase induction activities of commercial PCBs in Wistar rats and rat hepatoma H-4-II-E cells in culture. Chemosphere 13:695-701.

Sawyer TW, Safe S (1985) In vitro AHH induction by polychlorinated biphenyl and dibenzofuran mixtures: Additive effects. Chemosphere 14(1):7 9-84.

Tanabe S, Kannan N, Subramanian A, Watanabe S, Tatsukawa R

(1987) Highly toxic coplanar PCBs: Occurrence source, persistency and toxic implications to wildlife and humans. Environ Pollut 47:147-163.

Trick JA (1982) Reproductive success and foraging success as related to populations of Forster's terns (Sterna fosteri) on Green Bay, Lake Michigan. MS thesis, University of Wisconsin, Green Bay, 49 p.

Trotter WJ, Young SJV, Casterline JL, Bradlaw JA, Kamps LR (1982) Induction of aryl hydrocarbon hydroxylase activity in cell cultures by aroclors, residues from Yusho Oil samples, and polychlorinated biphenyl residues from fish samples. J Assoc Off Anal Chem 65:838-841.

Tumasonis CF, Bush B, Baker FD (1973) PCB levels in egg yolks associated with embryonic mortality and deformity of hatched chicks. Arch Environ Contam Toxicol 1:312-324.

Weber H, Harris MW, Haseman JK, Birnbaum LS (1985 Teratogenic potency of TCDD, TCDF and TCDD-TCDF combinations in C57BL/6N mice. Toxicol Lett 26:159-167.

Weseloh DV, Struger J, Kubiak TJ (1985) The occurrence of

congenital anomalies in Double-crested Cormorants and Herring Gulls in the Great Lakes, 1983-1984. Proc. 28th Conf. Great Lakes Res.

Whitlock JP (1987) The regulation of gene expression by 2,3,7,8-tetrachlorodibenzo-p-dioxin. Pharmacological Rev. 39(2):147-161.

Zar JH (1974) Biostatistical Analysis. Prentice Hall Inc, Englewood Cliffs, NY.

CHAPTER 3

Comparison of the Relative Potencies of PCB Mixtures from Environmental Samples with the H4IIE Rat Hepatoma Bioassay.

Introduction

Polychlorinated biphenyls (PCBs) are an important group of environmental contaminants consisting of up to a theoretical 209 individual congeners. Even though there has been a great deal of research into the toxicological effects of these compounds, assessment of the potential environmental effects of PCBs has been hindered. Part of the problem encountered in the environmental assessment of PCBs stems from the fact that the individual PCB congeners inherently have different physical and chemical properties (Mackay et al., 1983). The differences in physical and chemical properties among the PCB congeners results in variable rates of transport and transformation of these congeners in the environment. Environmental fate properties such as volatilization, sorption, bioaccumulation, photolysis, and biodegradation can exhibit vastly different rates for each of the PCB congeners (Hutzinger et al., 1974). The result of these differential rates of environmental transport and transformation for the individual PCB congeners is that patterns or ratios of PCBs are drastically different among certain environmental matrices (Schwartz et al., 1987). Conventional analysis of PCBs in environmental samples calls for standardization against the technical mixtures which were originally released into the environment (Pesticide Analytical Manual, 1979; Ribick et al., 1981; Burkhard and Weininger, 1987). In the U.S. these mixtures were largely the Aroclors 1232, 1242, 1248, 1254, and

1260 produced by Monsanto Corporation (St. Louis, MO) which contain 32%, 42%, 54%, and 60% chlorine by weight, respectively. Comparison of the technical PCB standards with environmental extracts of PCB becomes a difficult task because of the gross differences which can occur among the patterns of individual PCB congeners found in samples and standards. At best, these measurements of total PCBs are accurate but toxicologically irrelevant. At worst, such quantitation of total PCBs against a technical standard is completely inaccurate.

The differences in PCB patterns among environmental matrices has been demonstrated by several groups using congener specific PCB analysis. Norstrom (1988) compared patterns of PCB accumulation in relation to predator-prey trophic interactions to demonstrate the variation in PCB congener patterns. Similar work has shown selective uptake, retention, metabolism, and depuration of PCBs across trophic levels (Tanabe et al., 1987; Oliver and Niimi, 1988). Changes in PCB congener patterns have also been investigated on a temporal basis in sediment profiles (Oliver et al., 1989). These authors observed an increase in the relative percentage of the tetra- and pentachlorobiphenyls in sediment cores from Lake Ontario at depths corresponding to between the mid 1960s and latter 1980s. This has implications on the overall toxicity of the PCB mixtures, because the most toxic PCB congeners are tetra- and penta-chlorobiphenyls (Safe, 1986;

1987). The use of pattern recognition techniques has also been helpful in distinguishing the similarities and differences between PCB congener patterns (Stalling et al., 1987; Schwartz et al., 1987).

Dramatic improvements in the analytical methods associated with separation, clean-up, and enrichment of PCB extracts have been made in recent years (Smith, 1981; Huckins et al., 1988). These improvements have allowed for quantitation of nearly all of the PCB congeners and particularly the non-ortho- and mono-ortho-substituted congeners which can readily assume a planar configuration (McKinney and Pedersen, 1986). A planar configuration and halogen substitution at the lateral position are common traits of the most toxic PCB congeners (Poland and Knutson, 1982). Although these planar PCB congeners only comprise a minor fraction of the technical standards (Kannan et al., 1987; Huckins et al., 1988) they are found in much greater concentrations in humans and wildlife relative to that of the technical mixture (Tanabe et al., 1987). In samples from the Great Lakes, the planar PCBs are thought to comprise the major portion of the total PHH toxic potency (Niimi and Oliver, 1989a; 1989b; Kubiak et al., 1989). Even with these powerful new methods for determination of the toxic PCB congeners, additional information on the interactions of these complex PCB mixtures is necessary before a proper assessment of their potential hazards can be made.

PCBs, dioxins, and dibenzofurans have been variously shown to exhibit synergism, antagonism, or additive effects dependent on the combinations and molar ratios of the contaminants (Birnbaum et al., 1985; Weber et al., 1985; Eadon et al., 1986; Keys et al., 1986; Bannister and Safe, 1987; Bannister et al., 1987; Leece et al., 1987; Haake et al., 1987; Davis and Safe, 1988; Pluss et al., 1988a;b; Biegel et al., 1989; Bannister et al., 1987). A system which has been suggested as a possible tool for integration of these complex interactions of polyhalogenated hydrocarbons (PHHs) is the H4IIE rat hepatoma cell bioassay (Bradlaw and Casterline, 1979; Sawyer et al., 1984; Sawyer and Safe, 1985). The inductive response of the H4IIE cells in vitro, was highly correlated to the toxicity of PHH congeners and mixtures in vivo to rats (Safe, 1987). In other words, the bioassay response was predictive of the actual toxic response in the whole organism. Therefore, we felt that this bioassay may be helpful to bridge the gap between residue analysis in environmental samples and potential environmental effects.

It was the objective of this study to use the H4IIE bioassay in conjunction with residue analysis to examine the relationships between PCB residues and the bioanalytical results of the bioassay. In particular, total PCB residues in a variety of environmental samples were compared with H4IIE bioassay results on the same samples to evaluate this relative potency of total PCB in these samples. Additionally, a set

of Forster's tern eggs were analyzed with the H4IIE bioassay for comparison with congener specific PCB analysis which has been reported for egg samples from the same colonies.

Methods

Samples, Extractions, Bioassay

Samples for total PCB and bioassay analysis were; 28 double-crested cormorant (DCC, Phalacrocorax auritis), 3 Caspian tern (CPT, Hydroprogne caspia), and 2 Forster's tern (FST, Sterna forsteri) egg composites (> 5 eggs/composite). Two individual bald eagle (Haliaetus leucocephalus) eggs were also analyzed for total PCB content and the bioassay. Additionally, dorsal muscle (fillet) and eggs from 10 chinook salmon (Oncorhynchus tshawytscha) collected at the Little Manistee weir on October 7, 1987 (see Ankley et al. 1989 for details) were analysed for total PCB content as well as utilized in the H4IIE bioassay.

Individual Forster's tern eggs collected in 1983 from Lake Poygan and Green Bay, WI were extracted and analyzed with the H4IIE bioassay. The bioassay results were compared to congener specific PCB analysis, reported by Kubiak et al. (1989), on eggs taken from the same colonies on the same year.

Extraction and clean-up protocols were according to either the Pesticide Analytical Manual PAM (1979) Sec. 212 or

Ribick et al. (1981). The two eagle eggs and 11 of the double-crested cormorant egg composites were extracted and analyzed for total PCBs by T.R. Schwartz and K. Feltz at the U.S. Fish and Wildlife Service National Fish Contaminant Research Center, Columbia, MO. These samples were extracted according to Ribick et al. (1981) and aliquots of the extracts were sent to our laboratory for H4IIE bioanalysis.

A detailed description of the H4IIE bioassay procedures were presented in Chapter 2. Briefly, the procedures were as follows. H4IIE rat hepatoma cells were maintained using standard published techniques (Bradlaw and Casterline, 1979; Sawyer and Safe, 1982). The rat hepatoma cells were exposed to four to six serial dilutions of the sample extracts. Extracts were dissolved in isooctane prior to addition to the cell cultures (Bradlaw and Casterline, 1979). Each dilution of extract was assayed in duplicate or triplicate. Hepatoma cells were harvested after 72 hours of incubation. Concurrent with each assay, 2,3,7,8-TCDD was added to another series of plates for the determination of a standard 2,3,7,8-TCDD dose-response relationship. Upon harvesting, the cultures were washed, centrifuged, and resuspended in Tris-sucrose buffer (Mason et al., 1986). EROD activities in the cell suspensions were measured using the fluorimetric techniques described by Pohl and Fouts (1980).

Residue Analysis

Total PCBs were determined by gas chromotography (Perkin Elmer Model 8500) against a standard 1:1:1:1 Aroclor 1242:1248:1254:1260 mixture. Gas chromatographic conditions were: [63]-nickel electron capture detector (ECD) at 230°C; injector temperature 240°C, 30 m x 0.25 mm i.d. DB-5 column, He carrier gas (20 psig), and nitrogen make-up gas (55 psig). Initial oven temperature was 120°C and programmed temperature ramp was 2°C/min after an initial 1 min hold. Quantitation of total PCBs in the extracts was against the 1:1:1:1 Aroclor mixture based on relative retention indices and using a computerized program with a regressive pattern matching algorithm (COMSTAR; Burkhard and Weininger, 1987).

Data Analysis

The determination of TCDD-EQ based on the H4IIE bioassay was either by comparison of probit derived ED_{50} values (Sawyer et al., 1984) or by the slope ratio assay (Finney, 1978) The method for TCDD-EQ determinations by ED_{50} values has been described in detail elsewhere (Chap. 2). The slope ratio assay for calculation of extract potency involves a model which assumes a linear response of the system to a known power of the dose (Finney, 1978). Therefore, only a linear portion of the dose-response curve was utilized with this type of data analysis. The slope ratio assay may be used in cases where a full range of response is not elicited in the dosed H4IIE

cells. The relative potency of the extract, "TCDD-equivalents" (TCDD-EQ), was determined by comparison of the extract and TCDD standard dose-response curves (equation 1).

Extract potency (pg TCDD-EQ/ul) = Sample slope/TCDD slope (1)

The units of the sample and TCDD standard dose-response curve slopes are (EROD/ul of extract) and (EROD/pg TCDD), respectively. TCDD-EQ were then calculated based on extract volume and initial sample weight. All values of total PCBs and TCDD-EQ are reported on a weight weight basis. PCBs and TCDD-EQ values for the eagle egg samples were corrected for moisture and lipid loss based on initial egg volumes (Stickel et al, 1973).

Correlation and regression analysis was performed on PLOT IT (Scientific Programming Enterprises, Haslett, MI) statistical and graphical computer program. Comparison of means was by standard t-test (Zar, 1974).

Results

Total PCB Comparisons

A variety of environmental samples were analyzed for total PCB concentrations in addition to the H4IIE bioassay. The samples examined by both of these techniques include: Caspian Tern eggs, Forster's tern eggs, bald eagle eggs, double-crested cormorant eggs, and chinook salmon eggs and

dorsal muscle. These samples represent a wide range of total PCBs, 0.05 to 98.54 ug/g. The range of H4IIE-derived TCDD-EQ in these samples was 0 to 1065 pg/g. The average TCDD-EQ/PCB ratios among these species were from 7.44 to 35.32 ppm (pg TCDD-EQ/ug PCB, Table 8), while the range of these ratios within individual samples was 5.22 to 95.75 ppm.

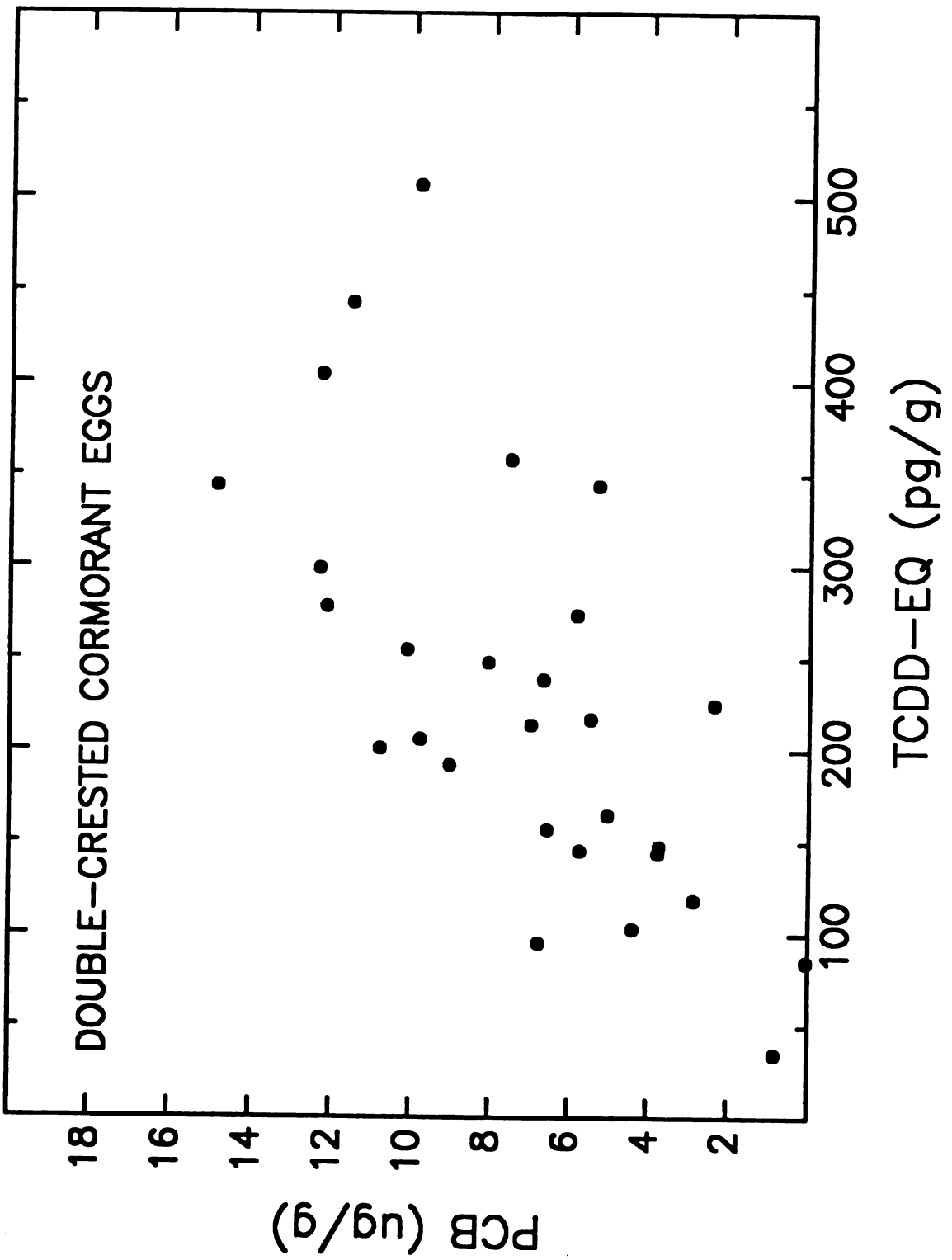
The ratio of H4IIE bioassay-derived TCDD-EQ to total PCBs varied among species, collection site, and tissue analyzed (Table 8). Double-crested cormorant eggs collected from locations around the Great Lakes and one site off the Great Lakes comprised the largest data set within a given species (n=27). The cormorant eggs were collected from 1984, 1986, 1987, and 1988, however there was no discernable difference in the relationship between TCDD-EQ and PCBs among these years. A complete data set from any one colony over all of these years was unavailable. The relationship of TCDD-EQ and total PCBs in double-crested cormorant eggs (Fig. 10) had a wide range of values. The range of TCDD-EQ was 35.0 to 506 pg/g and the range of total PCBs was 0.83 to 14.84 ug/g. The ratios of TCDD-EQ to total PCBs in these samples were from 14.05 to 95.75 ppm (pg TCDD-EQ/ug PCB). As would be expected, there was a significant positive correlation between total PCB concentrations and H4IIE-derived TCDD-EQ ($p = 0.0001$), however, the correlation is not very strong and the ability to predict one variable from the other is relatively poor ($r^2 = 0.464$). Similarly, Forster's terns and bald eagles had

Table 8. Average TCDD-EQ to Total PCB Ratios for Various Species from the Great Lakes

Species	Tissue	n	Average Ratio TCDD-EQ/PCB (SD)
Caspian Tern	Eggs	3	23.46 (1.26)
Forster's tern	Eggs	2	7.44 (3.14)
Double-crested cormorant	Eggs	27	35.32 (16.76)
Bald eagle	Eggs	2	15.73 (3.35)
Chinook salmon	Fillet	10	11.46 (5.06)
	Eggs	10	24.77 (4.42)

Ratio of H4IIE-derived TCDD-EQ/total PCB has units, (pg/g)/(ug/g) or ppm; SD = Standard deviation of ratio means

Figure 10. Corrlation Between Total PCB Concentrations and H4IIE Bioassay-Derived TCDD-EQ in Double-Crested Cormorant Eggs from the Great Lakes.


DOUBLE-CRESTED CORMORANT EGGS
PCB (ug/g)
2
4
6
8
10
12
14
16
18
100
200
300
400
500
TCDD-EQ (pg/g)

approximately 2-fold differences in TCDD-EQ/PCB ratios among eggs collected from different sites (Table 9). Variation in TCDD EQ/PCB ratios seen within a species when the samples are from different sites appears to be the rule with the exception of Caspian tern eggs. Caspian tern eggs collected in 1986 and 1987 had similar ratios of TCDD-EQ to total PCBs among the three different collection sites (Table 9). The TCDD-EQ/PCB ratios were within 10% of one another, even though there was greater than 2-fold differences among total PCBs and among TCDD-EQ in the Caspian terns eggs. The reason for the similarity of TCDD-EQ/PCB ratios in the Caspian tern eggs is unknown, however, dietary preference, metabolism, and genetics may be regulating factors.

Samples from a single species collected within the same area can have a stronger correlation between TCDD-EQ and total PCB concentrations. Female chinook salmon collected October 7, 1987 at the Little Manistee weir near Manistee, MI had much stronger correlations between concentrations of TCDD-EQ and total PCBs (Fig. 11). TCDD-EQ and total PCB concentrations were correlated in fillets ($r = 0.801$) and eggs ($r = 0.802$) from these salmon. The significant correlation between TCDD-EQ and total PCB concentrations in these samples is not completely unexpected due to the schooling behavior, similarity in diet, size, sex, sexual maturity of these fish, and the genetic homogeneity of the chinook from this site. It is also interesting to note the differences in relative PCB

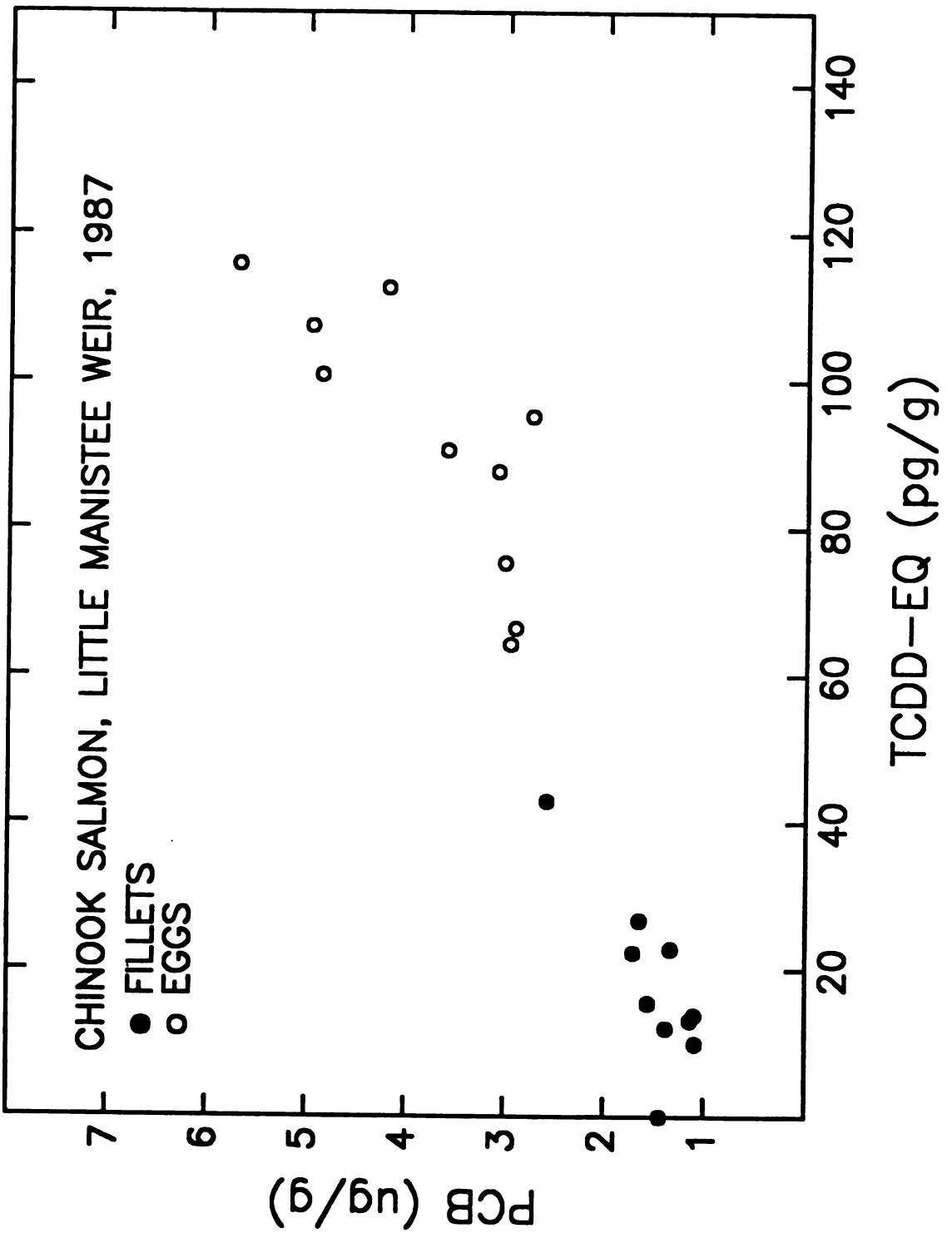
Table 9. H4IIE Bioassay TCDD-EQ to Total PCB Ratios in Egg Composites Collected from the Great Lakes

Species	TCDD-EQ (pg/g)	Σ PCB (ug/g)	TCDD-EQ/PCB ppm
Caspian Tern			
Beaver Is.	143.3	5.76	24.88
Green Bay	222.3	9.66	23.01
Saginaw Bay	294.9	13.12	22.48
Forster's Tern[a]			
Green Bay	214.5	22.20	9.66
Lake Poygan	23.5	4.50	5.22
Bald Eagle[b]			
Alpena, MI	1065.0	58.85	18.10
Big Bay de Noc, MI	559.6	41.89	13.36

a) Mean of TCDD-EQ for nine individual eggs compared to average total PCB residue analysis values in eggs from the same colony (Kubiak et al., 1989).

b) Bald eagle samples are individual eggs.

Figure 11. Correlation Between Total PCB Concentrations and H4IIE Bioassay-Derived TCDD-EQ in Chinook Salmon Fillets and Eggs. Female salmon collected at the Little Manistee weir in 1987.


CHINOOK SALMON, LITTLE MANISTEE WEIR, 1987
FILLETS
EGGS
PCB (ug/g)
TCDD-EQ (pg/g)
1
2
3
4
5
6
7
20
40
60
80
100
120
140

potency between the fillets and eggs of these salmon (Table 8). The means of the TCDD-EQ/PCB concentration ratios from fillets (11.46 ppm) and eggs (24.77 ppm) were significantly different ($p < 0.001$). The eggs from these salmon contain PCBs which elicit two-fold greater activity in the H4IIE bioassay than an equivalent amount of PCBs extracted from the fillets of the same salmon.

Congener Specific Analysis Comparisons

Forster's tern eggs collected from Green Bay and Lake Poygan, WI (approximately 60 miles inland from Green Bay) in 1983 were extracted and analyzed with the H4IIE bioassay. The average TCDD-EQ concentration in Forster's tern eggs from Green Bay was nearly 10-fold greater than the average concentration found in Forster's tern eggs from Lake Poygan (Table 10). TCDD-EQ in the eggs from the reference site, Lake Poygan, had an average value of 23.4 pg TCDD-EQ/g and a range from 13.6 to 34.2 pg TCDD-EQ/g. TCDD-EQ in the eggs from the contaminated area, Green Bay, averaged 214.5 pg TCDD-EQ/g and ranged from 90.1 to 339.1 pg TCDD-EQ/g.

Congener specific PCB analysis has been reported for Forster's tern eggs collected the same year, from these same sites, Lake Poygan and Green Bay, WI (Kubiak et al., 1989). These authors used the H4IIE bioassay-derived potencies (Safe, 1987) of the individual PCB congeners relative to TCDD to

Table 10. H4IIE-derived TCDD-EQ in Forster's Tern Eggs from Lake Poygan and Green Bay, Wisconsin - 1983

Sample ID	Sample Slope(CV)	TCDD Slope (CV)	Extract Potency (CV) (pg TCDD-EQ/ul)	H4IIE TCDD-EQ (SD) (pg TCDD-EQ/g)
Lake Poygan				
LP 205	0.2035 (8.2)	0.9530 (3.4)	0.214 (8.9)	34.2 (3.0)
LP 208	0.1161 (6.7)	0.8088 (2.4)	0.144 (7.1)	23.0 (1.6)
LP 217	0.1019 (7.5)	0.8088 (2.4)	0.126 (7.9)	20.2 (1.6)
LP 241	0.0810 (9.2)	0.9530 (3.4)	0.085 (9.8)	13.6 (1.3)
LP 250	0.0543 (6.1)	0.5894 (12.4)	0.092 (13.8)	14.8 (2.0)
LP 259	0.1230 (8.4)	0.5894 (12.4)	0.209 (15.0)	33.4 (5.0)
LP 268	0.1098 (5.4)	0.9530 (3.4)	0.115 (6.4)	18.4 (1.2)
LP 277	0.1084 (17.5)	0.5894 (12.4)	0.184 (21.4)	29.4 (6.3)
LP MEAN	0.112 (8.6)	0.784 (6.1)	0.143 (11.3)	23.4 (8.1)

Table 10. (continued)

Sample ID	Sample Slope(CV)	TCDD Slope (CV)	Extract Potency (CV) (pg TCDD-EQ/ul)	H4IIE TCDD-EQ (SD) (pg TCDD-EQ/g)
Green Bay				
GB 006	0.8542 (10.5)	0.5894 (12.4)	1.449 (16.2)	231.9 (37.7)
GB 018	0.5384 (12.5)	0.5894 (12.4)	0.913 (17.6)	146.2 (25.7)
GB 021	1.2392 (9.8)	0.8088 (2.4)	1.532 (10.1)	245.2 (24.7)
GB 039	1.9651 (6.0)	0.9530 (3.4)	2.062 (6.7)	330.0 (22.8)
GB 075	2.0195 (5.5)	0.9530 (3.4)	2.119 (6.5)	339.1 (21.9)
GB 111	0.8303 (5.8)	0.9530 (3.4)	0.871 (6.7)	139.4 (9.4)
GB 117	0.5593 (12.7)	0.5894 (12.4)	0.949 (17.8)	151.8 (26.9)
GB 123	0.4555 (6.8)	0.8088 (2.4)	0.563 (7.2)	90.1 (6.5)
GB 126	1.3001 (5.5)	0.8088 (2.4)	1.607 (6.0)	257.2 (15.4)
GB MEAN	1.085 (8.3)	0.784 (6.1)	1.384 (10.3)	214.5 (87.8)

Ethoxyresorufin O-deethylase (EROD) activity expressed as pmol/mg/min.

Sample slope = EROD/ul extract; TCDD slope = EROD/pg TCDD; CV = coefficient of variation (%); Extract potency = (Sample slope)/(TCDD slope) = pg TCDD-EQ/ul; TCDD-EQ in a sample = (pg TCDD-EQ/ul)(Extract volume, ul)/(sample wt., g) = pg TCDD-EQ/g; SD = standard deviation = (TCDD-EQ) (CV/100).

convert the PCB concentrations to TCDD-EQ concentrations in the tern eggs. After the concentration of each congener was converted to TCDD-EQ concentrations, the TCDD-EQ for each PCB congener were summed to give total TCDD-EQ in the sample based on an additive model (Kubiak et al., 1989). These authors estimated the median concentrations of TCDD-EQ based on residue analysis to be 2175 and 201 pg TCDD-EQ/g for the Green Bay and Lake Poygan colonies, respectively. Their estimates of TCDD-EQ included actual TCDD in the eggs, however actual TCDD only accounted for < 2% of the total TCDD-EQ (Kubiak et al., 1989). If the same type of calculations of TCDD-EQ are made from these authors PCB residue concentrations using conversion factors from this laboratory, the resultant TCDD-EQ estimates are much closer to the values predicted with the bioassay (Table 11). TCDD-EQs calculated in this manner from their PCB residue analysis, conversion factors, and additive model of toxicity were 70.83 pg/g for Lake Poygan eggs and 483.32 pg/g for eggs from Green Bay. These residue analysis-based TCDD-EQ are 2-3 fold greater than the H4IIE bioassay derived TCDD-EQ we determined in Forster's tern eggs collected at the same site and year (Table 10). However, the difference between the bioassay and an additive model still indicates less than additive potency of the PCB mixtures in these egg extracts.

Table 11. PCB Concentrations (ug/g) and Resultant TCDD-EQ (pg/g) in Forster's Tern Eggs Based on an Additive Model of Potency[a]

PCB Congeners[b]	Green Bay PCB (ug/g)	Green Bay TCDD-EQ (pg/g)	Lake Poygan PCB (ug/g)	Lake Poygan TCDD-EQ (pg/g)
PCB 77*	5.63×10^{-4}	0.01	--	--
105	0.56	369.97	0.09	59.40
114	6.9×10^{-2}	9.69	2.0×10^{-3}	0.28
118	1.12	10.02	0.23	2.06
126*	3.84×10^{-3}	83.59	2.95×10^{-4}	5.76
156*	0.14	6.89	0.05	2.46
169	1.06×10^{-3}	3.15	2.95×10^{-4}	0.87
	Total	483.32	Total	70.83

a) PCB congeners concentrations as reported by Kubiak et al. (1989)
b) Conversion factors from this laboratory (*) or from Sawyer and Safe (1982) for the individual PCB congeners. For each PCB, TCDD-EQ = PCB concentration x conversion factor, total TCDD-EQ were then calculated based on the sum of the TCDD-EQ for each PCB. Values are in pg/g we weight. Dash = not detected.

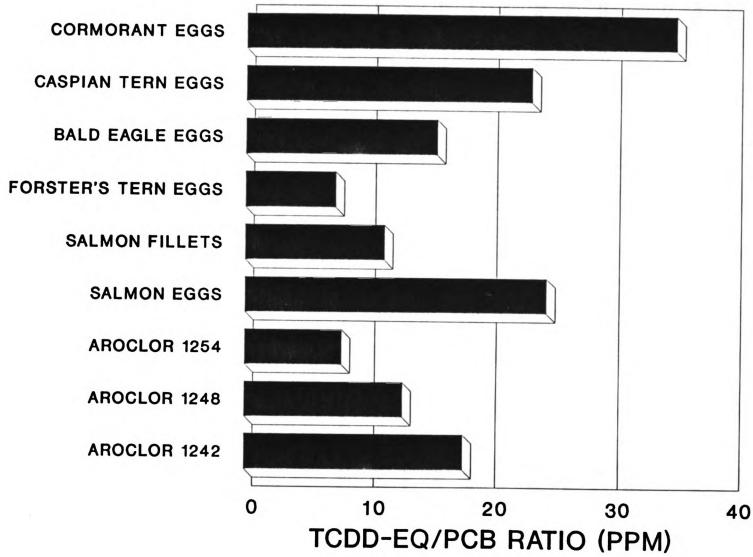
Discussion

Comparison of total PCB residues and H4IIE-derived TCDD-EQ in environmental samples has demonstrated the differences which can exist in the relative potencies of these contaminants among various matrices. The fact that patterns of PCBs are altered in the environment by biotic and abiotic transport and transformation processes is well known (Burkhard et al., 1985; Stalling et al., 1987; Schwartz et al., 1987; Oliver and Niimi, 1988; Oliver et al., 1989). In this study we demonstrate that these changes in PCB ratios can equate to differences in the potency of PCB mixtures in the environment. Even within a species, the relative potency of PCBs can have a wide range (Fig. 10). Double-crested cormorant eggs from different locations around the Great Lakes had vastly different ratios of TCDD-EQ concentrations to total PCB concentrations (14.05 - 95.75 ppm), suggesting that the difference in PCB patterns seen among regions within a given species can lead to significant differences in potential toxicity to the organisms. The source term for PCB exposure of these organisms appears to have a significant role in the ultimate overall potency of these mixtures of chemicals. In this regard, there were reasonably good correlations between total PCB concentrations and TCDD-EQ in chinook salmon fillets ($r = 0.81$) and eggs ($r = 0.82$) (Fig. 11). These fish were taken at the same time, from the same location, thus

minimizing the differences in exposure source terms. Similar results were seen when chemical residue analysis-derived TCDD-EQ were compared to total PCBs in fish from this same site a year earlier (Williams, 1989).

Another utility of these comparisons between H4IIE bioassay-derived TCDD-EQ and total PCBs is in development of an understanding of the potential enrichment of toxic potency which can occur in various species and within tissues of a given species. The average TCDD-EQ/PCB ratios for various species and tissues (from Table 8) are compared to the relative potencies of technical Aroclor standards determined with the H4IIE bioassay by Sawyer et al. (1984) (Fig. 12). From this graph, a few interesting relationships appear. First, there appears to be enrichment of PCBs as measured by induction potency for certain species compared to the technical standards. In particular, the cormorant eggs, Caspian tern eggs, and salmon eggs appear to have a more potent mixture of PCBs as compared to the standards (Fig. 12). These comparisons must be viewed with caution in that the potencies of the Aroclor standards were determined under different conditions which may affect the response of the bioassay and the resultant estimates of relative potency. However, a clear case for enrichment of PCB mixtures does exist in the TCDD-EQ/PCB ratios observed in salmon flesh and eggs. The relative potency of PCBs from the salmon eggs was two-fold greater than those extracted from fillets of the same

Figure 12. Average H4IIE Bioassay-Derived TCDD-EQ to Total PCB Ratios in Great Lakes Fish and Wildlife Extracts and Technical PCB Standards. PCB standards are as reported by Sawyer et al. (1984).


CORMORANT EGGS
CASPIAN TERN EGGS
BALD EAGLE EGGS
FORSTER'S TERN EGGS
SALMON FILLETS
SALMON EGGS
AROCLOR 1254
AROCLOR 1248
AROCLOR 1242
0
10
20
30
40
TCDD-EQ/PCB RATIO (PPM)

fish (Fig. 12). This may have implications on the reproductive problems that have been described in salmonid species from the Great Lakes (Mac et al., 1981; Berlin et al., 1981; Giesy et al., 1986; Mac, 1988).

The comparison of H4IIE bioassay results with congener specific PCB analysis indicates that additive or less than additive potency exists in these mixtures. The TCDD-EQ values derived by the bioassay were 2-3 fold less than what would be expected based on residue analysis, the potency factors of the individual PCB congeners derived under the same conditions, and an additive model of potency. The only other data of this type available for comparison are the congener specific PCB analysis of chinook salmon eggs from the Little Manistee weir collected one year prior to the samples we analyzed with the bioassay (Williams, 1989). When the PCB congener data from the 1986 chinook eggs are converted to TCDD-EQ with potency factors from our bioassay system, the average TCDD-EQ are 130.7 pg/g. This is very close to the bioassay estimated 91.6 pg TCDD-EQ/g in chinook salmon eggs collected from the same location in 1987. This also points out another use of this bioassay system, which is an aid in understanding which congeners are contributing to the overall potency of the mixture. Based on a simple additive model one can readily tell which congeners are the most important and which should be of lower priority in subsequent analyses.

In summary, it was demonstrated that PCB mixtures may

have variable potency as described by the H4IIE bioassay. This bioassay may be a useful tool to help interpret chemical residue analysis in environmental samples. The utility of this bioassay as a cellular integrator of the complex interactions of PHHs is powerful when used in conjunction with chemical residue analysis. Although more work is required to understand the toxicological implications of the H4IIE bioassay results with respect to other species, it may serve as a rapid, inexpensive method for evaluating relative potencies and possibly for predicting environmental effects of PHHs.

References

Ankley GT, Tillitt DE, Gooch JW, Giesy JP (1989) Hepatic enzyme systems as biochemical indicators or the effects of contaminants on reproduction of chinook salmon (Oncorhynchus tschawytscha). Compar Biochem Physiol Series C (in press).

Bannister R, Biegel L, Davis D, Astroff B, Safe S (1989) 6-Methyl-1,3,8-trichlorodibenzofuran (MCDF) as a 2,3,7,8-tetrachlorodibenzo-p-dioxin antagonist in C57BL/6 mice. Toxicol 54:139-150.

Bannister R, Davis D, Zacharewski T, Tizard I, Safe S (1987) Aroclor 1254 as a 2,3,7,8-tetrachlorodibenzo-p-dioxin antagonist: effects on enzyme induction and immunotoxicity. Toxicol 46:29-42.

Bannister R, Safe S (1987) Synergistic interactions of 2,3,7,8-TCDD and 2,2',4,4'5,5'-hexachlorobiphenyl in C57BL/CJ and DBA/2J mice: role of the Ah receptor. Toxicology 44:159-169.

Berlin WH, Hesselberg RJ, Mac MJ (1981) Growth and mortality of fry of Lake Michigan Lake Trout during chronic exposure to PCB's and DDE. U.S. Fish and Wildlife Service Technical Paper No. 105, Chlorinated Hydrocarbons as a Factor in the Reproduction and Survival of Lake Trout (Salvelinus namaycush) in Lake Michigan. Great Lakes Fishery Laboratory, Ann Arbor, MI pp 11-22.

Biegel L, Harris M, Davis D, Rosengren R, Safe L, Safe S (1989) 2,2',4,4',5,5'-Hexachlorobiphenyl as a 2,3,7,8-tetra-chlorodibenzo-p-dioxin antagonist in C57BL/6J mice. Toxicol Appl Pharmacol 97:561-571.

Birnbaum LS, Weber H, Harris MW, Lamb JC, McKinney JD (1985)

Toxic interaction of specific polychlorinated biphenyls and 2,3,7,8-tetrachlorodibenzo-p-dioxin: Increased incidence of cleft palate in mice. Toxicol Appl Pharmacol 77:292-302.

Bradlaw JA, Casterline JL (1979) Induction of enzyme activity in cell culture: A rapid screen for detection of planar polychlorinated organic compounds. J Assoc Off Anal Chem 62:904-916.

Burkhard LP, Armstrong DE, Andren AW (1985) Partitioning behavior of polychlorinated biphenyls. Chemosphere 14:1703-1716.

Burkhard LP, Weininger D (1987) Determination of polychlorinated biphenyls using multiple regression with outlier detection and elimination. Anal Chem 59:1187-1190.

Davis D, Safe S (1988) Immunosuppressive activities of polychlorinated dibenzofuran congeners: quantitative structure - activity relationships and interactive effects. Toxicol Appl Pharmacol 94:141-149.

Eadon G, Kaminsky L, Silkworth J, Aldous K, Hilker D, O'Keefe P, Smith R, Gierthy J, Hawley J, Kim N, DeCaprio A (1986) Calculation of 2,3,7,8-TCDD equivalent

concentrations of complex environmental contaminant mixtures. Environ Health Perspect 70:221-227.

Finney DJ (1978) Statistical Methods in Biological Assay, 3rd ed. Charles Griffin and Company, Ltd. London. 333 pgs.

Giesy JP, Newsted JL, Garling DL (1986) Relationships between chlorinated hydrocarbon concentrations and rearing mortality of chinook salmon (Oncorhynchus tshawytscha) eggs from Lake Michigan. J Great Lakes Res 12:82-98.

Haake JM, Safe S, Mayura K, Phillips TD (1987) Aroclor 1254 as an antagonist of the teratogenicity of 2,3,7,8-tetrachloro-dibenzo-p-dioxin. Toxicol Lett 38:299-306.

Huchins JN, Schwartz TR, Petty JD, Smith LM (1988) Determination, fate, and potential significance of PCBs in fish and sediment samples with emphasis on selected AHH-inducing congeners. Chemosphere 17:1995-2016.

Hutzinger O, Safe S, Zitko V, eds. (1974) The Chemistry of PCB's. CRC Press, Cleveland, OH.

Kannan N, Tanabe S, Wakimoto T, Tatsukawa R (1987) Coplanar polychlorinated biphenyls in Aroclor and Kanechlor mixtures. J Assoc Off Anal Chem (70:451-454.

Keys B, Piskorska-Pliszczynska J, Safe S (1986) Polychlorinated dibenzofurans as 2,3,7,8-TCDD antagonists: in vitro inhibition of monooxygenase induction. Toxicol Lett 31:151.

Kubiak TJ, Harris HJ, Smith LM, Schwartz T, Stalling DL, Trick JA, Sileo L, Docherty D, Erdman TC (1989). Microcontaminants and reproductive impairment of the Forster's tern in Green Bay, Lake Michigan - 1983. Archives of Environmental Contamination and Toxicology 18: 706-727.

Leece B, Denomme MA, Towner R, Li A, Landers J, Safe S (1987) Nonadditive interactive effects of polychlorinated biphenyl congeners in rats: role of the 2,3,7,8-tetrachlorodibenzo-p-dioxin receptor. Can J Physiol Pharmacol 65:1908-1912.

Mac MJ (1988) Toxic substances and survival of Lake Michigan salmonids: Field and laboratory approaches. In: MS Evans (ed) Toxic Contaminants and Ecosystem Health: A Great Lakes Focus. John Wiley and Sons, New York, NY.

pp. 389-401.

Mac MJ, Berlin WH, Rottiers DV (1981) Comparative hatchability of Lake Trout eggs differing in contaminant burden and incubation conditions. In: U.S. Fish and Wildlife Service Technical Paper No. 105, Chlorinated Hydrocarbons as a Factor in the Reproduction and Survival of Lake Trout (Salvelinus namaycush) in Lake Michigan. Great Lakes Fishery Laboratory, Ann Arbor, MI. pp 8-10.

Mackay D, Shiu WY, Billington J, Huang JGL (1983) Physical chemical properties of polychlorinated biphenyls. In: D. Mackay, S Patterson, SJ Eisenreich, MS Simmons (eds.) Physical Behavior of PCBs in the Great Lakes. Ann Arbor Science, Ann Arbor, MI. pp 59-70.

McKinney JD, Pedersen LG (1986) Biological activity of polychlorinated biphenyls (PCBs) related to conformational structure. Biochem J. 240:621-622.

Niimi AJ, Oliver BG (1989a) Distribution of polychlorinated biphenyl congeners and other halocarbons in whole fish and muscle among Lake Ontario salmonids. Environ Sci Technol 23:83-88.

Niimi AJ, Oliver BG (1989b) Assessment of relative toxicity of chlorinated dibenzo-p-dioxins, dibenzofurans, and biphenyls in Lake Ontario salmonids to mammalian systems using toxic equivalent factors (TEF). Chemosphere 18:1413-1423.

Norstrom RJ (1988) Bioaccumulation of polychlorinated biphenyls in Canadian wildlife. In: JP Crine (ed.) Hazards, Decontamination and Replacement of PCB. A Comprehensive Guide. Plenum Press, New York, NY. pp. 85-100.

Oliver BG, Charlton MN, Durham RW (1989) Distribution, redistribution and geochronology of polychlorinated biphenyl congeners and other chlorinated hydrocarbons in Lake Ontario sediments. Environ Sci Technol 23:200-208.

Oliver, Niimi AJ (1988) Trophodynamic analysis of polychlorinated biphenyl congeners and other chlorinated hydrocarbons in the Lake Ontario ecosystem. Environ Sci Technol 22:388-397.

Pesticide Analytical Manual (1979) Volume I. Food and Drug Administration, Washington, DC, Sec. 212.13a

Pluss N, Poiger H, Hohbach C, Schlatter C (1988a) Subchronic toxicity of some chlorinated dibenzofurans (PCDFs) and a mixture of PCDFs and chlorinated dibenzodioxins (PCDDs) in rats. Chemosphere 17:973-984.

Pluss N, Poiger H, Hohbach C, Suter M, Schlatter C (1988b) Subchronic toxicity of 2, 3, 4, 7, 8-pentachlorodibenzofuran (PeCDF) in rats. Chemosphere 17:1099-1110.

Pohl RJ, Fouts JR (1980) A rapid method for assaying the metabolism of 7-ethoxyresorufin by microsomal subcellular fractions. Analyt Biochem 107:150-158.

Poland A, Knutson JC (1982) 2,3,7,8-tetrachlorodibenzo-p-dioxin and related halogenated aromatic hydrocarbons: examination of the mechanism of toxicity. Ann Rev Pharmacol Toxicol 22:517-554.

Ribick MA, Smith IM, Dubay GR, Stalling DL (1981) Applications and results of analytical methods used in monitoring environmental contaminants. In: Branson DR, Dickson KL (eds) Aquatic Toxicology and Hazard Assessment: Fourth Conference. ASTM STP 737, Philadelphia, p 249-269.

Safe S (1986) Comparative toxicology and mechanism of action

of polychlorinated dibenzo-p-dioxins and dibenzofurans. Ann Rev Pharmacol Toxicol 26:371-399.

Safe S (1987) Determination of 2,3,7,8-TCDD toxic equivalent factors (TEFs): support for the use of the in vitro AHH induction assay. Chemosphere 16: 791-802.

Sawyer T, Safe S (1982) PCB isomers and congeners: induction of aryl hydrocarbon hydroxylase and ethoxyresorufin O-deethylase enzyme activities in rat hepatoma cells. Toxicol. Lett 13:87-94.

Sawyer TW, Vatcher AD, Safe S (1984) Comparative aryl hydrocarbon hydroxylase induction activities of commercial PCBs in Wistar rats and rat hepatoma H-4-II-E cells in culture. Chemosphere 13:695-701.

Sawyer TW, Safe S (1985) In vitro AHH induction by polychlorinated biphenyl and dibenzofuran mixtures: Additive effects. Chemosphere 14(1):7 9-84.

Schwartz TR, Stalling DC, Rice CL (1987) Are polychlorinated biphenyl residues adequately described by Aroclor mixture equivalents? Isomer-specific principal components analysis of such residues in fish and turtles. Environ Sci Technol 21:72-76.

Smith LM (1981) Carbon dispersed in glass fibers as an adsorbent for contaminant enrichment and fractionation. Anal Chem 53:2152-2154.

Stalling DL, Schwartz TR, Dunn WJ, Wold S (1987) Classification of polychlorinated biphenyl residues: Isomer vs. homologue concentrations in modeling Aroclors and polychlorinated biphenyl residues. Anal Chem 59:1853-1859.

Stickel LF, Wismeyer SN, Blas LJ (1973) Pesticide residues in eggs of wild birds: adjustment for loss of moisture and lipid. Bull Environ Contam Toxicol 9:193-196.

Tillitt DE, Ankley GT, Giesy JP, Kevern NR (1988) H4IIE rat hepatoma cell extract bioassay-derived 2,3,7,8-tetrachlorodibenzo-p-dioxin-equivalents (TCDD-EQ) from Michigan waterbird colony eggs, 1986 and 1987. Report to the Michigan Toxic Substance Control Commission and Department of Natural Resources. September 30, 1988.

Tanabe S, Kannan N, Subramanian A, Watanabe S, Tatsukawa R (1987) Highly toxic coplanar PCBs: Occurrence source,

persistency and toxic implications to wildlife and humans. Environ Pollut 47:147-163.

Weber H, Harris MW, Haseman JK, Birnbaum LS (1985 Teratogenic potency of TCDD, TCDF and TCDD-TCDF combinations in C57BL/6N mice. Toxicol Lett 26:159-167.

Williams LL (1989) Correlations of individual polychlorinated biphenyl congeners in eggs of Lake Michigan chinook salmon (Oncorhynchus tshawytscha) with rearing mortality. Master thesis. Department of Fisheries and Wildlife, Michigan State University, E. Lansing, MI pp 61.

Zar JH (1974) Biostatistical Analysis. Prentice Hall Inc, Englewood Cliffs, NJ.

CHAPTER 4

PCB Residues and Egg Mortality in Double-Crested Cormorants from the Great Lakes.

Introduction

In this study we have evaluated the overall potency of PCB extracts from double-crested cormorant eggs with an *in vitro* bioassay system, the H4IIE rat hepatoma cell bioassay. Results from the H4IIE bioassay are highly correlated with the hatching success in the colonies, while conventional methods of PCB analysis were not significantly correlated with hatching success in the colonies. Further significance of the relationship between the bioassay results and egg mortality comes from the fact that even though PCB residues have declined in almost all compartments of the Great Lakes environment, their effects are still being seen.

Great Lakes fish-eating waterbirds show a variety of symptoms similar to those seen in laboratory studies of avian species administered polychlorinated biphenyls (PCBs, 1). The general symptoms of PCB toxicity are altered biochemical homeostasis, gross anatomical deformities, and the reproductive effects of fetotoxicity and teratogenesis (2). Individually these symptoms could be caused by a variety of chemical, physical, or biological agents, but collectively the same suite of toxic effects is seen across phyla when organisms are exposed to planar halogenated hydrocarbons (PHHs, 3).

PHHs include a subset of the PCBs, the dibenzo-p-dioxins, and dibenzofurans, all of which are believed to

elicit their toxic effects through the same receptor-mediated mode of action (4). Environmental contamination by PHHs in the Great Lakes stems from regional industrial and urban activities along with atmospheric deposition of these compounds (5). The combination of various sources, hundreds of individual congeners with different potencies and environmental fate properties, and only a meager understanding of the complex interactions of PHH mixtures has made evaluation of environmental PHH contamination difficult from a toxicological standpoint. Routine methods of residue analysis group all of the PCBs as equipotent and use commercial mixtures as standards. However, the ratios of the individual congeners vary among environmental samples and do not reflect the same ratios found in the commercial standards which initially entered the environment (6). This may be a significant factor why PCB contaminant burdens in fish and wildlife measured in this fashion have not been highly correlated with the toxicological symptoms even though qualitatively the symptoms of PHH-like poisoning are greater in those areas of greater contamination. That is, more severe effects are seen in species from contaminated areas and little or no effects are seen in "cleaner" areas. However, a gradient of biological response and PHH chemical contamination has not been established in the Great Lakes (7). The problem of assessment of PHH mixtures is further confounded by the occurrence and effects of other environmental contaminants

such as agricultural pesticides. DDT (2,2-bis(p-chlorophenyl)-1,1,1-trichloroethane) and its environmental metabolites, co-contaminants in the Great Lakes basin, are the agents suspected to be responsible for egg shell thinning related mortalities of many fish-eating waterbirds (8).

It was therefore the purpose of this study to determine the role and extent to which PHHs may be responsible for the symptoms seen in Great Lakes fish-eating birds. In particular, we evaluated the overall potency of PCB extracts of double-crested cormorant eggs with the H4IIE rat hepatoma cell bioassay system. Bioassay results were then compared with reproductive success of the cormorant colonies from the various regions of the Great Lakes.

The H4IIE bioassay has been utilized by other researchers to evaluate the relative potency of complex PHH mixtures in environmental samples (9). The H4IIE rat hepatoma cells have the characteristics of low basal P450IA1-associated catalytic activity, yet they are highly inducible by PHHs (10). *In vitro* induction potency of various PHH congeners in this cell line is highly correlated with *in vivo* potency for the toxic endpoints of body weight loss and thymic atrophy in rats (11). The H4IIE bioassay shows promise as an integrative tool to assess the relative potency of complex mixtures of PHH. The synergistic, antagonistic, and additive interactions which are known to occur *in vivo* among PHHs may be accounted for by the H4IIE bioassay system.

Double-crested cormorants from 11 colonies representing 5 regional areas around the Great Lakes and a reference site outside the Great Lakes (Table 12) were monitored for reproductive performance in 1986, 1987, and/or 1988. Site selection criteria was based on the ability to find established ground nesting colonies with approximately 250 nests total. Although various groups were involved with the field collections and reproductive assessments at the different sites, field protocols were similar for all colonies (12). Initial visits were designed to census and map colonies, select nests to be observed (25-50 nests, except where the entire colony was monitored), collect eggs from the test area for residue analysis (6-12 eggs/colony), and mark nests and the remainder of the eggs within the experimental zone. We used pie-shaped test areas which included a portion of the outer border and extended into the center of the colony so as to normalize edge effects where predation by gulls can be the greatest. This first visit was staged at a time such that 85-90% of the eggs from the first nesting attempt had been laid. Archived eggs were weighed, placed in separate solvent rinsed jars, returned to the laboratory and stored at 10° C until extraction.

There were generally three subsequent visits to the colonies at time intervals of 10-14 days. Egg viability, hatching success, lost eggs, and new nesting attempts were monitored. Chicks were banded, checked for gross deformities

Table 12. Colony, Collection Years, and Regional Locations of Double-Crested Cormorants.

I. Green Bay, Lake Michigan

1986
Little Gull Island
Snake Island
1987
Gravelly / Little Gull Islands
1988
Spider Island

II. North Central Lake Michigan

1986
Hat Island
Pismjire Island
West Grape Island

III. Northwestern Lake Huron

1986
St. Martin's Shoal
1987
St. Martin's Shoal

IV. Southeastern Lake Superior

1986
Tahquamenon Island
1987
Tahquamenon Island

V. Lake Ontario

1988
Pigeon Island

VI. Lake Winnepegosis, Manitoba, Canada

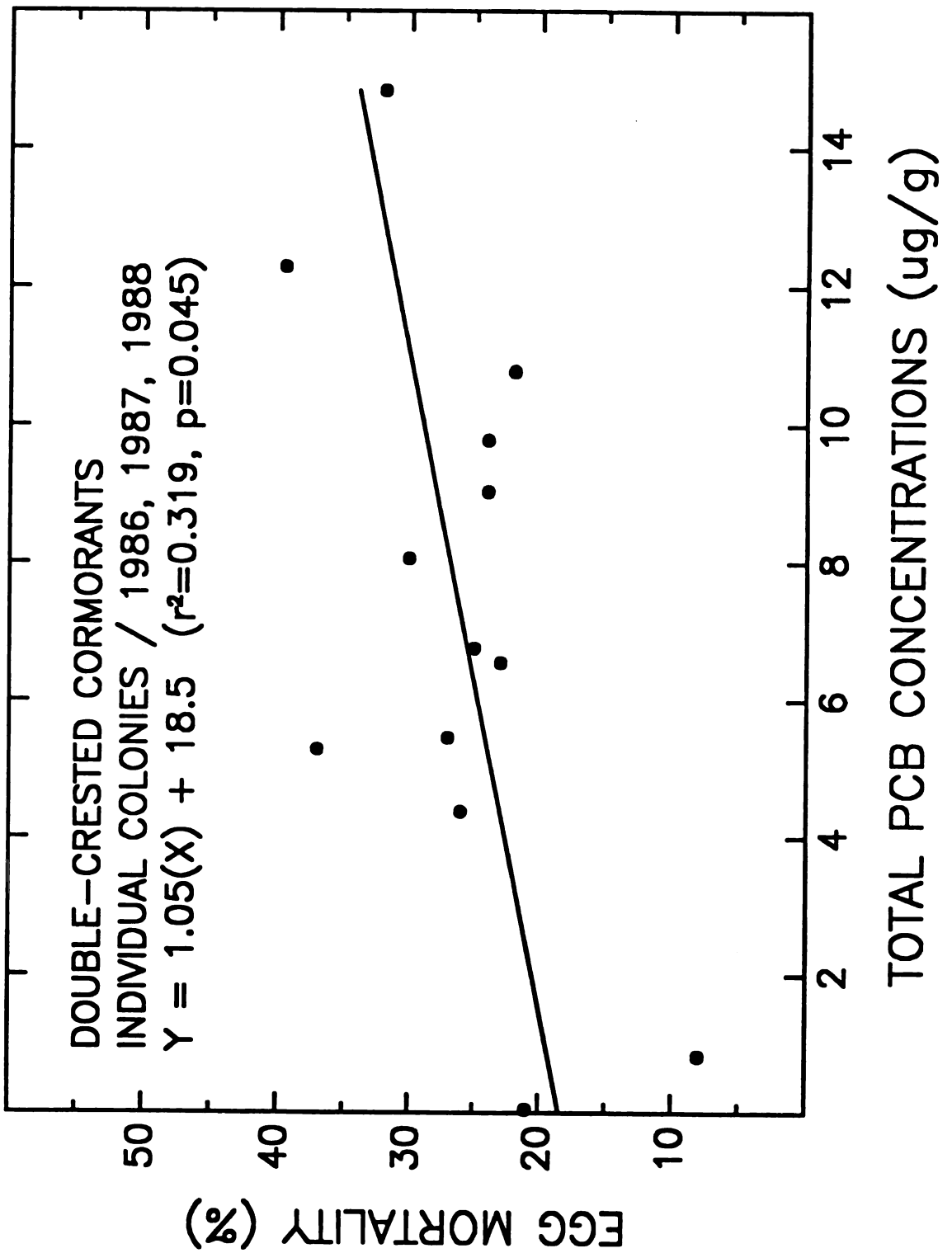
1988
Hay Reef

and counted to determine fledging rates. Additionally, 25 day old eggs from outside the experimental plot were collected and later dissected to determine rates of deformities.

Composites of 5-12 eggs were extracted and the extracts used to dose the H4IIE cells as previously described (9). The potency of PCB extracts was calibrated against a 2,3,7,8-tetrachlorodibenzo-p-dioxin standard dose-response curve in the H4IIE cells to determine TCDD-equivalents (TCDD-EQ) in the samples (13). Total PCBs in the same samples were analyzed by GC-ECD.

The H4IIE bioassay-derived TCDD-EQ in cormorant eggs were in the range 35-344 pg TCDD-EQ/g, while total PCBs had a range of 0.05-14.84 ug/g. Egg mortality in the cormorant colonies was between 8 and 39% of the eggs laid. There was a significant correlation ($p = 0.045$) between total PCB concentrations in composites of cormorant eggs from a colony and egg mortality rates from the same colony as a percent of those laid, as would be expected if these contaminants are exerting an influence on hatching success (Fig. 13). However, the relationship between total PCB concentrations and egg mortality rates was not very strong ($r^2 = 0.319$), making prediction of effects from this type of data rather inaccurate. In contrast, the correlation between H4IIE bioassay-derived TCDD-EQ in cormorant egg composites and egg mortality rates from these colonies was not only significant ($p = 0.003$), but TCDD-EQ were much better than total PCBs as

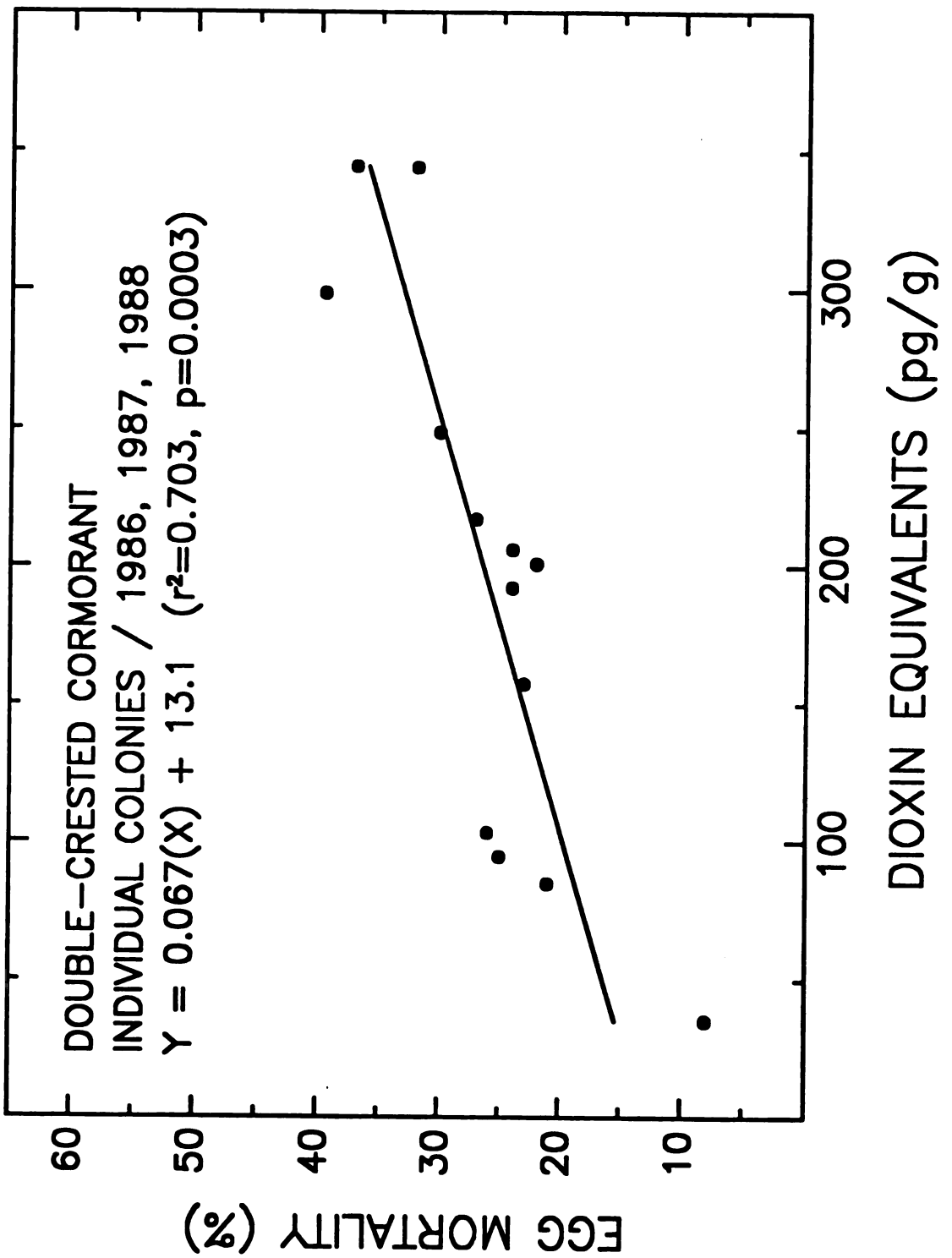
Figure 13. Correlation Between Total PCB Concentrations in Double-Crested Cormorant Eggs and Egg Mortality Rates.


DOUBLE-CRESTED CORMORANTS
INDIVIDUAL COLONIES / 1986, 1987, 1988
Y = 1.05(X) + 18.5 (r²=0.319, p=0.045)
EGG MORTALITY (%)
10
20
30
40
50
2
4
6
8
10
12
14
TOTAL PCB CONCENTRATIONS (ug/g)

a predictor of egg mortality rates (r^2 = 0.703) (Fig. 14). There appears to be a dose-response relationship between the bioassay-derived TCDD-EQ in the cormorant eggs and hatching success in these colonies. These data support the studies that suggest that PHHs may have a causal role in the reproductive impairment seen in double-crested cormorants in the Great Lakes (1). The biological gradient observed for these samples is an important relation which has not been established previously in suspected cases of environmental contamination in waterbirds (7-8).

The significance of this relationship of H4IIE bioassay-derived TCDD-EQ and reproductive success in cormorants has implications on three aspects of the environmental contamination issues in the Great Lakes. First, it suggests that even though concentrations of PCBs and other persistent compounds are going down in virtually all compartments of the environment, they continue to elicit effects. The concentrations of PCBs in Great Lakes biota are believed to have declined to an asymptote that will not be reduced significantly for some time (14). In the 1960s and 1970s both PCBs and p,p'-DDE residues were elevated in cormorants and other compartments of the Great Lakes environment. The decline of Double-crested cormorant populations seen during this period has largely been attributed to egg shell thinning by p,p'-DDE. The mean residue values of p,p'-DDE in cormorant eggs from Lake

Figure 14. Correlation Between H4IIE Bioassay-Derived TCDD-EQ in Double-Crested Cormorant Eggs and Egg Mortality Rates.


DOUBLE-CRESTED CORMORANT
INDIVIDUAL COLONIES / 1986, 1987, 1988
Y = 0.067(X) + 13.1 (r²=0.703, p=0.0003)
EGG MORTALITY (%)
10
20
30
40
50
60
100
200
300
DIOXIN EQUIVALENTS (pg/g)

Michigan colonies in 1977-78 was 3-5 ppm and these colonies had 7-14% egg shell thinning (15). A concentration of 10 ppm DDE is associated with 20% egg shell thinning, an amount believed to cause severe and possibly complete reproductive failure in cormorants (16). The large DDE concentrations found in Great Lakes cormorant eggs during the 1960s-1970s may have masked the effects of elevated PCBs in those same eggs. Now that DDE concentrations have declined , expression of the fetotoxic and teratogenic effects of PCBs and other PHHs may be occurring in the water birds. These effects would also be enhanced due to enrichment of the PCB mixtures to more toxic combinations (17). The second implication of the relationship between TCDD-EQ and egg mortality rates in the cormorant, is that it appears that the reproductive anomalies reported in Great Lakes cormorants are due to PHH, and largely PCBs, and not other pesticides. The last point of significance of this relationship is that it appears that the relative potency of PHHs in rats is similar to that in double-crested cormorants. That is, the rank order of PHH potency may be the same in both of these species, even though the absolute potency of species sensitivity may differ. This would be expected in the case of a receptor-based mode of action in which the receptor is phylogenetically conserved (18).

We have demonstrated a dose-response relationship between PCBs as measured by the H4IIE bioassay and egg mortality in double-crested cormorants in the Great Lakes.

Probit analysis of the data estimates an ED_{50} = 1007 pg TCDD-EQ/g (confidence interval 495 - 2048 pg/g). This is above the values seen in this study, and the ecological significance is not completely understood. Currently the populations of double-crested cormorants in the Great Lakes are experiencing a large increase and PCB residues have declined (19). The amount of egg mortality attributable to PCBs does not appear to be sufficient to decrease cormorant populations in the Great Lakes. However, it does suggest that PCBs are eliciting effects at the organismal level and that similar effects could occur in other species at the top of the aquatic food chain.

References and Notes

1. M. Gilbertson, Chemosphere 12, 357 (1983); M. Gilbertson, in Halogenated Biphenyl Terphenyls, Naphthalenes, Dibenzodioxins and Related Products, R.D. Kimbrough and A.A. Jenson, Eds. (Elsevior, Amsterdam, 1989), pp. 103-127; Kubiak et al., Arch. Environ. Contam. Toxicol. 18, 706 (1989).

2. S. Safe, CRC Crit. Rev. Toxicol. 13, 319 (1984); S. Safe, Annu. Rev. Pharmacol. Toxicol. 26, 371 (1986).

3. J.A. Goldstein, in Halogenated Biphenyls, Terphenyls, Napthalenes, Dibenzodioxins, and Related Products, R.D. Kimbrough, Ed. (Elsevier, Amsterdam, 1989), pp. 151-190.

4. A. Poland and J.C. Knutson, Annu. Rev. Pharmacol. Toxicol. 22, 517 (1982).

5. D. Mackey, S. Patterson, S.J. Eisenreich, M.S. Simmons, Eds. Physical Behavior of PCBs in the Great Lakes (Ann Arbor Science, ann Arbor, 1983).

6. R.J. Norstrom, in Hazards, Decontamination and Replacement of PCBs, J.P. Crine, Ed. (Plenum Press, New York, 1988), pp. 85-100; D. Stalking et al. Annal. Chem. 59, 1853 (1987); T.R. Schwartz et al. Environ. Sci. Technol. 21, 72 (1987).

7. D.B. Peakall and G.A. Fox, Environ. Health Perspect. 71, 187 (1987).

8. D.B. Peakall, in Chronic Effects of Toxic Contaminants

in Large Lakes, Proceedings of a technical session of the World Conference on Large Lakes, held May 18-21, 1986 (Lewis Publishers, Chelsea, MI, 1988) pp. 39-54.

9. J.A. Bradlaw and J.L. Casterline, J. Assoc. Offic. Anal. Chem. 62, 904 (1979); W.J. Trotter, et al., J. Assoc. Offic. Anal. Chem. 65, 838 (1982); J.L. Casterline, et al., J. Assoc. Offic. Anal. Chem. 66, 1136 (1983); T. Zacharewski, et al., Environ. Sci. Technol. 23, 730 (1989).

10. A. Niwa, et al., Mol. Pharmacol. 11, 399 (1975).

11. S. Safe, Chemosphere 16, 791 (1987).

12. H. Karita et al. Results of the 1987 Michigan Colonial Waterbird Monitoring Project on Caspian Terns and Double-Crested Cormorants: Egg Incubation and Field Studies of Colony Productivity, Embryonic Mortality and Deformities. Report to the Michigan Department of Natural Resources. (Ecological Research Services, Bay city, MI, 1987).

13. T. Sawyer et al., Chemosphere 7, 695 (1984).

14. P.C. Baumann and D.M. Whittle, Aquat. Toxicol. 11, 241 (1988).

15. G.H. Heinz et al., Environ. Monitor. Assess. 5, 223 (1985).

16. P.A. Pearce et al., Pest. Monitor. J. 13, 61 (1979).

17. S. Tanabe et al., Environ. Pollut. 47, 147 (1987).

18. M.S. Denison et al., Chemosphere 15, 1665 (1988).

19. J.P. Ludwig, Jack-Pine Warbler **62**, 91 (1984); W.C. Scharf and G.W. Shugart, Amer. Birds **35**, 910 (1981).

SUMMARY

The studies conducted on the characterization of the H4IIE bioassay have demonstrated that this *in vitro* technique can be useful in the assessment of complex mixtures of PHHs. It is a rapid, cost-efficient screening tool for PHHs in environmental extracts. The H4IIE bioassay has excellent precision, is compatable with a variety of extraction protocols, and has reasonably good accuracy when tested with fortified samples. It is a simple procedure which can compliment chemical residue analysis and environmental effects data in experiments designed to understand the possible effects of PHHs in the environment. The experiments performed here demonstrate that the H4IIE bioassay can help interpret chemical residue analysis by acting as an integrator of PHH effects at the cellular level. The overall potency of PHH mixtures can be evaluated with this bioassay and compared in relative terms with one another. The absolute toxic potency of PHHs extracted from an organism may not be predicted with this bioassay, and the experiments to understand this relationship in most species have not been undertaken. However, the results presented here suggest that the H4IIE bioassay may indeed be predictive of toxic potency for one species of waterbird, the double-crested cormorant. The correlation between H4IIE-derived TCDD-EQ in double-crested

cormorant eggs and egg mortality rates in the colony was highly significant ($p = 0.0003$) and the predictive power of this relationship was fairly robust ($r = 0.84$).

Complex mixtures of contaminants will continue to be a problem facing environmental toxicologists and chemists. We will need ways to assess complex mixtures in an attempt to predict or understand potential effects which may be elicited by these mixtures of contaminants. The H4IIE bioassay appears to be such a tool for fast and efficient assessment of PHHs. It is not being suggested that this bioassay is a panacea for studying PHHs. Quite the contrary, its strength is as an integrative, data reduction biomonitoring tool for use in conjunction with residue analysis and environmental effects data. The H4IIE bioassay has a firm molecular/biomolecular basis which appears to be consistent across certain phylogenetic lines. Validation of the H4IIE bioassay response in rats, the similarity in PHH toxic symptoms across phyla, and the successful use of the HeIIE bioassay in these studies suggest that this system may be useful for predicting environmental effects in other species.

Exactly how useful the H4IIE bioassay will be in the future is dependent on the results of the various characterization studies of this bioassay. Clearly, more work is needed in this area to calibrate the H4IIE bioassay and answer the critical questions. Is the rank order of potency of various PHHs similar among phyla? Does the H4IIE bioassay

successfully integrate the response of complex PHH mixtures in a predictive manner? Is the acute potency of PHH mixtures, which is predicted by this bioassay, equivalent to the chronic potency of the same PHH mixtures? Studies designed around these questions are crucial to the development of the H4IIE bioassay as a predictive biomonitoring tool.

Further studies with the H4IIE bioassay should include:

1) Comparison of the H4IIE bioassay response to simple 2 or 3 component PHH mixtures with the toxicity information from the literature on those mixtures in vivo. The ratios of the PHHs appears to be important in relation to synergistic, antagonistic, or additive responses. This will help understand if the responses of the H4IIE cells mimic the in vivo responses.
2) Calibrate the H4IIE bioassay to other species in terms of their structure-activity relationships of the PHHs. If the same QSARs hold true among species, the use of this rat cell line to predict effects in the other species may be justified.
3) Comparisons of the H4IIE bioassay with chemical residue analysis to further elucidate enrichment/dilution phenomenon in biota and sediments on both a trophic and temporal basis. These comparisons may also be aided by the use of pattern recognition techniques to group similar PHH

patterns and determine which contribute to toxicity or modulate it.

4) Selective chemical additions and/or deletions to environmental and synthetic PHH mixtures for bioassay scanning. These experiments would also lead to a better understanding of the compounds controlling or modulating PHH toxicity. Additionally, a partitioning and testing of various fractions in an extraction protocol could identify groups within PHHs that are more important based on the H4IIE bioassay potency (i.e. PCDDs vs. PCBs). These studies could also be informative about overall synergistic, antagonistic, or additive responses.