Arch. Environ. Contam. Toxicol. 39, 273–281 (2000)
DOI: 10.1007/s002440010105

ARCHIVES OF
Environmental
Contamination
and Toxicology

Relative Potencies of Individual Polychlorinated Naphthalenes to Induce Dioxin-Like Responses in Fish and Mammalian *In Vitro* Bioassays

D. L. Villeneuve,[1] K. Kannan,[1] J. S. Khim,[1] J. Falandysz,[2] V. A. Nikiforov,[3] A. L. Blankenship,[1] J. P. Giesy[1]

[1] Department of Zoology, National Food Safety and Toxicology Center, and Institute for Environmental Toxicology, Michigan State University, East Lansing, Michigan 48824, USA
[2] Department of Environmental Chemistry and Ecotoxicology, University of Gdañsk, Ul. J. Sobieskiego 18, 80-953 Gdañsk, Poland
[3] St. Petersburg University, Petrodvorets, Universitetski 2, 198904 St. Petersburg, Russia

Received: 6 December 1999/Accepted: 29 March 2000

Abstract. A growing body of evidence suggests that polychlorinated naphthalenes (PCNs) may be fairly widespread environmental contaminants. This may be cause for concern because exposure to PCNs has been linked to dioxin-like biological responses in a wide variety of species. This study used three *in vitro* bioassays to characterize the dioxin-like potency of 18 individual PCN congeners and 1 PCN metabolite. The PLHC-1 fish hepatoma cell bioassay was relatively insensitive to PCNs. At the concentrations tested, only 1,4 di-CN and 2,4-dichloro-1-napthol caused significant induction of ethoxyresorufin *O*-deethylase (EROD) activity in the PLHC-1 assay. *In vitro* EROD and luciferase assays using recombinant H4IIE rat hepatoma cells were more responsive to PCNs. Structure-activity relationships were observed both in terms of the degree of chlorination and the positions of chlorine substitutions. Hexa-chlorinated naphthalenes (CNs), exhibiting relative potencies (REPs) around 10^{-3} (relative to TCDD), were the most potent congeners tested. Penta-CNs were also rather potent, yielding REPs between 10^{-3} and 10^{-7}. Tetra-, tri-, di-, and mono-CNs were less active. REPs for the active congeners were similar to those for some PCBs. The relative potency estimates reported here contribute to an emerging body of information that will aid determination of the relative contribution of PCNs to the total dioxin-like activity associated with environmental samples.

Polychlorinated naphthalenes (PCNs) are a group of compounds composed of two fused benzene rings (naphthalene) with one to eight chlorine substitutions (Figure 1). There are a total of 75 possible PCN congeners. In general, they are hydrophobic waxy solids with high thermal stability and inertness (Kover 1975; Kannan *et al.* 1998). PCNs are structurally similar to PCBs and exhibit similar physical and chemical properties (Kover 1975; Kannan *et al.* 1998; Falandysz *et al.* 2000). Technical mixtures of the 75 possible PCN congeners have been used in dielectric fluids, engine oil additives, electroplating masking compounds, wood preservatives, lubricants, and dye production (Kover 1975; Falandysz *et al.* 2000). PCN mixtures have been sold under the trade name Halowaxes (Koppers Company, PA) in the United States and as Nibren waxes (Bayer, Germany), Seekay waxes (ICI, U.K.), Clonacire waxes (Prodelec, France), and Cerifal (Caffaro, Italy) (Kover 1975; Kannan *et al.* 1998). Global production of PCN mixtures has been estimated to be approximately 150,000 tons (Falandysz 1998). PCNs may have been released to the environment as byproducts of solid waste combustion and as co-contaminants in other industrial mixtures, such as PCB formulations (Benfenati *et al.* 1991; Imagawa and Yamashita 1996).

There is a growing body of evidence that suggests that PCN contamination may be fairly widespread in the environment (Harner and Bidleman 1997; Kannan *et al.* 2000). Prior to 1975 there were few reports of PCNs in environmental samples (Kover 1975). Such reports have increased with the development of sensitive, congener-specific analytical methods and increasing availability of standards. PCNs have now been detected in air (Dorr *et al.* 1996; Harner and Bidleman 1997), water (Crookes and Howe 1993; Espadaler *et al.* 1997), sediment (Järnberg *et al.* 1993; Furlong *et al.* 1988; Kannan *et al.* 1998), and biota (Asplund *et al.* 1990; Järnberg *et al.* 1993; Falandysz *et al.* 1996; Falandysz and Rappe 1996; Kannan *et al.* 1998, 2000). Thus, environmental exposures to PCNs may be occurring in a variety of locations.

Potential environmental exposures to PCNs may be cause for concern. Occupational exposure to PCNs has been linked to serious health problems in humans, including chloracne and liver disease (Kleinfeld *et al.* 1972; Kover 1975; Brinkman and Reymer 1976). PCNs have also been identified as causitive agents of X-disease in cattle (Bell 1954; Kover 1975). In rats, PCNs induce hepatic ethoxyresorufin *O*-deethylase (EROD) activity (Campbell *et al.* 1981, 1983; Mantyla and Ahotupa 1993) and oxidative stress resulting in increased lipid peroxi-

Correspondence to: D. L. Villeneuve

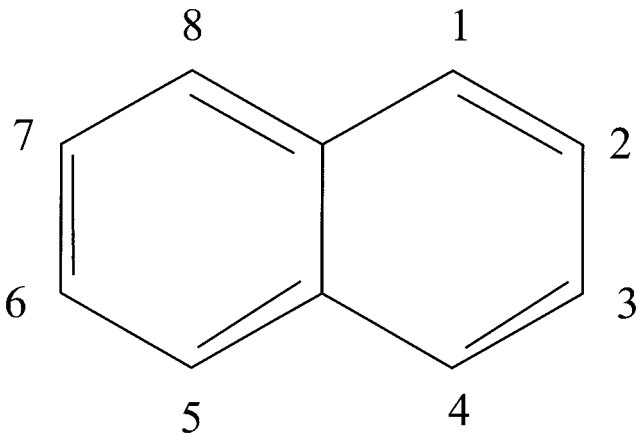

Fig. 1. Generalized structure and numbering system for polychlorinated naphthalenes (PCNs)

dation, decreased hepatic vitamin A and E, and decreased catalase and superoxide dismutase activities (Mantyla and Ahotupa 1993). Certain PCN congeners were also shown to bioaccumulate selectively in rats exposed to Halowax 1014 (Asplund *et al.* 1986, 1994). In birds, exposure to PCNs has been linked to edema in chickens (Pudelkiewicz *et al.* 1959) and both embryolethality and EROD induction in chicken and eider duck embryos (Engwall *et al.* 1994). Similar effects have been observed in fish. Microinjection of yolk-sac embryos with Halowax 1014 induced hepatic EROD activity in rainbow trout (Norrgren *et al.* 1993). Exposure to food contaminated with PCNs induced EROD activity and hepatic lipid accumulation in three-spined sticklebacks (*Gasterostus aculeatus*) (Holm *et al.* 1993). Halowaxes 1014 and 1013 were shown to be toxic to medaka (*Oryzias latipes*) at various early life stages (Villalobos *et al.* 2000). Thus, PCN exposure may lead to both adverse effects and biomarker responses in a broad range of species.

The profile of biological responses suggests that at least a portion of the toxic responses associated with PCNs may be mediated through an aryl hydrocarbon receptor (AhR)–dependent mechanism of action (Poland and Knutson 1982; Blankenship *et al.* 2000). As a result, *in vitro* bioassays that measure AhR-dependent reporter gene activation or enzyme induction may be useful tools for characterizing the relative potencies (REPs) of individual PCN congeners and PCN mixtures (Blankenship *et al.* 2000). Such assays have been widely used to characterize the mechanism-specific toxic potencies of related compounds, such as coplanar PCBs, polychorinated dibenzo-*p*-dioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs), all of which have been shown to exert adverse effects through an AhR-dependent pathway (Poland and Knutson 1982; Safe 1990; Sanderson and Giesy 1998).

This study used *in vitro* EROD assay with PLHC-1 fish hepatoma cells (Hightower and Renfro 1988; Hahn *et al.* 1993, 1996) and *in vitro* EROD and luciferase assays with H4IIE-luc recombinant rat hepatoma cells (Sanderson *et al.* 1996) to characterize the AhR-dependent, dioxin-like potency of 18 individual PCN congeners and 1 PCN metabolite, 2,4-dichloro-1-napthol (2,4-OH-CN; Crookes and Howe 1993). REPs, based on luciferase induction in H4IIE-luc cells, have been reported for 20 PCN congeners (Blankenship *et al.* 1999). This study expanded the characterization of 10 of these compounds by examining another endpoint and cell type. One representative congener from each group of PCN isomers (mono- through hepta-chlorinated) was selected for additional characterization. Three additional hexa-chlorinated congeners, which had been shown to be active in the H4IIE-luc bioassay, were also selected for further characterization. Nine of the congeners excluded from further characterization were shown to be inactive in the H4IIE-luc assay, and thus were unlikely to respond in similar bioassays (Blankenship *et al.* 1999). The remaining congener was a 1:1 mixture of two congeners that had been tested independently (Blankenship *et al.* 1999). In addition to previously analyzed congeners, this study determined REPs for eight PCN congeners and one PCN metabolite for which REPs were not previously available. The assay-specific REPs reported here add to a growing database that characterizes the potential toxic potency of individual PCNs. This information should support increasing efforts to assess risks associated with environmental exposures to PCNs, which may manifest their effects through a dioxin-like mechanism of action.

Materials and Methods

Chemicals

1,4-DiCN 98.3% pure was obtained from Accustandard (New Haven, CT). 2-MonoCN (99% pure) was obtained from ICN Chemicals (Irvine, CA). 1,2,3,4,5,6,7-HeptaCN was synthesized and purified at the Wallenberg Laboratory, Stockholm University, Sweden (Jakobsson *et al.* 1994). PCN congeners 1,2,7-triCN, 2,3,6,7-tetraCN, 1,2,3,6,7-pentaCN, and all the hexaCN congeners tested were obtained (>99% pure) from Promochem (GmbH, Wesel, Germany). PCN congeners 1,2,4,7-tetraCN, 1,3,5,7-tetracCN, 1,2,4,6,8-pentaCN, 1,2,4,6,7-pentaCN, 1,2,4,5,6-pentaCN, 1,2,3,7,8-pentaCN, 1,2,3,6,8-pentaCN, and 1,2,3,5,7-pentaCN were synthesized in the Nikiforov laboratory, St. Petersburg University, Russia, by general methods, which involved the nitration or chlorosulfonation of commercially available chloronaphthalenes, followed by substitution of nitro chlorosulfonyl groups by chlorine. In some cases additional hydrodechlorination was a final step to a target PCN isomer (Nikiforov and Wightman 1997; Miltsov *et al.* 1999). The congeners were > 99% pure and supplied in crystalline form. 2,4-OH-CN, a metabolite of some CNs (Crookes and Howe 1993), was obtained (> 95% pure) from Ultra Scientific (N. Kingstown, RI). PCN standards were analyzed for contamination with 2,3,7,8-substituted PCDDs, PCDFs, or unwanted PCN congeners using selected ion monitoring by low-resolution gas chromatography-mass spectrometry (GC-MS). PCDD and PCDF congeners were not detected at the 100 pg/g level in the standards examined. At this level the maximum response contributed by PCDD/DF congeners would have been at least 600-fold below the bioassay detection limits. Concentrations tested in the bioassay varied and were limited by the mass of standard available. All standards were prepared in high purity isooctane (Burdick and Jackson, Muskegon, MI) prior to dosing cells.

Cell Culture

PLHC-1 cells are desert topminnow (*Poeciliopsis lucida*) hepatoma cells, which have been shown to have inducible cytochrome P4501A1 activity (Hightower and Renfro 1988; Hahn *et al.* 1993, 1996). H4IIE-luc cells are rat hepatoma cells, which were stably transfected with a luciferase reporter gene under control of dioxin-responsive enhancers

(DREs) (Sanderson *et al.* 1996). H4IIE-luc cells were used for both luciferase assays (H4IIE-luc) and EROD assays (H4IIE-EROD). All cells were cultured in 100-mm disposable petri plates (Corning, Corning NY) and were incubated in a humidified 95:5 air:CO_2 atmosphere. PLHC-1 and H4IIE-luc cells were grown at 30°C and 37°C, respectively. H4IIE-luc were cultured in Dulbecco's Modified Eagle Medium (Sigma D-2902, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT). PLHC-1 were cultured in Minimum Essential Medium Eagle (MEM) supplemented with 292 mg/L L-glutamine (Life Technologies, Grand Island, NY) and 10% FBS (Hyclone). Cells were passaged when plates became confluent, and new cultures were started from frozen stocks after less than 30 passages.

Exposure

Cells were trypsinized from petri plates containing 80–100% confluent monolayers and resuspended in media. The number of cells per milliliter was estimated using a hemacytometer. H4IIE-luc cells were diluted to a concentration of approximately 7.5×10^4 cells/ml and seeded into the 60 interior wells of 96-well flat bottom microplates (ViewPlates, Packard Instruments, Meriden, CT for luciferase assays; Corning 25860 for EROD assays). PLHC-1 were diluted to a concentration of approximately 1.25×10^5 cells/ml and seeded into the 60 interior wells of 96-well flat bottom microplates (Corning 25860). All cells were seeded in 250 μl per well. The 36 exterior wells were filled with 250 μl culture media. Cells were incubated overnight to allow for cell attachment, then dosed. Test and control wells were dosed with 2.5 μl of the appropriate sample or solvent. Blank wells received no dose. A minimum of three control wells and three blank wells were tested on each plate. A minimum of three replicate wells of each sample concentration were tested. Dose-responses consisted of six concentrations prepared by threefold serial dilution from the maximum concentration tested (Table 1). All exposures were 72 h.

Luciferase Assays

Luciferase assays with H4IIE-luc cells have been detailed previously (Sanderson *et al.* 1996). Briefly, exposed wells were inspected using a microscope, and condition relative to control wells was noted. Culture media was then removed, and cells were rinsed with phosphate buffered saline (PBS). Cells were lysed, and luciferase assay reagent (containing a luciferin substrate) was added to the wells. Plates were incubated for 10 min at 30°C, then scanned with an ML3000 microplate reading luminometer (Dynatech Laboratories, Chantilly, VA). Following the luminometer scan a 1.08 mM solution of fluorescamine (Sigma) in high-purity acetonitrile (Burdick and Jackson) was added to each well and plates were assayed for protein (Kennedy and Jones 1994) using a Cytofluor 2300 (excitation 400 nm, emission 460 nm) after a 15-min incubation at room temperature. Total protein content per well was calculated by regression against a bovine serum albumin (BSA; Sigma A-2153) standard curve. For luciferase assays, total protein in the wells was used as an index of cell number to detect outliers that were not apparent by visual inspection with a light microscope. Relative luminescence units (RLUs) were not adjusted for protein.

EROD Assays

In vitro EROD assays with PLHC-1 and H4IIE-luc cells were performed using a modified version of an H4IIE-wild type EROD assay procedure (Sanderson and Giesy 1998). Exposed wells were inspected using a microscope, and condition relative to control wells was noted. Culture media was removed by vacuum manifold, and the cells were rinsed with PBS. Cells were lysed by freeze-thaw in 30 μl nanopure water then treated with 100 μl 0.05 M Na_2HPO_4 buffer containing 60 μM dicumarol (3,3′-methylene-bis[4-hydroxy-coumarin], Sigma M-1390), 50 μl 10 μM ethoxyresorufin (ER; Molecular Probes, Eugene, OR), and 20 μl 0.5 mM β-NADPH (Sigma N-1630) and incubated at 30°C for exactly 60 min. Reactions were stopped by addition of 75 μl 1.08 mM fluorescamine in acetonitrile. Plates were incubated for another 15 min, then scanned using a Cytofluor 2300 (excitation 530 and 400 nm, emission 590 and 460 nm, sensitivity 3). Resorufin (Molecular Probes) and protein (BSA; Sigma A-2153) standard curves were prepared by serial dilution and run in the same manner as sample plates (no ER was added to resorufin standard wells). Relative fluorescence units (RFUs) were converted to pmol resorufin produced per min per mg protein (pmol/min/mg) by regression against the resorufin and protein standard curves.

Bioassay Data Analysis

Sample responses expressed as mean RLU or mean pmol/min/mg protein (three replicate wells, among well CV generally $< 10\%$) were converted to a percentage of the mean maximum response observed for TCDD standard curves generated on the same day (%-TCDD-max). This was done to normalize for day-to-day variability in response magnitude and to make response magnitudes comparable from assay to assay. The mean solvent control response was subtracted from both the sample and TCDD standard responses, prior to conversion to a percentage, in order to scale values from 0% to 100%-TCDD-max.

In cases where the magnitude of induction was sufficient to allow a reasonable estimate, assay-specific relative potencies were calculated. The linear portion of each dose response (%-TCDD-max plotted as a function of log dose) was defined by dropping points from the tails until an $R^2 \geq 0.95$ was obtained and a linear regression model was fit to the remaining points. At least three points were used in all cases. The linear regression equations for the samples and corresponding TCDD standard were used to estimate the concentration associated with responses expressed as %-TCDD-max.

For point estimates of relative potency to be valid, the sample and standard dose-response must be statistically parallel and have the same maximum achievable response (Finney 1978; Putzrath 1997; Villeneuve *et al.* 2000). These conditions were tested empirically. The efficacy of most samples was either unknown or less than that of the standard. Thus, equal efficacy could not be assumed. The parallel slopes assumption was tested by calculating relative potencies (REP_i) at multiple levels of response (Y_i) ranging from 20–80%-TCDD-max. For parallel dose-responses, REP estimates are independent of the response level selected (Putzrath 1997). The minimum and maximum REP_i values generated (a relative potency band or RP-band) were reported as an estimate of the uncertainty in the REP estimate due to deviations from parallelism between the standard and sample curves (Villeneuve *et al.* 2000). In cases where the observed maximum response for the sample was less than 80%-TCDD-max, extrapolation beyond the range of the empirical results was used to estimate REP_i at Y_i greater than the observed maximum. This was done to make the RP bands comparable from sample to sample, since the width of the band is dependent on the range of responses over which it is calculated. In all cases, the maximum response observed was reported along with the REP estimate.

Results

Nineteen PCN congeners were analyzed using the PLHC-1, H4IIE-EROD, and H4IIE-luc bioassays. These included repre-

Table 1. Maximum concentrations of individual PCN congeners tested in PLHC-1, H4IIE-EROD, and H4IIE-luc *in vitro* bioassays, relative potency (REP) estimates,[a] and observed maximum responses[b]

		PLHC-1		H4IIE-EROD		H4IIE-luc	
PCN Congener	Max. Conc. (nM in well)	REP[a]	Obs. Max.[b]	REP[a]	Obs. Max.[b]	REP[a]	Obs. Max.[b]
2-CN	1.2×10^5	$<7.1 \times 10^{-7}$	0	$<1.1 \times 10^{-7}$	0	—	—
1,4 di-CN	5.1×10^5	$<1.6 \times 10^{-7}$	11	2.6×10^{-8}–3.6×10^{-10}	33	1.2×10^{-7}–1.0×10^{-8}	47
2,7-diCN	5.1×10^4	$<1.6 \times 10^{-6}$	0	$<2.6 \times 10^{-7}$	1	$<4.2 \times 10^{-7}$	3
2,4-OH-CN	1.1×10^5	$<7.4 \times 10^{-7}$	19	5.5×10^{-8}–7.6×10^{-9}	29	$<1.9 \times 10^{-7}$	2
1,2,7-triCN	2.2×10^4	$<3.8 \times 10^{-6}$	0	$<6.1 \times 10^{-7}$	−1	—	—
2,3,6,7-tetraCN	3.8×10^1	$<2.2 \times 10^{-3}$	−1	$<3.5 \times 10^{-4}$	3	—	—
1,2,4,7-tetraCN	3.8×10^4	$<2.2 \times 10^{-6}$	0	$<3.5 \times 10^{-7}$	3	$<5.7 \times 10^{-7}$	4
1,3,5,7-tetraCN	3.8×10^3	$<2.2 \times 10^{-5}$	0	$<3.5 \times 10^{-6}$	15	$<5.7 \times 10^{-6}$	−3
1,2,3,6,7-peCN	3.3×10^1	$<2.5 \times 10^{-3}$	1	2.7×10^{-4}–2.2×10^{-5}	35	$<6.4 \times 10^{-4}$	2
1,2,3,5,7-peCN	3.3×10^3	$<2.5 \times 10^{-5}$	0	$<3.9 \times 10^{-6}$	3	—	—
1,2,3,7,8-peCN	3.3×10^3	$<2.5 \times 10^{-5}$	0	3.9×10^{-5}–1.3×10^{-5}	61	4.6×10^{-5}–4.5×10^{-5}	66
1,2,4,5,6-peCN	3.3×10^4	$<2.5 \times 10^{-6}$	0	7.8×10^{-6}–3.1×10^{-7}	97	8.1×10^{-6}–1.5×10^{-6}	56
1,2,4,6,7-peCN	3.3×10^4	$<2.5 \times 10^{-6}$	0	$<3.9 \times 10^{-7}$	37	2.6×10^{-5}–3.6×10^{-7}	60
1,2,4,6,8-peCN	3.3×10^4	$<2.5 \times 10^{-6}$	0	$<3.9 \times 10^{-7}$	30	—	—
1,2,3,4,6,7-hxCN	3.0×10^2	$<2.8 \times 10^{-4}$	0	1.5×10^{-3}–2.6×10^{-4}	68	2.2×10^{-3}–3.0×10^{-3}	80
1,2,3,5,6,7-hxCN	3.0×10^3	$<2.8 \times 10^{-5}$	0	3.1×10^{-4}–2.7×10^{-4}	44	—	—
1,2,3,5,6,8-hxCN	3.0×10^1	$<2.8 \times 10^{-3}$	−1	$<4.4 \times 10^{-4}$	20	—	—
1,2,3,6,7,8-hxCN	3.0×10^2	$<2.8 \times 10^{-4}$	0	1.7×10^{-3}–2.6×10^{-3}	89	2.2×10^{-2}–4.5×10^{-3}	91
1,2,3,4,5,6,7-hpCN	2.7×10^1	$<3.1 \times 10^{-3}$	0	3.8×10^{-4}–5.6×10^{-4}	84	1.3×10^{-3}–3.8×10^{-4}	67

[a] REPs reported as the range of REP estimates generated from multiple point estimates over a response range from 20–80%-TCDD-max (RP-band). Extrapolation was used for samples that yielded maximum responses less than 80%-TCDD-max. All REP estimates were based on molar concentrations. REPs < x were calculated by dividing the maximum concentration tested by the EC-50 of the TCDD standard.
[b] Maximum response observed expressed as a percentage of the mean maximum response observed for the TCDD standard (%-TCDD-max). Maximum response was not necessarily achieved at the maximum concentration tested.

sentatives of monochlorinated through heptachlorinated congeners. PCNs generally did not elicit significant activity in the PLHC-1 bioassay (Figures 2–5). Only 1,4-diCN and 2,4-OH-CN elicited a response significantly different from controls in the PLHC-1 bioassay (Figure 2). In both cases the greatest magnitude of response was less than 20%-TCDD-max (Table 1). At the concentrations tested, none of the tri- through heptaCN congeners elicited a significant response in the PLHC-1 assay (Figures 2–5).

PCNs, particularly the more chlorinated congeners, were more active in the H4IIE assays (Figures 2–5). As in the PLHC-1 assay, 1,4-diCN and 2,4-OH-CN induced significant responses in the H4IIE-EROD assay (Figure 2). The magnitudes of response were slightly greater, reaching 33%- and 29%-TCDD-max for 1,4-diCN and 2,4-OH-CN, respectively. 1,4-DiCN was even more active in the H4IIE-luc assay, yielding a response of 47%-TCDD-max (Figure 2; Table 1). 2,4-OH-CN did not yield a response in the H4IIE-luc assay, however (Figure 2). REP estimates for 1,4-di and 2,4-OH-CN were around 10^{-8} (Table 1). 1,3,5,7-TetraCN produced a weak response (15%-TCDD-max) in the H4IIE-EROD assay but was not active in the H4IIE-luc assay (Figure 3; Table 1). No other tetraCN congeners were active at the concentrations tested (Figure 3). Significant activity was observed for most penta- through heptaCN congeners tested using the H4IIE assays (Figures 4 and 5). Magnitudes of induction ranged up to 97%-TCDD-max (Figures 3 and 4; Table 1). REPs were estimated to range from approximately 10^{-3} to 10^{-6}, with the hexa-chlorinated congeners generally being one to two orders of magnitude more potent than the pentachlorinated congeners (Table 1). The least active of the penta- through hepta-chlorinated congeners were 1,2,3,5,7- and 1,2,4,6,8-pentaCN (Figure 4). One congener (1,2,4,6,7-pentaCN) produced a dose-dependent response in the H4IIE-luc assay but not the H4IIE-EROD assay (Figure 4). The lack of a dose-dependent response in the H4IIE-EROD assay was confirmed with four additional replications of the H4IIE-EROD assay with 1,2,4,6,7-pentaCN (Figure 4).

Discussion

Relative Potency Estimates

A fundamental assumption behind REP estimation is that the sample being characterized responds in the assay as if it were simply a dilution of the prototypical compound used as the standard or reference compound to which all other compounds or samples are compared (Finney 1978; Putzrath 1997). This implies that the slopes and efficacy of the sample and standard dose-responses should be the same (Finney 1978; Putzrath 1997). This could not be demonstrated for all samples tested in this study. The observed maximum responses for the samples were often less than 100%-TCDD-max. Only 1,2,4,5,6-peCN and 1,2,3,4,5,6,7-hpCN in the H4IIE-EROD assay and 1,2,3,6,7,8-hxCN in both H4IIE assays yielded responses that were approximately equal to the efficacy of TCDD (Table 1; Figures 4 and 5). REP estimates may have been biassed by extrapolation of the slope beyond the observed maximum for the sample. The slopes of sample dose-responses were generally not dramatically different from that of the TCDD standard.

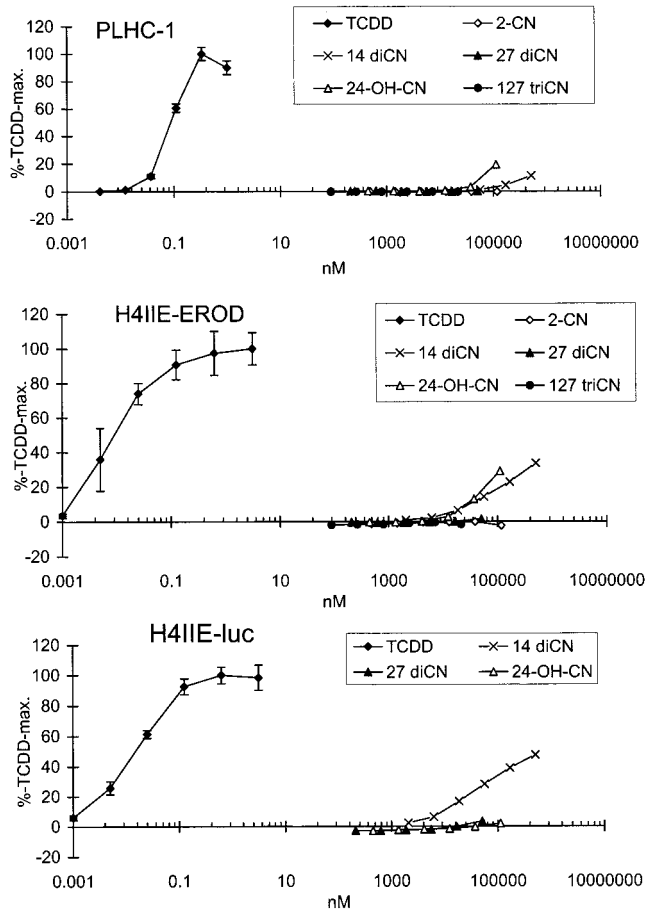

Fig. 2. Response of PLHC-1 fish (*Poeciliopsis lucida*) hepatoma cell bioassay and H4IIE rat hepatoma cell ethoxyresorufin *O*-deethylase (EROD) assay and luciferase (luc) assay to mono-, di-, and trichlorinated naphthalenes and a 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) standard. Doses were expressed as pmol PCN/ml media (nM) present in the test well. Responses expressed as a percentage of the maximum response observed for the TCDD standard (%-TCDD-max)

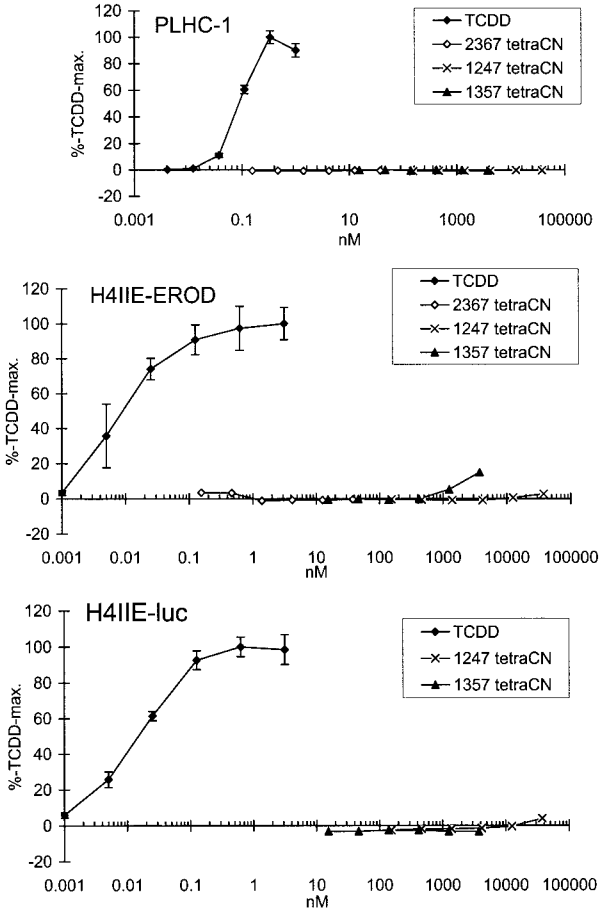

Fig. 3. Response of PLHC-1 (*Poeciliopsis lucida*) fish hepatoma cell bioassay and H4IIE rat hepatoma cell EROD assay and luc assay to tetrachlorinated naphthalenes and a TCDD standard. Doses expressed as pmol PCN/ml media (nM) present in the test well. Responses expressed as a percentage of the maximum response observed for the TCDD standard (%-TCDD-max)

There was only one compound (1,4-diCN) for which deviation from parallelism yielded more than one order of magnitude of variation in the REP estimate over the range of 20–80%-TCDD-max (Table 1). All other REP estimates varied by less than an order of magnitude over the range from 20–80%-TCDD-max (Table 1). The precision of most estimates derived in this study should be sufficient for most applications for which REPs are used. It should be noted, however, that uncertainty in the REP estimates will contribute uncertainty to toxic or TCDD equivalents (TEQ) estimates calculated using these values. The magnitude of uncertainty in TEQ estimates must be considered in mass balance analyses, which compare TEQs based on an additive model and analyte concentrations to bioassay-derived TCDD equivalents (TCDD-EQ) in an attempt to determine whether the known composition of the sample accounts for the activity observed and/or whether nonadditive interactions may be occurring (Giesy *et al.* 1997; Villeneuve *et al.* 1999).

REP estimates derived in this study were similar to those reported previously. H4IIE-EROD-based estimates were consistently within one order of magnitude of the estimates reported by Hanberg *et al.* (1990, 1991) (Table 2). The same was true for H4IIE-luc-based estimates reported in this study versus those reported by Blankenship *et al.* (1999, 2000) (Table 2). Thus, there is evidence to suggest that the assay-specific REPs reported may serve as a reasonable basis for the formulation of consensus values that may be applied in risk assessment until a greater database of *in vivo* relative potencies are available for PCNs.

REPs for penta-, hexa-, and heptaCN congeners range from approximately 10^{-3} to 10^{-6} (Table 2). This is similar to REPs or toxic equivalency factors (TEFs) reported for some PCB congeners (Van den Berg *et al.* 1998). Thus, PCNs may contribute significantly to total TEQs associated with environmental samples from some locations. Based on application of previously reported PCN REPs, PCNs accounted for approximately 1% to 15% of the total TEQs in pike muscle and Guillemot eggs collected as part of a Swedish survey (Järnberg *et al.* 1993; Blankenship *et al.* 2000). In fishes from the Detroit River, PCNs accounted for up to 50% of the total TEQs (Kannan *et al.* 2000). A study of sediments and biota collected near the site of a former chlor-alkali plant also found that PCNs accounted for over 50% of the total TEQs associated with the samples (Kannan *et al.* 1998). As REPs for additional active

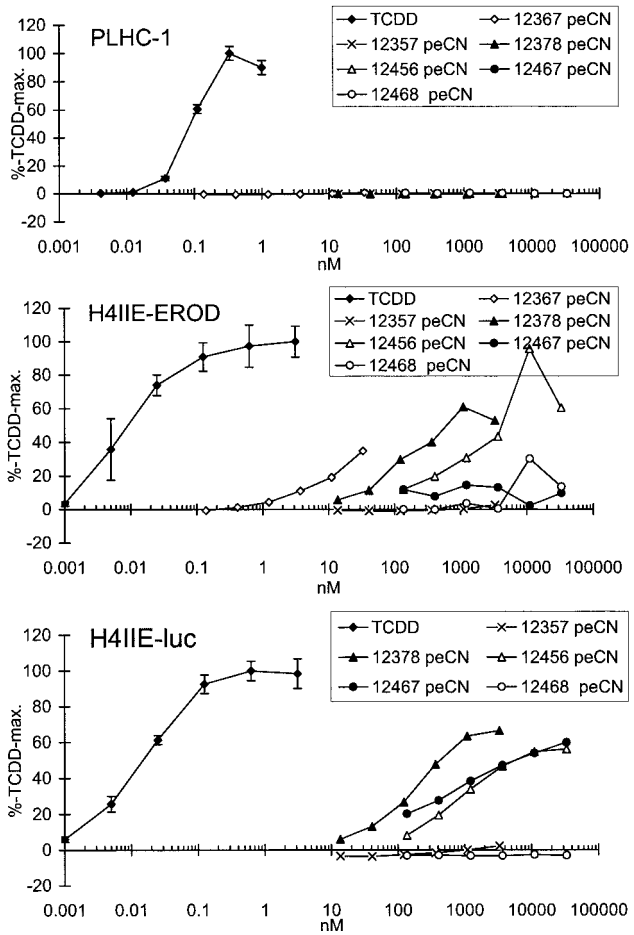

Fig. 4. Response of PLHC-1 (*Poeciliopsis lucida*) fish hepatoma cell bioassay and H4IIE rat hepatoma cell EROD assay and luc assay to pentachlorinated naphthalenes and a TCDD standard. Doses expressed as pmol PCN/ml media (nM) present in the test well. Responses expressed as a percentage of the maximum response observed for the TCDD standard (%-TCDD-max)

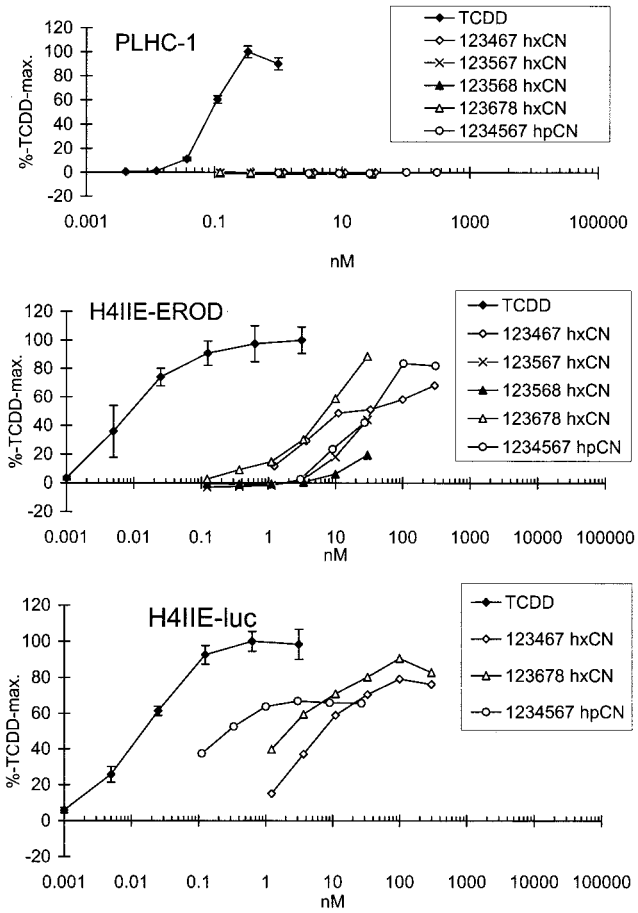

Fig. 5. Response of PLHC-1 (*Poeciliopsis lucida*) fish hepatoma cell bioassay and H4IIE rat hepatoma cell EROD assay and luc assay to hexa- and hepta-chlorinated naphthalenes and a TCDD standard. Doses expressed as pmol PCN/ml media (nM) present in the test well. Responses expressed as a percentage of the maximum response observed for the TCDD standard (%-TCDD-max)

PCN congeners become available, this contribution may increase even further. Thus, PCNs may be an important consideration when characterizing the overall dioxin-like potency of environmental samples containing complex mixtures of halogenated aromatic hydrocarbons.

The results of this study support the concept that the dioxin-like activities of individual PCN congeners are related chemical structure in terms of both the degree and the position of chlorine substitution. As observed in previous studies, potency generally increased with increasing chlorine substitution. Mono-, di-, tri-, and tetra-chlorinated congeners were relatively inactive. Penta-chlorinated congeners typically had REPs around 10^{-4} to 10^{-7}, whereas the hexa- and hepta-chlorinated congeners tested had REPs around 10^{-3} to 10^{-4} (Table 1). Among the penta-chlorinated congeners, the approximate rank order of potency was 1,2,3,6,7 > 1,2,3,7,8 > 1,2,4,5,6 > 1,2,3,5,7 ≈ 1,2,4,6,7 ≈ 1,2,4,6,8. Among the hexa-chlorinated congeners, the approximate rank order was 1,2,3,6,7,8 > 1,2,3,4,6,7 > 1,2,3,5,6,7 > 1,2,3,5,6,8. In both cases, the presence of a meta-substituted ring appears to be linked to decreased potency. The disparity between H4IIE-luc and H4IIE-EROD responses for 1,2,4,6,7-pentaCN suggests that structure may affect the mechanism through which PCNs elicit a dioxin-like response. The patterns of substitution associated with greater AhR-mediated potency may provide the steric bulk needed to fit into the roughly 10 × 3 Å recognition site on the AhR (Poland and Knutson 1982). Alternatively, the substitution patterns may be associated with polarization of the carbon-halogen atoms, which may be important for receptor binding (Poland and Knutson 1982). Molecular modeling of the steric and molecular orbital parameters associated with various PCN congeners could help elucidate the relationships between PCN structure and dioxin-like potency.

Fish vs. Mammalian Assays

The PLHC-1 fish hepatoma cell bioassay was consistently less responsive to individual PCNs than the H4IIE rat hepatoma cell bioassays. It was also approximately 10-fold less sensitive to TCDD. In the H4IIE assays, TCDD concentrations around 0.001 nM were able to elicit a significant response (Figures

Table 2. Comparison of relative potencies reported for individual PCN congeners. Expressed relative to a 2,3,7,8-TCDD standard. Values reported were calculated as a ratio of EC50s ($EC50_{TCDD}/EC50_{PCN}$)

PCN Congener	H4IIE-EROD[a]	H4IIE-EROD[b]	H4IIE-luc[a]	H4IIE-luc[c]
1,4-diCN	3.1×10^{-9}*	—	3.5×10^{-8}	2.0×10^{-7}*
2,4-diCN	2.0×10^{-8}	—	—	—
1,2,3,6,7-peCN	7.6×10^{-5}	—	$<6.4 \times 10^{-4}$	1.7×10^{-4}
1,2,3,7,8-peCN	2.2×10^{-5}	—	4.6×10^{-5}	—
1,2,4,5,6-peCN	1.6×10^{-6}	—	3.5×10^{-6}	—
1,2,3,4,6,7-hxCN	6.3×10^{-4}	—	2.6×10^{-3}	3.9×10^{-3}
1,2,3,5,6,7-hxCN	2.9×10^{-4}	2.0×10^{-3}	—	1.0×10^{-3}
1,2,3,5,6,8-hxCN	—	2.0×10^{-3}	—	1.5×10^{-4}
1,2,3,6,7,8-hxCN	2.1×10^{-3}	—	9.9×10^{-3}	5.9×10^{-4}
1,2,4,5,6,8-hxCN	—	7.0×10^{-6}*	—	—
1,2,3,4,5,6-hxCN	—	2.0×10^{-3}	—	—
1,2,3,4,5,7-hxCN	—	2.0×10^{-5}	—	—
1,2,3,5,7,8-hxCN	—	2.0×10^{-3}	—	—
1,2,3,4,5,6,7-hpCN	4.6×10^{-4}	3.0×10^{-3}	6.9×10^{-4}	1.0×10^{-3}

[a] This study. See Table 1 for uncertainties associated with estimates.
[b] Hanberg *et al.* (1990, 1991), Falandysz (1998).
[c] Blankenship *et al.* (1999).
* High uncertainty.

2–5). In the PLHC-1 assay, TCDD concentrations greater than 0.01 nM were required to elicit a significant response. These results suggest that the PLHC-1 fish hepatoma cell bioassay was approximately an order of magnitude less sensitive to TCDD equivalents than the analogous H4IIE assays. Due to the difference in sensitivity to the TCDD standard, PLHC-1-based REP estimates (expressed as $<$ X) were consistently greater than corresponding H4IIE-based REPs. Greater concentrations of PCN standards were not available to be tested in the PLHC-1 assay. As a result, this study could not resolve any differences between PLHC-1-based REP estimates and H4IIE-based REP estimates for individual PCNs (Table 1). Thus, there was not sufficient evidence to support a hypothesis that REPs and/or structure-activity relationships for individual PCNs were different between fish or fish cells and mammals or mammalian cells, despite the differences in responsiveness observed.

Lower sensitivity in the PLHC-1 assay did not appear to be due to the endpoint measured. The PLHC-1 and H4IIE-EROD assays both measure CYP1A1 catalytic activity as an endpoint. Furthermore, both assays were performed using the same protocol. This suggests that some difference in the biological properties of the cells, rather than a difference in the assay protocol or endpoint was responsible for the sensitivity difference. Cell line–specific differences in membrane permeability, AhR levels or structure, AhR nuclear translocator (ARNT) protein levels, and/or other transcription factors and co-factors may all affect the relative sensitivity of the PLHC-1 and H4IIE bioassays. Although PLHC-1 cells were shown to possess cytosolic AhR (Hahn *et al.* 1993, 1994), the concentrations of AhR present in the cells may be different from that in H4IIE. Furthermore, heterogeneity in the apparent molecular mass of AhR among species suggests that structural differences in the AhR protein could also contribute to taxa-specific differences in sensitivity to TCDD and other dioxin-like compounds (Hahn *et al.* 1994). At this time, it is unclear which factors were responsible for the difference in sensitivity observed.

Conclusions

Among the individual PCNs tested, penta-, hexa-, and heptachlorinated congeners were the most potent. In general, they were three to six orders of magnitude less potent than TCDD. This is rather similar to the relative potency of many PCB congeners (Van den Berg *et al.* 1998). The lower sensitivity of the PLHC-1 assay precluded the ability to resolve finite PLHC-1-based REP estimates for individual PCNs. Thus, the results of this study neither support nor reject the hypothesis that REPs for PCNs may vary among species. Additional studies are needed to address the question of potential taxa-specific differences in relative potency of PCNs. The congeners tested in this study have been identified in environmental samples, including fish and sediment (Kannan *et al.* 2000). Thus, the REPs generated in this study have an immediate and useful role to play in determining the relative contribution of PCNs to the total dioxin-like activity associated with environmental samples.

Acknowledgment. This work was supported by the U.S. Environmental Protection Agency (U.S. EPA) Biology Exploratory Grants Program, grant R85371-01-0; cooperative agreement CR 822983-01-0 between Michigan State University and the U.S. EPA; the National Institute of Environmental Health Sciences Superfund Basic Research Program NIH-ES-04911; and a Michigan State University Distinguished Fellowship to D. L. Villeneuve. We thank Emily Nitsch for her technical assistance; and Dr. Jac Aarts, University of Wageningen, The Netherlands; Cathy Richter, University of Missouri; and Dr. Lawrence Hightower, University of Connecticut, for developing and/or providing the cells used for this study.

References

Asplund L, Jansson B, Sundstrom G (1986) Characterization of a strongly bioaccumulating hexachloronaphthalene. Chemosphere 15:619–628

Asplund L, Grafstrom AK, Haglund P, Jansson B, Järnberg U, Mace D, Strandell M, de Wit C (1990) Analysis of non-*ortho* polychlorinated biphenyls and polychlorinated naphthalenes in Swedish dioxin survey samples. Chemosphere 20:1481–1488

Asplund L, Jakobsson E, Haglund P, Bergman A (1994) 1,2,3,5,6,7-Hexachloronaphthalene and 1,2,3,4,6,7-hexachloronaphthalene—selective retention in rat liver and appearance in wildlife. Chemosphere 28:2075–2086

Bell WB (1954) The production of hyperkeratosis (X disease) by a single administration of chlorinated naphthalenes. J Am Vet Med Assoc 124:289–290

Benefenati E, Mariani G, Fanelli R, Zuccotti S (1991) "De novo" synthesis of PCDD, PCDF, PCB, PCN, and PAH in a pilot incinerator. Chemosphere 22:1045–1052

Blankenship A, Kannan K, Villalobos S, Villeneuve D, Falandysz J, Imagawa T, Jakobsson E, Giesy J (1999) Relative potencies of halowax mixtures and individual polychlorinated naphthalenes (PCNs) to induce Ah receptor-mediated responses in the rat hepatoma H4IIE-Luc cell bioassay. Organohal Comp 42:217–220

Blankenship A, Kannan K, Villalobos S, Villeneuve D, Falandysz J, Imagawa T, Jakobsson E, Giesy J (2000) Relative potencies of halowax mixtures and individual polychlorinated naphthalenes (PCNs) to induce Ah receptor–mediated responses in the rat hepatoma H4IIE-Luc cell bioassay. Environ Sci Technol (in press)

Brinkman UA, Reymer HGM (1976) Polychlorinated naphthalenes. J Chromatogr 127:203–243

Campbell MA, Bandiera S, Robertson L, Parkinson A, Safe S (1981) Octachloronaphthalene induction of hepatic microsomal aryl hydrocarbon hydroxylase activity in the immature male rat. Toxicology 22:123–132

Campbell MA, Bandiera S, Robertson L, Parkinson A, Safe S (1983) Hepta-, hexa-, tetra-, and dichloronaphthalene congeners as inducers of hepatic microsomal drug-metabolizing enzymes. Toxicology 26:193–205

Crookes MJ, Howe PD (1993) Environmental hazard assessment: halogenated naphthalenes. Report TSD/13, Department of the Environment, London

Dorr G, Hippelein M, Hutzinger O (1996) Baseline contamination assessment for a new resource recovery facility in Germany. Part V: Analysis and seasonal/regional variability of ambient air concentrations of polychlorinated naphthalenes (PCNs). Chemosphere 33:1563–1568

Engwall M, Brunstrom B, Jakobsson E (1994) Ethoxyresorufin *O*-deethylase (EROD) and aryl hydrocarbon hydroxylase (AHH)-inducing potency and lethality of chlorinated naphthalenes in chicken (*Gallus domesticus*) and eider duck (*Somateria mollissima*) embryos. Arch Toxicol 68:37–42

Espadaler I, Eljarrat E, Caixach J, Rivera J, Marti I, Ventura F (1997) Assessment of polychlorinated naphthalenes in aquifer samples for drinking water purposes. Rapid Commun Mass Spectrom 11:410–414

Falandysz J (1998) Polychlorinated naphthalenes—an environmental update. Environ Pollut 101:77–90

Falandysz J, Rappe C (1996) Spatial distribution in plankton and bioaccumulation features of polychlorinated naphthalenes in a pelagic food chain in southern part of the Baltic proper. Environ Sci Technol 30:3362–3370

Falandysz J, Strandberg L, Bergqvist PA, Kulp SE, Strandberg B, Rappe C (1996) Polychlorinated naphthalenes in sediments and biota from the Gdañsk Basin, Baltic Sea. Environ Sci Technol 30:3266–32274

Falandysz J, Kawano M, Morita N, Matsuda M, Kannan K, Giesy JP, Wakimoto T (2000) Composition of chloronaphthalene isomers and congeners in technical PCN formulations of the Halowax series. J Environ Sci Health A35:281–298

Finney DJ (1978) Statistical method in biological assay. Charles Griffin and Company Ltd., London

Furlong ET, Carter DS, Hites RA (1988) Organic contaminants in sediments from the Trenton channel of the Detroit River, Michigan. J Great Lakes Res 14:489–501

Giesy JP, Jude DJ, Tillitt DE, Gale RW, Meadows JC, Zajieck JL, Peterman PH, Verbrugge DA, Sanderson JT, Schwartz TR, Tuchman ML (1997) Polychlorinated dibenzo-*p*-dioxins, dibenzofurans, biphenyls and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalents in fishes from Saginaw Bay, Michigan. Environ Toxicol Chem 16:713–724

Hahn ME, Lamb TM, Schultz ME, Smolowitz RM, Stegeman JJ (1993) Cytochrome P4501A induction and inhibition by 3,3,4,4-tetrachlorobiphenyl in an Ah receptor–containing fish hepatoma cell line (PLHC-1). Aquat Toxicol 26:185–208

Hahn ME, Poland A, Glover E, Stegeman JJ (1994) Photoaffinity labeling of the Ah receptor: phylogenetic survey of diverse vertebrate and invertebrate species. Arch Biochem Biophys 310:218–228

Hahn ME, Woodward BL, Stegeman JJ, Kennedy SW (1996) Rapid assessment of induced cytochrome P4501A (CYP1A) protein and catalytic activity in fish hepatoma cells grown in multi-well plates. Environ Toxicol Chem 15:582–591

Hanberg A, Waern F, Asplund L, Haglund E, Safe S (1990) Swedish dioxin survey—determination of 2,3,7,8-TCDD toxic equivalent factors for some polychlorinated-biphenyls and naphthalenes using biological tests. Chemosphere 20:1161–1164

Hanberg A, Stahlberg M, Georgellis A, de Wit C, Ahlborg UG (1991) Swedish dioxin survey: evaluation of the H-4-II E bioassay for screening environmental samples for dioxin-like enzyme induction. Pharmacol Toxicol 69:442–449

Harner T, Bidleman TF (1997) Polychlorinated naphthalenes in urban air. Atmos Environ 31:4009–4016

Hightower LE, Renfro JL (1988) Recent applications of fish cell culture to biomedical research. J Exp Zool 248:290–302

Holm G, Norrgren L, Andersson T, Thuren A (1993) Effects of exposure to food contaminated with PBDE, PCN, or PCB on reproduction, liver morphology and cytochrome P450 activity in the three-spined stickleback, *Gasterosteus aculeatus*. Aquat Toxicol 27:33–50

Imagawa T, Yamashita N (1996) Estimation of emission sources of polychlorinated naphthalenes using finger-print method for isomer composition. J Environ Chem 6:495–501 (in Japanese)

Jakobsson E, Lonnberg C, Eriksson L (1994) Synthesis and crystallography of two hexachloronaphthalenes. Part II 1,2,4,5,6,8-hexachloronaphthalene and 1,2,4,5,7,8-hexachloronaphthalene. Acta Chem Scand 48:891–898

Järnberg U, Asplund L, de Wit C, Grafstrom AK, Haglund P, Jansson B, Lexen K, Strandell M, Olsson M, Jonsson B (1993) Polychlorinated-biphenyls and polychlorinated naphthalenes in swedish sediment and biota—levels, patterns, and time trends. Environ Sci Technol 27:1364–1374

Kannan K, Imagawa T, Blankenship A, Giesy JP (1998) Isomer specific analysis and toxic evaluation of polychlorinated naphthalenes in soil, sediment, and biota near the site of a former chloralkali plant. Environ Sci Technol 32:2507–2514

Kannan K, Yamashita N, Imagawa T, deCoen W, Khim JS, Day RM, Summer CL, Giesy JP (2000) Polychlorinated naphthalenes and polychlorinated biphenyls in fishes from Michigan waters including the Great Lakes. Environ Sci Technol 34:566–572

Kennedy SW, Jones SP (1994) Simultaneous measurement of cytochrome P4501A catalytic activity and total protein concentration with a fluorescence plate reader. Anal Biochem 222:217–223

Kleinfeld M, Messite J, Swenciki R (1972) Clinical effects of chlorinated naphthalene exposure. J Occup Med 14:377–379

Kover F (1975) Environmental hazard assessment report: chlorinated naphthalenes. EPA 560/8-75-001, US Environmental Protection Agency, Washington, DC

Mantyla E, Ahotupa M (1993) Polychlorinated biphenyls and naph-

thalenes: long-lasting induction of oxidative stress in the rat. Chemosphere 27:383–390

Miltsov S, Karavan V, Nikiforov V, Tribulovich V, Varentsov V (1999) Selective reduction of polychloronaphthalenes with zinc. Zh Org Khim 5:724–727 (in Russian)

Nikiforov V, Wightman R (1997) Reaction of nitroarenes and arsenesulfonylchlorides with hexachlorocyclopentadienes. Chimia 7:452

Norrgren L, Andersson T, Bjork M (1993) Liver morphology and cytochrome P450 activity in fry of rainbow trout after microinjection of lipid-soluble xenobiotics in the yolk-sac embryos. Aquat Toxicol 26:307–316

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

Pudelkiewicz WJ, Boucher RV, Cullenbach EW, Miller RC (1959) Some physiological responses of New Hampshire chickens to a mixture of penta- and hexachloronaphthalenes. Poultr Sci 38:474

Putzrath RM (1997) Estimating relative potency for receptor-mediated toxicity: reevaluating the toxicity equivalence factor (TEF) model. Regulat Toxicol Pharmacol 25:68–78

Safe S (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. Crit Rev Toxicol 21: 51–88

Sanderson JT, Giesy JP (1998) Wildlife toxicology, functional response assays. *In* Meyers RA (ed) Encyclopedia of environmental analysis and remediation. John Wiley and Sons, New York, NY, pp 5272–5297

Sanderson JT, Aarts JMMJG, Brouwer A, Froese KL, Denison MS, Giesy JP (1996) Comparison of Ah receptor–mediated luciferase and ethoxyresorufin-*O*-deethylase induction in H4IIE cells: implications for their use as bioanalytical tools for the detection of polyhalogenated aromatic hydrocarbons. Toxicol Appl Pharmacol 137:316–325

Van den Berg M, Birnbaum L, Bosveld BTC, Brunström B, Cook P, Feeley M, Giesy JP, Hanberg A, Hasegawa R, Kennedy SW, Kubiak T, Larsen JC, van Leeuwen FXR, Djien Liem AK, Nolt C, Peterson RE, Poellinger L, Safe S, Schrenk D, Tillitt D, Tysklind M, Younes M, Waern F, Zacherewski T (1998) Toxic equivalency factors (TEFs) for PCBs, PCDDs, and PCDFs for humans and wildlife. Environ Health Perspect 106:775–792

Villalobos SA, Papoulias DM, Meadows J, Blankenship AL, Pastva SD, Kannan K, Hinton DE, Tillitt DE, Giesy JP (2000) Toxic responses of medaka (d-rR strain) to polychlorinated naphthalene mixtures after embryonic exposure by *in ovo* nanoinjection: a partial life cycle assessment. Environ Toxicol Chem 19:432–440

Villeneuve DL, Blankenship AL, Giesy JP (2000) Derivation and application of relative potency estimates based on *in vitro* bioassay results. Environ Toxicol Chem (in press)

Villeneuve DL, Richter CA, Blankenship AL, Giesy JP (1999) Rainbow trout cell bioassay-derived relative potencies for halogenated aromatic hydrocarbons: comparison and sensitivity analysis. Environ Toxicol Chem 18:879–888