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THE GLOBAL HEPATIC TRANSCRIPTIONAL RESPONSE OF MALE FISHER RATS TO DIETARY AROCLOR 1254 EXPOSURE

by

Trisha M. Basford

A Dissertation
Submitted to the
Faculty of The Graduate College
in partial fulfillment of the
requirements for the
Degree of Doctor of Philosophy
Department of Chemistry
Dr. Jay C. Means, Advisor

Western Michigan University
Kalamazoo, Michigan
June 2007

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ACKNOWLEDGMENTS

I would like to thank my family for all of their love, support, and encouragement through the years; your steadfast belief in my abilities gave me the will to persevere. Mom, especially, words cannot express my gratitude. Munk, thanks for coming out to Michigan with me, you helped to keep me sane. Dad and Penny, thanks for all the help and encouragement. Tara, for being such a good friend and for all you've done through the years; Angie and Taty for all the babysitting; Christine for editing and babysitting besides the moral support. Aunt Shirlene, this work is dedicated to you—I miss you.

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Trisha M. Basford

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CHAPTER I

INTRODUCTION

Polychlorinated biphenyls (PCBs) are stable, lipophilic compounds of biphenyls that were first manufactured on an industrial scale in the U.S. by Monsanto in 1929. PCBs were used extensively from 1930's through the 1970's: as non-conductive insulators in transformers and capacitors, as hydraulic and heat transfer fluids, in building materials, paints, carbonless copy paper, and as a carrier for application of pesticides; world production has been estimated at 1.3-1.5 million metric tons. 209 possible congeners can be formed by chlorine substitution on the biphenyl backbone, and complex mixtures of congeners classified by weight percent of chlorine in the mixture (e.g., Aroclors in the U.S., Clophens in Germany) were typically deployed in industrial uses (de Voogt & Brinkman, 1989; Fiedler, 1998).

Japan banned manufacture of PCBs in 1972, after humans were intoxicated by contaminated cooking oil containing very high concentrations of PCBs. The possibility of injuries to humans and wildlife, coupled with growing concern over the rising levels of PCBs in biotic and abiotic media, led to a ban on PCB manufacture in the U.S. in 1977. Several European countries followed suit soon afterwards, and strict regulatory controls on PCB use, reuse, and disposal have been in effect world wide for more than two decades. Because of the

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molecular hydrophobicity conferred by aromatic rings and chlorine, PCBs released to the environment partition preferentially in animal adipose and lipid-rich tissue, and in sediments and soils; their slow degradation causes them to accumulate in these phases. Persistent organic pollutants (POPs), which include PCBs and several other aromatic, halogenated compounds such as tetrachlorinated dibenzo-p-dioxins and -furans, some polyaromatic hydrocarbons, and polybrominated diphenyl ethers all share the following common traits 1) chemical inertness, 2) hydrophobicity, 3) bioaccumulation in animal tissues and food chains, 4) environmental persistence, repeated cycling through environmental phases and accumulation in phases rich in organic carbon (International Programme on Chemical Safety, 1993).

The POP congeners capable of assuming a planar configuration can also bind to the AhR, a nuclear receptor that lacks an endogenous ligand, and induce transcription of genes active in Phase I and Phase II drug metabolism. Effects observed downstream of AhR-induced gene expression *in vivo* include increases in benign and cancerous tumors of the liver and adrenals, reproductive and developmental abnormalities, thymic atrophy, and impaired immune function. Besides toxic endpoints common to AhR ligands, pre and post natal PCB exposure has been associated with cognitive deficits and other neurotoxicities, suppression of immune functions, depletion of thyroid hormone, diabetes, and initiation of inflammatory responses in vascular endothelia (ATSDR, 2000; S. Safe, 1993; Wilson & Safe, 1998).

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PCBs were produced, used, and dumped most extensively in the eastern U.S., especially in the highly industrialized Great Lakes basin states (WI, IL, MI, IN, NY, OH), the Hudson River in NY, and in Alabama in the south. Although levels of PCBs in water, air, sediments, and biota have declined from peaks observed in the late 1970's to early 1980's, several highly contaminated "hot spots" still exist in the U.S. and elsewhere . In the Great Lakes region, sediment-bound PCBs have been deposited into Lake Michigan by several contaminated tributaries, including the Kalamazoo River, and the lake has the highest concentrations of PCBs in water, biota, and sediments. Trout and coho salmon, top predator species in the Lake Michigan trophic webs, have average PCB body burdens at or close to 2 ppm, the "No Consumption" limit recommended for protection of human health. Fish from the lake and tributaries are also an important food source for many birds and land mammals in the Great Lakes basin, and animals in this region often have very high PCB levels compared to animals from less polluted regions. PCBs and other POPs present in these animals have been implicated in decreased reproductive success due to changes in behavior, physical reproductive and developmental abnormalities, thyroid deficiency, and extirpation of the mink from the Lake Michigan shoreline (Environment Canada, U.S. EPA, 2005; Fox, 2001).

Inappropriate disposal of tons of PCBs used in the manufacture of carbonless copy paper at the former Allied Paper plant in Kalamazoo, MI, in 1960's, is the source of contamination in the Kalamazoo River. Highly

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contaminated sediments were transported from riverbank dump sites at Kalamazoo and 12th St. by the river, and eventually deposited into Lake Michigan at the river outlet in Saugatuck. High PCB concentrations in riverine fish and wildlife in the watershed, the continuous addition of contaminated sediment to Lake Michigan, and periodic flooding along the river were all factors contributing to the decision to designate approximately eighty miles of the river between Kalamazoo and Lake Michigan a Superfund site (KRAOC) in 1996. Remediation of the site is currently in progress, but PCB levels in Kalamazoo River fish remain well above the MDCH 'No Consumption' advisory level, and levels in wildlife in the surrounding areas are elevated when compared to upriver reference sites in the southwestern Michigan area (Stratus Consulting, 2005).

The body of literature describing levels of PCB contamination of environmental media, effects associated with PCBs in wildlife in the Great Lakes basin, and the toxic effects of high doses of PCBs in laboratory animals is extensive. There are considerably fewer reports of the effects of lower, more environmentally relevant dietary exposures to PCBs, or of transcriptional changes that may occur in response to low level exposures. Diet is the most important pathway of exposure for POPs, and PCBs are ubiquitous in meat, fish and dairy products. Infant and fetal exposures also occur via placental and breast milk transfer from mothers, and these transfers can be critical determinants of the lifetime body burden PCB (ATSDR, 2000).

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The use of microarrays permits the simultaneous quantification of thousands of genes. At sub toxic exposure levels, measurements of transcriptional changes due to continuous, dietary exposure to a mixture of PCBs similar to actual human and wildlife exposures may lead to the discovery of genes crucial to the origin or perpetuation of subtle physiological effects in mammals. To date, the only reports of global profiles of changes in hepatic gene expression following Aroclor 1254 exposure are for intraperitoneal injection with high, toxic doses (Waring et al., 2001); post natal, lifetime gavage exposure to single PCB congeners have also been reported (NTP, 2006a; NTP, 2006c). The liver is a target organ for PCBs in rats and other mammals, and the effects of PCBs on this organ have been widely studied. While no single study can provide a complete picture of the impact of low level dietary PCB or POP exposure, we propose to investigate the hepatic effects of sub chronic and sub acute exposures to Aroclor 1254, a mixture of PCBs with a congener profile similar to that detected in the KRAOC.

The specific aims of this project, listed below, will help to fill in the existing information gap between laboratory studies of the effects of well-defined exposures to single congeners or to mixtures that induce overt toxicities, and the field observations that result from complex and ill-defined environmental exposures:

1. Characterize changes in rat hepatic global gene expression due to sub acute (1 week) dietary exposure to high and low doses of Aroclor 1254

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2. Characterize changes in rat hepatic global gene expression due to sub chronic dietary (12 week) exposure to high and low doses of Aroclor 1254
3. Compare transcriptional profiles at sub-acute and sub chronic exposure times to characterize anticipated changes in gene expression profile due to duration of exposure

Aroclor 1254 has a high percentage of penta- and hexachlorinated congeners similar to PCB profiles determined in fish and small mammals in the Kalamazoo area (Camp Dresser & McKee, 1997), and was chosen for this work because of this similarity, and because of the large body of existing literature describing the effects of Aroclor 1254 exposure in rats. Rats have been the most commonly used animal model for toxicological studies of PCBs effects, and will provide a wealth of information for comparisons.

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CHAPTER II

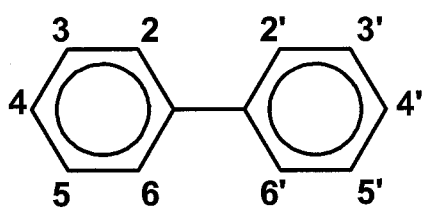
LITERATURE REVIEW

Background on Polychlorinated Biphenyls

Physical and Chemical Properties

Polychlorinated biphenyls (PCBs) are a thermally stable class of chemicals of the general formula $C_{12}H_{10-n}Cl_n$, where n is equal to the total number of chlorines on the biphenyl rings. Figure 1 shows the basic biphenyl structure of all PCBs, the number of chlorine substituents on the biphenyl framework can vary from one to ten, for a total of 209 possible congeners. All possible Cl substitution sites are indicated by number (2-6, 2'-6') on the aromatic rings in Figure 1. Chlorine substituted for hydrogen at two or more of the four available positions adjacent (ortho) to the biphenyl bond (2,2',6,6') will hinder rotation about the bond to some extent, if there are one or no chlorines at any of these positions, then the molecule is able to assume a planar or coplanar configuration.

Figure 1. Biphenyl Structure



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Substitution of Cl for the smaller hydrogen atoms not only determines the extent of rotation about the bond connecting the two benzene rings, it also increases the chemical inertness and hydrophobicity of the molecule. PCB's are thermally stable, resistant to acid and base attack, and freely soluble in non-polar organic solvents and biological lipids. Some of the physical and chemical properties of mixtures of PCBs that made them highly suitable for a broad range of industrial applications include: liquid at room temperature (density: 1.182-1.566 kg/L), high flash point (170-380°C), non-explosive, low electric conductivity, and very high thermal conductivity.

Vapor pressure and water solubility decrease as the molecular weight and the degree of chlorination increase; a recent critical review of existing physical data for trichlorinated through octachlorinated PCBs estimates vapor pressures for the supercooled liquid range from 10^{-1}-10^{-5}, and water solubilities from 10^{-2}-10^{-6} (N. Q. Li, Wania, Lei, & Daly, 2003). The polychlorinated biphenyls photolyze readily and most congeners undergo reductive dechlorination (K. H. Wong & Wong, 2006) upon absorption. PCBs are combustible at very high temperatures (ATSDR, 2000), and products of combustion include persistent, bioaccumulative polychlorinated dibenzodioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs).

Production, Use, and Disposal of PCBs

The chemical inertness, low electrical conductivity, and high thermal

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conductivity of liquid PCBs made them ideal coolants for electrical transformers and dielectric fluid in capacitors, and use in these two applications accounts for approximately 70% of all PCBs manufactured in the United States (de Voogt & Brinkman, 1989); applications in other closed systems include hydraulics (Fiedler, 1998) and heat exchange fluids (ATSDR, 2000). Polychlorinated biphenyls were also used in lesser quantities in open systems such as carbonless copy paper, lubricants, and pesticide extenders (ATSDR, 2000). They were also used in a variety of building materials: as a plasticizer in caulking (Herrick, McClean, Meeker, Baxter, & Weymouth, 2004), and to improve the flexibility, durability, and adhesive properties of concrete and plaster (Andersson, Ottesen, & Volden, 2003).

PCBs were synthesized industrially by reacting biphenyl with anhydrous Cl_2 in the presence of iron catalyst; the degree of chlorination was controlled by reaction times, which varied from 12-36 hrs. The complex liquid mixtures of PCB congeners yielded by this process were classified according to percent chlorine by mass, and were sold under a variety of technical trade names, for example: Aroclors were manufactured in the U.S. by Monsanto; Clophens were made in Germany by Bayer A.G., and Sovols were produced in the former U.S.S.R. by Orgsteklo. Mass production of PCBs began in the 1930's in the U.S., France, Russia, and Germany; Japan and several other European countries didn't start producing PCBs until the 1950's (Breivik, Sweetman, Pacyna, & Jones, 2002).

Monsanto was the major manufacturer of PCBs in the world, with a total

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PCB output of 641,246 ton--four times greater than that of Bayer AG, the second major global producer (de Voogt & Brinkman, 1989). Within the U.S., industrial use of PCBs was almost exclusively (99%) technical mixtures synthesized by Monsanto, among the most widely used were Aroclors 1242, 1254, 1260 (ATSDR, 2000). The last two numbers in the Aroclor designations indicate the percent chlorine by weight in the mixtures; ortho-substituted congeners are prominent constituents of all (detailed composition data for Aroclor 1242, 1254, 1260 are given in Appendix B). Cumulative production and end use of polychlorinated biphenyls within the U.S. between 1930 and 1975 has been estimated to be 1400 million pounds: 46-50% in capacitors, 24-27% in transformers, 9.2% as plasticizers, 6% in heat transfer fluids, 4% in carbonless copy paper, 1% as petroleum additives, and the remainder for miscellaneous industrial applications (de Voogt & Brinkman, 1989). In U.S. electrical equipment manufacturing, Aroclor 1260 was used more prior to 1950, as was Aroclor 1254; Aroclor 1242 was the dominant mixture used in the 1950s and 1960s (Fiedler, 1998).

The environmental and biological persistence of PCBs in animal tissue was first reported in the 1960's, following the invention and application of electron capture detectors in gas chromatography (Lovelock, 1958a; Lovelock, 1958b), the first analytical technique sensitive enough to detect the presence of trace amounts of chlorinated compounds (parts per million or less) in tissues (Erickson, 1997). By 1972, Monsanto, the major American producer of PCBs, had voluntarily limited PCB production to ~18% each of Aroclor 1254 and Aroclor

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1242, and less than 1% of Aroclor 1221. Aroclor 1016, a fractional distillate of Aroclor 1242 with a higher proportion of mono-, di-, and tri- chlorinated biphenyls than the parent Aroclor (Appendix B), accounted for the remainder of Monsanto production, and was the predominant technical mixture used in electrical applications in the 1970's (Fiedler, 1998).

A congressional ban on the manufacture, processing, use and distribution of PCBs in 1976 under the Toxic Substances Control Act (TSCA) and the Resource Conservation and Recovery Act (RCRA) (ATSDR, 2000) led to the cessation of PCB production in the United States in 1977. The majority of PCB manufacture and (new) use--in North America and Europe--was either voluntarily halted or banned by the early 1980's, however, Russia, the last known industrial manufacturer did not cease production until 1993 (Ivanov & Sandell, 1992). Historical global production of PCBs during the 20th century has been estimated to be 1.5 million metric tons (de Voogt & Brinkman, 1989), and more recently 1.324 million metric tons (Breivik et al., 2002), however the latter is likely an underestimation of actual production, since contributions from minor manufacturers in Europe and the U.S. were not included.

Unused or waste PCBs and materials containing PCBs were dumped or buried in landfills, discharged into rivers and coastal waters, released accidentally and via incineration throughout Europe and the U.S. (Erickson, 1997; Hansen, DeCaprio, & Nisbet, 2003; McFarland & Clarke, 1989; Ratanen, 1992; WHO, 1992), with little or no governmental oversight or control, for nearly

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fifty years. In 1978, after production in the U.S. had ended, the EPA began to regulate the storage and disposal of PCBs in the U.S. (ATSDR, 2000). More than half of the PCBs disposed of between 1930 and 1970--an estimated 0.3 million metric tons--entered the environment via landfills or dumps in the United States (Hammond, Nisbet, & Sarofim, 1972).

Reclamation of steel and other valuable, recyclable materials from large, ocean-going ships (Anonymous, 2005) and dumping of municipal waste (Minh et al., 2006; Watanabe et al., 2005) currently serve as an uncontrolled sources for the release of PCBs into the environment in sites throughout southeast Asia.

Environmental Distribution and Cycling of PCBs

Polychlorinated biphenyls can be found in nearly every phase of the abiotic environment--sediments (Butcher & Garvey, 2004; Gevao et al., 1997; Hermanson, Christensen, Buser, & Chen, 1991; VanMetre, Callender, & Fuller, 1997), soils (Meijer et al., 2003), water (Hornbuckle, Carlson, Swackhamer, Baker, & Eisenreich, 2006), and atmosphere (Jaward, Meijer, Steinnes, Thomas, & Jones, 2004) --and in biota worldwide: humans (Anderson et al., 1998; Kalantzi et al., 2004; Koizumi et al., 2005; Loaiza-Perez et al., 1999), mammals (Christensen, MacDuffee, MacDonald, Whiticar, & Ross, 2005; Tanabe, Niimi, Minh, Miyazaki, & Petrov, 2003; Verreault et al., 2005), birds (Hebert, Norstrom, Zhu, & Macdonald, 1999; Schoeters & Hoogenboom, 2006), fish (Barnthouse, Glaser, & Young, 2003; Carlson & Hites, 2005; Hornbuckle et al., 2006; Shaw et al., 2006)

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and shellfish (Gorni & Weber, 2004; Kannan, Tanabe, Giesy, & Tatsukawa, 1997; Nakata et al., 2002), vegetation (McLachlan, 1996), and plankton and bacteria (Borga et al., 2005; Sobek, Olli, & Gustafsson, 2006).

There have been no major new inputs to the environment since most of the manufacture and industrial use of PCBs was phased out more than two decades ago, and PCB levels have declined in many biotic and abiotic compartments (Heidtke, Hartig, Zarull, & Yu, 2006; Schneider, Stapleton, Cornwell, & Baker, 2001; P. G. Tee et al., 2003; van Metre & Mahler, 2005; Verreault et al., 2005) since this time. Concomitantly, the regulatory emphasis is now on minimizing 'new', uncontrolled environmental release by monitoring waste handling, cleanup, and disposal, EPA,WHO. Quantifying amounts and the temporal trends in PCB transfers among abiotic phases, and accumulation in biota, has become increasingly important in the study and modeling of environmental fate and transport of this class of persistent organic pollutants (POPs).

Because the United States was by far the largest producer and consumer of PCBs in the world (Breivik et al., 2002; de Voogt & Brinkman, 1989), and this work is focused on problems arising from contamination at the Kalamazoo River Superfund sites, this review will concentrate primarily on characterizing environmental contamination in the U.S., and Lake Michigan in the Great Lakes region.

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PCBs in Soils, Groundwater, and Sediments

As might be expected considering the historical methods of disposal mentioned in the previous section, the bulk (possibly >70%) of the total PCBs produced are estimated to be stored in the world's sediments and soils (Ockenden et al., 2003). Adsorption to organic carbon present in soils, to organic particulates, or to coatings on inorganic sediments are major mechanisms of environmental sequestration of hydrophobic POPs, including polychlorinated biphenyls (Mackay, 2001). Table 1 shows the breakdown, by abiotic media, of the total number of PCB-contaminated sites on the EPA's National Priority List (NPL) of Superfund sites in 2006. Of the 300 PCB-contaminated sites on the final NPL, almost all (290) contain contaminated soil, and a third of the sites have contaminated sediments. Groundwater contamination, which occurs primarily by migration or leaching of PCBs from the surface soil dumping sites or landfills, is also a concern in over half of the Superfund sites (Table 1). Meijer and coworkers (Meijer et al., 2003) investigated the role of surface soils in the global PCB budget by measuring PCBs in background surface (0-0.5 cm) and subsurface (0.5-20 cm) soil samples collected from rural or remote regions on every continent except Antarctica, and detected PCBs at surface and subsurface depths at all 191 sites. The highest concentrations of PCBs were detected in soils between 30°-60°N, the same latitudes where the bulk of PCBs were produced and used (Breivik et al., 2002). The mean for total PCBs in the background soils was 19.5 ng/g soil organic matter, with penta- and hexa- congeners present in

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the largest quantities, on average (Meijer et al., 2003).

Table 1

Number of Superfund Sites Per EPA Region with PCB-Contaminated Environmental Phases

	I	II	III	IV	V	VI	VII	VIII	IX	X	Total # Sites
Air	5	13	5	1	16	2	1	1	2	1	47
Surface Water	13	19	10	6	22	5	1	1	1	7	85
Sediment	17	20	13	13	21	4	2	2	2	10	104
Groundwater	24	31	18	14	43	4	2	4	4	11	155
Soil	33	57	33	32	77	9	6	6	17	20	290

Diffusion rates of POPs through soils are only 1-30 μm/year (Mackay, 2001), so burial of PCBs at depths below the top half centimeter of soil is likely a significant sink for PCBs globally. Based on the results of the background surface soils study ((Meijer et al., 2003), Ockenden and coworkers (2004) estimate that 21,000 metric tons are present in the labile top 0.5 cm of surface soils globally.

Unlike the case for sorption to soils, PCBs adsorbed to sediments can be transported miles from a contaminated source by rivers, streams, and lake currents, creating widespread contamination from a single point source. A USGS national water quality assessment comparing PCB burdens in sediment cores from four reservoirs in the Apalachicola-Chattahoochee-Flint River Basins (south of Atlanta, GA) with two remote, natural lakes determined that regional atmospheric sources made insignificant contributions to the total PCB burdens

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for the reservoirs compared to fluvial inputs, and that local and non-point sources of PCBs were increasingly important in more urban areas (VanMetre et al., 1997). Lacustrine sediments can also be a significant source of dissolved PCBs, particularly of more soluble, lighter weight congeners (Gevao et al., 1997), and/or following disturbance of sedimentary layers.

Flood events may also deposit sediment contaminants into soil reservoirs, this is illustrated by the relatively high levels of PCB-contaminated soils (15-25 ppm), in the Trowbridge Dam area of the Kalamazoo River, which is downriver from the Superfund sites in Kalazmazoo. Soil PCB levels at Plainwell, closer to the Kalamazoo site, ranged from 6-10 ppm, and sediment concentrations at sampling points along the river from Kalamazoo to the Trowbridge dam ranged from 10-16 ppm (Camp Dresser & McKee, 1997). The higher soil PCB concentrations indicate a burden left by repeated flooding in the Trowbridge Dam area.

PCB-contaminated sediments enter Lake Michigan from several tributaries, including the Kalamazoo RIVER , and sediment burdens in the lake reportedly ranged from 0.138-219 ng/g, with the highest PCB burdens (>100 ng/g) only in the southern and central basins of the lake (McCarty et al., 2004).

PCBs in the Atmosphere

Long range, abiotic transport of PCBs from contaminated to remote regions occurs primarily through the atmosphere. In a study designed to track

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long range atmospheric transport (LRAT) of selected POPs in northern Europe, and to investigate the global fractionation hypothesis--the theory that PCB molecules will vaporize from temperate and equatorial zones, be transported northward by prevailing air currents, and travel distances inversely proportional to their molecular mass, semipermeable membrane devices were deployed, distant from local PCB sources, on a latitudinal transect from southern UK to northern Norway (Jaward et al., 2004). Air samples were collected for this study in 2000-2002 (Jaward et al., 2004) and previous studies at the same sites in 1994-1996 (Ockenden, Sweetman, Prest, Steinnes, & Jones, 1998) and 1998-2000 (Meijer et al., 2003) were combined to estimate an average atmospheric clearance rate for PCBs of 3.5 ± 0.6 yrs, assuming a first order rate of loss. Chlorine number congener analyses from all three studies supported the fractionation hypothesis in that amounts of tri- and tetra- PCBs detected clearly increase with latitude, and hexa-, hepta-, and octa-PCBs levels decrease with latitude. The steady decline in atmospheric PCB concentrations at all sites over the three sampling times, however, suggests that the atmospheric concentrations of PCBs in this area of Europe are still controlled by emissions from primary sources, and not by deposition and revolatilization.

In the Great Lakes region, the IADN monitors atmospheric PCBs and several other POPs from five stations throughout the area (Hillery et al., 1998). Vapor phase total PCB are highly variable around Lake Michigan, from 21-2600 pg/m^3, with highest concentrations generally observed in the Chicago area

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(McCarty et al., 2004). PCB concentrations in the vapor phase were nine or more times greater than in the particulate phase, and PCB concentrations in precipitation (360-16000 pg/L) were greater than PCB in the lake water at all stations (McCarty et al., 2004).

PCBs in Surface Waters

While some PCBs were discharged directly into lakes and rivers, the most important routes for maintaining present levels of dissolved PCBs in surface waters are contact with other contaminated phases: soil, sediments, particulate matter, and the atmosphere. Regional and sub regional atmospheric deposition of particulate or gas phase PCBs, from urban areas or point sources, can be an important source of PCBs in surface waters (Pearson, Hornbuckle, Eisenreich, & Swackhamer, 1996). LRAT can also move PCBs (and other POPs) to remote areas, where surface water contamination can occur by direct deposition, and via drainage from the surrounding watershed (Jeremiason et al., 1999).

Along the Kalamazoo River, downstream from the Allied Superfund site, aqueous PCB concentrations ranged 31-120 ng/L, and the highest average concentration (75 ng/L) was reported for water above the Trowbridge Dam (Camp Dresser & McKee, 1997). In the tributaries emptying into Lake Michigan, total dissolved PCBs ranged from 0.43-35 ng/L; with an average of 23 ng/L from the more polluted sources: the Kalamazoo, Fox, Sheboygan, Milwaukee, Green and Grand Calumet rivers (McCarty et al., 2004). In the water column of Lake

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Michigan in 1994, the average dissolved PCB concentration was 0.18 ng/L and average particulate concentration was 0.073 ng/L. Trends over the entire lake indicate that PCB concentrations in the aqueous phase, in the top layer of sediments found on the lake bottom, and in the atmosphere above the water are highest in the southern basin of the lake--between Chicago on the west and the Kalamazoo RIVER outlet on the east side of the Lake

Food-Chain Bioaccumulation of PCBs

Absorption and Biomagnification

Dietary intake is the major route by which persistent pollutants, such as organochlorines, organobromines, and mercury, are bioaccumulated in mammals. Bioaccumulation, the increase in an organism's concentration of a chemical due to all possible routes of environmental exposure, can also occur by absorption through the skin and transport across respiratory surfaces. Fish, for example, are able to absorb organic chemicals with octanol-water partition coefficients (K_{ow}) less than ~10^5 directly from their aqueous environment through the skin or gills, but dietary intake is also their primary route of exposure to more hydrophobic organic compounds ($K_{ow} > 10^5 - 10^6$) (Russell, Gobas, & Haffner, 1999). Pollutants can be removed from an organism by several mechanisms: egestion in feces and urine, respiration, dermal diffusion, growth dilution, reproductive loss, and metabolic conversion, and the amount of a pollutant in any organism depends on the difference between uptake (diet,

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dermal absorption, respiration) and clearance. If a compound is not readily cleared from an organism by one of these methods, as is the case for many PCBs and other POPs, it will tend to accumulate in lipid-rich compartments, and so the body, organ, or tissue burden of the chemical is usually reported as mass (g) of chemical/mass (g) of lipid.

Fugacity (f), which is basically the partial pressure of a particular chemical in each phase of the environment, can be used as a surrogate for concentration (C) to develop a mechanistic picture of the food-chain bioaccumulation of a chemical (Gobas, 1993). The fugacity capacity (Z) is a constant specific to a particular chemical, the phase it is in (dissolved or sorbed), and the temperature, and is related to concentration by: $f = C/Z$. If two (or more) phases are at equilibrium, their fugacities will be equal.

Gobas and coworkers (1993) were the first to demonstrate experimentally that the physiological basis for the phenomenon of biomagnification (when the fugacity of a bioaccumulated chemical in an organism exceeds the fugacity of the chemical in the diet) was the increase in fugacity that occurs in the gastrointestinal tract during the process of dietary absorption. Guppies and goldfish were fed diets contaminated with a series of increasingly hydrophobic compounds that are not metabolized by the fish: tetra-, penta- and hexa-chlorobenzenes, a tetra- and a hexachlorobiphenyl, and mirex, and the fugacities of the chemicals in the diet (f_D), fecal matter (f_G), and the whole fish (f_F) were measured by GC headspace (Gobas, 1993). After 14 days of exposure to

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contaminated food, the gastrointestinal magnification factors (f_G/f_D) were less than one for all of the chlorobenzenes (log K_{ow} 4.50-5.47), and greater than one for the chlorobiphenyls and for mirex (log K_{ow} 6.10-7.50), with f_G/f_D increasing proportional to the increase in log K_{ow}s. The observed increases in f_G were attributed to a decrease in food volume as food is digested and absorbed, a decrease in Z_G following lipid absorption, and the slower elimination of the more hydrophobic compounds in urine and feces. The decrease in fugacity capacity that occurs during digestion provides the thermodynamic force that forces the chemical to diffuse passively through the intestinal membrane to a region of lower fugacity.

Distribution and Disposition

The rat has been used frequently in pharmacokinetic studies to model mammalian distribution of PCBs throughout the body following feeding, gavage, and intraperitoneal injection (*i.p.*) exposures (Matthews & Dedrick, 1984). Following exposure, PCBs are initially distributed by the blood to highly perfused organs such as the liver, spleen, lungs, and kidneys (Kania-Korwel et al., 2005). Concentrations in poorly perfused tissue (adipose) and subcutaneous fat rise later in time as the PCBs are redistributed into fatty storage depots. In rats continuously exposed to 1, 3.3, 10, 33, or 100 ppm Aroclor 1254 diets for 7, 28, or 84 days, total PCBs in livers rose two to three fold, whereas in the adipose tissue of the same animals, the increases in PCB concentration were four to six

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times greater over the same exposure periods (Dragnev et al., 1994). Blood and adipose total PCB concentrations increased in an approximately linear fashion for the 1-10 ppm dose groups in this study, and blood burdens were three to five times higher than the diet concentrations, at the end of the 84 day exposures, in this group. Mean blood: liver: adipose tissue total PCB ratios were 1: 22: 359 in rats after four weeks of oral gavaging with Aroclor 1254 in corn oil (P. R. S. Kodavanti et al., 1998), and 1: 21: 659 after one, four, and twelve weeks of continuous exposure to 1-100 ppm Aroclor 1254 diet, which may indicate either a difference in distribution or retention due to the method of exposure and the vehicle or may just be due to the difference in total accumulated dose, which was much higher for the gavage exposure.

Rats fed 10 and 100 ppm Aroclor 1254 diet for 7, 28, or 84 days (Nims, Fox, Issaq, & Lubet, 1994), or orally gavaged with 30mg A1254/kg body weight 5 days/week for 4 weeks (P. R. S. Kodavanti et al., 1998), tended to bioaccumulate ortho-substituted, non-coplanar congeners with five or more chlorines in their livers to a greater extent than the di-, tri-, and tetrachlorobiphenyls, presumably because the lower chlorinated congeners are more readily metabolized in mammals.

Half-lives of PCBs in livers (2-3 weeks) and in adipose tissue (~7 weeks) were calculated from two different sets of recovery animals: one week of Aroclor 1254 diet and three weeks control diet, and four weeks of Aroclor 1254 diet followed by eight weeks of control diet (Dragnev et al., 1994). They found that in

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animals exposed to a higher dose, more PCBs were lost from all three tissues, and animals that had been fed 1-10 ppm Aroclor 1254 lost PCBs at a slower rate.

PCBs can also cross the blood-brain barrier and accumulate in the rat brain (P. R. S. Kodavanti et al., 1998), in male and female reproductive organs, and are transferred from mother to young during gestation in mammals (Jacobson et al., 1989; Jordan & Feeley, 1999), and egg laying in birds, amphibians, and fish (Blankenship et al., 2005; Fox, 2001). Post-natal, lactational transfer is also a major source of PCBs and associated hydrophobic xenobiotics for nursing mammal infants (Arnold et al., 1997; Fein, Jacobson, & Jacobson, 1984; Patandin, Dagnelie, Mulder, Op De Coul, Van Der Veen, Weisglas-Kuperus, & Sauer, 1999; Solomon & Weiss, 2002), and is an important means by which adult females decrease their total PCB body burden (Ayotte, Muckle, Jacobson, Jacobson, & Dewailly, 2003; Fitzgerald et al., 2004; Nadon, Kosatsky, & Przybysz, 2002).

PCBs in Biota

There are currently a number of fish consumption advisories for sports caught fish (SCF), due to PCB contamination, throughout the Great Lakes watersheds, particularly for Lake Michigan. In the Kalamazoo River Area of Concern (KRAOC), which covers the 80 mile stretch from Morrow Lake to Lake Michigan, a variety of advisories either limiting or precluding consumption of fish caught in the Kalamazoo River are in effect. Between Morrow and Allegan

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Dams "No Consumption" advisories are in effect for carp, catfish, and small and largemouth bass (longer than 14"), for all ages and genders, because PCB concentrations in those fish typically exceed the 2 ppm advisory level (MCDH Environmental & Occupational Epidemiology Division, 2004). While there are numerous fish advisories in effect throughout the Great Lakes basin EPA, levels of PCBs in Lake Michigan fish (Hickey, Batterman, & Chernyak, 2006) and waters continue to exceed those of the other Great Lakes, in large part because of historical and current PCB sediment loading from contaminated tributaries including the Grand Calumet, Fox, Sheboygan, Milwaukee, Green, and Kalamazoo rivers (McCarty et al., 2004). In the early 1980's, the Michigan Department of Public Health established the Michigan Fish Eater (MFE) cohort consisting of 572 fish eaters (26 lbs/yr of), and 419 age, sex, and region-matched non-fish eaters (consuming 6 lbs/yr), to track exposure to PCBs and other environmental contaminants from consumption of SCF.

Several earlier reports have been released detailing MFE cohort characteristics and presenting results from cross-sectional studies reporting positive association between SCF consumption and mean serum PCB levels of constituent groups of the cohort e.g. (Hovinga, Sowers, & Humphrey, 1993; H. E. B. Humphrey & Budd, 1996; H. E. B. Humphrey, 1983). More recently, results from two longitudinal studies of the subsets of the original cohort indicate that MFE mean fish consumption and mean serum PCBs declined from 1978 to 1990, mean fish consumption continued to decline from 1990 to 1994, and found that

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consumption of SCF, increasing age, and male sex were all predictors of serum PCBs (He, Stein, Humphrey, Paneth, & Courval, 2001; P. G. Tee et al., 2003). Tee and coworkers (2003), using mixed linear modeling, found that total serum PCBs continued to decline from 1990 to 1994 (from 24 ppb to12 ppb over entire study period), but He, et. al. (2001), using random effects regression modeling, reported that PCBs levels did not change significantly from 1990 to 1994. This difference is probably due to different analytical approaches, and to the difference in subsets sampled in the two studies--Tee and coworkers reduced their data set to include only those cohort members with serum, demographic, and dietary information from at least two of the MCDH's three cohort characterizations (P. G. Tee et al., 2003). Preference for lake trout, a top predator in the Lake Michigan food web, has been reported to be associated with increased serum PCBs in Lakes Michigan (He et al., 2001; Hovinga et al., 1993), Huron,Erie (Falk et al., 1999), and in Lake Ontario (Gerstenberger, Dellinger, & Hansen, 2000), but Tee and coworkers (2003) found heavy fish consumption prior to 1980 to be the most important predictor in the MFE, and in a recent study of a cohort of Great Lakes charter boat captains, having fished and consumed SCF of any type from Lake Michigan was the best predictor of serum PCBs (M. Turyk et al., 2006).

Average whole-body PCB concentrations in lake trout caught from Lake Michigan decreased from 23 ± 5 ppm in 1975 to 1.80 ± 0.17 ppm in 1998, but are still the highest for all of the Great Lakes (Hickey et al., 2006). None of the

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pooled samples of piscivorous Coho salmon and lake trout (5 fish/pool, caught in 1994-1995) analyzed for the Lake Michigan Mass Balance (LMMB) study, were in the unrestricted consumption category (PCB < 50 ng/g), and slightly more than half of the lake trout samples had PCBs > 2 ppm (McCarty et al., 2004).

Hickey and coworkers (2006) used time series data collected under the auspices of the EPA Great Lakes National Program Office from 1977 to 1998 to model changes in PCB concentration in lake trout across the Great Lakes. The data generated from this continuous monitoring of fish PCB levels in the Great Lakes basin is especially valuable to ecosystem modelers, as locations and methodology used for sampling and analysis are consistent through time. A simple first order model with a constant term yielded the best fit to the trends of decreasing PCB concentrations for all the lakes, and calculated half-lives for PCB loss in fish from lakes Ontario andErie were 9.1-12.4 yrs. In two of the most polluted lakes, decline in PCB concentrations has slowed considerably since about 1988 (the loss curve became asymptotic), and the best fit to the existing data resulted when a term for 'irreducible concentration' was included in the rate equation. This irreducible, or refractory, level of PCBs is 1.82 ppm for Lake Michigan fish, and 1.12 ppm for fish from Lake Huron.

PCB concentrations were most variable in Lake Superior trout, which may be partially due to fluctuations in their lipid content over the sampling time, and were the poorest fit to the first order model (Hickey et al., 2006). The major cause of fluctuations in the Lake Superior fish, however, have been attributed to

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changes in their diet due to food web changes—in the late 1980's the relatively lean sculpins that were a major part of the trout diet became increasingly rare, and they began to consume more of the fatty lake herring and, thus, more of the hydrophobic POPs, including PCBs, stored in the lipid-rich fish (Bronte et al., 2003). A subsequent change to a much leaner diet, including more insects, decreased the lipid levels in the lake trout, and also decreased their body burden of several POPs: PCBs, toxaphene, and dieldrin.

Fish play a key role in many temperate and arctic ecosystems by providing food for predatory fish at higher trophic levels within freshwater and marine food webs, and for piscivorous animals including a wide variety of birds and marine mammals, bears, minks, otters, and humans. In the Great Lakes basin, fish consumption has also played a key role in moving contaminants out of the aquatic food chain and into terrestrial food webs, where they can be biomagnified to an even greater extent than in lacustrine food webs.

In the decades following World War II, the widespread use of the bioaccumulative pesticide DDT led to a dramatic decline in the reproductive success of eagles in the 1960's to 1970's. This decline was associated primarily with the bioaccumulation of DDT in the birds, and the eggshell thinning effect of *p,p'*-DDE, a metabolite DDT. Bald eagles, one of the top predators in freshwater, aquatic-based food chains in North America, were nearly extirpated from the continent due to their susceptibility to the effects of DDT/DDE; since that time, bald eagles have served as a sentinel species for monitoring regional trends in

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the biomagnification of organochlorines in the U.S. and Canada (Environment Canada, U.S. EPA, 2005).

The bald eagle has made a comeback in many areas of the Great Lakes (e.g., northern Minnesota, Wisconsin, etc), but fails to thrive in lower region of southwestern Michigan (Environment Canada, U.S. EPA, 2005). On average, 63 ppm (wet weight) PCBs were measured in failed bald eagle eggs collected from the Ottawa Marsh in the coastal area of the KRAOC in 1994, 1996, and 2000—nearly four times the PCB level in failed eggs from other Lake Michigan coastal sites, and sixteen times that of inland, lower peninsula sites (Stratus Consulting, 2005). Total PCB concentration of 532.5 ppm, estimated from the sum of 20 congeners, was reported in serum from three nestlings sampled in the Allegan State Game Area of the KRAOC in 1999 (2 birds), and in 2000 (1 bird). These levels are the highest in Great Lakes region, three to five times the estimated serum burdens of nestling bald eagles from LakesErie, Superior, and Huron, and 20-30 times greater than serum levels from other sites in both the Upper and Lower Peninsulas of Michigan.

Fish are probably the most well known feral species monitored for PCB contamination in the Great Lakes area and elsewhere, but fish consumption is not the only route by which PCB-contaminated sediments can affect terrestrial animals. The tree swallow, a passerine bird that feeds on emerging aquatic insects (those that hatch from the sediments deposited on river and stream banks), has been monitored for trophodynamic studies of PCB biota-sediment

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accumulation in the KRAOC. Mean, lipid-normalized total PCB concentrations in adult tree swallows (157 ppm), nestling tree swallows (46.5), and tree swallow eggs (68.7) were approximately 6-9 times greater in KRAOC study site (Trowbridge Dam) than in the reference site (Fort Custer), and both lipid-normalized and wet weight (ww) PCB concentrations in benthic invertebrates, aquatic emergent insects, crayfish, fish, and muskrats were also higher at Trowbridge dam (Neigh et al., 2006). The PCB body burden in fish sampled from the KRAOC were ~7 times greater than fish taken from the Kalamazoo RIVER in Fort Custer, however, PCBs in mink liver were slightly higher at the reference site (60.5 ± 40.6 μg/g lipid) than at contaminated site (68.8 ± 25.7 μg/g lipid). Neigh and coworkers (2006) attribute this to the observation that, regionally, mink diets consist of only about 14% fish, but the much smaller sample size from Fort Custer (n = 3) versus KRAOC (n = 9) may also bias the results.

PCBs have also affected terrestrial food webs in the floodplain of Trowbridge Dam. Total PCB bioaccumulation in a soil-based food web In this area of the KRAOC has been reported (Blankenship et al., 2005), with apparent biomagnification (13-15x) occurring at the last trophic transfer in two of the constituent food chains: Soil? Plant? Small mammals (except shrews)? Great horned owl eggs, and in Soil? Terrestrial insects? Shrews. Fort Custer was also the reference area in this study, and, as with their previous report on aquatic based bioaccumulation from sediments in KRAOC, total PCBs (ww) were higher

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in soil and in animals (at all trophic levels) in the contaminated area than in the reference area.

Several factors determine contaminant loads in animals at the top trophic level, these include: contaminant load and lipid content in each organism at lower trophic levels; lipid content, age, size, and the metabolic capacity and digestive efficiencies of all the organisms in the food web. While the contaminant load and the potential for biomagnification of PCBs and other POPs in humans is higher when carnivorous or omnivorous animals at or near the top of a food chain are consumed, PCBs are also bioaccumulated from agricultural food chains where only herbivorous (or grain-fed) animals or animal products are consumed.

Mclachlan (1995), measured the concentrations of selected PCB and PCDD/F congeners in air, soil, rye grass, corn leaves, and cow's milk all collected from the same remote region of rural Germany, where atmospheric PCBs are the predominant input, and applied a fugacity-based analysis to derive biomagnification factors for the air/soil? grass? cow? cow's milk? human (breast milk). The local data set for POP contamination of human breast milk was limited to only three individuals, so PCB, PCDD/F, and HCB levels, and lipid content from a Dutch cohort were used as a proxy. The fugacities of each di-ortho, Cl_4-Cl_7 biphenyl were bioconcentrated in plants compared to air and soil, biomagnified slightly in cow's milk, and were biomagnified 14 to 54 times in human breast milk.

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Kalantzi and coworkers (2004) collected commercial butter samples from the U.S., Australia and New Zealand, Africa, and from several Asian and European countries to test the utility of butter from grazing cows as a surrogate for quantifying atmospheric PCBs to determine possible effects of global fractionation and cold condensation on atmospheric PCB distribution. Polychlorinated biphenyls were detected in each of the 63 samples at concentrations ranging from 0.23 - 14.1 ng/g lipid, and levels were much lower in samples from the southern hemisphere than from the northern temperate zone. In general, concentrations of the higher chlorinated congeners were highest in the areas of Europe and the U.S. that were closest to contaminated sites or industrialized regions.

The literature is rich with reports of quantifying PCBs in foods for human consumption, generally the mean or average concentrations range from in tens of nanograms to picograms on a lipid-normalized basis. Besides the dairy studies discussed above (Kalantzi et al., 2001; McLachlan, 1996) PCBs have been reported in poultry and eggs (Lindström, Småstuen Haug, Nicolaysen, & Dybing, 2002; Schoeters & Hoogenboom, 2006), in farmed fish and shellfish from the Maine, eastern Quebec, Norway, western Canada and the Pacific Northwest (Carlson & Hites, 2005; Rawn et al., 2006; Shaw et al., 2006) and China (Kannan et al., 1997; Nakata et al., 2002). In the U.S., meat for human consumption has recently been surveyed for content of dioxin-like compounds by the USDA, including the coplanar congeners, PCB 77, 126, and 169 (Hoffman et al., 2006). Average

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concentrations of these congeners in fatty tissues of steers, hogs, chickens, and turkeys did not exceed 5 pg/g lipid. Conspicuously absent from the USDA survey of dioxin-like compounds in market meat are some of the most abundant PCB congeners found in dairy, fish (wild and farmed), human serum, and human breast milk: PCB 138, 153, 118, and 180 (ATSDR, 2000; Hansen et al., 2003; McFarland & Clarke, 1989). The current regulatory emphasis in the sphere of public health on PCB congeners having dioxin-like toxicity, particularly carcinogenicity (P. S. Ross & Birnbaum, 2003; Van den Berg et al., 1998; Van den Berg et al., 2006), fails to fully capture the extent of chronic effects of PCB exposure.

Hepatic Transcriptional Responses to PCB Exposure

Nuclear Receptor Transcription Factors

Nuclear receptors play a key role in mediating the hepatic transcriptional response to PCB exposure by inducing expression of genes that regulate the development and maintenance of health in an organism. These include genes and transcription factors involved in the cell growth, proliferation, redox status, differentiation, and metabolism, as well as ones coding for enzymes that metabolize xenobiotics and transport them out of the cell. Inappropriate initiation or suppression of gene transcription leads to disruption of signaling pathways, which can in turn affect gene transcription, and can cause or contribute to a suite of health problems *in vivo*--immunosuppression, endocrine

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disruption, carcinogenesis, neurotoxicity, and developmental toxicity (Boas, Feldt-Rasmussen, Skakkebæk, & Main, 2006; Hurst & Waxman, 2005; Mandal, 2005; Puga et al., 2002).

The nuclear receptor (NR) superfamily includes two types of ligand-activated receptor proteins with hydrophobic 'pockets' for ligand binding that are particularly important in the organismal response to polychlorinated biphenyls--steroid hormone receptors (SHRs), and the aryl hydrocarbon receptor (AhR). Endogenous ligands for the SHRs are small molecules, many of which have rigid, polycyclic structures similar to those of hydrophobic pollutants. Many orphan receptors (receptors whose endogenous ligands are not known), such as the AhR, CAR, and PXR, are also capable of binding hydrophobic xenobiotics with cyclic structures (Janosek, Hilscherova, Blaha, & Holoubek, 2006).

The general mode of action for modulation of gene expression by ligand-bound NRs begins with the inactive cytosolic NRs binding to a ligand and typically undergoing a conformational change which may release chaperones and expose domains required for DNA binding or dimerization. Once the ligand is bound, homo- or heterodimerization with another receptor protein, such as RXR, or complexation with coactivating proteins, may be required for translocation into the nucleus. Alternatively, as with AhR, dimerization with ARNT, another member of the bHLH PAS superfamily, to form a transcriptionally active complex, occurs within the nucleus. Inside the nucleus,

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the ligand-bound NR may require other coactivator protein(s) for DNA binding, or an NR-NR dimer may bind directly to response elements in the DNA and activate gene transcription. The DNA binding sequences of the SHRs are highly conserved, consisting of two four-cysteine Zn finger domains that bind palindromic DNA sequences (Wilson & Safe, 1998), whereas the AhR nuclear binding domain consists of a series of basic amino acids followed by a helix-loop-helix domain (bHLH) adjacent to the Per-ARNT-Sim dimerization interface (Hahn, 1998).

AhR is by far the most widely studied NR with reference to polyhalogenated aromatic hydrocarbon exposure. Planar 2,3,7,8-tetrachlorinated dibenzo-p-dioxin is the ligand with the highest (known) affinity for the AhR, coplanar PCBs 77, 126, and 169 are also very high affinity AhR ligands (S. H. Safe, 1995; Van den Berg et al., 2006). Reversible AhR-PCB binding is the result of more than hydrophobic interactions, however. The favorable change in Gibb's free energy that results from the "stacking" interactions of TCDD and PCBs lacking ortho chlorines with the AhR outweighs the unfavorable change in enthalpy required for these molecules to assume a coplanar conformation. These molecules can act as electron acceptors in charge-transfer associations with the AhR, and donor- acceptor complexation may also occur between the AhR and the sterically accessible, planar portion of a mono-ortho substituted PCB, such as 2,3,4,4',5-pentachlorobiphenyl (McKinney & Waller, 1994). Charge transfer interactions in non-ortho substituted PCBs such as PCB 77 and 126 (Appendix D)

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are strengthened further by polarizability vectors along the longitudinal axis that result from lateral chlorine substitutions at meta and para positions on the biphenyl ring (McKinney & Waller, 1994). The polarizability of laterally-substituted chlorines may be important in enhancing the non-covalent interactions of PCBs with nuclear receptors or other enzymes that have hydrophobic, C-shaped ligand binding regions (Chauhan, Kodavanti, & McKinney, 2000; McKinney, 1996)

Laboratory exposure to TCDD induces a remarkably wide range of non-genotoxic carcinogenic and non-carcinogenic health effects *in vivo*: cancer, thymic involution, abnormalities in male and female reproductive organs and function, endometriosis, embryonic teratogenesis, immune suppression, chloracne, hepatic damage and steatosis, gastric epithelial hyperplasia, oxidative stress, and induction of a lethal, irreversible wasting syndrome, most of which appear to be mediated by AhR-ARNT binding to DREs (Mandal, 2005). The broad range of pathological and toxicological effects induced by interaction of AhR with TCDD and related compounds, including the coplanar PCBs, has been the subject of intense scrutiny for over thirty years, and the associated literature has been reviewed extensively (Mandal, 2005; Marlowe & Puga, 2005; Puga et al., 2002; S. Safe, Wormke, & Samudio, 2000; S. H. Safe, 1994b; Schecter, Birnbaum, Ryan, & Constable, 2006; Wilson & Safe, 1998). The detailed mechanism for AhR pre-transcriptional complexation and ligand binding, nuclear translocation and dimerization with ARNT, binding to ARE, XRE/DRE has also been investigated

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extensively, and serves as a model for NR-mediated modulation of gene expression by xenobiotics (Hankinson, 1995; Janosek et al., 2006).

In human hepatocytes, upregulation of genes important in cell cycle and apoptosis, including several transcriptional factors (TF), was reported recently in response to 20 μM A1254 (Reymann & Borlak, 2006). Their framework analysis reveals three phases of interaction of this mixture of PCBs with the AhR. In the first phase, the PCB-activated AhR influences transcription factor expression by binding to AhR recognition sites in TF promoter regions. Once these TFs are translated, all of their associated gene regulatory networks may, in turn, be activated by the increase in TF proteins. Secondly, the identification of several (37 pairs) TF binding sites (e.g., NF-κβ, TBP, and CREB-1) proximal to the AhR promoter sites suggests that the AhR may act in concert with other TFs to regulate transcription. Thirdly, coexpression of a gene with an AhR promoter site, and a neighboring gene with a promoter site for a different TF, may occur because AhR activation gives the other TF access to the neighboring site on the chromosome. Because they used AhR specific cDNA arrays, their work does not exclude the possibility that other NRs and TFs may be primary targets for A1254-activated gene expression. If the expression of other NRs is also increased as a consequence of PCB-initiated AhR induction, the likelihood that phenotypes resulting from pleitropic gene expression will be observed is also much higher.

Xenobiotic ligands may also exert toxic effects by preventing endogenous ligands from binding to their receptors and repressing gene transcription, or by

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binding to NRs but failing to induce necessary gene transcription. These interceptions could both occur as a result of binding interactions with a receptor--a xenobiotic ligand with higher binding affinity could outcompete an endogenous ligand, or a weak affinity ligand could block the hormone-receptor binding interaction. To date, high affinity PCB-binding has only been demonstrated for the coplanar-AhR interaction, however, there are several mono ortho PCBs that are weak AhR ligands (Van den Berg et al., 2006), and weak binding to the ER has also been demonstrated (Sonnenschein & Soto, 1998). Proteasomal and ubiquitination protein degradation pathways will also be activated as a result of the increased gene expression and subsequent protein production, and this could deplete intracellular concentrations of cofactors or heterodimerizing NRs required for SHR-regulated transcription.

Xenobiotic Metabolism

SHRs and AhR maintain cellular homeostasis by regulating the transcription of genes that are translated into enzymes capable of metabolizing biologically active endogenous and exogenous ligands so that they can be excreted from the cell. This process occurs in three steps: detoxification or bioactivation (Phase I), conjugation (Phase II), and transport out of the cell (Phase III), and has been well-defined for many drugs, pollutants, and for the endogenous ligands of the nuclear receptors reviewed in (Handschin & Meyer, 2003; S. Safe, 1993; S. H. Safe, 1994a; Xu, Li, & Kong, 2005). Emphasis here will be

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on the known components of PCB metabolism, but it is important to keep several things in mind. Many PCBs, particularly the more highly chlorinated congeners with para substitutions (Hansen, 1999), are resistant to degradation by xenobiotic metabolizing enzymes (XMEs). Some metabolites may be more bioactive, and thus more toxic, than their parent compounds, and finally, not all metabolites will be excreted.

During Phase I metabolism, the polarity of the PCB structure is increased by enzymatic hydroxylation and epoxidation, and phenolic derivatives of PCBs have been reported in the breast milk and blood of humans and other mammals (ATSDR, 2000; Bergman, Klasson-Wehler, & Kuroki, 1994). Adaptive upregulation of genes coding for CYPs, monooxygenase proteins that catalyze epoxidation and hydroxylation of PCBs, is the most significant and widely observed Phase I response to PCB exposure in humans, laboratory rodents, fish and birds, as well as in wild mammals, fish, and birds. The cytochrome P450 superfamily of genes has thousands of known substrates, and a range of substrate specificities and polymorphisms (Nelson et al., 2004). The cytochrome families CYP1-CYP4 are primarily responsible for the metabolism of foreign chemicals, the remaining 13 gene families catalyze hydroxylation of endogenous substrates in a number of biosynthetic pathways: SH biosynthesis from cholesterol (CYPs11, 17, 19, 21), hydroxylation of retinoic acid (CYP 26), synthesis of bile acids, activated Vitamin D3, cholesterol (CYPs 7, 24, 27, 51), and thromboxane and prostacyclin synthesis (CYPs 5 and 8) (Waxman, 1999).

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Exposure to complex mixtures such as Aroclors introduces a variety of PCB congeners (and their metabolites), and thus is capable of inducing a suite of CYPs. CYP1A1 and 1A2 are both induced by AhR ligand-activated transcription, whereas 2B and 3A CYPs are induced by the binding of CAR and PXR, respectively. Coplanar and ortho-substituted PCBs can engage in non-covalent stacking interactions with the heme center of P450s (McKinney & Waller, 1994; Poulos & Raag, 1992), and epoxidize the biphenyl. Epoxidation by P450s can occur on PCBs with meta and para chlorines (e.g., PCB 77), but the enzymes will preferentially oxidize the most accessible sites: meta and para with vicinyl hydrogens (Hansen, 1999; S. Safe, Bandiera, & Sawyer, 1985).

The oxidized product generated in Phase I, typically an arene oxide for PCBs (Hansen, 1999), can be conjugated in Phase II metabolism. Conjugation generally introduces more electronegativity onto the molecule, which increases water solubility for excretion in urine, and changes the properties of the acceptor so that it can penetrate cell membranes or interact with Phase III transport proteins. Two of the most important enzymatic reactions for PCBs include attachment of glucuronic acid to phenols and hydroxyl groups by UDPGT's, and conjugation of GSH to an electrophilic center by the GSTs (Barter & Klaassen, 1994; Brouwer et al., 1998; Klaassen & Hood, 2001; Lubet et al., 1992). As is the case for the CYPs, expression of different variants of these Phase II enzymes will depend on the PCB substrates present, and on the species. Sulfotransferases, enzymes that transfer SO_3^- from PAPs (3'-phosphoandenosine-5-phosphosulfate)

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to a nucleophilic group on the acceptor are also active in conjugation of oxidized PCBs (Liu, Idil Apak, Lehmler, Robertson, & Duffel, 2006). Methylsulfonyl metabolites of PCBs bioaccumulate in fatty tissue, and have been detected at relatively high concentrations in human breast milk (Kato, Haraguchi, Shibahara, Masuda, & Kimura, 1998).

The recognition of the third phase of protein-mediated xenobiotic metabolism--transport out of the cell--is a fairly recent addition to the xenobiotic metabolism pathway (Xu et al., 2005), and specific PCB metabolite transporter proteins have not yet been reported. The membrane transport proteins identified thus far include the well-studied P-glycoprotein or multidrug resistant transporter (Kim, 2002), and the organic ion transporters. Proteins that are potential candidates for PCB metabolite transport include the bile acid transporters (OATPs) that move large organic anions out of cell, and OCTs, large organic cation transporting proteins (Battaglia & Gollan, 2001).

Oxidative Stress

Besides the generation of detoxified or toxified metabolites, the increase in Phase I and Phase II enzymes during xenobiotic metabolism may also have harmful secondary effects. The oxidizing capability of the iron center in heme monooxygenase cytochromes is regenerated in a cyclical fashion following O_2 transfer, by a one electron transfer from NADPH-cytochrome P450 reductase (Por). In the presence of lower chlorinated PCBs (e.g., PCB 77), uncoupling of the

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NADPH-dependent reaction has been reported for CYP1A1 in liver microsomes of rats treated with A1254, in insect microsomes transfected with recombinant human CYP1A1 (Schlezinger, White, & Stegeman, 1999) and for several species of fish and birds (Schlezinger, Keller, Verbrugge, & Stegeman, 2000). O_2 may be reduced directly by Por to form superoxide, hydrogen peroxide, and possibly other reactive oxygen species (ROS) such as hydroxyl radicals. In research led by Schlezinger (1999, 2000), the rate of Cyp1A reduction of PCB 77 was reduced ~50%, and ROS (H_2O_2, $O_2^{-\bullet}$) release was stimulated, in all models, by addition NADPH. ROS are capable of oxidizing membrane lipids, proteins, and DNA, and thus could damage cell components and interfere with basal cell functions. Genotoxic mutations resulting from the formation of DNA-OH adducts have not been shown to be important in the development of PCB-related cancers, however (S. Safe, 1989). Besides initiating oxidation of cellular components, hydrogen peroxide produced by the uncoupling of CYP1A1 can suppress the transcription of CYP1A1 and CYP1A2 (Barker, Fagan, & Pasco, 1994), possibly by direct interaction with the XRE (Xu & Pasco, 1998). Increases in hydroxylated PCB metabolites from Phase I metabolism could also directly affect the intracellular redox potential, as some may cycle from phenols to quinones (Bolton, Trush, Penning, Dryhurst, & Monks, 2000).

Cytokine, hormone, and growth factor action and secretion can all be stimulated by oxidative stress, which can, in turn, affect signaling pathways such as MAP kinase and NF-κβ that regulate transcription (Allen & Tresini, 2000).

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The oxidative-stress related response to a mixture of PCB congeners will be complex--CYP1A metabolism of the lower chlorinated PCBs can be a direct source of oxidized PCBs and ROS, and higher chlorinated planar and non-planar PCBs can activate the expression of oxidative-stress sensitive transcription factors, such as AP-1, by transcriptional mechanisms (Eum, Lee, Hennig, & Toborek, 2004; Twaroski, O'Brien, Larmonier, Glauert, & Robertson, 2001). Apoptosis is also stimulated by oxidative stress, and Slim and coworkers (2000) demonstrated that PCB 77-induced activation of JNK/SAPK and, subsequently, upregulation of Caspase 3 and Annexin V of the caspase 3 apoptotic pathway was preceded by a transient reduction in intracellular GSH in porcine endothelial cells. Protein sulfhydryls are key modulators of redox potential, and upregulation of glutathione-S-transferases, proteins integral to maintaining intracellular GSH levels, are widely observed in response to exposure to PCBs and other AhR ligands (Nebert et al., 2000; S. H. Safe, 1994b; Slim, Toborek, Robertson, Lehmler, & Hennig, 2000).

PCBs can interfere with cellular homeostasis by binding with NRs and inducing transcription or by interfering with the normal transcriptional regulatory functions of the receptor. Metabolism of PCBs can produce ROS and increase the overall toxicity by producing reactive metabolites with different toxic effects. Initiation of oxidative stress responses and subsequent abnormal up or down regulation of intracellular signaling proteins such as inflammatory mediators and growth factors can have profound effects on the adult and may

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result in permanent changes in developing organisms. Stressed cells may undergo apoptosis, and inappropriate release of cytokines can have harmful, systemic consequences on the health of the whole organism. Upon exposure to mixtures of PCBs, affected cells are likely to be reacting to several of these stresses simultaneously, and a wide range of health effects have been observed in humans and animals.

Health Effects Related to Dietary PCB Exposure

Accidental human exposure to mixtures containing high concentrations of PCBs, such as the Yusho and Yu-Cheng incidents in which rice oil used for cooking was contaminated with PCB-rich oil from a heat exchanger, have been associated with development of chloracne (pustules) accompanied by hyperpigmentation (development of patches of darkening of skin), and hepatic effects including induction of drug metabolizing enzymes and hepatomegaly (Aoki, 2001). Accidental exposures to PCBs can also occur via combustion, as with the 1981 capacitor fire in a state building in Binghamton, NY (Fitzgerald, Standfast, & Youngblood, 1986). Both of these accidents resulted in human exposure to mixtures of PCBs and other compounds, in the case of the Yusho/Yu-Cheng, the contaminated rice oil used for cooking contained not only PCBs, but also poly-quarternaryphenyls (PCQs), and chlorinated dibenzofurans (CDFs) formed within the heat exchanger, and possibly during the cooking process. The most serious health effects reported from the Yusho/Yu-Cheng---

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an increase in deaths from liver disease, and reproductive and developmental problems--have since been attributed primarily to CDFs present in the rice oil but the concentrations of PCBs were still very high in this incident (ATSDR, 2000). The combustion of PCBs during the capacitor fire in Binghamton resulted in the deposition of a large quantity of highly toxic products of PCB combustion: CDFs, PCDDs, and PCDFs, but no long term health effect were reported from this exposure (Fitzgerald et al., 1986).

Epidemiological evidence for (and against) human susceptibility to a variety of health effects from PCBs comes from two sources: typically small occupational cohorts in which workplace exposures were usually via inhalation or dermal, or environmental cohorts with elevated dietary exposures (e.g., Michigan Fish eaters Cohort). Serum PCB levels are used as the most practical means of gauging total PCB exposures in humans, because persistent compounds such as PCBs equilibrate among adipose, serum lipids, and organs, and serum levels should be a good indication of levels in other target tissues, such as liver and adipose tissue (ATSDR, 2000).

Clinical manifestations associated with high serum levels of PCBs in humans following occupational exposures include elevated levels of liver enzymes such as γ-glutamyltransferase (GGT) in serum, increases in serum lipids and cholesterol, induction of drug-metabolizing enzymes, and respiratory and eye irritations. Serum cholesterol was also reportedly higher in a contaminated-fish-eater cohort in Alabama, though this has not been observed consistently in

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the GL cohort (ATSDR, 2000).

The rat liver is the target organ for PCB toxicity, and significant increases in serum enzymes indicative of PCB-induced hepatotoxicity include GGT, ALP, AST and ALT, serum cholesterol, triglycerides, and bilirubin have been reported (ATSDR, 2000). Decreased body weights, in males and females, for intermediate (one year) and chronic (two years) exposure times to 25-200 ppm dietary Aroclors (Mayes et al., 1998; Smialowicz et al., 1989; Ward, 1985), as well as increases in absolute and relative liver and thyroid weights compared to controls have been reported. At high doses and chronic exposure times, increases in rat liver weights may be accompanied by non-neoplastic histopathological changes in the liver such as hepatomegaly, fatty and necrotic lesions, and/or by neoplastic changes such as increased foci of alteration, lesions, or tumors (Moore, Hardisty, Banas, & Smith, 1994; Ward, 1985). Other negative health effects associated with PCB exposure in humans, rats, and wildlife include significant increases in incidence of liver or gastrointestinal tract neoplasias, immune suppression (Dallaire et al., 2004; Dewailly et al., 2000; Tryphonas et al., 1989), neurotoxic behavioral effects (Jacobson, Jacobson, & Humphrey, 1990; Schantz & Widholm, 2001; Tilson & Kodavanti, 1998), alterations in thyroid hormone levels (Brouwer et al., 1998; Koopman-Esseboom et al., 1994), reproductive and developmental problems (Carpenter, 2006; Gray Jr. et al., 1999), and increases in inflammatory responses (Everett et al., 2007; Hennig, Oesterling, & Toborek, 2007; Slim et al., 2000). Rats and other animal models have been used extensively

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to test the toxicity and carcinogenicity of Aroclor mixtures and individual PCB congeners for over thirty years, and a comprehensive and detailed review of the range of health effects particularly earlier, comprehensive laboratory studies is available through the ATSDR review (2000). The primary focus of this portion of the literature review will be to find commonality between Aroclor 1254-induced health effects observed in laboratory experiments with the male Fisher rat and effects of environmental PCB exposures in human and some wild animal populations.

Overview of Carcinogenicity

The tumor promoting potential of Aroclor 1254 has long been known (Preston, Van Miller, Moore, & Allen, 1981); tumor promotion has also been observed for the structurally-related compound TCDD (Bock & Kohle, 2005), individual coplanar (3,3',4,4'-tetrachlorobiphenyl) and ortho-substituted (2,2',4,4',5,5'-hexachlorobiphenyl) congeners (Tharappel, Lee, Robertson, Spear, & Glauert, 2002), and for other technical PCB mixtures (ATSDR, 2000). PCBs are listed as a probable human carcinogen by the IARC, and the National Toxicology Program (NTP) has classified the evidence from animal studies as sufficient to anticipate that PCBs are a human carcinogen, however the EPA has designated the carcinogenic potential of PCBs as 'B2' to indicate that animal evidence is sufficient, but epidemiological evidence is not. Retrospective mortality epidemiological studies typically use occupational cohorts, and can be limited

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because of small study groups, uncertainties in amount and duration of exposure and/or employment, short-term follow up, and a lack of information or background on individual subjects. The designation of PCBs as probable human carcinogens remains controversial (Shields, 2006), but is thought to be protective of public health (Carpenter, 2006).

Two years of consumption of 100 ppm Aroclor 1254 diets, in the National Cancer Institute's 1978 study, significantly increased both number and types of hepatic lesions in male and female Fisher 344 rats (Ward, 1985), and intestinal metaplasia and adenocarcinomas were also observed in the stomach sections from some of the high dose male rats in this study. Cancerous or precancerous hepatic lesions were also reported for Aroclor 1242 and 1260 in other early dietary studies of mixtures, though not consistently (ATSDR, 2000). In 1994, histological evidence from seven of the cancer studies conducted in previous decades were reexamined by a panel of pathologists, who applied new diagnostic criteria and nomenclature to liver sections (Moore et al., 1994). In this analysis, several diagnoses of neoplastic lesions were reclassified as hepatocellular hyperplasia or adenoma, and some diagnoses of neoplastic nodules were changed to "non-neoplastic". After reviewing data from all the studies in light of the new tissue classifications, the panel found that rats consuming PCB mixtures with 60% chlorine consistently had more liver tumors than ones consuming Aroclor 1254, and that dietary exposure to mixtures with 54% or 42% chlorination did not result in a statistically significant increase in

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liver tumors.

Mayes and coworkers published the results of a comprehensive lifetime study of PCB-induced carcinogenicity in rats in 1998. In this study, male and female Sprague-Dawley rats were fed diets containing high concentrations of Aroclor 1016 (50-200 ppm), Aroclor 1242 (50, 100 ppm), and Aroclors 1254 and 1260 (25, 50, 100 ppm) for two years. Most of the toxic responses to exposure were seen in the high dose Aroclor 1260 or 1254 dose groups, and were generally greater for the female rats than the male rats: the females had dose-related increases in group mean liver weights (24-120%), significant increases in GGT, AST, and ALT, and tumorigenic responses that were Aroclor and dose-dependent. In both sexes, benign hepatocellular adenomas were the major carcinogenic response to treatment, and these neoplasms had no effect on survival rates. Liver tumor rates for Aroclor 1254 exposure in females were 9 to 14 times greater than the adjusted rates reported by Moore (1994) for the female (Fisher) rats receiving the same dose in the 1978 NCI study (Ward, 1985).

The higher rate of tumors observed in the Mayes 1998 study may be due, in part, to different responses in the two breeds, but the lot of Aroclor 1254 (lot# KI-02-6024) used in this study was not produced prior to 1976 (P. R. S. Kodavanti & Ward, 2005), and may also have been a cause of the greater toxic response. The modified KI-02-6024 or "late" Aroclor 1254 (ATSDR, 2000) lot used in this study was free from CDFs, but the dioxin-like toxic equivalents (TEQs) from coplanar PCBs 77 and 126 in this modified batch were still about double that of

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the commercial product in use from 1958-1977. PCB 126, 3,3',4,4',5-pentachlorobiphenyl, is the PCB congener the most similar to 2,3,7,8-tetachlorodibenzo-p-dioxin (TCDD) in its ability to bind the aryl hydrocarbon receptor (AhR), and most potent in inducing dioxin-like toxic effects. Thus, the presence of relatively high concentrations of PCB 126 in the Aroclor 1254 used in the later study (Mayes et al., 1998), and the differences in breeds tested, could have increased the rate of tumors in females, compared to the report by Ward from the 1985 study.

The carcinogenicity of pure PCB 126 (30-1000 ng PCB/kg bw), and PCB 126 (62-3110 ng PCB/kg bw) in a binary mixture with 2,3',4,4',5-pentachlorobiphenyl (PCB 118, 10-500 µg/kg bw) were tested by gavage in female Harlan Sprague-Dawley rats (NTP, 2006c). Significant increases in incidences of liver cholangiocarcinomas and hepatocellular neoplasms (mostly adenomas) were observed in both of these 2-year studies, and were considered 'clear evidence of carcinogenic activity' by the NTP review panels. The hepatic cholangiomas observed in both studies were thought to be treatment related. Hepatic cholangiomas were also observed in a related NTP study of PCB 153 (2,2',4,4',5,5'-hexachlorobiphenyl) carcinogenicity and toxicity, in some of the female Harlan Sprague-Dawley rats gavaged with 1000-3000 µg/kg bw for two years (NTP, 2006a).

Rat and human responses to PCB exposure appear to differ substantially with respect to hepatic neoplasms and tumorigenicity. Several authors have

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analyzed existing reports of epidemiological data from PCB-exposed occupational cohorts for proof of carcinogenic effects in humans (M. P. Longnecker, Rogan, & Lucier, 1997; Shields, 2006), and agree that there is no obviously causal evidence for liver carcinogenicity in occupationally exposed populations. The evidence most suggestive of PCB-induced liver effects was an observation of 8 cases versus 2.8 expected in a cohort of 2572 highly exposed capacitor workers followed for 32 years (Brown, 1987). Shields (2006) pointed out, however, that liver cancer was grouped with biliary passage and gall bladder cancers in that study, and only four of the eight cases actually originated in the liver. Three additional deaths due to liver/biliary tract/gall bladder cancers were also reported for that cohort for 1983-1998 (Prince et al., 2006), but there was no clear relationship between duration of exposure or employment, and mortality. Increased incidence of liver cancer was reported for the 15 year follow up period, in Italian women exposed to TCDD in the 1976 industrial spill in Seveso (Landi et al., 2003), which suggests that coplanar congeners such as PCB 126 would be the most likely, of any of the PCBs, to have carcinogenic effects on human livers.

In his very brief review of human health effects of PCBs, Carpenter (2006) points out several other cancers that have been found to be associated with PCB exposure in industrial or disease cohorts--gastrointestinal/biliary, thyroid, brain, pancreas, breast and lung cancers, malignant melanoma, and non-Hodgkins lymphoma--however, many of the positive associations are found in only one or

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two occupational cohorts, and consistent correlative relationships between PCB exposure and cancer do not hold up in cross study comparisons. Pancreatic and breast cancers have been significantly correlated with serum PCBs (Hoppin et al., 2000), however, a clearly positive, consistent relationship between PCBs and human carcinogenicity is lacking in extant epidemiological studies. This could be for a number of reasons unrelated to causation: small study sizes, insufficient exposure (short term employment) or insufficient latency period for a cohort, lack of adequate controls, gaps in exposure measurements or subject history, inaccuracies in self-reported information, and inaccurate classification of cancer type or cause of mortality. In the absence of a clearly defined mechanism for PCB-induced carcinogenicity, the very close correlation between PCB body burdens, and the concentrations of other lipophilic POPs due to environmental exposures makes it difficult to exclude the possibility that observed effects are caused by agents other than PCBs, or that effects are due to interactions of PCBs with other pollutants present.

Overview of Non-Cancer Effects

Immunotoxicity

High body burdens of organochlorines, including PCBs, have been significantly associated with increased susceptibility to middle ear (Dewailly et al., 2000) and respiratory tract (Dallaire et al., 2006) infections in humans. PCBs and other POPs have been implicated as immunosuppressants in wild

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populations of marine mammals (Beland et al., 1993), and as T-cell mediated and cell-mediated suppressants in seals (P. S. Ross et al., 1995; P. S. Ross et al., 1996). Suppression of non-specific, cell-mediated, and humoral immunities, have been reported in rats, mice, and monkeys following oral exposure to Aroclor 1254 (ATSDR, 2000; Smialowicz et al., 1989; Tryphonas, 1994).

Male Fischer rats were exposed to 0.1-25 ppm Aroclor 1254 by gavage for 5, 10, and 15 weeks, and spleen, lymph nodes, and thymus were removed (Smialowicz et al., 1989). The mitogen-stimulated proliferative response of splenic lymphocytes was tested with T-cell mitogens phytohemagglutinin (PHA-P) and concanavalin A (ConA), B-cell mitogen *Salmonella typhimurium*, and T- and B-cell pokeweed mitogen (PWM). Treatment effects on non-specific immunity in the Fisher rats were evaluated by measuring the ^{51}Cr released by the splenic natural killer cell (NKC)-mediated lysis of ^{51}Cr-labelled lymphocytes from YAC-1 and Wistar rats. Lymphoma cells of the PCB-treated and control rats (responder cells) were incubated with radio-labeled lymphoma cells from Wistar rats (stimulator cells), then additional stimulator was added and the release of ^{51}Cr was used as a measure of the effector response of cytotoxic T-lymphocytes (CTL). To test the capacity of T-cells to respond to non T-cells, responder cells were also combined with stimulator cells that had been treated with mitomycin C in a one-way, mixed-lymphocyte reaction (MLR).

Aroclor 1254 treatment had no effect on Fisher rat lymphocyte response to CTL or to non T-cells, or on the splenic proliferative response to PWM, ConA, or

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S. typhimurium in these experiments (Smialowicz et al., 1989). Significant, dose-related decreases in thymus weights were observed in rats gavaged with 10 and 25 ppm Aroclor 1254 at all treatment times, and significant reductions in whole body weight were observed at 10 and 15 weeks for highest dosed rats. NKC activity towards Wistar and YAC-1 rat lymphocytes was suppressed at 15 weeks in the 10 and 25 ppm groups, and the in vitro response of splenocytes to PHA-P was increased in rats treated with 25 ppm PCBs for 15 weeks. Increase in mitogen-induced splenocyte proliferation indicates that the high dose of Aroclors may actually have induced a protective immune response in the Fisher rats. The composition of lymphocyte subsets were not measured in this study, however, and the increase in mitogen-stimulated proliferation may reflect a change in lymphocyte phenotypes due to Aroclor treatment. The limited immunotoxic responses in these experiments occurred only in conjunction with significant body weight loss, which suggests that they may be secondary responses stemming from systemic, treatment-related stress on the organism (Vos & Van Loveren, 1998), rather than a primary immunotoxic response to PCBs.

Rats may not be a sensitive model for immunotoxicity of PCBs or related POPs in mammals, no changes in cell-mediated immunity (total or differential white blood cell counts) or in the histology of immune system organs (thymus, spleen, and lymph nodes) were observed in the male and female Sprague-Dawley rats exposed to Aroclor 1254, 1016, 1242, 1260 in the Mayes, et.al. lifetime

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study (1998). *In vitro* treatment of Fisher rat leukocytes with mixtures of Aroclor 1242, 1254, and 1260 (0.1-2 μg/g) or PCDD/PCDF (1-15 pg/g), or with a combination of the Aroclor and PCDD/PCDF mixtures at environmentally relevant concentrations did not affect peritoneal or peripheral NKC or phagocytotic activity, or the splenic T-cell mediated MLR (Omara et al., 1998). The immune systems of adult rats were not significantly affected by eating a diet containing Great Lakes trout or salmon (Tryphonas, 1994), or herring from the highly contaminated Baltic Sea (P. S. Ross, Van Loveren et al., 1996). There were no differences in immune responses, between treated and control animals, for lymphocyte proliferation or phenotyping, oxidative burst, NKC, phagocytosis, or in the presence of plaque-forming cells in Fisher 344 rats that had been fed a diet containing 5% lyophilized blubber lipids from Arctic or St. Lawrence River beluga whales (Lapierre et al., 1999). The possibility of antagonism of immunosuppressive effects cannot be ruled out in the whale blubber and fish feeding experiments, as these environmental matrices contain a complex mixture of organochlorines including PCBs, dioxins, and PCDD/Fs.

Marine mammals that feed on fish often have very high POP body burdens, and these pollutants have been hypothesized to have immunosuppressive effects in wild populations (Deguise, Martineau, Beland, & Fournier, 1995; P. S. Ross, 2002). The population of belugas from the St. Lawrence River estuary used by Lapierre, et.al. (1999) has widespread bacterial infections, and a very high incidence of skin lesions and tumors compared to

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belugas from the Arctic and elsewhere. A diet of contaminated fish impaired the overall capacity of seal immune systems to mount a defense against a foreign protein, as captive seals from the North Atlantic fed Baltic herring for two years reportedly had a significantly suppressed response to subdermal ovalubumin challenge, and changes in T-cell subsets, compared to seals fed cleaner N. Atlantic herring (P. S. Ross et al., 1996). In the wild, bioaccumulative methylmercury (MeHg) is also a component of total marine mammal contaminant burdens (P. S. Ross, 2002), and may play a critical role in immunosuppression. In the *in vitro* experiments of Omara (1999), significant suppression of NKC, phagocytosis and MLR were observed only when 2 μg/g MeHg was added to mixtures of Aroclors and/or PCDD/F's in rat leukocyte cultures.

The most responsive laboratory animal model for Aroclor 1254-induced immunotoxicity found to date is the monkey (ATSDR, 2000). Adult female rhesus monkeys were fed gelatin capsules containing Aroclor 1254 (Lot #KA634) in oil at 0, 5, 20, 40, and 80 μg PCB/kg bw/day for 23 months, and immunological studies were initiated when serum and adipose PCB concentrations had equilibrated (Tryphonas et al., 1989). Antibodies to sheep red blood cells (SRBC, administered on day 1 and day 8), lymphocyte transformation (PHA-P and ConA), T and B lymphocytes, and serum hydrocortisones and total immunoglobulins were measured in peripheral leukocytes were isolated from blood withdrawn at 1, 7, and 14 days. Significantly, as the dose increased, the

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antibody titers for T-cell dependent IgM and IgG both decreased. Aroclor 1254 suppressed the monkeys' ability to raise the Ig type antibodies to SRBC even at the lowest, most environmentally relevant exposure level (5 μg/ kg bw/day). Increased T-suppressor (T_S) and decreased T-helper (T_H) cells were also observed in the high dose group; a similar shift in the lymphocyte subsets ratio (T_H/T_S) was also observed as a persistent effect following oral exposure to PCB and co-contaminant mixtures in the Yu-Cheng cohort (ATSDR, 2000). Treatment with PHA-P, but not ConA mitogen increased the lymphocyte proliferative response in these experiments, similar to the apparently immunosupportive effects observed by Smialowicz (1989) in Aroclor 1254-treated rat splenocytes, and in Yu-Cheng patients (ATSDR, 2000).

Trends in human immune responses to PCBs and, by default, a mixture of common environmental organochlorine co-contaminants such as PCDD/Fs, DDE/DDT, TCDD, and HCB are derived entirely from accidental exposures such as Yu-Cheng, and from environmentally exposed cohorts such as fish and marine mammal eaters. One of the most consistently reported immunotoxic associations for PCBs and other POPs is an increased incidence in infections in children or infants due to prenatal exposure (ATSDR, 2000; Dallaire et al., 2004; Dewailly et al., 2000). Marine mammals and fish are staples of the traditional Inuit diet, and their POP body burdens are among the highest in the world (Van Oostdam et al., 2005). Increased incidence of otitis media, respiratory tract, and gastrointestinal tract infections were associated with higher prenatal maternal serum levels of

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POPs, particularly PCBs and DDE, in a cohort of Inuit infants (Dallaire et al., 2004). In an earlier study of another Inuit infant cohort from the same region (Dewailly et al., 2000), the risk of otitis media was higher for infants whose mothers had higher organochlorine burdens, but there were no significant differences in lymphocyte subsets, white blood cell counts, or Ig antibodies (G, A, M) between breast- and bottle-fed infants. Prenatal exposure to PCBs was associated with increased middle-ear infections and chicken pox in Dutch cohort of preschoolers, and numbers of lymphocytes and cytotoxic, memory, and activated T-cells were reportedly higher in the formula-fed subset of children with higher prenatal PCBs (Weisglas-Kuperus et al., 1995). Similarly, non-specific and specific immune responses to RCMV virus challenge were impaired in rat pups exposed *in utero* to TCDD and oil extracts of Baltic herring lipids, with the most pronounced impairment occurring at the earliest ages (P. Ross et al., 1996/8/16). These results suggest that prenatal exposure to sub toxic levels of PCBs and related organochlorines has a significantly suppressive effect on the ability of infant or immature mammal immune systems to mount a defense against infectious agents, even in less susceptible organisms.

Thyroid Endocrine Effects

PCBs have been hypothesized to act as endocrine disruptors in humans and wildlife by binding with thyroid hormone receptors (TR) and changing gene expression in target tissue (Zoeller, 2005), or through alterations of thyroid

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homeostasis (Bowerman, Best, Grubb, Sikarskie, & Giesy, 2000; Hagmar, 2003; Schell et al., 2004). The thyroid hormone system is thought to play an important role in the mechanism(s) of PCB toxicity because of the consistently observed depression in levels of circulating thyroid hormone in fetal or neonate rats following *in utero* or lactational exposures to PCBs (Bowers et al., 2004; Ulbrich & Stahlmann, 2004). A decrease in serum thyroid hormones --specifically in triiodothyronine (T_3), the active form of the hormone, and in thyroxine (T_4), the inactive form that can be rapidly converted to T_3 in tissues by iodothyronine deiodinases (DI's) in response to changes in hormonal status--can lead to a hypothyroid state in the developing organism. Normal development of brain and sex organs in mammals, amphibians, and birds are dependent on thyroid hormone status, and a change in thyroid hormone levels during or prior to a critical stage of development may permanently alter neurological and reproductive capabilities (Brouwer et al., 1999; T. Colborn, vom Saal, & Soto, 1994; M. A. Longnecker et al., 2003; Schantz, 1996).

Aroclor 1254 exposure at high doses causes thymic atrophy in a manner consistent with an overt toxic response (Smialowicz et al., 1989; Ward, 1985) in laboratory rats. Histological changes in the rat thyroid gland indicative of 1) gland stimulation and 2) disruption of normal follicular colloid processing that is required for production and secretion of TH, have also been observed at these and lower, sub-acute or sub-chronic exposures to Aroclor 1254 and other PCBs (ATSDR, 2000; Barter & Klaassen, 1994; Bowers et al., 2004). Chronic stimulation

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of the thyroid by TSH release can lead to follicular cell proliferation and hyper- or neoplastic lesions in the organ (Barter & Klaassen, 1994; Brouwer et al., 1998). Higher degrees of interfollicular fibrosis also interferes with the ability of the thyroid to produce TH, and has been associated with PCB and POP concentrations in marine mammal blubber from animals in highly polluted locales: St. Lawrence River belugas (Mikaelian, Labelle, Kopal, De Guise, & Martineau, 2003), North and Baltic Sea porpoises (Das et al., 2006), and seals from the North Sea (Tabuchi et al., 2006), compared to cleaner reference sites.

Wild mammals, birds, and fish in heavily polluted regions have been reported to have abnormal TH ratios, depression in circulating thyroxine levels, and/or abnormalities in thyroid gland development, compared to animals living in less polluted reference areas (Brouwer et al., 1998; T. Colborn, 2002; Fox, 2001; Rolland, 2000). Significant decreases in T_4 were reported in rats following a two month feeding exposure to coho salmon taken from Lakes Michigan,Erie, and Ontario, compared to rats fed rat chow and rats fed coho salmon from the Pacific Ocean (Sonstegard & Leatherland, 1979). Thyroid weights and height of thyroid follicular epithelial cells were both significantly higher in the GL exposures, and decreases in serum thyroxine were not affected by iodide supplementation, suggesting that GL salmon (at that time) contained a goitrogenic chemical or mixture of chemicals. Numerous field studies have reported thyroid gland enlargement in colonies of herring gulls and other fish-eating birds in the GL basin compared to reference areas (Fox, 2001; Rolland, 2000), and comparison of

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thyroid effects in several colonies throughout the basin showed that the most severe depletion of vitamin A levels and the most significant changes in thyroid histology were observed in the same colony on western Lake Erie(Moccia, Fox, & Britton, 1986). A diet of polluted fish also induces hypothyroidism in fish-eating mammals--plasma and total free T_4 (FT_4), total T_3 (TT_3), and retinol were significantly reduced in seals fed herring from the Baltic Sea (Brouwer, Reijnders, & Koeman, 1989), compared to seals fed Icelandic herring.

Decreased T_4 levels have also been recently correlated to human serum PCBs in GL fish eaters (M. E. Turyk et al., 2006), and in populations with lower 'background' exposures in the GL region of New York state (M. Bloom, Vena, Olson, & Moysich, 2006; Schell et al., 2004). Persky and coworkers (2001) reported that fish consumption, but not PCBs, was negatively correlated with serum TH in the GL consortium, however, a recent reanalysis, in which data from the WI Angler Cohort was excluded, showed that serum PCBs were significantly negatively correlated with T_3 in men, and correlation with decreases in FTI and TSH were close to significance (M. E. Turyk et al., 2006). In their most recent study of the consortium of male GL charter boat captains (2006), Turyk and coworkers report T_3, T_4, and TSH were all negatively correlated with serum PCBs, and positively correlated with TEQs. Higher serum TCDDs have been significantly correlated with increased TSH, but not TT_4, T_3 uptake, or thyroid disease, in the highly exposed group of veterans responsible for aerial spraying of the dioxin-contaminated defoliant Agent Orange during the Vietnam war (the

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Ranch Hand cohort) (Pavuk, Schecter, Akhtar, & Michalek, 2003). In a cohort with lower, background exposures, dioxin-like compounds (DLC) in serum did not correlate with TSH (M. S. Bloom, Weiner, Vena, & Beehler, 2003). Circulating T_3 was not correlated to total PCBs in Akwesasne youth (Schell et al., 2004) or GL anglers (Persky et al., 2001), nor to PCB TEQs in the New York angler cohort (M. S. Bloom et al., 2003). Unlike the generally consistent negative correlation between serum T_4 and either measured serum PCBs or exposure to dose-related increases in PCBs, comparisons across laboratory studies and species have not yielded a consistent correlation between PCB exposures and serum T_3 (ATSDR, 2000; Brouwer et al., 1998; Rolland, 2000).

Induction of liver UDP-GTs in Phase II metabolism following dietary exposure to PCBs can deplete serum thyroxine by increasing it's rate of elimination, and significant correlations between UDPGT induction and depletion of total and free T_4 has been observed in rats treated with Aroclor 1254 (Barter & Klaassen, 1994; Morse, Wehler, Wesseling, Koeman, & Brouwer, 1996). Following glucuronodation, T_4 is excreted, thus decreasing the amount of thyroxine available for rapid conversion to T3 in peripheral tissues. Male Sprague-Dawley rats were fed diets containing high concentrations of UDPGT inducers: 1200 ppm phenobarbital (PB), 1000 ppm pregnenolone-16α-carbonitrile (PCN), 250 ppm 3-methylcholanthrene (3-MC), and 200 ppm Aroclor 1254, for 21 days to determine effects of the enzyme inducers on circulating TH and gland morphology (Barter & Klaassen, 1994). Liver UDPGT levels were significantly

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greater than controls for all treatments, and the extent of induction was greatest for the animals fed Aroclor. Total and free T_4 were reduced 30-40% by PCN, 3-MC, and PB, but exposure to dietary Aroclor 1254 had the most pronounced effect, with 80-90% reductions in total and free thyroxine. Free T_3 was unaffected, and changes in total T_3 were intermittent and modest for all treatments. TSH levels were increased for PCN, PB, and Aroclor 1254 on days 14 and 21, but the increase in TSH for Aroclor treatment was smaller than for PCN treatment, which would not be expected if TSH release alone was compensating for TH depletion in A1254-treated animals. Thyroidal ^{131}I capture was significantly higher in rats treated with PCN, PB, and Aroclor 1254 than controls, and thyroid weights were significantly increased by PCN and A1254 treatment. The increased TSH, thyroid weights, and ^{131}I capture provide good qualitative evidence for thyroid gland stimulation in response to UDPGT induction during Phase II metabolism, but fall short of quantitatively accounting for the marked decrease in thyroxine observed with PCB treatment.

Some hydroxylated PCB metabolites may also lower circulating T_4 by binding reversibly to TH transport proteins with hydrophobic molecular clefts such as transthyretin (TTR) and thyroxine binding globulin (TBG) (McKinney & Waller, 1994), thus decreasing the number of carriers available to transport thyroxine. Cheek and coworkers (1999) tested TH transport protein binding for several types of POPs known to decrease TH and induce UDPGT's: two coplanar PCBs (77 and 126), six hydroxylated PCB metabolites, DDE and DDT isomers,

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and two chloracetanilide pesticides. As a class, the hydroxylated PCBs were the only effective competitors against T_4, they found that three of the six the hydroxylated PCBs had higher affinities for TTR binding than thyroxine, while none of the compounds could compete effectively for TBG at nanomolar concentrations. TTR is the primary TH carrier in rats, while TBG is the major TH transporter in primates (Cheek, Kow, Chen, & McLachlan, 1999). TTR is the only TH transport protein within the primate brain, however, and TTR binding to PCB-OH in the brain could conceivably cause local depletion of TH during development that might have lasting effects. Transthyretin gene expression was reportedly significantly increased in *X. laevis* tadpoles treated with 50 ppb Aroclor 1254 compared to controls (Lehigh Shirey, Jelaso Langerveld, Mihalko, & Ide, 2006), which might be a response to compensate for increased levels of PCB-OH's.

Significant fetal and neonatal accumulation of a 4-OH-pentachlorinated biphenyl was reported for the offspring of dams gavaged with 25 mg/kg of Aroclor 1254 on gestational days (GD) 10-16 (Morse et al., 1996). Metabolite transport across the placental barrier appeared to be uni-directional, by GD 20, the concentration of PCB-OH in the fetus exceeded that in the mother. Brain T_4 was severely reduced in the fetal cerebella but not in the fetal forebrains in this study, however, only small, non-significant decreases in T_3 concentration were observed. The maintenance of euthyroid T_3 levels in the fetal brain is likely due, at least in part, to the significantly higher activity of fetal brain type II deiodinase

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that was simultaneously observed. TTR was not quantified in this study, so it is not known if protein levels increased with PCB-OH concentration, or if PCB-OH binding to the protein lowered the amount of TTR available to transport T4 to the fetal cerebella.

Maternal treatment with Aroclor 1254 during gestation also had sex-specific effects on the offspring--21 day old females had significantly lower brain serum thyroxine (-44%) in this experiment (Morse et al., 1996), but TH in their male littermates was not different from controls. UDPGT was induced in treated dams and reduced in the offspring of treated dams, compared to controls, but by 90 days postpartum protein levels in males were indistinguishable from controls, while in the females UDPGT levels were still 40% lower than controls. Thyroxine was significantly depleted in the offspring of Sprague-Dawley rats that had received daily oral doses of 15 mg/kg Aroclor from gestational day 1 through postnatal day (PD) 23, but no gender-related differences in circulating TH were observed on PD 35 (Bowers et al., 2004).

Methlysulfonyl metabolites of ortho-substituted PCBs have been detected in many species, including in human milk, liver, and adipose tissue, and 3- and 4- $MeSO_2$ derivatives are the most commonly reported in mammals (Gauger et al., 2004; Haraguchi, Koga, & Kato, 2005; Kato et al., 1998). Effects of these metabolites on TH homeostasis were evaluated after male rats were injected *i.p.* with 20 μmol/kg of four of the most prevalent methylsulfonyls: 3-$MeSO_2$-CB132, 3-$MeSO_2$-CB141, 3-$MeSO_2$-CB149 and 4-$MeSO_2$-CB149, or with PB at 430 μmol/kg

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for four consecutive days (Kato et al., 1998). Seven days after the last treatment, total T_4 and T_3 were reduced by treatment with para substituted $MeSO_2$-CB149, but only T_4 was decreased in the serum of rats injected with meta-substituted methylsulfonyl CB's. Overall, TH reductions were significantly more pronounced following injection with the PCB metabolites treatment than with PB. TSH was not measured in these experiments, but thyroid weights were significantly higher in rats treated with 3-$MeSO_2$-CB141 than in the controls, which suggests that some $MeSO_2$ PCB metabolites could exert a stimulatory action on the thyroid.

Zoeller and several collaborators have been at the forefront of some of the most cogent research into mechanisms of thyroid-mediated neurological disruption in the developing rat brain. Using in situ hybridization to quantify changes in gene expression, they have reported TH-like upregulation of several genes in spite of depression in maternal levels of circulating T_4 and T_3 induced by treatment with A1254 (Bansal, You, Herzig, & Zoeller, 2005; Dowling & Zoeller, 2000; Gauger et al., 2004). Postpartum levels of RC3 (neurogranin) mRNA were increased, MBP mRNA levels were similar, and serum T_4 was reduced, compared to controls, in rats whose mothers had been fed 4 or 8 mg/kg A1254; whereas expression of these genes was uniformly reduced in rats born to mothers treated with goitrogenic propylthiouracil or methimazole (Dowling & Zoeller, 2000). Expression of RC3, Oct-1, NSP-A, and HES were all increased in the rat fetal cortex in a manner consistent with TR promoter-activated gene expression

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following TH binding, by maternal exposure to 4 or 8 mg/kg A1254 (Bansal et al., 2005; Gauger et al., 2004).

Because of the paradoxical effects of A1254 treatment on fetal gene expression and serum thyroxine levels observed in this series of experiments, they also tested rat hepatic TRα and TRβ binding to THs, coplanar PCBs 77 and 126, mono-ortho substituted PCBs 105 and 118, di-ortho substituted PCBs 138 and153, as well as an array of hydroxylated and methylsulfonylated metabolites of these PCBs (Gauger et al., 2004). Neither the PCBs nor any of their derivatives were capable of displacing ^{125}I-T_3 from the TR in a competitve assay, and the binding affinity of ^{125}I-T_3 for TR was unaltered by any of the parent PCBs. Recently, 4-OH-PCB 106 was reported to act as a direct agonist of TR interaction with the growth hormone promoter in vitro (You, Gauger, Bansal, & Zoeller, 2006). In this study,they found that GH mRNA was increased by addition of the hydroxylated metabolite to cultures of GH3 cells transfected with a TR--luciferase reporter construct, similar to the effect of T_3 addition. A single base pair substitution in the promoter construct effectively blocked upregulation of GH, suggesting that the hydroxylated PCB may be blocking access to the promoter sequence.

Detrimental behavioral and cognitive effects have been reported in rats and monkey following or concurrent with perinatal exposures to Aroclor 1254. One of the most consistently reported outcomes following prenatal and

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lactational exposure to Aroclor 1254, defined mock-environmental mixtures of PCBs rich in ortho-substituted congeners, or individual, ortho-substituted PCB congeners are deficits or delays in learning spatial tasks. Provost and coworkers (1999) reported that development of "mapping skills" required to navigate the Morris water maze was impaired in rats exposed to environmentally relevant dietary levels of A1254 (12.5 ppm) during gestation and lactation, in particular they found the treated rats performed worse than controls in the later trials. Higher error rates have been reported for radial arm maze navigation for rats that were exposed perinatally to higher doses of A1254 (Corey, De Ku, Bingman, & Meserve, 1996), suggesting that spatial learning and memory can be affected by PCB exposure. Post-natal exposure to higher doses of Aroclors 1254, 1260, 1242, and 1016, (25-250ppm Aroclors, described previously by Mayes et.al., 1998) did not have any measurable effect on a battery of neurological endpoints, including sensorimotor, CNS, and autonomic functions, muscle tone and equilibrium, motor activity or nervous system histopathology, measured in adult Sprague-Dawley male and female rats after one year of dietary exposure (Freeman et al., 2000). Higher cognitive functioning was not evaluated in these studies.

Jacobson and Jacobson (1990) were the first to report neurotoxic effects of prenatal exposure to PCBs in a cohort of infants born to women who consumed two to three meals of Lake Michigan fish per month, for at least six years prior to conception. In this longitudinal study, high levels of prenatal PCBs were

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significantly associated with poor recognition memory on the Fagan Test of Infant Intelligence (FTII) at birth and lower general IQ scores at age four, as measured by the McCarthy Scales of Children's Abilities (Jacobson & Jacobson, 1993; Jacobson & Jacobson, 1996). By eleven years of age, children with highest prenatal PCBs (cord blood 1.25 µg/g lipid) scored an average of 6.2 points less on the WISC-R IQ test, and were 7 months behind in reading comprehension (Jacobson & Jacobson, 2003). In the mid 1990's, when the only published studies were the Michigan Cohort and a similar study in a cohort of North Carolina infants (Rogan, Gladen, & McKinney, 1986), in which no significant differences in cognitive abilities were found between high and low PCB exposures had been published, concerns with small sample size, selection bias, and failure to control adequately for potential confounders such as methylmercury exposure in the Michigan studies (Schantz, 1996). While results from several subsequent cohort studies in Rotterdam-Groningen, the Netherlands (Patandin, Lanting et al., 1999), and Oswego, NY (Stewart, Reihman, Lonky, Darvill, & Pagano, 2004), cannot be compared quantitatively due to differences in design and measurements, convergent results (except for the NC study) indicate a clear association between neurological deficits in infants and children (4 yrs) and *in utero* exposure to elevated levels of PCBs (Ribas-Fitó Fitó(Ribas-Fito, al., 2001; Schantz et al., 2004). Postnatal exposure to PCBs through breastmilk does not appear to be associated with neurological deficits in infants (Patandin, Dagnelie, Mulder, Op De Coul, Van Der Veen, Weisglas-Kuperus, & Sauer et al., 1999; Ribas-Fitó et al., 2001), and

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preschoolers that had been breastfed actually scored better on intelligence tests than their bottle-fed counterparts (Huisman et al., 1995; Patandin, Lanting et al., 1999).

In addition to disruption of TH homeostasis, several other mechanisms for neurotoxicity have been investigated. Non-coplanar PCBs with ortho (2,2') or ortho-lateral (2,2',5,5') chlorine substitutions and intermediate K_{ow}s are reportedly the most neurotoxic congeners (Fischer, Seegal, Ganey, Pessah, & Kodavanti, 1998). Decreased brain dopamine levels have been reported in rats following oral exposure to high doses (500 and 1000 ppm) A1254 (R. F. Seegal, Bush, & Brosch, 1991), and in rat and primate brains, and neuronal cell cultures, following exposure to ortho-substituted PCBs (Chu et al., 1995; R. F. Seegal, Bush, & Shain, 1990). Some ortho-substituted PCBs and PCB-OH's have been reported to interfere directly with neuronal function by altering intracellular Ca^{2+} homeostasis in vitro (P. R. Kodavanti & Tilson, 1997; P. R. S. Kodavanti & Ward, 1998) and in vivo (P. R. S. Kodavanti et al., 1998; Sharma, Derr-Yellin, House, & Kodavanti, 2000). Circulating total T_4 dropped by 95%, cerebellar Ca^{2+} buffering capacity was decreased, and PKC translocation to neuronal membranes was significantly increased in male Long-Evans rats exposed to 30 mg/kg/day of Aroclor 1254 for four weeks (P. R. S. Kodavanti et al., 1998). Tyrosine hydroxylase was unaffected in these experiments, however, indicating that in vivo exposure to A1254 can potentially affect second messenger systems in the absence of an effect on dopamine synthesis. A common mode of action for

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ortho-substituted PCB neurotoxicity has not emerged from existing studies, and environmental exposure to mixtures of PCBs may affect several potentially neurotoxic pathways simultaneously (ATSDR, 2000).

An array of neurological and cognitive effects arising from prenatal or lactational exposure to PCBs have also been observed in laboratory studies of rats and a thorough survey of the literature generated by decades of these studies is beyond the scope of this review. The reader is referred to several reviews (Dietrich et al., 2005; Schantz & Widholm, 2001; Stewart, Reihman, Lonky, Darvill, & Pagano, 2004; Weiss, 2005) for an overview of the effects of PCBs on brain neuronal growth and organization in the developing hippocampus of fetal and neonate rats, and for discussions of cognitive and psychobehavioral effects of developmental PCB exposure.

Emerging Concerns

PCBs, dioxins, and other, structurally-related POPs present in human blood may play a role in the development of atherosclerosis (Hennig et al., 2002; Hennig et al., 2007), and thus be a contributing factor to cardiovascular disease. Atherosclerosis is a chronic vascular inflammatory state characterized by the accumulation of migratory smooth muscle cells and and cholesterol-laden macrophages or "foam cells" to form fibrous plaques rich in oxidized cholesterol and lipids. Vascular endothelial cell dysfunction (within the walls of the blood vessel) leading to upregulation of cell adhesion molecules such as VCAM-1,

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ICAM-1, PECAM-1, integrins, and selectins, is now thought to be the initiating event in plaque development. Immune cells attach to the adhesion molecules and "activate" the endothelial cells to release pro-inflammatory cytokines, surface receptors, and proteinase enzymes such as interleukins, interferons, matrix metalloproteinases (MMPs) and monocyte chemoattractant proteins (MCP-1, MCP-4) that increase the permeability of the endothelial wall. Once the barrier is compromised, lipids and monocytes can migrate through to the intima, form foam cells, and release inflammatory cytokines that activate the smooth muscle cell layer to proliferate and migrate to the lesion. Apoptotic and necrotic foam cells in the interior release cholesterol as plaques age, which, in turn recruits more macrophages to the site and causes the plaque to grow and the blood vessels to constrict. Plaques weaken and rupture, partially due to the action of the MMPs, exposing blood components to tissue factor, and initiating coagulation, platelet recruitment, and ultimately thrombosis (Glass & Witztum, 2001)

Oxidative stress due to contact with coplanar PCBs in circulating blood may be an precursor to vascular endothelial cell dysfunction and the initiation of atherosclerotic lesions. Slim and coworkers (2000) found that PCB 77-induced activation of JNK/SAPK and, subsequently, upregulation of Caspase 3 and Annexin V of the caspase 3 apoptotic pathway was preceded by a transient reduction in intracellular GSH in porcine endothelial cells. In porcine endothelial cells treated with coplanar PCBs 77, 126, and 169, oxidative stress

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evaluated by DCF flourescence, and DNA-binding activity of NF-? ß were all increased but not with ortho-substituted PCB 153 (Hennig et al., 2002). The proinflammatory coplanar PCBs increased IL-6 production and VCAM-1 expression *in vitro* in these experiments, consistent with increased transcriptional activity of NF-? ß, and the endothelial barrier function (evaluated by albumin transfer) was significantly decreased by addition of coplanar PCBs. VCAM-1 expression was also greater in the aortic tissue of wild type, but not AhR $^{-/-}$ mice, following injection of 170 µmol/kg PCB 77.

Inflammatory sequelae in vascular endothelium have also been implicated in the metastasis of cancer cells to sites distant from a malignant tumor. Choi and coworkers (2003) investigated the hypothesis that an ortho substituted PCB (2,2',4,6,6'-pentaCB, PCB 104) could induce oxidative stress in human umbilical vascular endothelial cells (HUVEC) and increase production of adhesion molecules and inflammatory mediators. Consistent with their hypothesis, oxidative stress measured by Rhodamine 123 and DCF fluorescence was higher in PCB-treated cells, and E-selectin, ICAM-1, and MCP-1 expression were all significantly upregulated at 10 and 20 µM PCB 104. In addition, adherence to human leukemic cells (THP-1) was significantly increased in a PCB dose-related manner in HUVEC, even at the lowest (5 µM) exposure. Addition of as little as 10 µM PCB 104 to cultured monolayers of human microvascular cells (HMEC-1) significantly increased the permeability and allowed metastatic breast cancer cells to migrate across the cell layer (Eum et al., 2004). This increase in

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permeability was regulated by the phosphotidylinositol-3-kinase mediated upregulation of vascular endothelial growth factor (VEGF). Most noteworthy, perhaps, was the observation that passage of the metastatic cells across the monolayer was not altered at all by the addition of antioxidants, thus implying that blood-borne PCBs may also cause endothelial dysfunction by pathways entirely independent of AhR-mediated oxidative stress.

Interactions *in vivo* between PCBs and dietary lipids that demonstrate that PCB treatment can alter lipid metabolism and increase hepatic lipid content, as well as increase adhesion protein expression in blood vessel walls have also recently been reported (Hennig, Reiterer, Toborek et al., 2005). VCAM-1 expression in aortic endothelium and subendothelial space was much higher in LDL-$R^{-/-}$ mice maintained on a diet rich in corn oil (linoleic acid) than for those fed olive oil, following exposure to PCB-77 (170 μmol/kg, *i.p.*). In PCB-treated mice fed corn oil, clearance of linoleic, oleic, palmitic, stearic, and arachidonic acids from circulating blood was significantly decreased compared to rats on the corn oil control diet, and the hepatic neutral lipid content, as measured by Oil Red O staining, was also significantly greater in these animals. There was no significant difference in liver lipid content or in circulating levels of fatty acids between corresponding treatments for the olive oil diet. The literature is rife with reports of hepatic steatotosis resulting from PCB (particularly coplanar) and TCDD gavage exposures using corn oil as a vehicle , and this finding of significantly fattier livers with corn oil versus olive oil raises the issue that the

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use of corn oil as a vehicle may have been an essential, contributing factor in the hepatic accumulation of lipids in many experiments.

Microarray gene expression profiles in livers of PCB-treated animals also differed according to dietary fat content--in general, dietary linoleic acid resulted in downregulation of fatty acid synthesis genes, whereas fatty acid binding and transport genes were affected by oleic acid but not by linoleic acid. Cd36, a long-chain fatty acid transporter normally expressed at low levels in the liver (Abumrad, Coburn, & Ibrahimi, 1999), was upregulated 1.5 fold by PCB treatment only in animals fed dietary linoleic acid. Increased hepatic expression of Cd36 in LDL-$R^{-/-}$ mice may be an overcompensating response to the lack of an LDL receptor; plasma lipids were lowered, and hepatic Cd36 was upregulated approximately 8x, following conjugated linoleic acid feeding, in $LDLR^{-/-}$, apo $^{100/100}$ mice, a model for human hypercholesterolemia (Degrace et al., 2006).

Exposure to relatively high concentrations of TCDD has been positively associated with Type II diabetes in Vietnam veterans in the Ranch Hand cohort, and in small industrial cohorts from phenoxy herbicide manufacturing (M. P. Longnecker & Michalek, 2000; Remillard & Bunce, 2002b). Non-coplanar PCBs and POPs other than TCDD have been positively associated with insulin resistance in nondiabetic adult populations (D. H. Lee & Jacobs, 2006), and with the prevalence of Type II diabetes mellitus in a cohort of Michigan women (Vasiliu, Cameron, Gardiner, DeGuire, & Karmaus, 2006). Receptor-mediated mechanisms involving coplanar ligands and the ligand-activated PPARs have

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been posited (Remillard & Bunce, 2002a) as the basis for a causal relationship between exposure and diabetes. Dioxin-like compounds inhibit preadipocyte differentiation (Hanlon, Cimafranca, Liu, Cho, & Jefcoate, 2005), which is controlled by PPAR? (Gregoire, 2001), suggesting that AhR-mediated interference with normal PPARγ functions could be a contributing factor to the development of diabetes.

The major, biologically plausible mechanisms proposed by Remillard and Bunce (2002) for diabetogenic activity of TCDD and structurally related compounds are based on observations in rodents or from *in vitro* studies, these include 1) reduced glucose transporter expression (GLUT4, GLUT1), 2) AhR dependent increases in TNF-α expression that repress PPAR? expression, 3) cross-talk between AhR- and PPAR? -mediated signaling pathways through LXXLL domains. However, mechanisms in which an initiating step involves interactions of DLC with the AhR are inadequate explanations for positive correlations between noncoplanar PCBs and type II diabetes (D. Lee et al., 2006). Several authors have reviewed the close interconnection of obesity, inflammation, and type II diabetes and noted that while increased adiposity is not obligatory for development of diabetes, as was previously thought (Chen, 2006; Guzik, Mangalat, & Korbut, 2006; Shoelson, Lee, & Goldfine, 2006; Smith, 2002), inflammatory macrophage infiltration of adipose and increased production of IL-6 by adipocytes do seem to be consistently present prior to the onset of insulin resistance. In a sequence of events analogous to the development

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of aortic atherosclerosis outlined above, endothlial dysfunction could develop in vasculature surrounded by adipose tissue following the secretion of inflammatory adipokines, and this, in turn, could lead to onset of Type II diabetes (Guzik et al., 2006; Shoelson et al., 2006).

Adipose tissue has long been viewed as a storage compartment for persistent organic pollutants, and the potential for *in vivo* alteration of endocrine, nervous, or immune system functions (Kershaw & Flier, 2004; Lawrence & Coppack, 2000) by POPs has only rarely been explored experimentally (Mullerova & Kopecky, 2006). Given that adipocytes are the major site for steroid metabolism, that they secrete hormones and inflammatory mediators, and that they express several receptors that play a crucial role in the organismal response to bioaccumulative OCs, and are also the major repository for hydrophobic xenobiotics, it would be highly surprising if PCBs did *not* exert effects on the functioning of adpose tissue.

Microarray Profiling of Gene Expression

Toxicogenomics

The sequencing of human genome and genomes of prototypical human toxicological models such as the mouse and the rat, coupled with the development of microarray technology that permits evaluation of the expression of thousands of genes simultaneously in a single sample, has led to a new paradigm in the study of organismal response to toxicological insult.

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Toxicogenomics, or "the study of the structure and output of the entire genome as it relates and responds to adverse xenobiotic exposure" (Ulrich, 2003), has enabled investigators to evaluate the effects of well-known toxicological responses, such as hepatic microsomal enzyme induction, in the wider context of cellular or tissue genomic expression. Measuring expression of a wide spectrum of genes simultaneously gives investigators insight into the transcriptional changes occurring in biochemical pathways and networks that contribute to the overall physiological response in an organism or cell to pharmaceuticals, non-chemical environmental stressors such as heat and pH, and synthetic and natural chemical toxicants (Bustin & Dorudi, 2002; Chiappini et al., 2006; Waring, Ciurlionis, Jolly, Heindel, & Ulrich, 2001; Waring et al., 2002). The U.S. and other countries have devoted considerable resources to the development of appropriate statistical modeling and bioinformatic infrastructures that support the use of gene expression data collected using high-throughput methods such as microarray technology (Barrett et al., 2007; Dudoit, Gendeman, & Quackenbush, 2003; Gentleman et al., 2004; R. M. Simon & Peng Lam, 2004; Tong et al., 2003)

Prior to the development of microarrays, measurements of mRNA expression in a single experiment were limited to only a few genes, using labor-intensive radio-labeling and hybridization of mRNA in northern blots. Radio-labeled nucleic acid sequences are also used in nylon membrane spotted arrays, but probe hybridization to nucleic acids incorporating a fluorescent biotin or Cy3/Cy5 dye-swap are used in oligo and cDNA arrays, respectively. Oligo and

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cDNA microarray technology differ with respect to the type of probe used to bind sequences from a sample, but both are based on the immobilization of nucleic acid sequences onto a solid surface. The cloned cDNA used for cDNA, or spotted arrays, is prepared from DNA extracted from the organism of interest and then attached to the slide, whereas probes on oligo arrays are small sequences designed using EST and gene sequence data from public databases that are synthesized directly on the array. These sequences are then reacted with fluorescently labeled target sequences of complementary RNA (cRNA) or complementary DNA (cDNA) prepared by reverse transcription from purified total or message RNA isolated from the sample of interest. Once the labeled target sequences have been hybridized to their complementary sequences on the array, the array is rinsed to remove excess sample, the chip is irradiated, and the fluorescence of the remaining, bound sequences is measured. The intensity of the fluorescence in the image generated is proportional to the number of copies of bound target sequence, and these intensities are used to quantify expression of individual genes on the array.

Affymetrix was one of the first companies to report successful preparation of high-density arrays (GeneChip™); they employed a photolithographic technique to activate the surface of glass slides for chemical coupling to 5'-capped nucleosides (G, A, T, or C). Iterative steps of masking, photo activation, and binding are used to anchor nucleosides to specific areas on the slides, and iterative steps of 5'-uncapping and binding are used to extend the probe, or

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oligonucleotide, to a sequence of bases (20-25 for RGU34A) that will bind to a particular cRNA. One 'feature' on a microarray contains millions of copies of a single oligonucleotide probe. Sixteen to twenty different probe pairs containing one perfect (PM) and one mismatch (MM) probe are designed to bind to different segments of the target cRNA sequence, and fluorescence intensities of all the probe sets are considered together to derive an expression summary for each gene on an array. MMs differ from PM probes by only one base, and MM intensities may be subtracted from the hybridization signal for the probe pair to correct for non-specific hybridization.

Analysis of Microarray Data

Because of the high density of probes on the microarray, vast quantities of data are generated from a single GeneChip™, and this image-based data must be processed prior to comparisons with other arrays (control and treated) generated by a single toxicological experiment. Collecting, managing, and analyzing microarray data remains one of the biggest challenges in toxicogenomics (Dudoit et al., 2003; R. Simon & Lam, 2004). Signal variance increases proportional to mean probe signal intensities, so the fluorescence intensities are typically log2 transformed, and a statistical model may be developed to reduce the dependence of the variance on the mean (Gautier, Cope, Bolstad, & Irizarry, 2004; Irizarry et al., 2003). Processing probe set data into gene expression summaries involves 1) background correction to reduce signal noise within probesets on a single chip,

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2) normalization to reduce non-biological variance among chips, 3) fitting adjusted signal to a model from which an expression summary is calculated for the entire probe set (Bolstad, Collin, Simpson, Irizarry, & Speed, 2004). Several statistical models and data processing pipelines are currently used for background correction and normalization of Affymetrix microarray data. Among the most popular are the Affymetrix Microarray Analysis Suite (MAS 5.0), and Robust Multiarray Averaging (RMA) (Irizarry et al., 2003) followed by quantile normalization (Bolstad, Irizarry, Astrand, & Speed, 2003).

Background correction in Affymetrix MAS 5.0 is based on the anti-log of the robust (Tukey biweight) average of (PM - CT), where CT is equal to MM intensities when MM<PM, but is adjusted to be less than PM if MM > PM (Irizarry, Hobbs et al., 2003). A significant difference between the Affymetrix (MAS 5.0) approach and other, model-based approaches (Irizarry et al., 2003; C. Li & Hung Wong, 2001) is that the Affymetrix method does not account for probe effects across multiple arrays (McShane, Shis, & Michalowska, 2003). Li and Wong (2001) described a "probe affinity effect", namely that the variation of a specific probe across multiple arrays was often much smaller than the variation within a probe set on a single array, and developed their multiplicative dChip model to account for this probe affinity effect. More recently, Irizarry and coworkers (2003) developed RMA, an additive model in which the PM transformation is the sum of the log2 signals, the log scaled terms accounting for probe affinities, and the error. Log transformed, background corrected and

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normalized PM intensities are for summarized into a single value for probe expression using median polish. Comparisons of specificity, sensitivity and consistency of the three summary algorithms (MAS 5.0, dChip, and RMA) were conducted using freely available spike in and dilution data sets (Irizarry et al., 2003; Irizarry, Hobbs et al., 2003). In general, fold changes calculated using RMA and dChip expression summaries showed less dependence on initial concentration of cRNA hybridized to the array than did MAS, and the number of false positives as determined by RMA was less than for the other two methods, indicating increased sensitivity and specificity. Fold changes calculated from RMA summaries were depressed 10-20% compared to MAS, however, indicating that some accuracy is sacrificed for the increase in precision (Irizarry et al., 2003).

Once expression summaries have been calculated for each gene on each array, this information must be compared to determine if there are any statistically relevant changes between controls and treatments. The aims of microarray studies generally fall into one of three categories: class discovery, class prediction, and class comparison (R. M. Simon et al., 2003). In class discovery, the analytical goal is to find out whether or not the samples analyzed fall into natural groupings and what are the similarities within a class; classes are not known in advance in this type of study. Class prediction is the primary goal of (for example) many cancer tumor studies; genes that will reliably predict clinical outcomes, for example, whether a tumor is benign or malignant, and if it is malignant, how fast does it progress to metastasis, is the type of information

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sought in these studies (R. M. Simon et al., 2003). In toxicogenomic studies, one of the primary goals is to determine whether average global gene expression patterns in controls and treated tissue or cells are distinctly different. The identity of class discriminators--genes that distinguish among dose classes, and the relative magnitude of differences in gene expression between control and treated tissue are the other major results sought in class comparison analyses.

The magnitude of the multiple comparison problem presented by the testing of thousands of null hypotheses generated by the comparison of control arrays versus treated arrays can be addressed using multivariate permutational methods (R. M. Simon et al., 2003; Tusher, Tibshirani, & Chu, 2001). Random variance, non-parametric tests (t- or F-tests) such as these test whether or not the experimental results could be randomly distributed, and differ from parametric tests based on Gaussian distributions (Wright & Simon, 2003). Neither the assumptions of pooled common variance, nor completely random variance, may be useful for microarray data when the number of independent specimens per treatment or control group (2-5) (McShane et al., 2003), as groups of genes may be co regulated. Random variance tests that allow sharing of gene information within a class without assuming all variation is equivalent present a sensible compromise between the two extremes, particularly for data (e.g., inbred strains) which is expected to have lower individual gene variances (Wright & Simon, 2003).

Control of the false discovery rate, or the probability that a class

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discriminator will be wrongly determined to be significant by multiple hypothesis testing (Benjamini & Hochberg, 1995), is important for limiting the results of microarray experiments to a more statistically meaningful list of genes (Storey & Tibshirani, 2003; Tusher et al., 2001). The higher the proportion of false positives in a list of genes generated by a toxicogenomic study, the greater the chances of wasting time and resources investigating false leads. Global permutation tests conducted by comparing random permutations of class labels with the actual class labels can be used to determine whether or not the gene expression profiles, as a whole, differ among control and treatment classes (McShane et al., 2003).

Once the raw data has been processed and filtered for significance, the list of significantly changed, annotated genes can be "mined" or examined for biologically significant information. The gene ontologies (GO), controlled vocabularies developed to codify the cellular location, physiological functions, and molecular reactions of gene products, are critical to this endeavor. The ontologies are based on several sources of information including experimental results published in the literature, and inferences drawn from sequence similarities across several species (Currie et al., 2005; Ma, Morrow, Fernandes, & Walbot, 2006), and are constantly being refined and updated. Gene ontologies are also linked to biochemical gene (e.g., Biocarta) and protein (e.g., KEGG) pathway databases, and several open source online and free-standing programs (e.g., ArrayTools, TMEV, Mapp, NetAffyx, Bioconductor packages) are designed

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to access the gene ontologies and related gene and gene product databases and link current information with lists of significantly changed genes obtained from of statistical analyses. In a class comparison context, permutation tests can be used to find GO categories that have more genes differentially expressed among the classes than might be expected by chance (R. M. Simon & Peng Lam, 2004). Using the gene ontologies in this way, experimenters can place their results into a biologically meaningful context.

The wide variability in approaches to microarray data analysis, and the underreporting of RNA extraction and hybridization protocols, as well as image processing and analysis, has led some skepticism regarding the reliability or reproducibility of microarray results (McShane et al., 2003; R. Simon, Radmacher, Dobbin, & McShane, 2003). In an effort to impose more rigor on the reporting of microarray data, the MEGD has worked to make the MIAME convention--Minimum Information About Microarray Experiments--the standard for publication of results from microarray experiments (Frueh, Huang, & Lesko, 2004). MIAME-compliant reports include detailed information on the platform and molecular protocols used, or reference to standard methods and platforms, such as Affymetrix, as well as methods used for image processing and statistical approaches to data analysis (Burgoon, Boutros, Dere, & Zacharewski, 2006; Tong et al., 2003).

The Toxicogenomics Research Consortium (2005) recently addressed the issue of cross-platform and cross-laboratory reproducibility of microarray results

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by distributing aliquots of a mouse liver RNA standard and a pooled sample containing RNA isolated from mouse liver, lung, kidney, brain, and spleen tissue samples to eight Consortium laboratories. A total of twelve different platforms, commercial and in-house oligo and cDNA arrays, were used according to the laboratory's standard practice; correlation of resulting gene intensities among labs and array type was poor (median R^2~.60) for liver and pooled samples. The most consistent inter-laboratory results were produced when the experiment was repeated in all the Consortium labs with commercial arrays and standard manufacturer labeling and hybridization protocols, in fact the microarray platform used accounted for more than 50% of the technical variability. Standardization of image analysis and data entry, storage, and retrieval processes also helped to reduce inter-laboratory variation. An interesting result of this study was the qualitative similarity in results--enrichment in gene ontology nodes (categories) was similar, even when the individual member gene intensities were not consistently changed across labs.

The initial dissimilarities in microarray results, and increased convergence when standardized protocols and platforms were implemented, are a reflection of the multiple, complex steps required to prepare targets for microarray analysis. The first step in the preparation of labeled target cRNA, the conversion of mRNA (or total) to cDNA, requires the use of reverse transcriptase, an enzyme with poor processivity. The use of oligo dT primers to selectively bind mRNA for first strand synthesis can introduce 3' bias into the analysis, because reverse

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transcriptase tends to produce cDNA strands of only 1000-1500 bp, thus limiting the number of splice variants that can be detected. First and second strand synthesis, and the *in vitro* transcription used to produce fluorescently-labeled cRNA are all reactions whose outcome is dependent on enzymes, and the use of standard, optimized conditions, reagents, and enzymes for these reactions is important for reproducible results. Quality control or assurance of the starting RNA is also essential, an $A_{260/280}$ of 1.8-2.0 has been recommended for aqueous RNA, and more recently standardized microcapillary electrophoresis-based evaluation of total RNA quality has been encouraged for ensuring the quality of total RNA for microarray and quantitative PCR applications (Schroeder et al., 2006).

Verification by Quantitative RT-PCR

Fluorescent microarrays have a relatively narrow dynamic range ($<10^3$) that can limit the ability to accurately quantify gene expression levels, and so replication of results using an alternative method is desirable. Genes are selected for confirmation based on the results of the microarray data analysis and the objectives of the investigators; real time, or quantitative RT-PCR (qPCR), is the most common technique used to confirm the changes in selected gene expression observed with microarrays (Dallas et al., 2005). In the first step of qPCR, the RNA used for microarray analysis is reverse transcribed (RT) into a cDNA template for qPCR using random, oligo-dT, or gene specific primers. The 5'→3'

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exonuclease nick translation activity of a thermally stable DNA polymerase, such as *Thermus aquaticus* (Taq), is exploited by extending the sequence between 3' and 5' oligonucleotide primers (Holland, Abramson, Watson, & Gelfand, 1991). A probe oligonucleotide that binds the sequence of interest is tagged with a 3' quencher and 5' reporter dye (e.g., 6-FAM) is used to detect amplification. The sequence of interest, or amplicon, is produced by the enzyme in the presence of excess nucleosides, in repeated cycles of cDNA denaturation and annealing/extension, at elevated temperatures. Taq hydrolysis of the probe sequence releases the reporter dye during amplification, and the increase in signal is proportional to the increase in amplicon in each cycle. The technique is high-throughput, many reactions are carried out simultaneously in plates with many wells (e.g., ABI Sequence Detection Systems) or in capillary tubes in rotor-like carousels (e.g., Roche Lightcyclers), and automated systems record the changes in fluorescence with each amplification cycle (Watzinger & Lion, 2003).

The quantitative, or kinetic aspect of the analysis makes use of the relationship between the amount of starting material (cDNA template), and the amount of PCR product at the end of a PCR cycle. In the log linear phase of amplification, the change in fluorescence increases linearly with each cycle, and the amount of amplicon and thus the change in fluorescence, should double in each subsequent cycle. This relationship is indicated by $P = P_0(1+E)^n$ where n is the number of cycles, P is the amount of product, and E is the efficiency of the PCR reaction. Taking the log of this equation converts it to linear form, and the

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initial target concentration (P_0) can be estimated by the y-intercept of the plot of log P versus number of cycles. Two types of quantitation can be done, 1) absolute quantification, based on an internal or external calibration curve, and 2) relative expression ratio, based on comparison of target and reference gene expression in treated and control samples. Absolute quantification requires the generation of precisely quantified recombinant DNA or RNA standard material for the standard curve, as well as normalization to an endogenous reference gene, and thus is very labor intensive (Bustin, 2000). The efficiencies of the recombinant standard and the target must also be identical, and the labor and extensive validation required for accurate quantification makes the method unwieldy for experiments where the objective is to validate physiological changes in expression of several genes (Applied Biosystems, 2001; Pfaffl, 2001).

Relative quantification is widely used in toxicogenomic and other applications, in this method target gene expression in each tissue is normalized to that of an endogenous reference gene whose expression does not change across control and sample tissues. Standard curves may be used to quantify gene expression--in this case serial dilutions of target and reference gene assays must be run on each plate, and initial concentrations are found using the standard curve. Several models have been developed to calculate relative gene expression (Applied Biosystems, 2001; Peirson, Butler, & Foster, 2003; Pfaffl, 2001), all are based on the idea that the log linear phase of amplification commences when the fluorescence rises above background levels, threshold (C_t or C_p) is crossed. The

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cycle number (n) at which this occurs is often fractional, and the log-linear equation for quantification can be rewritten as $P_{Ct} = P_0 + (1 + E)^{Ct}$. The ratio P_{Ct}(samples)/P_{Ct}(controls) is the basis for relative quantification of differences in gene expression due to treatments (Livak & Schmittgen, 2001; Pfaffl, 2001).

Among the most widely used models for relative PCR quantification are the 2^{-Ct}, which requires the assumption of perfect amplification efficiencies in target and reference genes (Applied Biosystems, 2001), and the Pfaffl model, in which the efficiencies of target and reference genes may differ (Pfaffl, 2001). Both methods depend on reproducible and constant (across samples and tissue type) reference gene expression and amplification kinetics. Reaction efficiencies may be determined from standard curves (Applied Biosystems, 2001), or by using the increase in absolute fluorescence during the log-linear phase to define reaction kinetics (Peirson et al., 2003; Ramakers, Ruijter, Deprez, & Moorman, 2003; Tichopad, Dilger, Schwarz, & Pfaffl, 2003). The selection and validation of an appropriate reference gene is one of the most important factors in obtaining reliable and meaningful results from qPCR relative quantitation (Bustin, 2002; M. L. Wong & Medrano, 2005). The reference gene is intended to serve as an endogenous control in relative quantitation, similar to the use of constitutively expressed (housekeeping) genes in low throughput biomolecular techniques (Bustin, 2002; Thellin et al., 1999). The variability of many so-called housekeeping genes, such as β-actin and GAPDH, in a variety of tissues or

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treatments has been investigated by many practitioners of qPCR (e.g., (Dheda et al., 2004; Pohjanvirta et al., 2006; Silvia et al., 2006; Thellin et al., 1999), and verification of reference gene expression is now considered to be an essential prerequisite of any qPCR determination of changes in relative gene expression (Dheda et al., 2005; Huggett, Dheda, Bustin, & Zumla, 2005; Pfaffl, Tichopad, Prgomet, & Neuvians, 2004).

As with microarray based analyses, the reverse transcription reaction in qPCR is one of the least understood in the process, and another potential source of variation that has recently come under scrutiny (Bustin & Mueller, 2005). Stahlberg and coworkers (2004) used the dsDNA intercalating dye SYBR Green I and amplicon-specific T_m's to quantify the qPCR expression of five genes (GAPDH, β-tubulin, GLUT2, Insulin2, and CaV1D). Five replicates (each) of four different RT priming strategies--random hexamers, oligo-dT, a single gene-specific primer, and a mixture of all the gene-specific primers--were used with Invitrogen's Superscript II reverse transcriptase reagent set. By comparing standard deviations for the PCR versus the RT reactions, they found that the RT step accounted for most of the variability in their results. The efficiency of the RT reactions were better as the template concentration increased-- Ct's for the different priming methods ranged from 0.8 for the gene present in greatest quantity (GAPDH), to 4.4 for the lowest expressed gene (GLUT2)--but none of the priming strategies clearly outperformed the others. They recommend that RT be done at least in duplicate for each individual sample in a qPCR study to

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ensure that the cDNA population is reproducible (Stahlberg, Hakansson, Xian, Semb, & Kubista, 2004).

The verification of a minute fraction of all the genes present in a biological mixture does not prove that an entire RNA population has been faithfully reverse transcribed by the enzyme, but the use of a single priming strategy aids in minimizing variability. RNA is very sensitive to degradation, as well, so the possibility of RNase contamination should be minimized. The qPCR reaction can be enhanced by organics such as DMSO and formamide, and inhibited by biological impurities such as hemoglobin and urea, by phenols, and even by RT left over from cDNA synthesis (Suslov & Steindler, 2005). Good technique, sterile and DNase/RNase-free disposables, regular lab and pipettor decontamination, and scrupulous sample handling are all imperative for successful RT-PCR based analyses, as any contaminating DNA or RNA can be readily reverse transcribed or amplified (Bustin, 2002). In spite of the many possible pitfalls of real-time qPCR, its sensitivity and ease of use are unmatched, and with planning, consistent, careful technique, proper controls and data analysis, it is particularly useful for the relative quantification of changes in gene expression.

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CHAPTER III

CHANGES IN THE FISHER RAT HEPATIC TRANSCRIPTOME FOLLOWING LOW-LEVEL DIETARY PCB EXPOSURE

Introduction

Polychlorinated biphenyls (PCBs) are stable, lipophilic compounds of biphenyls that were first manufactured on an industrial scale in the U.S. by Monsanto in 1929 (de Voogt & Brinkman, 1989). Complex mixtures of the 209 possible congeners, classified by weight percent of chlorine in the mixture (e.g., Aroclors in the U.S., Clophens in Germany) were typically deployed in industrial uses. PCBs were used extensively from 1930's through the 1970's: as non-conductive insulators in transformers and capacitors, as hydraulic and heat transfer fluids, in building materials, paints, carbonless copy paper, and as a carrier for application of pesticides (Fiedler, 1998; Herrick et al., 2004); world production has been estimated at 1.3-1.5 million metric tons (Breivik et al., 2002). Persistent organic pollutants (POPs) are a class of chemical compounds that includes PCBs and several other aromatic, halogenated compounds such as TCDDs, PCDD/Fs, some PAHs, and PBDEs are all chemically inert, hydrophobic, bioaccumulate in food chains and persist in the abiotic environment . The POP congeners capable of assuming a planar configuration can also bind to the arylhydrocarbon receptor to some degree, and induce transcription of genes active in Phase I and Phase II drug metabolism in the

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nucleus. Effects observed downstream of AhR-induced gene expression *in vivo* include increases in benign and cancerous tumors of the liver and adrenals, reproductive and developmental abnormalities, thymic atrophy, and impaired immune function (Mandal, 2005; NTP, 2006b). Besides toxic endpoints common to AhR ligands, pre and post natal exposure to ortho-substituted PCBs has been associated with cognitive deficits in human populations (Jacobson et al., 1990; Jacobson & Jacobson, 2003), neurotoxic effects in rats (Schantz & Widholm, 2001), and suppression of immune functions and depletion of thyroid hormone (ATSDR, 2000; Carpenter, 2006).

PCBs were produced, used, and dumped most extensively in the eastern U.S., especially in the highly industrialized Great Lakes basin states (WI, IL, MI, IN, NY, OH). Although levels of PCBs in water, air, sediments, and biota have declined from peaks observed in the late 1970's to early 1980's, several highly contaminated "hot spots" still exist in the U.S. Great Lakes region, particularly around Lake Michigan (Environment Canada, U.S. EPA, 2005). Fish from the lake and tributaries are an important food source for many birds and land mammals in the Great Lakes basin, and animals in this region typically have very high PCB levels compared to animals from less polluted regions (Fox, 2001). PCBs and other POPs present in these animals have been implicated in decreased reproductive success due to changes in behavior, physical reproductive and developmental abnormalities, thyroid deficiency, and extirpation of the mink from the Lake Michigan shoreline (Fox, 2001; Giesy, Ludwig, & Tillitt, 1994;

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Hickey et al., 2006).

Measuring expression of a wide spectrum of genes simultaneously gives investigators insight into the transcriptional changes occurring in biochemical pathways and networks that contribute to the overall physiological response in an organism or cell to stressors such as synthetic chemical toxicants (Balbus, 2005; Lettieri, 2006; Waring et al., 2001). At sub toxic exposure levels, measurements of transcriptional changes due to continuous, dietary exposure to a mixture of PCBs similar to actual human and wildlife exposures may lead to the discovery of genes crucial to the origin or perpetuation of subtle physiological effects in mammals. To date, the only reports of global profiles of changes in hepatic gene expression following Aroclor 1254 exposure are for intraperitoneal injection with high, toxic doses (Waring et al., 2001); post natal, lifetime (two year) gavage exposure to TCDD and single PCB congeners have also been reported (NTP, 2006a; NTP, 2006b; NTP, 2006c). The liver is a target organ for PCBs in rats and other mammals, and the effects of PCBs on this organ have been widely studied (ATSDR, 2000; Wilson & Safe, 1998).

Diet is the most important pathway of exposure for POPs, and PCBs are ubiquitous in meat, fish, dairy products, and breast milk (Carlson & Hites, 2005; Kalantzi et al., 2001; Solomon & Weiss, 2002). Much of the existing toxicological data for PCBs relies on measurements made in animals gavaged with PCBs in oil, and this route of exposure may result in different health outcomes than environmentally relevant dietary exposures (Arnold et al., 2000; Yuan et al.,

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1995). Measurement of the global transcriptional responses to dietary exposure to A1254 has not been reported to date, and is important given the persistence of PCBs, and range of effects associated with exposure. No single study can provide a complete picture of the impact of low level dietary PCB or POP exposure, but investigation into the hepatic effects of sub chronic and sub acute exposures to Aroclor 1254, a mixture of PCBs with a congener profile similar to that detected in animals in the KRAOC (Camp Dresser & McKee, 1997), will provide data on health effects associated with this area of the Great Lakes.

The aims of this study were to obtain gene expression profiles by microarray analyses for rat livers following sub acute and sub chronic dietary PCB exposures, in order to characterize the global rat hepatic transcriptional response to dietary PCBs. Aroclor 1254 was chosen for this work because of the large body of existing literature describing the effects of Aroclor 1254 exposure in rats (ATSDR, 2000), and an inbred rat strain was chosen to minimize genetic variability. Male Fisher 344 rats were fed control diet or diet containing 5.6 ppm or 18.6 ppm A1254, for 7 or 84 days. The mixture has a high percentage of penta- and hexa-chlorinated congeners, and is similar to PCB congener profiles found in animals in the Kalamazoo River area (Camp Dresser & McKee, 1997). Results from this study will help to fill in the existing information gap between laboratory studies of the hepatic global transcriptional responses to well-defined gavage exposures to single congeners (NTP, 2006a), or to intraperitoneal concentrations of PCB mixtures that induce overt toxicities (Waring et al., 2001),

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and the field observations that result from dietary environmental exposures.

Methods and Materials

Four-week-old male weanling Fisher 344 rats (Charles River Labs) were acclimated for three weeks at the Western Michigan University animal facility prior to beginning experimentation. All animal experiments were conducted under an approved Institutional Animal Use and Care Protocol Number 99-09-02. Following acclimation, rats were randomly assigned by weight to control, low, and high dose Aroclor 1254 (A1254) treatment groups (n = 8), and exposed via diet for one week. The animals were housed in wire-bottom cages, with a 12 hour dark/light cycle, and allowed free access to prepared diets and to reverse osmosis (RO)-purified water. A 12 week long dietary exposure study was also conducted; animal conditions and feeding regimes for this experiment were identical to those of the short-term exposures, except for the duration of the experiment.

Diets were prepared by mixing aliquots of a 100 mg/L stock solution of Aroclor 1254 (Lot 124-191-B, Accustandard) in isooctane with ethanol vehicle to yield final target concentrations in food of 0 (vehicle control), 10, and 50 mg/kg rat chow (Purina), respectively. The PCBs in solution were added to the pelleted rat diet and incorporated by shaking for 4h, then air-dried in a chemical ventilation hood ≥ 48h. The animals were allowed to eat weighed portions of prepared diets freely, and food intake was estimated by weight. The Aroclor 1254 diet was prepared and handled in a chemical fume hood; latex gloves,

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masks, and disposable jackets were worn at all times when handling food and when cleaning animal cages.

Following each exposure period, rats were sacrificed between 9:00 am and 11:00 am by euthanizing with CO_2; blood was then collected immediately for serum analysis by cardiac puncture. Serum analysis for direct and total bilirubin, ALT, AST, ALP, triglycerides, cholesterol, and GGT was performed on the day of collection by Regional Medical Laboratories in Battle Creek, MI. Livers, kidneys, hearts, thymus, and spleens were excised, snap frozen in liquid N_2, and stored at -80 °C until processing; brains were removed by decapitation for a separate, neurobiological study.

Organ somatic indices were calculated for individual animals, and blood serum parameters were normalized to liver weights. Parametric tests for statistically significant differences were done between control and A1254-dosed groups using the single-factor ANOVA or 2 sample t-tests implemented in Microsoft Excel, assuming unequal variances.

In laboratory exposed rat tissues, liver tissue was excised from the total liver (approximately 0.5 g) using a scalpel. Liver and food samples were analyzed for PCBs using a matrix solid phase dispersion method as previously reported (Fisher et al., 2003) and the PCB congeners were quantified by the multiple selected ion monitoring GC/MS method adapted from previous work (McMillin & Means, 1996; Means, 1998). Analysis was performed on 2µl samples of the extracts using an Agilent Technologies (Palo, Alto, CA USA)

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6890A gas chromatograph equipped with the capillary column (AG DB-5MS) (30m X 0.025mm i.d.), which was directly interfaced to an AG 5973N Mass Selective Detector, equipped with a 7683 autosampler. The congener groups were quantified using a calibration standard containing a representative congener from each chlorination group (Accustandard, New Haven, CT USA). A select list of specific congeners was also determined using authentic standards for these compounds from the same supplier.

Detection limits for each analyte in the sample matrix type were estimated from statistical information derived from standard calibration curves (Taylor, Jain, & George, 1987). For liver tissue, this limit was typically approximately 5-12 ng/g wet wt. (ppb) with a mean value of 9 μg/g. Samples were spiked immediately before injection with 10 μl of a 100 μg/ml solution of 4,4'-dibromo-octafluoro biphenyl (DBOFP) as an internal standard.

Measured total PCB concentrations in the prepared diet were as follows: control: 0.231 mg/kg; low dose: 5.618 ± 1.509 mg/kg food; high dose: 18.597± 0.741 mg/kg food. Presumably, some PCBs were lost during the 2 day drying period in the ventilation hood. To insure that the animals were receiving a diet representative of the original PCB mixture, the PCB congener content of the PCB-impregnated food was also estimated, and is shown as percent total PCBs in Table 2. Except for slightly elevated levels of hexachlorobiphenyls in the low dose diet (~10% greater than standard A1254), the distribution of chlorine-number isomer fractions in the rat chow prepared in our lab is very similar to

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the distribution of congeners typically found in Aroclor 1254 (Table 2).

All glassware, mortar and pestles, and stainless steel instruments used for tissue extraction and handling, and for RNA isolation, were soaked in distilled water and Contrad 70 detergent (Decon Laboratories, Inc., King of Prussia, PA), rinsed in RO-purified water, soaked overnight in 10% DEPC (Midwest Scientific, Valley Park, MO), then wrapped and autoclaved before use. Disposable plastic ware (disposable pipettes, centrifuge tubes) was purchased RNase and DNase free; aerosol barrier RNase and DNase free disposable micropipettor tips were used exclusively (Midwest Scientific, Valley Park, MO). All reagents and water used were molecular biology grade.

Less than 1 g (0.75-0.95 g) of frozen rat liver tissue was pulverized in liquid N_2, rapidly transferred to a homogenizer, and ground with 10 ml guanidine isothiocyanate buffer. The Ambion Poly (A^+) Pure® kit (Ambion, Inc., Austin, TX) reagents and manufacturer's protocol was used for all mRNA extractions in this project. Messenger RNA was extracted directly from centrifuged, washed homogenate diluted in binding buffer by hybridizing the poly A tails to 100 mg of oligo-dT cellulose beads, washing, then eluting mRNA from the oligo-dT, and precipitated overnight at -20°C in 3M NaOAc and 2.5 volumes 80% ethanol. The precipitates were centrifuged for 20 min at 12000 x *g*, washed twice with ice-cold 80% ethanol, and spun in a vacuum centrifuge to evaporate the solvent. The pellet was dissolved in 20 µL DEPC-treated water,

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and quantified spectroscopically at A_{260}. Spectroscopic analysis was also used for quality control--only mRNA with A_{260}/A_{280}= 1.8-2.0 was used as starting material for microarray analysis. The mRNA was visualized by electrophoresis with ethidium bromide on a denaturing agarose gel. Purified mRNA in DEPC water was stored at -80°C until use.

Affymetrix RGU34A microarrays, which probe for 8799 genes and ESTs, were processed for three animals in each dose/time group, for a total of 18 arrays. Details of the molecular and quality assurance methods used for target labeling, array hybridization, and scanning are given in the following section, these methods conformed to those recommended for mRNA in the third revision of the Genechip® Expression Analysis Manual.

First strand synthesis started with 2.0 µg of mRNA, 400 U superscript II reverse transcriptase, 100 pmol T_7-$(dT)_{24}$ primer (Proligo), 1x first strand buffer and 0.5 mm dNTP's for 1 h at 42° C. First strand cDNA was incubated with 10 U E. Coli ligase, 40 U DNA polymerase I, 2 U RNase H, 1x second strand buffer and 0.2 mm dNTP's at 16 ° C for 2h to synthesize the second strand of cDNA. Invitrogen reagents (Carlsbad, CA) were used exclusively for first and second strand synthesis.

Biotin-labeled nucleotides were incorporated into cRNA by *in vitro* transcription, using the dsDNA created by first and second strand synthesis as a template. Bioarray High Yield RNA transcript labeling kit (Enzo Diagnostics, Farmingdale, NY) was used for this reaction, 8 µl of dsDNA was mixed with

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dNTP's (with Bio-UTP and Bio-CTP), 1x HY reaction buffer, 1x DTT, 1x RNase inhibitor mix and 1x T7 RNA polymerase, and incubated for 5 h at 37 °C. The labeled cRNA was then purified (Affymetrix or Qiagen RNA clean up kits, according to manufacturer's instruction), precipitated in ethanol, and dissolved in RNase-free water. The purified cRNA was quantified spectroscopically then fragmented by incubating in a high salt fragmentation buffer (Affymetrix, Santa Clara, CA) at 94 °C for 35 min.

For hybridization to the RG-U34A Genechip, 17-20 μg fragmented cRNA (A_{260}/A_{280}= 1.8-2.1) was incubated with 0.5 mg/ml acetylated BSA (Invitrogen, Carlsbad, CA), 0.1 mg/ml herring sperm DNA (Promega, Madison, WI), and 1x hybridization buffer and the controls manufactured and recommended by Affymetrix: 50 pM oligo-B2 control and a 1x eukaryotic hybridization control consisting of 1.5 pM bioB, 5 pM bioC, 25 pM bioD and 100 pM Cre, at 45 °C for 16 h in a rotisserie oven. Microarray washing and staining, including a streptavidin-phycoerythrin (Molecular Probes Eugene, OR) antibody amplification step, was performed with an Affymetrix Genechip® fluidic station, as recommended by the manufacturer. The amplification step, in which the array is first stained with SAPE, then with SAPE antibody followed by additional SAPE, was used to ensure sufficient fluorescent signal for image capture. Image files of the phycoerythrin-stained and scanned chips (.cel files) were used to calculate expression measurements.

Probe level data was summarized into a single expression value for each

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gene on each array using the analysis recommended by Bolstad, et. al. (Bolstad et al., 2003; Bolstad et al., 2004), which is included in the affy package available from the Bioconductor Project (Gautier et al., 2004). Specifically, Robust Multi-array Averaging (RMA) developed by Irizarry, etal. (Irizarry et al., 2003), was used to carry out background correction for perfect match (PM) probes only, resulting in an observed PM equal to the sum of an exponential signal and linear noise term. Quantile normalization (Bolstad et al., 2004) which reduces non-biological variance by making the distribution of probe intensities the same for all chips, was applied and median polish was used to fit a linear model to background corrected, normalized, log2 probe intensities and calculate a single intensity value for each gene on the array.

Gene expression values were then imported into BRB ArrayTools (R. Simon & Lam, 2004), where class comparisons employing multivariate permutation F-tests (R. M. Simon et al., 2003) were used to identify genes that were differentially expressed among dose classes. Although t-statistics were used, the multivariate permutation test is non-parametric and does not require the assumption of Gaussian distributions. The random variance test allows sharing of information among genes about within-class variation without assuming that all genes have the same variance. We also performed a global test of whether the expression profiles differed between the classes by permuting the labels of which arrays corresponded to which classes. The significance level of the global test is the proportion of the permutations that gave at least as many

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significant genes as the actual data.

Class comparisons were performed independently for 1 week and for 12 week data with 0 ppm (control), 5.6 ppm (low dose), and 18.6 ppm (high dose) dietary A1254 doses as the classes. For each treatment time, two separate comparisons--control versus low dose and control versus high dose--were done. Each comparison yielded a list of class discriminators, genes whose expression was determined to be significantly different between the classes. A significance threshold of $p \leq 0.005$ for the random variance t-test was used to limit the number of false positive findings for class discriminators with 90% confidence in the calculated probability of false discoveries. We also performed a global test of whether the expression profiles differed between the classes by permuting the array and corresponding class labels. For each permutation, the p values were re-computed and the number of genes significant at the 0.005 level was noted. The proportion of the permutations that gave at least as many significant genes as with the actual data was the significance level of the global test.

Pathway class comparisons were also done using the random variance t-tests implemented in BRBArrayTools. The permutational tests employed in these analyses are similar in principle to the class comparisons described in the previous paragraph, except in this case the test evaluates whether or not expression of each group of genes in the pathway, as a whole, is different among the classes. This reduces the number of tests that have to be conducted. Samples of n genes are randomly selected from genes represented on the array and the

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summary statistic computed for those random samples. For each group of related genes, the number of genes represented on the microarray in that group, and the statistical significance p*i* value for each gene (*i*) in the group are computed. These p*i* values reflect differential expression among classes, and were computed based on random variance t-tests (R. Simon & Lam, 2004). A significant statistic (Fisher (LS) < 0.005) is the proportion of the random samples giving as large a value of the summary statistic as in the actual *n* genes in the related group.

Results

Juvenile male Fisher rats were fed rat chow impregnated with ethanol vehicle, low, or high doses of polychlorinated biphenyls as Aroclor 1254. Average total PCB concentrations in the rat livers after one week and twelve weeks, respectively, of dietary exposure are shown in Table 3. The total PCB content of the high and low dose groups are further delineated into fractions according to the number of chlorines detected on each congener group (Table 4). As would be expected from dietary exposure to our Aroclor diet (see Table 2) tetra-, penta-, and hexachlorobiphenyls accounted for more than 85% of the total PCBs accumulated in the livers. The nominal amounts of PCBs detected in the control diet (0.231 mg/kg), and in the livers from the control groups (Table 3), were almost entirely mono-chlorinated biphenyls.

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Table 2

PCB Homolog Fractions in Prepared Aroclor 1254 Diet

Number of Cl	% Total PCBs Low Dose	% Total PCBs High Dose	% Total PCBs* in Aroclor 1254
2	0	0	<0.1
3	0	0	1.8
4	14	17	17.1
5	48	53	49.3
6	38	29	27.8
7	1	1	3.9

* Concise Chemical Assessment Document 55, WHO, 2003

Table 3

PCB Concentrations in Rat Livers At Time Of Sacrifice Following 1 Week and 12 Weeks Dietary Exposure

1 WEEK AROCLOR DIET		12 WEEK AROCLOR DIET	
Control:	0.04 ppm ±0.027	Control:	0.10 ppm ±0.039
Low:	2.62 ppm ±0.512	Low:	3.45 ppm ±0.701
High:	9.81 ppm ±2.013	High:	8.57 ppm ±1.190

Mean serum alkaline phosphatase (ALP) levels for the 1W high dose rats (39.31 U/L) were significantly lower than observed in the corresponding control (46.95 U/L) and low dose (46.85 U/L) groups. The concentration of serum enzymes linked to liver damage (AST, ALT), and other indicators of liver toxicity (total and direct bilirubin) were not significantly affected by Aroclor 1254 exposure in this study. Serum factors typically increased by exposure to PCBs (triglycerides and cholesterol) were statistically similar between controls and treated animals. Gamma-glutamyltransferase (GGT) protein was not

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detected in the rat serum in either of these experiments.

Table 4

Tetra, Penta, and Hexachlorinated PCBs Detected In Rat Livers As % Total PCBs

Cl#	12W/High	12W/Low	1W/High	1W/Low
4	5.9%	5.5%	7.4%	22.9%
5	40.0%	50.9%	52.3%	41.4%
6	38.6%	31.4%	33.7%	24.6%

Rates of weight gain during the long-term exposure periods were similar for all study groups, and average animal body weights were nearly identical at the time of sacrifice (Figure 2). Livers were significantly enlarged only in animals consuming the 18 ppm A1254 diet by approximately 12% after one week exposure, and by approximately 20% after 12 weeks exposure. The average liver weights (n = 8) are shown below (Table 5), values that differ significantly from controls by two sample t-test, assuming unequal variance are marked with an asterisk (*, $p<.05$).

Table 5

Rat Liver Weights and Liver Somatic Indices

	1 WEEK A1254 EXPOSURE		12 WEEKS A1254 EXPOSURE	
Liver/Body Dose	Liver (grams)	Liver/Body (%)	Liver (grams)	Liver/Body (%)
CONTROL	10.168 ± 1.20	5.06 ± 0.32	14.308 ± 2.093	4.15 ± 0.40
5 PPM	10.604 ± 1.34	4.95 ± 0.42	15.322 ± 1.678	4.43 ± 0.27
18 PPM	12.034 ± 1.26*	5.65 ± 0.39*	17.162 ± 1.990*	4.96 ± 0.51*

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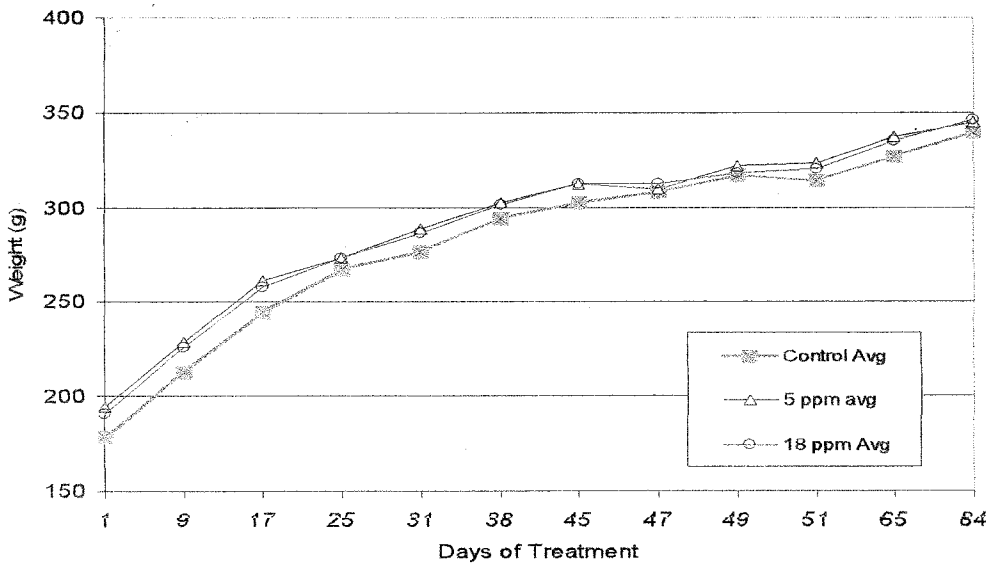
Organ to whole body mass ratios for thymus, kidneys, heart, and spleen tissues were also calculated (results not shown), but no significant differences were observed.

Figure 2

Animal Weight Gain Throughout 84 Day Exposure To Aroclor 1254



One week of the low dose A1254 diet (1W5) had a relatively small effect on hepatic gene expression in the Fisher rats with only six genes showing a significant change, compared to controls, at the $p < .005$ threshold level of the class comparison (Table 6). The five genes listed for 1W5 in Table 6 (Cyp1a1, Cyp1a2, Psmb2, Sds, Udpgt2) are those that increased the most compared to controls; these genes were also significantly changed by the 1W18 treatment.

The majority of significant changes in gene expression due to short-term

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PCB exposure were inductions; consumption of the high dose Aroclor diet for one week (1W18) corresponded to a near tripling of the number of class discriminators, compared to the 1W5 class comparison. Expression levels of three genes common to the two doses: Cyp1a1, Cyp1a2, and Udpgtr2 also increased, the most remarkable difference was the ~9 fold increase in the probeset coding for the Cyp1a1 protein (e00778cds_s_at, Table 6). Cyp1a1 was the most significant class discriminator for both doses at one week, having the lowest p-values (both probesets, 1W18: $p < 1 \times 10^{-7}$; 1W5: $p < 9 \times 10^{-5}$) for the multivariate t-test and the largest fold changes (Table 6) of all probesets in their respective data sets.

Table 6

Fold Changes in Gene Expression

Probe set	Gene	12W18	12W5	1W18	1W5
J05210_g_at	Acly	-1.4	-2.2		
E00717UTR#1_s_at	Cyp1a1	11.5	3.2	32.4	6.7
E00778cds_s_at	"	15.3	3.0	29.7	3.4
M26127_s_at	Cyp1a2	2.6		5.1	2.2
K03241cds_s_at	"	6.6	2.2	12	
E01184cds_s_at	"	5.1		9.3	
D17349cds_f_at	Cyp2b15	2.2		2.6	
L00320cds_f_at	Cyp2b2	5.6	1.8		
J00728cds_f_at	"	4.5		5.8	
K00996mRNA_s_at	"	4.8		6.9	
K01721mRNA_s_at	"	4.4		6.6	
L00320cds_f_at	"	5.6		7.2	
M11251cds_f_at	"	5.6		7.5	
M13234cds_f_at	"	4.4		5.1	
D21799_at	Psmb2			1.9	1.9
J03863_at	Sds			-1.8	-1.8
M13506_at	Udpgtr2			2.9	1.9

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Cyp1a1 is also the top discriminator for 12W5 ($p < 2 \times 10^{-5}$), with fold change ratios of about 3 for both probesets. The probesets listed in Table 6 include all the 12W5 class discriminators with fold changes greater than 1.7, four genes were increased, and two were decreased compared to controls. The changes in expression for 12W5 were in the same direction, though much smaller in magnitude, than the corresponding 12W18 class discriminators. Changes in hepatic gene expression following twelve weeks of feeding the high dose Aroclor diet (12W18) resulted in the most statistically significant class comparison of the four treatments. The false discovery rate (FDR), or probability that the same list of class discriminators could be randomly generated by permutation, was only 10% for 12W18, compared to 50% for 1W18, and 60-70% for the low dose regimes (Table 7, below).

Table 7

Class Comparison Summary

	12W18	12W5	1W18	1W5
# Class Discriminators UP	101	5	23	9
# Class Discriminators DOWN	8	9	4	1
False Discovery Rate (%)	10	70	50	60

The diminished FDR is due, in large part, to the fact that class comparison for 12W18 data yielded 109 class discriminators at $p < .005$, a much greater

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number than any of the other groups. The false discovery rates (expressed as percentage in Table 7) are synonymous with the *p* for the global multivariate permutation tests in these analyses (R. Simon & Lam, 2004), thus the FDRs can also be used to decide whether or not the dose classes represent distinctly different classes. In this case, the p-values for the global permutation tests are as follows: *p* (12W18) = .01, *p* (1W18) = .05, *p* (12W5) = .07, *p* (1W5) = .06. If a significance criteria of $p < .05$ is applied to the comparisons, only the 12W18 treatment group can be considered to be a distinct class from the controls. The 1W18 treatment is very close to the $p < .05$ cutoff, but both low dose treatments are particularly non-significant. The lower total number of class discriminators and the smaller magnitude FCs for both 1W5 and 12W5 (Table 7) are both critical factors in the determination of higher FDRs for these groups.

Only a small subset of the 74 genes found to be significantly changed in gene expression in 12W18, compared to the 12W dietary control group, is shown in Table 6 above. Hepatic gene induction was predominant for 12W18 as in the one week exposures, and the two probesets coding for Cyp1a1 are the most significant ($p < 1 \times 10^{-7}$) and are the ones with the largest fold change ratios (expression 11-15x greater than controls), as with all the other treatment groups. All of the cytochrome P450s induced by 1 week exposure to the high dose of PCBs (Cyp1a1, Cyp1a2, Cyp2b15, and Cyp2b2) were also significantly increased over controls at 12 weeks, but to a lesser extent.

Functional annotations and descriptions for all of the 12W class

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discriminator genes in Table 6, along with several other genes significant in the 12W18 set, are presented in Table 8. Functional annotations for the genes were collected from several integrated or connected sources: Biocarta and KEGG gene lists annotated in the BRB ArrayTools output for dose and pathway class comparisons, DAVID functional annotations, the Gene Ontologies, Rat Genome Database, Pubmed, and CGAP. Netaffyx was used to select probesets with grade A, unambiguous annotations for inclusion in this table. A single, best probeset was selected for genes identified by several representative probesets (see Supplemental Table 1, Appendix C for other probesets), based on which could fulfill the majority of the following criteria: annotated with a rat gene symbol and description, or a mouse or human homolog, fewest (or no) cross-hybridizing probes in the probeset, mRNA coding, and no known pseudogenes for the rat.

Table 8

12W18 Class Discriminators

Probe sets	FC	Gene Description	Gene symbol	Functional Gene Annotations
J03637_at	1.44	aldehyde dehydrogenase family 3, member A1	Aldh3a1	FA, GG, Trp, X
M00001_i_at	1.52	apolipoprotein A-I	Apoa1	IM
AF037072_at	-2.92	carbonic anhydrase	Ca3	OX
rc_AA799326_s_at	2.75	cd36 antigen	Cd36	IM, LC, TA
U23056_at	5.01	CEA-related cell adhesion molecule 10	Ceacam10	
U26033_at	1.80	carnitine O-octanoyltransferase	Crot	LC

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Table 8--Continued

Probe sets	FC	Gene Description	Gene symbol	Functional Gene Annotations
rc_AA818144_at	1.64	C-reactive protein, petaxin related	Crp	IM
rc_AI176595_s_at	1.71	cathepsin L	Ctsl	IM
E01184cds_s_at	5.09	cytochrome P450, family 1, subfamily a, polypeptide 2	Cyp1a2	AC,AH,FA,H IM,NR,Trp,X
D17349cds_f_at	2.18	cytochrome P450, family 2, subfamily b, polypeptide 15	Cyp2b15	GH, X, Trp
K00996mRNA_s_at	4.85	Cytochrome P450, family 2, subfamily b, polypeptide 2	Cyp2b2	PB-induced, nicotine metabolism
rc_AI011998_at	2.44	DnaJ (Hsp40) homolog, subfamily B, member 9	Dnajb9	AA
M26125_at	1.92	epoxide hydrolase 1	Ephx1	EIC, IM, X
M76767_s_at	-1.56	fatty acid synthase	Fasn	FA, INS
X53588_at	-1.93	glucokinase	Gck	GG, IM
rc_AI233261_i_at	2.24	glutamate cysteine ligase, modifier subunit	Gclm	GSH
rc_AA893189_at	1.50	glutathione reductase	Gsr	FRA, GSH, IM
rc_AI138143_at	1.61	Glutathione S-transferase, theta 2	Gstt2	GSH, X
U81186_at	1.52	17 beta-hydroxysteroid dehydrogenase type 3	Hsd17b12	AA, TCE
rc_AA998683_g_at	-1.78	heat shock 27kDa protein 1	Hspb1	OX
M26594_at,	2.93	malic enzyme 1	Me1	IM, PP
J02679_s_at	2.87	NAD(P)H dehydrogenase, quinone 1	Nqo1	AH, OX
E01524cds_s_at	1.73	P450 (cytochrome) oxidoreductase	Por	ON
rc_AA799650_g_at	1.63	peroxiredoxin 3	Prdx3	OX
E03859cds_s_at	1.64	Rab11a, member RAS oncogene family	Rab11a	RAS, RE
M94043_at	1.48	Rab38, member RAS oncogene family	Rab38	RAS
rc_AA944856_at	1.63	RAS related protein 1b	Rap1b	RAS
S56936_s_at	2.95	UDP glycosyltransferase 1 family, polypeptide A6	Ugt1a6	HM, ON
U06273_i_at	1.77	UDP glycosyltransferase 2 family, polypeptide B4	Ugt2b4	HM, X
S82820mRNA_s_at	5.62	glutathione S-transferase Yc2 subunit (Gsta3)	Yc2	GSH, X

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Table 8--Continued

Key for Genelists and Functional Annotations

AA: negative regulation of apoptosis

AC: Mechanism of Acetaminophen Activity/ Toxicity (BioCarta:m/h_Acetaminophen‡)

AH: Ah-Battery genes; EIC: eicosanoid metabolism (Biocarta)

FA: Fatty Acid Metabolism (Kegg: 00071‡); FRA: Free Radical-Induced Apoptosis

GG: glycolysis/gluconeogenesis (Kegg: hsa00010)

GH: gamma-Hexachlorocyclohexane degradation (Kegg: 00361‡)

GSH: Glutathione metabolism (Kegg:)

HM: androgen and estrogen metabolism (Kegg: 00150); IM: Immunology (CGAP)

INS: Insulin Signaling (Kegg: 04910); LC: Long-chain fatty acid transport

NR: Nuclear Receptors in Lipid Metabolism and Toxicity (BioCarta: m/h_nuclearRs‡)

ON: Oxidative Stress Induced Gene Expression via Nrf2 (Biocarta: h_arenrf2‡)

OX: Oxidative stress (*PharmGKB pathway)

PP: Mechanism of Gene Regulation by Peroxisomal Proliferators (BioCarta: h_ppara‡)

RAS: Ras-related/member of RAS oncogene family

RE: Rab GTPases mark targets in the endocytotic machinery (Biocarta: h_rabPathway)

TA: TSP-1 Induced Apoptosis in microvascular endothelial cells (Biocarta: h_tsp1‡)

TCE_tetrachloroethene degradation (Kegg: 00625)

Trp: Tryptophan metabolism (Kegg: 00380‡)

X: Xenobiotic metabolism by Cytochrome P450's (Kegg: 00980)

‡ = *pathway significant by pathway class comparison ($p < .005$)*

Table 9

Pathway Analysis Class Comparison Results

Pathway Description	12W18	12W5	1W18	1W5
gamma-Hexachlorocyclohexane degradation	< .0001	< .005	< .0001	< .0001
Fatty acid metabolism	< .0001	NC	< .0001	< .0001
Mechanism of Acetaminophen Activity and Toxicity	< .0001	NC	< .005	NC
Nuclear Receptors in Lipid Metabolism and Toxicity	< .0001	NC	< .005	NC

The results of the pathway class comparisons are presented in Table 9

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above. Pathways in the 12W18 treatment group are changed most significantly compared to the 12W controls ($p<.0001$), which is not surprising considering that there were many more genes with significant changes in expression in this group than in any of the others.

Discussion

The changes in gene expression reported here occurred in the absence of any overt signs of toxicity: body weights were not affected, and increases in serum enzymes that would indicate liver toxicity, such as ALP and AST, were not observed. Twelve weeks exposure to 18.6 ppm dietary Aroclor 1254 resulted in a global gene expression profile significantly different from the corresponding gene expression profile for the control group; the global gene expression profiles resulting from the other three treatments were not sufficiently different overall to comprise statistically distinct classes. In this report, we have shown that hepatic expression of genes involved in Phase I and Phase II xenobiotic metabolism and proteasomal protein degradation were significantly changed in male Fisher 344 rats by short term dietary exposures to relatively low levels of Aroclor 1254 (Table 6). Though the changes in global gene expression observed after one week of the high dose were not sufficient for 1W18 to be considered a distinct class, identical CypP450 probesets were upregulated in 1W18 and 12W18 (Table 6). The marked overexpression of this set of cytochrome P450s in the high dose groups is likely a primary factor in the similarity of pathway comparison results

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between these two treatment groups (Table 9), since the global profile of 1W18 was insignificant by the test, and 1W18 had only about a quarter of the number of class discriminators as 12W18 (Table 7). AhR battery genes Cyp1a1 and Cyp1a2 (Nebert et al., 2000) were the most significantly changed genes in the pathways analyses. The increase in Cyp2b2 expression indicates phenobarbital-like gene induction (Waxman, 1999) by interaction of CAR, and possibly TRβ with a phenobarbital response element (Paquet, Trottier, Beaudet, & Anderson, 2000; Tabb et al., 2004). Cyp2b2 induction has been widely observed in rats following Aroclor 1254 exposure (Lubet et al., 1992; Nims et al., 1994), but detailed functional information cannot be linked to the Cyp2b2 probesets affected, because they are provisionally annotated.

The expression of the Phase I (Cyps) and Phase II (Udpgts) genes decreased as the treatment was prolonged; Cyp1a1 expression, in particular, was decreased three-fold between 7 and 84 days exposure (Table 6). Similar trends in hepatic Cyp1a1 enzyme expression have been reported in male Fisher 344 rats following 7 and 84 day feeding exposures to A1254 (Dragnev et al., 1994; Nims et al., 1992). Downregulation of Cyp1a1 following an initial period of PCB-induced microsomal enzyme induction has been demonstrated in recombinant human CYP1A1, as well as in rats, fish, and birds, and may be an adaptive response to oxidative stress brought on by Cyp1a1 redox cycling in some species (Schlezinger et al., 1999; Schlezinger et al., 2000). Cytochrome P450 reductase

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(Por, Table 8) reduces O_2 in an NADPH-dependent reaction to regenerate the oxidizing iron center in heme monooxygenases Cyp1a1, and ROS such as superoxide and H_2O_2 have been shown to be generated *in vitro* by the uncoupling of mammalian Por from Cyp1a redox cycle, following induction of Cyp1a with AhR agonists PCB 77 (Schlezinger et al., 1999; White, Shea, Schlezinger, Hahn, & Stegeman, 2000) and PCB 126 and PCB 169 (Schlezinger, Struntz, Goldstone, & Stegeman, 2006). ROS generated by the enzymatic activity of human cytochrome P450 reductase are capable of oxidizing DNA (Heine, Glatt, & Epe, 2006), and ROS can damage membrane lipids and proteins directly by oxidation (•OH) and peroxidation (HOO•, ROO•) (Beckman & Ames, 1998). Increases in ROS can increase the oxidation potential of the intracellular redox environment and initiate apoptosis (Valko et al., 2007), alter intracellular signaling pathways (Dro¨ge, 2002), and participate directly in the regulation of gene transcription (Sen & Packer, 1996).

Expression levels of genes coding for proteins with a variety of cellular functions were affected after 12 weeks, including oxidative stress response genes, metabolism of xenobiotic and endogenous molecules, immunology, negative and positive regulation of apoptosis, cell signaling, intracellular vesicle trafficking, and lipid transport and metabolism (Table 8). Nqo1 provides a defense against intracellular oxidative stress by catalyzing the two electron reduction of quinones to less oxidative phenols (Jaiswal, 1994), and Gst and Udpgt2 proteins

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catalyze Phase II conjugations of oxidized PCB metabolites so that they may be excreted from the cell (Xu et al., 2005). The GST enzymes may also reduce peroxyl and hydroperoxyl radicals; though the reaction is not as efficient as GSH-peroxidase reduction, these proteins are much more abundant than the selenium-dependent peroxidases in most tissues (Hayes & McLellan, 1999b). In addition to its role as a Phase II conjugate, glutathione (GSH) plays a key role in maintaining intracellular redox homeostasis by scavenging free radicals (Hayes & McLellan, 1999a), and by coordinately repairing protein sulfhydryl oxidation with thioredoxin (Biswas, Chida, & Rahman, 2006; Hayes & McLellan, 1999b). Intracellular GSH was reduced following *in vitro* addition of PCB 77 to porcine endothelial cells (Slim et al., 2000), JNK/SAP and the AP-1 expression was increased. The increase in hepatic Gsr expression in this study is consistent with reduction of ROS by GSH to form G-S-S-G, followed by reduction of GSSG back to GSH. The increase in peroxiredoxin 3 expression may be a compensatory response to increased intracellular H_2O_2 (Nonn, Berggren, & Powis, 2003).

Carbonic anhydrase III gene expression was decreased more than any other gene (~3x, Table 8) in response to high dietary A1254; this gene was also decreased in rats exposed to AhR ligands in the NTP studies (Vezina, Walker, & Olson, 2004). The cytosolic protein is abundant in skeletal muscle, liver, and adipocytes (Lynch, Brennan Jr., Vary, Carter, & Dodgson, 1993; Wistrand, 2002), and decreases in rat hepatic protein expression have been reported following *in vitro* exposure (Ishii, 2005), and *in vivo* exposure to 10 and 25 mg/kg PCB 126

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(Ikeda et al., 2000). Compared to other family members (e.g., CaI and CaII), the protein is a weak CO_2 esterase and hydrolase (Wistrand, 2002), and is unique in that it has phosphoserine/threonine activity when glutathiolated at Cys^{186} (Cabiscol & Levine, 1996). The cellular function of the protein has not been fully elucidated, but Wistrand (2002) proposes that high levels of Ca3 in muscle and perivenous hepatocytes (Carter, Lönnerholm, Meyerson, & Wistrand, 2001) may protect proteins in these tissues from oxidative stress induced by hemoglobin and myoglobin degradation products. The mechanism by which AhR ligands depress Ca3 levels are not known, but the redox-dependent covalent modification by GSH suggests that it could be involved in responses related to oxidative stress (Cabiscol & Levine, 1996), perhaps as a redox sensor via glutathionation (Fratelli et al., 2005) or by GSH-conferred phosphorylase. In culture, transfection of Ca3 into cells lacking endogenous expression of the protein blocked H_2O_2-induced apoptosis (Räisänen et al., 1999). This ability to directly scavenge radicals may be an important source of intracellular reducing equivalents in hepatocytes, where the protein has been reported to comprise as much as 8% of total cytosolic proteins (Wistrand, 2002).

Hepatic microarray gene expression profiles have been reported for male Sprague-Dawley rats acutely exposed to a cumulative dose of 1200 mg/kg A1254 via *i.p.* injection over the course of three days (Waring et al., 2001). Similar to our results for high dietary exposure, (average cumulative dose of 87.3 mg/kg A1254 over 84 days), Cyp1a1, Gst, and Me1 were increased, though

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expression of all three genes was at least a magnitude higher following the acute toxic exposures (Waring et al., 2001). Waring and coworkers (2001) compared the global gene expression profiles of several hepatotoxins, and found that 3-methylcholanthrene and A1254 consistently clustered together, and that upregulation of Me1, Cyp1a1, and Gst was characteristic of this cluster. In our study, serum triglycerides were not affected by treatment, and livers were enlarged 20% compared to controls; whereas livers were enlarged ~140% compared to controls, and serum triglycerides were significantly doubled in the acute exposure (Waring et al., 2001). The finding of common upregulation of Me1, Cyp1a1, and Gst between our sub chronic results and the toxicological profile based on the acute study of Waring (2001) imply that measurements of gene expression may be a more sensitive indicator of toxic exposure effects than routinely measured clinical parameters, particularly at environmentally relevant exposure levels. Expression of Cyp1b1 was significantly increased by toxic doses of A1254 (Waring et al., 2001), and by exposure to 1 mg/kg/day (via gavage) to AhR ligands TCDD, PCB 126, and PeCDF (Vezina et al., 2004). In this study, Cyp1b1 expression was increased approximately three fold by the high A1254 dose, but the signals were all below the cutoff (data not shown).

An interesting correspondence between this report and the results of the microarray analysis of livers from female rats dosed by gavage with 1 mg/kg /day PCB 126 or 100 ng/kg/day TCDD for 13 weeks, is the marked increase in Ceacam10 expression (Table 8) (Vezina et al., 2004). Ceacam10 protein is a

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soluble protein capable of homodimerization in a model system, and may mediate cell-cell adhesion *in vivo* (Lin, Cheng, Earley, Luo, & Chou, 1998). The liver tissues used in the microarray study by Vezina and coworkers (2004) were obtained from recent NTP toxicological studies, and 2 years of TCDD and PCB 126 PeCDF treatments resulted in cholangiocarcinoma and hepatocellular adenomas in female Sprague-Dawley rats in these studies (NTP, 2006b; NTP, 2006c), and the authors speculate that upregulation of this cell adhesion molecule may mediate Ceacam1 cell-cell adhesion, thus the gene may be an early requirement for the subsequent development of lesions (Vezina et al., 2004). The incidence of hepatocellular neoplasms in male and female Sprague-Dawleys treated for 2 years with Aroclor 1254 was higher than for Aroclors 1260, 1242, and 1016, and cancerous lesions were significantly more prevalent in females than in males (Mayes et al., 1998). Long term TCDD treatment has also reportedly increased the incidence of hepatocellular carcinomas in females more than males (NTP, 2006b), so it is not clear whether hepatic overexpression of Ceacam10 in male rats, as observed here, could conceivably be linked to subsequent development of carcinogenicity.

We were unable to measure serum PCBs in these experiments, but assuming that approximate equilibration between liver and blood PCB concentrations has been reached by twelve weeks of dietary exposure, and using mean blood to liver partitioning ratios (1:22) reported for A1254 distribution in adult rats (P. R. S. Kodavanti et al., 1998), total PCB serum levels at 84 days (this

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study) are estimated to be 1.82 ppb in controls, 119 ppb in low dose, and 446 ppb for rats eating the high dose A1254 diet. These estimates are within the range of total wet weight serum PCBs reported in cohorts of Great Lakes Sports Caught Fish Eaters (GLSCFE) defined as those who consumed 24 or more pounds of Great Lakes fish/year for at least six years prior to the study (P. G. Tee et al., 2003). Thus, changes in gene expression reported here may be especially applicable to populations with past, high fish consumption levels from contaminated regions such as the Great Lakes (M. Bloom et al., 2006; M. E. Turyk et al., 2006). The results presented in this study show that continuous, dietary exposure to PCBs results in hepatic gene expression consistent with ongoing oxidative stress. PCBs in Lake Michigan fish have declined since the peak in late 1970's, but in seem to have reached low, refractory levels in popular species such as trout and coho salmon, and thus still have the potential to affect humans and wildlife that consume them.

PCB-induced oxidative stress has been shown to upregulate the stress activated protein kinases JNK/SAPK (Slim et al., 2000) and increase NF-κβ/DNA binding (Hennig et al., 2002) in endothelial cells. In rats injected with coplanar PCB 77 and non-coplanar PCB 153, hepatic levels of oxidized Vitamin E and AP-1 binding to DNA were significantly increased compared to controls, and, similar to our gene expression results, Nqo1 and Cyp1a1 enzyme levels were also higher (Twaroski et al., 2001). Over time, oxidative stress may damage cellular components such as DNA and membrane lipids, and changes in

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the intracellular redox balance may directly affect gene transcription by activating genes, or by altering signal transduction. Inappropriate regulation of gene transcription and intracellular signaling or apoptotic cascades initiated by ROS from Cyp redox cycling, or possibly by glutathiolated Ca3, may contribute to chronic effects in older organisms resulting from increased production of inflammatory cytokines and vascular endothelial cell dysfunction, such as the initiation of atherosclerotic lesions or the development of diabetes.

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CHAPTER IV

SHIFTS IN RAT HEPATIC TRANSCRIPTIONAL RESPONSES RELATED TO SUBACUTE AND SUBCHRONIC DIETARY AROCLOR 1254 EXPOSURE

Introduction

Polychlorinated biphenyls (PCBs) are stable, lipophilic compounds of biphenyls that were first manufactured on an industrial scale in the U.S. by Monsanto in 1929 (de Voogt & Brinkman, 1989). Complex mixtures of the 209 possible congeners, classified by weight percent of chlorine in the mixture (e.g., Aroclors in the U.S., Clophens in Germany) were typically deployed in industrial uses. PCBs were used extensively from 1930's through the 1970's: as non-conductive insulators in transformers and capacitors, as hydraulic and heat transfer fluids, in building materials, paints, carbonless copy paper, and as a carrier for application of pesticides (Fiedler, 1998; Herrick et al., 2004); world production has been estimated at 1.3-1.5 million metric tons (Breivik et al., 2002). Persistent organic pollutants (POPs) are a class of chemical compounds that includes PCBs and several other aromatic, halogenated compounds such as TCDDs, PCDD/Fs, some PAHs, and PBDEs are all chemically inert, hydrophobic, bioaccumulate in food chains and persist in the abiotic environment. The POP congeners capable of assuming a planar configuration can also bind to the arylhydrocarbon receptor to some degree, and induce transcription of genes active in Phase I and Phase II drug metabolism in the

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nucleus (Xu et al., 2005) . Effects observed downstream of AhR-induced gene expression *in vivo* include increases in benign and cancerous tumors of the liver and adrenals, reproductive and developmental abnormalities, thymic atrophy, and impaired immune function (Mandal, 2005; NTP, 2006b). Besides toxic endpoints common to AhR ligands, pre and post natal exposure to ortho-substituted PCBs has been associated with cognitive deficits in human populations (Jacobson et al., 1990; Jacobson & Jacobson, 2003), neurotoxic effects in rats (Schantz & Widholm, 2001), and suppression of immune functions and depletion of thyroid hormone (ATSDR, 2000; Carpenter, 2006).

PCBs were produced, used, and dumped most extensively in the eastern U.S., especially in the highly industrialized Great Lakes basin states (WI, IL, MI, IN, NY, OH). Although levels of PCBs in water, air, sediments, and biota have declined from peaks observed in the late 1970's to early 1980's, several highly contaminated "hot spots" still exist in the U.S. Great Lakes region, particularly around Lake Michigan (Environment Canada, U.S. EPA, 2005). Fish from the lake and tributaries are an important food source for many birds and land mammals in the Great Lakes basin, and animals in this region typically have very high PCB levels compared to animals from less polluted regions (Fox, 2001). PCBs and other POPs present in these animals have been implicated in decreased reproductive success due to changes in behavior, physical reproductive and developmental abnormalities, thyroid deficiency, and extirpation of the mink from the Lake Michigan shoreline (Fox, 2001; Giesy et al.,

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1994; Hickey et al., 2006).

The use of microarrays permits the simultaneous quantification of thousands of genes from a single tissue, and can be used to determine toxicant-induced changes in global gene expression profiles (Balbus, 2005; Lettieri, 2006). At sub toxic exposure levels, measurements of transcriptional changes due to continuous, dietary exposure to a mixture of PCBs similar to actual human and wildlife exposures may lead to the discovery of genes crucial to the origin or perpetuation of subtle physiological effects in mammals. To date, the only reports of global profiles of changes in hepatic gene expression following Aroclor 1254 exposure are for intraperitoneal injection with high, toxic doses (Waring et al., 2001); post natal, lifetime gavage exposure to TDDD and single PCB congeners have also been reported (NTP, 2006a; NTP, 2006b; NTP, 2006c). The liver is a target organ for PCBs in rats and other mammals, and the effects of PCBs on this organ have been widely studied (ATSDR, 2000; Wilson & Safe, 1998).

Diet is the most important pathway of exposure for POPs, and PCBs are ubiquitous in meat, fish, dairy products, and breast milk (Carlson & Hites, 2005; Kalantzi et al., 2001; Solomon & Weiss, 2002). Much of the existing toxicological data for PCBs relies on measurements made in animals gavaged with PCBs in oil, and this route of exposure may result in different health outcomes than environmentally relevant dietary exposures (Arnold et al., 2000). Measurement of the global transcriptional responses to dietary exposure to A1254 has not been

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reported to date, and is important given the persistence of PCBs, and range of effects associated with exposure. No single study can provide a complete picture of the impact of low level dietary PCB or POP exposure, but investigation into the hepatic effects of sub chronic and sub acute exposures to Aroclor 1254, a mixture of PCBs with a congener profile similar to that detected in animals in the KRAOC (Camp Dresser & McKee, 1997), will provide data on health effects associated with this area of the Great Lakes. The aims of this study were to compare hepatic transcriptional profiles previously determined by microarray analyses following at sub acute and sub chronic PCB exposures to characterize the anticipated shift in gene expression profiles, and to use quantitative RT-PCR to confirm changes in selected gene expression. Fisher rats were fed control diet or diet containing 5.6 ppm or 18.6 ppm A1254, for 7 or 84 days. Aroclor 1254 was chosen for this work because of the large body of existing literature describing the effects of Aroclor 1254 exposure in rats (ATSDR, 2000). The mixture has a high percentage of penta- and hexa-chlorinated congeners, and is similar to PCB congener profiles found in animals in the Kalamazoo River area (Camp Dresser & McKee, 1997). Results from this study will help to fill in the existing information gap between laboratory studies of the effects of well-defined gavage or exposures to single congeners (NTP, 2006a), or to intraperitoneal concentrations of PCB mixtures that induce overt toxicities (Waring et al., 2001), and the field observations that result from dietary environmental exposures.

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Methods and Materials

Seven week old male Fisher 344 rats were exposed to PCB's via diets impregnated with 0 ppm, 5.6 ppm, or 18.6 ppm Aroclor 1254 (Lot 124-191-B, Accustandard), for one week (sub-acute exposure) or for twelve weeks (sub-chronic exposure). Livers of these animals were removed; flash frozen at -80 °C upon sacrifice, and mRNA was subsequently extracted from the hepatic tissue using the Ambion Poly(A+) Pure® kit. Affymetrix RGU34A microarrays (Affymetrix, Santa Clara, CA) were processed for three animals in each dose/time group, for a total of 18 arrays, as described previously. Molecular techniques and quality controls used for RNA extraction, target labeling, and array hybridization have already been described in detail (previous manuscript), and conform to those recommended for mRNA processing in the third revision of the Affymetrix GeneChip® Expression Analysis Manual.

Fluorescent image files of the scanned microarrays were imported into the affy package available from the Bioconductor Project (Gautier et al., 2004). Probeset expression summaries were calculated by first background-correcting PMs with RMA (Irizarry et al., 2003), then quantile normalization to reduce non-biological variation among chips (Bolstad et al., 2003; Bolstad et al., 2004). Median polish was used to fit a linear model to background corrected, normalized, log2 probe intensities and calculate a single intensity value for each gene on the array. Gene expression values were then imported into BRBArrayTools (R. Simon & Lam, 2004) in which class comparisons employing

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random variance or multivariate permutation F-tests were used to identify genes differentially expressed among classes. A global test for differing expression profiles among or between the classes was also conducted by permuting which array labels corresponded to which classes. For both tests, the test statistic (p-value) is a measure of the probability that random permutations will yield the same class discriminators as the experimental data. Tests were done at p =.005 significance level. To remove the confounding effect of transcriptional changes due to animal growth, a class comparison of 1 week and 12 week controls at the p =.005 significance level was done. The 37 probesets generated by this analysis were filtered out of all microarray data presented here. The three class (control, low, and high A1254 dose) comparisons reported here were all conducted using this adjusted data set. The sets of class discriminators generated by these analyses were compared to see if there was a shift in gene expression profiles due to time of exposure. Genes chosen for real-time RT-PCR quantification were selected from the results of these analyses.

Gene ontology (GO) class comparisons were also done, to identify groups of genes whose expression was differentially regulated among the classes, using the permutational tests implemented in BRBArrayTools. These analyses are similar in principle to the class comparisons described above, except in this case the test evaluates whether or not expression of each GO category as a whole is different among the classes (R. Simon & Lam, 2004). For each GO group we computed the number n of genes represented on the microarray in that group,

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and the statistical significance *pi* value for each gene *i* in the group (Wright & Simon, 2003). These *p* values reflect differential expression among classes and were computed based on random variance F-tests. A significant statistic (Fisher (LS) < 0.005) is the proportion of the random samples giving as large a value of the summary statistic as the actual *n* genes of the GO category. Only GO categories with between 5 and 100 genes represented on the array were considered, and some of the categories were overlapping. In the event that two categories had identical gene members, only the more specific category was considered for this analysis.

Two-step kinetic RT-PCR (qPCR) reactions using TaqMan RT reagents (Applied Biosystems, Foster City, CA) were done to measure changes in gene expression. Reverse transcription reactions were carried out in 100 µL total reaction volume for a final cDNA concentration of 10 ng/ µL. In these reactions, 1.0 µg mRNA was primed with 2.5 µM random hexamers and reverse transcribed with MultiScribe™ Reverse Transcriptase (1.25 U/µL) in a master mix containing 0.4 U/µL RNase Inhibitor, 4µM dNTP mixture, 1X RT Buffer, and 5.5 mM MgCl2 solution. TaqMan Gene Expression Assays on Demand (Applied Biosystems, Foster City, CA) containing two gene specific primers and fluorescent probes, were used to measure the expression of both reference and target genes (Table 2) on an ABI 7700 Sequence Detection System. PCR reactions were performed in 25 µL total volume containing 7.5 ng of cDNA, 900 pM of each sequence-specific primer, 250 pM FAM-labeled Taqman probe, and 1x PCR

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Mastermix (without uracil N-glycosylase) comprised of AmpliTaq Gold® DNA Polymerase, dNTPs with dUTP, ROX™ as the passive reference, and optimized buffer components (Applied Biosystems, Foster City, CA), and nuclease-free distilled water (Sigma, St. Louis, MO). The thermal cycler program started with a 10 min hold at 95 °C to activate the DNA polymerase, then 40 cycles consisting of denaturing (95 °C for 15s) and primer annealing and extension (1 min at 60°C).

Examination of microarray signal data showed that Ywhaz gene expression was among the least variable of all the potential reference genes considered in these experiments (Table 12), making it a likely candidate gene for qPCR normalization. Ywhaz gene expression was measured in all samples on three consecutive days to evaluate the variability of gene expression across the sample set, and to test the reproducibility of the Ywhaz gene assay. Kinetic data collected from these runs (benchmark data) was used for the normalization of target gene expression. To determine whether or not the RT reactions would be influenced by the amount of starting material, reverse transcriptions were conducted with 100 ng and 500 ng. Similar quantities of Ywhaz were detected from these qPCR reactions, indicating that the amount of (Ywhaz) cDNA produced did not depend on the quantity of initial mRNA.

Ywhaz gene expression in a calibrator mRNA, which was extracted from liver taken from a rat in the one week control group and reverse-transcribed as described above, was used to track the reproducibility of the reference gene

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expression assay throughout the course of the analyses. For the most part, reactions to determine individual target gene expression levels were conducted on a single PCR plate with an optical cover (Applied Biosystems, Foster City, Ca); each plate contained all samples in triplicate, two no-template controls for every gene, and triplicates of the target and reference gene expression assays for the calibrator mRNA. Pooled no amplification control reactions, containing 7.3 ng/μL of each mRNA in a treatment-time group, were run in triplicate for each treatment group and gene combination to check for genomic DNA contamination.

All fluorescence data was collected at threshold = 0.20 to ensure that kinetic data for the reference gene would be comparable to data collected for all the target genes. The optimum threshold setting ($\Delta Ct < 0.5$ among replicates) was determined by comparing ΔCt at several threshold settings above the background and within the linear phase. Kinetic data was imported into DART-PCR (Peirson et al., 2003), to calculate qPCR efficiencies for the reference and target genes based on individual reaction amplification curves of all the samples. These efficiencies, and Cts for the target and reference genes, were imported into Q-Gene (Muller, Janovjak, Miserez, & Dobbie, 2002) for calculation of Ywhaz-normalized target gene expression and the associated standard errors.

Results

Class comparison analyses of microarray RMA expression values showed that A1254 in the diet induced genes coding for microsomal enzymes involved in

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Phase I and Phase II detoxification, oxidative stress, energy production, protein processing and degradation, and long-chain fatty acid transport and metabolism. Results from the gene ontology class comparisons, which incorporate only biological process (BP) and molecular function (MF) GO categories, are summarized by the two pie graphs in Figure 3. Color versions of these charts, as well as the tabulated results of the 1W and 12W GO class comparisons (GO numbers and descriptions, the test statistics, and the number of genes in each category) used for Figure3 are available in supplemental information (Supplemental Table 2, Appendix C).

After one week of dietary exposure to A1254, Phase I (hepatic responses to drugs/xenobiotic enzyme inducers, Figure 3), and Phase II (sulfur metabolism and redox, UDP-glycosyl transferase activity, Fig. 3) metabolic responses accounted for 47% of all significant changes; after prolonged feeding these same GO categories accounted for only 37% of all significant changes. Changes in expression of sets of related genes involved in cofactor metabolism, other transferase activity (not UDP-glycosyl and GSH), and protein catabolism were affected by Aroclor 1254 feeding at both times, however the relative importance of all these categories decreased by ~50% as time of exposure increased. Transferase activity, in particular, became a less significant component of overall GO change; at 1W expression of groups of genes involved in the transfer of nitrogenous, glycosyl, acyl, alkyl (not methyl) or aryl, and hexosyl groups were all significantly affected, at 12W just genes in the latter two categories were

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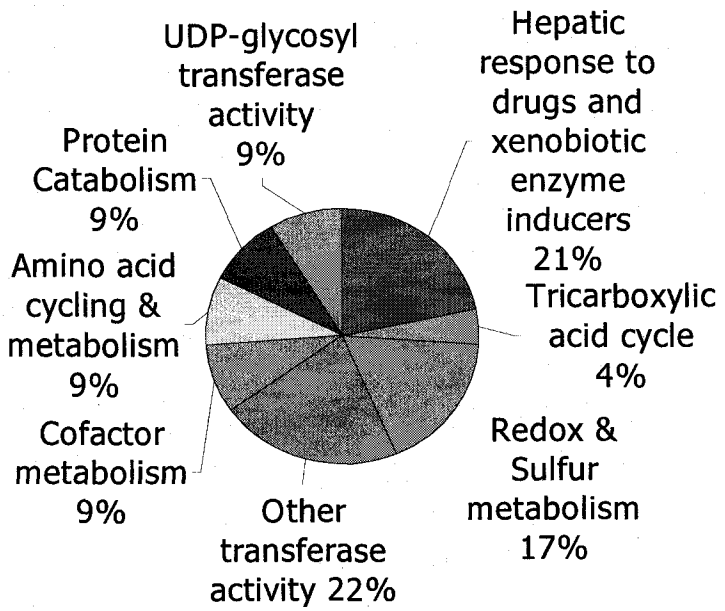
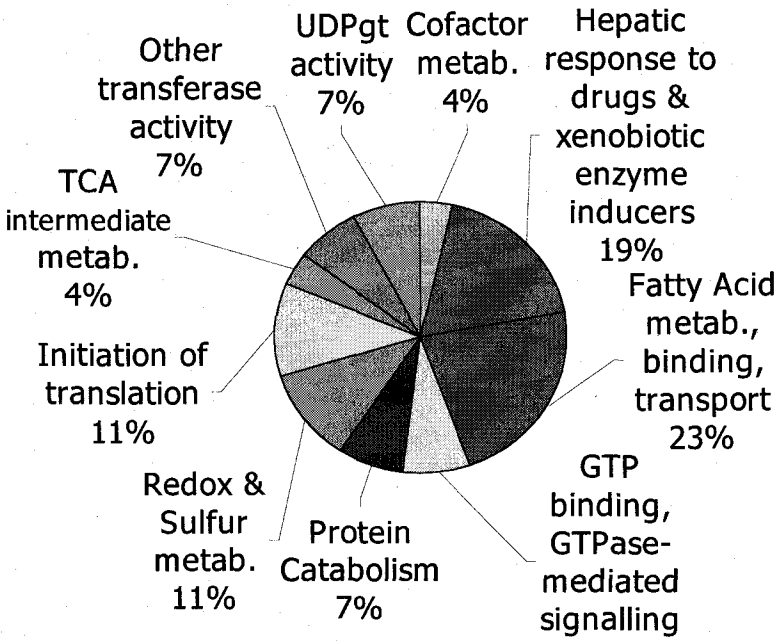
Figure 3

Changes in Rat Hepatic Gene Ontology Profiles Associated with Length Of Dietary Exposure To Aroclor 1254

A. 1W Exposure



B. 12W Exposure



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(Supplemental Table 2, Appendix C). Processes involving fatty acid metabolism, binding, and long-chain fatty acid transport, small GTPases, and initiation of translation became significant only after long-term exposure (12 W, Fig. 3).

Results from the 1W and 12W GO class comparisons can also be ranked in terms of the multivariate permutation p value (LS statistic) calculated for the individual GO categories (Supplemental Table 2, Appendix C). For the 1W class comparison, only three categories: glutathione transferase activity, monooxygenase activity, and response to chemical substance, were significant at the $p \leq .00001$ level. After long-term exposure to A1254, gene expression had changed to the extent that 12 of the 27 GO categories had a multivariate permutation $p \leq .00001$. All of the categories comprising fatty acid metabolism, binding, and transport were significant at $p \leq .00009$; expression of groups of related genes involved in glutathione metabolism and transferase activity, GTP binding, monooxygenase activity and response to chemical substance were also highly significant ($p \leq .00001$) after 12 weeks dietary exposure to Aroclor 1254.

The number of class discriminators determined by 3 (dose) class comparisons increased nearly four-fold, from 20 to 73, as the length of Aroclor 1254 exposure increased from one week to twelve weeks. The p-value of the global test for the 12W class comparison was also much more significant than for 1W; the probability of generating the same list of 73 genes (significant at the 0.005 level) by random permutation of class labels was only 10%, but the

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probability of the same test using the 1W microarray data was 50%. The decrease in the FDR and the increase in the number of highly significant GO categories mentioned above are due, to some extent, to the fact that class comparison for 12W data yielded a much greater number of class discriminators than the 1W group.

Monooxygenase expression (Cyp2b2, Cyp1a2, Cyp2b15) was prominent in the hepatic responses to drugs/xenobiotic enzyme inducers GO categories of Figure 3, and was greatly increased following one week exposure to both doses of A1254 (Table 11). Genes coding for the xenobiotic-conjugating enzymes glutathione S-transferase (Yc2) and Phenobarbital-inducible UDP-glucuronosyltransferase (Udpgtr2), important members of GO categories in Redox & sulfur metabolism and UDP-glucuronosyltransferase activity (respectively, Fig. 3), were also highly induced in livers of animals exposed to A1254 for one week. Expression levels of the Phase I (cytochrome P450s), Phase II (GSH and UDP-glucuronsyl transferases), and another AhR battery gene (Nqo1) also increased in a dose-dependent manner, with the lowest hepatic mRNA levels in the animals fed the 5 ppm diet, and the highest in rats fed the 18 ppm Aroclor 1254 diet (Table 11). Only three of the 20 class discriminators for 1W were decreased by the Aroclor diet—Sds, Hmgcs2, and Hal. Fold decreases in Sds levels were nearly identical in 1W5 and 1W18 (-1.81 and -1.85, respectively, Table 10); but seemed to be modestly dose-dependent for Hmgcs2 and Hal, a larger decrease in gene expression corresponded to a higher dose.

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Table 10

Class Discriminator Genes[a] from 1W Filtered Class Comparison Ranked by Significance of F-test

p-value	FC 5ppm	FC 18ppm	Description	Gene symbol
6.20E-06	1.74	7.51	Cytochrome P450, family 2, subfamily b, polypeptide 2	Cyp2b2*
2.30E-05	1.91	2.95	liver UDP-glucuronosyltransferase, phenobarbital-inducible form	Udpgtr2
1.58E-04	2.57	9.33	cytochrome P450, family 1, subfamily a, polypeptide 2	Cyp1a2*
1.24E-03	1.86	1.93	proteasome (prosome, macropain) subunit, beta type 2	Psmb2*
1.24E-03	0.55	0.54	serine dehydratase	Sds
2.02E-03	1.36	2.65	NAD(P)H dehydrogenase, quinone 1	Nqo1
2.89E-03	0.879	0.693	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2	Hmgcs2
3.76E-03	1.34	3.71	glutathione S-transferase Yc2 subunit	Yc2
4.05E-03	1.54	2.63	cytochrome P450, family 2, subfamily b, polypeptide 15	Cyp2b15

[a]Genes: transcripts have gene symbol and functional description
All probesets except Cyp2b2 have Grade A annotation

* indicates that multiple probesets for gene were present in list, see Supplemental Table 2

† indicates that additional probesets related to gene were present in list, see Supplemental Table 2, Part B

After 12 weeks of dietary A1254 exposure, several genes representative of important GO categories in Figure 3 are significant at $p \leq .002$ (Table 11, Supplemental Table 2). The same Phase I (Cyp1a2, Cyp2b2, Cyp2b15) and Phase II (Yc2, Udpgtr) genes important in hepatic responses to drugs/xenobiotic enzyme inducers, sulfur metabolism/redox, and UDPgt activity (Figure 3) at 1 week were among the top 20 class discriminators at 12 weeks (Table 11). Genes

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coding for proteins active in GTP binding and small GTPase-mediated signal transduction (Rab11a), fatty acid metabolism, transport, and binding (Me1, Cd36), and protein catabolism (Psmb5, Psma1) were also among the most significant of the 12W class discriminators (Table 11).

Table 11

Class Discriminator Genes[a] from 12W Filtered Class Comparison Ranked by Significance of F-test

p-value	FC 5ppm	FC 18ppm	Description	Gene symbol
3.00E-07	1.13	5.01	CEA-related cell adhesion molecule 10	Ceacam10
2.60E-06	1.16	2.93	malic enzyme 1	Me1
5.10E-06	1.82	5.58	Cytochrome P450, family 2, subfamily b, polypeptide 2	Cyp2b2*
1.36E-05	2.18	6.64	cytochrome P450, family 1, subfamily a, polypeptide 2	Cyp1a2*
1.82E-04	1.59	5.62	glutathione S-transferase Yc2 subunit	Yc2
2.87E-04	1.33	2.75	cd36 antigen	Cd36†
5.36E-04	1.27	2.24	liver UDP-glucuronosyltransferase, phenobarbital-inducible form	Udpgtr2
5.49E-04	1.08	1.63	RAB11a, member RAS oncogene family	Rab11a
8.49E-04	1.14	2.18	cytochrome P450, family 2, subfamily b, polypeptide 15	Cyp2b15
1.07E-03	0.81	1.48	proteasome (prosome, macropain) subunit, beta type 5	Psmb5
1.30E-03	1.16	1.92	epoxide hydrolase 1	Ephx1
1.84E-03	1.15	1.52	proteasome (prosome, macropain) subunit, alpha type 1	Psma1
1.91E-03	0.80	2.24	glutamate cysteine ligase, modifier subunit	Gclm
1.92E-03	0.96	0.34	carbonic anhydrase 3	Ca3

[a]Genes: transcripts have gene symbol and functional description; all probesets except Cyp2b2 have Grade A annotation

* indicates that multiple probesets for gene were present in list, see Supplemental Table 2

† indicates that additional probesets related to gene were present in list, see Supplemental Table 2, Part B

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Most target genes chosen for kinetic RT-PCR quantitation were from the 12 week class comparison, as one of our main objectives was to characterize shifts in global hepatic gene expression due to long-term exposure to Aroclor 1254. The 12W class comparison was much more statistically robust than 1W, so our chances of making a false positive discovery among candidates in the 12W list of 73 genes ($p \leq 0.10$) is much less than in the 1W list of 20 genes ($p \leq 0.50$). The first and second most significant of the class discriminators for 12W dietary Aroclor 1254 exposure (Table 11) were selected. The first, Ceacam10, is a secretory, extracellular paralog to Ceacam1 (S. Li, Lee, Hsiao, & Chen, 2005) and a recently reported novel dioxin-responsive gene (Vezina et al., 2004). Malic enzyme (Me1), the second most significant member of the 12W discriminator list, generates NADPH reducing equivalents essential to fatty acid synthesis; its mRNA expression has been reported to be increased after exposure to PCBs (Boll, Weber, Messner, & Stampfl, 1998) and related, dioxin-like compounds (Loaiza-Perez et al., 1999). Cd36, a key fatty acid transport gene in muscle tissues, that has also recently been reported to increase in LDL$^{-/-}$ mouse livers following exposure to PCB 77 (Hennig, Reiterer, Majkova et al., 2005), was chosen as representative of fatty acid transport and binding GO categories, and Rab11a was selected because of its significance in the GTP-related GO categories. Very few of the class discriminators were decreased by short or long-term Aroclor exposure, and only two of the target genes selected were decreased

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following exposure: Ca3 at 12W (Table 12) and Hmgcs2 at 1W (Table 11). Exposure to PCB 126 has been reported to suppress Ca3 protein in rat livers after a single i.p. injection (Ikeda et al., 2000), and the protein is also decreased in human hepatocellular carcinomas (Kuo et al., 2003). Hmgcs2 was significant in the 1W cofactor metabolism GO category (Fig.3); the enzyme catalyzes the condensation of acetyl-CoA with acetoacetyl CoA in the first, rate-limiting step of ketogenesis (Casals et al., 1992), and is reportedly downregulated by insulin (Nadal, Marrero, & Haro, 2002). At the time of target gene selection and qPCR analysis, all the probesets now annotated as Cyp2b2 were identified as Cyp2b15, and Cyp2b15 was selected because of it's frequency and prominence in both sets of class discriminators (Table 10-11, Supplemental Table 3).

Quantitative PCR requires normalization to either an exogenous standard, or to a reference—an endogenous gene whose expression does not change across treatments or tissues. Microarray signal data of several representative probesets was examined to select a reference gene for qPCR normalization (Table 1); the candidate genes screened were chosen from journal publications (Dheda et al., 2004; Thellin et al., 1999) and manufacturer's recommendations (e.g., Ambion, Applied Biosystems, Invitrogen). Ideally, reference gene expression should be unaffected by experimental conditions, and should be invariant across all tissues (Applied Biosystems, 2001; Bustin & Nolan, 2004). A brief list of reference gene candidates screened, their biological functions, and variability in microarray

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signal detected, is given in Table 12. The ranges in microarray signal for each gene refer to the range in mean (geometric) signal among the six treatment groups, and the variation is this range calculated as the percent of mean signal in all tissues.

Microarray expression of the Ywhaz gene (tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein, zeta polypeptide), a 30kDa component of the mitochondrial import stimulation factor, a protein complex that targets precursor proteins to the mitochondrion (Alam et al., 1994), was the least variable of all the gene candidates considered for this study, so this gene was chosen as the reference gene for normalization in quantitative RT-PCR.

Table 12

Candidate Reference Genes

Gene	GO Biological Process Description	Signal Range	Variation
Alb	transport, negative regulation of cell death, body fluid osmoregulation	5562	29%
B2m	antigen processing, endogenous antigen via MHC class I	1681	22%
Gapdh	glycolysis, apoptosis	2199	75%
Hmbs	heme biosynthesis	286	58%
Hprt	purine nucleotide biosynthesis and salvage	890	62%
Ppia	protein folding, regulation of viral genome replication	4325	42%
Sdha	tricarboxylic acid cycle, electron transport	656	66%
Ubc	ubiquitin-dependent protein catabolism	1008	26%
Ywhaz	protein targeting to mitochondrion	80	10%

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The qPCR reaction efficiencies for the target and reference genes ranged from 0.835 for Me1, to 1.003 for Hmgcs2; standard deviations for the efficiency determinations were < .035, and mean coefficients of variation were between 1.3 and 3.3% (Table 13). The intergroup p-values for all genes are greater than 0.5 (Table 13), indicating that there were no statistically significant differences in the PCR amplification efficiency of any gene among the six treatment groups;

Table 13

Quantitative PCR Efficiencies of TaqMan Assays for Target and Reference Genes

Gene	Assay ID	Mean Efficiency	± SD	Mean CV Efficiency	Intergroup p-values
Ywhaz	Rn00755072_m1	0.948	0.019	2.5%	0.123
Ca3	Rn00695939_m1	0.870	0.014	3.0%	0.257
Cc10	Rn00597983_m1	0.963	0.021	3.2%	0.606
Cd36	Rn00580728_m1	0.982	0.027	3.3%	0.211
Cyp2b15	Rn01457880_m1	0.880	0.031	1.3%	0.058
Hmgcs2	Rn00597339_m1	1.003	0.032	2.0%	0.073
Me1	Rn00561502_m1	0.835	0.026	3.0%	0.100
Rab11a	Rn00579853_m1	0.979	0.018	1.8%	0.112

therefore, these mean efficiencies were used to calculate relative changes in gene expression for all the target genes (Tables 14 and 15). The ratio of Ywhaz expression in the benchmark analyses to Ywhaz expression in all subsequent analyses varied from 1.01-1.05 for the calibrator sample, demonstrating that the reference gene assay was reproducible and comparable across analyses. Low levels of genomic DNA for Ywhaz, Cyp2b2, Ca3, Hmgcs2, Me1, and Cd36 were found only in one low dose sample from the 1W treatment group, but this RNA

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was not treated with DNase because even with the DNA contamination, expression of target genes in 1W5 was not significantly different from controls (Tables 14 and 15). Also, treatment with a DNase might have altered the sample, rendering it different from the original mRNA used for microarray analysis.

Table 14

Real-time RT-PCR Quantitation of Ca3, Cd36, Hmgcs2, Me1, and Rab11a Gene Expression

FC	Ca3	Cd36	Hmgcs2	Me1	Rab11a
1W5	1.55	1.02	0.752	0.967	1.08
1W18	1.04	0.995	0.553*	1.66	0.882
12W5	1.26	1.92**	1.27	1.55**	1.30
12W18	0.343	2.44**	0.938	3.08**	1.58*

**p< 0.005, *p< 0.05 by student's t-test

The results from the kinetic RT-PCR determination of hepatic levels of the target genes in rats exposed to dietary Aroclor 1254 are expressed as fold change (FC), where FC = gMNE (treated): gMNE (control), and gMNE is the geometric mean of the Mean Normalized Expression of the three samples in each treatment group. Fold changes determined for Ca3, Cd36, Hmgcs2, Me1, and Rab11a are in Table 14; FCs for Ceacam10 and Cyp2b15 are in Table 15. Five of the class discriminators: Cd36, Me1, Rab11a; Ceacam10, Cyp2b15, selected from the microarray data as indicative of long-term dietary Aroclor 1254 exposure were confirmed to be significantly increased over 12W controls ($p < .05$, two-sample student's t-test) in the 12W18 group by qPCR. However, in the 12W5 group, only Me1 and Cd36 (Table 14) and Cyp2b15 (Table 15) gMNE levels were significantly

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increased over the 12W controls. For the 1W measurements of changes in target gene expression, significant change in expression levels of both class discriminators was confirmed by qPCR in the 18 ppm group only: Cyp2b15 was induced, Hmgcs2 was decreased. There were no statistically significant changes in gMNE, for any of the target genes, in the 1W5 group.

Quantitative PCR measurements of normalized expression levels for several of the target genes in 12W18 are very close to those measured by microarray. Comparison of the fold changes determined by the two methods shows close correspondence in 12W18 between microarray and qPCR values (Table 11, chip vs Table 14, qPCR) for Rab11a (1.63 vs 1.58), Me1 (2.93 vs 3.03), and Cd36 (2.75 vs 2.44), and are identical for Ca3 (-2.92). In the 12W5 group, the closest agreement between chip and PCR FC's of 12W class discriminators was for Rab11a expression (1.08 vs 1.26), Ca3 and Me1 were within 0.4, but the FC difference for Cd36 (1.33 vs 1.92) was greater than 0.5. For Hmgcs2, the 1W class discriminator whose hepatic expression was apparently decreased by Aroclor feeding, comparison of Table 11 and Table 14 shows that FC's for this gene were within 0.4 fold of each other, with (-1.14 chip vs -1.33 qPCR) for 1W5 and (-1.44 vs -1.81) for 1W18.

Comparison of the microarray results presented in Tables 9 and 10 and the corresponding quantitative RT-PCR results presented in Table 15 (columns with "a" superscript) show that the discrepancies in measurements of fold change

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between the two methods are much greater for Ceacam10 and Cyp2b15 than for any of the other target genes. Cyp2b15 levels in 1W5 and 12W5 were 2-3 times greater when measured by qPCR than when measured by microarray; in 1W18 and 12W18, the qPCR measurements were ~7x greater than the microarray. For Ceacam10, qPCR-determined expression levels in the 1W samples were only 1.3-2.0 times greater than the quantities measured by chip, but for 12W, measured Ceacam10 levels were 3 (12W5) and ~10 (12W18) times greater by qPCR than by microarray. To see if the qPCR measurements were truly representative of gene expression in the treatment groups, mRNA from six additional experimental animals, (one from each of the control, low, and high dose groups), was extracted, reverse-transcribed, and Ceacam10 and Cyp2b15 gene expression was quantitated by real-time RT-PCR. Fold changes for Cyp2b15 and Ceacam10 were recalculated with the additional samples (n = 4 per group), and are presented in Table 15 in the columns with "b" superscripts.

Table 15

Relative Mean Normalized Expression of Cyp2b15 and Ceacam10

FC	Ceacam10[a]	Ceacam10[b]	Cyp2b15[a]	Cyp2b15[b]
1W5	1.54	1.53	3.50	2.31
1W18	2.66*	2.99*	19.0*	12.3**
1W5: 1W18	*1.7*	*2.0*	*5.4*	*5.3*
12W5	3.53	3.43*	3.22*	2.88*
12W18	49.3*	49.5**	14.1*	12.7**
12W5:12W18	*14.0*	*14.4*	*4.4*	*4.4*

[a] FC with microarray samples only, [b] FC with additional sample
**p< 0.005, *p< 0.05 by student's t-test

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Inclusion of extra samples had a nominal effect on the group FC's of Ceacam10 shown in Table 15, only for 12W5 was the FC lowered (0.1 fold). The magnitude of increase in FC for remaining groups was trivial, the largest being the increase from 2.66 to 2.99 for 1W18. With an additional sample in each group, the difference in means of Ceacam10 gMNE in 12W controls and 12W5 became statistically significant ($p < .05$), and p of the t-test for the difference in 12W control versus 12W18 group means decreased to <.005 (Table 15). The dose-dependent changes in Ceacam10 expression for each time point were estimated by calculating the ratios 1W5:1W18 and 12W5:12W18. Table 15 shows that both of these ratios were increased slightly when 4 samples were used, but Ceacam10 levels were still approximately 2x greater in 1W18 than 1W5, and 14x greater in 12W18 than 12W5.

Fold changes in Cyp2b15 gene expression were lowered, for all treatment groups, by inclusion of a fourth sample in each group (Table 15). Most noticeable is the decrease in FC for 1W18 from 19.0 with 3 samples, to 12.3 with 4 samples; reductions in Cyp2b15 FCs for the other three groups ranged from -1.4 for 12W18 to -0.34 for 12W5. Because the Cyp2b15 gMNE levels were all lower with the extra sample, the disparity between the qPCR and microarray measurements was also diminished. Comparison of fold changes in Cyp2b15 in Tables 10 and 11 with those in the "b" columns in Table 15 show that the qPCR measurements of Cyp2b15 were only 1.5-2.0 times greater for lose dose groups,

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and ~5-6x greater than the microarray for 1W18 and 12W18. Even though all the group mean levels of Cyp2b15 expression were lower for n = 4 samples, the Cyp2b15 expression ratios used to approximate the dose-dependent differences for each treatment time remained remarkably constant. Following the short term exposure to A1254, Cyp2b15 levels were approximately 5.4 times greater in animals fed the high dose than in animals fed the low dose diet, after 12 weeks exposure to the Aroclor mixture, Cyp2b15 gene expression levels were 4.4 times higher in the high dose group.

Discussion

After 12 weeks dietary exposure to Aroclor 1254, the global hepatic gene expression profiles of male inbred rats were significantly different from one week hepatic gene expression profiles. These comparisons are between rats of different ages, and the information acquired from this study can be thought of as a time series that models changes in gene expression due to dietary A1254 that occur initially (sub acute) and later in the rat lifetime, following extended, but sub chronic, feeding. We have presented results that confirm our microarray measurements for a small subset of discriminators selected from the 1W and 12W class comparisons. Only in the long term exposures were the rat hepatic transcriptomes for the low, high, and control groups sufficiently different so as to be considered distinct classes in the three way class comparison. The most noticeable difference between 1W and 12W global gene expression profiles is the

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significant changes in fatty acid-related GO categories following long term A1254 feeding.

In the liver, PPARα may bind fatty acids, eicosanoids, NSAIDs, or fibrate ligands (Maloney & Waxman, 1999), and form a transcriptionally active complex by dimerizing with RXR bound to retinoic acid. The PPARα/RXR dimer upregulates genes involved in lipogenesis, lipid transport, ketogenesis, FA transport, and FA oxidation in liver and skeletal muscle. Expression of Cd36, Me1, and Hmgcs2, three of the class discriminators determined to be significant in these experiments by microarray class comparisons and by qPCR, is also regulated by the PPARα/RXR nuclear receptor dimer in the liver (Hertz, Nikodem, Ben-Ishai, Berman, & Bar-Tana, 1996; Meertens, Miyata, Cechetto, Rachubinski, & Capone, 1998; Patsouris, Reddy, Muller, & Kersten, 2006). Hmgcs2, or 3-hydroxy-3-methylglutaryl-CoA synthase, catalyzes the condensation of Ac-CoA and acetoacetyl-CoA, the rate-limiting step in ketogenesis (Casals et al., 1992). Ketogenesis provides lipid-derived fuel from fatty acid oxidation to peripheral tissues during starvation and diabetes, and to the brain in normal conditions (Fukao, Lopaschuk, & Mitchell, 2004). Mitochondrial Hmgcs2 contains a PPAR response element with an LXXLL consensus sequence, and is capable of regulating its own transcription by interacting directly with PPARα in the nucleus *in vitro* and *in vivo* (Meertens et al., 1998). In this work, mitochondrial Hmgcs2 expression was significantly

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decreased after one week of dietary A1254. Insulin inhibits the synthesis of HmgCoA by repressing the forkhead transcription factor (FKHRL1)-mediated upregulation of Hmgcs2 gene expression (Nadal et al., 2002), and could possibly have played a role in the decreased expression of this gene.

Cd36 and malic enzyme were unaffected after one week of the PCB diets, but expression of both genes was increased after 12 weeks of Aroclor 1254 feeding. Malic enzyme is a lipogenic enzyme that provides NADPH reducing equivalents required for fatty acid synthesis, and induction of the enzyme has been reported in rats following dietary exposure to high doses of Clophens (Boll et al., 1998). The rat malic enzyme gene contains a thyroid hormone response element (Petty, Desvergne, Mitsuhashi, & Nikodem, 1990), and is upregulated by thyroid hormone (Song, Grieco, Rall, & Nikodem, 1989). Addition of thyroid hormone (T_3) decreases the rate of Me1 mRNA degradation and increases gene transcription (Song, Dozin, Grieco, Rall, & Nikodem, 1988), so the increase in malic enzyme observed at 12 weeks may be due to thyroid hormone activation as well as stabilization of the transcript. The increase in Me1 reported here was not accompanied by increases in other genes, such as glucose-6-phosphate dehydrogenase, or 6-phosphogluconate dehydrogenase, as has been reported previously for lipogenic gene induction initiated by PCB feeding (Boll et al., 1998). Fatty acid synthase mRNA in treated animals was lower than controls at 12 weeks (data not shown), and serum triglycerides were not affected by any

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A1254 treatments in our feeding studies (previous manuscript). Malic enzyme was an chosen as a representative of the FA-related GO analysis categories, but corroborating evidence for increased hepatic fatty acid synthesis was not observed in this study. Upregulation of Me1 may occur in response to oxidative metabolism of PCBs (Braun, Eigenbrodt, Laib, & Brunn, 1991); NADPH produced by the Me1-catalyzed conversion of malate to pyruvate could also be utilized to maintain intracellular GSH levels and counter PCB-induced oxidative stress (Ayala, F-Lobato, & Machado, 1986), or by Por to recycle P450s (Schlezinger et al., 1999).

The gene Cd36 upregulation has been reported in mouse liver following exposure to PCB-77 (170 μmol/kg, *i.p.*), in LDL-R$^{-/-}$ mice maintained on a diet enriched with linoleic acid (Hennig, Reiterer, Majkova et al., 2005), but this is the first report of significant hepatic upregulation of Cd36 following dietary exposure to a mixture of PCBs. Cd36 is a 88 kDa glycosylated, transmembrane protein whose long chain fatty acid (LCFA) transport activity is important in muscle tissue, supplying LCFA for immediate conversion into energy (Abumrad et al., 1999; Luiken et al., 2002). Cd36 expression levels are controlled by PPARγ in heart and skeletal muscle, and in adipocytes, and by PPARα in the liver (Patsouris et al., 2006). In the obese Zucker rat model, fixation of the protein in the cell membranes of muscle and adipose tissues helped to maintain the insulin resistant state in the animal (Luiken, Bonen, & Glatz, 2002/8), but Cd36 was not

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found to be an important LCFA transporter in the diabetic rat liver (Luiken et al., 2002). The recent report of hepatic overexpression of Cd36 in the LDL-R$^{-/-}$ mice exposed to PCB 77, by Hennig and coworkers (2005), occurred only with a linoleic acid diet, and not in mice fed oleic acid. The increase in the LCFA transporter gene was accompanied by significant increases in liver neutral FA content as measured by Oil Red O staining. We were unable to conduct a similar analysis on the liver tissue in this study, but it seems unlikely that we would have observed a similar, significant increase in liver FAs in animals' constitutively expressing the LDL receptor.

There is growing evidence linking Cd36 to vascular endothelial damage, and to participation in the atherosclerotic cascade. Overexpression of the gene and the protein in microvascular endothelial cells has been linked to erectile dysfunction in diabetic Fisher 344 rats (Sullivan et al., 2005). Cd36 is expressed on monocytes and macrophages, including Kupffer cells in the liver, where it serves as a scavenger for oxidized LDL, and as a collagen type I and thrombospondin receptor (Schneiderhan et al., 2001). Accumulation of oxLDL in macrophages is the critical step in foam cell formation and the subsequent development of atherosclerotic plaques in blood vessels. Deficiency in macrophage CD36 in the ApoE$^{-/-}$ mouse model of atherosclerosis reduced development of inflammatory lesions by nearly 90% (Febbraio, Guy, & Silverstein, 2004), and significant overexpression of the mRNA and the protein

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has been reported in peripheral monocytes of patients with poorly controlled Type 2 diabetes compared to a reference group (Zhang, Zhang, Zhou, & Li, 2005).

The top 12W class discriminator in this study was the Ceacam10 gene. The protein is a soluble, extracellular paralog of membrane cell adhesion molecule (CAM), Ceacam1, a common tumor marker protein (Obrink, 1997), but very little is known about the physiological function of Ceacam10. The Ceacam10 gene is expressed in spleen, lung, large intestine (Lin et al., 1998), and in mouse seminal vesicles, where it enhances sperm motility in a testosterone-controlled fashion (S. Li et al., 2005). Unlike many soluble CAMs, Ceacam10 is capable of weak homodimerization to the membrane-expressed protein (Lin et al., 1998). The increase in expression of this gene was also very high in female Sprague-Dawley rats following 13 weeks of oral gavage dosing with 1 mg/kg/day of TCDD, PCB 126, and PeCDF, but not with PCB 153 (Vezina et al., 2004), and its increased expression in these studies has been attributed specifically to transcriptional regulation by the AhR (Ovando, Foxenberg, Kransler, Vezina, & Olson, 2005). In the liver expression of Ceacam10 is normally low, whereas basal levels of Ceacam1 are much higher (Lin et al., 1998). The marked increase in expression in this tissue in response to A1254 feeding (this study), and to AhR ligands (Vezina et al., 2004) suggest that dioxin-like

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compounds have the potential to alter cell-cell adhesion or adhesion-mediated intercellular communication by increasing production of soluble Ceacam10.

The overall expression of gene members of the GTP binding and small GTPase-mediated signaling GO categories (Figure 3 and Supplemental Table 2) were significantly different after 12 weeks of A1254 exposure, but not after only one week of treatment. The expression of the small Ras-related GTPase Rab11a, a member of this GO group that directs intracellular vesicle trafficking during exocytosis and endocytosis, was increased significantly by the high dose diet at 12 weeks. The role of this protein in directing the recycling of receptors from late endosomes back to the plasma membrane has come under scrutiny recently, and Rab11a has been shown to be important in recycling, and thus controlling the cell membrane expression, of many GPCRs, including human protease-activated receptor 2 (Roosterman, Schmidlin, & Bunnett, 2003), the neurokinin 1 receptor (Roosterman, Cottrell, Schmidlin, Steinhoff, & Bunnett, 2004), and the transmembrane receptor (β) for thromboxane A2 (Hamelin, Thériault, Laroche, & Parent, 2005). Calcium-sensing receptor (CaR) intracellular trafficking and secretion of parathyroidhormone-related protein was blocked in a Rab11a$^{-/-}$ mutant (Reyes-Ibarra et al., 2007). Restoration of Rab11a and the associated resumption of CaR endosomal recycling stimulated hormone secretion from the cell in this study indicate that this protein serves as a signal transducer in addition to its role in vesicular trafficking. The increase in Rab11a in this study

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was minor compared to the upregulation of Rab11a observed in human epithelial skin tumors (Gebhardt et al., 2005), and in breast ductile carcinomas (Palmieri, Bouadis, Ronchetti, Merino, & Steeg, 2006). However, given the prominent regulatory function of this protein in the intracellular routing and exocytosis of cell surface receptors and secretion of their associated soluble factors, such as hormones (Reyes-Ibarra et al., 2007) and growth factors (Palmieri et al., 2006), even small increases in expression of this gene may be sufficient to alter the extracellular environment and initiate pro-carcinogenic events via a paracrine mechanism.

In this work, shifts in global hepatic gene expression responses to short and long term dietary PCB exposures were qualitatively identified using gene ontology class comparisons. With short term exposure, the majority of responses in the rat livers were related to drug or xenobiotic metabolism; after 12 weeks of dietary Aroclor 1254, GTP-related and FA-related gene ontology categories and genes emerged as significant characteristics of the global transcriptome.

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CHAPTER V

CONCLUSION

This is the first report of global transcriptional response to PCBs administered at relatively low concentrations, by an environmentally relevant route. Rat hepatic transcriptional responses to a mixture of dietary PCB were obtained from microarray profiling in this work. Twelve weeks exposure to 18.6 ppm Aroclor 1254 in the diet produced a global expression profile that was significantly different from the control group, while one week of the same diet resulted primarily in large changes in Phase I and Phase II xenobiotic metabolizing enzymes. Gene expression changes between the low dose groups and their respective controls were nominal, though dose-related changes were apparent for common expression of Cyp1a1 and Cyp1a2 at a given time.

A shift in global expression profiles due to time of feeding was apparent in this work. The difference in animal ages at the time the livers were harvested prohibits a direct, rigorous comparison of time of treatment effects on dose groups. This was controlled for by filtering out any genes changed between controls at the two time points, and by treating each group independently, but the possibility of age-related effects in individual animals, independent of treatment, cannot be entirely ruled out in this analysis. Another limitation of this study, in terms of environmental relevance, is that animals were not treated until

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after birth. Humans and animals are exposed to PCBs throughout their lifetimes, including the prenatal period. No single study can capture the complexities of environmental exposures, however, and results from this work may be used as a starting point for probing initiation or perpetuation of chronic health effects due to PCB exposure.

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APPENDIX A

IACUC Research Protocol Clearance

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WESTERN MICHIGAN UNIVERSITY

Institutional Animal Care and Use Committee

Centennial 1903-2003 Celebration

Date: June 11, 2004

To: Jay Means, Principal Investigator
Lisa Baker, Co-Principal Investigator
Alhaji N'Jai, Student Investigator for dissertation
Trisha Basford, Student Investigator for dissertation

From: Robert Eversole, Chair

Re: IACUC Protocol No. 03-04-02

Your protocol entitled "Investigation of Markers of Cell Death and Immune Function in Rats Exposed to Selected Chemicals" has received approval from the Institutional Animal Care and Use Committee. The conditions and duration of this approval are specified in the Policies of Western Michigan University. You may now begin to implement the research as described in the application.

The Board wishes you success in the pursuit of your research goals.

Approval Termination: June 11, 2004

Walwood Hall, Kalamazoo, MI 49008-5456
PHONE: (269) 387-8293 FAX: (269) 387-8276

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APPENDIX B

Percent Composition by Mass of Aroclors

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IUPAC Number	Chlorine Substitutions		Name	A1016	A1242	A1254	A1260
1	2	-	monochlorobiphenyl	0.52	0.54		0.02
2	3	-	monochlorobiphenyl	0.02	0.03		
3	4	-	monochlorobiphenyl	0.15	0.18		
4	2,2'	-	dichlorobiphenyl	3.62	3.08	0.06	0.02
5	2,3	-	dichlorobiphenyl	0.17	0.14		
6	2,3'	-	dichlorobiphenyl	1.64	1.43	0.02	0.01
7	2,4	-	dichlorobiphenyl	0.29	0.26		
8	2,4'	-	dichlorobiphenyl	8.29	7.05	0.13	0.04
9	2,5	-	dichlorobiphenyl	0.58	0.50		
10	2,6	-	dichlorobiphenyl	0.23	0.20		
12	3,4	-	dichlorobiphenyl	0.07	0.06		
13	3,4'	-	dichlorobiphenyl	0.24	0.22		
15	4,4'	-	dichlorobiphenyl	2.40	2.10	0.03	0.01
16	2,2',3	-	trichlorobiphenyl	3.88	3.14	0.09	0.01
17	2,2',4	-	trichlorobiphenyl	3.98	3.13	0.08	0.02
18	2,2',5	-	trichlorobiphenyl	10.86	8.53	0.25	0.05
19	2,2',6	-	trichlorobiphenyl	0.99	0.80		
20	2,3,3'	-	trichlorobiphenyl	0.88	0.72		
22	2,3,4'	-	trichlorobiphenyl	3.50	2.84	0.04	0.01
23	2,3,5	-	trichlorobiphenyl	0.01	0.01		
24	2,3,6	-	trichlorobiphenyl	0.16	0.13		
25	2,3',4	-	trichlorobiphenyl	0.72	0.59		
26	2,3',5	-	trichlorobiphenyl	1.57	1.28	0.03	
27	2,3',6	-	trichlorobiphenyl	0.51	0.41		
28	2,4,4'	-	trichlorobiphenyl	8.50	6.86	0.19	0.03
29	2,4,5	-	trichlorobiphenyl	0.10	0.08		
30	2,4,6	-	trichlorobiphenyl	trace			
31	2,4',5	-	trichlorobiphenyl	9.32	7.34	0.28	0.04
32	2,4',6	-	trichlorobiphenyl	2.37	1.90	0.05	0.01
33	2,3',4'	-	trichlorobiphenyl	6.21	5.01	0.16	0.03
34	2,3',5'	-	trichlorobiphenyl	0.03	0.02		
35	3,3',4	-	trichlorobiphenyl	0.05	0.08		
37	3,4,4'	-	trichlorobiphenyl	1.02	2.03	0.07	0.01
40	2,2',3,3'	-	tetrachlorobiphenyl	0.58	0.76	0.12	
41	2,2',3,4	-	tetrachlorobiphenyl	0.76	0.68	0.01	
42	2,2',3,4'	-	tetrachlorobiphenyl	1.59	1.19	0.15	0.01
43	2,2',3,5	-	tetrachlorobiphenyl	0.28	0.18		
44	2,2',3,5'	-	tetrachlorobiphenyl	4.47	3.55	2.31	0.03
45	2,2',3,6	-	tetrachlorobiphenyl	1.23	0.89	0.05	
46	2,2',3,6'	-	tetrachlorobiphenyl	0.49	0.36		

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47	2,2',4,4'	-	tetrachlorobiphenyl	1.26	0.93	0.14	
48	2,2',4,5	-	tetrachlorobiphenyl	1.61	1.18	0.12	
49	2,2',4,5'	-	tetrachlorobiphenyl	3.35	2.53	1.10	0.01
50	2,2',4,6	-	tetrachlorobiphenyl	0.01	trace		
51	2,2',4,6'	-	tetrachlorobiphenyl	0.32	0.23		
52	2,2',5,5'	-	tetrachlorobiphenyl	4.63	3.53	5.38	0.24
53	2,2',5,6'	-	tetrachlorobiphenyl	0.95	0.71	0.12	
54	2,2',6,6'	-	tetrachlorobiphenyl	0.01	0.01		
55	2,3,3',4	-	tetrachlorobiphenyl		0.10		
56	2,3,3',4'	-	tetrachlorobiphenyl	0.07	1.81	0.55	0.02
57	2,3,3',5	-	tetrachlorobiphenyl	0.01	0.02		
59	2,3,3',6	-	tetrachlorobiphenyl	0.41	0.32	0.02	
60	2,3,4,4'	-	tetrachlorobiphenyl	0.04	1.18	0.18	0.04
63	2,3,4',5	-	tetrachlorobiphenyl	0.06	0.12	0.02	
64	2,3,4',6	-	tetrachlorobiphenyl	1.87	1.70	0.59	0.01
65	2,3,5,6	-	tetrachlorobiphenyl				
66	2,3',4,4'	-	tetrachlorobiphenyl	0.39	3.39	1.01	0.02
67	2,3',4,5	-	tetrachlorobiphenyl	0.06	0.16		
69	2,3',4,6	-	tetrachlorobiphenyl	trace			
70	2,3',4',5	-	tetrachlorobiphenyl	0.59	3.73	3.49	0.04
71	2,3',4',6	-	tetrachlorobiphenyl	1.16	1.03	0.15	0.01
72	2,3',5,5'	-	tetrachlorobiphenyl	trace	0.01		
73	2,3',5',6	-	tetrachlorobiphenyl	trace	trace		
74	2,4,4',5	-	tetrachlorobiphenyl	0.33	1.81	0.84	0.05
75	2,4,4',6	-	tetrachlorobiphenyl	0.06	0.04		
76	2,3',4',5'	-	tetrachlorobiphenyl		0.08	0.02	
77	3,3',4,4'	-	tetrachlorobiphenyl		0.31	0.03	
81	3,4,4',5	-	tetrachlorobiphenyl		0.01		
82	2,2',3,3',4	-	pentachlorobiphenyl		0.26	1.11	
83	2,2',3,3',5	-	pentachlorobiphenyl		0.11	0.48	0.01
84	2,2',3,3',6	-	pentachlorobiphenyl	0.05	0.41	2.32	0.11
85	2,2',3,4,4'	-	pentachlorobiphenyl		0.31	1.28	0.01
86	2,2',3,4,5	-	pentachlorobiphenyl		0.03	0.06	
87	2,2',3,4,5'	-	pentachlorobiphenyl		0.46	3.99	0.41
88	2,2',3,4,6	-	pentachlorobiphenyl		trace		
89	2,2',3,4,6'	-	pentachlorobiphenyl		0.09	0.09	
91	2,2',3,4',6	-	pentachlorobiphenyl	0.06	0.21	0.93	0.01
92	2,2',3,5,5'	-	pentachlorobiphenyl		0.09	1.29	0.30
93	2,2',3,5,6	-	pentachlorobiphenyl		trace		
94	2,2',3,5,6'	-	pentachlorobiphenyl		0.01	0.02	
95	2,2',3,5',6	-	pentachlorobiphenyl	0.31	0.61	6.25	2.45
96	2,2',3,6,6'	-	pentachlorobiphenyl	0.04	0.03	0.04	
97	2,2',3,4',5'	-	pentachlorobiphenyl		0.38	2.62	0.09

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99	2,2',4,4',5	-	pentachlorobiphenyl	0.01	0.46	3.02	0.04
101	2,2',4,5,5'	-	pentachlorobiphenyl	0.04	0.69	8.02	3.13
102	2,2',4,5,6'	-	pentachlorobiphenyl	0.04	0.07	0.15	
103	2,2',4,5',6	-	pentachlorobiphenyl			0.03	
105	2,3,3',4,4'	-	pentachlorobiphenyl	trace	0.47	2.99	0.22
107	2,3,3',4',5	-	pentachlorobiphenyl		0.06	0.37	0.01
110	2,3,3',4',6	-	pentachlorobiphenyl		0.83	9.29	1.33
114	2,3,4,4',5	-	pentachlorobiphenyl		0.04	0.18	
115	2,3,4,4',6	-	pentachlorobiphenyl		0.04	0.20	
117	2,3,4',5,6	-	pentachlorobiphenyl		0.03	0.23	
118	2,3',4,4',5	-	pentachlorobiphenyl		0.66	7.35	0.48
119	2,3',4,4',6	-	pentachlorobiphenyl			0.08	
122	2,3,3',4',5'	-	pentachlorobiphenyl		0.01	0.10	
123	2,3',4,4',5'	-	pentachlorobiphenyl		0.03	0.15	
124	2,3',4',5,5'	-	pentachlorobiphenyl		0.03	0.29	0.01
125	2,3',4',5',6	-	pentachlorobiphenyl		0.02	0.02	
126	3,3',4,4',5	-	pentachlorobiphenyl			trace	
128	2,2',3,3',4,4'	-	hexachlorobiphenyl		0.02	1.42	0.53
129	2,2',3,3',4,5	-	hexachlorobiphenyl			0.38	0.14
130	2,2',3,3',4,5'	-	hexachlorobiphenyl			0.60	0.22
131	2,2',3,3',4,6	-	hexachlorobiphenyl			0.19	0.07
132	2,2',3,3',4,6'	-	hexachlorobiphenyl		0.04	2.29	2.90
133	2,2',3,3',5,5'	-	hexachlorobiphenyl			0.11	0.07
134	2,2',3,3',5,6	-	hexachlorobiphenyl			0.37	0.34
135	2,2',3,3',5,6'	-	hexachlorobiphenyl			0.61	1.08
136	2,2',3,3',6,6'	-	hexachlorobiphenyl			0.70	1.46
137	2,2',3,4,4',5	-	hexachlorobiphenyl			0.42	0.02
138	2,2',3,4,4',5'	-	hexachlorobiphenyl		0.10	5.80	6.54
139	2,2',3,4,4',6	-	hexachlorobiphenyl			0.15	
141	2,2',3,4,5,5'	-	hexachlorobiphenyl		0.01	0.98	2.62
144	2,2',3,4,5',6	-	hexachlorobiphenyl			0.24	0.61
145	2,2',3,4,6,6'	-	hexachlorobiphenyl				
146	2,2',3,4',5,5'	-	hexachlorobiphenyl			0.67	1.15
147	2,2',3,4',5,6	-	hexachlorobiphenyl			0.10	
148	2,2',3,4',5,6'	-	hexachlorobiphenyl				
149	2,2',3,4',5',6	-	hexachlorobiphenyl		0.06	3.65	8.75
150	2,2',3,4',6,6'	-	hexachlorobiphenyl				
151	2,2',3,5,5',6	-	hexachlorobiphenyl			0.69	3.04
152	2,2',3,5,6,6'	-	hexachlorobiphenyl				
153	2,2',4,4',5,5'	-	hexachlorobiphenyl		0.06	3.77	9.39
154	2,2',4,4',5,6'	-	hexachlorobiphenyl			0.04	
156	2,3,3',4,4',5	-	hexachlorobiphenyl		0.01	0.82	0.52
157	2,3,3',4,4',5'	-	hexachlorobiphenyl			0.19	0.02

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158	2,3,3',4,4',6	-	hexachlorobiphenyl	0.01	0.81	0.58
163	2,3,3',4',5,6	-	hexachlorobiphenyl	0.01	1.03	2.42
164	2,3,3',4',5',6	-	hexachlorobiphenyl		0.40	0.69
166	2,3,4,4',5,6	-	hexachlorobiphenyl		0.05	
167	2,3',4,4',5,5'	-	hexachlorobiphenyl		0.27	0.19
170	2,2',3,3',4,4',5	-	heptachlorobiphenyl		0.52	4.11
171	2,2',3,3',4,4',6	-	heptachlorobiphenyl		0.14	1.11
172	2,2',3,3',4,5,5'	-	heptachlorobiphenyl		0.07	0.70
173	2,2',3,3',4,5,6	-	heptachlorobiphenyl			0.10
174	2,2',3,3',4,5,6'	-	heptachlorobiphenyl		0.34	4.96
175	2,2',3,3',4,5',6	-	heptachlorobiphenyl			0.17
176	2,2',3,3',4,6,6'	-	heptachlorobiphenyl		0.04	0.59
177	2,2',3,3',4,5',6'	-	heptachlorobiphenyl		0.20	2.57
178	2,2',3,3',5,5',6	-	heptachlorobiphenyl		0.03	0.83
179	2,2',3,3',5,6,6'	-	heptachlorobiphenyl		0.10	2.03
180	2,2',3,4,4',5,5'	-	heptachlorobiphenyl		0.67	11.38
181	2,2',3,4,4',5,6	-	heptachlorobiphenyl			0.01
183	2,2',3,4,4',5',6	-	heptachlorobiphenyl		0.18	2.41
185	2,2',3,4,5,5',6	-	heptachlorobiphenyl			0.55
187	2,2',3,4',5,5',6	-	heptachlorobiphenyl		0.25	5.40
189	2,3,3',4,4',5,5'	-	heptachlorobiphenyl		0.01	0.10
190	2,3,3',4,4',5,6	-	heptachlorobiphenyl		0.07	0.82
191	2,3,3',4,4',5',6	-	heptachlorobiphenyl			0.17
193	2,3,3',4',5,5',6	-	heptachlorobiphenyl		0.03	0.53
194	2,2',3,3',4,4',5,5'	-	octachlorobiphenyl		0.01	2.07
195	2,2',3,3',4,4',5,6	-	octachlorobiphenyl			0.84
196	2,2',3,3',4,4',5,6'	-	octachlorobiphenyl			1.09
197	2,2',3,3',4,4',6,6'	-	octachlorobiphenyl			0.07
198	2,2',3,3',4,5,5',6	-	octachlorobiphenyl			0.10
199	2,2',3,3',4,5,5',6'	-	octachlorobiphenyl		0.01	1.78
200	2,2',3,3',4,5,6,6'	-	octachlorobiphenyl			0.25
201	2,2',3,3',4,5',6,6'	-	octachlorobiphenyl			0.24
202	2,2',3,3',5,5',6,6'	-	octachlorobiphenyl			0.33
203	2,2',3,4,4',5,5',6	-	octachlorobiphenyl		0.02	1.40
205	2,3,3',4,4',5,5',6	-	octachlorobiphenyl			0.10
206	2,2',3,3',4,4',5,5',	-	nonachlorobiphenyl		0.03	0.53
207	2,2',3,3',4,4',5,6,6	-	nonachlorobiphenyl			0.05
208	2,2',3,3',4,5,5',6,6	-	nonachlorobiphenyl		0.01	0.13

Data condensed from: Frame, G. M., Cochran, J. W., and Boewadt, S.S., *J. High Res. Chromatogr.*, Vol. 19, pp 657-668 (1996); *No Detect* congeners omitted from table

†IUPAC#s 107, 108, 109, 199, 200, & 201 correspond to BZ #s 108, 109, 107, 201, 199 & 200, respectively.

*Aroclor 1254 composition is "normal", not late lot.

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APPENDIX C

Supplemental Data Tables

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Supplemental Table 1

Class Comparison Results for 1W and 12W

	p-value	FC 5ppm	FC 18ppm	Probe set	Gene symbol
1W	2.78E-04	2.64	12.01	K03241cds_s_at	Cyp1a2
	3.54E-05	1.75	7.23	L00320cds_f_at	Cyp2b2
	1.15E-04	2.05	6.89	K00996mRNA_s_at	" "
	5.96E-04	1.77	5.77	J00728cds_f_at	" "
	2.07E-03	2.23	6.62	K01721mRNA_s_at	" "
	2.14E-03	1.70	5.08	M13234cds_f_at	" "
	4.38E-03	0.67	0.55	M58308_at	Hal
	2.25E-03	1.36	1.74	X96437mRNA_g_at	Ier3
	4.46E-03	1.55	1.62	D21799_g_at	Psmb2
	3.66E-03	1.47	1.96	K00136mRNA_at	LOC494499
	1.58E-03	1.49	1.83	M14776_f_at	Cyp2c37 /// Cyp2c6 /// LOC293989
12W	4.04E-04	1.24	2.14	AF072411_at	Cd36/// RGD1562323 /// RGD1565355
	4.87E-04	1.22	2.09	AF072411_g_at	" "
	7.60E-06	1.70	4.85	K00996mRNA_s_at	Cyp2b2
	1.70E-05	1.82	4.41	K01721mRNA_s_at	" "
	1.11E-03	2.01	4.37	M13234cds_f_at	" "
	1.45E-03	0.87	1.31	rc_AA892805_g_at	
	5.26E-05	0.89	1.67	D13912_s_at	Cyp3a3 /// Cyp3a1
	1.19E-04	1.90	5.63	M11251cds_f_at	" "
	2.33E-04	2.32	5.09	E01184cds_s_at	Cyp1a2
	3.45E-04	1.71	4.54	J00728cds_f_at	" "
	5.09E-04	0.76	1.41	rc_AA893307_at	Ncbp2_predicted

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6.68E-04	0.70	1.63	rc_AA799650_g_at	Prdx3
9.56E-04	1.17	1.87	U23055cds_s_at	Ceacam1 ///Ceacam10
1.03E-03	0.97	1.56	D28560_g_at	Enpp2
3.07E-04	0.66	0.97	J03583_at	Cltc
1.86E-03	0.81	1.14	rc_AA800169_at	Tmem69
1.89E-03	0.69	1.10	rc_AA892422_at	mrpl11
1.82E-03	0.79	1.30	rc_AA892863_at	Mtch2_predicted
1.67E-03	0.62	1.10	rc_AA892642_at	
1.78E-03	0.70	1.24	rc_AA892547_at	RGD:1303272
9.44E-04	0.75	1.65	rc_H33149_at	RGD1311161
3.55E-04	0.70	1.19	rc_AA891221_at	LOC293103
5.54E-04	0.73	1.39	rc_AA894282_at	

Supplemental Table 2

A.

GO category	LS	1W GO_DESCRIPTION	# genes in GO category	Pie Chart Group
4497	1.00E-05	monooxygenase activity	83	D
42221	1.00E-05	response to chemical substance	65	D
4364	1.00E-05	glutathione transferase activity	22	R
5839	9.00E-05	proteasome core complex (sensu Eukaryota)	28	PC
15020	9.00E-05	glucuronosyltransferase activity	22	U
6805	1.00E-04	xenobiotic metabolism	19	D
6511	1.50E-04	ubiquitin-dependent protein catabolism	36	PC
16765	3.10E-04	transferase activity\, transferring alkyl or aryl (other than methyl) groups	41	T
16758	3.30E-04	transferase activity\, transferring hexosyl groups	63	T
51186	3.70E-04	cofactor metabolism	53	C
42493	3.90E-04	response to drug	18	D
16769	8.70E-04	transferase activity\, transferring nitrogenous groups	23	T
46912	1.16E-03	transferase activity\, transferring acyl groups\, acyl groups converted into alkyl on transfer	7	T

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16651	1.31E-03 oxidoreductase activity\, acting on NADH or NADPH	19	R
6084	1.47E-03 acetyl-CoA metabolism	10	G
8483	1.49E-03 transaminase activity	22	AA
	oxidoreductase activity\, acting on paired donors\, with incorporation or reduction of molecular oxygen\, reduced flavin or flavoprotein as one donor\, and		
16712	1.76E-03 incorporation of one atom of oxygen	19	D
6099	2.74E-03 tricarboxylic acid cycle	12	C
8194	2.79E-03 UDP-glycosyltransferase activity	47	U
16746	2.80E-03 transferase activity\, transferring acyl groups	56	T
6790	2.94E-03 sulfur metabolism	23	R
6520	3.57E-03 amino acid metabolism	64	AA
6749	3.84E-03 glutathione metabolism	12	R

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B.

GO category	LS	12W GO_DESCRIPTION	# genes in GO category	Pie Chart Group
4497	1.00E-05	monooxygenase activity	83	D
42221	1.00E-05	response to chemical substance	65	D
42493	1.00E-05	response to drug	18	D
5504	1.00E-05	fatty acid binding	14	F
6631	1.00E-05	fatty acid metabolism	85	F
6869	1.00E-05	lipid transport	39	F
15909	1.00E-05	long-chain fatty acid transport	13	F
5525	1.00E-05	GTP binding	99	GTP
4470	1.00E-05	malic enzyme activity	6	M
4364	1.00E-05	glutathione transferase activity	22	R
6749	1.00E-05	glutathione metabolism	12	R
16765	1.00E-05	transferase activity\, transferring alkyl or aryl (other than methyl) groups	41	T
8289	9.00E-05	lipid binding	92	F
6100	9.00E-05	tricarboxylic acid cycle intermediate metabolism	6	TCA
5839	1.00E-04	proteasome core complex (sensu Eukaryota)	28	PC
16615	1.00E-04	malate dehydrogenase activity	7	TCA
15020	1.60E-04	glucuronosyltransferase activity	22	U
16712	1.80E-04	oxidoreductase activity\, acting on paired donors\, with incorporation or reduction of molecular oxygen\, reduced flavin or flavoprotein as one donor\, and incorporation of one atom of oxygen	19	D
6790	4.30E-04	sulfur metabolism	23	R
51186	8.90E-04	cofactor metabolism	53	C
6446	1.26E-03	regulation of translational initiation	5	t
6511	1.58E-03	ubiquitin-dependent protein catabolism	36	PC
8194	1.78E-03	UDP-glycosyltransferase activity	47	U
6805	2.00E-03	xenobiotic metabolism	19	D
16758	2.44E-03	transferase activity\, transferring hexosyl groups	63	T
3743	2.46E-03	translation initiation factor activity	12	t
8135	2.84E-03	translation factor activity\, nucleic acid binding	22	t
7264	3.67E-03	small GTPase mediated signal transduction	86	GTP

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Key for Pie Chart Group Codes

Group Code	Description of Pie Chart Group
AA	Amino acid cycling & metabolism
C	Cofactor metabolism
D	Hepatic response to drugs/xenobiotic enzyme inducers
F	Fatty Acid metabolism, binding, and transport
G	Tricarboxylic acid cycle
GTP	GTP binding & small GTPase-mediated signal transduction
PC	Protein Catabolism
R	Redox & Sulfur metabolism
t	Initiation of translation
T	Other transferase activity
TCA	TCA intermediate metabolism
U	UDP-glycosyl transferase activity

Supplemental Table 3

p-value	FC	Probe set	Description	Gene symbol
5E-06	2.93	M26594_at	malic enzyme 1	Me1
4E-05	2.43	rc_AI008020_at	malic enzyme 1	Me1
3E-04	2.48	rc_AI171506_g_at	malic enzyme 1	Me1
3E-03	2.82	rc_AI171506_at	malic enzyme 1	Me1
8E-05	2.75	rc_AA799326_s_at	cd36 antigen	Cd36
3E-03	1.43	AB005743_at	cd36 antigen	Cd36
1E-03	2.56	rc_AA925752_at	cd36 antigen /// similar to fatty acid translocase/CD36	Cd36 /// LOC499984
1E-04	2.14	AF072411_at	cd36 antigen /// similar to fatty acid translocase/ CD36 /// similar to fatty acid	Cd36 /// LOC499984 /// LOC499985
1E-04	2.09	AF072411_g_at	translocase/CD36	Cd36 /// LOC499984 /// LOC499985

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2E-05	2.95	S56936_s_at	UDP glycosyltransferase 1 family, polypeptide A6	Ugt1a6
2E-03	1.96	D38061exon_s_at	" "	Ugt1a6
3E-03	1.71	D38062exon_s_at	UDP glycosyltransferase 1 family, polypeptide A7	Ugt1a7
2E-03	1.76	S56937_s_at		Ugt1a1 /// Ugt1a6 /// Ugt1a7 /// Ugt1a8 ///Ugt1a2///Ugt1a4// / Ugt1a11 /// Ugt1a5
1E-05	3.98	rc_AA945082_at	glutathione S-transferase Yc2 subunit	Yc2
1E-05	5.62	S82820mRNA_s_at	glutathione S-transferase Yc2 subunit	Yc2
3E-05	5.98	S72506_s_at	glutathione S-transferase Yc2 subunit	Yc2
1E-03	1.54	S35751_f_at	3-α-hydroxysteroid dehydrogenase	RGD:708361
3E-03	1.40	D17310_s_at	3-α-hydroxysteroid dehydrogenase	RGD:708361
6E-04	1.87	U23055cds_s_at	CEA-related cell adhesion molecule 1 /// CEA-related cell adhesion molecule 10	Ceacam1 /// Ceacam10
2E-03	1.40	X86178mRNA_g_at	alpha-2-glycoprotein 1, zinc	Azgp1
3E-03	1.51	X86178mRNA_at	alpha-2-glycoprotein 1, Zn	Azgp1
CYP 450s				
2E-03	1.88	rc_AI169735_g_at	cytochrome P450IIB3	RGD:628627
4E-03	1.39	M14776_f_at	cytochrome P450, 2c37 /// Cytochrome P450, subfamily IIC6 /// cy P450- like	RGD:620377 /// RGD:619934 /// LOC293989

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4E-04	1.65	X64401cds_s_at	cytochrome P450, subfamily 3A, polypeptide 3 /// cytochrome P450, family 3, subfamily a, polypeptide 1	Cyp3a3 /// Cyp3a1
1E-04	1.67	D13912_s_at	" "	Cyp3a3 /// Cyp3a1
3E-03	1.52	L24207_i_at	" "	" "
4E-03	1.42	M13646_s_at	cytochrome P450, family 3, subfamily a, polypeptide 11	Cyp3a11
6E-04	2.08	X62086mRNA_s_at	cytochrome P450, subfamily 3A, polypeptide 3 /// cytochrome P450, family 3, subfamily a, polypeptide 11 /// cytochrome P450, family 3, subfamily	Cyp3a3 /// Cyp3a11 /// Cyp3a1

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APPENDIX D

PCB Structures

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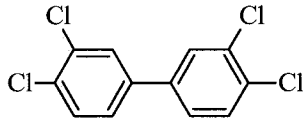
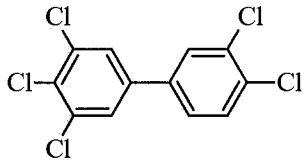
COPLANAR COMPOUNDS

PCB 77



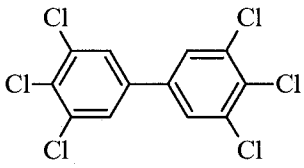
3,3',4,4'-tetrachlorobiphenyl

PCB 126



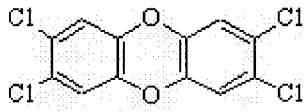
3,3',4,4',5-pentachlorobiphenyl

PCB 169



3,3',4,4',5,5'-hexachlorobiphenyl

TCDD



2,3,7,8-tetrachlorooxanthrene

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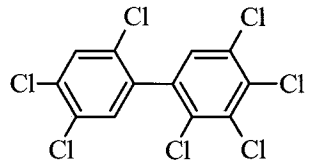
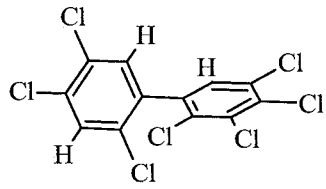
MOST PREVALENT PCBs IN HUMAN AND ANIMAL TISSUES

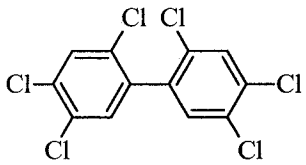
FLAT VIEW



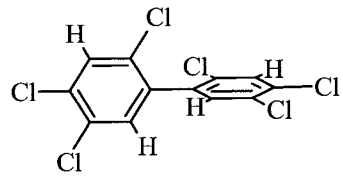
2,2',3,4,4',5,5'-heptachlorobiphenyl

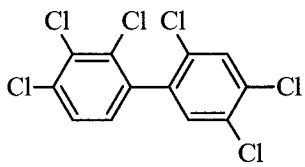
3D ORIENTATION



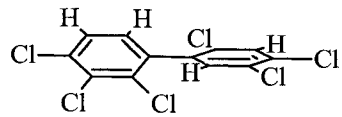


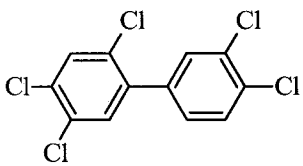
2,2',4,4',5,5'-hexachlorobiphenyl



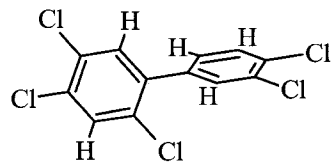


2,2',3,4,4',5'-hexachlorobiphenyl





2,3',4,4',5-pentachlorobiphenyl



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