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H4IIE Rat Hepatoma Cell Bioassay-Derived 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin Equivalents in Colonial Fish-Eating Waterbird Eggs from the Great Lakes[1]

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Abstract. Fish-eating waterbirds from the Great Lakes of North America have shown symptoms of poisoning similar to those observed in laboratory exposures of various avian species to planar halogenated hydrocarbons (PHHs). PHHs, include among others, polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs) and have been implicated in some of the reproductive problems of Great Lakes waterbirds. The objectives of this study were to assess the overall potencies of PCB-containing extracts from colonial waterbird eggs taken from the Great Lakes and to compare the potencies with the location and spatial distribution of the colonies. The potencies of the extracts were assessed by their ability to induce cytochrome P450IA1-associated ethoxyresorufin O-deethylase (EROD) activity in H4IIE rat hepatoma cells as compared to the standard, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). The H4IIE bioassay-derived TCDD-equivalents (TCCD-EQs) in the waterbird eggs concur with residue analyses and biological data from other studies. The greatest concentrations of TCDD-EQs were found in waterbird eggs from historically polluted, industrialized or urbanized areas in which the reproductive impairment of colonial waterbirds was most severe. However, significant concentrations of TCDD-EQs were detected at all sites tested; with a range of 49 to 415 pg TCDD-EQ/g egg, uncorrected for extraction efficiencies. The H4IIE bioassay proved to be a useful biomonitoring tool to assess the overall potency of complex PHH mixtures in environmental samples.

Certain populations of fish-eating birds in the Great Lakes of North America have had incidences of elevated mortality in chicks, altered reproductive behavior, increased incidence of teratogenesis, gross physical and histopathological anomalies, immune suppression, and altered biochemical homeostasis. These problems with fish-eating birds have occurred primarily 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 revealed differences in reproductive success and embryological defects based upon regional distribution of the waterbird colonies (Kurita *et al.* 1987). At one geographic area known to be contaminated, the Saginaw Bay Confined Disposal Facility (CDF), Caspian terns had greatly reduced hatching success (28%) and no survival past fledging (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 at various locations 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 (Tumasonis *et al.* 1973; Cecil *et al.* 1974; Ax and Hansen 1975). It has been suggested that PHHs are, at least in part, responsible for some of the reproductive problems in fish-eating waterbirds at contaminated locations, including the Great Lakes (Gilbertson and Fox 1977; Gilbertson 1983; Kubiak *et al.* 1989).

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, per-

[1] Some of these results were presented at the 1989 International Symposium on Responses of Marine Organisms to Pollutants, Plymouth, England (Tillitt *et al.* 1989)

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sistence, 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). 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, subcutaneous edema, immune suppression, hormonal alterations, liver P450IA1-associated enzyme induction, and the reproductive effects of embryo/fetotoxicity and teratogenesis (see reviews by Safe 1986; Whitlock 1987). It is the presence of PHHs in fish-eating waterbirds from contaminated areas around the Great Lakes (Gilbertson 1983; Fox *et al.* 1988; Kubiak *et al.* 1989) along with the ability of PHHs to impair reproduction of avian species in laboratory studies. (Tumasonis *et al.* 1973; Cecil et al. 1974; Ax and Hansen 1975) 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 are extremely costly and time-consuming, particularly when samples may theoretically contain up to 209 different PCB, 75 PCDD, 135 PDF congeners (Safe 1987). Moreover, even if reliable determination of PHH concentrations are 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; Davis and Safe 1988).

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) and birds (Goldstein 1980). It is generally accepted that the toxic properties of different PHHs are expressed via a common mode of action; therefore, it is 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; Safe 1987; Kubiak *et al.* 1989; Niimi and Oliver 1989; Safe and Phil 1990).

One approach for the calculation of TCDD-equivalents of PHH mixtures involves congener specific analysis and the application of toxic equivalency factors (TEFs) developed from the ability of each PHH 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 multiplying the concentration of each congener by its relative potency factor. Total toxic potency of the mixture is determined by summing the equivalents atrributable to 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 (Tanabe *et al.* 1987; Kubiak *et al.* 1989; Niimi and Oliver 1989). However, this technique is limited by the fact that it assumes strict additivity 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. Additionally, the TEFs reported in the literature and used in the calculation of the TCDD-equivalents very greatly, and thus can significantly affect the calculated TCDD-equivalents (Safe and Phil 1990).

The H4IIE rat hepatoma cell line was first suggested by Nebert and coworkers (Niwa et al. 1975) as a method to detect minute (pg) quantities of TCDD in a fast, inexpensive manner. 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 (Bannister *et al.* 1987). H4IIE rat hepatoma cells have low basal AHH and EROD enzyme activities, yet are highly inducible by PHHs. The advantage of the bioassay is that it can integrate the complex interactions of PHHs which are known to occur at the cellular level. Investigators at the U.S. Food and Drug Administration (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 (Sawyer and Safe 1982; Bandiera *et al.* 1984; Sawyer *et al.* 1984; Leece *et al.* 1985; Sawyer and Safe 1985; 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 *in vivo* toxicity of these isomers 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). We felt that this bioassay could be used to screen waterbird eggs for bioactive PHHs.

In this study we use the H4IIE bioassay system to evaluate the relative potency of extracts from fish-eating waterbird eggs. In particular, our objectives were: (1) To determine TCDD-equivalents (TCDD-EQs) in PCB-containing extracts of waterbird egg composites from various colonies of double-crested cormorants and Caspian terns based on

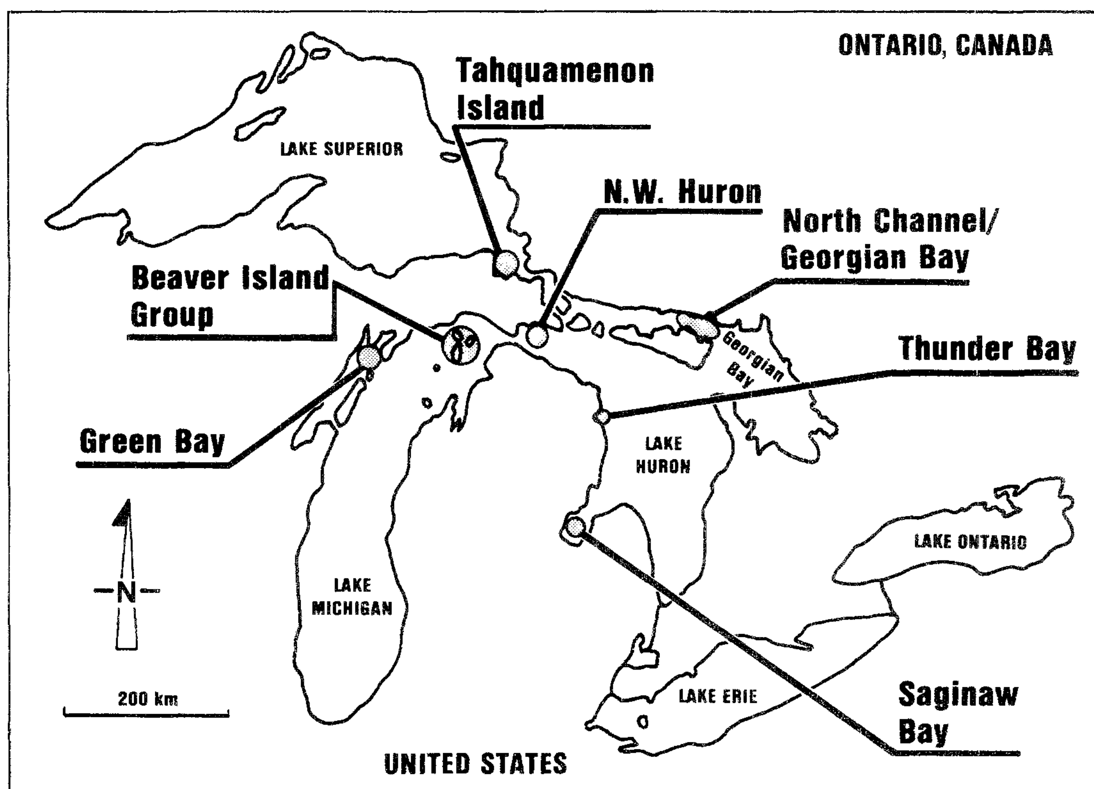

Fig. 1. Area map of the Great Lakes with regional collection sites

relative induction of cytochrome P450-dependent EROD activity in H4IIE rat hepatoma cells; (2) To compare H4IIE bioassay-derived TCDD-EQs in composites of waterbird eggs from various locations with the biological effects observed within those colonies.

Materials and Methods

Samples

Individual waterbird eggs (217), from seven regional areas in the Great Lakes (Figure 1), were collected in 1986 and 1987, and stored frozen prior to extraction. The eggs were from 14 double-crested cormorant (DCC), 10 Caspian tern (CPT), 2 common tern (CMT, *Sterna hirundo*), 1 ring-billed gull (RBG, *Larus delawarensis*), and 1 black-crowned night heron (BCH, *Nycticorax nycticorax*) colonies, representing 41 different collection sites, dates, and/or species (Table 1). 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 CT). Equal portions (generally 5–6 ml) of each egg within a colony (5–6 eggs/colony) were combined to make a single 30g composite sample from each collection site/date. The composite samples and the individual eggs were stored at −20°C until they were extracted.

Extractions

Extraction of the composited eggs was done according to the Pesticide Analytical Manual Sec. 212 (US FDA 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% diethyl ether/petroleum ether (v/v, 6% fraction), the eluate collected, and solvent transferred to isooctane. The resultant 6% fraction contains PCBs (along with a number of organochlorine pesticides) and not PCDDs or PCDFs (Trotter *et al.* 1982; Tillitt *et al.* 1991). Therefore, since the cells were dosed with the 6% fraction (after solvent exchange to isooctane), the TCDD-EQs 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]-2,2',4,4',5,5'-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 addition of internal standards was not possible because of the unknown effect an internal standard may have on the bioassay response. Therefore, the PCB 153 external standard was used for comparative purposes and the results of the bioassay reported here were not corrected for extraction efficiencies from these or any other external standards.

Bioassay Procedures

Bioassay procedures were as previously reported (Tillitt *et al.* 1991). The carrier solvent of the extracts was isooctane at a volume of 100 μl/plate (1.0%). This amount of isooctane had no effect on either survival or on the basal EROD activity of the H4IIE cells under standard culture conditions. Each extract dose was performed in triplicate 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 and

Table 1. Collection site, region and date, species and numeric designation of egg composites

Colony ID	Collection site (region, date)	Species[a]
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

[a] Species abbreviations given in Materials and Methods

used to calculate TCDD-EQs. Therefore, bioassay-to-bioassay variations in cell response were taken into account. Protein determinations (Lowry *et al.* 1951) were made in duplicate for each cell suspension. Cell suspensions were diluted to 1 mg protein/ml and P450IA1-associated activity was measured by the indirect spectrofluorometric EROD assay described by Pohl and Fouts (1980). Duplicate EROD assays were performed on each cell suspension. Fluorescence of resorufin in the samples was determined with an SLM 4800 spectrofluorometer (Urbana, IL) at an emission wavelength of 585 nm and an excitation wavelength of 550 nm. This instrument was first calibrated with a standard rhodamine B solution. Consequently, the fluorescence in samples was measured relative to this internal standard. 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, MI). 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. The potency of each sample extract was calculated according to 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 to the sample extract ED_{50} (ul/plate) with the resultant extract potency, expressed in units of pg TCDD-EQ/ul of extract. TCDD-EQs were then calculated by

$$\text{TCDD} - \text{EQ (pg/g)} = \frac{\text{(extract potency) (extract vol., } \mu\text{l)}}{\text{(sample weight, g)}} \quad (2)$$

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

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

where

CV_T = coefficient of variation for TCDD-EQs
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 CT_T by the estimated TCDD-EQs for that sample.

Differences in TCDD-EQs from composites of eggs among regional areas were 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 1982; 1987). The value for the single egg collected at the Saginaw Bay CDF (June 11, 1986) was not included in these calculations.

Results

The extracts of all waterbird eggs tested significantly induced EROD activity in the H4IIE cells. Representative extract (Figure 2) and TCDD standard (Figure 3) dose-response curves obtained the same day are presented. All extracts had a complete range of responses, from minimal to maximal, within the dosing scheme of 1–100 µl of extract/plate. This volume of extract represents 0.015–3.0 g-equivalents of waterbird egg. The range of ED_{50} values for extracts was 10.14–34.34 µl extract/plate while the TCDD ED_{50} values had a range of 40.14–82.59 pg TCDD/plate. Coefficients of variation for ED_{50} estimates were in the range of 3.83–11.42% and 1.40–2.81% for extract ED_{50} and TCDD ED_{50} values, respectively. The resultant estimates of TCDD-EQs in the waterbird eggs generally had coefficients of variation less than 15%. Thus, the analytical error associated with the

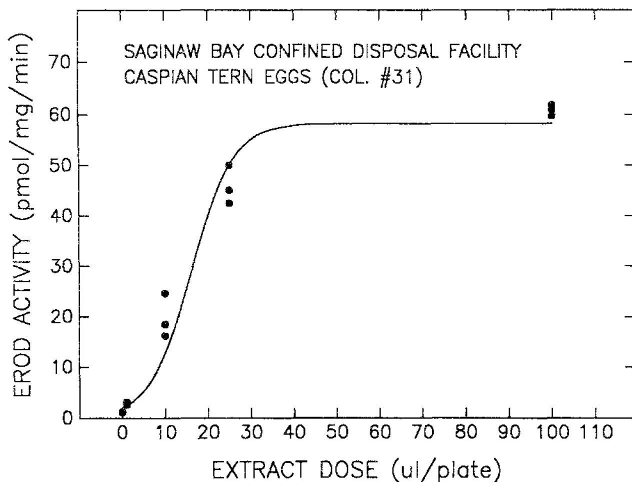

Fig. 2. Representative H4IIE bioassay dose-response curve for a waterbird egg extract. EROD = ethoxyresorufin O-deethylase. Composite (30g) of waterbird eggs were from a single colony and were extracted and concentrated to 2 ml final volume in isooctane. The seeding rate was 1×10^6 cells/plate 24 h prior to dose, and cells were incubated 3 days after dosage

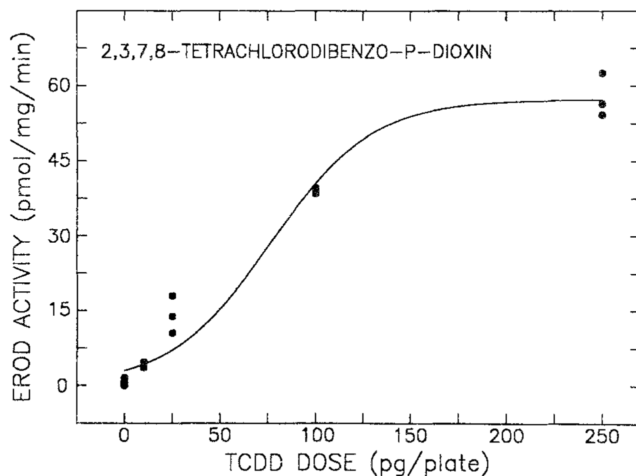

Fig. 3. Representative H4IIE bioassay dose-response curve for the TCDD standard. EROD = ethoxyresorufin O-deethylase. TCDD delivered in 100 μl isooctane. Seeding rate 1×10^6 cells/plate 24 h prior to dose, incubated 3 days after dosage

cell culture, dosing, and enzyme assay of this bioassay system was relatively small.

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 (Figure 4) and Caspian tern egg composites (Figure 5) 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 in Caspian tern eggs collected in 1986 from the High Is. colony (No. 6) to a high of 415.7 pg TCDD-EQ/g in Caspian tern eggs collected from the Saginaw Bay Confined Disposal Facility in 1986 (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 in which both species were present, cormorant eggs had slightly greater TCDD-EQs values (12%), but these differences were not statistically significant (GLM, p = 0.57–081). Additionally, there was no difference between TCDD-EQs in waterbird eggs collected in 1986 and 1987 (GLM SAS, $p > 0.80$).

TCDD-EQs in waterbird eggs from the various regions differed. When the TCDD-EQs in cormorant and Caspian tern eggs were ranked in descending order of TCDD-EQs and the colony sites were classified according to regional distribution in the Great Lakes, a significant regional effect was observed. The average rank of the cormorant and Caspian tern egg samples for the regions was calculated (Table 2). Comparison of ranks showed a regional effect (Kruskal-Wallis, $0.10 > p > 0.05$) as did comparison of TCDD-EQs in the waterbird eggs (GLM SAS, $p = 0.0058$). Regional averags of TCDD-EQs for cormorant and tern egg composites are also presented (Table 3). The variation of TCDD-EQs 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 was 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 TCDD-EQs in these eggs.

Three other species of waterbird eggs were collected and analyzed with the H4IIE bioassay for comparative purposes (Table 4). TCDD-EQs in the eggs of these species were within the range of values found for cormorants and Caspian terns. Because of the limited number of samples from these species it was 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 fall in the lower half of the overall ranks.

Discussion

Significant amounts of TCDD-EQs were detected in PCB-containing extracts of colonial fish-eating waterbird eggs 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 μl/plate) in all cases. This translates to maximal induction by 1–3 g-equivalents of the egg composites. This has not always been 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 extraction 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 cannot be accounted for by any of these factors. The fact that fairly high TCDD-EQs were detected even in remote, non-urban, nonindustrialized areas is

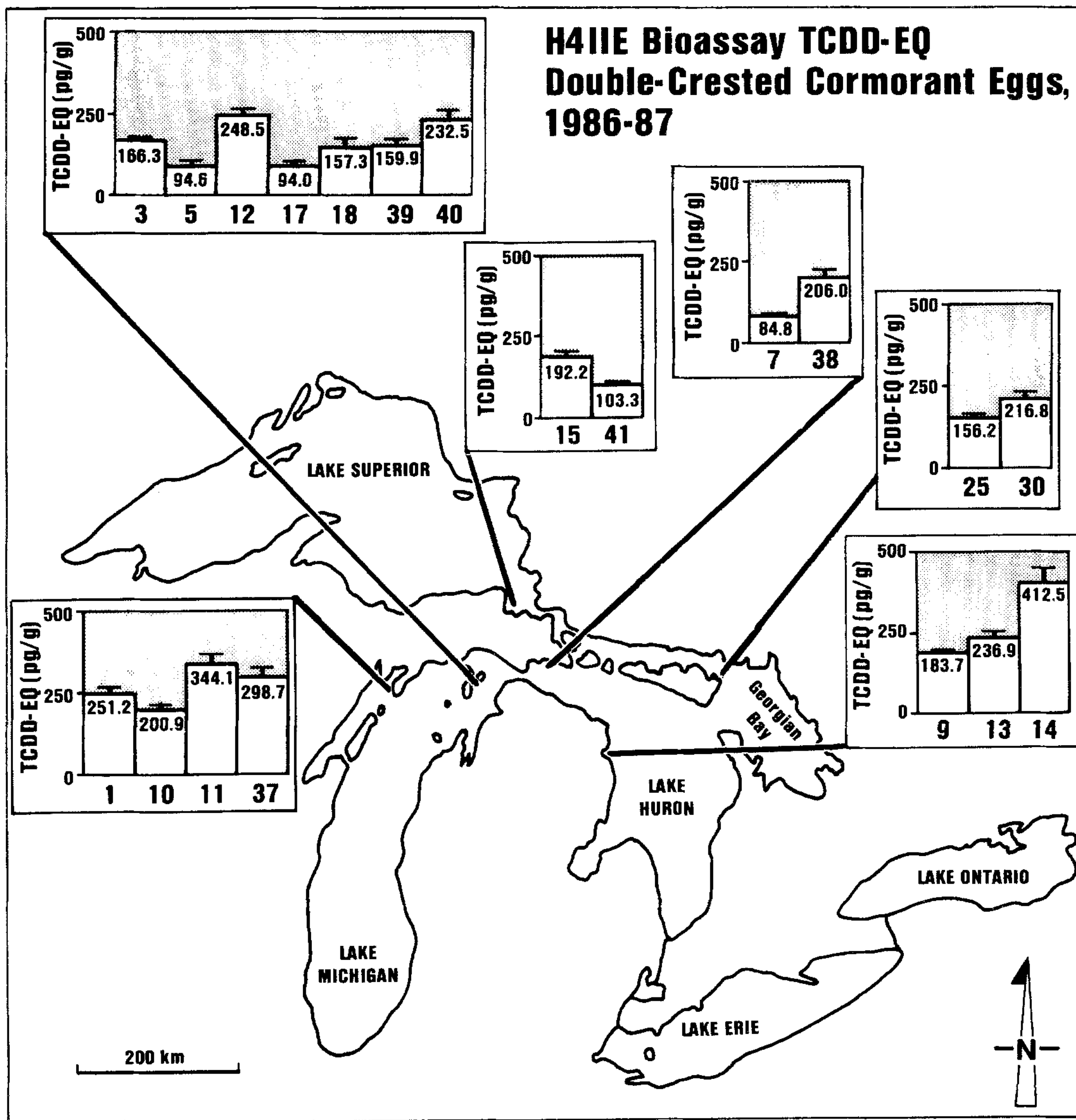

Fig. 4. H4IIE bioassay-derived TCDD-EQ (pg/g) in composites of double-crested cormorant eggs. Standard deviation bars presented. Numbers on the x-axis of the bar graph inserts refer to colony identification numbers given in Table 1

not completely unexpected based on the recalcitrant nature of these environmental contaminants and the estimates that a large amount of PCBs currently enter the Great Lakes through atmospheric transport and deposition (Eisenreich *et al.* 1981).

H4IIE bioassay-derived TCDD-EQs 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 understood at this time. However, it has been established that the inductive response in the H4IIE bioassay is quantitative with respect to PHH dose (Bradlaw and Casterline 1979; Sawyer and Safe 1982; Tillitt *et al.* 1991). 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; Tillitt *et al.* 1991). The H4IIE bioassay can act as a data reduction tool that integrates the overall interactions of complex PHH mixtures at the cellular level. Even if the values obtained are not always 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 the *in vitro* enzyme induction response and the toxic responses of rats *in vivo* to individual PHH congeners (Safe 1987). If the H4IIE bioassay is to become a tool for predicting environmental effects in various species, an understanding of the relative structure-activity relationships within those species as well as an understanding of the acute versus chronic potency within the same species will be required. In other words, it must be demonstrated that the rank order of PHH potencies is similar

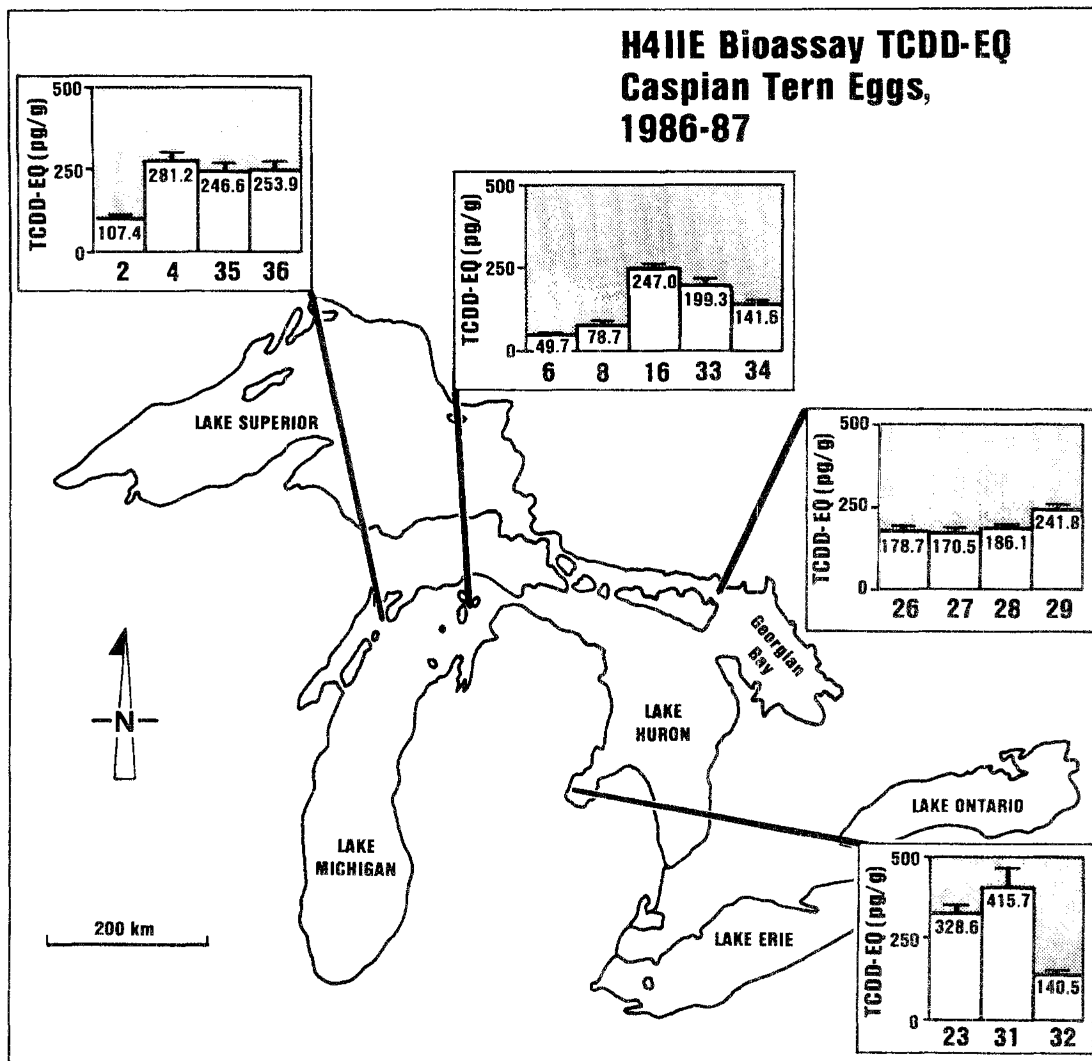

Fig. 5. H4IIE bioassay-derived TCDD-EQ (pg/g) in composites of Caspian tern eggs. Standard deviation bars presented. Numbers on the x-axis of the bar graph inserts refer to colony identification numbers given in Table 1

among rats and the species of interest. There are no such data for the waterbirds tested in this study, however, the limited data available for the chicken indicate that the relative rank order of potency for PCDDs (Poland and Glover 1973; Bradlaw and Casterline 1979) and PCBs (Brunstrom 1986; Brunstrom and Andersson 1988; Brunstrom 1989; 1990) is similar to that of rats.

The relative ranks of the TCDD-EQs in egg composites from this study are concordant with those areas known to have PHH contamination and known to have the greatest rates of deformities in fish-eating waterbirds. Forster's terns (*Sterna forsteri*) from Green Bay have exhibited impaired reproduction including an increased incidence of embryo toxicity, increased incidence of deformities, and altered parental behavior (Hoffman *et al.* 1987; Kubiak *et al.* 1989). 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. Kubiak *et al.* (1989) found decreased hatching success (both in the field and in laboratory-incubated eggs), decreased body weights of the hatchlings, and increased liver to body weight ratios in Green Bay terns

Table 2. Average rank by region of H4IIE-derived TCDD-EQs[c] in composites of double-crested cormorant and Caspian tern eggs[a]

Region	N[b]	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

[a] Concentrations of H4IIE TCDD-EQ in composites of eggs were ranked from greatest to smallest and the average ranks, within regions, were determined. The greatest concentration received a rank of 1 and thus the smaller the average rank the greater the concentration

[b] N = number of egg composites tested from each region.

Table 3. Regional averages of TCDD-EQs[d] in composites of double-crested cormorant and Caspian tern eggs[a]

Region	Species[b]	N[c]	Mean TCDD-EQ (SD)
Green Bay, Lake Michigan	DCC	4	273.7 (62)
	CPT	4	222.3 (78)
Saginaw Bay, Lake Huron	CPT	3	294.9 (141)
Thunder Bay, Lake Huron	DCC	3	277.7 (120)
Georgian Bay/North Channel	DCC	2	186.5 (43)
Lake Huron	CPT	4	194.3 (32)
Beaver Island, Lake Michigan	DCC	7	164.7 (60)
	CPT	5	143.3 (82)
N.W. Lake Huron	DCC	2	145.4 (86)
Tahquamenon Island, Lake Superior	DCC	2	147.8 (63)

[a] Arithmatic means and standard deviations (SD) of H4IIE bioassay-derived TCDD-EQs (pg/g) in waterbird egg composites by region and species
[b] Species abbreviations given in Materials and Methods
[c] N = number of egg composites tested from each region
[d] TCDD = 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

Table 4. TCDD-EQ[c] in individual composites of black crowned night heron, ring-billed gull and common tern eggs[a]

Location (species)	Colony ID[b]	TCDD-EQ (SD) (pg/g)
Saginaw Bay, Lake Huron (BCH)	24	221.8 (20)
Saginaw Bay, Lake Huron (RBG)	22	208.1 (17)
N. Beaver Is, Lake Michigan (CMT)	19	187.4 (17)
Thunder Bay Is, Lake Huron (CMT)	20	104.3 (5.0)

[a] H4IIE bioassay-derived TCDD-EQs (pg/g) and standard deviations (SD) in waterbird egg composites. Species abbreviations given in Materials and Methods
[b] Colony ID number from Table 1
[c] TCDD-2,3,7,8-tetrachlorodibenzo-*p*-dioxin

as compared to those from the reference site, Lake Poygan. A similar etiology of chick edema disease was described by Gilbertson (1983) for the severe reproductive failure of herring gulls (*Larus agenatus*) in PHH contaminated areas of Lake Ontario. Elevated TCDD-EQs 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 showed depressed hatching success and no survival of chicks beyond fledge (Kurita *et al.* 1987) in some years. Additionally, Fox *et al.* (1991) found the incidence of congenital anomalies in double-crested cormorant chicks from Green Bay (1979–1987) to be 52 per 10,000 (60 of 11,520) compared to 0–12.3 per 10,000 for the other study areas within the Great Lakes and 0.6 per 10,000 (1 of 16,788) in colonies from Alberta and Saskatchewan during this same period. Fox *et al.* (1988) 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. Vitamin A metabolism is also adversely affected by PHHs (Thunberg 1984) and is believed to be related to the developmental anomalies associated with PHH poisoning (Abbott and Birnbaum 1989). Spear *et al.* (1990) measured retinol to retinyl palmitate ratios in herring gull eggs from around the Great Lakes and observed the smallest ratios in eggs taken from Saginaw Bay. Depressed retinol to retinyl palmitate molar ratios and a corresponding reduction in egg viability were observed in a laboratory study of dove eggs injected with 3,3′, 4,4′-tetrachlorobiphenyl, a potent PHH (Spear *et al.* 1989).

Laboratory studies (Higginbotham *et al.* 1968; Cecil *et al.* 1974; Poland and Glover 1973; Tumasonis *et al.* 1968; Ax and Hansen 1975; Brunstrom and Andersson 1988; Spear *et al.* 1989, 1990; Brunstrom 1990) 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 colonial fish-eating waterbirds (Fox and Weseloh 1987). 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 remains circumstantial. Regardless, the evidence for such a relationship is strong. Whether the TCDD-EQs obtained from this bioassay quantitatively reflect the effects of a comparable amount of TCDD in the organism was beyond the scope of the present study and has not been addressed by other investigators.

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 conge-

ner'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, Kubiak *et al.* (1989) 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-EQs in Green Bay waterbird eggs reported in our 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 (Bannister *et al.* 1987). 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. Thus, the discrepancies between the H4IIE bioassay-derived TCDD-EQs from this study and chemical residue analysis-derived TCDD-EQs from waterbirds from the same (Kubiak *et al.* 1989) are not likely to be due to the fact that this study only assessed the potency of the PCB fraction and not PCDDs or PCDFs. Clearly further studies are required to elucidate this phenomenon. Currently, we have studies underway to evaluate various fractions of environmental extracts for their ability to modulate EROD activity in the H4IIE bioassay.

In conclusion, the H4IIE bioassay is 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 (i.e., the egg in this case), 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 potency of PHHs in rat cells are similar to their relative potencies in other species. The potential utility of this simple, fast, and integrative bioassay in environmental risk/hazard assessment is clearly apparent.

Summary

The H4IIE bioassay was used to assess the potency of PCB-containing extracts from colonial fish-eating waterbird eggs from around the Great Lakes. Significant concentrations of TCDD-EQs 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 double-crested cormorants and Caspian terns. Waterbird eggs from urban and industrialized areas had the greatest amount of TCDD-EQs while the more remote areas had less contaminated eggs. 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

Abbott BD, Birnbaum LS (1989) Cellular alterations and enhanced induction of cleft palate after coadministration of retinoic acid and TCDD. Toxicol Appl Pharmacol 99:287–301

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, Zmudzka 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

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

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

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 Offic Anal Chem 62:904–916

Brunstrom B (1986) Activities in chick embryos of 7-ethoxycoumarin O-deethylase and aryl hydrocarbon (benzo[a]pyrene) hydroxylase and their induction by 3,3′,4,4′-tetrachlorobiphenyl in early embryos. Xenobiotica 16:865–872

——— (1989) Toxicity of coplanar polychlorinated biphenyls in avian embryos. Chemosphere 19:765–768

——— (1990) Mono-ortho-chlorinated chlorobiphenyls: toxicity and induction of 7-ethoxyresorufin O-deethylase (EROD) activity in chick embryos. Arch Toxicol 64:188–192

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

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 Offic 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

Davis D, Safe S (1988) Immunosuppressive activities of polychlorinated dibenzofuran congeners: quantitative structure-activity

relationships and interactive effects. Toxicol Appl Pharmacol 94:141–149

Elder GH (1978) Porphyria caused by hexachlorobenezene and other polyhalogenated aromatic hydrocarbons. In: DeMatteis F, Aldridge WD (eds) Heme and hemoproteins. Springer-Verlag, NY, pp 157-200

Eisenreich SJ, Looney BB, Thorton JD (1981) Airborne organic contaminants in the Great Lakes ecosystem. Environ Sci Technol 15:30–38

Finney DJ (1978) Statistical methods in biological assay. 3rd ed. Charles Griffin and Co, London

Fox GA, Collins B, Hayakawa E, Weseloh DV, Ludwig JP, Kubiak TJ, Erdman TC (1991) Reproductive outcomes in colonial fish-eating birds: a biomarker for developmental toxins in Great Lakes food chains II. Spatial variation in the occurrence and prevalence of bill defects in young double-crested cormorants in the Great Lakes, 1979–1987. J Great Lakes Res (in press)

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

Fox GA, Weseloh DV (1987) Colonial waterbirds as bioindicators of environmental contamination in the Great Lakes. In: Diamond AW, Filion FL (eds) The value of birds. ICBP Tech Publ 6, Cambridge UK, pp 209–216

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

Goldstein JA (1980) Structure-activity relationships for the biochemical effects of relationship to toxicity. In: Kimbrough RD (ed) Topics in environmental health: Vol 4, Halogenated biphenyls, terphenyls naphthalenes, dibenzodioxins and related products. Elsevier North-Holland, 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

Harris HJ (1988) Persistent toxic substances and birds and mammals in the Great Lakes. In: Evans MS (ed) Toxic contaminants and ecosystem health: A Great Lakes focus. John Wiley and Sons, NY, pp 557–569

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 220:702–703

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

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 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, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with Folin phenol reagent. J Biol Chem 193:265–275

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

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

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. Chap 4

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

——— (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

Safe S, Phil D (1990) Polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), and related compounds: Environmental and mechanistic considerations which support the development of toxic equivalency factors (TEFs). Crit Rev Toxicol 21:51–88

SAS Institute Inc. (1982) SAS users guide: Statistics. Box 8000, Cary, NC

——— (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 activites 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 14:79–84

Spear PA, Bourbonnais DH, Peakall DB, Moon TW (1989) Dove reproduction and retinoid (vitamin A) dynamics in adult females and their eggs following exposure to 3,3′, 4,4′-tetrachlorobiphenyl. Can J Zool 67:908–911

Spear PA, Bourbonnais DH, Norstrom RJ, Moon TW (1990) Yolk retinoids (vitamin A) in eggs of the herring gull and correlations with polychlorinated dibenzo-*p*-dioxins and dibenzofurans. Environ Toxicol Chem 9:1053–1061

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

Thunberg G (1984) Effects of TCDD on vitamin A and its relation to TCDD-toxicity. In: Poland A, Kimbrough R (eds) Bradbury Re-

port 18: Biological mechanisms of dioxin action. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp 333–344

Tillitt DE, Ankley GT, Giesy JP (1989) Planar chlorinated hydrocarbons (PCHs) in colonial fish-eating waterbird eggs from the Great Lakes. Mar Environ Res 28:505–508

Tillitt DE, Giesy JP, Ankley GT (1991) Characterization of the H4IIE rat hepatoma cell bioassay as a tool for assessing toxic potency of planar halogenated hydrocarbons (PHHs) in environmental samples. Environ Sci Technol 25:87–92

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 Offic 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

U.S. Food and Drug Administration (1979) Pesticide analytical manual, Vol I. Washington, DC, Sec. 212.13a

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

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

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

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