Cytochrome P4501A induction by highly purified hexachlorobenzene in primary cultures of avian hepatocytes

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Abstract

Hexachlorobenzene (HCB) is a persistent organic pollutant that was primarily produced for use as a fungicide dating back to the 1940s. Worldwide emissions have declined steadily over the past forty years, but HCB is still produced as a by-product of a number of industrial processes and is still detected in remote locations around the globe. Many studies have been conducted to determine the toxic and biochemical effects of HCB, but it has been suggested that reported toxic and biochemical effects initially attributed to HCB exposure may have actually been elicited by contamination of HCB by polychlororinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and dioxin-like polychlorinated biphenyls (PCBs).

This thesis investigates whether highly purified HCB (HCB-P; defined as HCB containing < 0.2 ppb of any PCDD, PCDF, or co-planar PCB congener [the detection limit of current analytical methods]) can induce cytochrome P4501A (CYP1A) in three avian species *in vitro*. Primary cultures of chicken (*Gallus gallus domesticus*), ring-necked pheasant (*Phasianus colchicus*) and Japanese quail (*Corturnix japonica*) embryo hepatocytes were used to compare the potencies of reagent-grade (RG-HCB), HCB-P and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) as inducers of ethoxyresorufin *O*-deethylase (EROD) activity, CYP1A4 messenger ribonucleic acid (mRNA) and CYP1A5 mRNA. The potencies of two mono-*ortho* substituted PCBs, 2,3,3',4,4'-pentachlorobiphenyl (PCB 105) and 2,3',4,4',5-pentachlorobiphenyl (PCB 118) were also assessed in chicken embryo hepatocytes using the same endpoints. All compounds induced EROD activity and up-regulated CYP1A4/5 mRNAs in the hepatocytes of each species. The potency of HCB relative to the potency of TCDD (ReP) was 0.0001, 0.001 and 0.01 in chicken, ring-necked pheasant and Japanese quail embryo hepatocytes, respectively. $EC_{threshold}$ values were suggested to be more appropriate than EC_{50} values because $EC_{threshold}$

values account for differences in maximal EROD and CYP1A4/5 mRNA levels that are observed with HCB exposure in avian embryo hepatocytes more so than EC_{50} values. Differences in species sensitivity to HCB were also assessed, and did not vary as greatly as the listed ReP values. The results presented herein suggest that HCB is capable of inducing effects downstream of activation of the aryl hydrocarbon receptor, and may warrant its inclusion in the World Health Organization's toxic equivalency concept.

Résumé

L'hexachlorobenzène (HCB) est un polluant organique persistant qui a été surtout produit pour servir de fongicide à partir des années 1940. Les émissions à l'échelle mondiale ont diminué régulièrement au cours des quarante dernières années, mais l'hexachlorobenzène demeure un sous-produit d'un certain nombre de procédés industriels et il est toujours détecté dans des régions éloignées du monde entier. De nombreuses études ont été menées en vue de déterminer les effets toxiques et biochimiques de l'hexachlorobenzène, mais on laisse entendre que les effets toxiques et biochimiques signalés qu'on a initialement attribués à l'exposition à l'hexachlorobenzène auraient pu être provoqués, en fait, par la contamination de l'hexachlorobenzène au moyen de polychlorodibenzoparadioxines (PCDD), de polychlorodibenzofuranes (PCDF) et de polychlorobiphényles (PCB; aussi appelés « biphényles polychlorés » ou BPC) de type dioxine.

Cette thèse examine si l'hexachlorobenzène fortement purifié (HCB-P; défini comme étant de l'hexachlorobenzène dont la teneur en PCDD, en PCDF ou en congénère de PCB présentant une structure coplanaire est inférieure à 0,2 ppb [c'est-à-dire la limite de détection de la méthode analytique actuelle]) est susceptible de provoquer les effets de l'induction du cytochrome P4501A (CYP1A) chez trois espèces *in vitro*. Des hépatocytes d'embryons de cultures primaires de poulet (*Gallus gallus domesticus*), de Faisan de Colchide (*Phasianus colchicus*) et de Caille du Japon (*Corturnix japonica*) ont été utilisés pour comparer la puissance de l'hexachlorobenzène (HCB) de qualité réactif avec celle du HCB-P et du 2,3,7,8-tétrachlorodibenzo-*p*-dioxine (TCDD) considérés comme inducteurs d'activité de l'éthoxyrésorufine-*O*-déséthylase (EROD) et de l'acide ribonucléique messager (ARNm) du CYP1A4 et du CYP1A5. La puissance de deux produits de remplacement mono-*ortho* des PCB, soit le 2,3,3',4,4'-pentachlorobiphényle (PCB-105) et le 2,3',4,4',5-pentachlorobiphényle

(PCB-118), ont été mesurés dans les hépatocytes des embryons de poulet en utilisant les mêmes paramètres. Tous les composés ont entraîné une activité de l'enzyme EROD et ont augmenté l'ARNm du CYP1A4 dans les hépatocytes de chaque espèce. La puissance de l'hexachlorobenzène, par rapport à la puissance relative du TCDD, s'élevait à 0,0001, à 0,001 et à 0,01 dans les hépatocytes des embryons de poulet, de Faisan de Colchide et de Caille du Japon, respectivement. Les valeurs de la CE_{seuil} seraient plus appropriées que celles de la CE_{50}, car, comparativement à ces dernières, elles tiennent davantage compte des différences entre les niveaux maximums de l'EROD et de l'ARNm du CYP1A4/5 relevés lors de l'exposition à l'hexachlorobenzène dans les hépatocytes des embryons des espèces aviaires. La différence entre les niveaux de sensibilité à l'hexachlorobenzène chez ces espèces a également été mesurée et les écarts enregistrés ne se sont pas révélés aussi élevés que ceux enregistrés entre les valeurs de puissance relative. Les résultats présentés ci-après semblent indiquer que l'hexachlorobenzène peut entraîner des effets en aval de l'activation du récepteur de l'aryl-hydrocarbone, ce qui peut justifier son ajout à la liste des facteurs d'équivalence toxique de l'Organisation mondiale de la santé.

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List of Abbreviations

AHR	aryl hydrocarbon receptor
AHH	aryl hydrocarbon hydroxylase
ARNT	aryl hydrocarbon receptor nuclear translocator
BMF	biomagnification factor
cDNA	complementary DNA
CEH	chicken embryo hepatocytes
CYP1A	cytochrome P4501A
CYP1A1	cytochrome P4501A1
CYP1A2	cytochrome P4501A2
CYP1A4	cytochrome P4501A4
CYP1A5	cytochrome P4501A5
DLC	dioxin-like compound
DMSO	dimethyl sulfoxide
EC_{50}	median effective concentration
$EC_{threshold}$	threshold effective concentration
EROD	ethoxyresorufin *O*-deethylase
HAH	halogenated aromatic hydrocarbon
HCB	hexachlorobenzene
HCB-N	"new" reagent-grade hexachlorobenzene
HCB-O	"old" reagent-grade hexachlorobenzene
HCB-P	highly purified hexachlorobenzene
HCH	hexachlorocyclohexane
HRGC	high-resolution gas chromatography
HRMS	high-resolution mass spectrometry
HSP90	heat shock protein 90
IARC	International Agency for Research on Cancer
JEH	Japanese quail embryo hepatocytes
LBD	ligand binding domain
LD50	median lethal dose
Log K_{ow}	log octanol-water partitioning coefficient
LRMS	low-resolution mass spectrometry
3-MC	3-methylcholanthrene
mRNA	messenger ribonucleic acid
OCDD	octachlorodibenzo-*p*-dioxin
OCDF	octacholorodibenzofuran
P23	prostaglandin E synthase 3
PAS	PER-ARNT-SIM
PBS-EDTA	phosphate buffered saline-ethylenediaminetetraacetic acid
PCB	polychlorinated biphenyl
PCB 37	3,4,4'-trichlorobiphenyl
PCB 77	3,3',4,4'-tetrachlorobiphenyl
PCB105	2,3,3',4,4'-pentachlorobiphenyl
PCB118	2,3',4,4',5-pentachlorobiphenyl
PCB126	3,3',4,4',5-pentachlorobiphenyl

PCB 156	2,3,3',4,4',5-hexachlorobiphenyl
PCB 157	2,3,3',4,4',5'-hexachlorobiphenyl
PCB169	3,3',4,4',5,5'-hexachlorobiphenyl
PCDD	polychlorinated dibenzo-*p*-dioxin
PCDF	polychlorinated dibenzofuran
PCP	pentachlorophenol
PCT	porphyria cutanea tarda
PeCDF	2,3,4,7,8-pentachlorodibenzofuran
PEH	ring-necked pheasant embryo hepatocytes
Q-PCR	quantitative RT-PCR
ReP	relative potency
ReS	relative sensitivity
RG-HCB	reagent-grade hexachlorobenzene
RT-PCR	reverse transcriptase-polymerase chain reaction
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TCDF	2,3,7,8-tetrachlorodibenzofuran
TEF	toxic equivalency factor
TEQ	toxic equivalency
UROD	uroporphyrinogen decarboxylase
U.S. EPA	United States Environmental Protection Agency
WHO	World Health Organization
XAP2	immunophilin-like protein hepatitis B virus X-associated protein 2
XRE	xenobiotic response element

Statement of Contributions

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Experimental design, results analysis and manuscript preparation	Lukas Mundy Sean Kennedy
HCB chemical analysis and purification	Alex Konstantinov Fiona Utley David Potter
Chicken embryonic cell cultures	Lukas Mundy Jessica Hervé Doug Crump Stephanie Jones
EROD assays	Lukas Mundy Jessica Hervé
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Chapter 1

Introduction

1.1. General Introduction

Hexachlorobenzene (HCB), initially reported to be a nontoxic benzene derivative, was first introduced as a fungicide in 1933 and has since been used in a variety of other applications such as for the manufacture of synthetic rubber, fireworks, ammunition and other commercial products. HCB is also a by-product of several chemical manufacturing processes, including the production of other chlorine-containing chemicals (Bailey 2001; Barber *et al.* 2005; Courtney 1979; Wang *et al.* 2010; Zitko 2003). The toxic properties of HCB only became apparent after it was found to be responsible for an outbreak of porphyria cutanea tarda in Turkey from 1955-1960. An estimated 3000 – 5000 cases of porphyria and a large number of neonatal and infant deaths were the direct result from the consumption of HCB treated grain (Jarrell *et al.* 2002). Due to its toxicity and environmental persistence, commercial production of HCB was banned many years ago. Global emissions peaked during the 1970s to an estimated 10,000 tonnes/year but have since fallen 70-95% (Barber *et al.* 2005; Courtney 1979). At present, no single source dominates current emissions, but HCB remains present in the environment and is still being released unintentionally into the environment as an unintended byproduct in chemical processes, incomplete combustion, and as an impurity in pesticides (Bailey 2001). It is still detected globally, in air, soil, water, sediment, biota, and in human tissues.

In 1998, van Birgelen (1998) suggested that HCB be a candidate for inclusion in the World Health Organization's (WHO) toxic equivalency concept (TEF), and be regulated as a dioxin-like compound. The central premise of my thesis has focused on determining whether

highly purified HCB does in fact possess dioxin-like properties and whether it warrants inclusion in the TEF concept.

1.2. Environmental fate of HCB

1.2.1. Past and present sources

HCB was first introduced as a fungicide and seed disinfectant for onions, sorghum and grain products like wheat, barley, oats and rye. Agricultural use dominated the emissions of HCB during the 1950s and 1960s and its application was expanded briefly during the early 1970s to replace mercury-based fungicides (Courtney 1979; Zitko 2003). During this time period, global HCB production was estimated at 1000 – 2000 tonnes/year, later peaking in the neighbourhood of 10,000 tonnes/year from 1978-1981 (Barber *et al.* 2005; Courtney 1979; Rippen and Frank 1986). During the mid-1970s that HCB production and application was largely discontinued in most countries worldwide, but it was used in Tunisia until at least 1986 (IARC 1997). In Canada, the production and use of HCB was restricted in 1976 and it was fully banned by 2003. Similarly, HCB production was discontinued in the late 1970s and it was deregistered as a pesticide in 1984 in the United States (ATSDR 2002; Barber *et al.* 2005). Aside from its primary application as a fungicide, HCB has also been used in military pyrotechnic smokes, carbon anode treatment, aluminum fluxing and degassing, as a synthetic rubber peptizing agent, as a wood preserving agent, in the manufacture of fireworks, and as a chemical intermediate in the production of hexachlorocyclohexane (HCH) and pentachlorophenol (PCP) in China (ATSDR, 2002; Bailey 2001; Barber *et al.* 2005; Wang *et al.* 2010; Wei *et al.* 2007).

Overall global production of HCB has exceeded 100,000 tonnes, but since restrictions have been imposed, present day emissions are currently estimated to be 70 – 95% lower than emissions from 1970 (Barber et al., 2005). Levels in the environment have also dropped substantially over the past 20 years (Braune *et al.* 2005; Braune *et al.* 2007; Kannan *et al.* 1992;

Norstrom and Hebert 2006; Wang *et al.* 2010). Currently no single source dominates emissions, however HCB is still being generated and inadvertently released into the environment as a by-product or impurity in several chemical processes, such as the manufacture of chlorinated solvents, chlorinated aromatics, and pesticides (Bailey 2001; Barber *et al.* 2005; Jacoff *et al.* 1986).

HCB has been detected as a trace contaminant in several chlorine containing pesticides like atrazine, simazine, picloram, pentachloronitrobenzene, chlorothalonil, dacthal, lindane, technical HCH, and PCP (Bailey 2001; Pacyna *et al.* 2003; van Birgelen 1998; Wang *et al.* 2010) and it was estimated a decade ago that approximately 6500 kg/year of HCB was being released globally through pesticide application (Bailey 2001). This value may be underestimated since information on HCB contamination levels in pesticides manufactured and used in developing countries such as India and China was not included in this assessment. Meanwhile, it has also been speculated that weak regulations regarding HCB impurities in pesticides, and their overall widespread use of some pesticides in China may be an important current environmental source of HCB. In addition, high concentrations of HCB have been detected in the vicinity of lindane and PCP manufacturing plants in China (Wu *et al.* 1997; Wang et *al.* 2010).

HCB, much like polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs), is also produced as a by-product of incomplete combustion of all kinds of waste incineration (hazardous waste, municipal waste, medical waste, and sewage sludge) where chlorine is present (Bailey 2001; Barber *et al.* 2005; Wang *et al.* 2010) and is also formed as a by-product of magnesium and nickel production (Oehme *et al.* 1989; Deutscher and Cathro 2000). Oehme et al. (1989) reported that about 7 kg/week (350 kg/year) of HCB was emitted to water at a magnesium factory situated near Porsgrunn, Norway.

In the United States, high HCB emissions were reported in the area surrounding a magnesium production facility on the south-western shore of Great Salt Lake in Utah (Beltman and Stackhouse 2007). The site is currently listed as a U.S. EPA Superfund site for potential clean up and remediation and is of particular ecological interest due in part to the large number of avian species that migrate through, and live in the area. HCB was detected at inordinately high concentrations in soil and sediment, sampled from within and surrounding chemical waste ponds on site. The mean concentration of HCB from 16 soil samples was 16,100 ppb with a maximum detected concentration of 134,000 ppb. In sediment (n = 49), HCB was detected with mean and maximum concentrations of 29,400 and 140,000 ppb, respectively. HCB was also found at elevated concentrations in bird eggs. Eggs from snowy plovers (*Charadrius alexandrinus*) contained a range of 500 to 900 ppb HCB, and a single horned lark (*Eremophila alpestris*) egg had a measured concentration of 7,190 ppb HCB (Beltman and Stackhouse 2007).

1.2.2. Global distribution

HCB is a stable, lipophilic, and volatile compound. Based on its physicochemical properties HCB has been widely distributed across the globe and is detected in air, water, soil, sediment, wildlife and humans across the globe. It is even detected in remote locations like the Arctic and the middle of the Pacific Ocean, with concentrations higher than those for many other organochlorine compounds (Zitko 2003).

Due in part that atmospheric degradation of HCB is very slow and that HCB in the atmosphere is found almost exclusively in the gaseous phase, it is thought that HCB can undergo long-range atmospheric transport via global distillation (Wania and Mackay 1996). HCB has been detected in higher concentrations in snow on mountains in northern Europe than in snow on peaks in southern Europe, thus propagating the hypothesis that HCB is undergoing global

distillation (Barber *et al.* 2005; Carrera *et al.* 2000). It has also been proposed that HCB, and other persistent organic pollutants, can undergo bio-vector transport. A study conducted by Blais et al. (2005) showed a 10-fold increase in HCB concentrations in Arctic pond sediments adjacent to large colonies of northern fulmars (*Fulmarus glacialis*) compared to isolated reference ponds. This finding suggests that these predatory fish eating birds are capable of transporting and depositing anthropogenic contaminants from a marine food web to terrestrial/fresh water ecosystems (Blais *et al.* 2005).

1.2.3. Bioaccumulation and biomagnification

Regulatory authorities have identified bioaccumulative compounds as hydrophobic, fat-soluble chemicals with high octanol-water coefficients (i.e. log $K_{ow} \geq 5$) (Kelly *et al.* 2007). Accordingly, HCB is a persistent lipophilic (log $K_{ow} = 5.5$) compound that can bioaccumulate, and possesses characteristics that enable it to potentially biomagnify. A recent study showed that HCB was capable of biomagnifying in a marine food web with a food web biomagnification factor (BMF) of 2.5. HCB concentrations ranged from 21 to 30 ppb in lower trophic level organisms such as molluscs and fish, and increased to 283 ppb in fish eating seabirds (black guillemot, *Cepphus grylle*) (Skarphedinsdottir *et al.* 2009). HCB has also been shown to accumulate and biomagnify in terrestrial food webs, such as lichen-caribou-wolf food chains. BMFs of HCB in male caribou in Canada's Western Arctic region ranged from 4.0 to 6.9 and 2.1 to 6.5 in wolves (Kelly and Gobas 2001). BMF values are expressed as the concentration of a persistent compound within an organism divided by the concentration of the compound present in its food. Thus, a BMF value greater than 1 implies the potential for biomagnificiation (Conder *et al.* 2008).

1.2.4. Environmental persistence

HCB degrades at a slow rate in all environmental compartments. Atmospheric HCB is thought to be degraded by photolysis and by chemical reactions with hydroxyl radicals (OH^-). Half-life estimates range from 0.63 years in tropical regions, to 1.94 years in temperate regions to 6.28 years in polar regions (ATSDR 2002). In other media, the half-life of HCB is estimated 3-6 years in soils and surface water, and 5.3 to 11.3 years in groundwater (ATSDR 2002). In humans, the half-life of HCB is not generally known, however the half-life of HCB in human breast milk lipids is approximately 13 years (van Birgelen 1998; Zitko 2003).

1.3. Toxic equivalency factor (TEF) and toxic equivalent (TEQ) concentrations

1.3.1. Overview

The TEF concept was developed to facilitate risk assessments for PCDD, PCDF and polychlorinated biphenyl (PCB) mixtures by relating the toxicity of these dioxin-like compounds (DLCs) to the toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. According to the most recent guidance on the subject by the WHO (summarized in van den Berg et al., 1998) and the U.S. EPA (2008), the inclusion of a compound into the TEF concept requires that the compound must: (a) have a structural similarity to TCDD, (b) bind to the aryl hydrocarbon receptor (AHR), (c) elicit AHR-mediated biochemical and/or[*] toxic effects and (d) be persistent and accumulate in the food chain. As such, the term "dioxin-like" is used to refer to PCDDs/Fs and PCBs that share a similar chemical structure and a common mechanism of toxic action with TCDD. In total there are 75 PCDD, 135 PCDF and 209 PCB congeners, of which only 29 (7 PCDDs, 10 PCDFs, 12

[*] A discrepancy exists between the WHO and the U.S. EPA criteria for the inclusion of a compound within the TEF concept. The WHO states that a DLC must elicit AHR-mediated biochemical and toxic effects, whereas the U.S. EPA states that a DLC must elicit AHR-mediated biochemical and/or toxic effects. No differences exist for the other criteria.

PCBs) are currently included in the TEF concept (Larsen 2006; Poland and Knutson 1982; Van den Berg *et al.* 1998; Van den Berg *et al.* 2006).

The TEF value for a particular DLC is based on the approximated ratio of a given endpoint, such as binding affinity to the AHR, cytochrome P4501A (CYP1A) enzyme induction or even the lethal dose which kills 50% of a given population (LD_{50} value) compared with that same endpoint value obtained for TCDD (i.e. TEF = EC_{50} [TCDD] /EC_{50} [test compound]). TCDD has been arbitrarily assigned a TEF of 1.0 in humans and animals as it is generally considered to be the most potent dioxin-like compound with regard to AHR binding (Safe 1986). However there are reports that 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) has been shown to be more potent than TCDD in some animals which certainly complicates the risk assessment of such mixtures (Cohen-Barnhouse *et al.* 2011; Hervé *et al.* 2010b; Hervé *et al.* 2010c; Rankouhi *et al.* 2005; Sanderson *et al.* 1998). TEF values are used in combination with chemical residue data to calculate the total dioxin toxic equivalent concentrations (TEQ) in various environmental samples, including animal tissues, soil, sediment and water (Van den Berg *et al.* 1998). The TEQ for a mixture is defined as the sum of the concentrations of individual dioxin-like congeners multiplied by their corresponding TEF as exemplified in the following equation: $TEQ = \sum_{n1} [PCDD_i \times TEF_i] + \sum_{n2} [PCDF_i \times TEF_i] + \sum_{n3} [PCB_i \times TEF_i]$ (Ahlborg *et al.* 1994; Safe 1998; Van den Berg *et al.* 1998).

It should also be noted that TEF values are decided through a consensus scientific building process and are ideally based on both *in vivo* and *in vitro* data when available. In situations where a TEF has been obtained from a single study, the term "relative potency" (ReP) should be used instead (van den Berg *et al.* 1998). There have also been cases when only a ReP

was available and has been used in lieu of a TEF for risk assessment purposes (Van den Berg *et al.* 1998).

The TEF and TEQ concept is a useful tool to address the potential impact of DLCs in the environment, however it is not infallible, and has been soundly criticized because: 1) additive effects are not always observed, 2) the TEFs assigned by the WHO are not always consistent with the RePs and TEFs derived from experimental studies, and 3) dose-response curve shapes are different among compounds (Giesy and Kannan 1998; Harper *et al.* 1995; Harris *et al.* 1993; Toyoshiba *et al.* 2004). Furthermore, TEFs are assigned for a broad group or organisms (i.e. mammal, birds, fish), but are usually based on experiments carried out using a limited number of species (i.e. laboratory rodents, chicken and rainbow trout) (Van den Berg *et al.*, 1998). To further complicate matters, it has been repeatedly shown that the sensitivities to DLCs can vary greatly among species, and even among species of the same group. For example, the LD_{50} in guinea pig for TCDD is 5000 times higher than the LD_{50} in hamster (1 µg/kg bw guinea pig vs. 5000 µg/kg bw hamster) (Olson *et al.* 1980; Poland and Knutson 1982; Schwetz *et al.* 1973). This variability in the sensitivity of DLCs is not a phenomenon observed only in mammals, but has also been observed with a wide degree of variation among avian species (Cohen-Barnhouse *et al.* 2011; Head *et al.* 2008; Hervé *et al.* 2010a; Hervé *et al.* 2010b; Hervé *et al.* 2010c; Karchner *et al.* 2006; Kennedy *et al.* 1996a; Yang *et al.* 2010). Furthermore, TEFs for DLCs can therefore vary depending on the species as well as depending on the endpoints used to derive said TEFs (Behnisch *et al.* 2001; Bosveld *et al.* 1992). As such, scientific regulators and risk assessors should use caution when extrapolating TEF/ReP results from one species to another.

1.3.2. van Birgelen's TEF proposal for HCB

In 1998, van Birgelen (van Birgelen 1998) proposed that HCB should be classified as a 'dioxin-like' compound and assigned a TEF because the compound meets the four criteria for inclusion within the TEF concept as categorized above. Based on her review of the scientific literature, she recommended that HCB should be assigned a TEF of 0.0001, which is similar to the TEFs for some of the mono-*ortho* substituted PCBs. The suggested TEF was based on the results of a limited number of *in vitro* studies that determined effects that are thought to be mediated by the AHR.

1.3.3. Purity issues

To date van Birgelen's suggestion has not been accepted by the WHO or the U.S. EPA due to a number of reasons, perhaps the most important being that it is not absolutely certain if HCB *itself* binds to the AHR, and induces CYP1A, because some batches of HCB have been known to be contaminated with PCDDs, PCDFs and dioxin-like PCBs (Goldstein 1979; Van den Berg *et al.* 2006). Octachlorodibenzo-*p*-dioxin (OCDD) and octacholorodibenzofuran (OCDF) are among the most prevalent dioxin and furan contaminants detected in HCB (Courtney 1979; Goldstein *et al.* 1978; Goldstein *et al.* 1979; Mundy *et al.* 2010; Villanueva *et al.* 1974; Zitko 2003). As such, the similarities between some of the effects of HCB and those of PCDDs and PCDFs suggest that these impurities may be responsible for some of the symptoms and signs of HCB exposure. One example where the trace contamination of HCB with DLCs has been questioned was generated from a study examining the developmental effects of CD-1 mice exposed to HCB *in utero*. Pups of dams that were treated with 10 or 50 mg/kg bw HCB exhibited structural malformations of the cleft palate and enlarged kidneys, which happen to be consistent with malformations attributed with TCDD exposure *in utero* (Andrews and Courtney 1986;

IARC 1997). Consequently, a dose of 10 mg/kg bw HCB, even at 99% purity could result in the TCDD exposure of 100 µg/kg bw, which is well within the range of concentrations needed to induce TCDD related teratogenecity in rodents (Dienhart *et al*. 2000; IARC 1997). Therefore, before HCB can be included in the TEF concept, priority should be given to confirm the compound's dioxin-like properties using highly purified HCB with measured absence of DLCs (Van den Berg *et al.* 2006).

1.3.4. Additional concerns

Aside from the purity issue, other concerns have also been raised with regard to the inclusion of HCB in the TEF concept. Vos (2000) has pointed out that while TCDD and HCB share target organs of toxicity, the effects produced in these systems and organs can and do differ. One example is that neurotoxic effects like tremors are common following HCB exposure in rodents and birds, but are not observed after the exposure to TCDD (Michielsen *et al.* 1999; Vos 2000). However, HCB is described as a mixed-type cytochrome P450 inducer, and effects that are not typical for TCDD poisoning are to be expected (Goldstein *et al.* 1982; van Birgelen 1998). Most inducers of hepatic drug-metabolizing enzymes (CYP enzymes) are divided into two classes. One group induces CYP enzymes identical to those induced by phenobarbital, while the other group induces CYP enzymes consistent with 3-methylcholanthrene (3-MC) exposure (Conney 1967; Goldstein *et al.* 1982). A mixed-type inducer can therefore induce CYP enzymes associated with both phenobarbital and 3-MC exposure. It is important to note that mono-*ortho*-substituted PCBs, like HCB, are also mixed-type CYP inducers that happen to be included within the TEF concept (Bohnenberger *et al.* 2001; van Birgelen 1998).

The logic of the suggested TEF value *itself*, 0.0001, has also been criticized because this value is only based on the results from two *in vitro* studies (Schwab 1999), the first of which

measured the binding affinity of HCB to the AHR in mice (Hahn *et al.* 1989) and the other measured ethoxyresorufin *O*-deethylase (EROD) and uroporphyrin accumulation in chicken hepatocytes (Sinclair *et al.* 1997b). PCDD/F impurities within the HCB samples used in these studies may also have influenced the results and conclusions made.

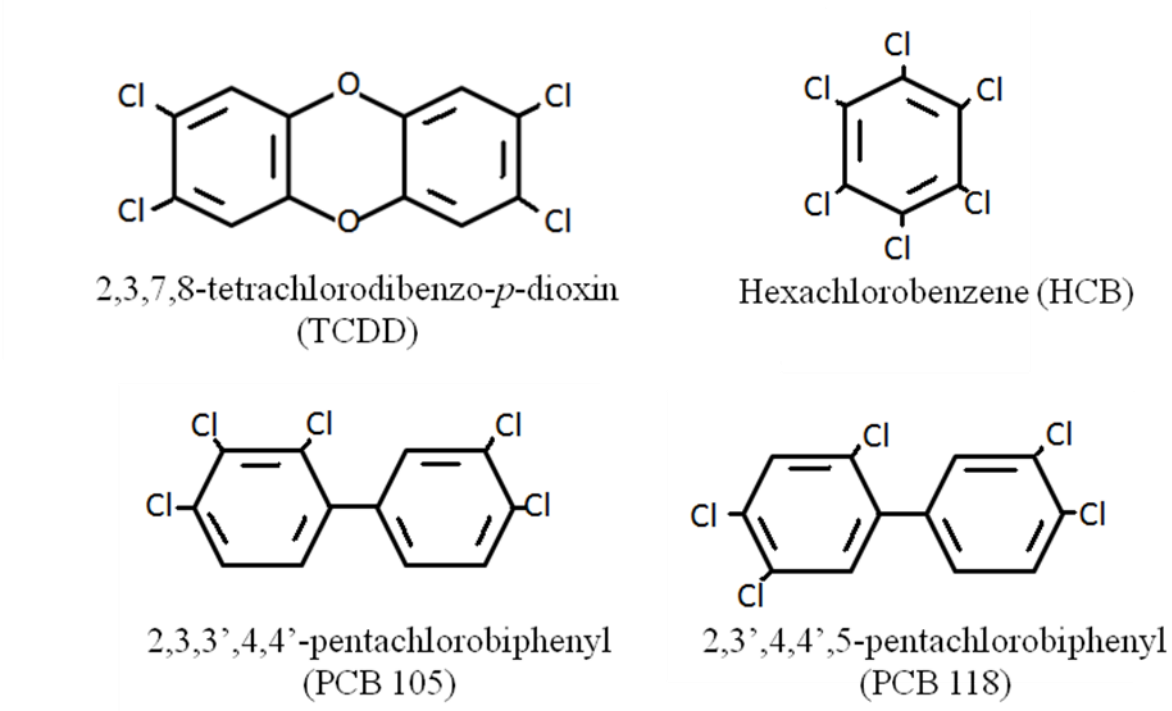

Figure 1.1. Chemical structures of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), hexachlorobenzene (HCB) and two mono-*ortho*-substituted polychlorinated biphenyls, 2,3,3',4,4'-pentachlorobiphenyl (PCB 105) and 2,3',4,4',5-pentachlorobiphenyl (PCB 118).

1.4. Toxicity of HCB

1.4.1. General overview

Prior to the large scale accidental HCB poisoning of Turkish residents in the 1950s (discussed in detail below), HCB was considered to have very limited or no toxicity (acute toxicity LD_{50} estimates ranging from 1 g/kg bw to over 10 g/kg bw in some species) (Courtney 1979; Zitko 2003). Since the unfortunate incident in Turkey, HCB toxicity has been studied extensively and has been shown to give rise to a broad spectrum of species-dependent biochemical and toxic responses. In man, HCB has been shown to cause death, systemic, neurological, developmental, endocrine, and immunological toxicity and is currently listed by the International Agency for Research on Cancer (IARC) as a possible human carcinogen (Group 2B). In animals, HCB has also been shown to cause reproductive toxicity and cancer (ATSDR 2002; IARC 1997; Queiroz *et al.* 1997; Queiroz *et al.* 1998).

1.4.2. Dioxin-like effects defined

TCDD is generally considered the most potent DLC, and has accordingly been the most extensively studied. The exact mechanism by which TCDD elicits its wide array of biochemical and toxic effects is unclear, but appear to stem from TCDD - AHR coupling (White and Birnbaum 2009). TCDD induces an array of toxic endpoints in humans and experimental animals, some of which are also observed in HCB poisoning. Similar to TCDD, HCB has also been shown to cause induction of CYP1A1 and CYP1A2, hepatic porphyrin accumulation and excretion, alterations in thyroid hormone levels and metabolism, alterations in retinoid levels, liver damage, reduction in reproduction, splenomegaly, increase in mortality, neurological alterations, teratologic effects, and immunotoxic effects (Carthew and Smith 1994; den Besten *et al.* 1993; Ezendam *et al.* 2004; Goldstein *et al.* 1978; Goldstein *et al.* 1982; Gorman *et al.* 2007;

Li *et al.* 1989; Linko *et al.* 1986; Michielsen *et al.* 1999; Smith and Cabral 1980; van Birgelen 1998). However, one should still exercise caution on a study by study basis as these similarities may be subject to PCDD/F impurities present within HCB.

1.4.3. Mass poisoning in Turkey

Porphyria is considered the hallmark of HCB exposure in humans and animals and is characterized by a deficiency of the enzyme uroporphyrinogen decarboxylase (UROD) and the subsequent accumulation of porphyrins in the liver (Smith and Elder 2010). Porphyria, and measurement of porphyrins as a biomarker for halogenated aromatic hydrocarbon (HAH) exposure, is described in more detail in section 1.5.2 of this chapter.

In Turkey during the 1950s, 3000 – 5000 residents developed the photosensitive skin disease porphyria cutanea tarda (PCT) resulting from HCB ingestion and the over-production and accumulation of porphyrins in the liver. Symptoms of PCT include fragile skin, superficial erosions, subepidermal bullae, hypertrichosis, and patchy pigmentation on areas of skin exposed to sunlight like the back of hands and face (Smith and Elder 2010). Children between the ages of 6 – 16 years of age were those primarily affected, and there was high mortality (> 95%) among infants exposed to HCB via placental or maternal milk transfer (Cam 1960; Ezendam *et al.* 2004; Michielsen *et al.* 1999). Other symptoms associated with PCT consisted of weight loss, muscle wasting, weakness, hepatomegaly, thyromegaly, and arthritic changes (Daniell *et al.* 1997). Symptoms persisted decades following HCB poisoning. Twenty to thirty years following the onset of PCT by HCB, a large number of patients (n =252) still showed dermatological, neurological, and orthopaedic symptoms and signs. Many had scarring of the face and hands (83.7%), hyperpigmentation (65%), hypertrichosis (44.8%), pinched facies (40.1%), painless arthritis (70.2%), small hands (66.6%), sensory shading (60.6%), myotonia (37.9%),

cogwheeling (41.9%), enlarged thyroid (34.9%) and enlarged liver (4.8%) (Gocmen *et al.* 1989). Other epidemiological studies corroborated these clinical observations and also reported enlarged thyroids found in 25% of men and 60% of women (Cripps *et al.* 1984; Peters *et al.* 1986).

1.4.4. Mechanism of action

The mechanism by which DLCs exert their toxic and biochemical effects on vertebrate species is through activation of the AHR, a ligand-activated basic helix-loop-helix transcription factor, and a member of the PER-ARNT-SIM (PAS) superfamily of transcription factors (Mandal 2005) (Figure 1.2.). The AHR is present in the cytosol in a complex with two molecules of heat shock protein (Hsp90), an immunophilin-like protein hepatitis B virus X-associated protein 2 (XAP2), and a small prostaglandin E synthase 3 protein (p23). Upon binding with a DLC, the complex translocates into the nucleus where the AHR dissociates from the Hsp90 complex and forms a heterodimer with its partner molecule AHR nuclear translocater (ARNT). This heteromeric complex then binds with its respective DNA xenobiotic response element (XRE) located in the promoter region of the CYP1A1 gene, resulting in increased expression of the gene (Mandal 2005; Mimura and Fujii-Kuriyama 2003; White and Birnbaum 2009; Whitlock, Jr. 1993).

In addition to the induction of the CYP1A gene, DLCs also cause a broad spectrum of biochemical and toxicological effects in a variety of different animal species. Extensive studies using AHR-deficient mice have revealed that the binding action of a DLC with the receptor is responsible for most, if not all, of the toxic effects attributed with dioxin-like toxicity (Mimura and Fujii-Kuriyama 2003). Therefore, this signal transduction pathway is generally accepted as the mechanistic model by which DLCs elicit their broad spectrum of AHR-mediated biochemical and toxic responses (Safe 1998). HCB has been shown to be a weak AHR agonist (Hahn *et al.*

1989) and its inclusion to the TEF concept hinges on whether highly purified HCB can bind with, and elicit AHR-mediated effects, such as CYP1A induction.

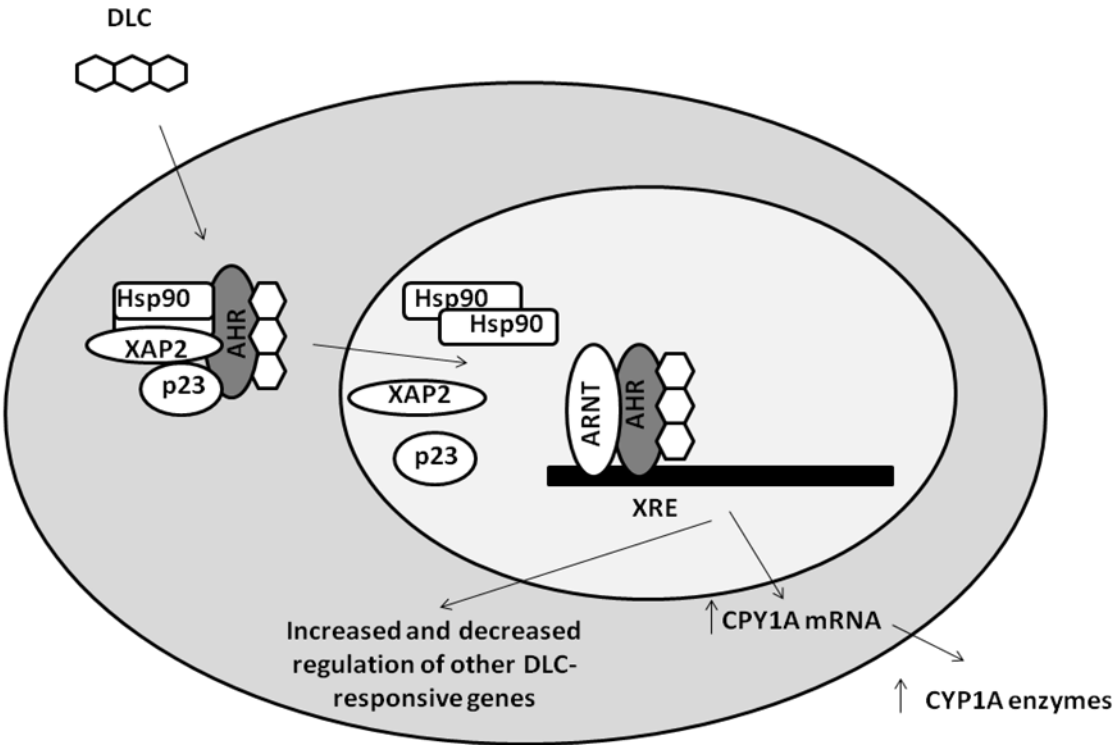

Figure 1.2. Simplified diagram of the aryl hydrocarbon receptor (AHR) pathway. When bound to a dioxin-like compound (DLC), the AHR-DLC complex translocates to the nucleus and forms a heterodimer with its partner molecule the aryl hydrocarbon receptor nuclear translocator (ARNT). This heteromeric complex then binds with its respective DNA xenobiotic response element (XRE) located in the promoter region of the cytochrome P4501A (CYP1A1) gene. The complex, attached to DNA, interacts with coactivators and induces the transcription of various genes including CYP1A1. This diagram is adapted from a figure in Mimura and Kuriyama (2003).

1.5. Biomarkers of dioxin exposure

1.5.1. CYP1A: predicting avian sensitivity to DLCs

CYPs have been predominantly described as liver-localized enzymes that metabolize drugs and other xenobiotics, but they are also present in the heart, kidneys and the small intestine (Gilday *et al.* 1996; Nebert and Dalton 2006). Aside from being mainly involved in xenobiotic metabolism, CYP enzymes have also been shown to be involved in a number of other cellular functions, such as the metabolism of ecosanoids, the biosynthesis of cholesterol and bile acids, and steroid synthesis and metabolism (Nebert and Dalton, 2006). In humans, 57 CYP genes in 18 families have been characterised, of which members of the CYP1 to CYP4 families are involved in the metabolism of thousands of exogenous chemicals. The metabolism of xenobiotics by xenobiotic-metabolizing enzymes has been classified into phase I functionalization and phase II conjugation reactions, of which, CYP enzymes comprise 70 – 80% of all phase I enzymes. Some CYPs participate in the detoxification and metabolism of xenobiotics, whereas others paradoxically, form reactive intermediates of chemicals that can damage lipids, proteins and DNA (Denison and Nagy 2003; Guengerich 2008).

When mammals are exposed to TCDD and structurally related HAHs, two isoforms of CYP1A (CYP1A1 and CYP1A2) are induced whereas, in birds exposed to dioxins, the CYP enzymes CYP1A4 and CYP1A5 are up regulated (Gilday *et al.* 1996; Rifkind *et al.* 1994; Rifkind 2006). Avian CYP1A4 and CYP1A5 are not direct orthologs of mammalian CYP1A1 and CYP1A2 and both CYP1A4/5 amino acid sequences are more similar to CYP1A1 than CYP1A2. In birds, CYP1A4 has been found to be responsible for EROD and aryl hydrocarbon hydroxylase (AHH) activity, whereas CYP1A5 is preferentially active in TCDD-induced

arachadonic acid epoxygenation and the oxidation of uroporphyrinogen to uroporphyrin (Rifkind *et* al., 1994; Rifkind 2006; Sinclair *et al*. 1997).

The induction of CYP1A is not a toxic endpoint *per se*, since the relationship between increases in CYP1A gene expression and the occurrence of toxic effects is not well understood (Nukaya *et al.* 2009). As phase I xenobiotic metabolising enzymes, the induction of CYP1A enzymes are thought to aid an organism in the metabolism and excretion of exogenous chemicals, however, they may lead to adverse effects through the generation of free radicals and reactive oxygen species (Denison and Nagy 2003; Guengerich 2008). Regardless of the mechanistic uncertainties, there is an excellent correlation between the potency of a particular dioxin congener to cause CYP1A induction and their toxic potency in a number of species (Poland and Knutson 1982). Of particular interest and importance for the work presented herein, the induction of CYP1A has been shown to be a useful predictive marker of relative dioxin toxicity in avian embryos as demonstrated from a number of studies (Brunstrom *et al.* 1991; Cohen-Barnhouse *et al.* 2011; Head and Kennedy 2010; Kennedy *et al.* 1996a). CYP1A induction is an excellent marker of dioxin-like toxicity and can be measured with enzymatic assays (Bosveld *et al.*, 1992; Hahn *et al.*, 1996; Kennedy *et al.*, 1996a; Poland and Knutson, 1982), through CYP1A protein induction (Hahn *et al.*, 1996; Kennedy *et al.*, 1995), and through the expression of CYP1A mRNA (Ezendam *et al.*, 2004; Head and Kennedy, 2007b; Hervé *et al.*, 2010b; Jones and Kennedy, 2009).

The use of CYP1A induction as a predictive tool of avian embryo lethality was assessed in a recent review by Head and Kennedy (2010). In that paper, EROD EC_{50} values obtained from chicken embryo hepatocytes (CEH) cultures were compared with LD_{50} values obtained from chicken egg injection studies using TCDD and six non-*ortho* (PCB126, PCB77, PCB169), and

mono-*ortho*-substituted PCBs (PCB 105, PCB 156, PCB 157). The correlation between the *in vitro* and *in ovo* potency of these DLCs was significant ($r^2 = 0.93$, $p < 0.005$). This relationship was further validated *in ovo* by Cohen-Barnhouse et al. (2011). The authors injected TCDD, PeCDF and 2,3,7,8-TCDF (TCDF) into the air cell of chicken, ring-necked pheasant, and Japanese quail eggs, and confirmed that *in vitro* findings regarding the sensitivity of these species to these particular DLCs was upheld *in ovo*. The relative potencies of these compounds *in ovo* were also similar to those determined *in vitro* (Cohen-Barnhouse *et al.* 2011; Hervé *et al.* 2010b; Kennedy *et al.* 1996a). Therefore, EROD induction (CYP1A induction) in embryonic hepatocyte cultures is a useful tool that can be used to predict the sensitivity of avian species to embryo lethality caused by DLCs (Figure 1.3.).

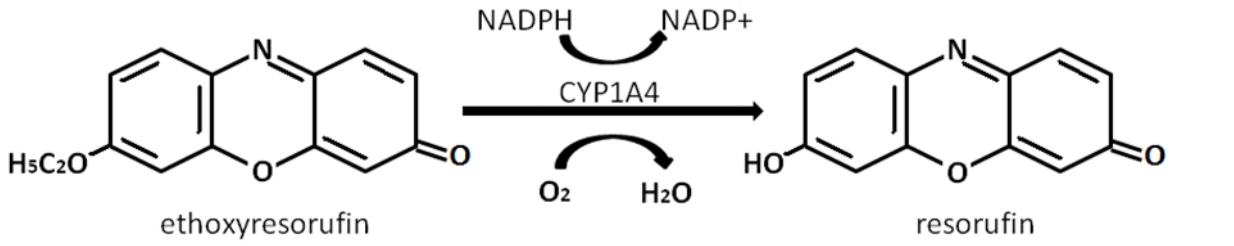

Figure 1.3. Conversion of ethoxyresorufin by cytochrome P4501A4 (CYP1A4) in the ethoxyresorufin *O*-deethylase (EROD) assay.

1.5.2. Porphyrias

Porphyrins are tetrapyrrolic pigments that are involved in the heme biosynthetic pathway and can give rise to a group of disorders known as porphyrias (Marks 1985; Smith and Elder 2010). Porphyrias are inherited or acquired metabolic disorders which are attributed to the overproduction of heme precursors and their subsequent accumulation in certain tissues because of decreased activity of a specific enzyme(s) in the heme synthesis pathway. The majority of

porphyrias in humans are inherited and are the result of a genetic deficiency that affects a single enzyme in the heme synthesis pathway (De Matteis and Lim 1994; Daniell *et al.* 1997). However, one such disorder, porphyria cutanea tarda can be acquired through xenobiotic exposure to a number of chemicals, including HAHs and metals. PCT is caused by a marked decreased in uroporphyrinogen decarboxylase (UROD) activity, which causes an accumulation of the heme intermediate uroporphyrinogen (Daniell *et al.* 1997).

CYP1A1 induction is the biochemical endpoint most commonly measured to assess the toxic potencies of dioxin-like compounds; however, as described above, the deregulation of the heme biosynthetic pathway has also been associated with dioxin exposure (Lorenzen *et al.* 1997; Marks 1985). Since porphyrins also possess fluorescent characteristics they can be detected in different biological materials at very low concentrations and have therefore been proposed as sensitive biomarkers of xenobiotic exposure (Casini *et al.* 2003; De Matteis and Lim, 1994). In birds, DLCs have been shown to elevate levels of highly carboxylated porphyrins, such as uroporphyrins, hepta- and hexa-carboxylic acid porphyrins in order to induce porphyria. This has been observed in both wild bird populations and in embryonic cell cultures exposed to dioxin-like compounds (Fox *et al.* 1998; Kennedy and Fox 1990; Lorenzen *et al.* 1997). Interestingly, Japanese quail appear to be very sensitive to porphyrin accumulation with HCB exposure (Carpenter *et al.* 1984; Vos *et al.* 1971).

There has also been some light shed on the mechanistic relationship between the onset of porphyria and the AHR pathway. It has been hypothesized that the induction of CYP1A isoenzymes can catalyze oxidation of porphyrinogens to porphyrins (Jacobs *et al.* 1989; Smith and De Matteis 1990). In birds, CYP1A5 catalyzes oxidation of uroporphyrinogen to uroporphyrin, resulting in the reduced conversion of uroporphyrinogen to copoporphyrinogen,

thus leading to the subsequent accumulation of the heme precursor, uroporphyrin (Rifkind 2006). It has also been suggested that uroporphyrin accumulation is perhaps the result of reactive intermediates or reactive oxygen species formed by CYP1A metabolism of xenobiotics which directly inhibit UROD activity (Casini *et* al. 2003; den Besten *et al*. 1993; Rifkind 2006). Moreover, Davies et al. (2008) recently demonstrated that the AHR pathway may be involved in TCDD-induced uroporphyria. TCDD exposed AHR knock-out mice showed no increase in hepatic uroporphyrin whereas substantial amounts were detected in $AHR^{+}/^{+}$ and the $AHR^{+}/^{-}$ mice (964- and 60-fold increase, respectively) (Davies *et al.* 2008). Additionally, it has been shown that $CYP1A2^{-}/^{-}$ null mice were also protected against TCDD-induced uroporphyria and hepatoxicity (Rifkind 2006; Smith *et al.* 2001).

1.6. Thesis overview

1.6.1. Rationale

Despite the fact that HCB emissions and concentrations have decreased dramatically worldwide since the mid-1970s, there are still point sources where HCB is being unintentionally released into the environment at appreciable amounts (Beltman and Stackhouse 2007). While it has been suggested that HCB should be a candidate for inclusion in the TEF regulatory concept, questions surrounding the purity of HCB used in previous toxicological studies has been cause for why HCB is not yet classified as a DLC.

My thesis focused on determining whether highly purified HCB (defined as HCB containing < 0.2 ppb of any PCDD, PCDF, or co-planar PCB congener [the detection limit of current analytical methods]) does in fact possess dioxin-like properties and examined whether HCB should indeed be subject for inclusion in the TEF concept. As such, my research assessed whether highly purified HCB was truly capable of activating AHR-responsive genes (i.e.

CYP1A4/5) in avian embryonic hepatocyte cultures of three species: chicken (*Gallus gallus domesticus*), ring-necked pheasant (*Phasianus colchicus*) and Japanese quail (*Cortunix japonica*).

1.6.2. Hypotheses and predictions

The three hypotheses associated with these objectives were:

1 - Highly purified HCB will induce an AHR-mediated response (CYP1A induction) in avian embryo hepatocytes.

2 - Relative potency values will be equal for reagent-grade and highly purified HCB in each test species as PCDD/F impurities are not at sufficient quantities to elicit AHR-dependent biochemical effects in reagent-grade HCB.

3 - Differences in species sensitivity to EROD induction and CYP1A4/5 mRNA expression are expected with HCB exposure. The rank order of sensitivity predicted for HCB was predicted to be: chicken > ring-necked pheasant > Japanese quail.

1.6.3. Study objectives

The main research objectives were:

1 - To determine whether highly purified HCB could induce an AHR-mediated response (i.e. CYP1A4/5 induction) in embryo hepatocytes of three avian species.

2 - To determine relative potency values for reagent-grade and purified HCB for three avian species using three different experimental endpoints (EROD activity, CYP1A4/5 mRNA expression).

3 - To determine whether species differences in sensitivity to HCB exposure exist.

1.6.4. Species selected

These three avian species were chosen for this research because of the results obtained from previous work which demonstrated that differences in species sensitivity to DLCs are apparent (Head and Kennedy 2008; Karchner *et al.* 2006; Kennedy *et al.* 1996a). Karchner et al., (2006) recently uncovered a mechanistic link between the amino acid sequence of the AHR ligand binding domain (LBD) and the degree to which the receptor is activated by TCDD. It was shown that *in ovo* sensitivity of these three species to DLCs was correlated with their respective AHR LBD genotype (Head and Kennedy 2008). Chickens are highly sensitive, ring-necked pheasant are moderately sensitive, and Japanese quail have low sensitivity to DLCs. As such, these three species were used because they are representative of a range of sensitivities found in birds.

1.6.5. Cell culture and CYP1A induction

Primary avian embryo hepatocyte cultures were used because validated methodologies exist for determining CYP1A induction potencies of DLCs using either the EROD assay or CYP1A4/5 mRNA expression in a controlled setting devoid of physiological and environmental factors (i.e. age, stress, diet) (Head and Kennedy 2007a,b; Kennedy *et al.* 1996a,b). Cell cultures are also beneficial since they require a smaller number of individuals and are a cost and time effective alternative to *in vivo* exposure studies. Furthermore, the fact that CYP1A induction in embryonic cultured hepatocytes has been shown to be significantly correlated with embryo-lethality was an important determinant.

CHAPTER 2

Highly Purified Hexachlorobenzene Induces Cytochrome P4501A in Primary Cultures of Chicken Embryo Hepatocytes[†]

2.1. Introduction

Hexachlorobenzene (HCB) is a persistent organic pollutant that was used as a fungicide and for the manufacture of synthetic rubber, fireworks, ammunition and other commercial products. HCB is also a byproduct of several chemical manufacturing processes, including the production of other chlorine-containing chemicals. Due to its toxicity and environmental persistence, commercial production of HCB was banned many years ago. Globally, HCB restrictions began as early as the 1960s (Barber et al., 2005). Nevertheless, HCB remains present in the environment, and is still detected in wildlife tissues and human serum in some locations around the globe (Bailey, 2001; Barber et al., 2005; Zitko, 2003).

In 1998, van Birgelen (van Birgelen, 1998) proposed that HCB should be classified as a 'dioxin-like' compound and assigned a toxic equivalency factor (TEF) because the compound meets the criteria for inclusion within the TEF concept as it is formally described by the World Health Organization (WHO) (van den Berg et al., 1998; van den Berg et al., 2006). The TEF concept relates the toxicity of dioxin-like compounds to the toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), which is generally considered to be the most toxic of all dioxin-like compounds. According to the most recent guidance on the subject by the WHO and the United States Environmental Protection Agency (U.S. EPA, 2008), a dioxin-like compound must: (a) have a structural similarity to TCDD, (b) bind to the aryl hydrocarbon receptor (AHR),

[†] Adapted from Mundy, L.J., Jones, S.P., Crump, D., Hervé, J.C., Konstantinov, A., Utley, F., Potter, D. and Kennedy, S.W., 2010. Highly purified hexachlorobenzene induces cytochrome P4501A in primary cultures of chicken embryo hepatocytes. Toxicol. App. Pharmacol. 248(3), 185-193.

(c) elicit AHR-mediated biochemical or toxic effects and (d) be persistent and accumulate in the food chain to be included in the TEF concept. Based on her review of the scientific literature, van Birgelen (1998) recommended that HCB should be assigned a TEF of 0.0001, which is similar to the TEFs for some of the mono-*ortho* substituted polychlorinated biphenyls (PCBs). The suggested TEF was based on the results of a limited number of *in vitro* and *in vivo* studies that determined effects that are thought to be mediated by the AHR.

However, van Birgelen's recommendation for including HCB within the TEF concept has been criticized (Pohl et al., 2001; Schwab, 1999; Vos, 2000). Part of the criticism is due to uncertainty regarding the purity of the HCB that was used in the studies cited in her paper. For example, some batches of HCB are known to be contaminated with polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs), and it was suggested that PCDDs and/or PCDFs may have elicited the dioxin-like effects observed in the HCB studies cited, and not the HCB itself. A WHO expert committee stated that before HCB can be included in the TEF concept, "Priority should be given to confirm the compound's dioxin-like properties using highly purified HCB with measured absence of 2,3,7,8-chlorine-substituted dioxins and dibenzofurans or dioxin-like PCBs" (van den Berg et al., 2006).

A series of *in vivo* studies carried out by Goldstein et al. (1978, 1982) and Linko et al. (1986) addressed this issue by using HCB which contained non-detectable levels of PCDDs and PCDFs. While the authors concluded that the observed effects in rats and mice were attributed to HCB exposure, it is plausible that dioxin-like contaminants may still have contributed to the reported toxic and biochemical effects. The detection limit employed (< 500 ppb) may have been too high to rule out effects caused by PCDD/F and dioxin-like PCB impurities that may have been present, but undetected.

The goal of this study was to determine if highly purified HCB is an inducer of cytochrome P4501A (CYP1A; an AHR-mediated response) in primary cultures of chicken embryo hepatocytes (CEHs) and to determine an estimate of relative potency (ReP) of HCB compared to TCDD. The purified HCB used herein contained no detectable amounts of PCDDs/Fs or dioxin-like PCBs (< 0.2 ppb). Validated methodologies exist for determining CYP1A induction potencies of compounds using either the ethoxyresorufin *O*-deethylase (EROD) assay or CYP1A messenger ribonucleic acid (mRNA) expression in CEH cultures (Head and Kennedy, 2007a,b; Kennedy et al., 1996a,b). We report for the first time for any test system or any avian species that highly purified HCB is an inducer of EROD activity and CYP1A4/5 mRNA expression. The relative potency value of the purified HCB was similar to that of two mono-*ortho* PCBs, 2,3,3',4,4'-pentachlorobiphenyl (PCB 105) and 2,3'4,4',5-pentachlorobiphenyl (PCB 118).

2.2. Materials and Methods

2.2.1. HCB analysis and purification

Wellington Laboratories, Inc. (Guelph, ON, Canada) analyzed two samples of HCB for contamination by PCDDs, PCDFs, non-*ortho* substituted PCBs and mono-*ortho* substituted PCBs. An older lot of HCB, referred to in this paper as HCB-O (97% pure: Aldrich Cat. #171050; lot number: 2104EJ) and a newer lot of HCB (HCB-N: 99% pure: Aldrich Cat. #171050; lot number: 1420664) were used for these analyses. Because HCB is a known human carcinogen, all procedures were carried out to ensure no possible exposure. In brief, duplicate 10 mg subsamples of HCB-O and HCB-N were spiked with 13-C labeled surrogates of PCDDs, PCDFs and PCBs (500 pg of each congener) and dissolved in hexane. These solutions were

applied to carbon columns (PX21/Celite) that were then eluted with a series of solvents to remove the bulk of the HCB. The PCDDs, PCDFs and PCBs were then back eluted with toluene. The toluene eluants were evaporated to dryness and the residues were transferred to micro vials. After addition of recovery/injection standards, analysis was conducted by high-resolution gas chromatography-high-resolution mass spectrometry (HRGC-HRMS) according to EPA Method 1613B protocols which were adapted for the inclusion of the PCBs. The HCB samples were also analyzed by HRGC-LRMS for other major contaminants. The identification and quantification of PCDD, PCDF and PCB congeners was performed using an Agilent (Santa Clara, CA, USA) 6890 series high-resolution gas chromatograph with direct capillary interface to a Waters (Milford, MA, USA) Autospec Ultima HRGC-HRMS. Chromatographic separations were either carried out on a 60 m DB5 (0.25 mm ID, 0.25 µm film thickness) column or a 30 m DB225 (0.25 mm ID, 0.25 µm film thickness) column, depending on the congener of interest**.**

HCB-O was selected for purification since it was found to contain lower levels of PCDD/Fs than HCB-N. HCB-O was purified by first dissolving it in hexane and passing it through a column containing a layer of PX21 on Celite on top of a layer of silica gel. HCB was then eluted with hexane and was concentrated to dryness. The HCB was then further purified by recrystallization from hexane to yield purified HCB (HCB-P). HCB-P was subsequently analyzed for PCDDs, PCDFs and PCBs using the procedures described above for HCB-N and HCB-O. In order to obtain data for the mono-*ortho* PCBs, a solution of HCB-P was spiked with 13C-labeled mono-*ortho* PCBs and analyzed directly by HRGC-HRMS. The detection limits for mono-*ortho* PCBs are thus higher than the detection limits for the non-*ortho* PCBs and the PCDD/Fs.

ALS Laboratory Group (Burlington, ON, Canada) independently (under a contract with Booz Allen Hamilton Inc.) analyzed HCB-P for PCDD/Fs using EPA Method 8290A protocols. The 8290A and 1613B EPA methods are similar in that both methods involve spiking with 13C-labelled PCDDs and PCDFs, extraction and cleanup, addition of recovery/injection standards and final analysis by HRGC-HRMS (10000 resolution). Neither of the EPA methods address neat HCB as the matrix, but both the ALS Laboratory Group and Wellington Laboratories decided independently that the methods were appropriate for HCB analysis for this study.

2.2.2. Preparation of solutions of TCDD, HCB and PCBs

A detailed description of the preparation of the TCDD solutions is provided elsewhere (Hervé et al., 2010). In brief, serial dilutions of TCDD were prepared from a dimethyl sulfoxide (DMSO) stock solution with a nominal concentration of 80 µg/ml. Identification and quantification of TCDD in the stock solution was determined by isotope dilution following EPA Method 1613 (U.S. EPA, 1994) by use of HRGC-HRMS. The measured concentration of the stock solution was 72.9 µg/ml. HCB-P, HCB-O, HCB-N, PCB 105 (Accustandard Inc., New Haven, CT, USA; lot # 101905AG-AC; purity stated to be 100%), and PCB 118 (Accustandard; lot # 032494; purity stated to be 100%) were weighed (0.5 -1.0 mg) and stock solutions were prepared in DMSO. The nominal concentrations of the stock solutions were: HCB-P, 57.0 µg/ml; HCB-O, 57.0 µg/ml; HCB-N, 57.0 µg/ml; PCB 105, 83 µg/ml; PCB 118, 160 µg/ml. The stock solutions were heated, sonicated and vortexed prior to the preparation of serial dilutions.

2.2.3. Preparation of cultured hepatocytes

Primary cultures of chicken embryo hepatocytes were prepared as described in detail elsewhere (Head et al., 2006; Kennedy et al., 2003). All procedures were conducted according to protocols approved by the Animal Care Committee at the National Wildlife Research Centre.

Reagents were obtained from Sigma (St. Louis, MO, USA) unless otherwise specified. Fertilized, un-incubated chicken eggs were obtained from the Canadian Food Inspection Agency (Ottawa, ON, Canada). Eggs were incubated for 19 d at 37.5°C and 60% relative humidity. Eggs were candled periodically throughout development and removed from the incubator if found to be unfertilized or to contain dead embryos. In brief, 24 chicken embryos were euthanized 1-2 days pre-hatch by decapitation; livers were removed and pooled. Hepatocyte cultures were prepared from this pool of livers using collagenase digestion, filtration, and centrifugation as described in detail elsewhere (Head et al., 2006). Percoll (Amersham Bioscience, Uppsala, Sweden) was used to separate erythrocytes from hepatocytes and DNAse (Roche, Laval, QC, Canada) treatment was carried out to prevent cell clumping. The resulting cell pellet was weighed and suspended in a volume of Medium 199 equal to 32 times its mass. Cell homogenates were plated in 48-well plates by adding 25 µl of the cell suspension to 500 µl of medium into each well. Hepatocytes were allowed to acclimatize at 37.5°C, 5% CO_2 for 24 h prior to the addition of serial dilutions of TCDD, HCB, and PCBs. Serial dilutions of DMSO solutions (2.5 µl/well) of TCDD, HCB-P, HCB-N, HCB-O, PCB 105 or PCB 118 were added to hepatocytes. TCDD was administered such that concentrations in cell culture medium ranged from 0.0003 nM to 10 nM, whereas HCB and PCB concentrations were from 0.03 nM to 1000 nM. Plates were returned to the incubator for 24 h exposure at the same conditions indicated above. Following this 24 h exposure time point, cell culture medium was removed and plates were flash-frozen in powdered dry ice and stored at -80 °C until the time of analysis. Plates used for EROD assays were rinsed with approximately 200 µl/well of phosphate buffered saline-ethylenediaminetetraacetic acid (PBS-EDTA) prior to being flash-frozen.

The data presented herein were obtained from two separate cell culture studies. The first study (CEH-1) compared the concentration-dependent effects of HCB-P, HCB-O, HCB-N and TCDD on EROD activity and CYP1A4/5 mRNA expression. The goals of the second study (CEH-2) were to: (a) repeat the CEH-1 study for HCB-P and TCDD and (b) compare the concentration-dependent effects of HCB-P, TCDD, PCB 105 and PCB 118 on EROD activity and CYP1A/5 mRNA expression.

2.2.4. Cell viability

Cell viability was determined using the Calcein-AM assay (Invitrogen-Molecular Probes, Eugene, OR, USA). Vehicle (DMSO) treated cells were included as a positive control and 99% ethanol-killed cells were used as a negative control. A working solution was prepared by adding 3 µl of Calcein-AM to 10 mL of PBS-EDTA. The culture medium was removed and 200 µl of the Calcein-AM solution was added to each well. Plates were incubated in the dark for 45 min at room temperature and fluorescence was then measured using a Cytofluor 2350 (Millipore, Bedford, MA, USA) fluorescence plate reader with a 485 nm excitation wavelength and a 530 nm emission wavelength. Calcein-AM, a membrane-permeant, is hydrolyzed by intracellular esterase into green fluorescent calcein. Fluorescent calcein is retained in live cells. Mean cell viability was assessed after 24 h exposure for the range of concentrations administered for each compound. CEH plates used for cell viability were not used for RNA isolation.

2.2.5. EROD assays

EROD assays were conducted as described in detail elsewhere (Head and Kennedy, 2007b; Kennedy et al., 1995). In brief, hepatocytes were incubated at 37.5°C in the presence of nicotinamide adenine dinucleotide phosphate (NADPH, reduced) and 7-ethoxyresorufin for 7 minutes. Reactions were stopped by the addition of cold acetonitrile containing fluorescamine.

Standard curves of resorufin and protein were prepared on each 48-well plate for each run. Plates were analyzed for both EROD activity (excitation wavelength: 530 nm, emission wavelength: 590 nm) and total protein concentration (excitation wavelength: 400 nm, emission wavelength 460 nm) using a Cytofluor 2350 fluorescence plate reader.

2.2.6. RNA isolation and complementary DNA synthesis

Total RNA was extracted from 48-well plates using RNeasy 96 kits (Qiagen, Mississauga, ON, Canada) according to the manufacturer's instructions with modifications described elsewhere (Head and Kennedy, 2007a). An on-column DNAse treatment to remove genomic DNA contamination was also incorporated into the protocol. Following RNA collection, an additional DNAse treatment was performed using the Ambion DNA-*free* kit (Ambion, Austin, TX, USA) per the manufacturer's instructions. The absence of genomic DNA was confirmed using quantitative polymerase chain reaction (QPCR). A standard volume of RNA (11.5 µl) was reverse-transcribed to complementary DNA (cDNA) using Superscript II and random hexamer primers (Invitrogen, Burlington, ON, Canada).

2.2.7. Quantitative polymerase chain reaction (QPCR)

A multiplex QPCR assay using dual-labeled fluorescent hydrolysis probes (Head and Kennedy, 2007b) was used to quantify chicken CYP1A4, CYP1A5 and β-actin (normalizer gene) mRNA abundance. With the exception of primers and probes, all QPCR reactions were carried out using reagents acquired from Stratagene and either the Stratagene Mx3000P or Mx3005 instrument (Stratagene, La Jolla, CA, USA). All primers (Invitrogen) and probes (Biosearch, Novato, CA, USA) were designed and validated as previously described (Head and Kennedy, 2007b). Taqman reactions were performed using Brilliant QPCR Core Reagent kits. Each 25 µl reaction contained forward and reverse primers and probes for the genes of interest (CYP1A4,

CYP1A5) and the normalizer gene (β-actin), 1x Core RT-PCR buffer, 5 mM $MgCl_2$, 0.8 mM dNTPs, 0.08% v/v glycerol, 25 nM reference dye (ROX), 0.05 U Surestart Taq DNA polymerase and 5 µl of diluted cDNA. The thermal profile for all reactions was as follows: 10 min at 95°C, followed by 40 cycles of 95°C for 30 s and 60°C for 1 min. Data collection was at the end of the 60°C phase. Each assay was performed once with samples in duplicate. Fold changes of CYP1A4/5 mRNA expression in TCDD-, HCB- and PCB-treated hepatocytes was quantified using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.2.8. EROD and CYP1A4/5 mRNA data analysis

Fluorescence data were imported into GraphPad (GraphPad Prism 5.0 software, San Diego, CA, USA) for curve fitting. EROD activity data were fit to a modified Gaussian curve as previously described (Kennedy et al., 1993). For each treatment, three EROD curves were generated from data originating from three separate cell culture plates, each dosed with triplicate concentrations of HCB, TCDD or PCBs. EC_{50}, $EC_{threshold}$ and maximal values represent the mean value of replicate values obtained from three 48-well plates ± standard error (SE). The $EC_{threshold}$ represents the concentration of a compound that elicited an EROD response greater than DMSO-treated cells that was statistically significant using a one-way analysis of variance (ANOVA) with a Bonferroni correction.

CYP1A4 and 1A5 mRNA expression data were fit to a four parameter logistic model as described elsewhere (Head and Kennedy, 2007b). The equation integrates the EC_{50}, baseline response and maximal response as parameters. A single curve fit was generated for mRNA induction, using data from the average of three wells from the same cell culture plate, assessed in duplicate. EC_{50} is represented by the values derived from the curve fit ± SE. $EC_{threshold}$ was determined as it was for EROD data. Statistical differences among EC_{50} and differences in

mRNA expression were tested using a one-way ANOVA with a Bonferroni correction. $p < 0.05$ was considered statistically significant in all cases.

2.2.9. Calculation of relative potencies

The concept of using relative potencies to compare the potencies of dioxin-like compounds to TCDD is well established (van den Berg et al., 1998). As defined by the U.S. EPA (U.S. EPA, 2008), relative potency (ReP) is an estimate based on a single *in vitro* or *in vivo* study of the potency, relative to 2,3,7,8-TCDD, of an individual chemical to cause a particular AHR-mediated toxicological or biological effect. In the present study, ReP is defined as EC_{50} (or $EC_{threshold}$) of TCDD / EC_{50} (or $EC_{threshold}$) of HCB and/or PCBs.

2.3. Results

2.3.1. HCB analysis and purification

Chemical analysis of the two different lots of reagent-grade HCB (HCB-N and HCB-O) revealed detectable concentrations of PCDDs and PCDFs; HCB-N contained more PCDD/F contaminants than HCB-O (Table 2.1). With the exception of octachlorodibenzofuran (OCDF), the concentrations of all other measured PCDD/Fs were higher in HCB-N than in HCB-O. A non-*ortho* PCB (3,3',4,4'-PCB; PCB 77) was also detected in HCB-O. Recoveries of the 13-C-labeled mono-*ortho* PCBs were not acceptable (less than 10%); therefore, data for these compounds in HCB-O and HCB-N are not reported. The WHO avian TEFs are indicated in Table 2.1, and toxicity equivalents (TEQs) of PCDDs, PCDFs and PCBs in HCB-N and HCB-O were, at most, 76 ng/g and 8.0 ng/g, respectively. The analysis for PCDDs, PCDFs and PCBs in HCB-P by Wellington Laboratories indicated that all compounds were below the detection limits; as such the total TEQ was, at most, 0.56 ng/g (calculated using detection limits for non-

detected compounds). The ALS Laboratory Group did not analyze HCB-P for PCBs, but their PCDD and PCDF data indicated that the total TEQ was, at most 0.092 ng/g (Table 2.1). The lower ALS TEQ compared to the Wellington TEQ was due to detection limit differences for PCDD/Fs between the two laboratories.

Table 2.1. PCDD, PCDF, non-*ortho* PCB and mono-*ortho* PCB concentrations in two reagent-grade HCB samples (HCB-N and HCB-O) and purified HCB (HCB-P). Wellington Laboratories analyzed all samples; the ALS Laboratory Group analyzed only HCB-P. All concentrations are in units of ng/g (ppb). Avian WHO-TEF values are those indicated in Van den Berg *et al.* (1998). TEQ values are calculated as the product of each compound concentration and its associated WHO-TEF. For non-detected compounds, detection limits were used as the compound concentration.

		Wellington Laboratories*						ALS	
	AVIAN WHO-TEF	HCB-N	HCB-N TEQ	HCB-O	HCB-O TEQ	HCB-P	HCB-P TEQ	HCB-P	HCB-P TEQ
DIOXINS									
2,3,7,8-TCDD	1	5.1	5.1	ND (0.1)	<0.1	ND (0.1)	<0.1	ND (0.038)	<0.038
1,2,3,7,8-PeCDD	1	25.2	25.2	ND (0.1)	<0.1	ND (0.1)	<0.1	ND (0.011)	<0.011
1,2,3,4,7,8-HxCDD	0.05	22.9	1.145	ND (1.9)	<0.095	ND (0.1)	<0.005	ND (0.027)	<0.0014
1,2,3,6,7,8-HxCDD	0.01	29	0.29	ND (2.0)	<0.02	ND (0.1)	<0.001	ND (0.0086)	<0.000086
1,2,3,7,8,9-HxCDD	0.1	64.5	6.45	ND (2.2)	<0.22	ND (0.1)	<0.01	ND (0.014)	<0.0014
1,2,3,4,6,7,8-HpCDD	0.001	424	0.424	ND (0.4)	<0.0004	ND (0.1)	<0.0001	ND (0.021)	<0.000021
OCDD	0.0001	4520	0.452	2045	0.2045	ND (0.1)	<0.00001	ND (0.13)	<0.000013
FURANS									
2,3,7,8-TCDF	1	NDR (6.6)	<6.6	NDR (1.2)	<1.2	ND (0.1)	<0.1	ND (0.022)	<0.022
1,2,3,7,8-PeCDF	0.1	55.4	5.54	43.5	4.35	ND (0.1)	<0.01	ND (0.014)	<0.014
2,3,4,7,8-PeCDF	1	3	3	ND (0.1)	<0.1	ND (0.1)	<0.1	ND (0.01)	<0.01
1,2,3,4,7,8-HxCDF	0.1	44.6	4.46	ND (0.2)	<0.02	ND (0.1)	<0.01	ND (0.015)	<0.0015
1,2,3,6,7,8-HxCDF	0.1	28.8	2.88	NDR (9.8)	<0.98	ND (0.1)	<0.01	ND (0.012)	<0.0012
2,3,4,6,7,8-HxCDF	0.1	116	11.6	ND (0.2)	<0.02	ND (0.1)	<0.01	ND (0.012)	<0.0012
1,2,3,7,8,9-HxCDF	0.1	6.8	0.68	ND (0.3)	<0.03	ND (0.1)	<0.01	ND (0.015)	<0.0015
1,2,3,4,6,7,8-HpCDF	0.01	215	2.15	2.3	0.023	ND (0.1)	<0.001	ND (0.055)	<0.00055
1,2,3,4,7,8,9-HpCDF	0.01	61.7	0.617	ND (0.4)	<0.004	ND (0.2)	<0.002	ND (0.064)	<0.00064
OCDF	0.0001	1330	0.133	4210	0.421	ND (0.2)	<0.00002	0.609	0.000061
BIPHENYLS									
Non-*ortho*									
PCB #81	0.1	ND (0.3)	<0.03	ND (0.2)	<0.02	ND (0.2)	<0.02	NA	NA
PCB #77	0.05	NDR (0.5)	<0.025	0.6	0.03	ND (0.2)	<0.01	NA	NA
PCB #126	0.1	ND (0.1)	<0.01	ND (0.1)	<0.01	ND (0.3)	<0.03	NA	NA
PCB #169	0.001	NDR (0.3)	<0.0003	ND (0.1)	<0.0001	ND (0.1)	<0.0001	NA	NA
Mono-*ortho*									
PCB #105	0.0001	NA	NA	NA	NA	ND (130)	<0.013	NA	NA
PCB #114	0.0001	NA	NA	NA	NA	ND (110)	<0.011	NA	NA
PCB #118	0.00001	NA	NA	NA	NA	ND (110)	<0.0011	NA	NA
PCB #123	0.00001	NA	NA	NA	NA	ND (110)	<0.0011	NA	NA
PCB #156	0.0001	NA	NA	NA	NA	ND (30)	<0.003	NA	NA
PCB #157	0.0001	NA	NA	NA	NA	ND (30)	<0.003	NA	NA
PCB #167	0.00001	NA	NA	NA	NA	ND (30)	<0.0003	NA	NA
PCB #189	0.00001	NA	NA	NA	NA	ND (30)	<0.0003	NA	NA
TOTAL TEQ**	ng/g		**76**		**8.0**		**0.56**		**0.092**

ND – not detected; detection limit indicated in brackets, NDR – not detected based on peak ratio (outside ± 15 % of theoretical ratio), NA – not analyzed

* Data from Wellington Laboratories are the mean concentrations in two replicate samples

** Total TEQs are the sum of TEQs concentrations for each compound, and represent the maximum possible TEQs because detection limit concentrations for all non-detected compounds were used in TEQ calculations

2.3.2. CEH-1 study

HCB-P, HCB-O, HCB-N and TCDD induced EROD activity in a concentration-dependent manner in CEH cultures (Figure 2.1; top two panels). Maximal EROD activity was followed by a decrease in activity at the higher concentrations of each of the compounds. This decrease in activity at the higher concentrations was not due to overt toxicity as measured by the Calcein-AM assay (results not shown). The maximal EROD activity was similar for HCB-O and HCB-N (10-11 pmol/min/mg protein; Figure 2.1, Table 2.2), but was lower for HCB-P (7 pmol/min/mg protein; Figure 2.1, Table 2.2). The maximal EROD activity induced by the different lots of HCB was 29- to 45-fold lower than that observed for TCDD (318 pmol/min/mg protein; Figure 2.1, Table 2.2). The three lots of HCB elicited maximal EROD activity at a concentration of 100 nM, whereas TCDD induced maximal EROD activity at 0.03 nM (3333-fold difference between HCB and TCDD; Figure 2.1).

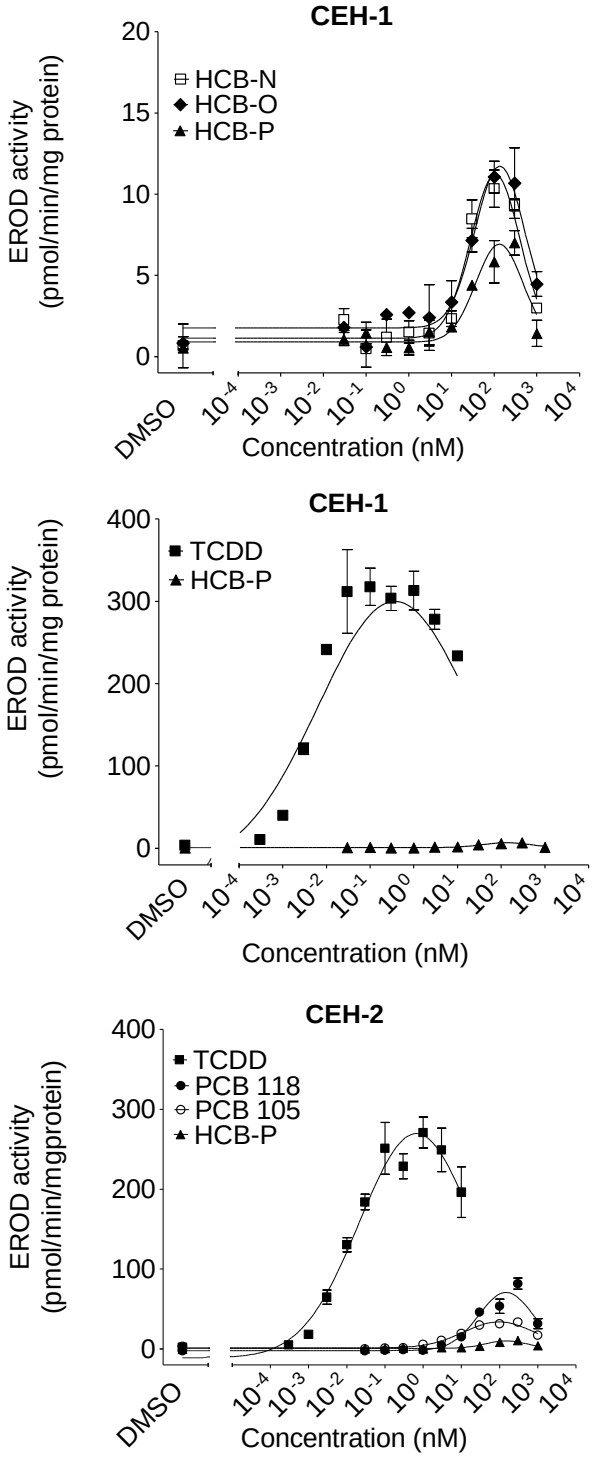

Figure 2.1. Concentration-dependent effects of HCB-P, HCB-O, HCB-N, PCB 105, PCB 118 and TCDD on EROD activity in chicken embryo hepatocytes exposed for 24 h from two separate cell cultures. The top two panels represent EROD activity from the CEH-1 study and the bottom panel represents EROD activity from the CEH-2 study. Points represent mean EROD activity for results obtained from 3 replicate cell culture plates. Each plate received triplicate doses for each compound concentration that was administered. Values before the axis break indicate EROD activity observed for DMSO-treated control. Bars represent standard error.

There were no statistical differences among EC_{50} values obtained for HCB-N, HCB-O and HCB-P (EC_{50} values were between 22 nM and 30 nM; Table 2). The EC_{50} value for TCDD was 0.0048 nM. Based upon EROD EC_{50} values, TCDD was approximately 5000 – 6000 times more potent than either reagent-grade or purified HCB, and the EC_{50}-based RePs were 0.00021, 0.00019 and 0.00016 for HCB-N, HCB-O and HCB-P, respectively. The $EC_{threshold}$-based RePs for the three lots of HCB were 0.0001.

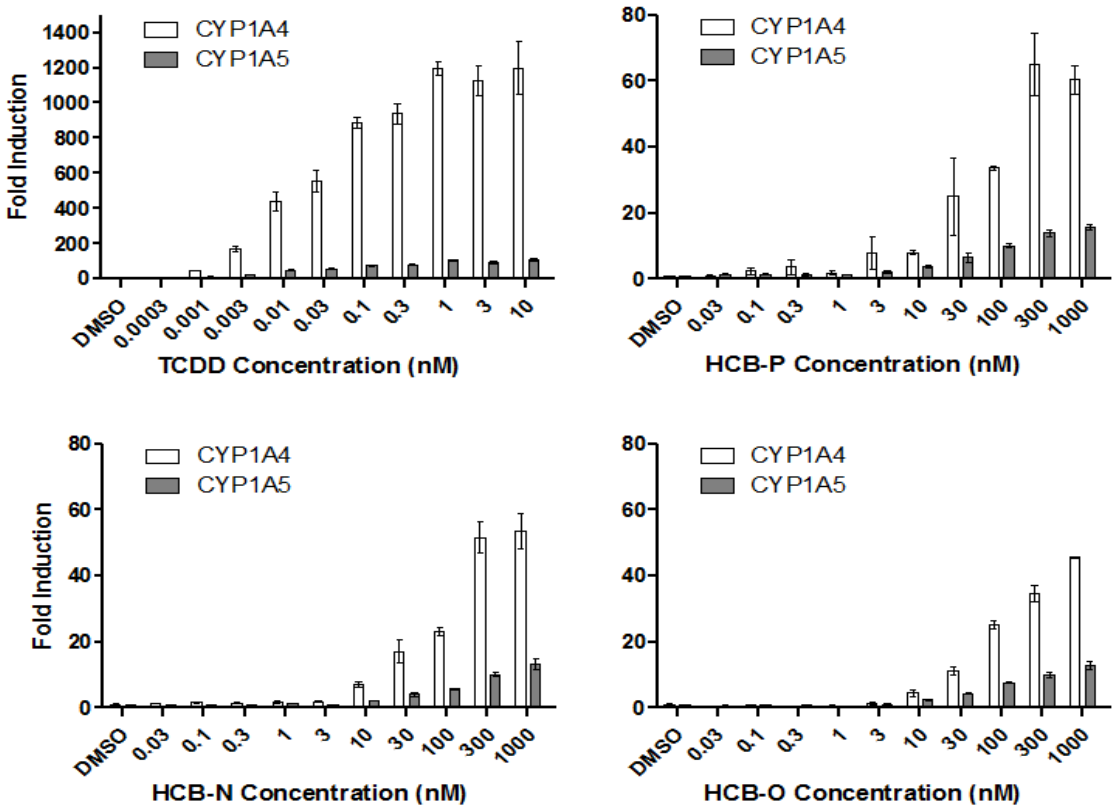

Figure 2.2. Concentration-dependent effects of TCDD, HCB-P, HCB-N and HCB-O on CYP1A4 and CYP1A5 mRNA expression in chicken embryo hepatocytes exposed for 24h in the CEH-1 study. Means and standard errors indicate data obtained from three replicate wells, assessed in duplicate, on a single 48-well cell culture plate.

There were no statistically significant differences in β-actin mRNA expression for all compounds tested over the range of concentrations administered. As such, all reported

differences in mRNA expression were the result of changes in CYP1A4 and 1A5 mRNA expression, and were not caused by changes in the expression of the normalizer gene. Expression of CYP1A4 and 1A5 mRNA was induced in a concentration-dependent manner by TCDD and HCB, but maximum expression of CYP1A4 was greater than that of CYP1A5 (Figure 2.2, Table 2.2). TCDD induced CYP1A4 and 1A5 mRNA to maximum levels of 1200- and 110-fold, respectively. CYP1A4 and 1A5 mRNA levels were maximally induced by the different lots of HCB as follows: HCB-P: 65- and 16-fold; HCB-N: 54- and 13-fold; HCB-O: 46-and 13-fold. The EC_{50}-based RePs for the three lots of HCB for both CYP1A4 and 1A5 mRNA expression were similar. CYP1A4 derived RePs ranged from 0.00025 to 0.0004, whereas RePs for CYP1A5 ranged from 0.000068 to 0.00027. $EC_{threshold}$-based RePs for the three lots of HCB were also similar (0.00033 or 0.0001).

2.3.3. CEH-2 study

The CEH-2 study compared the EROD-inducing potencies of two mono-*ortho* substituted PCBs (PCB 105 and PCB 118) to the potencies of HCB-P and TCDD. Both PCBs caused concentration-response effects that were similar in shape to those of TCDD and HCB (i.e., bell-shaped curves) as observed from both CEH studies (Figure 2.1, lower panel). In addition, the EROD curves for these PCB congeners were similar to those observed in earlier studies (Kennedy et al., 1996a,b). The maximal EROD activity for PCB 105 and 118 was 34 pmol/min/mg and 71 pmol/min/mg, respectively. The maximal activities for HCB-P and TCDD were similar to those found in the CEH-1 study (HCB-P: 10 pmol/min/mg vs.7 pmol/min/mg; TCDD: 270 pmol/min/mg vs. 318 pmol/min/mg). The EC_{50}-based RePs for HCB-P, PCB 105 and PCB 118 were 0.00035, 0.0015 and 0.00057, respectively. The $EC_{threshold}$-based RePs for HCB-P, PCB-105 and PCB118 were 0.0001, 0.001 and 0.0003, respectively.

Consistent with CEH-1 results, expression of both the CYP1A4 and 1A5 mRNAs was induced in a concentration-dependent manner by TCDD and HCB-P. PCB 105 and PCB 118 also induced CYP1A4 and 1A5 mRNAs in a concentration-dependent manner. Maximum expression of CYP1A4 was greater than that of CYP1A5 for all compounds tested (Figure 2.3, Table 2.2). PCB 105 induced CYP1A4 and 1A5 mRNA to a maximum of 208- and 48-fold, respectively, while PCB 118 induced mRNA levels by 680- and 79-fold, respectively. The maximal CYP1A4 and 1A5 mRNA levels induced by TCDD in the CEH-2 study were approximately double the levels in the CEH-1 study, but maximal levels were similar for HCB-P in both studies. The EC_{50}-based and $EC_{threshold}$-based RePs values are summarized in Table 2.2, and are in general

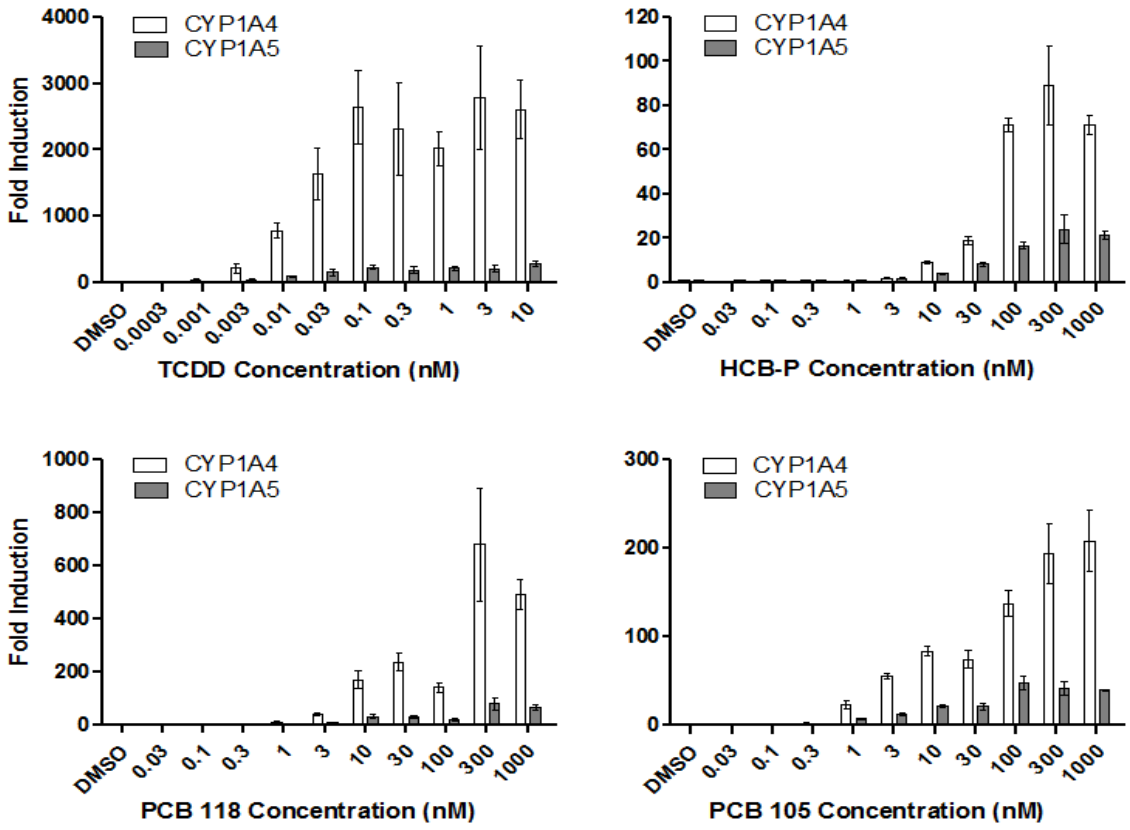

Figure 2.3. Concentration-dependent effects of TCDD, HCB-P, PCB 118 and PCB 105 on CYP1A4 and CYP1A5 mRNA expression in chicken embryo hepatocytes exposed for 24h in the CEH-2 study. Means and standard errors indicate data obtained from three replicate wells on a single 48-well cell culture plate, assessed in duplicate.

concordance with ReP values calculated from EROD concentration-response curves. The EC_{50}-based RePs for HCB-P, PCB 105 and PCB 118 for both CYP1A4 and 1A5 mRNA expression were similar. CYP1A4 derived RePs were 0.00034, 0.001 and 0.00055 for HCB-P, PCB 105, and PCB 118, respectively. Similarly, CYP1A5 derived RePs for said compounds were 0.00028, 0.0015 and 0.00055, respectively. $EC_{threshold}$-based RePs were either 0.00033 or 0.0001 for HCB-P and either 0.0033 or 0.001 for PCB 105 and PCB 118 (Table 2.2).

Table 2.2. EC_{50}, $EC_{threshold}$, ReP values and maximal responses determined from EROD and CYP1A4/5 mRNA data obtained from chicken embryo hepatocyte cultures exposed to TCDD, HCB-N, HCB-O, HCB-P, PCB 105 and PCB 118 for 24 h from two separate cell culture studies (the CEH-1 and CEH-2 studies are denoted with superscripts). Standard errors (±) are indicated.

Compound	End point	EC50 (nM)	EC50-based ReP	ECthr (nM)	ECthr-based ReP	Maximal response (EROD: pmol/min/mg protein, CYP1A4/5: fold induction)
TCDD[1]	EROD	0.00478 ± 0.0020	1.0	0.0010	1.0	318 ± 23
TCDD[1]	CYP1A4	0.0284 ± 0.0073	1.0	0.0010	1.0	1200 ± 150
TCDD[1]	CYP1A5	0.0190 ± 0.0051	1.0	0.0010	1.0	110 ± 8
HCB-N[1]	EROD	22.4 ± 4.3	0.00021	10	0.00010	10 ± 1
HCB-N[1]	CYP1A4	112 ± 32	0.00025	3.0	0.00033	54 ± 5
HCB-N[1]	CYP1A5	279 ± 190	0.000068	10	0.00010	13 ± 2
HCB-O[1]	EROD	25.4 ± 3.7	0.00019	10	0.00010	11 ± 1
HCB-O[1]	CYP1A4	115 ± 16	0.00025	10	0.00010	46 ± 0.2
HCB-O[1]	CYP1A5	105 ± 32	0.00018	10	0.00010	13 ± 1
HCB-P[1]	EROD	29.7 ± 7.3	0.00016	10	0.00010	7 ± 0.8
HCB-P[1]	CYP1A4	71.6 ± 28	0.00040	10	0.00010	65 ± 10
HCB-P[1]	CYP1A5	69.5± 18	0.00027	3.0	0.00033	16 ± 1
TCDD[2]	EROD	0.0118 ± 0.0040	1.0	0.0010	1.0	270 ± 11
TCDD[2]	CYP1A4	0.0175 ± 0.007	1.0	0.0010	1.0	2790 ± 788
TCDD[2]	CYP1A5	0.0136 ± 0.0071	1.0	0.0010	1.0	288 ± 50
HCB-P[2]	EROD	34.5 ± 9.0	0.00034	10	0.00010	10 ± 1
HCB-P[2]	CYP1A4	47.0 ± 7.7	0.00037	10	0.00010	89 ± 18
HCB-P[2]	CYP1A5	48.6 ± 13	0.00028	3.0	0.00033	24 ± 6
PCB-105[2]	EROD	7.90 ± 1.7	0.0015	1.0	0.0010	34 ± 2
PCB-105[2]	CYP1A4	17.6 ± 12	0.0010	0.30	0.0033	208 ± 34
PCB-105[2]	CYP1A5	9.25 ± 3.0	0.0015	0.30	0.0033	48 ± 8
PCB-118[2]	EROD	21.2 ± 4.4	0.00056	3.0	0.0003	71 ± 5
PCB-118[2]	CYP1A4	31.6 ± 15	0.00055	0.30	0.0033	680 ± 212
PCB-118[2]	CYP1A5	24.9 ± 15	0.00055	1.0	0.0010	79 ± 23

2.4. Discussion

For at least 30 years it has not been certain which of the toxic and biochemical effects of HCB were caused by HCB itself, and which effects were caused by PCDD and/or PCDF contaminants in the HCB that was used for multiple *in vivo* and *in vitro* studies (Ezendam *et al.*, 2004; Goldstein, 1979; Pohl et al., 2001; Schwab, 1999; van den Berg et al., 2006; Vos, 2000). Much of the uncertainty has centered on questions regarding the ability of HCB to elicit effects mediated by the AHR, including induction of CYP1A. The purpose of the present study was to prepare highly purified HCB and compare the CYP1A-inducing potencies of highly purified HCB and reagent-grade HCB. We used CEH cultures for this work because this system is useful for measuring CYP1A induction in birds, and because data obtained from CEH cultures were used to help the WHO establish TEF values for avian species (WHO-TEF) (van den Berg et al., 1998). To our knowledge, this is the first study to involve the preparation of highly purified HCB (quantifiably pure enough to rule out any effects attributable to PCDD/F impurities) for the determination of *any* AHR-mediated biochemical response.

Both studies with CEH cultures (CEH-1 and CEH-2) revealed that HCB-P induced EROD activity and CYP1A4/5 mRNA expression. The ReP values for HCB-P, HCB-O and HCB-N were similar. The PCDD, PCDF and dioxin-like PCB data in Table 1 were used to determine the maximum possible TCDD concentrations and TEQs in CEH cultures that were dosed with graded concentrations of HCB. These concentrations are indicated in columns 4 and 5 of Table 2.3. For example, the maximum TCDD concentration in HCB-P ranged from 1.0E-09 nM to 1.0E-07 nM over the range of HCB-P concentrations (10 nM – 1000 nM) that were administered to CEH cultures (Table 2.3, column 5). The lowest concentration of TCDD that elicited EROD activity above basal activity was 1.0E-03 nM (Figure 1, centre and lower panels).

Therefore the concentration of TCDD in HCB was 1.0E-06-fold lower (1.0E-03 ÷ 1.0E-09 = 1.0E-06) than that required to cause EROD induction. Even at the highest concentration of HCB administered to CEH cultures (1000 nM), TCDD was 1.0E-04-fold lower (1.0E-03 ÷ 1.0E-07 = 1.0E-04) than the concentration needed to cause EROD induction. Therefore, TCDD concentrations and total TEQs were too low in all three lots of HCB to elicit an EROD response. Additional clarification in the form of figures and calculations are included in Appendix 1.

Table 2.3. Maximum possible TCDD and TEQ concentrations (indicated in the 4th and 5th columns, respectively) in HCB-P, HCB-O and HCB-N that were administered to CEH cultures at the concentrations indicated in column 3 (10 nM -10000 nM). Sample calculation: Max. TEQ for HCB-P administered at 10 nM = 5.6E-10 X 10 nM = 5.6E-09 nM.

Max. [TCDD] in HCB-P* (fractional amount)	Max. [TEQ] in HCB-P* (fractional amount)	[HCB-P] in CEH nM	Max. [TCDD] in CEH nM	Max. [TEQ] in CEH nM
1E-10	5.60E-10	10	1.00E-09	5.60E-09
1E-10	5.60E-10	30	3.00E-09	1.68E-08
1E-10	5.60E-10	100	1.00E-08	5.60E-08
1E-10	5.60E-10	300	3.00E-08	1.68E-07
1E-10	5.60E-10	1000	1.00E-07	5.60E-07
Max. [TCDD] in HCB-O* (fractional amount)	**Max. [TEQ] in HCB-O* (fractional amount)**	**[HCB-O] in CEH nM**	**Max. [TCDD] in CEH nM**	**Max. [TEQ] in CEH nM**
1E-10	8.00E-09	10	1.00E-09	8.00E-08
1E-10	8.00E-09	30	3.00E-09	2.40E-07
1E-10	8.00E-09	100	1.00E-08	8.00E-07
1E-10	8.00E-09	300	3.00E-08	2.40E-06
1E-10	8.00E-09	1000	1.00E-07	8.00E-06
Max. [TCDD] in HCB-N* (fractional amount)	**Max. [TEQ] in HCB-N* (fractional amount)**	**[HCB-N] in CEH nM**	**Max. [TCDD] in CEH nM**	**Max. [TEQ] in CEH nM**
5.10E-09	7.60E-08	10	5.10E-08	7.60E-07
5.10E-09	7.60E-08	30	1.53E-07	2.28E-06
5.10E-09	7.60E-08	100	5.10E-07	7.60E-06
5.10E-09	7.60E-08	300	1.53E-06	2.28E-05
5.10E-09	7.60E-08	1000	5.10E-06	7.60E-05

* These data are from Table 1

ReP values are generally calculated using only EC_{50} values, and are ideally obtained from comparisons made with parallel EROD curves that share the same maximal response. The present study, however, revealed that HCB, PCB 105 and PCB 118 all elicited lower maximal EROD activity than TCDD. As such, the overestimation of a compound's potency can occur when a compound elicits lower maximal EROD activity than TCDD. When only EC_{50} values are applied, the lower maximal EROD activity causes a leftward shift of the EC_{50} for HCB, PCB 105 and PCB 118, thereby creating an overestimation of the potency for these compounds (Hahn et al., 1993; Head and Kennedy, 2007b; Hervé et al., 2010b; Hestermann et al., 2000). Therefore, ReP values were calculated by comparing both EC_{50} and $EC_{threshold}$ values in an attempt to obtain a less biased estimate of ReP. As mentioned above, the $EC_{threshold}$ value represents the first concentration at which a compound produces a statistically significant ($p < 0.05$) response (EROD activity or CYP1A4/5 mRNA expression) that is greater than the DMSO control group.

The inherent difference in relative potency and maximal EROD activity of HCB, PCB 105 and PCB 118 relative to TCDD may be partially explained by the affinity and efficacy of the ligand-receptor interaction (Hestermann et al., 2000; Jenkinson et al., 1995). Ligands with a lower binding affinity for the AHR will be less potent and may have a lower maximal response than strong AHR agonists such as TCDD (Hestermann et al., 2000; Poland and Knutson, 1982**)**. Additionally, competitive inhibition can also lead to a decrease in maximal EROD activity. At high concentrations of inducer, dioxin-like compounds compete with ethoxyresorufin for the CYP1A4 active site and inhibit enzymatic activity. When inhibition is much stronger than binding affinity, an overestimate of sensitivity is likely (Head and Kennedy 2007b; Petrulis and Bunce, 1999). Empirical evidence exists demonstrating that HCB, PCB 105, and PCB 118 have been shown to possess lower affinity for the AHR than TCDD (Hahn et al., 1989; Kafafi et al.,

1993a; Kafafi et al., 1993b). Hahn et al. (1989) found that HCB had an affinity for rat AHR that was approximately 10,000 times less than TCDD, and showed that HCB was able to competitively inhibit TCDD-AHR binding at higher concentrations (K_I = 2.1 µM HCB). Although the detection limit used by Hahn et al. (1989) to quantify the absence of PCDD/F impurities within the HCB sample was 1000 times higher than that used here, both the Hahn study and the present study suggests that CYP1A induction by HCB is mediated by the AHR. Additionally, on-going research to determine the carcinogenic and porphyrinogenic effects of HCB in humans and experimental mammal models have also shed light on HCBs ability to act through the AHR (Gorman et al., 2007; Smith and Elder, 2010). Traditionally CYP1A induction has been widely used as a biomarker for dioxin-like toxicity based on a strong correlation observed between AHR binding affinity, CYP1A induction, and dioxin-like toxicity (Behnisch et al., 2001; Behnisch et al., 2002; Head and Kennedy, 2010; Hu et al., 2007). While it is plausible that the observed effects are attributed to HCB's intrinsic ability to bind to the AHR, CYP1A is a non-specific indicator of AHR activation (Hu et al., 2007). In all likelihood, binding assays will need to be repeated using highly purified HCB to truly determine whether HCB binds to the AHR.

ReP values calculated with EC_{50} and $EC_{threshold}$ were similar, however the $EC_{threshold}$-based RePs were generally lower than those obtained from using EC_{50} values. The $EC_{threshold}$-based RePs for highly purified HCB, in both studies, range from 0.0001 to 0.00033 based on the endpoints examined. Thus, we suggest that the appropriate ReP for HCB in chicken embryo hepatocytes cultures is 0.0001 because this is the most conservative estimate of the ReP values obtained. This $EC_{threshold}$-based ReP value also incorporates, to a degree, the major difference in HCB's maximal EROD activity compared to TCDD, unlike EC_{50}-based ReP values.

Nonetheless, this value may still be overestimating the true ReP for HCB based on these obvious differences in maximal EROD activity and CYP1A4/5 mRNA expression compared to TCDD. CYP1A4/5 mRNA results are well reflected by EROD activity. RePs calculated from mRNA results reinforce the suggestion of a ReP of 0.0001 for highly purified HCB. A ReP value of 0.0001 in CEH cultures is consistent with the TEF suggested by van Birgelen for HCB (1998), but studies with other systems and organisms are needed to obtain a TEF. While TEF values are determined through a scientific consensus-building process, the ReP calculated herein provides a useful estimate of the potency of highly purified HCB in one *in vitro* system. Moreover, ReP values determined by CYP1A induction in cultured avian hepatocytes appear to be predictive for the relative toxicity in the developing embryo (Head and Kennedy, 2010; Kennedy et al., 1996a; van den Berg et al., 1998). *In vitro* techniques are valuable prescreening tools that provide direction for subsequent *in vivo* experimentation in order to obtain sufficient evidence to generate reliable TEFs that are widely accepted for regulatory purposes. Egg injection and other *in vivo* studies with highly purified HCB are clearly warranted.

In summary, this study confirmed that reagent-grade HCB was contaminated with PCDDs, PCDFs, and dioxin-like PCBs. However, the presence of dioxins and furans did not have an observed effect on CYP1A4/5 induction using the endpoints measured. Highly purified HCB also increased both EROD activity and CYP1A4/5 mRNA expression in a concentration-dependent manner in CEHs. Based upon a comparison of the EC_{50} and $EC_{threshold}$ values for EROD and CYP1A concentration-response curves, the potency of HCB relative to the potency of TCDD was 0.0001. Additionally, HCB elicited similar EROD activity and CYP1A4/5 mRNA expression to that of two mono-*ortho* substituted PCBs, 105 and 118. Both PCB 105 and 118 are weak AHR agonists that have been characterized as dioxin-like compounds and have avian TEFs

of 0.0001 and 0.00001, respectively. These TEF values are consistent with the ReP value for HCB obtained in the present study. We recommend that future work should be directed to expose different avian species to highly purified HCB both *in vitro* and *in vivo* in order to provide RePs for HCB in birds. The methods to purify and analyze HCB for trace levels of PCDD/Fs described in this paper indicate how such work could be conducted.

Chapter 3:

Induction of cytochrome P4501A by highly purified hexachlorobenzene in primary cultures of ring-necked pheasant and Japanese quail embryo hepatocytes

3.1. Introduction

The toxic equivalency (TEF) concept was developed to facilitate risk assessments for polychlorinated dibenzo-*p*-dioxin (PCDD), polychlorinated dibenzofuran (PCDF) and polychlorinated biphenyl (PCB) mixtures by relating the toxicity of these dioxin-like compounds (DLCs) to the toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). In 2005, the World Health Organization (WHO) reevaluated human and mammalian TEFs for dioxins and dioxin-like compounds and also addressed the possible inclusion of other chlorinated compounds and brominated compounds into the TEF scheme. The brominated compounds considered were polybrominated dibenzo-*p*-dioxins and furans, polybrominated biphenyls, polybrominated diphenyl ethers and polybrominated naphthalenes. The chlorinated compounds considered were chlorinated naphthalenes, 3,4,4'-trichlorobiphenyl (PCB 37) and hexachlorobenzene (HCB) (van den Berg *et al.* 2006).

As indicated in Chapter 1, multiple studies have suggested that HCB causes dioxin-like effects, including induction of hepatic cytochrome P4501A (CYP1A), uroporphyria and thymic atrophy (Goldstein *et al.* 1978, 1982; Li *et al.* 1989; Linko *et al.* 1986). In addition, HCB was reported to bind to the AHR (Hahn et al., 1989). In 1998 it was suggested that HCB should be included within the TEF concept (van Birgelen, 1998). However, this recommendation has not been accepted by the WHO or the U.S. EPA. Perhaps the most important reason why HCB is not currently included within the TEF concept is because some, if not all, of the HCB used for AHR binding studies and toxicology studies was contaminated with trace concentrations of PCDDs,

PCDFs and dioxin-like PCBs (Goldstein, 1979; van den Berg et al., 2006). Difficulties associated with the possible inclusion of HCB within the TEF concept will not be resolved until *in vivo* and *in vitro* studies are conducted with HCB that is sufficiently free of trace levels of PCDDs, PCDFs and/or PCBs to allow scientific consensus on the toxic and biochemical properties of HCB itself.

The first HCB study of my thesis (Chapter 2) determined the concentration-dependent effects of highly purified (HCB-P) and reagent-grade HCB (RG-HCB) on CYP1A4 messenger RNA (mRNA) and CYP1A5 mRNA expression and ethoxyresorufin *O*-deethylase (EROD) activity in chicken (*Gallus gallus domesticus*) embryo hepatocyte (CEH) cultures. That study found that the concentration-dependent effects of HCB-P and RG-HCB in CYP1A4/5 mRNA expression and EROD activity were similar, and the relative potency (ReP; potency of HCB compared to the potency of TCDD) was 0.0001 (Chapter 2; Mundy *et al.* 2010). Chicken was chosen to investigate the effects of highly purified HCB because of three prevailing factors: 1) chicken has been shown to be the most sensitive species to DLC exposure (Head *et al.* 2008), 2) the majority of avian TEFs are derived from chicken (van den Berg *et al.* 1998), 3) RePs ranging from 0.00009 to 0.0002 had been previously estimated in CEH using unpurified HCB and EROD activity as an endpoint (Sinclair *et al.* 1997b).

While the TEF concept was created to facilitate the risk assessment of DLC mixtures in the environment it is not infallible (general issues associated with the concept are reviewed briefly in Chapter 1). As such, the experimental work carried out following the CEH study was done in large part to address a few of these issues with one major concern being that the majority of avian RePs and TEFs are derived using only chicken. Differences in sensitivity to DLCs

among avian species are apparent and as such, additional species should be tested in order to get a better understanding of how HCB may adversely affect wild birds.

The mechanism by which avian species elicit differential sensitivity to DLCs is not completely understood, but evidence of a molecular basis has been suggested. Karchner et al. (2006) recently uncovered a mechanistic link between the amino acid sequence of the AHR ligand binding domain (AHR LBD) and the degree to which the receptor is activated by TCDD. The chicken was shown to possess a high affinity receptor for TCDD, whereas the common tern (*Sterna hirundo*) was found to have a low affinity receptor. It was determined that two key amino acid sites are crucial in dictating the difference in TCDD binding affinity with the AHR. Residues in chicken (Ile324 and Ser380) differed from those found in common tern (Val324 and Ala380). Head et al. (2008) elaborated on this concept and found that differences at these two residue sites are sufficient for predicting categories of TCDD sensitivity in a number of different of avian species. The chicken's Ile/Ser genotype is generally considered the most sensitive, while the ring-necked pheasant's (*Phasianus colchicus*) Ile/Ala and the Japanese quail's (*Cortunix japonica*) Val/Ala genotypes are classified as moderately and least sensitive to DLCs, respectively.

The present study expanded on the work done with CEH with the goal of extending our findings to other bird species. Primary cultures of ring-necked pheasant and Japanese quail embryo hepatocytes (PEH and JEH, respectively) were used to determine if HCB-P exposure induced EROD activity and CYP1A4/5 mRNA expression. As mentioned, ring-necked pheasant and Japanese quail were used based their different AHR LBD genotypes and because of their differential sensitivity *in vitro* and *in ovo* to DLCs (Cohen-Barnhouse *et al.* 2011; Head *et al.* 2008; Hervé *et al.* 2010b, Yang *et al.* 2010).

3.2. Materials and Methods

3.2.1 HCB Analysis and Purification

Wellington Laboratories Inc. (Guelph, ON, Canada) analyzed reagent-grade HCB (RG-HCB; stated purity of 97%: Aldrich Cat. #17,105-0; lot number: 2104EJ) for contamination by PCDDs, PCDFs, non-*ortho* substituted PCBs and mono-*ortho* substituted PCBs. A complete description of the analysis and purification of RG-HCB is provided in detail in Chapter 2. It should be noted that RG-HCB referred to in this chapter is synonymous with HCB-Old (HCB-O) from Chapter 2. Since there were no observed differences in the relative potencies of the two lots of reagent-grade HCB (HCB-N and HCB-O) employed in Chapter 2 it seemed logical to continue experimentation using only one batch of reagent-grade HCB. HCB-O was selected because it was purified to yield HCB-P.

3.2.2. Preparation of TCDD and HCB solutions

Detailed accounts of how TCDD and HCB stock and serial solutions were prepared are described in Chapter 2.

3.2.3. Preparation and dosing of cultured hepatocytes

Primary cultures of ring-necked pheasant and Japanese quail hepatocytes were prepared as described in detail elsewhere (Kennedy *et al.* 1995) including subsequent modifications (Head and Kennedy 2007b; Kennedy *et al.* 2003). All procedures were conducted according to protocols approved by the Animal Care Committee at the National Wildlife Research Centre. Reagents were obtained from Sigma (St. Louis, MO, USA) unless otherwise specified.

Fertilized, un-incubated ring-necked pheasant and Japanese quail eggs were obtained from Couvoir Simetin (Mirabel, QC, Canada) and were incubated at 37.5 °C and 60% relative humidity for 1-2 days pre-hatch (23 and 16 days for pheasant and quail, respectively). Eggs were

candled periodically throughout development and removed from the incubator if found to be unfertilized or to contain dead embryos. In brief, 51 ring-necked pheasant and 85 Japanese quail embryos were euthanized 1-2 days pre-hatch by decapitation; livers were removed and pooled. Hepatocyte cultures were prepared from these pools of livers using collagenase digestion, filtration, and centrifugation as described in detail in Chapter 2 and elsewhere (Head *et al.* 2006). Serial dilutions of DMSO solutions (2.5 µl/well) of TCDD, RG-HCB or HCB-P were added to hepatocytes. TCDD was administered such that concentrations in cell culture medium ranged from 0.0003 nM to 10 nM, whereas HCB concentrations ranged from 0.03 nM to 1000 nM. Plates were returned to the incubator for 24 h exposure at the same conditions indicated in Chapter 2. Following this 24 h exposure, cell culture medium was removed and plates were flash-frozen in powdered dry ice and stored at -80 °C until the time of analysis. Plates used for EROD assays were rinsed with approximately 200 µl/well of phosphate buffered saline-ethylenediaminetetraacetic acid (PBS-EDTA) prior to being flash-frozen.

3.2.4. Cell Viability

Cell viability was determined using the Calcein-AM assay (Invitrogen-Molecular Probes, Eugene, OR, USA) and was conducted exactly as described in Chapter 2.

3.2.5. EROD assays

EROD assays were conducted as described in Chapter 2.

3.2.6. RNA isolation and complementary DNA synthesis

The protocol used to isolate RNA and synthesize complementary DNA (cDNA) from ring-necked pheasant and Japanese quail embryo hepatocytes was the same method described for chicken embryo hepatocytes in Chapter 2.

3.2.7. Quantitative polymerase chain reaction (QPCR)

Multiplex QPCR assays using dual-labeled fluorescent hydrolysis probes (Hervé *et al*. 2010b) were used to quantify ring-necked pheasant and Japanese quail CYP1A4, CYP1A5 and β-actin (normalizer gene) mRNA abundance. With the exception of primers and probes, all QPCR reactions were carried out using reagents acquired from Stratagene and either the Stratagene Mx3000P or Mx3005 instrument (Stratagene, La Jolla, CA, USA). All primers (Invitrogen) and probes (Biosearch, Novato, CA, USA) were designed and validated as previously described (Hervé et al. 2010). Taqman reactions were performed using Brilliant QPCR Core Reagent kits using the same reaction conditions as in Chapter 2.

3.2.8. EROD and CYP1A4/5 mRNA data analysis

Data analysis for EROD activity and CYP1A4/5 mRNA expression was conducted as described in Chapter 2.

3.2.9. Calculation of relative potency and relative sensitivity

The concept of using relative potencies to compare the potencies of dioxin-like compounds to TCDD is well established (van den Berg *et al.* 1998; U.S. EPA 2008). Relative potency (ReP) is based on a single *in vitro* or *in vivo* study of the potency, relative to TCDD, of an individual chemical to cause a particular AHR-mediated toxicological or biological effect. In the present study, ReP is defined as EC_{50} (or $EC_{threshold}$) of TCDD ÷ EC_{50} (or $EC_{threshold}$) of HCB.

A measure of relative sensitivity (ReS) among species was calculated. Because chicken is generally considered the most sensitive species to dioxin-like compounds (Head and Kennedy 2008), comparisons were made with chicken data. ReS is defined as EC_{50} or ($EC_{threshold}$) of compound *x* in chicken ÷ EC_{50} or ($EC_{threshold}$) of compound *x* in the species of interest. Chicken

EROD and CYP1A4/5 mRNA data (Chapter 2; Mundy et al., 2010) were used herein to calculate ReS values.

3.3. Results

3.3.1. Concentration-dependent effects of TCDD and HCB on EROD activity

General Observations

TCDD, HCB-P and RG-HCB induced EROD activity in a concentration-dependent manner in both PEH and JEH (Figure 3.1.). Maximal EROD activity was followed by a decrease in activity in cell exposed to the higher concentrations of each compound. This decrease in activity at the higher concentrations of HCB and TCDD was not due to overt toxicity, as measured by the Calcein-AM assay (results not shown). The maximal EROD activity elicited by TCDD and HCB was lower in PEH (174 and 46-66 pmol/min/mg protein for TCDD and HCB, respectively; Figure 3.1., Table 3.1.) than in JEH (280 and 142-167 pmol/min/mg protein for TCDD and HCB, respectively Figure 3.1., Table 3.2.). A paired t-test was used to compare EROD activity induced by RG-HCB and HCB-P in PEH and JEH at the concentrations administered. Maximal EROD activity did not statistically differ for RG-HCB and HCB-P in the hepatocytes of either species ($p > 0.05$).

Intercompound comparisons: relative potencies of TCDD and HCB

The maximal EROD activity induced by RG-HCB in PEH (46 pmol/min/mg protein) was lower than that induced by HCB-P (66 pmol/min/mg protein). There was no statistical difference among EC_{50} values obtained for RG-HCB and HCB-P (23.6 nM vs 21.8 nM; Table 3.1.). The EC_{50} value for TCDD was 0.045 nM. As such, the EC_{50}-based RePs were 0.0019 and 0.0021 for RG-HCB and HCB-P, respectively. $EC_{threshold}$ values for RG-HCB and HCB-P were 10 nM and

3nM, respectively, resulting in an $EC_{threshold}$ -based RePs of 0.0003 for RG-HCB and 0.001 for HCB-P.

In JEH, the maximal EROD activity induced by RG-HCB (167 pmol/min/mg protein) was higher than that induced by HCB-P (142 pmol/min/mg protein). There was no statistical difference among EC_{50} values obtained for RG-HCB and HCB-P (6.9 nM vs 6.8 nM; Table 3.2.). The EC_{50} value for TCDD was 0.081 nM. Therefore, EC_{50}-based RePs were 0.012 for both RG-HCB and HCB-P. $EC_{threshold}$ values were 3 nM for both RG-HCB and HCB-P. The$EC_{threshold}$-based ReP for both RG-HCB and P-HCB was 0.003.

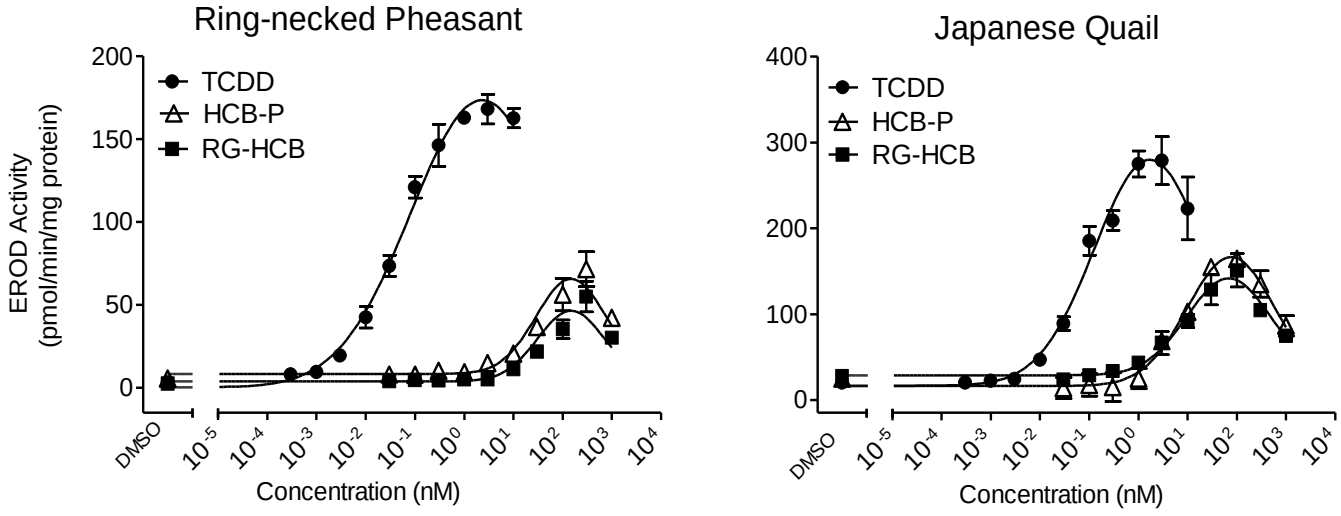

Figure 3.1. Concentration-dependent effects of TCDD, HCB-P and RG-HCB on EROD activity in ring-necked pheasant and Japanese quail embryo hepatocytes exposed to the compounds for 24 h. Points represent mean EROD activity for results obtained from 3 replicate cell culture plates. Each plate received triplicate doses of each concentration of TCDD, HCB-P and RG-HCB indicated. Values before the axis break indicate EROD activity observed for DMSO-treated control. Bars represent standard error.

3.3.2. Concentration-dependent effects of TCDD and HCB on CYP1A4/5 mRNA expression

There were no statistically significant differences in β-actin mRNA expression in hepatocytes treated with HCB or TCDD. Therefore all reported differences in mRNA expression were the result of changes in CYP1A4 and 1A5 mRNA expression, and were not caused by changes in the expression of the normalizer gene. Expression of CYP1A4/5 mRNA was induced in a concentration-dependent manner by TCDD and HCB, but maximum expression of CYP1A4 mRNA was greater than that of CYP1A5 mRNA in all cases (Figure 3.2.).

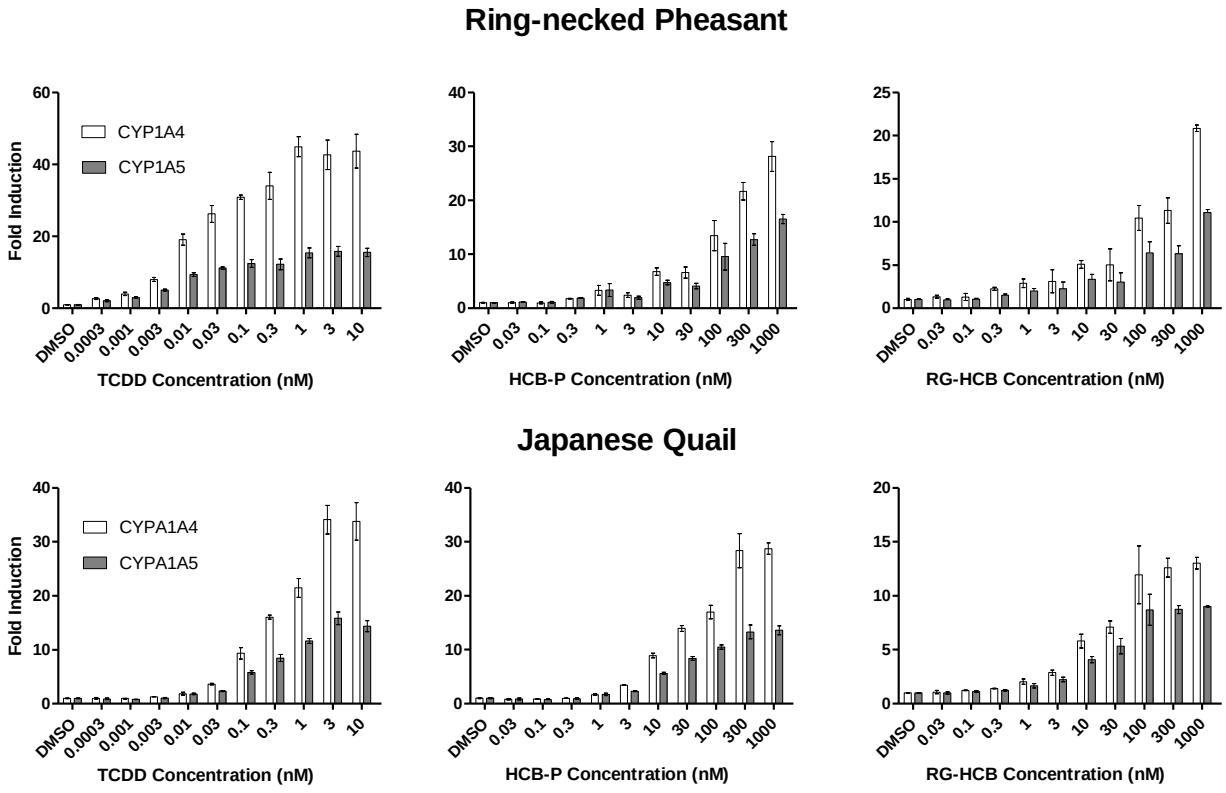

Figure 3.2. Concentration-dependent effects of TCDD, HCB-P and RG-HCB on CYP1A4 and CYP1A5 mRNA expression in ring-necked pheasant and Japanese quail embryo hepatocytes exposed for 24h. Means and standard errors indicate data obtained from three replicate wells, assessed in duplicate, on a single 48-well cell culture plate.

In ring-necked pheasant hepatocytes, TCDD induced CYP1A4/5 mRNA to maximum levels of 45- and 16-fold, respectively. HCB-P induced CYP1A4 and CYP1A5 to a maximum of 28- and 17-fold, respectively, while RG-HCB induced mRNA levels by 21- and 11-fold, respectively (Figure 3.2.). The EC_{50}-based RePs for RG-HCB and HCB-P for both CYP1A4 and 1A5 mRNA expression were similar. CYP1A4 derived RePs ranged from 0.00015 to 0.00021 for RG-HCB and HCB-P, respectively. RePs obtained from CYP1A5 concentration-response curves were 0.000083 and 0.00011 for RG-HCB and HCB-P, respectively. $EC_{threshold}$-based RePs were 0.001 for CYP1A4/5 mRNA expression for RG-HCB and HCB-P in ring-necked pheasant embryo hepatocytes (Table 3.1.).

Table 3.1. EC_{50}, $EC_{threshold}$, ReP values and maximal response calculated from EROD and CYP1A4/5 mRNA data obtained from ring-necked pheasant embryo hepatocyte cultures exposed to TCDD, RG-HCB and HCB-P for 24 h. Standard errors (±) are indicated.

Compound	End point	EC50 (nM)	EC50-based ReP	Ecthr (nM)	ECthr-based ReP	Maximal response (EROD: pmol/min/mg protein, CYP1A4/5: fold induction)
TCDD	EROD	0.0451 ± 0.0076	1.0	0.0030	1.0	170 ± 3.6
TCDD	CYP1A4	0.0224 ± 0.0060	1.0	0.00030	1.0	45 ± 2.8
TCDD	CYP1A5	0.0101 ± 0.0032	1.0	0.00030	1.0	16 ± 1.3
RG-HCB	EROD	23.6 ± 6.3	0.0019	10.0	0.00030	46 ± 3.8
RG-HCB	CYP1A4	152 ± 31	0.00015	0.30	0.0010	21 ± 0.37
RG-HCB	CYP1A5	122 ± 33	0.000083	0.30	0.0010	11 ± 0.34
HCB-P	EROD	21.8 ± 5.0	0.0021	3.0	0.0010	66 ± 4.2
HCB-P	CYP1A4	108 ± 16	0.00021	0.30	0.0010	28 ± 2.8
HCB-P	CYP1A5	94.6 ± 18	0.00011	0.30	0.0010	17 ± 0.87

In Japanese quail hepatocytes, TCDD induced CYP1A4/5 mRNA to maximum levels of 34- and 16-fold, respectively. CYP1A4/5 mRNA levels were induced by purified and reagent-grade HCB as follows: HCB-P: 29- and 14-fold; RG-HCB: 13- and 9 fold (Figure 3.2., Table 3.2.). The EC_{50}-based RePs for RG-HCB and HCB-P for both CYP1A4/5 mRNA expression were similar. CYP1A4 derived RePs ranged from 0.01 (HCB-P) to 0.019 (RG-HCB). RePs for CYP1A5 were 0.013 (HCB-P) and 0.015 (RG-HCB), consistent with the observed EROD EC_{50}-based RePs (Table 3.2.). $EC_{threshold}$-based RePs for HCB were 0.01 for CYP1A4/5 mRNA expression for RG-HCB and HCB-P in Japanese quail embryo hepatocytes (Table 3.2.).

Table 3.2. EC_{50}, $EC_{threshold}$, ReP values and maximal response calculated from EROD and CYP1A4/5 mRNA data obtained from Japanese quail embryo hepatocyte cultures exposed to TCDD, RG-HCB and HCB-P for 24 h. Standard errors (±) are indicated.

Compound	End point	EC50 (nM)	EC50-based ReP	Ecthr (nM)	ECthr-based ReP	Maximal response (EROD: pmol/min/mg protein, CYP1A4/5: fold induction)
TCDD	EROD	0.0807 ± 0.017	1.0	0.010	1.0	280 ± 9.6
TCDD	CYP1A4	0.377 ± 0.056	1.0	0.010	1.0	34 ± 2.6
TCDD	CYP1A5	0.266 ± 0.035	1.0	0.010	1.0	16 ± 1.2
RG-HCB	EROD	6.82 ± 1.3	0.012	3.0	0.0030	170 ± 6.9
RG-HCB	CYP1A4	19.5 ± 4.2	0.019	1.0	0.010	13 ± 0.55
RG-HCB	CYP1A5	18.3 ± 3.2	0.015	1.0	0.010	9.0 ± 0.055
HCB-P	EROD	6.86 ± 1.6	0.012	1.0	0.0030	140 ± 6.8
HCB-P	CYP1A4	36.4 ± 5.7	0.010	1.0	0.010	29 ± 1.1
HCB-P	CYP1A5	20.1 ± 2.4	0.013	1.0	0.010	14 ± 0.83

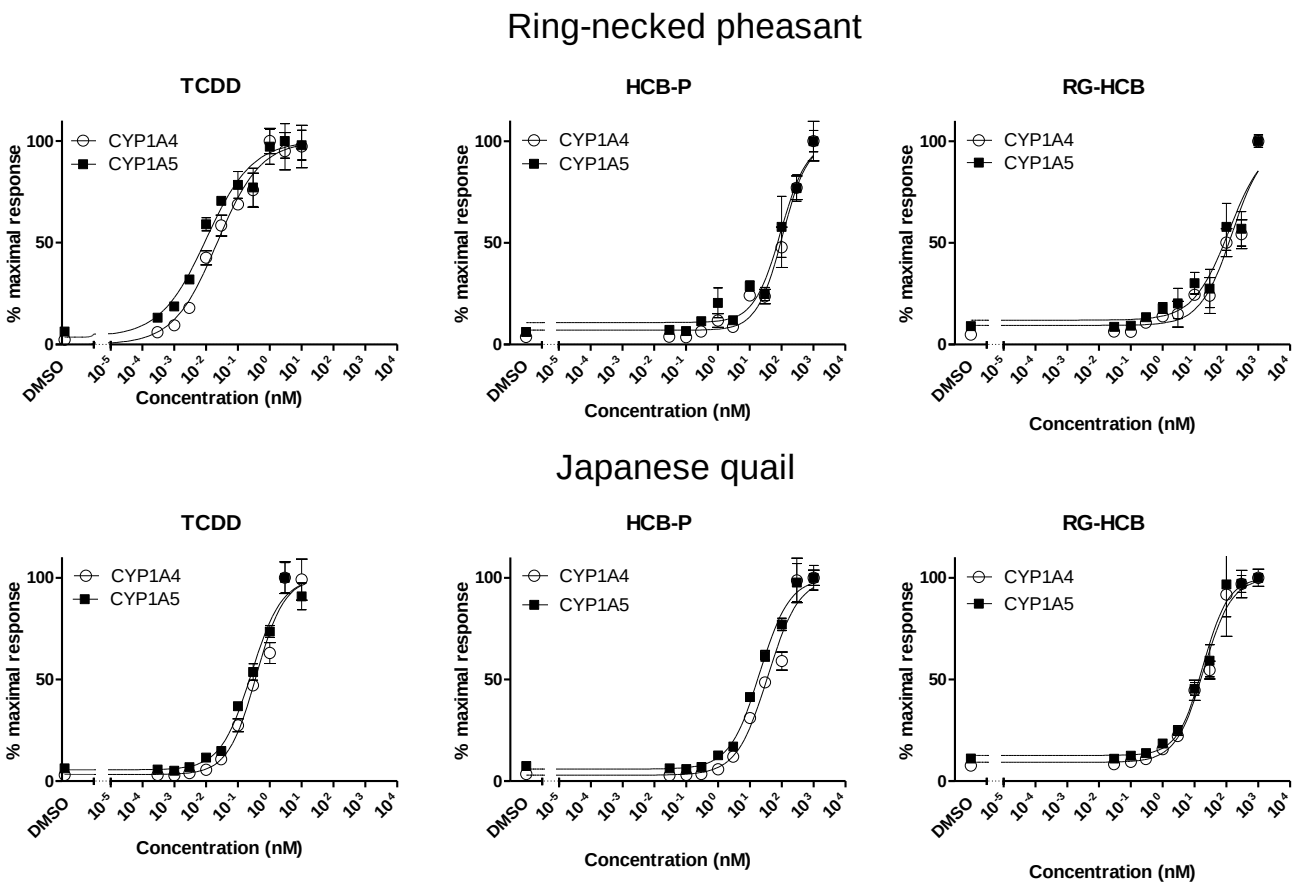

Figure 3.3. Concentration-dependent effects of TCDD, HCB-P and RG-HCB on CYP1A4 mRNA expression (○) and CYP1A5 mRNA expression (■) in ring-necked pheasant and Japanese quail embryo hepatocytes cultures exposed for 24 h. Data are expressed as percent maximal response for comparative purposes. For mRNA expression, each point represents the mean of three replicate wells, assessed in duplicate, from a single 48-well cell culture plate. Bars represent standard error of the means.

Table 3.3. Maximal EROD response, EC_{50}, EC_{thr}, and ReS values calculated from EROD data obtained from chicken, ring-necked pheasant and Japanese quail embryo hepatocyte cultures exposed to TCDD or HCB-P for 24 h. Standard errors (±) are indicated.

Compound	Species	EC_{50} (nM)	EC_{50}-based ReS	EC_{thr} (nM)	EC_{thr}-based ReS	Maximal EROD response (pmol/min/mg protein)
TCDD	Chicken	0.0048 ± 0.002	1.0	0.0010	1.0	320 ± 23
TCDD	R-n. pheasant	0.045 ± 0.008	0.10	0.0030	0.30	170 ± 3.6
TCDD	J. Quail	0.081 ± 0.02	0.060	0.010	0.10	280 ± 9.6
HCB-P	Chicken	29.7 ± 7.3	1.0	10.0	1.0	7.0 ± 0.8
HCB-P	R-n. pheasant	21.8 ± 5.0	1.4	3.0	3.3	66 ± 4.2
HCB-P	J. Quail	6.86 ± 1.6	4.4	3.0	3.3	140 ± 6.8

*Values for chicken embryo hepatocytes are from CEH-1 in Chapter 2

3.4. Discussion

The purpose of the present study was to compare the CYP1A-inducing potencies of highly purified HCB (all PCDD/Fs were < 0.2 ppb; non-*ortho* substituted PCBs were <0.03 ppb; mono-*ortho* substituted PCBs were < 130 ppb) and reagent-grade HCB in primary cultures of ring-necked pheasant and Japanese quail embryo hepatocytes. Comparisons of sensitivities between these species and chicken (Chapter 2) exposed to HCB was also conducted. As previously mentioned, avian embryo hepatocyte cultures were used for this work because the relative potencies of DLCs in hepatocytes are predictive of *in ovo* toxicity in birds (Head and Kennedy 2010; Kennedy *et al.* 1996a). Furthermore, hepatocytes from these three species of Galliformes (chicken, ring-necked pheasant, Japanese quail) were used for the present study because these species are useful for defining differential toxic and biochemical sensitivity to DLCs (Cohen-Barnhouse *et al.* 2011; Head *et al.*, 2008; Hervé *et al*., 2010a; Hervé *et al*., 2010b). The highest possible TEQ concentration present within purified HCB at the highest concentration administered to hepatocytes (1000 nM) was 10,000 times lower than the

concentration needed to elicit EROD induction. TCDD concentrations and total TEQs were also too low in reagent-grade HCB to elicit an EROD response. A detailed description of the analytical chemistry, HCB purification, and evidence that PCDD/F impurities played no role in the effects of HCB-P in CEH is provided in Chapter 2 and Appendix 1.

ReP values are often calculated using only EC_{50} values from both EROD and CYP1A4/5 mRNA concentration-response curves. From EROD curves, EC_{50} values are ideally obtained from comparisons made with parallel curves that share the same maximal response (Kennedy *et al.* 1996a). The present study, however, revealed that HCB-P and RG-HCB elicited lower maximal EROD activity than TCDD in both PEH and JEH. If only EC_{50} values are applied for HCB, the lower maximal EROD activity causes a leftward shift of the EC_{50}, resulting in an overestimation of HCB's ReP (Hahn *et al.* 1993; Hestermann *et al.* 2000). To mitigate such issues, ReP values were calculated using both EC_{50} and $EC_{threshold}$ values; greater emphasis in our conclusions was placed on $EC_{threshold}$ values. As previously defined, the $EC_{threshold}$ value represents the first concentration at which a compound produces a statistically significant ($p < 0.05$) response (EROD activity or CYP1A4/5 mRNA expression) that is greater than the DMSO control group.

The $EC_{threshold}$-based RePs for HCB-P and RG-HCB in PEH ranged from 0.0003 to 0.001 based on the endpoints examined. We suggest an ReP value of 0.001 for HCB in PEH cultures since this value was representative of five of six $EC_{threshold}$ values obtained for HCB-P and RG-HCB as calculated from EROD and CYP1A4/5 mRNA concentration-response curves (Fig 3.1., Fig 3.3., Table 3.1.). In JEH, the $EC_{threshold}$-based RePs for HCB-P and RG-HCB ranged from 0.003 to 0.01. An ReP value of 0.01 for HCB in Japanese quail is warranted since this value was

consistent with four of six $EC_{threshold}$ values and all EC_{50}-based estimates obtained from HCB-P and RG-HCB (Fig 3.1., Fig 3.3., Table 3.2.).

The RePs obtained for PEH and JEH were higher than the ReP value obtained for HCB-P from the previous study using CEH (0.0001: Chapter 2; Mundy *et al*. 2010). One interpretation of the differences observed in ReP values for HCB-P from these three species is that HCB is 10 and 100 times more potent in JEH, than in PEH and CEH, respectively. However, ReP values differ because of differences in species sensitivity to TCDD, rather than a differences in the absolute potency of HCB. For example, the $EC_{threshold}$ values for HCB-P obtained from CYP1A4/5 mRNA expression data in PEH and JEH (0.3 nM in PEH and 1 nM in JEH) differed only slightly (i.e. < 10 times). As such, it appears that these two species share similar sensitivity to HCB exposure. Conversely, TCDD $EC_{threshold}$ values for these two species (0.0003 nM in PEH vs. 0.01 nM in JEH) differed substantially from one another. Based on these values, PEH are demonstrated to be 33 times more sensitive to TCDD exposure than JEH.

In addition to ReP values, ReS values were calculated in order to investigate the relative sensitivity of a given species to HCB exposure with respect to chicken. Chicken is used as the reference because this species is generally considered the most sensitive species to dioxin-like compounds. As previously defined, the ReS value is defined as EC_{50} or ($EC_{threshold}$) of compound *x* in chicken / EC_{50} or ($EC_{threshold}$) of compound *x* in the species of interest.

Consistent with previous *in vitro* and *in ovo* results (Cohen-Barnhouse et al. 2011; Hervé et al. 2010a,b; Yang et al. 2010), TCDD ReS values, notably EC_{50}-based values (Table 3.3.), demonstrated that the rank order of sensitivity to EROD induction by TCDD exposure was: chicken > ring-necked pheasant > Japanese quail. While there are several hypotheses for the variability in TCDD potency among species, the major hypothesis is that the greater the binding

affinity of a compound to the AHR, the greater the potency (Hestermann et al. 2000; Poland and Knutson, 1982). As mentioned, differences in amino acid residues at specific sites within the AHR LBD of chicken, ring-necked pheasant and Japanese quail can alter the binding affinity of TCDD to the AHR, thereby altering its toxic response (Head et al. 2008). Differences in ligand-specific AHR-coactivator interactions and metabolism have also been suspected of playing a role in species sensitivity differences (Head et al. 2008; Zhang et al. 2008).

With regard to differences in HCB sensitivity in CEH, PEH and JEH, interestingly, no drastic differences (i.e. $\geq$ 10 times) in species sensitivity to EROD induction by HCB exposure were observed as exemplified by their similar ReS values (Table 3.3.). Indeed, there was a small difference in sensitivity to EROD induction in JEH and CEH which accounted for quail being approximately 3 to 4 times more sensitive to HCB exposure than chicken. Past studies measuring hepatic porphyrin accumulation in Japanese quail and chicken have hinted that quail may be more sensitive to HCB toxicity than chicken (Carpenter *et al.* 1984; Vos *et al.*, 1971), however the marginal difference in ReS observed herein is not fully conclusive, and only minimally accounts for the 100 times difference in their respective ReP values (0.01 vs 0.0001). As stated before, these differences were primarily due to the varying degree of sensitivity to TCDD exposure in these three species.

While the ReS values for the three species were similar, CEH were less responsive (i.e. lower maximal response) to EROD induction by highly purified HCB compared to pheasant and quail embryo hepatocytes. The maximal EROD activity achieved by HCB-P in CEH was (7 pmol/min/mg protein; Chapter 2) which is 10 and 20 times lower than the maximal EROD activity achieved by HCB-P in PEH and JEH, respectively. The same issues (i.e. making comparisons from non-ideal concentration-response curves) that impact ReP comparisons are

perpetuated here. As such, the overall sensitivity of chicken is potentially being overestimated when the efficacy (i.e. magnitude) of the response is taken into account. It appears that PEH and JEH are equally sensitive to HCB exposure and chicken may be slightly less sensitive based on EROD induction. The rank order of sensitivity from most to least for HCB is estimated as follows: Japanese quail = ring-necked pheasant ≥ chicken. The chicken is generally regarded as the most sensitive species to DLCs, however our studies indicate that chicken may be less sensitive than ring-necked pheasant and Japanese quail to the effects of HCB. This finding was surprising given that chicken is highly sensitive to DLCs. Recently, it was reported that chicken may be marginally less sensitive to 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) exposure *in vitro* than Japanese quail (ReS = 1.8) (Hervé et al., 2010b), however these findings were not substantiated *in ovo* (Cohen-Barnhouse et al., 2011).

While the maximal EROD activity for TCDD and HCB was observed to be lower in PEH than JEH, this is considered inconsequential. Maximal EROD activities can vary among cell culture preparations. In fact, a 3-fold difference has been observed among different cell culture preparations of the same species, conducted in the same laboratory, using the same procedures (Hervé 2009 thesis/dissertation; Kennedy *et al.* unpublished results). The decreased EROD activity caused by HCB in CEH is significant since it was replicated twice, and the maximal response achieved by TCDD was the same in CEH and JEH.

In summary, consistent with CEH results (Chapter 2), this study confirmed that the presence of PCDDs, PCDFs and dioxin-like PCBs did not have an observed effect on CYP1A4/5 induction using the endpoints measured. Highly purified HCB also increased both EROD activity and CYP1A4/5 mRNA expression in a concentration-dependent manner in PEH and JEH. Based upon the comparison of the EC_{50} and more appropriately, $EC_{threshold}$ values for

EROD and CYP1A4/5 concentration-response curves, the potency of HCB relative to TCDD in PEH and JEH was 0.001 and 0.01, respectively. While the ReP values obtained for HCB differed in pheasant and quail, this could be attributed to species differences in sensitivity to TCDD rather than HCB. Consistent with results shown elsewhere, the rank order of sensitivity to EROD induction caused by TCDD from most to least was: chicken > ring-necked pheasant > Japanese quail. HCB was found to be a more potent inducer of EROD activity in pheasant and quail than in chicken. The estimated rank order of sensitivity of EROD activity followed by HCB exposure from most to least is: Japanese quail = ring-necked pheasant ≥ chicken. The results obtained from this study highlight the ability of HCB to induce a relatively uniform dioxin-like response in avian embryo hepatocytes; species sensitivity was not as variable as in TCDD-exposed cells. As such, predicting the sensitivity of a particular species to DLCs based on their AHR LBD sequence may be more appropriately reserved for strong AHR agonists. In order to truly elucidate the nature of species sensitivity to HCB, egg injection and other *in vivo* studies using highly purified HCB are clearly warranted. We also recommend that future work include *in vitro* binding assays to truly demonstrate whether highly purified HCB is acting via the AHR.

Chapter 4:
Conclusions, general discussion and future directions

4.1 General conclusions and discussion

The experiments conducted in this thesis were done to test the hypothesis that highly purified hexachlorobenzene (HCB-P; defined as HCB containing < 0.2 ppb of any PCDD, PCDF, or co-planar PCB congener [the detection limit of current analytical methods]) can induce CYP1A4/5 in the embryo hepatocytes of three avian species. The findings provide useful information regarding the dioxin-like effects of HCB and strengthen the case that HCB warrants consideration for inclusion into the WHO TEF concept.

The first finding from my line of experimentation corroborated with the findings of others: reagent-grade HCB contained detectable quantities of PCDDs, PCDFs and dioxin-like PCBs. As described in Chapter 2, two lots of reagent-grade HCB that were chemically analysed contained maximum total dioxin equivalents (TEQ) of 8.0 ng/g and 76 ng/g. OCDD and OCDF, both weak AHR agonists, were the most prevalent dioxin and furan congeners detected. Overall, these impurities played no role in CYP1A4/5 induction, or had any influence on the ReP estimates obtained for highly purified HCB or reagent-grade HCB in the embryonic hepatocytes of CEH, PEH or JEH, .

The second finding was that HCB-P was able to increase EROD activity and CYP1A4/5 mRNA expression in a concentration-dependent manner in CEH, PEH and JEH. Based upon a comparison of the EC_{50} and $EC_{threshold}$ values, for EROD and CYP1A4/5 mRNA concentration-response curves the potency of HCB relative to the potency of TCDD was estimated to be 0.0001, 0.001, and 0.01 in chicken, ring-necked pheasant and Japanese quail embryo hepatocytes, respectively (Chapters 2 and 3). Greater emphasis in our conclusions was placed on

$EC_{threshold}$ values as the use of these values may help account for differences in maximal EROD and CYP1A4/5 mRNA levels that are observed with HCB exposure in avian embryo hepatocytes more so than EC_{50} values. Consequently, HCB also elicited a similar concentration-dependent response compared to two mono-*ortho* PCBs (PCB 105 and PCB 118) in CEH that are currently included in the TEF concept (Chapter 2).

The third finding demonstrated that species differences in sensitivity to HCB are less apparent than differences to TCDD sensitivity in these three avian species (Chapter 3). Chickens are highly sensitive, ring-necked pheasant are moderately sensitive, and Japanese quail are least sensitive to TCDD exposure. With respect to HCB exposure, ring-necked pheasant and Japanese quail were equally sensitive. The disparity in their ReP values was simply due to species sensitivity differences to TCDD exposure. While chicken appears to share similar sensitivity to HCB exposure as ring-necked pheasant and Japanese quail, CEH were less responsive to EROD induction (lower maximal response) than both PEH and JEH. It is not certain why maximal EROD activity was lower in CEH, but lower binding affinity with the AHR is a potential explanation. Ligands with lower affinity for the AHR have been shown to be less potent and responsive (lower maximal response) than high affinity AHR ligands (Hestermann *et al.* 2000; Poland and Knutson 1982).

As mentioned previously, it has been suggested that HCB should be included in the WHO TEF concept and assigned a toxic equivalency factor of 0.0001 (van Birgelen 1998). In summary, the WHO and the U.S EPA state that a dioxin-like compound must: (a) have a structural similarity to TCDD, (b) bind to the AHR, (c) elicit AHR-mediated biochemical and/or toxic effects and (d) be persistent and accumulate in the food chain to be included in the TEF concept. The findings presented herein conclusively demonstrate that highly purified HCB is

capable of eliciting AHR-mediated biochemical effects, as measured by the induction of EROD activity and CYP1A4/5 mRNA expression in three avian species. HCB has also been shown to possess a low affinity for the rat AHR (0.0001) compared to TCDD (Hahn *et al.* 1989). This study has been widely cited in order to characterize HCB as a weak AHR agonist, however, the presence of PCDD/F impurities should not be overlooked. My findings, in conjunction with the Hahn et al (1989) study, do suggest however, that HCB may be capable of activating the AHR. Additionally, based on its ability to bioaccumulate and its potential for biomagnification, HCB appears to merit consideration for inclusion in the WHO TEF concept as outlined above.

Another facet of the experimentation I conducted included examination of the results obtained from EROD and CYP1A4/5 mRNA endpoints. Previous studies conducted in the Kennedy laboratory have suggested that EROD activity and CYP1A4/5 mRNA expression can be used interchangeably to predict potency of dioxin-like compounds *in vitro* (Head and Kennedy 2007b; Hervé *et al.* 2010b). As shown herein, EROD and CYP1A4/5 concentration-response curves were similar in appearance (i.e. similar slopes) and gave rise to similar ReP values for TCDD and HCB in CEH, PEH and JEH. Overall, the EROD bioassay is preferable in terms of cost, labour, and the ease of application to multiple species, whereas the CYP1A4/5 assay is a more time consuming approach where experimental conditions must be optimized for each species individually. CYP1A4/5 mRNA concentration-response curves are preferable because they may provide increased accuracy for relative potency estimates of weak AHR agonists. This is because mRNA concentration-response curves, unlike EROD curves, are not affected by competitive inhibition (Head and Kennedy 2007b). Furthermore, the analysis of CYP1A4/5 mRNA expression was very important in determining that Japanese quail and ring-necked pheasant were equally sensitive to HCB exposure, since PEH elicited lower maximal

EROD activity than JEH. As such, the use of both bioassays was deemed essential for the work conducted with HCB in avian embryo hepatocytes.

4.2 Future directions

There are a number of future studies that I recommend to further justify the inclusion of HCB into the WHO TEF concept. It has recently been suggested that CYP1A is a non-specific indicator of AHR activation (Hu *et al.* 2007). As such, AHR binding assays and luciferase reporter gene assays using highly purified HCB are essential in demonstrating that HCB is truly acting via the AHR. It would also be useful from a regulatory standpoint to determine whether the relative potency values determined *in vitro* are indicative of embryo lethality *in ovo*. Moreover, egg injection studies are essential in order to develop an accurate TEF (if HCB were to be included in the TEF concept) for HCB in birds.

Additionally, because the sensitivity of CEH, PEH and JEH to HCB exposure were similar, it would be worthwhile applying the same line of experimentation conducted herein using wild birds. The herring gull (*Larus argentatus*) would be an ideal candidate species because it is not overtly sensitive to TCDD exposure (Kennedy *et al.* 2003; Hervé *et al.* 2010c). Recently, Norstrom and Hebert (2006) speculated on the probable involvement of HCB in the poisoning of herring gull embryos in the early 1970s because HCB levels in gull eggs was detected in excess of 4500 ppb at certain sites (Norstrom and Hebert 2006). Applying relative potency values to chemical residue data from the Great Lakes during the 1960s and 1970s could yield interesting historical implications regarding the potential role that HCB may or may not have had on herring gull population declines. As mentioned in Chapter 1, there are point sources where HCB is being detected at very high concentrations in bird eggs. As such, these species could also be potential future candidates for HCB experimentation.

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Appendix 1:
Sample calculations, figures and explanations regarding HCB purity issues

One of the major components of the work conducted herein has focused on the use of highly purified hexachlorobenzene (HCB-P; defined as HCB containing < 0.2 ppb of any polychlorinated dibenzo-*p*-dioxin [PCDD], polychlorinated dibenzofuran [PCDF], or co-planar polychlorinated biphenyl [PCB] congener [the detection limit of current analytical methods]) to induce cytochrome P4501A (CYP1A) in birds with the purpose of demonstrating that CYP1A induction was indeed caused by HCB and not dioxin-like impurities.

The World Health Organization (WHO) avian toxic equivalency factors (TEFs) are indicated in Table 2.1, and toxicity equivalent (TEQs) concentrations of PCDDs, PCDFs and PCBs in two batches of reagent-grade HCB were, at most, 76 ng/g and 8.0 ng/g, respectively (Chapter 2). The analysis for PCDDs, PCDFs and PCBs in HCB-P by Wellington Laboratories indicated that all compounds were below the detection limits; as such the total TEQ was, at most, 0.56 ng/g (calculated using detection limits for non-detected compounds). The ALS Laboratory Group did not analyze HCB-P for PCBs, but their PCDD and PCDF data indicated that the total TEQ was, at most 0.092 ng/g (Table 2.1).

Sample Calculations:

<u>Amount of HCB at 1000 nM dose (highest concentration administered)</u>

Molecular weight of HCB = 284 g/mol
Concentration of HCB in medium = 1 μM (1 μmol/L)
Volume of medium = 0.5 ml (0.0005 L)

Total HCB in medium (per cell culture well) = (284 g/mol)(10^6 μg/g)(1μmol/L)(10^{-6} mol/μmol)(0.0005 L medium)
= 0.142 μg of HCB/well in the highest dosed concentration
= 142 ng/well

Amount of TCDD at 0.001 nM dose (EROD ECthreshold in CEH)

Molecular weight TCDD = 322 g/mol
Concentration of TCDD in medium = 0.000001 µM (0.000001 µmol/L)
Volume of medium = 0.0005 L
Total TCDD = 0.000161 ng/well

Table A.1. The theoretical amount of TCDD present in a cell culture well (ng/well), depending on the extent of contamination (%) in HCB at the highest concentration administered (1000 nM). The lowest amount of TCDD needed to induce an EROD response in chicken embryo hepatocytes is approximately 0.00016 ng.

TCDD Contamination (%)	Amount TCDD (ng/well)
1	1.4
0.1	0.14
0.01	0.014
0.001	0.0014
0.0001	**0.00014**
0.00001	0.000014

Therefore, as shown in Table A.1., the percent contamination where TCDD is present within HCB that could influence the results is estimated at 0.0001 % (0.00014 ng/well assumed to equal 0.00016 ng/well, which is the lowest amount of TCDD needed to induce EROD activity as calculated above). Figure A.1 below, demonstrates that 0.0001% TCDD contamination equates to 1000 ng/g (ppb) TCDD within HCB. The highest TEQ determined for reagent-grade HCB was 76 ppb, which is roughly 13 times lower than that required to induce EROD activity in chicken embryo hepatocytes (CEH). The TEQs for HCB-P were found to be at most 0.56 ppb (Wellington analysis) and 0.092 ppb (ALS analysis) which is approximately 1800 and 10,900 times lower than that required to induce an EROD response in CEH, respectively. As such, the observed induction of CYP1A4/5 is directly attributed to HCB (RG-HCB and HCB-P), and not the presence of PCDD/F impurities.

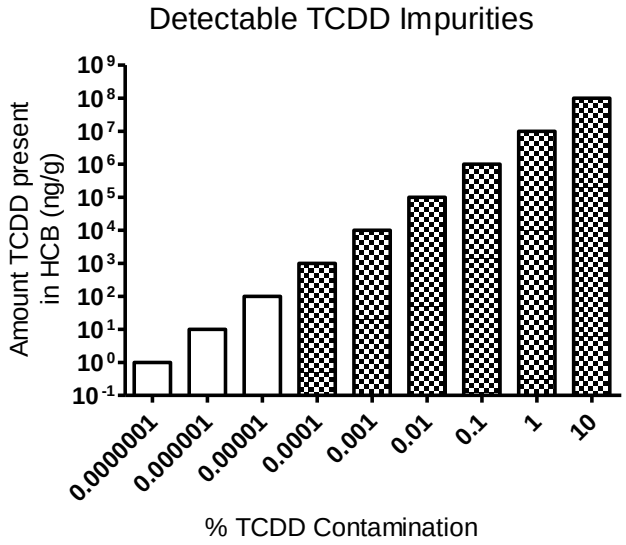

Figure A.1. The amount of TCDD present within HCB (ng/g) with respect to percent TCDD contamination. White bars indicate contamination percentages which are too low to elicit ethoxyresorufin *O*-deethylase activity (EROD) activity in chicken embryo hepatocytes. Checkered bars indicate percentages whereby EROD activity could be induced by the presence of TCDD contamination.

As mentioned in Chapter 2, a series of *in vivo* studies carried out by Goldstein et al. (1978, 1982) and Linko et al. (1986) conducted toxicological experiments using HCB which contained non-detectable levels of PCDDs and PCDFs. The detection limit employed by these authors was < 500 ppb and would have been too high to rule out effects caused by PCDD/F and dioxin-like PCB impurities that may have been present, but undetected. The TEQ with a detection limit of < 500 ppb for PCDDs and PCDFs (not including dioxin-like PCBs) would equate to 2340.60 ng/g, about double the amount of (1000 ng/g) needed to induce EROD activity in chicken embryo hepatoctyes.