Toxicant Equivalent Factors for selected PCBs measured by a fish, RTL-W1 and a rat, H4IIE, liver cell line

J.H. Clemons, D.G. Dixon and N.C. Bols
Dept. of Biology, University of Waterloo, Waterloo ON N2L 3G1

1. Introduction

Toxic equivalency factors (TEFs) are a potential tool for assessing the environmental risk of polychlorinated biphenyls (PCBs)[1]. PCBs belong to the halogenated aromatic group of environmental contaminants of which 209 exist[1]. Like polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs), many PCBs exert their toxic effects through the Ah (Aromatic hydrocarbon) receptor. This common mechanism supports the TEF approach, which has been used to assess the risk of PCDDs and PCDFs[1]. A TEF is the fractional potency of a compound relative to a standard toxin, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). The TEFs are used to convert analytical data for mixtures of PCDD, PCDF and PCBs into TCDD toxic equivalents (TEQs). Concentrations of individual compounds in a complex mixture, such as in environmental samples, are multiplied by their TEFs and the results summed to give a single TEQ.

A variety of Ah-mediated endpoints have been measured *in vivo* and *in vitro* to derive TEFs for mammals. Some *in vivo* responses have been tumour-promoting activity[2], immunotoxicity and teratogenicity[3]. The most common *in vitro* response has been the increased expression of the CYP1A1 gene. The result is enhanced levels or induction of the cytochrome P450 protein P4501A1[4] which is assayed as aryl hydrocarbon hydroxylase (AHH) or 7-ethoxyresorufin O-deethylase (EROD). This was first done, and continues to be done, with the rat hepatoma cell line, H4IIE[5]. The *in vitro* method provides a rapid, inexpensive way of screening large numbers of compounds and fulfills a societal desire to reduce the use of animals in research.

Among the PCBs, the non-ortho coplanar PCBs are thought to exert their actions through the Ah receptor[1]. The non-ortho coplanar PCBs are substituted in both para, at least two meta, and no ortho positions. Their most toxic members in mammals are 3,3',4,4'-tetraCB (77), 3,4,4',5-tetraCB (81) 3,3',4,4',5-pentaCB (126) and 3,3',4,4',5,5'-hexaCB (169). These congeners competitively bind with relatively high affinity to the Ah receptor and induce CYP1A1 gene expression.

Although TEFs are being used for aquatic environmental risk assessment[6][7][8][9], questions about the applicability of TEFs derived in mammalian systems to this purpose have resulted in research on the derivation of TEFs in piscine systems. Early life stage mortality in rainbow trout[10] and lake trout[11], embryotoxicity in medaka[12], and AHH or EROD induction in rainbow trout[12][13] are *in vivo* responses that have been used to develop piscine TEFs. The *in vitro* response has been the induction of EROD activity in cell lines from topminnow hepatoma (PLHC-1)[14][15]and from rainbow trout liver[16]. For some congeners of dioxins and furans, TEFs derived with the trout RTL-W1 were significantly higher that those derived with the rat H4IIE[16].

A limited amount of research intimates that the derivation of TEFs for PCBs could be more dependent on the species than is the case for other polyaromatic halogenated hydrocarbons[17].In this report we have investigated this directly by comparing the ability of three non-ortho coplanar PCBs to induce EROD activity in the rat cell line, H4IIE, and in the trout cell line, RTL-W1, and to use this information to derive TEFs.

2. Methods

Cells were maintained as per Lee et al.[18] for RTL-W1 and as per Clemons et al.[17] for H4IIE. EROD activity was measured in both RTL-W1 and H4IIE cell lines using a similar method as Kennedy et al.[19] For 2,3,7,8-

tetrachlorodibenzo-p-dioxin and 3 PCBs: 3,3',4,4'-tetrachlorobiphenyl (77), 3,4,4',5-tetrachlorobiphenyl (81), and 3,3',4,4',5-pentachlorobiphenyl (126). 48-well plates were seeded with the cells at high density and exposed to TCDD or the PCB congener at a 0.5% DMSO carrier solvent concentration. After a fixed incubation period the cells are assayed for EROD activity. All reagents for H4IIE are kept at 37°C while RTL-W1 assays were run at room temperature (approx. 22°C). Protein analysis follows as per Lorenzen and Kennedy[20]. EROD activity is expressed in pmol resorufin·mg protein^{-1}·min^{-1} (pmol·mgP^{-1}·min^{-1}). To determine whether time of incubation influenced results for PCB 77 with either cell line, time trials were run with TCDD and PCB 77 over time periods of 6, 12, 24, 48, 36, 48, 60, and 72 h. EROD assays were performed on RTL-W1 and H4IIE concurrently as described in Clemons et al.[21].

3. Results

3.1 EROD induction and TEF derivation

With standard exposure times of 24 to 48 h, the non-ortho-coplanar PCBs inducer EROD activity and yielded sigmoidal dose-response curves in H4IIE and RTL-W1 (Fig 1A&B). As a percentage of the maximum activity achieved with TCDD, the maximum activity for the PCBs varied and was in the 60-90% range for H4IIE and in the 50-75% range for RTL-W1. The shapes of the dose-response curves were similar for the three PCBs and TCDD in

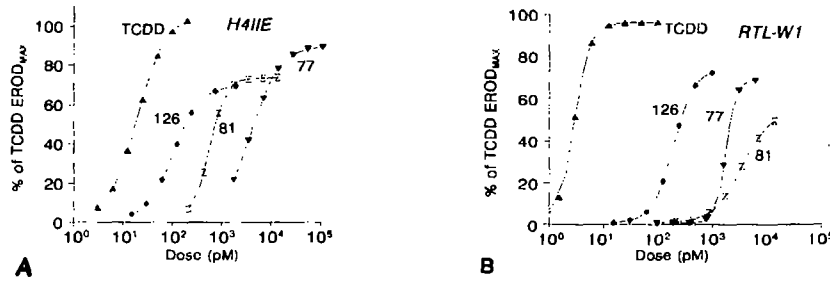

Figure 1. Representative dose-response curves for H4IIE (A) and RTL-W1 (B) illustrating the potency of the non-ortho-coplanar PCB congeners. EROD activity is expressed as a percent of the TCDD maximum EROD activity (TCDD EROD$_{max}$). H4IIE TCDD EROD$_{max}$ ranges between 250-600 pmol·mgP^{-1}·min^{-1}. while RTL-W1 TCDD ERODmax is between 80 and 150 pmol·mgP^{-1}·min^{-1}.

both H4IIE and RTL-W1 (Fig 2A&B). The PCBs were ranked for their relative induction potency by calculating their EC50s (Table 1) and comparing TEFs (Table 2). For both cell lines PCB 126 was the most potent: PCB77, the least.

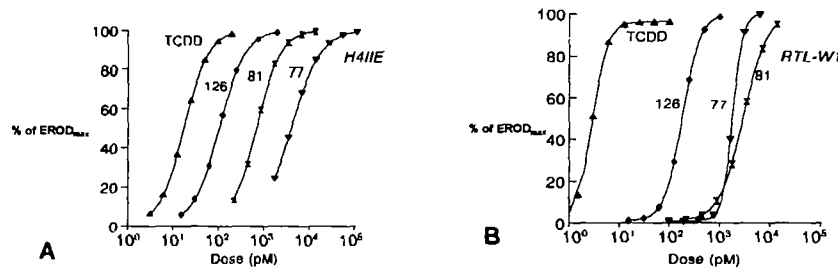

Figure 2. Representative dose-response curves for H4IIE (A) and RTL-W1 (B) illustrating the potency of the non-ortho coplanar PCB congeners. EROD activity is expressed as a percent of the maximum EROD activity for each individual congener (EROD$_{max}$). The shape of the curves are similar for all the congeners with both H4IIE and RTL-W1.

3.2. Effect of PCB 77 exposure time on EROD induction and TEF derivation

Exposure of H4IIE and RTL-W1 to either TCDD or PCB 77 was varied from 6 to 72 h and the effect on EROD induction was examined by comparing two parameters of the dose-response curves: the maximum activity and the EC50. The maximum EROD activity was achieved at the 36 h time point after which the maximum declined slightly. The EC50 values for the TCDD dose-response curve changed little over the 72 h time period for both H4IIE (Fig. 3) and RTL-W1. In contrast to TCDD, the dose-response curves or PCB 77 in H4IIE changed as the exposure time was extended. Five of the seven doses were at maximum at 6 h, but only three of the highest doses were at maximum at 72 h. The EC50 values for PCB 77 in H4IIE increased significantly measured at 6 h. In contrast to H4IIE, the dose-response curves for PCB 77 in RTL-W1 changed insignificantly over 72 hours. In preliminary experiments with the other PCBs, little change was observed in the dose response curve as the exposure times were extended in H4IIE and in RTL-W1.

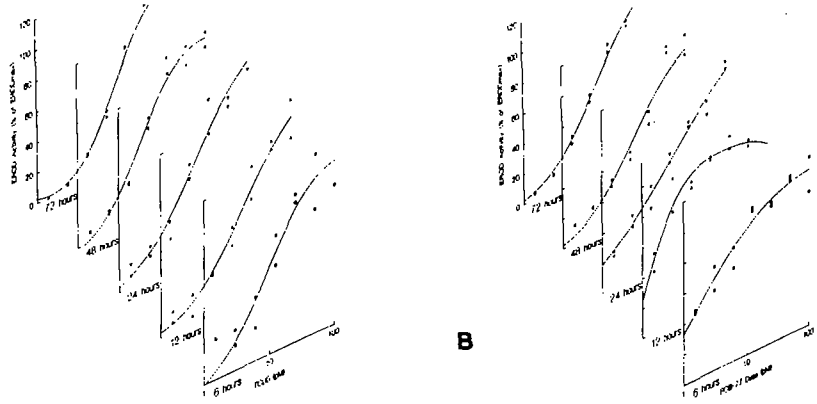

Figure 3. Representative dose-response curves illustrating the EROD activity as a percent of maximum activity for each congener at the specified time period (% of EROD $_{max}$) for H4IIE. TCDD (A) and PCB 77 (B) were exposed to the cells for 5 different time periods (6, 12, 24, 48, 72 h). The shape of the curves are similar for TCDD but differ significantly with time for PCB 77.

Table 1. Effective concentrations at 50% response (EC50s) values for selected polychlorinated biphenyls derived for RTL-W1 and H4IIE cell lines. Average EC50s are listed along with their standard deviations and number of assays from which the average was obtained.

Congener	*RTL-W1*		*H4IIE*	
	n	EC50 (S.D.)	n	TEF (S.D.)
2,3,7,8-tetraCDD (TCDD)		0.98 (0.42)		1.38 (0.62)
non-ortho PCBs				
3,3',4,4'-tetraCB (77)	15	317.99 (85.84)	24	1705.04 (763.70)
3,4,4',5-tetraCB (81)	13	196.83 (42.47)	10	256.94 (196.24)
3,3',4,4',5-pentaCB (126)	3	36.64 (2.77)	20	16.05 (6.625)

4. Discussion

The potencies of PCBs 77 and 126 for inducing EROD activity in H4IIE relative to 2,3,7,8-TCDD were similar to an earlier study by Sawyer and Safe[22] on EROD induction in the same rat hepatoma cell line and with similar PCB

Table 2. Toxic equivalent factors (TEFs) for selected polychlorinated biphenyls derived for the RTL-W1 and H4IIE cell lines. Average TEFs are listed along with their standard deviations and number of assays from which the average was obtained.

Congener	*RTL-W1*		*H4IIE*	
	n	TEF (S.D.)	n	TEF (S.D.)
2,3,7,8-tetraCDD (TCDD)		1.0		1.0
non-ortho PCBs				
3,3',4,4'-tetraCB (77)	15	0.0034 (0.0018)	24	0.00079 (0.00030)
3,4,4',5-tetraCB (81)	13	0.0064 (0.0018)	10	0.0072 (0.0028)
3,3',4,4',5-pentaCB (126)	3	0.023 (0.0046)	20	0.10 (0.045)

exposure times, but a third PCB (81) was quite different. The TEFs for PCBs 77 and 126 were 0.0091 and 0.323 respectively in the earlier study and 0.00079 and 0.100 in the current study. On the other hand, PCB 81 had a TEF of 0.0072, which was approximately 170 fold higher than in the previous study. This TEF puts PCB 81 in the same potency range as the other non-ortho-coplanar PCBs, but is higher than the I-TEF for this congener, which is listed sometimes as 0.001[8)]

Generally, little data is available for this PCB[23)] Although PCB 81 is worthy of further examination, the reassuring point is that the TEFs derived in the current study for PCBs 77 and 126 fall within the potency ranges for these congeners, which were defined by the results from in vivo and in vitro assays[1)23)], and are very similar to TEFs derived over a decade earlier by Sawyer and Safe[22)] who grew the H4IIE in a slightly different medium and used different instrumentation for measuring EROD activity.

When PCB exposure time was varied, only the TEF for PCB 77 changed with exposure time and only in H4IIE. The highest TEF was observed after a 12 h exposure, and the TEF declined as exposure to PCB 77 was extended up to 72 h which agrees with a previous study by Yu et al.[24)]. One possible cause of this problem is differential metabolism of this congener among the PCBs and between rats and rainbow trout. Of the toxic non-ortho- coplanar and mono-ortho-coplanar congeners, PCB 77 is the one most readily metabolized in mammals[1)]. Perhaps, PCB 77 metabolism is less active or absent in fish. Metabolism could make H4IIE cells less sensitive to induction by reducing the concentration of the inducer or producing metabolites that interfere with induction[25)] or with enzyme activity. This raises the question of which H4IIE TEF to choose for PCB 77. The TEF derived under the optimal induction conditions would seem most appropriate, in which case the H4IIE TEF for PCB 77 is more in the range of RTL-W1 at 0.00485±0.0021.

In comparison to the TEFs derived in this study with the rat H4IIE, the TEFs derived with the rainbow trout liver cell line (RTL-W1) were lower for PCBs 81 and 126 and possibly lower for PCB 77. The greatest difference was with PCB 126, which was approximately 4 fold lower in RTL-W1. For PCB 77, the RTL-W1 TEF was lower than the TEF derived from the optimal exposure time (12 h) to H4IIE but not to those TEFs derived from 48-72h exposures. If only the short exposure TEF is considered, the higher TEFs with H4IIE cells suggests that these cells are more sensitive than the RTL-W1 to the three non-ortho coplanar PCBs. This is in contrast to our previous study in which RTL-W1 was more sensitive than H4IIE to 6 dioxins and furans[16)].

The relative ability of the PCBs to induce EROD in RTL-W1 was similar to their activity upon injection into fish. In rainbow trout, PCB 77[12)13)26)27)], 81[12)], and 126[13)26)] induced either AHH, EROD or CYP1A1 protein. PCBs 77 and 126 also have been reported to induce EROD activity in carp[26)]. The TEFs derived with RTL-W1 compare favorably to TEFs derived by rainbow trout embryotoxicity[10)11)] and by AHH induction in rainbow trout[12)13)]. The TEFs derived with EROD induction in RTL-W1, AHH induction in whole rainbow trout, and toxicity in trout embryos were respectively 0.0034, 0.002, and 0.002 for PCB 77. Similarly for PCB 81 the three techniques yield TEFs of 0.0064,

0.004 and 0.006 for PCB 81 and 0.023, 0.005, and 0.005 for PCB 126. These values are surprisingly close to one another when the range of TEF values derived by different endpoints in mammals are considered[1].

A trend that emerges from the above studies is that rainbow trout are less responsive to the non-ortho coplanar PCBs than mammals, and mammalian derived TEFs probably overestimate the potency of these compounds to this species. This problem is most extreme if the I-TEFs are used. I-TEFs are approximately 10 fold higher than the TEFs derived with rainbow trout. On the other hand, H4IIE TEFs were relatively close to the RTL-W1 TEFs, with the exception of PCB 81. The ability of RTL-W1 to detect the active PCBs in fish should make this cell line a useful complement to H4IIE in screening environmental samples for halogenated aromatic contaminants. The greater sensitivity of RTL-W1 for dioxins and furans complements the greater sensitivity of H4IIE for PCBs.

5. References

1) Safe S.H. (1994): Polychlorinated biphenyls (PCBs): Environmental Impact, Biochemical and Toxic Responses, and Implications for Risk Assessment. Crit. Rev. Toxicol. 24, 87-149.
2) Flodström S., and U.G. Ahlborg (1992): Relative Liver Tumour Promoting Activity of Some Polychlorinated Dibenzo-*p*-Dioxin-, Dibenzofuran- and Biphenyl-Congeners in Female Rats. Chemosphere, 25, 169-173.
3) Mayura C., C.B. Spainhour, L. Howie, S. Safe, and T.D. Phillips (1993): Teratogenicity and Immunotoxicity of 3,3',4,4',5-Pentachlorobiphenyl in C57BL/6 Mice. Toxicol. 77, 123-129.
4) Bosveld B.A.T.C, M. Van den Berg, and R.M.C. Theelen (1992): Assessment of the EROD Potency of Eleven 2,3,7,8-Substituted PCDD/Fs and Three Coplanar PCBs in the Chick Embryo. Chemosphere, 25, 911-918.
5) Bradlaw J.A., and J.L. Casterline, Jr. (1979): Induction of Enzyme Activity in Cell Culture: A Rapid Screen for Detection of Planar Polychlorinated Organic Compounds. J. Assoc. Off. Anal. Chem. 62, 904-916.
6) Ankley G.T., D.E. Tillitt, J.P. Giesy, P.D. Jones, and D.A. Verbrugge (1991): PCB-Containing Extracts from the Flesh and Eggs of Lake Michigan Chinook Salmon (*Oncorhychus mykiss)* and Possible Implications for Reproduction. Can. J. Fish. Aquat. Sci. 48, 1685-1690.
7) Tillitt D.E., G.T. Ankley, D.A. Verbrugge, J.P. Giesy, J.P. Ludwig, and T.J. Kubiak (1991): H4IIE Rat Hepatoma Cell Bioassay-Derived 2,3,7,8-Tetrachlorodibenzo-p-dioxin Equivalents in Colonial Fish-Eating Waterbirds Eggs from the Great Lakes. Arch. Environ. Contam. Toxicol. 21, 91-101.
8) Williams L.L., J.P. Giesy, N. DeGalan, D.A. Verbrugge, D.E. Tillitt, and G.T. Ankley (1992): Prediction of Concentrations of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin Equivalents from Total Concentrations of Polychlorinated Biphenyls in Fish Fillets. Environ. Sci. Technol. 26, 1151-1159.
9) van den Heuvel M.R., K.R. Munkittrick, G.J. Van Der Kraak, M.E. McMaster, C.B. Portt, M.R. Servos and D.G. Dixon (1994): Survey of Receiving-Water Environmental Impacts Associated with Discharges from Pulp Mills. 4. Bioassay-Derived 2,3,7,8-Tetrachlorodibenzo-p-dioxin Toxic Equivalent Concentration in White Sucker (*Catostomus commersoni*) in Relation to Biochemical Indicators of Impact. Environ. Toxicol. Chem. 13, 1103-1115
10) Walker M.K., R.E. Peterson (1991): Potencies of Polychlorinated Dibenzo-*p*-dioxin, Dibenzofuran, and Biphenyl Congeners, Relative to 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin, for Producing Early Life-Stage Mortality in Rainbow Trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 21, 219-238.
11) Zabel E.W., and R.E. Peterson (1993): 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) Toxicity at Three Stages Lake Trout Egg Development. In: Society of Environmental Toxicology and Chemistry, 14th Annual Meeting. p153.
12) Metcalfe C.D., D.M. Janz, G.E. Harris, and Y. Kipirissis (1993): Toxic Equivalency Factors for Coplanar PCBs in Fish. In: Society of Environmental Toxicology and Chemistry, 14th Annual Meeting. p286.
13) Janz D.M., and C.D. Metcalfe (1991): Relative Induction of Aryl Hydrocarbon Hydroxylase by 2,3,7,8-TCDD and Two Coplanar PCBs in Rainbow Trout (*Oncorhynchus mykiss*). Environ. Toxicol. Chem. 10, 917-923.
14) Tillitt D.E., and S.M. Cantrell (1992): Planar Halogenated Hydrocarbon (PHH) Structure Activity Relationship in a Teleost (PLHC) Cell Line. In: Society of Environmental Toxicology and Chemistry, 13th Annual Meeting.
15) Hahn M.E., T.M. Lamb, M.E. Schultz, R.M. Smolowitz, and J.J. Stegeman (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.

16) Clemons J.H., M.R. van den Heuvel, N.C. Bols, and D.G. Dixon (1994): Comparison of Bioassay Derived Dioxin Equivalents for Polychlorinated Dibenzo-p-dioxins and Dibenzofurans Using the RTL-W1 and H4IIE Cell Lines. Can. J. Fish. Aquat. Sci. 51, 1577-1584.
17) Stegeman J.J, and M.E. Hahn (1994): Biochemistry and Molecular Biology of Monoxygenases: Current Perspectives on Forms, Functions, and Regulation of Cytochrome P450 in Aquatic Species. In: Aquatic Toxicology: Molecular, Biochemical, and Cellular Perspectives. Eds: D.C. Malins and G.K. Ostrander. CRC Press, Inc. 87-206.
18) Lee L.E.J., J.H. Clemons, D.G. Bechtel, S.J. Caldwell, K.B. Han, M. Pasitschniak-Arts, D.D. Mosser, and N.C. Bols (1993): Development and Characterization of a Rainbow Trout Cell Line Expressing Cytochrome P450-Dependent Monooxygenase Activity. Cell Biol. Toxicol. 9, 279-294.
19) Kennedy S.W., A. Lorenzen, C.A. James, and B.T. Collins (1993): Ethoxyresorufin-o-deethylase and Porphyrin Analysis in Chicken Embryo Hepatocyte Cultures With a Fluorescence Multiwell Plate Reader. Anal. Biochem. 211, 102-112.
20) Lorenzen A., and S.W. Kennedy (1993): A fluorescence-Based Protein Assay for Use With a Multiplate Reader. Anal. Biochem. 214, 346-348.
21) Clemons J.H., C.R. Myers, L.E.J. Lee, D.G. Dixon and N.C. Bols (in preparation): Toxicant Equivalent Factors for Selected PCBs Measured by a Fish, RTL-W1 and a Rat, H4IIE, Liver Cell Line. To be submitted to Can. J. Aquat. Sci.
22) Sawyer T., and S. Safe (1982): PCB Isomers and Congeners: Induction of Aryl Hydrocarbon Hydroxylase and Ethoxyresorufin-o-deethylase Enzyme Activity in Rat Hepatoma Cells. Toxicol. letters, 13, 87-94.
23) 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 Equivalent Factors (TEFs). CRC Crit. Rev. Toxicol., 21, 51-88.
24) Yu K., A. Burton, S. Channel, J. Fisher, J. Drerup, and D. Tillitt (1994): Carrier Effects of Dosing the H4IIE Cells With PCB#77 (3,3',4,4'-tetrachlorobiphenyl) in DMSO or Isooctane. . In: Society of Environmental Toxicology and Chemistry, 15th Annual Meeting. p155.
25) Kiyohara C., N. Mohri, T. Hirohta, K. Haraguchi, and Y. Masuda (1990): In Vitro Effects of Methylsulfonyl Polychlorinated Biphenyls and 7,8-Benzoflavone on Aryl Hydrocarbon Hydroxylase Activity in Human Lymphoblastoid Cells. Pharamacol. Toxicol. 66, 273-276.
26) Melancon M.J., and J.J. Lech (1982): Dose-Effect Relationships for Induction of Hepatic Monoxygenase Activity in Rainbow Trout and Carp by Aroclor 1254. Aquat. Toxicol. 4, 51-61.
27) Vodicnik M.J., C.R. Clifford, and J.J. Lech (1981): The Effect of Various Types of Inducing Agents on Hepatic Microsomal Monoxygenase Activity in Rainbow Trout. 59, 364-374.