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CHARACTERIZATION AND APPLICATION FOR A
SEMI-AUTOMATED SEPARATION AND ANALYSIS TECHNIQUE
FOR POLYCHLORINATED BIPHENYLS IN
GREAT LAKES WILDLIFE

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Lisa Lynn Williams

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CHARACTERIZATION AND APPLICATION OF A SEMI-AUTOMATED SEPARATION AND ANALYSIS TECHNIQUE FOR POLYCHLORINATED BIPHENYLS IN GREAT LAKES WILDLIFE

By

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ABSTRACT

CHARACTERIZATION AND APPLICATION OF A SEMI-AUTOMATED SEPARATION AND ANALYSIS TECHNIQUE FOR POLYCHLORINATED BIPHENYLS IN GREAT LAKES WILDLIFE

By

Lisa Lynn Williams

Polychlorinated hydrocarbons (PCHs) are toxic, persistent compounds in the environment. Classes of PCHs of special concern in the environment include polychlorinated dibenzo-*p*-dioxins, dibenzofurans, and biphenyls (PCBs). Quantitative structure-activity relationships have been developed for compounds within these classes which are stereochemically most similar to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and which act through the same cytosolic *Ah* receptor. The non-*ortho* substituted PCB congeners comprise most of the *Ah* receptor-mediated PCH toxicity in aquatic systems.

The proportion of the most toxic congeners to the total concentration of PCBs is suspected to vary in the environment, but these congeners are difficult to quantify. The non-*ortho* substituted congeners cannot be resolved from interfering congeners using single-column, high resolution gas chromatography. Alternative methods developed thus far have generally been expensive, labor-intensive, or both.

The studies described here focus on the development and use of a semi-automated separation of the non-*ortho* substituted congeners from interferences using porous graphitic carbon (PGC). The PGC method developed is faster and uses less solvent than previous separations based on carbon columns. This method is both precise and accurate, with no discernable interferences. Results obtained from this method can be used with TCDD equivalency factors (TEFs) to calculate TCDD-equivalents (TEQs) derived from PCBs.

The PGC method was used successfully to measure PCB congeners in eggs of fish-eating birds. Variability in TEQ and PCB concentrations among eggs within a double-crested cormorant colony was investigated. No significant differences in concentrations of PCBs or TEQs were observed among eggs from nests with different reproductive outcomes; therefore, concentrations of TEQs and PCBs could not be related to reproductive success of nests within a single colony. TEQs determined in an H4IIE bioassay for EROD activity were significantly greater than TEQs calculated from concentrations of PCB congeners. In another study, concentrations of TEQs and PCBs in red-breasted merganser eggs collected 13 years apart were compared. Concentrations of TEQs and PCBs declined significantly between 1977 and 1990, but the ratio of TEQ to total PCB did not change.

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I am also indebted to the other members of the Aquatic Toxicology Lab team, old and new, and the friendly, helpful folks in the Pesticide Research Center office. I must especially thank Dave Verbrugge. We've worked together so well for the many years we've been torturing GCs and little blue raquetballs together. I'm going to miss you, Dave.

One very special person has been my patient, supportive, understanding anchor as I've completed my doctoral program. Thanks for keeping an eye on my sanity level, S. Joy!

I could not have become the person that I am now without the foundation provided by my warm, loving, supportive parents. Their love has been a constant in the ups and downs of these last several years. Thanks, Mom and Dad.

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GENERAL INTRODUCTION

Increasing awareness of the persistence and potential toxicity of synthetic organic chemicals in the last 25 years has led to reduced production and use of many of these compounds. Organochlorine pesticides, for example, have been largely replaced with less persistent organophosphorus and carbamate pesticides in North America. Polychlorinated biphenyls (PCBs), formerly used in capacitors, transformers, hydraulics, carbonless copy paper and other applications in which their stability was an asset, have also been replaced. Neither these compounds nor the problems resulting from them have disappeared, however.

Polychlorinated biphenyls, in particular, continued to be measured in the environment at concentrations which impact fish and wildlife. PCB concentrations have decreased in many areas since they were banned in the 1970s, yet concentrations in tissues of fish-eating organisms in the Great Lakes are still generally greater than those which elicit adverse effects in the laboratory. Predicting toxicity of PCB mixtures in the field based on laboratory exposures is complicated by the nature of the PCB mixtures. PCBs were originally sold as several different technical mixtures, each of which contained approximately 100 different congeners.

Animals in the laboratory are usually exposed to these technical formulations, yet animals in the environment are exposed to PCB mixtures from a variety of sources and which may have been altered by degradation, partitioning, and metabolism.

Alterations in the pattern of PCB congeners within the mixture of PCBs may have toxicological consequences. PCB congeners elicit many of their toxicological effects through the same receptor-mediated mode of action as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). PCB congeners stereochemically most similar to TCDD are those which can assume a planar structure. These planar congeners elicit adverse effects at concentrations many orders of magnitude less than other congeners. Some of the questions being asked by ecotoxicologists today are the following: What are the concentrations of the planar congeners in biota? Are the patterns of PCB congeners changing over time in the environment? Do the decreased concentrations observed in biota over the last two decades represent a real decrease in toxicity?

Studies presented in this dissertation are designed to explore the extent and significance of changing patterns of PCBs in the environment. These questions have been difficult to answer in part because of the analytical difficulties inherent in measuring the concentrations of the most potent PCB congeners in complex mixtures. In the first chapter, an automated separation is described which allows concentrations of these congeners to be determined more efficiently than they have been previously. In the second chapter, variability in the ratio of the most potent congeners to the total concentration of PCBs is determined among groups of double-crested cormorants with different reproductive outcomes within the same colony. TCDD-equivalents calculated from concentrations of PCB congeners are compared with TCDD-equivalents determined in an H4IIE cell bioassay for ethoxyresorufin-*O*-deethylase (EROD). In the third chapter, the possibility of selective enrichment of the most potent congeners relative to the total concentration of PCBs in one area over a 13 year time span is examined.

CHAPTER 1

Characterization of a Semi-Automated Separation Method for Analysis of Non-*Ortho* Substituted Polychlorinated Biphenyl (PCB) Congeners in Environmental Samples

INTRODUCTION

Analysis of non-*ortho* substituted polychlorinated biphenyl (PCB) congeners is difficult because other PCB congeners co-elute with them in chromatographic methods like high resolution gas chromatography (HRGC). Although many HRGC columns have been tried, no single column is capable of adequately separating all of the environmentally relevant non-*ortho* substituted congeners from their PCB interferences (Larsen *et al.* 1993; Bowadt and Larsen 1992). Part of the problem is that the non-*ortho* substituted congeners may be present in environmental samples at concentrations 100 fold less than those of the interfering compounds. Two congeners may be mostly resolved when injected at equimolar concentrations, yet the quantitation of the congener at a significantly lesser concentration becomes extremely variable when the same two congeners are injected in the widely disparate concentrations in which they occur in environmental samples.

Several analytical approaches have been attempted to overcome these difficulties. Isotope dilution methods with HRGC/high resolution mass spectrometry have been used successfully to quantify the non-*ortho* substituted PCB congeners (Kuehl *et al.* 1991). Negative chemical ionization mass spectrometry has also been used with HRGC (Schmidt and

Hesselberg 1992), but compensation must be made for minor fragments of PCB 110 (2,3,3′, 4′6-pentachlorobiphenyl) which interfere with major ions of PCB 77 (3,3′,4,4′-tetrachlorobiphenyl). The PCB congener numbering system used here is adapted from Ballschmitter and Zell (1980). Mass spectrometry methods require significant investments in equipment and personnel that are not available in many laboratories, so other methods continue to be explored. Dual column HRGC with both heart-cutting (Duinker *et al*. 1988; Schulz *et al*. 1989; Kannan *et al*. 1991) and serial column (Zell and Ballschmiter 1980; Larsen *et al*. 1993) configurations have been used, but the methods most often used successfully with environmental samples use some sort of carbon column separation step prior to HRGC analysis (Passivirta *et al*. 1986; Tanabe *et al*. 1987a, b; Pruell *et al*. 1988; Hong and Bush 1990; Smith *et al*. 1990; Athanasiadou *et al*. 1991; Sericano *et al*. 1991; Wilson-Yang *et al*. 1991; Williams and Giesy 1992; Williams *et al*. 1992). The disadvantages of these methods are that they are labor-intensive, use large volumes of solvents, and use carcinogenic solvents like benzene or toluene. Furthermore, most carbon columns have been "home-made" from a variety of carbon types so technology transfer among laboratories is difficult.

The goal of this work was to develop an automated method using a porous graphitic carbon (PGC) column which has recently become commercially available. Early work with this PGC column showed that it could separate PCB congeners based on planarity in standards fortified with the non-*ortho* substituted congeners (Creaser and Al-Haddad 1989). Advantages of this column over activated carbon columns include its structural strength and small, controlled particle size--both of which allow the PGC to be used as an HPLC packing. Objectives of this research were to incorporate this column in an automated system, to determine its ability to separate mixtures of PCBs similar to those in environmental samples, and to determine elution characteristics of other environmental contaminants.

EXPERIMENTAL SECTION

Design of the Automated System

The PGC column was incorporated into a high performance liquid chromatography (HPLC) system equipped with an autosampler, fraction collector and switching valve to allow back-flushing of the column. The PGC column itself was a Hypercarb PCB column, 50 mm x 4.7 mm with 7 μm particle size (Shandon Scientific, Ltd., U.K., supplied by Keystone Scientific, Inc., Bellefonte, PA). The HPLC pump was a Perkin Elmer Series 410 with a quaternary solvent delivery system with solvent mixing on the low pressure side of a single pump. The flow range was 0.01 to 10.0 ml/min with a maximum pressure of 6100 psi. Surfaces in contact with solvents were stainless steel, glass, sapphire or fluorocarbon polymer. The Perkin Elmer ISS 100 autosampler was equipped with a Rheodyne model 7125 syringe loading sample injector. The injector was modified with a 2000 μL sample loop. Direction of flow through the column was controlled with an air-actuated 6-port, 2-position valve (Valco, Model A6UW) with a Digital Valve Interface. The Isco (Lincoln, NE) Foxy 200 fraction collector was a programmable, microprocessor controlled X-Y fraction collector equipped with a flow diverter valve. All components were synchronized with injection signals and timed events from the autosampler. More detailed descriptions of the components and connections can be found in Appendix A.

Standards and Reagents

All solvents were high purity solvents obtained from Burdick and Jackson (Muskegon, MI). PCB congener standards were obtained as crystalline solids (>95% purity) from either Ultra Scientific (North Kingstown, RI) or AccuStandard (New Haven, CT). Aroclor® standards (Aroclors® 1242, 1248, 1254 and 1260) were prepared from pure stocks which were originally from Monsanto Chemical Company, but were a gift to our laboratory from M. Zabik (Pesticide Research Center, MSU). Polychlorinated naphthalene (PCN, technical grade) and pesticide standards were obtained from EPA repository certified stocks. Authentic standards for individual polychlorinated diphenyl ethers (PCDEs) and PCNs were obtained as crystalline solids from Ultra Scientific. The 2,3,7,8-tetrachlorodibenzo-*p*-dioxin stock solution was calibrated with a certified standard from Restek Corporation (Bellefonte, PA). ^{13}C-labelled, 2,3,7,8-substituted PCDD and PCDF solutions were purchased from Chemsyn Science Laboratories (Lenexa, KS) through Wellington Laboratories (Guelph, ONT). All stock solutions were prepared in iso-octane and stored in the dark.

Elution Profiles of PCB Congeners: Initial Experiments

Elution profiles of selected PCB congeners were first investigated using elution parameters previously described for the Hypercarb PCB column by Creaser and Al-Haddad (1989). According to their protocol, all PCB congeners should have eluted in 100 mL of hexane at a flow rate of 5 mL/min. For these studies, a uv detector ($\lambda = 254$) was connected to the system between the column and the fraction collector. Hexane at a flow rate of 5 mL/min was used as the mobile phase. PCB congeners were injected in iso-octane at either 1 μg or 2 μg total loading (10 ng/μL solutions). A 1:1:1:1 mixture of Aroclors® 1242, 1248, 1254 and 1260 was injected at 5 μg loading (50 ng/μL solution).

Flow rates for the separation of mono-*ortho* substituted congeners from the bulk of the *ortho* substituted congeners were optimized with PCB 118 (mono-*ortho*) and PCB 149 (tri-*ortho*). Resolution (R_s) was calculated from retention times (t_i) and peak widths at one-half height (w_i) for the two peaks (Eq. 1).

$$R_s = \frac{2(t_2 - t_1)}{w_1 + w_2} \qquad (1)$$

Gradient Elution Tests

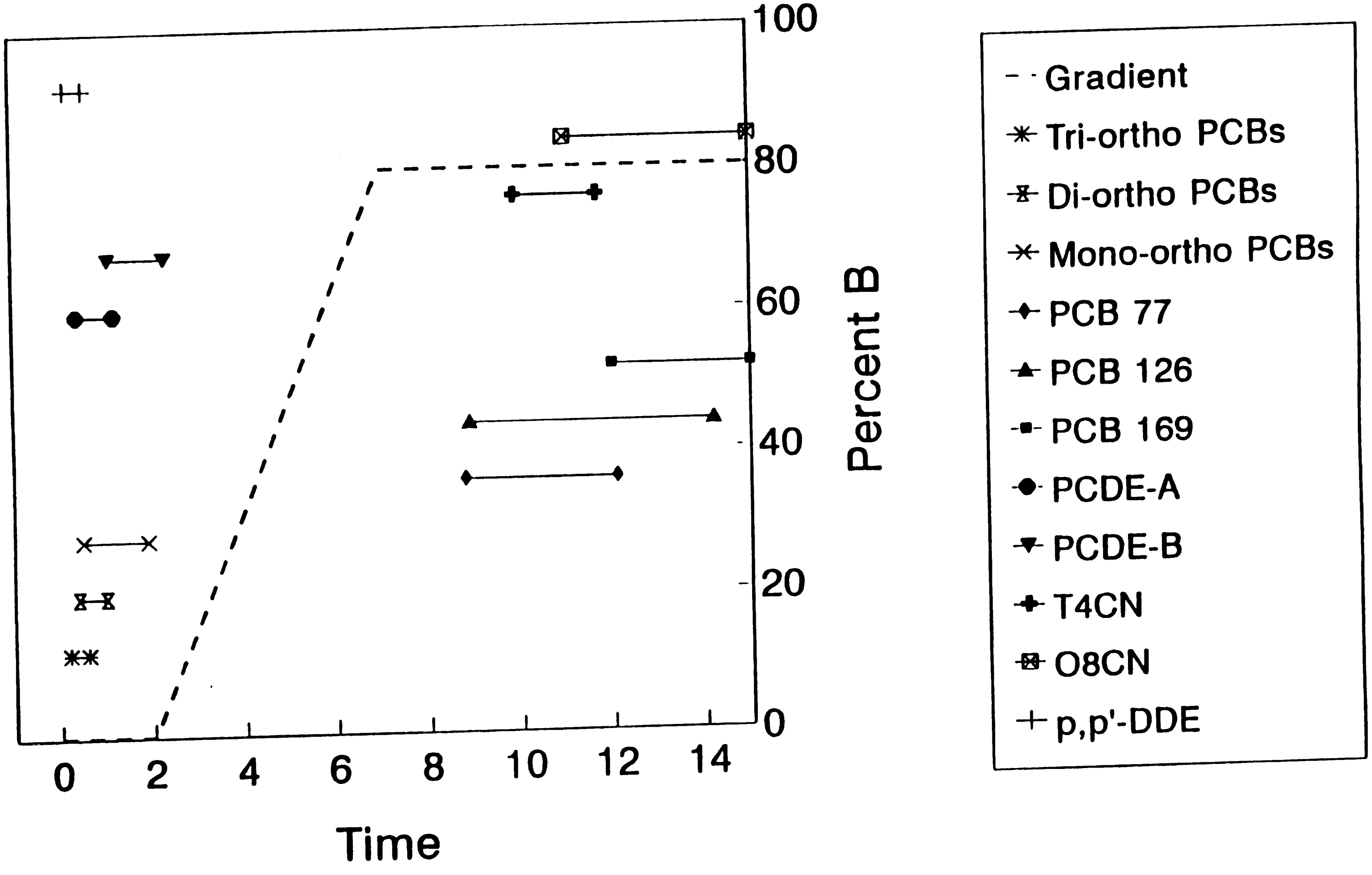
Because hexane alone was too weak a mobile phase to elute non-*ortho* substituted congeners in the forward direction within a reasonable volume, a gradient elution of hexane and dichloromethane (DCM) was used (Figure 1). The elution protocol developed was 100% hexane for 2 min, a linear gradient to 20% hexane/80% DCM in 5 min and then 15 min with 20% hexane/80% DCM. All other parameters were as described in the "Initial Experiments" section above. Standards containing PCB congeners, p,p′-DDE, PCNs, and PCDEs were injected in iso-octane at either 1 μg or 2 μg total loading (10 ng/μL solutions). The PCN isomers used were 1,2,3,4-tetrachloronaphthalene (T_4CN) and octachloronaphthalene (O_8CN). The PCDE isomers included in group PCDE-A were 2,4,4′-T_3CDE, 2,4,4′,5-T_4CDE, 2,3′,4,4′-T_4CDE and 2,2′,4,4′,5-P_5CDE and those in PCDE-B were 3,3′,4,4′-T_4CDE and 3,3′,4,4′,6-P_5CDE. Mixtures of compounds at loadings expected for extracts from environmental samples (3.5 - 35 ng) were also separated with the gradient elution protocol. Fractions from these separations were concentrated and then quantitated using GC-ECD (GC parameters are described in detail in Appendix A).

Recovery of 2,3,7,8-TCDD was determined by collecting fractions and analyzing by GC-ECD since 2,3,7,8-TCDD could not be detected by the uv detector at the concentrations used. 2,3,7,8-TCDD was eluted by reversing the direction of flow at 16 min in the gradient program described above and then

Figure 1. Elution Profiles for PCB Congeners and Other Halogenated Compounds on the Hypercarb PCB Column.

Other

Column.



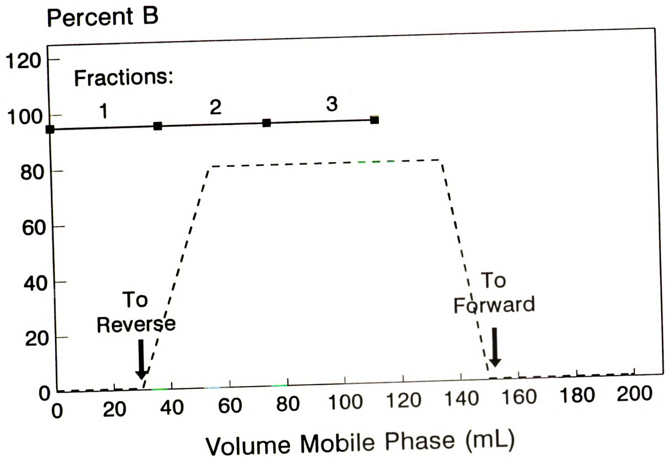
Flow rate = 5 mL/min
Solvent A = hexane
Solvent B = dichloromethane

employing another linear gradient to increase the proportion of DCM in the mobile phase to 100% DCM in 5 min.

Development of Protocol for Environmental Samples

The separation protocol developed for environmental samples employed a reversal in flow direction to elute the non-*ortho* substituted PCB congeners. This protocol is illustrated in Figure 2 and described in detail in Appendix A. Hexane at a flow rate of 5 mL/min for 6 minutes was used to elute the bulk of the PCB congeners. The flow direction was then switched and a linear gradient from 100% hexane to 20% hexane/80% DCM over 5 min followed by a 15 min elution at that final mobile phase composition was used to elute the non-*ortho* substituted PCB congeners. The rinsing volume of 20% hexane/80% DCM was greater than that used in the initial characterization studies. The fraction collector could only be configured for equal length collection windows, and no non-*ortho* substituted congeners elute before 40 mL, so 35 mL fractions were collected in round-bottom flasks. Non-*ortho* substituted PCB congeners were recovered in Fractions 2 and 3 and congeners with two or more *ortho* substituted chlorine atoms were recovered in Fraction 1. Mono-*ortho* substituted congeners were eluted in both fractions 1 and 2. The volume of solvent in the collected fractions was reduced by roto-evaporation. Fractions were transferred to 2 dram vials and evaporated just to dryness under a gentle stream of purified nitrogen. An aliquant of a solution containing an internal

Figure 2. Final Elution Protocol for Separation of PCB Congeners on the Hypercarb PCB Column.



Flow rate = 5 mL/min
Solvent A = hexane
Solvent B = dichloromethane

standard for GC quantitation (PCB 30 at 200 pg/μL) was added such that the concentrations of the non-*ortho* substituted congeners were within the linear range of the ECD detector.

Separation, recovery and loading capacity were determined for this protocol by injecting both standards containing mixtures of individual PCB congeners and standards containing technical Aroclors$^{\bullet}$. Complete separation of the non-*ortho* substituted congeners from those congeners with which they coelute using HRGC was confirmed by injecting a standard containing PCB 110 (interferes with PCB 77) and PCB 129 (interferes with PCB 126) onto the PGC column. An Aroclor$^{\bullet}$ mixture was separated to determine the loading capacity of the column for total PCBs. Apparent concentrations of PCB congeners 77, 81, 126 and 169 in Aroclor$^{\bullet}$ 1254 were determined by quantifying the peaks in the appropriate retention window (±0.002 units of retention time relative to PCB 30) with relative response factors for those congeners. The total mass of Aroclor$^{\bullet}$ 1254 loaded on the column ranged from 0.5 μg to 100μg.

Extraction and Clean Up of Matrix Spikes

The possibility of matrix interferences was investigated by adding both standards containing 20 individual congeners and Aroclor$^{\bullet}$ standards to biological tissues. Chicken eggs and bovine livers purchased at a local grocery store were used as surrogates for the bird eggs and mink livers for which this method was implemented. Tissues were homogenized in large batches and frozen until spiking and analysis. All standards added to tissue homogenates were dissolved in iso-octane at concentrations such that less than 100 μL were required. Congener standards were added to the thawed tissues to produce 10 ng/g concentrations of each congener. An Aroclor® 1:1:1:1 (1242:1248:1254:1260) standard was added to separate aliquants of tissue to produce concentrations of either 2, 5 or 10 μg/g. Some egg homogenates were also spiked with a mixture of ^{13}C-labelled, 2,3,7,8-substituted PCDDs and PCDFs to produce concentrations of 40 pg/g in the tissues. All of these concentrations were based on concentrations observed in eggs of fish-eating birds from the Great Lakes (Ankley *et al.* 1993). Samples of the tissue homogenates were also analyzed as blanks.

Spiked tissues were extracted and "cleaned up" according to methods modified slightly from those reported in Williams *et al.* (1992) and are described in detail in Appendix B. Briefly, spiked tissues were ground with five times their weight of anhydrous sodium sulfate. Lipids and relatively nonpolar xenobiotics were extracted from the resulting free-flowing powder using column elution with DCM. Gel permeation chromatography was used to remove lipids from the extract. PCBs were separated from more polar compounds with adsorbent column chromatography. Deactivated (8%) basic alumina was used in place of acidic silica gel as the top layer of the silica gel column, since lesser recoveries of non-*ortho* substituted congeners compared with other congeners were observed on the original acidic silica gel/silica gel column. The carbonaceous layer produced as residual lipids and other compounds were oxidized by the acidic silica gel was suspected of providing a retention site for non-*ortho* substituted congeners. PCB extracts were exchanged into iso-octane and the volume was adjusted to 1 mL. Recovery of Aroclor® mixes from matrix spikes was greater than 90%.

RESULTS AND DISCUSSION

Results of the initial elution profile experiments indicated that my column retained non-*ortho* substituted congeners more strongly than the one used by Creaser and Al-Haddad (1989). The bulk of the Aroclor® mixture and *ortho* substituted congeners PCB 110 and PCB 129 eluted in the first 6 mL of hexane, but the non-*ortho* substituted congeners PCB 77, 126 and 169 were not observed by uv detection within the first 170 mL of hexane eluted from the column. When the direction of flow through the column was reversed after elution of 7.5 mL, the non-*ortho* substituted congeners were detected within the following 15 mL. Other researchers corroborated this observation (M. Pyell, University of Bordeaux, France, pers. commun.; K. Echols, NFCRC, Columbia, MO, pers. commun.). The column used by Creaser and Al-Haddad (1989) was one of the first produced and apparently had different retention characteristics than those produced later.

Resolution of congeners PCB 118 (mono-*ortho*) and PCB 149 (tri-*ortho*) was constant within flow rates from 2.0 to 5.0

mL/min of 100% hexane in the forward direction (Table 1). PCB 149 eluted before PCB 118, as expected, but elution of PCB 149 overlapped that of another toxicologically significant mono-*ortho* substituted congener, PCB 105, over the entire range of flow rates studied (2-5 mL/min). Therefore, the Hypercarb PCB column could not be used to separate all mono-*ortho* substituted congeners from the bulk of the *ortho* substituted PCBs. Recent work by other researchers indicates that the longer Hypercarb S column (100 mm x 4.7 mm) can resolve these congeners (K. Echols, personal communication). This separation was not a priority of this research since the most significant mono-*ortho* substituted congeners in environmental samples based on concentration and potency (PCB 105 and 118; Williams *et al.* 1992) could be quantitated using HRGC alone.

Non-*ortho* substituted congeners were separated from most expected interferences using gradient elution in the forward direction. *Ortho* substituted congeners eluted within the first 10 mL (2 min), while non-*ortho* substituted congeners eluted in the fraction 45 mL - 75 mL (9-15 min; Figure 1). The pesticide p,p-DDE was not retained by the PGC column. PCDE congeners with *ortho* substituted chlorine atoms (PCDE-A in Figure 1), like their PCB analogs, were eluted within the first 7 mL of hexane . Non-*ortho* substituted PCDE congeners were eluted only slightly later, unlike their PCB analogs which were strongly retained by the PGC column. The oxygen bridge between the two phenyl rings in the PCDEs provides enough of a structural difference from the PCBs that their

Table 1. Resolution (R_s) of PCB Congeners 118 and 149 on Hypercarb PCB Column.

Mass Injected (μg)[1]	Flow rate (mL/min)	R_s
1.0	2.5	2.0
1.0	3.0	1.9
1.0	3.5	1.9
1.0	4.0	1.9
1.0	4.5	2.0
2.0	2.0	1.4
2.0	3.5	1.5
2.0	5.0	1.4

[1] Mass each congener injected. PCB 118 and 149 were injected separately in the 2.0 μg injections. A mixture of the two congeners was used in the 1.0 μg injections.

interactions with the porous graphitic carbon stationary phase is quite distinct. PCN compounds, as expected by their strictly planar structure, were eluted within the same fraction as the non-*ortho* substituted PCB congeners. 2,3,7,8-TCDD did not elute within the first 75 mL (15 min) of this program, but was recovered (102%) in the fraction immediately following the flow reversal and in those from the next 200 mL.

A faster separation of the non-*ortho* substituted PCB congeners was achieved by switching the direction of flow through the column after an initial forward elution of 30 mL hexane (Figure 2). Recoveries of non-*ortho* substituted congeners in fractions collected after the flow reversal were between 79 and 114% (Table 2). At loadings of 35 ng of each congener, PCB 110 and PCB 129 were recovered completely within the first 15 mL of hexane in the forward direction. A non-*ortho* substituted congener not found in Aroclor® mixtures or environmental samples (PCB 127) eluted only after the flow reversal (after at least 45 mL total elution volume) in the same trials. Thus, the non-*ortho* substituted PCB congeners were completely separated from the potentially interfering PCB congeners.

The apparent concentrations of the non-*ortho* substituted congeners in Aroclor 1254 were expected to increase when the column became saturated with PCBs and interfering, *ortho* substituted congeners began to elute in the non-*ortho* substituted PCB fraction (Fraction 2, Figure 2). This "break-through" point was observed between 80 and 100 μg of Aroclor®

Table 2. Recovery of PCB Congeners Injected on Hypercarb PCB Column (35 ng each).

Congener	Percent Recovery	CV (N=3)
77	79	19%
81	86	15%
126	97	9%
169	114	7%

1254 (Figure 3). Greater concentrations observed when 0.5 and 1.0 μg were loaded on the column were the result of carry-over from preceding experiments with greater loadings combined with insufficient rinsing. The rinsing section of the procedure was changed after these trials to that illustrated in Figure 2. As an added precaution, no samples were injected at loading less than 20 μg of total PCB without being preceded by a blank.

The automated system and the final protocol described above provided reproducible separations of non-*ortho* substituted congeners from interfering PCB congeners in extracts from matrix spikes. Better rinsing protocols used with matrix spikes resulted in more consistent recovery of the non-*ortho* substituted congeners, even though overall recovery was less than the recovery from the PGC column alone (Table 3). Concentrations of non-*ortho* substituted congeners in Aroclor® 1254 when 50-80 μg were loaded on the PGC column (Figure 3) were within the range of those reported by other laboratories (Kannan *et al.* 1987; Pruell *et al.* 1988; Larsen *et al.* 1993).

Only nonvolatile, nonpolar, planar compounds with strongly electronegative functional groups could persist through the entire extraction and separation protocol to possibly interfere with non-*ortho* substituted PCB congeners during HRGC-ECD analysis. Most possible interferences, including p,p′-DDE, were removed by this procedure. One class

Table 3. Recovery of PCB Congeners from Chicken Eggs Following Hypercarb PCB Column Separation. Expected Concentrations in Eggs were 10 ng/g.

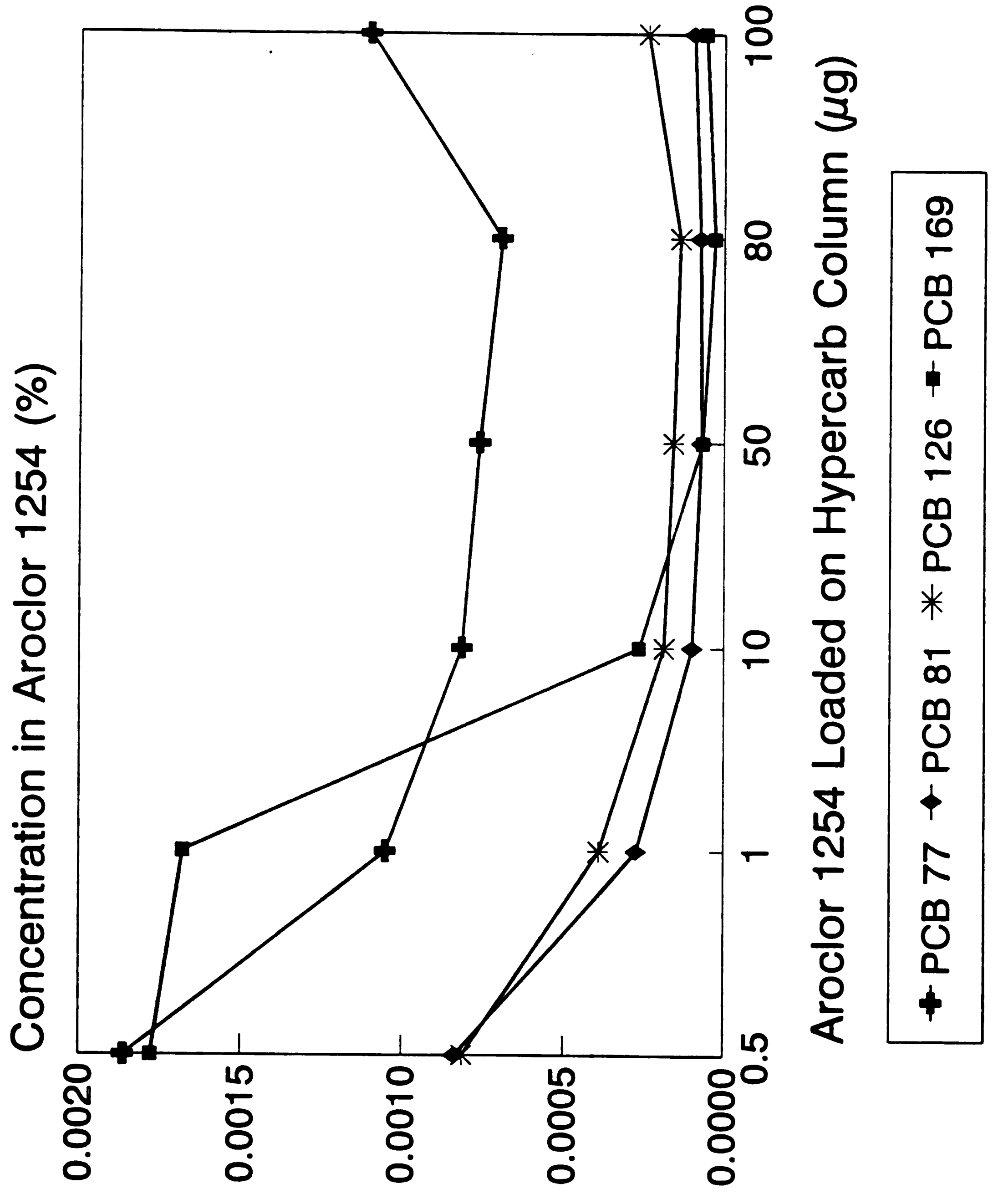
Congener	Percent Recovery	CV (N=5)
77	54	7.1%
81	51	5.2%
126	52	4.9%
169	52	8.7%

Figure 3. Apparent Concentrations of Non-*Ortho* Substituted PCB Congeners in Aroclor 1254 When Different Masses of Aroclor 1254 Are Loaded on the Hypercarb PCB Column.

titute:

Masse:

arb PC



of compounds that can interfere are the PCNs. PCN chromatographic patterns have not been observed in our laboratory in extracts of environmental samples from the Great Lakes region, but have been observed in significant concentrations in carp from Saginaw Bay (D.E. Tillitt, National Fishery Contaminants Research Center, Columbia, MO, pers. commun.). One constituent of Halowax 1014 elutes near PCB 77 on the DB-5 HRGC column and co-elutes with PCB 77 on the DB-1 column. The presence of typical Halowax peaks can be monitored in chromatograms. If they are detected in sample extracts, adsorbent chromatography procedures developed elsewhere can be used to separate the PCNs from the PCBs (Armour and Burke 1971; Beck *et al.* 1990) or a mass selective detector can be used with the HRGC analysis. 2,3,7,8-Substituted PCDD and PCDF isomers were quantitatively recovered in Fractions 2 and 3 of the final protocol (Table 4), but once again, concentrations of these compounds are negligible relative to those of non-*ortho* substituted PCB congeners in environmental samples (Ankley *et al.* 1993). Mono-*ortho* substituted PCB congeners elute partially in Fraction 2, but all of these are well-resolved from non-*ortho* substituted PCB congeners during HRGC analysis.

The sensitivity of the extraction and separation method depends on both the sensitivity of the HRGC-ECD instrument and level of matrix interference. Instrumental limits of detection (ILOD) and limits of quantitation (ILOQ) based on methods in Taylor (1987) were approximately 4 and 13 pg/μL for

Table 4. Recovery of 2,3,7,8-Substituted Isomers of PCDDs and PCDFs Injected on the Hypercarb PCB Column (4 ng each, except 8 ng of O_8-CDD).

PCDDs	Percent Recovery	PCDFs	Percent Recovery
2,3,7,8	66	2,3,7,8	73
1,2,3,7,8[1]	178	1,2,3,7,8	83
1,2,3,4,7,8	75	2,3,4,7,8	92
1,2,3,6,7,8	80	1,2,3,4,7,8	78
1,2,3,4,6,7,8	71	1,2,3,6,7,8	79
O_8CDD	77	1,2,3,7,8,9	77
		2,3,4,6,7,8	88
		1,2,3,4,6,7,8	89
		1,2,3,4,7,8,9	85

[1] An interference was observed at the GC retention time for this isomer in this and other fractions collected from the Hypercarb column in the same time period.

each congener, respectively (Table 5). These values correspond to ILODs and ILOQs of 0.007 and 0.023 ng/g, respectively, for 10 gram samples. Concentrations of congeners exceeding these values were detected in matrix blanks. The pattern of congeners suggests the presence of PCBs rather than a matrix interference effect (Table 5). Definitions of method limits of detection (MLOD) and limits of quantitation (MLOQ) are being revised by the American Chemical Society (Keith 1992), but are defined here as 2 and 6 times the concentration observed in matrix blanks, respectively. If the ILOD and ILOQ are greater than the MLOD and MLOQ, respectively, then the instrument-based limits are used in place of the method limits.

The porous graphitic carbon (PGC) column used in these studies is more retentive than the one used by Creaser and Al-Haddad (1989), but appears to be similar to PGC columns used more recently. A 100 mm PGC column which is twice as long as the one described here has been successfully used to separate PCB congeners and PCDD and PCDF isomers into several fractions (Echols 1992). As with the 50 mm PGC column, solvents stronger than hexane were required to elute strongly retained congeners from the 100 mm PGC column. The method developed with the longer column resulted in more fractions, but also required considerably more time and solvent for each sample. Reverse elution of mono- and non-*ortho* substituted congeners with hexane has recently been achieved with a 100 mm PGC column (Hong *et al.* 1992), but di-*ortho* substituted congeners

Table 5. Limits of Detection (LOQ) and Limits of Quantitation (LOQ) for Individual PCB Congeners.

	Instrumental (ng/g)		Concentration in Blank	Based on Blanks (ng/g)	
PCB #	ILOD	ILOQ	(ng/g)	MLOD	MLOQ
77	0.007	0.024	0.038	0.076	0.23
81	0.007	0.023	0.008	0.016	0.048
126	0.006	0.020	0.016	0.032	0.096
169	0.007	0.023	0.031	0.062	0.19
101	0.007	0.023	0.11	0.22	0.66
105	0.006	0.021	0.05	0.10	0.30
118	0.003	0.010	0.002	0.004	0.012
128	0.008	0.028	0.05	0.10	0.30
138	0.007	0.023	0.16	0.32	0.96
158	0.004	0.015	0.010	0.020	0.060
167	0.009	0.030	0.002	0.009	0.030
180	0.008	0.028	0.11	0.22	0.66
189	0.007	0.023	0.002	0.007	0.023
123, 149	0.006	0.019	0.39	0.78	2.3
153, 132	0.007	0.023	0.24	0.48	1.4
156, 171	0.004	0.012	0.04	0.080	0.24
157, 200	0.007	0.022	0.03	0.060	0.18
170, 190	0.009	0.029	0.05	0.10	0.30

and other, less planar compounds were also eluted in that fraction.

This automated separation with the PGC column has several advantages relative to the activated carbon system previously used (Williams 1989; Williams *et al*. 1992). The PGC column has a greater loading capacity for total PCBs (>80 μg) than the 150 mg AX-21 activated carbon column (40 μg; Williams *et al*. 1992), so lower detection limits were achieved for the non-*ortho* substituted congeners. The PGC method requires approximately half the volume of solvents that was required by the activated carbon column. Toluene is not used in the PGC method, so all fractions can be readily evaporated and the solvent exchanged to iso-octane. The PGC method is more reproducible since the flow rate is microprocessor-controlled and the injection, gradient elution, and fraction collection are automated. The activated carbon method was not automated and required manual additions of different solvents and manual switching of column flow direction. In contrast, the automated PGC method can operate unattended. Operator handling of solvents and analytes is also reduced in the automated system. The analysis time for a single sample including rinsing and equilibration is 40 min with the automated method as compared to approximately 3 hr for the activated carbon method.

The automated separation of non-*ortho* substituted PCB congeners described here provides an efficient, reproducible method for analyzing these persistent, toxic compounds in biological matrices. The limits of detection achieved allows measurement of the non-*ortho* substituted congeners in environmental samples containing a total concentration of PCBs which is less than 100 ng/g. The method was developed on a commercially available column, so the method will be able to be used by other laboratories.

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CHAPTER 2

Polychlorinated Biphenyls and 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin Equivalents in Eggs of Double-Crested Cormorants Near Green Bay, Wisconsin, USA

INTRODUCTION

Polychlorinated hydrocarbons (PCHs) including polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) are persistent environmental contaminants which are toxic and which bioaccumulate (Muir *et al.* 1986, 1990; Van den Berg *et al.* 1987; Landrum and Poore 1988; Kannan *et al.* 1989; Borgmann *et al.* 1990). The most toxic isomer within these three classes of compounds is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, Safe 1990). The toxicity of other PCHs is greatest for those compounds which are stereochemically most similar to TCDD. The planar PCBs, PCDDs and PCDFs with laterally substituted chlorine atoms appear to elicit their toxicity through the same mode of action as TCDD (Poland *et al.* 1979; Poland and Knutson 1982).

The potencies of the PCHs to elicit toxic effects relative to the potency of TCDD have been used to derive Toxic Equivalency Factors (TEFs, Safe 1990). The potency of a mixture of these compounds can then be expressed as TCDD-Equivalents (TEQs). TEQs for complex mixtures of PCHs are determined in two different ways. The TEFs for individual PCHs can be multiplied by their respective concentrations to obtain TEQs contributed by each compound in a sample. The total concentration of TEQs in a sample is then calculated by summing the TEQs contributed by each compound (Safe 1990).

TEQs determined by this method are referred to as calculated TEQs.

Alternatively, an extract containing the PCHs from a sample is used to dose H4IIE rat hepatoma cells. Planar PCHs which bind to the *Ah* receptor induce the cytochrome-requiring P450IA1 monooxygenase enzyme (Poland and Glover 1977; Denomme *et al.* 1986; Safe 1990). When oxidation of the substrate 7-ethoxyresorufin by this enzyme is measured, the enzyme activity is referred to as ethoxyresorufin-*O*-deethylase (EROD) activity (Tillitt *et al.* 1991). Induction of EROD activity is strongly correlated with whole-animal toxicity (Safe 1990). EROD activity in the cells dosed with the sample extract is compared to the induction in cells dosed with TCDD to determine the integrated, total concentration of TCDD-equivalents (TEQs). TEQs determined by this method are referred to in this paper as bioassay TEQs.

Some of the effects that PCHs elicit in birds in controlled studies include bill and foot deformities, microphthalmia, hydropericardia, subcutaneous edemas, induction of hepatic mixed function oxidases, nest inattentiveness and pre- and post-hatch mortality (Higginbotham *et al.* 1968; Tori and Peterle 1983; Fox and Weseloh 1987; Brunstrom 1988, 1991). These compounds are associated with many of the same symptoms, including impaired reproduction, in fish-eating birds in the Great Lakes basin (Fox and Weseloh 1987; Kubiak *et al.* 1989; Fox *et al.* 1991a, b; Giesy *et al.* 1993; Yamashita *et al.* 1993). In particular, greater productivity in different colonies of double-crested cormorants (*Phalocrocorax auritus*) is correlated with lesser concentrations of TEQs (Tillitt *et al*. 1992). Over 90% of the toxicity from PCHs identified in fish-eating waterbirds of the Great Lakes can be attributed to planar PCB congeners rather than PCDDs and PCDFs (Jones *et al*. 1993; Yamashita *et al*. 1993).

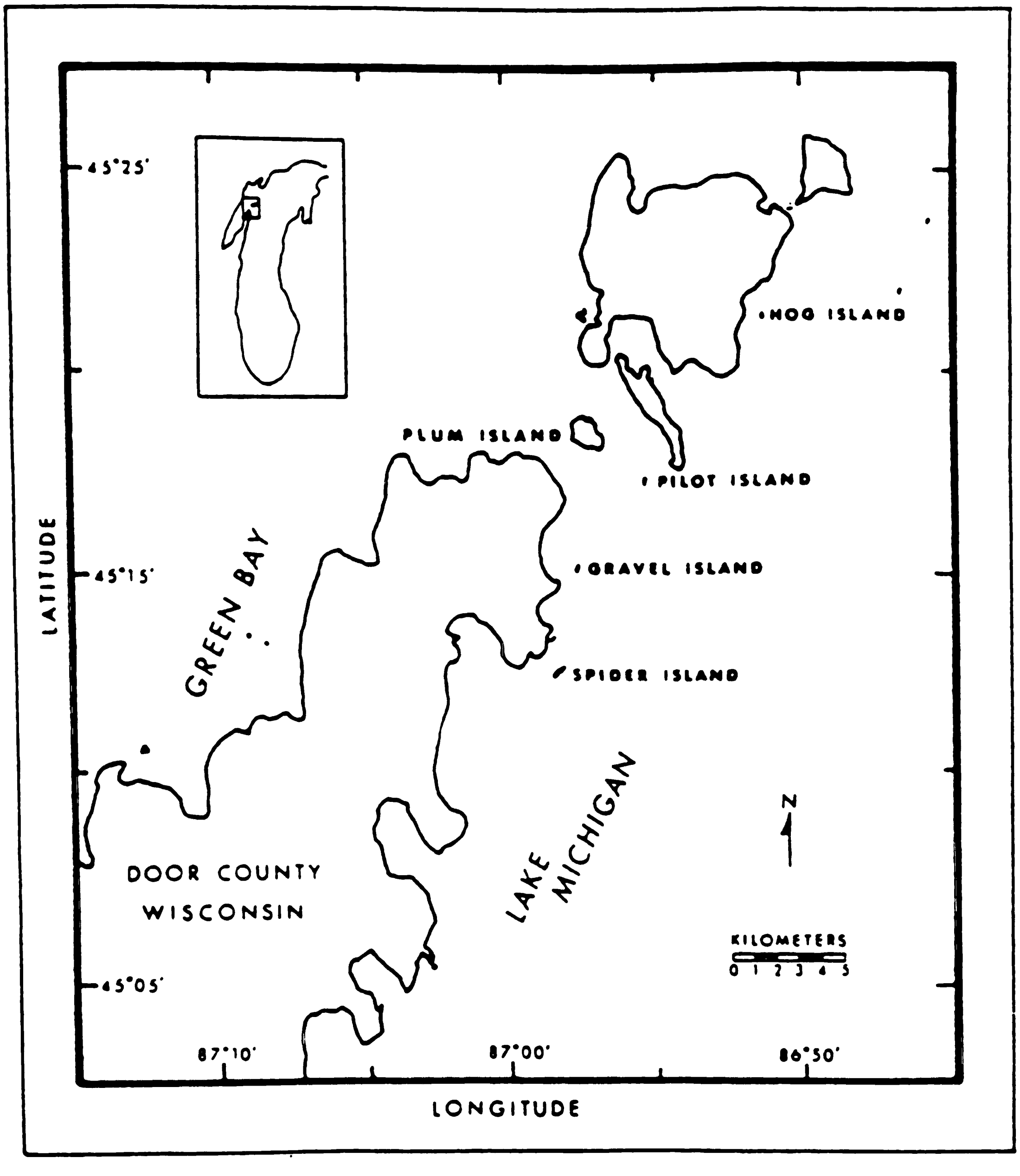
The objective of this study was to measure concentrations of PCB congeners, total concentrations of PCBs and both calculated and bioassay TEQs in eggs sampled from a single colony of double-crested cormorants in Green Bay, Wisconsin. This study provides important comparative data for the two methods for determining TEQs. Within-colony relationships between reproductive outcomes and concentrations of PCB congeners, calculated TEQs and bioassay TEQs were examined by pooling eggs from nests with known productivity rates and from nests which produced young with bill deformities.

MATERIALS AND METHODS

Sample Collection

An egg was collected from each of approximately 1,000 marked, double-crested cormorant (*Phalocrocorax auritus*) nests at Spider Island (1 km offshore in Lake Michigan, Door County, WI, Figure 4). The entire contents of each collected egg were emptied into a solvent-rinsed glass jar and frozen until the time of chemical analysis. Weekly observations of the nests were made to categorize productivity as High (every incubated

Figure 4. Map of Door County, Wisconsin, Area Showing Locations of Islands Which Support Nesting Colonies of Fish-eating Birds.


45°25'
45°15'
45°05'
LATITUDE
87°10'
87°00'
86°50'
LONGITUDE
PLUM ISLAND
PILOT ISLAND
HOG ISLAND
GRAVEL ISLAND
SPIDER ISLAND
GREEN BAY
LAKE MICHIGAN
DOOR COUNTY
WISCONSIN
N
KILOMETERS
0 1 2 3 4 5

egg produced a fledged young), Low (only one young fledged), or Cross-billed (one of the fledglings had a deformed bill).

Reagents and Standards

All solvents were high-purity grade solvents obtained from Burdick and Jackson (Muskegon, MI). PCB congener standards were obtained as crystalline solids (>95% purity) from either Ultra Scientific (North Kingstown, RI) or AccuStandard (New Haven, CT). Aroclor® standards (Aroclors® 1242, 1248, 1254 and 1260) were prepared from pure stocks which were originally from Monsanto Chemical Company, but were a gift to our laboratory from M. Zabik (Pesticide Research Center, MSU). All stock solutions were prepared in iso-octane and stored in the dark. Anhydrous sodium sulfate (Baxter Scientific, Ann Arbor, MI) was baked at 500°C overnight and then stored at 130°C. Activated basic alumina, Al_2O_3, (~150 mesh, 58 Å pore size, Aldrich, Milwaukee, WI) was deactivated with 8% (w/w) deionized water immediately prior to use.

Extraction and Initial Separation

Three eggs from each of the three productivity groups were randomly selected and pooled for PCB analysis. Pooled egg samples were homogenized with an Omni-mixer. Ten gram aliquants were ground with 50 g of sodium sulfate to obtain a free-flowing powder. Lipids and halogenated hydrocarbons were extracted by column elution with dichloromethane (DCM) at a flow rate of 3-5 mL/min. Lipid content was determined gravimetrically from an 80 μL aliquant of this crude extract.

Polar lipids and fine particulates were removed from the crude extract by hexane elution (25 mL) from an adsorbent chromatography column consisting of 1 g of sodium sulfate, 3 g of 8% deactivated basic alumina, 1 g of sodium sulfate, and glass wool. Polar lipids were then eluted from the alumina column with 15 mL acetone. Gel permeation chromatography (GPC) was used to remove the remaining lipids from the extract (See Appendix B for details). The volume of the extract collected from the GPC was reduced by roto-evaporation. The solvent was exchanged with iso-octane and the final volume adjusted to 1,600 µL.

PCBs were separated from interfering compounds on a nitrile HPLC column (S5 Nitrile Spherisorb, 25 cm x 4.6 mm, Phase Separations, Inc., Norwalk, CT). The HPLC system consisted of a Perkin Elmer Series 410 pump, a Perkin-Elmer ISS 200 autosampler with a Rheodyne injection valve equipped with a 2 mL sample loop, and an Isco (Lincoln, NE) Foxy 200 fraction collector. The flow rate was 3.0 mL/min. The column was equilibrated for 17 min with hexane before sample injection. The volume of sample injected was 1500 µL. Relatively nonpolar compounds eluted during a 20 min elution with hexane. The mobile phase was changed to 100% dichloromethane (DCM) with a linear gradient (3 min). Relatively polar compounds such as dieldrin were eluted during the subsequent 22 min elution with DCM. Column back pressure at a flow rate of 3 mL/min was approximately 1500 psig with 100% hexane and 2000 psig with 100% DCM. Six mL fractions were collected in calibrated glass tubes. The volume of the fractions were reduced to 1.0 mL under a gentle stream of nitrogen. PCBs were recovered in the first fraction collected after the void volume. This fraction was split for immediate GC analysis for PCBs, for the EROD bioassay, and for further fractionation of PCB congeners.

Total concentrations of PCBs were determined by gas chromatography with electron capture detection (GC-ECD). The GC used was a Perkin-Elmer 8500 equipped with a ^{63}Ni ECD (350°C) and a split/splitless injector operated in the splitless mode (250°C). PCBs were analyzed on a 30 m DB-5 column (0.25 mm i.d., 0.25 μm film thickness, J&W Scientific, Folsom, CA). The carrier gas was helium at a linear flow rate of 24 cm/sec and the makeup gas was nitrogen. Initial oven temperature was held at 120° for 6 min. The temperature was then increased to 260° at a rate of 2°/min. and then to 280°C at a rate of 10°/min. All injections were 1.0 μL and were made by an autosampler. All fractions injected contained PCB 30 at 200 pg/μL. Aroclor® performance standards were injected daily. Samples were diluted if their initial concentration exceeded the linear range of the detector. Additional PCB 30 was added during the dilution process so that the mass of PCB 30 injected was within the range of 194 - 210 pg.

A 1:1:1:1 mixture of Aroclors® 1242, 1248, 1254 and 1260 was used to quantitate total concentrations of PCBs and concentrations of selected individual peaks. All quantitation was based on peak areas relative to the area of PCB 30. Total concentrations of PCBs were determined with COMSTAR, a multiple regression software package which calculates a linear combination of Aroclor$^{\bullet}$ standards which best represents the observed chromatogram (Burkhard and Weininger 1987). Concentrations of selected peaks were calculated from response factors for the corresponding peaks in the 1:1:1:1 Aroclor$^{\bullet}$ standard. Response factors were determined by calibrating our 1:1:1:1 Aroclor$^{\bullet}$ standard mix with a 1:1:1:1 mix with known congener composition (Schwartz and Stalling 1991). The calibration Aroclor$^{\bullet}$ mix was a gift from T.R. Schwartz (National Fisheries Contaminants Research Center, Columbia, MO). Mean recovery of total concentration of PCBs was 53.6%, but very reproducible (CV = 8.9%). Similarly, recovery of selected congeners was 46.7% (CV = 6.7%, Table 6). All PCB concentrations were corrected for recovery and reported on a wet weight basis.

Separation of Non-*Ortho* Substituted PCB Congeners

Non-*ortho* substituted PCB congeners were separated from other PCB congeners in the PCB extract on a porous graphitic carbon (PGC) column. The column used was a 50 mm x 4.7 mm Hypercarb PCB column with 7 μm particle size (Shandon Scientific, UK, purchased from Keystone Scientific, Bellefonte, PA). This column was installed in the HPLC system which was used with the nitrile column (described above). A 6-port valve was added to allow automated reversal of flow direction. The characterization of this method is described

Table 6. Recovery of Selected Congeners from Chicken Eggs Spiked with an Aroclor Mixture and Separated on a Nitrile HPLC Column. Expected Total Concentration of PCBs was 2 $\mu g/g$.

PCB #	Mean	Standard Deviation	CV
101	44%	8%	19%
105	55%	7%	12%
118	49%	1%	3%
128	50%	5%	11%
138	47%	7%	15%
158	38%	3%	8%
167	40%	6%	14%
180	45%	8%	17%
189	59%	20%	33%
123, 149	39%	8%	20%
153, 132	38%	11%	28%
156, 171	50%	9%	18%
157, 200	40%	13%	34%
170, 190	48%	7%	14%
Total PCB	54%	5%	9%

in detail in Chapter 1 and in Appendix A. PCB extracts from the cormorant eggs and matrix spikes were injected onto the column in volumes ranging from 500 to 750 μL. The volume injected was optimized so that the total mass of PCB loaded on the column was between 20 and 60 μg while still reserving enough extract for the EROD bioassay.

Concentrations of non-*ortho* substituted PCB congeners were determined by GC-ECD. Internal standard calibration with authentic standards was used for quantitation. GC-ECD conditions were the same as described above with the following alterations. The initial oven temperature of 70°C was held for 1 min and then the oven temperature was increased to 140°C at 10°/min. Oven temperature was then increased to 240°C at 2°/min and from 240°C to 260°C at 10°C. One μL manual injections of standards and samples were made with a 10 μL Hamilton syringe. Mean recoveries of the non-*ortho* substituted PCB congeners ranged from 35% to 39% (CV $\leq$ 20%, N=3). Concentrations of individual PCB congeners were corrected for recovery and reported on a wet weight basis.

Calculation of TCDD-Equivalents (TEQs)

TEQs were calculated from concentrations of individual congeners and TEFs from Tillitt *et al.* (Tillitt *et al.* 1991 and pers. commun., Table 7). The Tillitt TEFs for individual congeners were determined in the same type of H4IIE cell bioassay for EROD activity as is described below for analysis of sample extracts. Concentrations of congeners were

Table 7. Toxic Equivalency Factors (TEFs) for PCB Congeners Used to Calculate TEQs in Samples.[1]

PCB #	TEF	PCB #	TEF
77	1.8×10^{-5}	105	7.6×10^{-6}
81	1.9×10^{-3}	118	3.5×10^{-7}
126	2.2×10^{-2}	156	5.5×10^{-5}
169	4.7×10^{-4}	157	1.5×10^{-5}

[1] TEF values are from Tillitt *et al.* (1991) and from D.Tillitt, pers. commun., 1993.

converted to molar concentrations before being multiplied by the TEFs since the TEFs represent the ratio of the response elicited by a pmole of a PCB congener to the response elicited by a pmole of TCDD. Total concentrations of TEQs are the sum of the TEQs for each congener. TEQs from PCB 156 and PCB 157 were not included in the total TEQs because they were not completely resolved from interfering PCB congeners. If the entire peaks for these congeners were assumed to be the mono-*ortho* substituted congeners and the corresponding TEFs applied, the combined contribution of these two peaks would still be less than 4% of the total concentration of TEQ.

Determination of TEQs with the EROD Bioassay

An EROD bioassay with H4IIE rat hepatoma cells was used as an alternative method to determine total concentrations of TEQ in the extracts from the cormorant eggs. The EROD activity in the cells was determined by measuring the rate at which 7-ethoxyresorufin was converted to resorufin. This bioassay has been modified from that of Tillitt *et al.* (1991). For complete details of the following procedure, see Crawford *et al.* (1993). The H4IIE cells were cultured in 96-well microtiter plates instead of petri dishes. The cells in some wells were dosed with a dilution series of the sample PCB extracts. Other wells were dosed with a dilution series of a TCDD standard. After a 72 hr incubation period, 7-ethoxyresorufin was added to all wells. Dicumerol was added to the cell cultures with the 7-ethoxyresorufin to prevent diaphorase interference. After a one hr incubation with NADPH, resorufin concentrations were determined directly in the 96-well plates with a Cytoflour 2300 scanning fluorometer (Millipore, Inc). The protein content in the wells was determined with Bradford Reagent dye on a BIO-TEK Model EL308 EIA Reader. EROD activity was calculated as pmol/mg protein/min. TCDD-equivalents (TEQs) in the sample extracts were calculated by comparing the mass of TCDD required to elicit the same response as a given volume of sample extract.

Statistical Analysis

All statistical analyses were performed with Statistical Analysis System (SAS, Version 6.04) procedures. The level of significance for all tests was 0.05. Means of analytical duplicates were used in all tests. All concentrations examined in statistical procedures were above limits of detection, so no corrections needed to be made. Both parametric and nonparametric statistical analyses were performed because of the small sample sizes, but results were similar.

RESULTS

Concentrations of total PCBs and PCB congeners 77, 81, 126, 169, 105, 118, 138 and 153 were above the limit of quantitation in all 9 pooled egg samples (Table 8). All concentrations are reported on a wet weight basis. The median

Table 8. Concentrations of PCB Congeners, Total PCBs, and TCDD-Equivalents in Eggs of Double-Crested Cormorants from Spider Island, Wisconsin. All Concentrations Are Expressed on a Wet Weight Basis and Are Corrected for Recovery.

Sample[1]	Total PCBs (μg/g)	Bioassay TEQ (pg/g)	Calculated TEQs (pg/g)	PCB Congener Concentrations (ng/g)[2]: 77	81	126	169	105	118	138	153[3]
H1	13.2	17.8	141	1.27	1.81	6.26	1.21	136	399	655	1370
H2	12.2	234	90.5	0.96	1.63	3.95	0.75	114	408	631	1050
H3	15.1	406	131	3.13	2.13	5.73	1.19	246	618	924	1470
L1	15.1	133	121	1.83	2.32	5.23	1.02	224	617	836	1370
L2-A	38.4	-	289	3.31	5.58	12.5	3.72	639	1640	2120	3580
L2-B	34.6	470	239	2.75	4.04	10.4	2.10	489	1370	1830	3120
L3	11.8	387	137	1.87	1.79	6.02	1.10	193	468	849	1220
X1	9.71	82.9	106	1.23	1.80	4.62	0.85	140	381	600	1060
X2	13.6	270	136	2.58	2.04	5.96	1.15	222	501	991	1510
X3-A	13.1	198	141	2.19	1.90	6.24	1.32	181	441	825	1400
X3-B	11.9	117	123	2.49	1.59	5.44	1.11	173	481	876	1280
Mean[4]	15.5	240	140	2.03	2.23	6.12	1.27	224	595	924	1530
Median[4]	13.2	235	132	1.87	1.81	5.84	1.15	193	468	849	1370

[1] Samples consist of a pool of three eggs from a productivity group. Productivity groups symbols: H= high productivity, L = low productivity, X = cross-billed chick.

[2] PCB congener numbers are adapted from Ballschmitter and Zell (1980).

[3] Congener 153 concentrations also include a co-eluting congener (PCB 132).

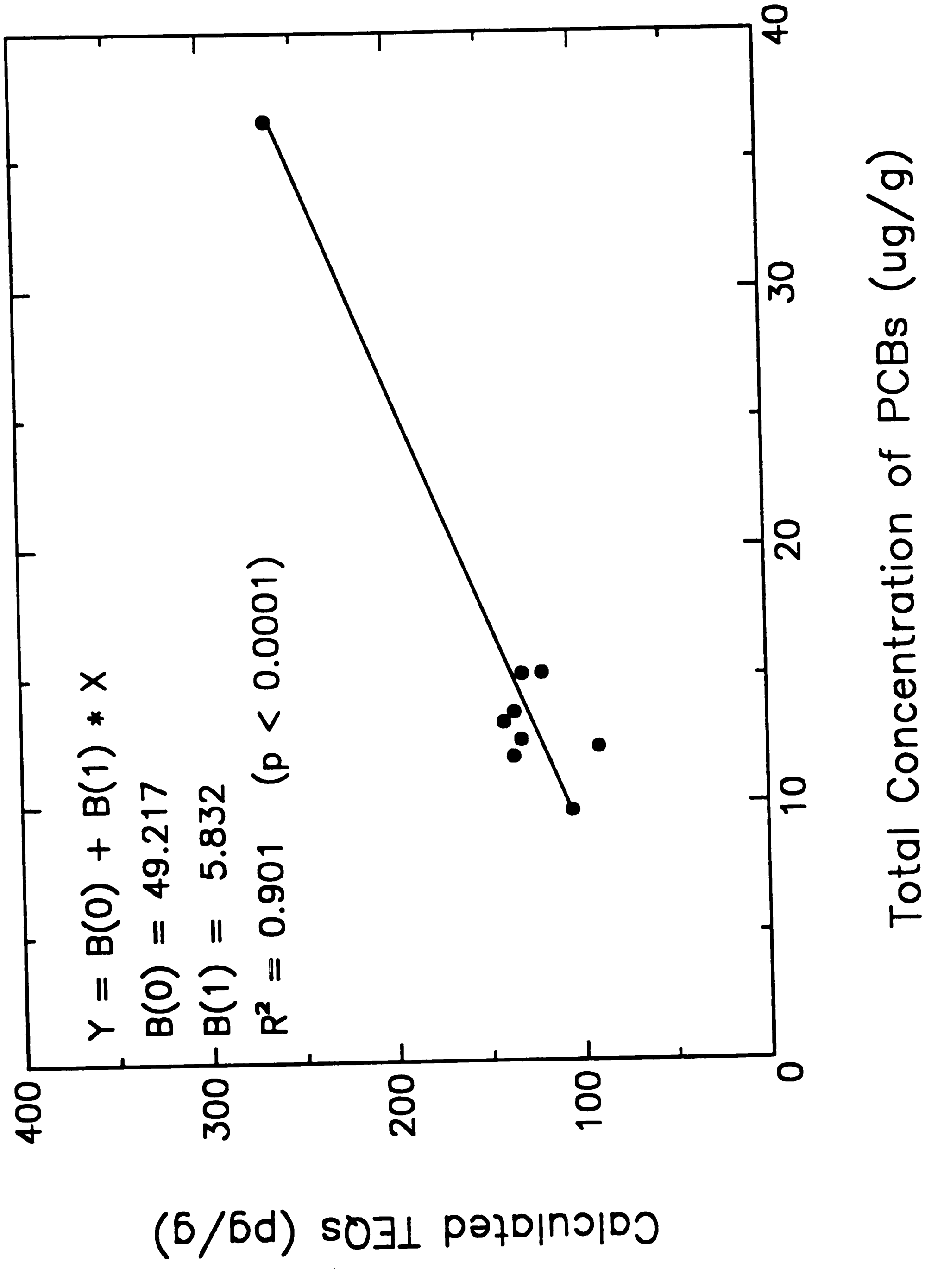
[4] The mean of analytical duplicates (L2 and X3) was used instead of using both values to compute the mean and median: N = 9 for both mean and median.

concentration of total concentrations of PCBs was 13.2 μg/g. The range of values observed among the 9 samples was 11.8 to 38.4 μg/g. Concentrations of the non-*ortho* substituted PCB congeners (PCB 77, 81, 126 and 169) ranged from 0.75 to 12.5 ng/g. Concentrations of the mono-*ortho* substituted PCB congeners 105 and 118 in the eggs were approximately 1000 fold greater. Neither total PCBs nor individual congeners were detected in matrix blanks at concentrations exceeding 5% of the least concentration measured in any of the samples. Mean percent difference between duplicates was less than 20% for total PCBs and all congeners except for 81 and 169; the mean percent difference in concentrations for these two congeners in duplicates were 25% and 35%, respectively.

Concentrations of TCDD-equivalents measured in the EROD bioassay were generally greater than those calculated from concentrations of the PCB congeners (Table 8). The median concentration of bioassay TEQs was 235 pg/g while the median concentration of calculated TEQs was 132 pg/g. This difference was significant at $p = 0.10$, but not $p = 0.05$ using either a paired t-test or Wilcoxon's signed rank test for paired data. Over 90% of the calculated TEQs were contributed by PCB congener 126.

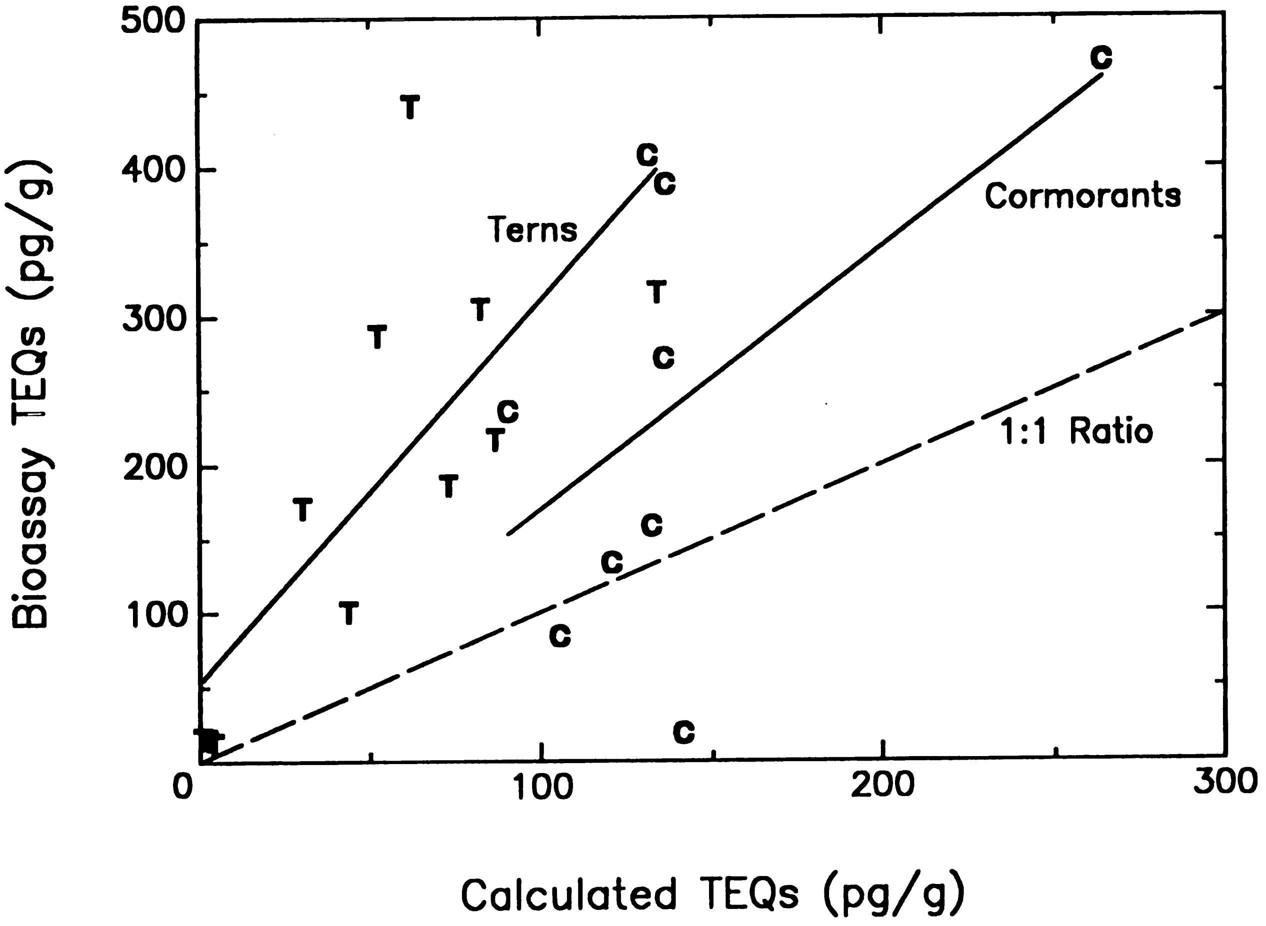
Calculated TEQs were correlated with total concentrations of PCB, but the correlation between bioassay TEQs and either of these two measures of contamination was weaker. Approximately 90% of the variability in calculated TEQs was explained by total concentration of PCBs (Figure 5) while only

Figure 5. Calculated TCDD-Equivalents (TEQs) as a Function of Total PCBs in Double-Crested Cormorants.


Y = B(0) + B(1) * X
B(0) = 49.217
B(1) = 5.832
R^2 = 0.901 ($p < 0.0001$)
Calculated TEQs (pg/g)
400
300
200
100
Total Concentration of PCBs (ug/g)
0
10
20
30
40

34% of the variability in bioassay TEQs was explained by total PCBs. The correlation between the two methods for determining TEQs explained 31% of the variability (Figure 6). No significant differences in concentrations of either PCBs or TEQs were observed among productivity groups using either parametric (ANOVA) or non-parametric (Kruskal-Wallace) techniques (Figures 7 and 8). The median ratio of calculated TEQs to total concentration of PCBs was slightly greater for the eggs from the cross-bill productivity group (Figure 9), but this difference was not statistically significant.

Figure 6. Concentrations of TCDD-Equivalents (TEQs) in Bird Eggs from Near Green Bay, Wisconsin: TEQs Measured in an H4IIE Bioassay for EROD Activity as a Function of TEQs Calculated from Concentrations of PCBs.



R^2 = 0.311 and slope = 1.76 for C (Williams et al.)

R^2 = 0.565 and slope = 2.58 for T (Jones et al.)

Figure 7. Total Concentrations of PCBs in Eggs of Double-Crested Cormorants from Nests with Different Reproductive Outcomes on Spider Island, Wisconsin.

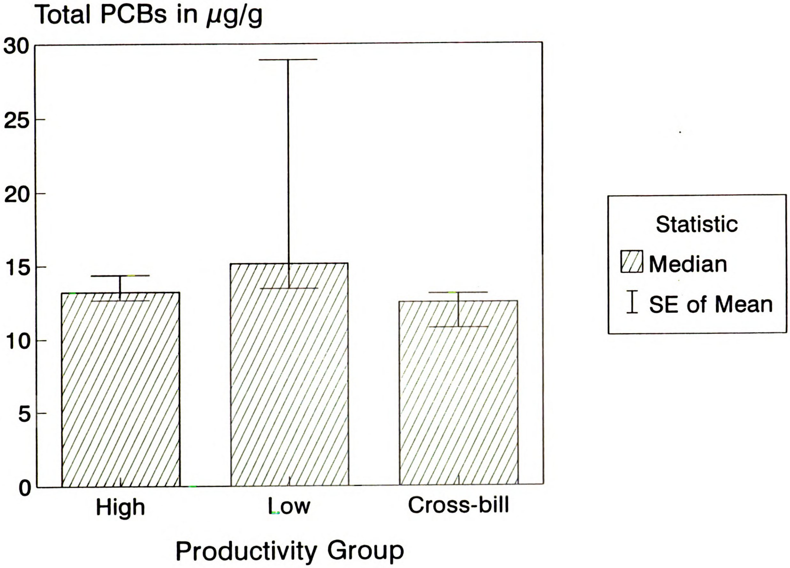

Total PCBs in μg/g
30
25
20
15
10
5
0
High
Low
Cross-bill
Productivity Group
Statistic
▨ Median
I SE of Mean

Figure 8. Concentrations of TEQs in Eggs of Double-Crested Cormorants from Nests with Different Reproductive Outcomes on Spider Island, Wisconsin.

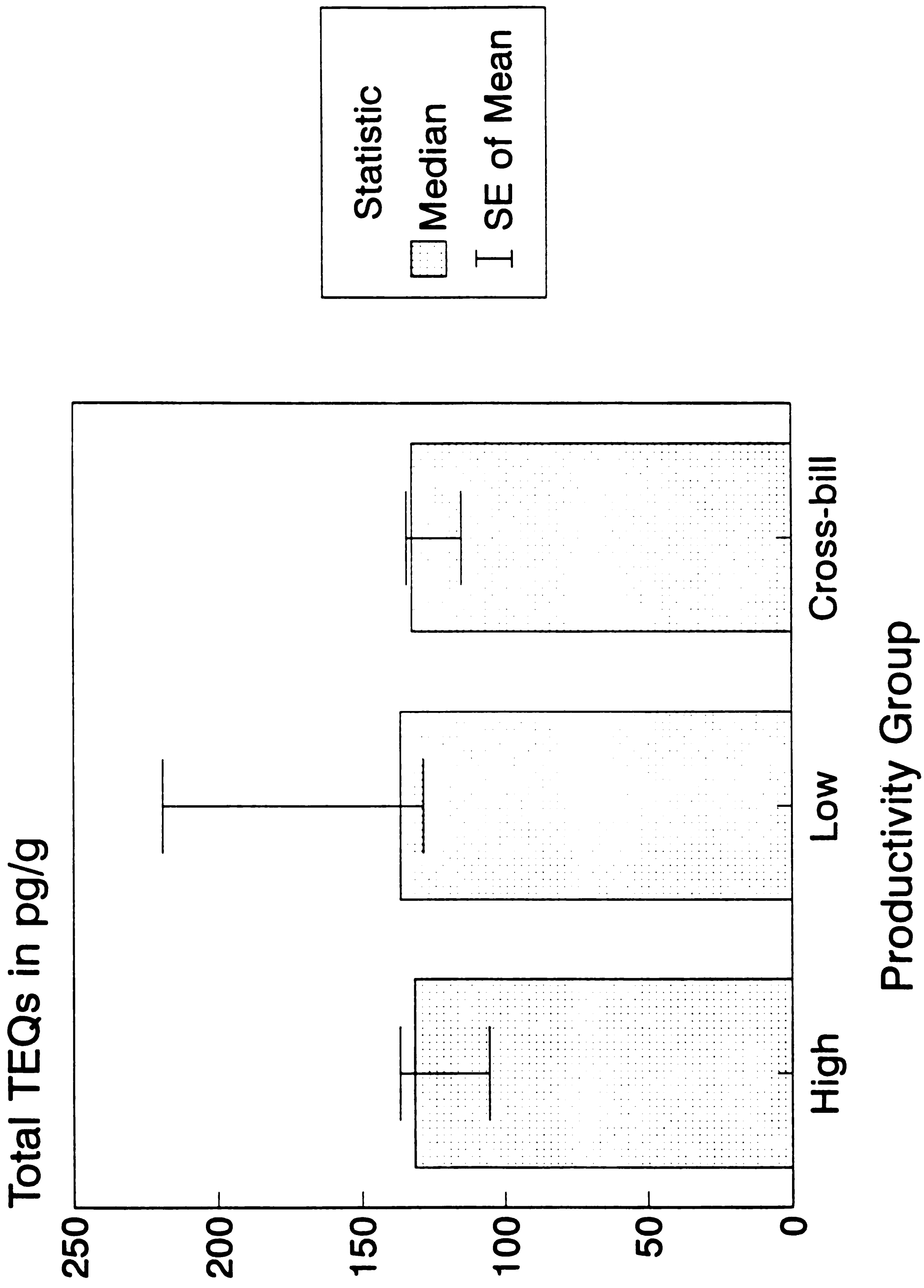
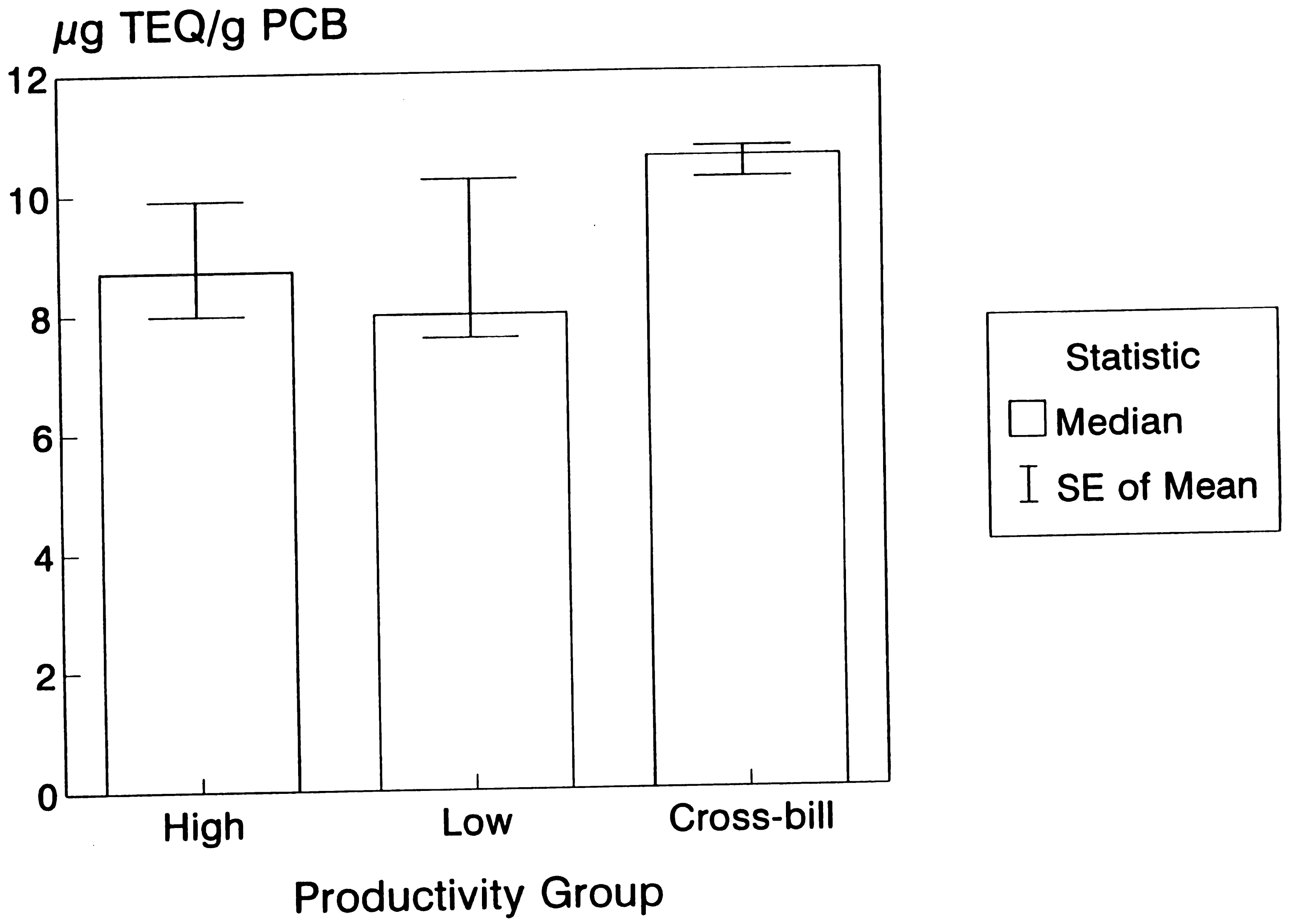

Total TEQs in pg/g
250
200
150
100
50
0
High
Low
Cross-bill
Productivity Group
Statistic
Median
SE of Mean

Figure 9. Ratio of Concentrations of TEQs to Concentrations of PCBs in Eggs of Double-Crested Cormorants from Nests with Different Reproductive Outcomes on Spider Island, Wisconsin.


μg TEQ/g PCB
12
10
8
6
4
2
0
High
Low
Cross-bill
Productivity Group
Statistic
Median
SE of Mean

DISCUSSION

Concentrations of PCBs in the eggs of double-crested cormorants studied here were similar to those measured in eggs of other bird species from the Green Bay area (Kubiak *et al.* 1989; Ankley *et al.* 1993; Jones *et al.* 1993; Yamashita *et al.* 1993). The observed concentrations were greater than lowest observed adverse effect levels (LOAEL) for PCBs in eggs of birds in the environment which are approximately 3-5 µg/g (Giesy *et al.* 1993). In the laboratory, chicken eggs dosed with 5 µg/g Aroclor® 1248 exhibit 33% embryo mortality (Brunstrom 1991). Thus, the presence of deformed chicks and overall reduced productivity of the Spider Island double-crested cormorant colony was expected given the total concentrations of PCBs of 13 µg/g observed.

The ratios of concentrations of individual congeners to total concentrations of PCBs in these double-crested cormorant eggs were similar to those in eggs of other bird species from the Great Lakes with the exception of PCB 77 (Table 9). The relative proportion PCB 77 in these samples was an order of magnitude less than that observed in other samples. A complete review of recoveries, chromatograms and data analysis did not reveal any analytical errors. PCB 77 is more easily metabolized than the other non-*ortho* substituted congeners because it has adjacent, unsubstituted, *meta-para* positions on both rings (Boon and Eijgenraam 1988; Borlakoglu *et al.* 1988). The double-crested cormorants which laid the eggs collected

Table 9. Mean, relative proportions of four planar PCB congeners in technical Aroclor mixtures and bird and fish tissues.

Sample	Total PCB ($\mu g/g$)	Relative Proportion of Congener (%) 77	105	126	169
Cormorant Eggs[1]	13.2	0.014	1.46	0.044	0.0087
Merganser Eggs ('77)[2]	25.3	0.11	2.08	0.055	0.0070
Merganser Eggs ('90)[2]	13.3	0.13	1.77	0.051	0.0077
Forster's Tern Eggs[3]					
Green Bay	22.2	0.002	2.52	0.017	0.0048
Lake Poygan	4.5	<0.001	2.00	0.007	0.007
Cormorant Eggs[4]	7.3	0.11	2.88	0.049	0.0074
Bird Tissues[5]	4.6	2.28	3.62	0.03	0.0014
Chinook Salmon Lake Michigan[6]	1.0	0.22	2.42	0.08	---
Aroclor 1242[7]	---	0.52	---	0.002	---
Aroclor 1248[7]	---	0.61	---	0.0062	---
Aroclor 1254[7,9]	---	0.06	---	0.0046	0.08-0.00005
Aroclor 1260[7,9]	---	0.03	---	0.0008	0.05
Aroclor 1242[8]	---	0.30	0.42	0.003	---
Aroclor 1254[8]	---	0.02	5.49	0.003	---
Aroclor 1260[8]	---	0.001	0.03	0.000	---
Aroclor 1254[10]	---	0.07	---	0.01	0.003

[1] This work, Chap. 2

[2] This work, Chap. 3

[3] Kubiak *et al.* 1989

[4] Yamashita *et al.* 1993

[5] Giesy *et al.* 1993

[6] Williams *et al.* 1992

[7] Kannan *et al.* 1987

[8] Pruell *et al.* 1988

[9] Larsen *et al.* 1993

[10] This work, Chap. 1

for this study may have had a different metabolic status than the other birds listed in Table 9, but this seems inadequate to explain the entire difference among sample sets. The relative proportion of PCB 126 in the total concentration of PCBs was consistent with other birds from the Green Bay area (Table 9). Tern eggs from Lake Poygan, a reference site away from the Great Lakes, contained a lesser proportion of PCB 126 (Table 9). The relative proportion of PCB 126 was also greater in the eggs of double-crested cormorants and other bird species from the Great Lakes than the relative proportion of PCB 126 in any of the technical Aroclors®. This is significant because it indicates that PCB 126, which contributes over 90% of the calculated TEQs in the cormorant eggs, was selectively enriched relative to the mixture of PCBs originally released into the environment.

The relationship between bioassay TEQs and calculated TEQs were similar to that observed in a similar study (Jones *et al.* 1993) in that calculated TEQs were unable to account for all of the TEQs measured in the bioassay. A slope of unity for the plot of bioassay TEQ as a function of calculated TEQ would be expected if the planar PCBs accounted for all of the activity in the bioassay and if interactions among congeners were strictly additive. The slope of this function was greater than unity for both sets of data (Figure 10). This indicates that other EROD-active compounds were present in the extracts or that significant supra-additivity was observed. Although supra-additivity is occasionally observed in biochemical assays comparing two congeners (Safe 1990), two planar PCH compounds which are supra-additive at one dose can be antagonistic (infra-additive) at a greater dose (Janz and Metcalfe 1991). The interactions of congeners in the complex mixture of PCBs are more likely to be competitive and antagonistic given the relatively great ratio of concentrations of congeners with low binding affinities for the *Ah* receptor to those with greater binding affinities. The more likely explanation for the difference between the bioassay TEQs and calculated TEQs is that compounds other than planar PCBs contributed to the bioassay TEQs. The most obvious candidates for these additional compounds are the PCDDs and PCDFs, but when these compounds were accounted for in the paper by Jones *et al.* (1993) the change in slope was insignificant. Additional compounds which might contribute to the TEQs measured in the bioassay include the following classes of polychlorinated compounds: naphthalenes (PCNs), diphenyl ethers (PCDEs), diphenyl toluenes (PCDT), phenoxy anisoles (PCPAs), biphenyl anisoles (PCBAs), xanthenes (PCXE), xanthones (PCXO), anthracenes (PCAn), fluorenes (PCFls), dihydroanthracenes (PCDHAs), diphenyl methanes (PCBMs), phenylxylylethanes (PCPXEs), dibenzothiophenes (PCDTh), quaterphenyls (PCQs), quaterphenyl ethers (PCQEs) and biphenylenes (PCBE). In addition to the chlorinated compounds, brominated and chloro/bromo-substituted analogues of PCDD and PCDF have been found in the environment (Haglund *et al.* 1988) and are known to induce ethoxyresorufin-odeethylase (EROD) activity *in vivo* and *in vitro* (Safe 1990).

The difference between the two slopes in Figure 6 could have several explanations. The most obvious is that the samples were simply different. Different protocols were used for both chemical analysis and the EROD assay. Systematic differences among laboratories and protocols could have contributed significantly to the observed difference if they happened to produce biases in the same direction. The different extraction protocols may have resulted in different recoveries of some of the other EROD-active classes of compounds listed above, or these compounds may have been present in different proportions relative to the planar PCBs in the two sets of bird egg samples.

Within-colony variation in concentrations appears to be insignificant relative to variations in sensitivity among birds. Teratogenesis, in particular, is a low probability event. In controlled laboratory studies, only a small fraction of individuals within a given dose group will show teratogenic effects. An ED-10 is a single dose at which only 10% of birds exhibit the adverse effect being measured even though all birds in that group were dosed at exactly the same level under controlled conditions. In this study, the entire colony represents a dose group rather than each group selected *a posteriori* based on productivity and presence of deformities. This view of the colony as a single dose group is substantiated by the lack of significant differences in concentrations of PCBs, TEQs or the TEQ to PCB ratio among the productivity groups. Metabolic differences among birds did not produce changes in the congener distribution which might have altered the potency of the PCB mixture to which their embryos were exposed.

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CHAPTER 3

Polychlorinated Biphenyls and 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin Equivalents in Eggs of Red-Breasted Mergansers Near Green Bay, Wisconsin, USA, in 1977-78 and 1990

INTRODUCTION

Polychlorinated hydrocarbons (PCHs) including polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) are persistent environmental contaminants which are toxic and which bioaccumulate (Muir *et al.* 1986, 1990; Van den Berg *et al.* 1987; Landrum and Poore 1988; Kannan *et al.* 1989; Borgmann *et al.* 1990). The most toxic isomer within these three classes of compounds is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, Safe 1990). The toxicity of other PCHs is greatest for those compounds which are stereochemically most similar to TCDD. The planar PCBs, PCDDs and PCDFs with laterally substituted chlorine atoms appear to elicit their toxicity through the same mode of action as TCDD (Poland *et al.* 1979; Poland and Knutson 1982).

The potencies of the PCHs to elicit toxic effects relative to the potency of TCDD have been used to derive Toxic Equivalency Factors (TEFs, Safe 1990). The potency of a mixture of these compounds can then be expressed as TCDD-Equivalents (TEQs). TEQs for complex mixtures of PCHs can be determined with an additive model. The TEFs for individual PCHs are multiplied by their respective concentrations to obtain TEQs contributed by each compound in a sample. The total concentration of TEQs in a sample is then calculated by summing the TEQs contributed by each compound (Safe 1990). TEQs determined by this method are referred to as calculated TEQs.

Some of the effects that PCHs elicit in birds in controlled studies include bill and foot deformities, microphthalmia, hydropericardia, subcutaneous edemas, induction of hepatic mixed function oxidases, nest inattentiveness and pre- and post-hatch mortality (Higginbotham *et al.* 1968; Tori and Peterle 1983; Fox and Weseloh 1987; Brunstrom 1988, 1991). These compounds are associated with many of the same symptoms, including impaired reproduction, in fish-eating birds in the Great Lakes basin (Fox and Weseloh 1987; Kubiak *et al.* 1989; Fox *et al.* 1991a, b; Giesy *et al.* 1993; Yamashita *et al.* 1993). Over 90% of the toxicity from PCHs identified in fish-eating waterbirds of the Great Lakes has been attributed to planar PCB congeners (Jones *et al.* 1993; Yamashita *et al.* 1993).

The pattern of the individual PCB congeners is not the same in sediments and aquatic biota as it is in the original technical Aroclors® (Schwartz *et al.* 1987; Schwartz and Stalling 1991). The planar PCB congeners, in particular, may make up a greater percentage of the total concentration of PCBs in some environmental samples than they do in technical Aroclors® (Giesy *et al.* 1993). One of the concerns in the Great Lakes basin has been whether or not the potencies of PCB mixtures to which biota are exposed has increased over time. The relative concentrations of PCB congeners in the environment have the potential to change because of differential degradation, volatility, adsorption, photolysis, bioaccumulation, biomagnification and metabolism. Egg mortality in double-crested cormorant colonies in the Great Lakes basin is more closely correlated with the potency of the PCB mixture than with the actual total concentration of PCBs (Tillitt *et al.* 1992).

The objective of this study was to measure concentrations of PCB congeners, total concentrations of PCBs and calculated TCDD-equivalents (TEQs) in red-breasted merganser (*Mergus serrator*) eggs collected from colonies near Green Bay, Wisconsin in 1977-78 and in 1990. The hypothesis that the ratio of TEQ to total PCB has increased over time was tested by comparing this ratio in the two sets of eggs collected 13 years apart.

MATERIALS AND METHODS

Sample Collections

Eggs were collected from red-breasted merganser nests on Spider, Pilot and Hog Islands in 1977-78 and from Pilot and Hog Islands in 1990 (Figure 4). One egg was collected from each marked nest and hatching success was later determined in those nests. Water loss at the time of collection was determined by the methods of Stickle *et al.* (1973). The entire contents of each collected egg were emptied into a solvent-rinsed glass jar and frozen until the time of chemical analysis. The 24 eggs selected for analysis were all those from nests which were incubated until hatching.

Reagents and Standards

All solvents were high-purity solvents obtained from Burdick and Jackson (Muskegon, MI). PCB congener standards were obtained as crystalline solids (>95% purity) from either Ultra Scientific (North Kingstown, RI) or AccuStandard (New Haven, CT). Aroclor® standards (Aroclors® 1242, 1248, 1254 and 1260) were prepared from pure stocks which were originally from Monsanto, but were a gift to our laboratory from M. Zabik (Pesticide Research Center, MSU). All stock solutions were prepared in iso-octane and stored in the dark. Anhydrous sodium sulfate (J.T.Baker Co., Phillipsburg, NJ) was baked at 500°C overnight and then stored at 130°C. Activated basic alumina, Al_2O_3, (~150 mesh, 58 Å pore size, Aldrich, Milwaukee, WI) was deactivated with 8% (w/w) deionized water immediately prior to use. Silica gel (70/230 mesh, Sigma Chemical Co., St. Louis, MO) baked at 130°C overnight and then stored at 130°C.

Extraction and Initial Separation

Complete details of the extraction and initial separation of PCBs from other xenobiotics are presented in Appendix B. In brief, approximately 10 g of a thawed, homogenized egg were ground with 5 times the sample weight of anhydrous sodium sulfate. Lipids and halogenated hydrocarbons were extracted from the resulting powder by column elution with DCM at a flow rate of 3-5 mL/min. Lipid content was determined gravimetrically from an 80 µL aliquant of this crude extract. Gel permeation chromatography was used to remove lipids from the extract. PCBs were separated from more polar compounds with a column consisting of deactivated (8%) basic alumina and silica gel. PCB extracts were exchanged into iso-octane and volume was adjusted to 1 mL.

Total concentrations of PCBs were determined by gas chromatography with electron capture detection (GC-ECD). The GC used was a Perkin-Elmer 8500 equipped with a ^{63}Ni ECD (350°C) and a split/splitless injector operated in the splitless mode (250°C). PCBs were analyzed on a 30 m DB-5 column (0.25 mm i.d., 0.25 µm film thickness, J&W Scientific, Folsom, CA). The carrier gas was helium at a linear flow rate of 24 cm/sec and the makeup gas was nitrogen. Initial oven temperature was held at 120° for 6 min. The temperature was then increased to 260° at a rate of 2°/min. and then to 280°C at a rate of 10°/min. All injections were 1.0 µL and were made by an autosampler. All fractions injected contained PCB 30 at 200 pg/µL. Aroclor® performance standards were injected daily. Samples were diluted if their initial concentration exceeded the linear range of the detector. Additional PCB 30 was added during the dilution process so that the mass of PCB 30 injected was within the range of 194 - 210 pg.

A 1:1:1:1 mixture of Aroclors® 1242, 1248, 1254 and 1260 was used to quantitate total concentrations of PCBs and concentrations of selected individual peaks. All quantitation was based on peak areas relative to the area of PCB 30. Total concentrations of PCBs were determined with COMSTAR, a multiple regression software package which calculates a linear combination of Aroclor® standards which best represents the observed chromatogram (Burkhard and Weininger 1987). Concentrations of selected peaks were calculated from response factors for the corresponding peaks in the 1:1:1:1 Aroclor® standard. Response factors were determined by calibrating our 1:1:1:1 Aroclor® standard mix with a 1:1:1:1 mix with known congener composition (Schwartz and Stalling 1991). The calibration Aroclor® mix was a gift from T.R. Schwartz (National Fisheries Contaminants Research Center, Columbia, MO). Mean recovery of total concentration of PCBs was 92% (CV = 12%). Recovery of selected congeners was similar, but slightly more variable (Table 10). Total concentrations of PCBs are reported on a wet weight basis (as received) and are not corrected for recovery.

These samples were previously analyzed for total PCBs and organochlorine pesticides by the Mississippi State Chemical Laboratory under contract with Patuxent Analytical control Facility (U.S. Fish and Wildlife Service, Laurel, MD). Total concentrations of PCBs determined at Mississippi State and here at Michigan State are in excellent agreement ($r^2 = 0.930$, slope = 0.948).

Table 10. Recovery of Selected Congeners and Total PCBs from Chicken Eggs.

PCB #	Mean	CV	Spiking Solution[1]
77	54%	7%	1
81	49%	3%	1
126	52%	5%	1
169	51%	8%	1
101	81%	20%	2
105	107%	10%	2
118	102%	18%	2
128	92%	27%	2
138	93%	20%	2
158	99%	26%	2
167	80%	32%	2
180	89%	22%	2
189	106%	41%	2
56, 60	94%	20%	2
77, 110	81%	19%	2
81, 87	89%	19%	2
123, 149	85%	17%	2
153, 132	86%	20%	2
156, 171	87%	60%	2
157, 200	108%	27%	2
170, 190	90%	21%	2
Total PCB	92%	12%	2

[1] Spiking solutions:
(1) Congeners 77, 81, 126 and 169 were added to chicken egg homogenate to produce a concentration of 10 ng/g of each congener.
(2) An Aroclor Mixture of 1242, 1248, 1254 and 1260 (1:1:1:1) was added to chicken egg homogenate to produce a total concentration of PCBs of 2 μg/g.

Separation of Non-*ortho* Substituted PCB Congeners

Non-*ortho* substituted PCB congeners were separated from other PCB congeners in the PCB extract on a porous graphitic carbon (PGC) column. The column used was a 50 mm x 4.7 mm Hypercarb PCB column with 7 μm particle size (Shandon Scientific, UK, purchased from Keystone Scientific, Bellefonte, PA). The column was incorporated into an HPLC system consisting of a Perkin Elmer (Norwalk, CT) Series 410 pump, a Perkin-Elmer ISS 200 autosampler with a Rheodyne injection valve equipped with a 2 mL sample loop, and an Isco (Lincoln, NE) Foxy 200 fraction collector. A 6-port valve was added to allow automated reversal of flow direction. The characterization of this method is described in detail in Chapter 1 and in Appendix A. PCB extracts from the merganser eggs and chicken egg matrix spikes were injected onto the column in volumes ranging from 200 to 800 μL. The volume injected was optimized so that the total mass of PCB loaded on the column was between 28 and 65 μg. The capacity for this column was approximately 80 μg (Chapter 1).

Concentrations of non-*ortho* substituted PCB congeners were determined by GC-ECD. Internal standard calibration using authentic standards was used for quantitation. GC-ECD conditions were the same as described above with the following alterations. The initial oven temperature of 70°C was held for 1 min and then the oven temperature was increased to 140°C at 10°/min. Oven temperature was then increased to 240°C at 2°/min and from 240°C to 260°C at 10°C. One μL manual injections of standards and samples were made with a 10 μL Hamilton syringe.

Mean recoveries of the non-*ortho* substituted PCB congeners ranged from 49% to 54% (Table 10). These recoveries were consistent (CV $\leq$ 8%, N=5). Concentrations of individual PCB congeners were corrected for recovery and reported on a wet weight basis.

Calculation of TCDD-Equivalents (TEQs)

TEQs were calculated from concentrations of individual congeners and TEFs from Tillitt *et al.* (1991 and pers. commun.; Table 7). The Tillitt TEFs for individual congeners were determined in an H4IIE cell bioassay for EROD activity. Concentrations of congeners were converted to molar concentrations before being multiplied by the TEFs since the TEFs represent the ratio of the response elicited by a pmole of a PCB congener to the response elicited by a pmole of TCDD. Total concentrations of TEQs are the sum of the TEQs for each congener. TEQs from PCB 156 and PCB 157 were not included in the total TEQs because they were not resolved from interfering PCB congeners. If the entire peaks for these congeners were assumed to be the mono-*ortho* substituted congeners and the corresponding TEFs applied, the combined contribution of these two peaks would still be less than 4% of the total concentration of TEQ. The actual contribution of PCB 156 and PCB 157 would be less than this, so only a small error is introduced by not using these peaks in the TEQ calculations.

Statistical Analysis

All statistical analyses were performed with Statistical Analysis System (SAS, Version 6.04) procedures. The level of significance for all tests was 0.05. Means of analytical duplicates were used in all statistical tests. All concentrations examined in statistical procedures were above limits of detection, so no corrections needed to be made. Both parametric and nonparametric statistical analyses were performed because of the small sample sizes, but results were similar.

RESULTS

Concentrations of total PCBs and PCB congeners 77, 81, 126, 169, 105, 118, 138 and 153 were above the limit of quantitation in all 23 egg samples (Table 11 and 12). (One sample of the original 24 had insufficient homogenate for re-analysis following recalibration of the GPC column.) The median total concentrations of PCBs were 25 and 13 μg/g in the eggs from 1977-78 and 1990, respectively. The range of values observed among the 23 samples was 5.5 to 44 μg/g. Concentrations of the mono-*ortho* substituted PCB congeners 105 and 118 in the eggs were approximately 10 to 400 fold greater. Neither total PCBs nor individual congeners were detected in matrix blanks at concentrations exceeding 5% of the least concentration measured in any of the samples. Mean percent difference between duplicates was less than 20% for total PCBs and all congeners. Duplicate determinations of the non-*ortho*

Table 11. Concentrations of PCB Congeners, Total PCBs, and TCDD-Equivalents in Red-Breasted Merganser Eggs from Lake Michigan, Door County, Wisconsin, Collected in 1977-78.

Sample	Total PCB µg/g	Calculated TEQs pg/g	PCB Congener Concentrations (ng/g)[1]:								
			77	81	126	169	105	118	138	153[2]	180
7701	40.5	434	34.6	7.69	18.7	1.66	778	1580	2290	3600	860
7701	-	400	31.3	7.07	17.2	1.58	-	-	-	-	-
7702	15.2	214	20.5	4.35	9.19	0.96	308	556	835	1370	341
7703	25.3	357	28.8	5.81	15.5	1.90	526	871	1510	2880	777
7704	16.5	233	25.4	4.35	10.1	1.20	257	668	969	1730	530
7705	16.3	247	28.8	4.48	10.7	1.46	247	631	980	1400	521
7705	19.8	228	26.7	4.20	9.82	1.12	400	668	1120	1960	546
7706	10.9	151	27.4	4.40	6.36	0.74	212	334	694	1130	288
7708	27.1	308	26.7	5.47	13.3	2.28	526	973	1880	3260	845
7809	23.0	320	16.5	5.89	13.8	1.77	451	808	1540	2610	733
7810	27.8	331	20.6	5.35	14.3	2.54	575	1110	1770	3070	762
7811	44.0	462	30.6	7.51	19.9	2.14	868	1660	2930	5190	1300
7811	-	559	38.8	9.67	24.2	2.62	-	-	-	-	-
7812	37.5	465	34.2	7.36	20.1	2.24	807	1360	2230	4200	1070
7812	-	543	34.0	8.99	23.6	2.67	-	-	-	-	-
Mean[3]	26.0	322	26.9	5.82	14.0	1.74	512	961	1610	2790	731
Median[3]	25.3	316	27.4	5.47	13.8	1.77	526	871	1540	2880	762

[1] PCB congener numbers are adapted from Ballschmitter and Zell (1980).

[2] Congener 153 concentrations also include a co-eluting congener (PCB 132).

[3] The mean of analytical duplicates was used instead of using both values to compute the means and median: N = 11 for both mean and median.

Table 12. Concentrations of PCB Congeners, Total PCBs, and TCDD-Equivalents in Eggs of Red-Breasted Mergansers from Lake Michigan, Door County, Wisconsin, Collected in 1990.

	Total PCB	Calculated TEQs	PCB Congener Concentrations (ng/g)[1]:								
Sample	µg/g	pg/g	77	81	126	169	105	118	138	153[2]	180
9001	12.5	207	27.0	3.74	8.97	1.19	239	590	747	1360	420
9001	13.9	203	24.9	3.35	8.78	1.19	278	531	860	1660	488
9002	15.8	166	15.3	1.97	7.19	1.14	387	628	1000	2010	480
9003	5.93	90.7	11.3	1.42	3.93	0.52	134	220	347	638	151
9004	13.3	156	15.8	2.72	6.72	0.88	264	466	845	1760	421
9005	14.6	216	32.3	3.77	9.35	1.44	269	534	929	1630	510
9006	9.43	141	16.6	2.15	6.13	0.94	205	403	614	1120	263
9007	14.2	188	23.0	2.86	8.16	1.37	254	594	925	1630	450
9008	5.53	82.8	17.4	2.03	3.54	0.43	75	184	328	530	150
9009	14.0	163	26.0	2.91	7.01	1.08	238	495	893	1630	511
9010	14.9	187	37.3	5.01	7.91	1.01	261	522	668	1250	296
9010	13.5	159	31.1	4.17	6.76	0.98	206	481	593	1090	263
9011	12.3	146	15.0	1.79	6.40	1.33	152	419	698	1230	344
9012	8.27	114	15.6	2.23	4.93	0.59	152	317	438	800	186
Mean[3]	11.7	151	20.4	2.63	6.58	0.99	218	443	704	1300	350
Median[3]	13.3	156	17.0	2.47	6.74	1.03	235	480	750	1370	382

[1] PCB congener numbers are adapted from Ballschmitter and Zell (1980).

[2] Congener 153 concentrations also include a co-eluting congener (PCB 132).

[3] The mean of analytical duplicates was used instead of using both values to compute the means and median: N = 12 for both mean and median.

substituted PCB congeners in complete extraction duplicates (including extraction and PGC column separation) were at least as precise as those in repeated PGC column separations of a single PCB extract.

Calculated TEQs were strongly correlated with total concentrations of PCB. Approximately 95% of the variability in calculated TEQs was explained by total concentrations of PCBs (Figure 10). Over 90% of the calculated TEQs were contributed by PCB congener 126.

Significant differences in concentrations of both PCBs and TEQs were observed between the two collection periods using both parametric (t-test) and non-parametric (median test) techniques (Figures 11 and 12). The median ratio of calculated TEQs to total concentration of PCBs was slightly greater for the eggs collected in 1990 than those from 1977-78 (Figure 13), but this difference was not statistically significant.

DISCUSSION

Concentrations of PCBs in the eggs of red-breasted mergansers collected in 1990 were similar to those measured in other bird eggs from the Green Bay area (Figure 14, Kubiak *et al.* 1989; Ankley *et al.* 1992; Yamashita *et al.* 1993). The observed concentrations were greater than lowest observed adverse effect levels (LOAEL) for PCBs in eggs of birds in the environment which are approximately 3-5 μg/g (Giesy *et al.*

Figure 10. Calculated TCDD-Equivalents (TEQs) as a Function of Total PCBs in Red-Breasted Mergansers.

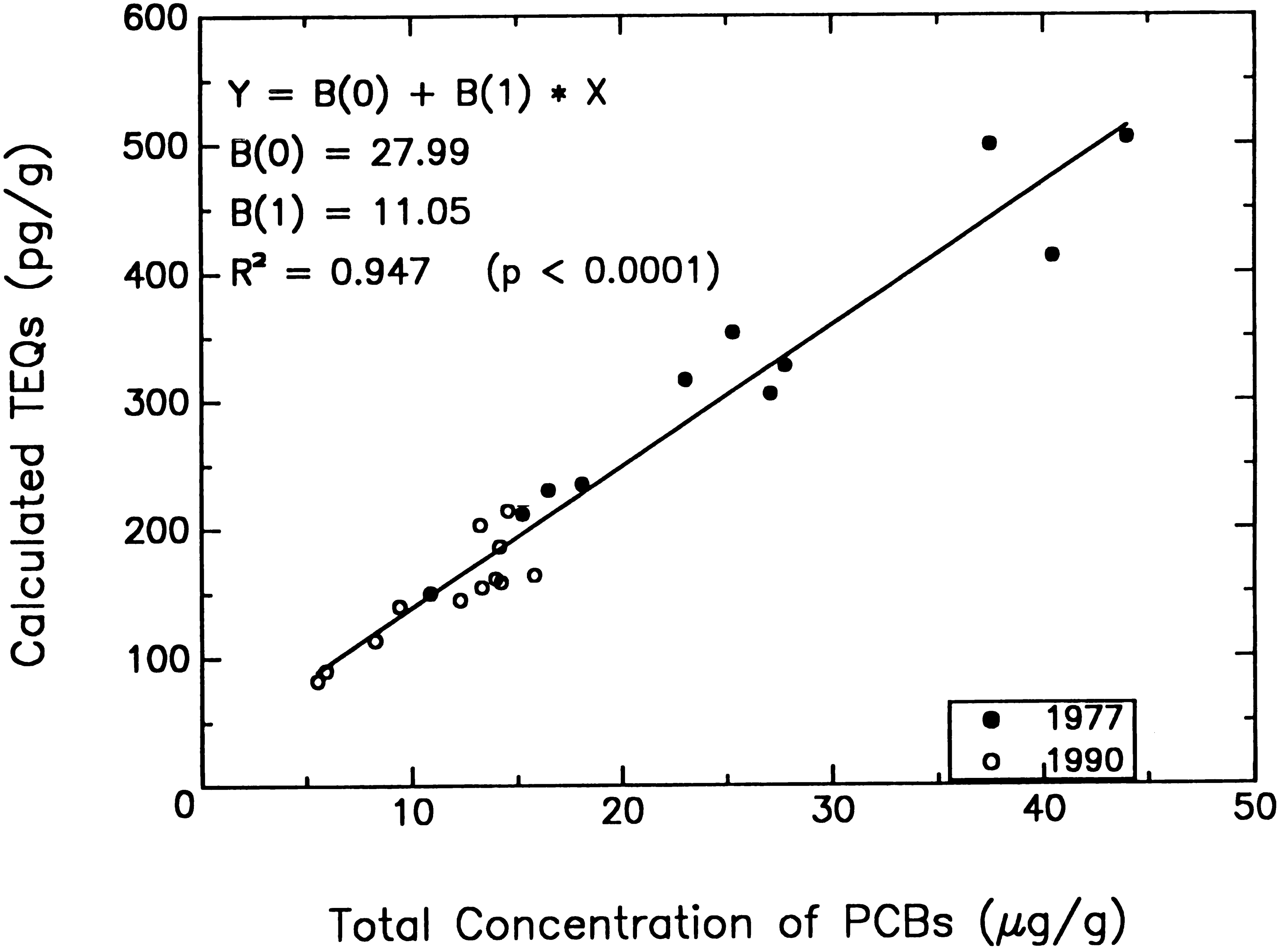

Y = B(0) + B(1) * X
B(0) = 27.99
B(1) = 11.05
R² = 0.947 (p < 0.0001)
Calculated TEQs (pg/g)
600
500
400
300
200
100
0
10
20
30
40
50
Total Concentration of PCBs (μg/g)
1977
1990

Figure 11. Total Concentrations of PCBs in Eggs of Red-Breasted Mergansers Collected in 1977-78 and in 1990.

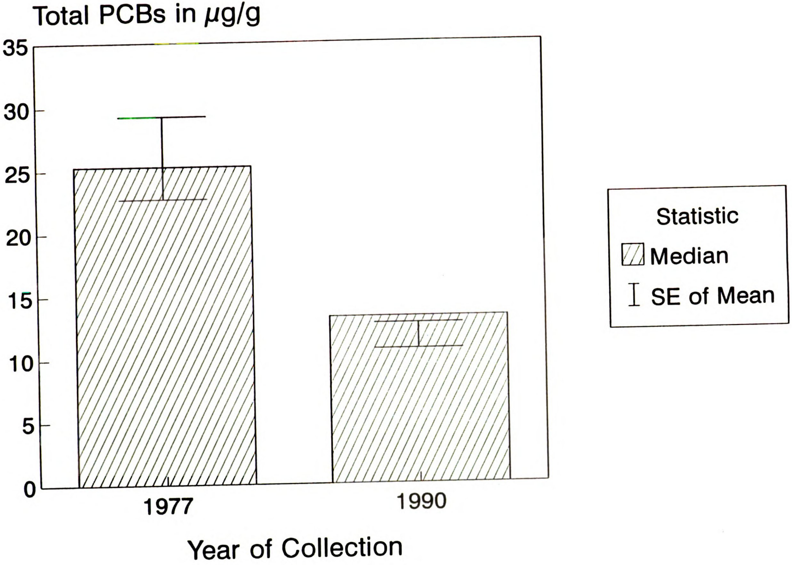

Total PCBs in μg/g
35
30
25
20
15
10
5
0
1977
1990
Year of Collection
Statistic
▨ Median
I SE of Mean

Figure 12. Concentrations of TEQs in Eggs of Red-Breasted Mergansers Collected in 1977-78 and in 1990.

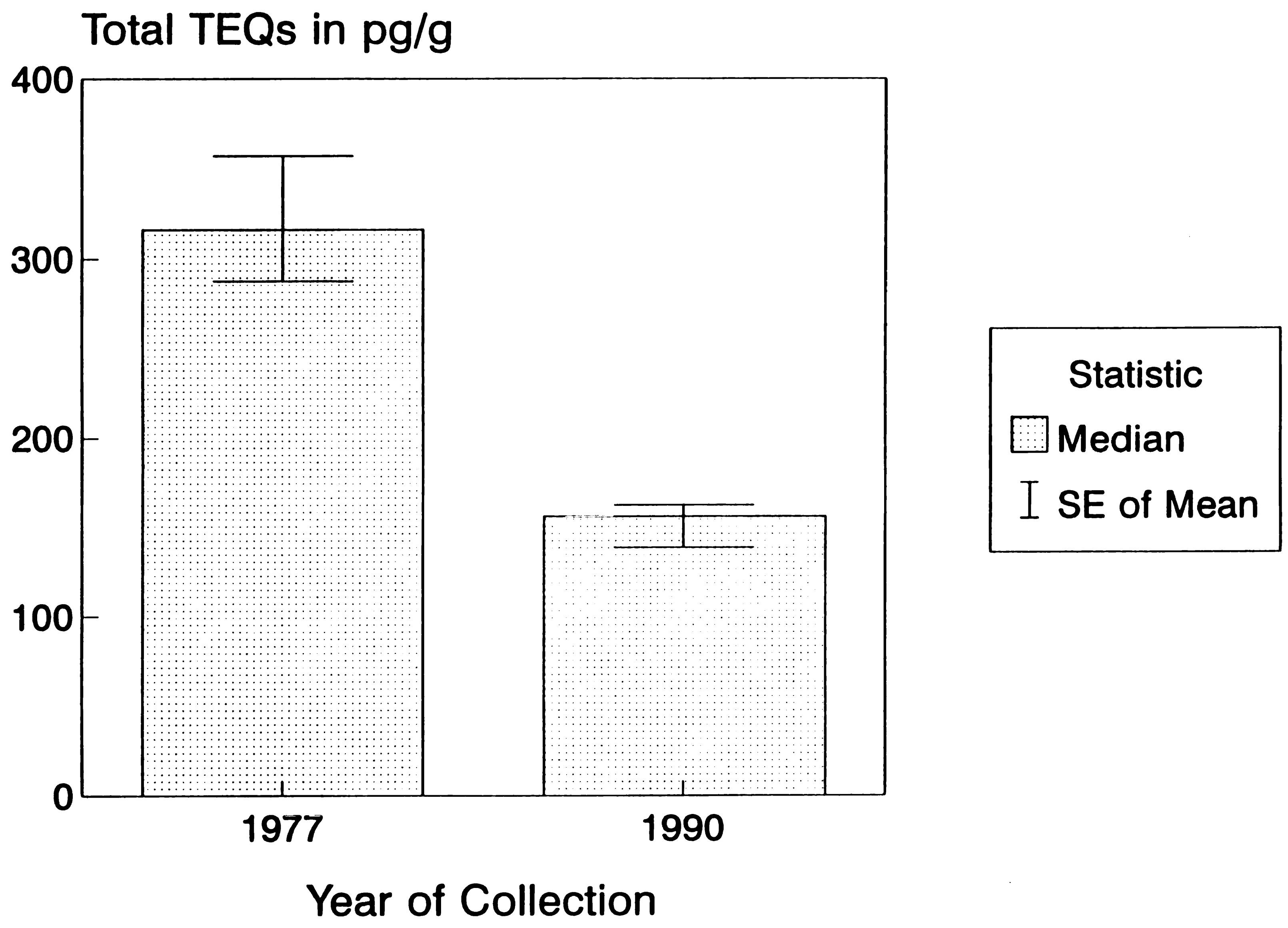

Total TEQs in pg/g
400
300
200
100
0
1977
1990
Year of Collection
Statistic
▨ Median
I SE of Mean

Figure 13. Ratio of Concentrations of TEQs to Concentrations of PCBs in Eggs of Red-Breasted Mergansers Collected in 1977-78 and in 1990.

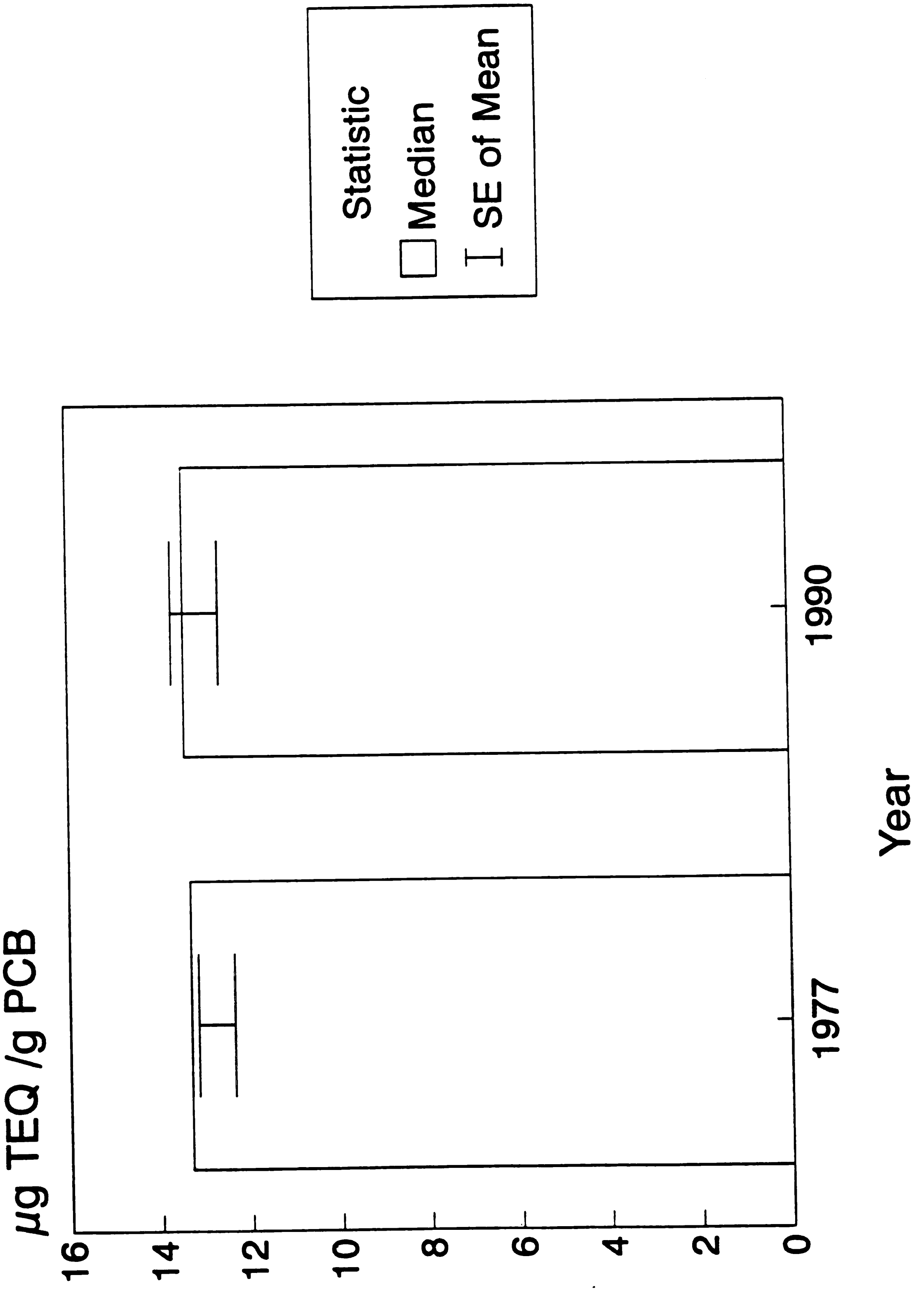
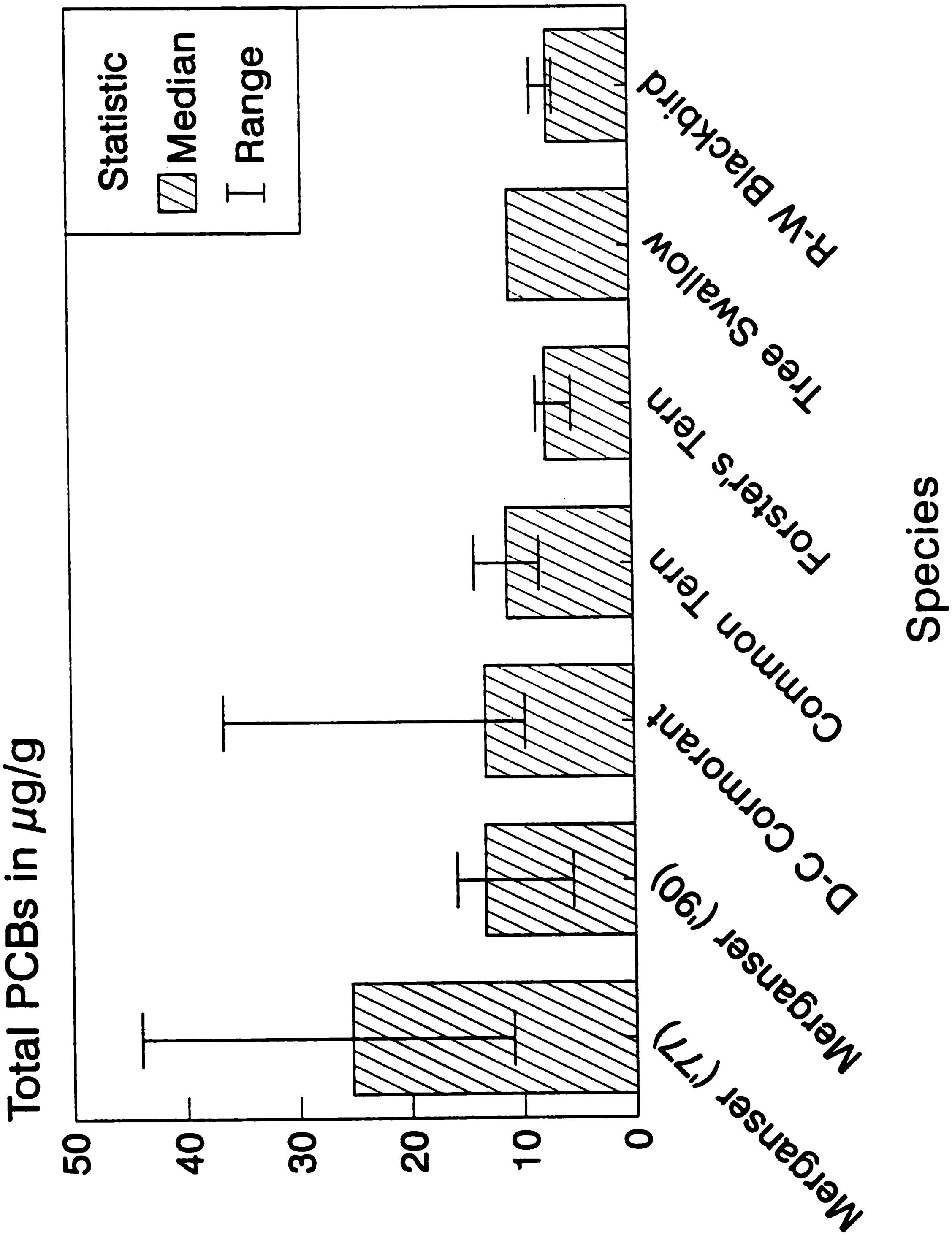

μg TEQ /g PCB
16
14
12
10
8
6
4
2
0
1977
1990
Year
Statistic
Median
SE of Mean

Figure 14. Total Concentrations of PCBs in Eggs of Birds from Near Green Bay, Wisconsin. Values for Red-Breasted Mergansers and Double-Crested Cormorants Are from This Work. Others Are Samples Collected in 1989 (Jones *et al.* 1993).

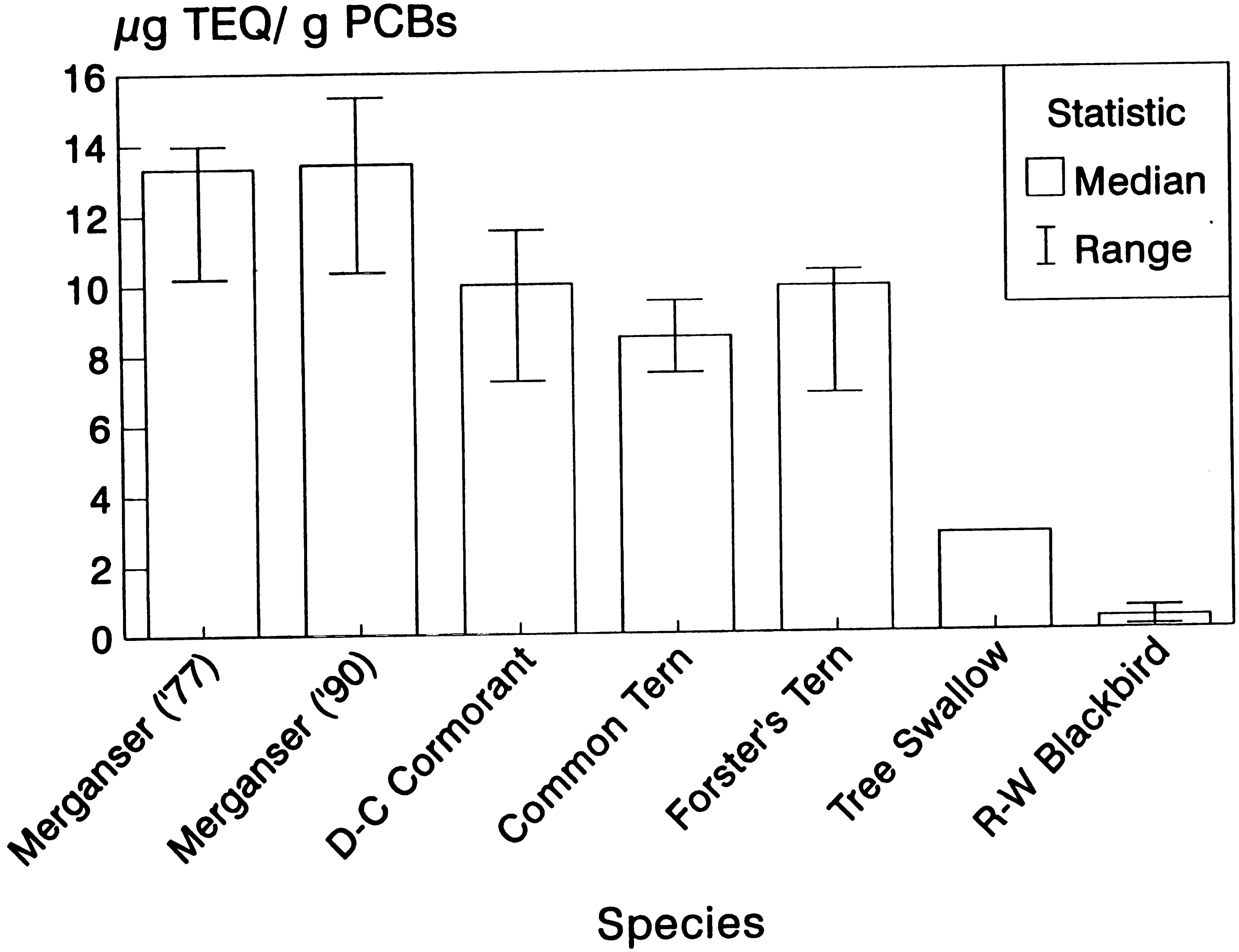

Total PCBs in μg/g
50
40
30
20
10
0
Statistic
Median
Range
Merganser ('77)
Merganser ('90)
D-C Cormorant
Common Tern
Forster's Tern
Tree Swallow
R-W Blackbird
Species

1993). In the laboratory, chicken eggs dosed with 5 µg/g Aroclor® 1248 exhibit 33% embryo mortality (Brunstrom 1991). Deformed chicks and overall reduced productivity of the Spider Island double-crested cormorant colony occurred at lesser concentrations in their eggs (13 µg/g) than present in the red-breasted merganser eggs. Red-breasted mergansers appear to be one of the least sensitive avian species to PCH reproductive toxicity, given 80% hatching success in both 1977-78 and 1990 (Haseltine *et al*. 1981; G. Heinz, U.S. Fish and Wildlife Service, pers. commun.).

Further evidence of the tolerance of red-breasted mergansers to toxicity from planar PCHs is provided by examining the potency of PCB mixture. The ratio of TEQs to total concentrations of PCBs was greater in the red-breasted merganser eggs from both 1977-78 and 1990 than it was in eggs from any other samples in Figure 15. Any possible antagonistic effects from non-planar PCB congeners would have been manifested the least in these eggs with the greatest TEQ to PCB ratio, yet no reproductive impairment was observed.

Ratios of concentrations of individual congeners to total concentrations of PCBs in these red-breasted merganser eggs were similar to those in eggs from other birds in the Great Lakes region (Table 9). The relative proportion of PCB 126 in the total concentration of PCB was consistent with other birds from the Green Bay area (Table 9). Tern eggs from Lake Poygan, a reference site away from the Great Lakes, contained

Figure 15. Ratio of Concentrations of TEQs to Concentrations of PCBs in Eggs of Birds from Near Green Bay, Wisconsin. Values for Red-Breasted Mergansers and Double-Crested Cormorants Are from This Work. Others Are Samples Collected in 1989 (Jones *et al.* 1993).


μg TEQ/ g PCBs
16
14
12
10
8
6
4
2
0
Statistic
Median
Range
Merganser ('77)
Merganser ('90)
D-C Cormorant
Common Tern
Forster's Tern
Tree Swallow
R-W Blackbird
Species

a lesser proportion of PCB 126 (Table 9). The relative proportion of PCB 126 was also greater in the eggs of red-breasted merganser and other bird species from the Great Lakes than the relative proportion of PCB 126 in any of the technical Aroclors®. This is significant because it indicates that PCB 126, which contributes over 90% of the calculated TEQs in the merganser eggs, was selectively enriched relative to the mixture of PCBs originally released into the environment. PCB 77 was not enriched in these samples relative to its proportions in Aroclors® 1242 and 1248. This was probably due to the fact that PCB 77 is more easily metabolized than the other non-*ortho* substituted congeners because of its adjacent, unsubstituted, *meta-para* positions on both rings (Boon and Eijgenraam 1988; Borlakoglu *et al*. 1988). The proportion of PCB 169 is difficult to evaluate relative to the technical Aroclors® because of the wide range of values reported for the proportion of PCB 169 in these mixtures.

The decrease in PCB concentrations observed between 1977-78 and 1990 continues a trend previously observed in eggs of red-breasted mergansers from this area (based on arithmetic means reported by Haseltine *et al*. (Haseltine *et al*. 1981). A first-order exponential decay curve fitted to total concentrations of PCBs in the eggs predicts a continued, but gradual, decrease in PCB concentrations (Figure 16). Based on the first-order model, the constant for depuration, k_d, was -0.132 (Equation 1) and the half-life ($t_{1/2}$) for loss of total concentration of PCB is 5.25 years (Equation 2).

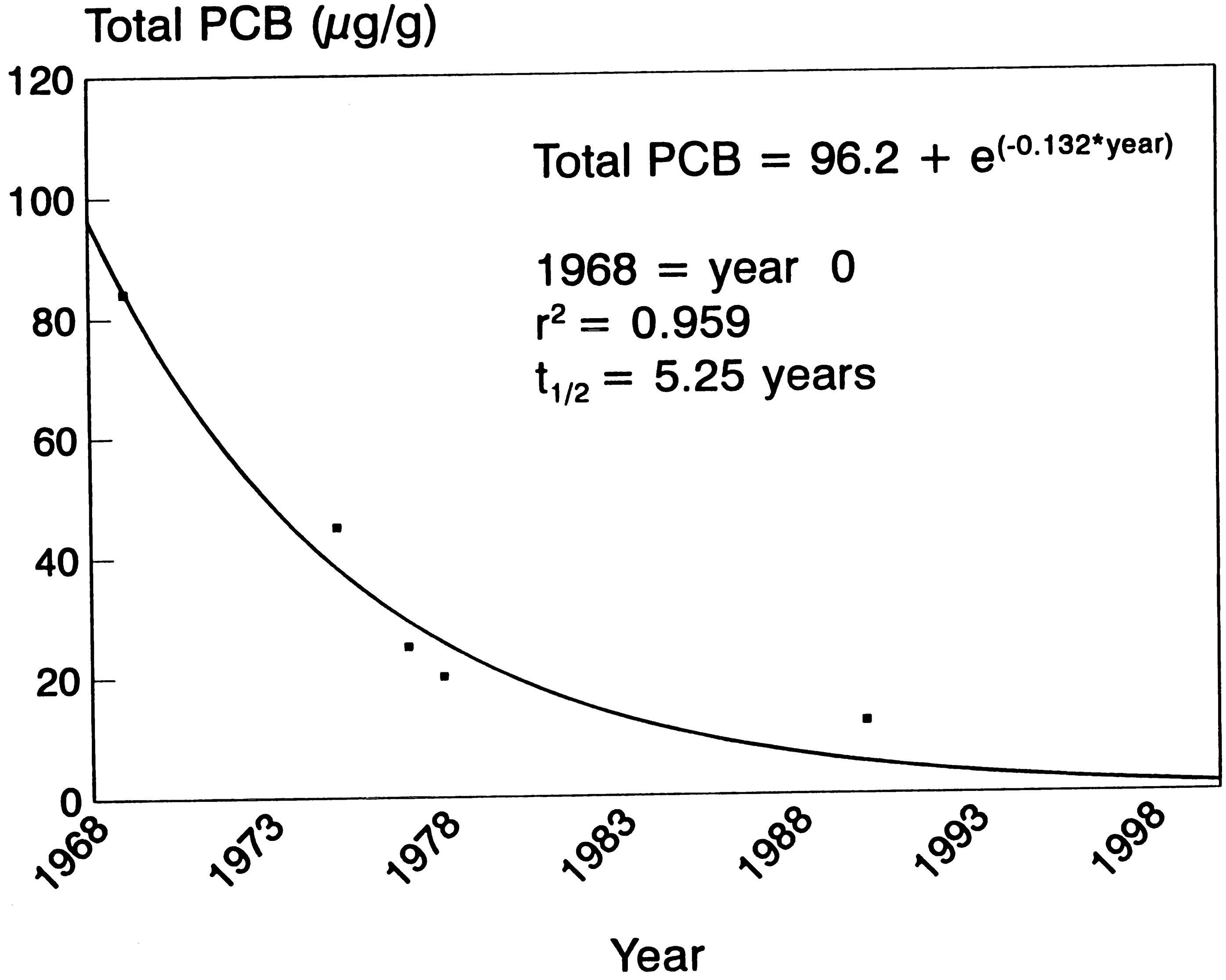
$$C_t = C_0 + e^{(k_d \cdot t)} \qquad (1)$$

$$t_{1/2} = \frac{\ln 2}{k_d} = \frac{0.693}{k_d} \qquad (2)$$

C_t and C_0 are concentrations at time t and time 0, respectively. Concentrations from bird eggs in the field should continue to follow this trend if no new sources are present. The similarity of TEQ to PCB ratios in the 1977-78 and 1990 samples suggests that the concentration of TEQs are declining in parallel with the PCB concentrations.

Several possibilities mitigate against this optimistic prediction. Approximately 65% of the total PCBs ever manufactured still have the potential to enter the aquatic environment (Tanabe 1988). PCBs continue to enter Green Bay and the Great Lakes from tributaries and point sources (Swackhamer and Armstrong 1988; Armstrong and Marti 1990; Hermanson *et al.* 1991). The Great Lakes region also receives atmospheric inputs of PCBs and other persistent compounds (Eisenreich *et al.* 1981). The observed concentration of PCBs in the eggs in 1990 was greater than that predicted from the exponential decay curve. More evidence is needed, however, before we can conclude that the rate of decline has slowed relative to the predictions of the model. Concentrations of

Figure 16. Decline in Total Concentrations of PCBs in Eggs of Red-Breasted Mergansers from Lake Michigan Colonies, Door County, Wisconsin.


Total PCB (μg/g)
120
100
80
60
40
20
0
1968
1973
1978
1983
1988
1993
1998
Year
Total PCB = 96.2 + e^(-0.132*year)
1968 = year 0
r² = 0.959
t1/2 = 5.25 years

PCBs in eggs of fish-eating birds should continue to be monitored at time intervals of several years.

The consistent ratio between TEQ and total concentration of PCB in eggs collected 13 years apart indicates that the potency of the PCB mixture to which embryos are exposed has not changed. This consistency was observed despite a significant decline in total PCB burden. Apparently, the source of PCBs to which the mergansers are exposed, primarily fish in their diet, has not changed over this time, since metabolic pathways in the mergansers are expected to be consistent. The consistency of the TEQ/PCB ratio indicates that any differential weathering of PCBs in the area of these colonies is insignificant. This is the first retrospective study that I know of that includes the non-*ortho* substituted PCB congeners.

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CONCLUSIONS AND RECOMMENDATIONS

The studies conducted with the porous graphitic carbon (PGC) column have demonstrated that it can be an efficient tool for separating complex mixtures of halogenated hydrocarbons on the basis of their planarity. Analysis of biological tissues fortified with mixtures of PCBs demonstrated both the precision and accuracy of this column in separating non-*ortho* substituted PCB congeners from other PCB congeners. The incorporation of this column in an automated HPLC system produced a more reliable and efficient method for this type of separation than had been previously available with "home-made" carbon columns. Although biochemical measurements which integrate the toxicity of complex mixtures are available and useful, this type of chemical residue analysis continues to be important in identifying specific toxicological agents. The identity and quantities of toxic components within complex mixtures is vital in understanding changes in the mixtures, identifying sources, and prioritizing prevention and remediation efforts.

The method developed with the PGC column was used successfully to measure concentrations of the non-*ortho* substituted PCB congeners in environmental samples. In one study, the analysis of the non-*ortho* substituted PCB congeners demonstrated that variation in reproductive success among birds within a single colony was the result of variation in sensitivity among birds rather than being the result of differences in the potency of the PCB mixture among bird eggs. The double-crested cormorant eggs from nests with low productivity or which produced a cross-billed chick did not have significantly different concentrations of PCBs or TCDD-equivalents from those which exhibited high productivity. This implies that metabolic difference among birds did not produce changes in the congener distribution which might have altered the potency of the PCB mixture to which the embryos were exposed.

The PGC separation method was also used to investigate changes in PCB composition and potency over time in eggs of red-breasted mergansers. PCB mixtures in eggs collected in 1977-78 and in 1990 contained a greater proportion of the most potent PCB congener, PCB 126, than did technical Aroclor® mixtures. However, the proportion of the total TCDD-equivalents in the PCB mixture in the eggs was not significantly different in the eggs collected 13 years apart. The decline in total concentrations of PCBs observed since the early 1970s appears to represent a real decline in toxic potency contributed by PCBs.

The PGC method described here has been optimized for separation and measurement of non-*ortho* substituted PCB congeners in samples from the Great Lakes. This method is fast and inexpensive. Additional work with this column and the longer, 100 mm PGC column may allow us to answer questions about the contributions of some the other planar, halogenated hydrocarbons which also are capable of acting through the *Ah* receptor and eliciting toxic responses. Some of these compounds were listed in Chapter 2. The significance of the contribution of these other classes of compounds to the integrated *Ah*-mediated response could be determined by comparing the compounds present in an extensive fractionation from the PGC column with the activity of these fractions in an EROD assay. Questions of additivity of response in environmental mixtures could also be investigated with these two tools. The EROD activity of extracts from environmental samples could be measured before and after fractionation on the PGC column.

APPENDIX A

STANDARD OPERATING PROCEDURE:

SEPARATION OF PCB CONGENERS WITH HYPERCARB PCB HPLC COLUMN

Michigan State University
Aquatic Toxicology Laboratory
Pesticide Research Center
Department of Fisheries and Wildlife

Prepared by: Lisa L. Williams

I. SCOPE

This procedure separates PCB congeners on the basis of affinity for porous graphitic carbon packed in a commercial HPLC column and quantitates PCB congeners using GC-ECD. The samples are PCB extracts from which lipids and pesticides have been removed.

II. REFERENCED DOCUMENTS:

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III. TERMINOLOGY:

PCB	polychlorinated biphenyl
congener	a PCB compound with a specific chlorine substitution pattern. PCB congeners are referred to by their IUPAC numerical designation [1]; e.g. 3,3',4,4'-tetrachlorobiphenyl is PCB 77.
coplanar	PCB congeners which can assume approximately planar configurations; primarily nonortho-substituted congeners, but also monoortho-substituted congeners.
GC-ECD	gas chromatography with electron capture detection
ECD-active	ECD-active compounds have strongly electronegative functional groups and are readily detected with an electron capture detector
HPLC	high pressure liquid chromatography

IV. SUMMARY OF METHOD:

An automated HPLC system is used to separate a PCB extract into two fractions. The automated system consists of an autosampler, quaternary pump, 6-port air-actuated switching valve and a fraction collector. Events in each of these components are coordinated by the autosampler. A known volume of PCB extract is loaded onto the Hypercarb PCB column in the forward direction. Most congeners are eluted in the forward direction with 100% hexane. Non-ortho-substituted congeners are eluted in the reverse direction following a linear gradient to 20% hexane in dichloromethane (DCM).

V. SIGNIFICANCE AND USE:

The coplanar congeners are the most toxic PCB congeners. They co-elute with less toxic congeners in gas chromatography (GC). This method allows the nonortho-substituted coplanar congeners to be separated from the interfering compounds and concentrated before GC analysis.

VI. INTERFERENCES:

The PCB extract loaded on the column in this procedure should already be free of polar compounds. ECD-active compounds that have been observed in significant concentrations in environmental PCB extracts include hexachlorobenzene, p,p'-DDE and occasionally trans-nonachlor. Loading studies with a variety of compounds have demonstrated that only planar compounds elute in the coplanar fraction. DDE, for example, elutes immediately following the void volume. Polychlorinated diphenyl ethers (PCDEs) deviate from coplanarity such that they also elute in the forward direction with 100% hexane. Studies with Aroclor 1254 indicated that interfering PCB congeners do not enter the coplanar fraction unless the total PCB loading is equal to or greater than 100ug. For comparison, the loading capacity of the hand-packed column MSU-1 was only 15 μg of total PCB.

Classes of planar compounds which can elute in the coplanar fraction include polychlorinated napthalenes (PCNs), polychlorinated dibenzofurans (PCDFs), and polychlorinated dibenzo-*p*-dioxins (PCDDs). The GC retention time of one of the components of the technical PCN Halowax 1014 is near that of PCB 77. The presence of Halowax components is determined by monitoring 8 other prominent Halowax peaks in the GC chromatogram of the coplanar fraction. If PCNs are determined to be present, the fraction must be analyzed by mass spectrometry or subjected to additional purification on deactivated alumina [4]. Both of these procedures are beyond the scope of this SOP. PCDFs and PCDDs are generally present in Great Lakes biota in concentrations approximately 1000 fold less than those of coplanar PCB congeners, so they should not be a measurable interference.

Benzene or toluene in the PCB extract will distort the elution profile. Extracts should be completely exchanged into iso-octane.

VII. APPARATUS

A. Instrumentation:

1. Perkin Elmer Series 410 HPLC pump:
   This microprocessor controlled quaternary solvent delivery system has 4 solvent gradient capability including step, linear, convex and concave gradients. Solvent mixing is accomplished on the low pressure side of a single pump. The flow

range is 0.01 to 10.0 mL/min with a maximum pressure of 6100 psi. Surfaces in contact with solvents are stainless steel, glass, sapphire or fluorocarbon polymer. Software control includes method chaining, 10 programming steps per method, storage of 9 methods, and the ability to edit active and inactive methods. Connections include those for autosamplers, data systems and a printer. Two timed events (TE1 and TE2), which are signaled by contact closures, can be programmed. Other contact closure signals include EXT RUN, EXT STOP, TRAY ADV, RUN OUT, RDY1 and RDY2.

2. Hypercarb PCB HPLC column:
   The Hypercarb PCB column is a porous graphitic carbon column manufactured by Shandon Scientific (U.K.) and available in the United States from Keystone Scientific (Bellefonte, PA) and Alltech (Deerfield, IL). The column used to develop this SOP was column serial #109. The dimensions of the column are 50mm x 4.6 mm ID with particle diameters of 7 μm.

3. Perkin Elmer ISS 100 autosampler (AS):
   This is a microprocessor controlled autosampler with a Rheodyne model 7125 syringe loading sample injector. The mobile needle assembly allows random sample access as well as positioning over the sample and flushing ports. The injector is equipped with a 2000 μL loop which is filled in $\leq$200 μL increments while not under pressure and is part of the flow path from the pump to the column except during this fill cycle. The AS can be programmed for a manual or automatic mode of operation, but no method storage is provided. The AS includes contact closures for 5 timed events (E1-E5), outputs for START, STOP, and 2 inject time signals (INJ1, INJ2) and relay inputs for READY, inject (INJ), and RUN/STOP. All contact closures are 500ms pulse time closures rated for 50V DC and 500 mA.

4. Isco Foxy fraction collector (FC):
   The FC is a programmable, microprocessor controlled X-Y fraction collector equipped with a flow diverter valve. The diverter valve directs flow to waste while the collection arm moves to prevent pressure build-up and also directs unwanted eluent to waste. Uniform fractions are set by time, drop count or volume and peaks can be detected by time windows, slope and threshold of

detector signal and combinations of these methods. Our system is not configured for peak detection based on detector signal. Four programs can be stored. Collection programs can be repeated each time a restart signal is received via the INJECT input port. Other connections are available for a detector, RS-232-C connection, a valve controller and a pump.

5. Valco 6-port valve with Digital Valve Interface: The Valco model A6UW valve is a 6-port, 2-position, helical drive, air-actuated valve with 1/16" fittings. It is rated for 5000 psig. The valve is configured to allow both forward and reverse flow through the column: ports 1 through 6 are connected to the AS, front of the column, port 4, port 3, the detector/FC, and the back of the column, respectively. For forward flow (inject position), the solvent goes from port 1 to 2 to the column to port 6 then to port 5. For reverse flow (load position), the solvent goes from port 1 to port 6 to the column to port 2 to 3 to 4 to 5.
   The digital valve interface (DVI) is an electronic controller which supplies air pulses to the valve's air actuator in response to either operation of the manual toggle switch or contact closure though a remote signal cable. A signal to the DVI causes the interface circuitry to provide a 2 second pulse to a 3-way solenoid which then allows an air pulse to flow from the compressed air tank to the air actuator. Separate connections and, therefore, signals are used to signal switches to the inject and load positions. Contact closures for timed events synchronized with the inject signal are provided on the DVI, but are not configured on our system.

B. Automation connections:

The system is set up so that the autosampler provides primary control of the system. It has contact closure connections to external devices which provide synchronization with injection (INJ1, INJ2) and timed events (E1, E2 .. E5), and accept synchronization with ready status of other devices (READY). All ports are set to the NO (normally open) position. The INJ1 and INJ2 ports are connected to external start ports on the fraction collector (INJECT) and pump (EXT RUN), respectively. The contacts in these ports close momentarily at the time the AS injection is made, so the FC and pump receive a signal to begin their programs. The E1 and E2 timed events ports on the AS are connected by multi-wire ribbon to the DVI. The common, or ground wire, on the ribbon is connected to E1 and a splice wire connects that to E2. The contact closure for E1 (black wire) and E2 (blue wire) are made at the times specified in the AS program and signal the DVI to switch the valve to the LOAD and INJECT positions, respectively. The LOAD position of the valve corresponds to reverse flow through the column and the INJECT position corresponds to forward flow. The READY port of the AS is connected to the RDY1 port on the pump. The AS program will not begin until the pump completes Step 0 of its method and sends a ready signal to the AS.

The automation connections on the pump include the EXT RUN and RDY1 ports which are connected to the INJ2 and READY ports on the AS, respectively. The pump also has timed event ports (TE1, TE2). TE1 is connected to the external stop (EXT STP) port on the pump so that an event can be programmed to shut the pump off after the last sample is injected. (See programming section.) For this connection, connections for common (C) and normally open (NO) on TE1 are connected to RET and SIG, respectively, on the EXT STP port.

The only automation connection on the fraction collector (FC) is the one for the external start (INJECT) which is connected to INJ2 on the AS. The ground wire for this is connected to the GND port on the FC.

C. Plumbing connections:

All connections are Parker style (also known as Parker-Hannifin), except possibly those on the Valco valve. These were the standard fittings used by Perkin-Elmer on their HPLC equipment at the time of purchase and installation of the system. Parker fittings also came standard on the Keystone PCB Hypercarb column and the Phenomenex CN column. Reusable stainless steel nuts with Vespel ferrules are used in some connections (Keystone Scientific, Bellefonte, PA, (800)437-2999, Part No. 36027 and 36024). These connections are universal, rated to 5000 psi and can be used over and over without fatigue. To attach to column, put nut and ferrule onto SS tubing, then place tubing into column until butted against frit. Finger tighten nut until very snug. No wrenches required and you probable can't over tighten with fingers alone.

Solvent filters (2 μm) purchased from Phenomenex (AFO-0359) are for 1/8" tubing. Filter size of original Perkin Elmer filters are unknown at this time.

D. **Operation in Automated Mode (with programming):**
All three instruments (pump, FC, and AS) must be programmed to execute automated runs with fraction collection. Method storage is provided for the FC and pump, so once methods are developed they need only be recalled to set up a queue. The following section describes the programming required to inject multiple samples, each run on the same gradient elution and multiple fractions collected for each.

The following conventions are used to describe programming options:

<Key>	(hard key) key actually present on keyboard
KEY	(soft key) option provided in display which is selected with a corresponding soft key on the keyboard (e.g. A, B, F1, F2 etc)

1. Pump programming:
The pump programming software has two modes: EDIT and ACTIVE. Methods are written, edited and stored off-line in the EDIT mode. The ACTIVE mode is used for making on-line changes in system conditions. EDIT appears as a softkey option in the ACTIVE mode and the <Use> key is used to get from EDIT to ACTIVE. <Return> goes to previous screen while retaining changes and <Quit> goes to previous screen without retaining changes. Programming begins in the EDIT mode.

Example:

NEW, PUMP

Step	Time	Flow	%A	%B	%C	%D	Curve
0	17	3	100	0	0	0	
1	20	3	100	0	0	0	
2	5	3	0	100	0	0	1
3	12	3	0	100	0	0	
4	3	3	100	0	0	0	1

Explanations:
Use <Insert> to add new lines.
"Time" refers to minutes required for that step.
Flows are in mL/min.
"Curve" determines the gradient. A gradient of +1 is a linear progression from the

conditions at the end of the previous step to the conditions specified in the current step.

T.E.
T.E. 1 *30.0
Explanations:
T.E. 1 is an automatic shut-off. The asterisk indicates the time that is allowed to elapse in Step 0. If Step 0 continues for 30 minutes without the pump receiving a start signal from the AS, the pump will shut off.

PRES
MIN 200 MAX 4000
Note: Setting MIN at less than 200 can result in the pump shutting off when it receives the start signal from the AS.

<Store>, <Use>

2. Fraction Collector Programming:
The fraction collector is programmed with a series of menus in a branching configuration. <Enter> is used to accept entries, <Back Step> is used to go to previous screen, and <Clear> is used to clear entries. A <Help> key is available. Example for collecting in test tubes:

EDIT A
TIME TIME
Windows? YES
Programming for: Rack 1
Tubes: 12/13mm 144 tubes/rack <Enter>
Collection options? Peak=Rack 1
Non-peak=Valve <Enter>
Size: 2:00 min. <Enter>
PEAK Windows only: Window 1 1:00 to 25:00
Window 2 25:00 to 39:00
Window 3 <Exit>
DELAY 0:00
RESTART Momentary, 1 collection, skip tube
INIT. WASTE No
<Back Step>
STORE AS: A
<Back Step>, <Back Step> to READY A

3. Autosampler Programming:
No methods storage is provided, so the AS must be programmed every time. Everything is cleared from memory by pressing <CE>, <Manual>. Then press <Auto> to begin programming. The keys for the

parameters themselves should not be pressed. Entering data or zeroes advances the display to the next parameter. Example for 2 samples:

Set	First	Last	Vol.	Rep.	Time A	Time E
1	3	4	900	1	0.1	0
0						

4. Starting the Queue:
   The order is important. Assume that all instruments are programmed and ready.

   FC: (After READY A) RUN ("Waiting for restart...." should be displayed)

   AS: <Run> (Ready light should now blink)

   Pump: (from EDIT mode) USE, QUE (specify the method that was stored and first and last vials such that the same number of samples are run as are listed in the AS method), LINK, <Return>, STRT

   The pump will now execute Step 0 while the AS waits for a ready signal from it. At the end of pump Step 0, the AS will receive a ready signal and start its injection cycle. Upon injection, signals will be sent to both the FC and the pump. The FC will start its program and the pump will proceed to Step 1.

E. Operation in Automated Mode (from stored programs):

Routine operation will require recalling methods on the fraction collector and pump followed by queue linking on the pump and programming of the autosampler:

Pump: RCL Method 2, USE, QUE (specify vials), LINK
AS: same as for example above
FC: LOAD A, READY A, RUN A
Pump: STRT

VIII. REAGENTS AND MATERIALS:

A. Solvents

All solvents are Burdick & Jackson high purity solvents. Solvents used are hexane, dichloromethane (DCM), acetone, iso-octane and methanol.

B. Materials

The following glassware is required to run one sample:

1 Pasteur pipette
3 250 mL round bottom flasks
1 screw-top 2 dram vial

Pipettes are used as received. Vials are baked overnight at 500 C and capped with Teflon-lined septa. Flasks and miscellaneous glassware are washed in detergent then rinsed at least 3 times each with warm tap water, Millipore deionized water, acetone and hexane.

Other equipment:
- Various capacity Rainen Pipet-Men
- Bucchi Roto-evaporator
- Organomation Associates N-Evap concentrator
  - The nitrogen used in the concentrator is purified with an in-line trap containing a desiccant and activated charcoal.

IX. HAZARDS AND PRECAUTIONS

The solvents used in this procedure are volatile, flammable and toxic. PCBs are toxic and teratogenic. All procedures should be carried out in an approved fume hood away from open flames. The operator should wear solvent-proof gloves when handling solvents, standards and extracts.

HPLC pumps operate at high pressures, so the system must be treated with care. Minute leaks are capable of subcutaneous injections of solvent. No such problems have been observed with the system described here. The only solvent/pressure problem experienced has been with the purging process. Occasionally, high pressures build up and solvent escapes explosively when the syringe is loosened. Gloves and goggles are mandatory for this process.

X. SAMPLE PREPARATION

Extracts containing a mixture of PCBs in iso-octane should be prepared using protocols which remove PCBs from environmental matrices and separate them from lipids and pesticides (e.g. MSU-ATL SOP for Analysis of Organochlorine Pesticides and PCBs in Muscle and Egg Tissues of Fish and Birds). The final extract should be prepared in iso-octane and the volume of extract adjusted to a known volume of one mL or less. The gram-equivalents of sample (g/mL) and the total concentration of PCBs (μg/mL) in the extract must also be known.

XI. PREPARATION OF APPARATUS

An HPLC separation begins with sparging of all solvents to be used that day. Sparging with helium removes dissolved gases which would form vapor bubbles under the pressure in the system. Helium is bubbled somewhat vigorously through each solvent. The sparging line must be rinsed between solvents with the next solvent to be sparged. The weakest solvent should be sparged first to lessen the impact of any possible carry-over. The sparging line must be removed from the solvent before turning off the gas because of an increase in helium flow. Sparge for approximately 5 min per liter of solvent. Sparging is most important for polar solvents and those used under high (>1000psig) pressures. Solvents are sparged in the hood with a loose-fitting aluminum foil cap. After sparging the solvent inlet lines from the HPLC are placed in the solvent bottles and they are capped as tightly as possible with aluminum foil. When not in use, all lines are stored in a large solvent-rinsed flask to prevent contamination. Parafilm can be used around the Al foil, but organic solvent will dissolve it and gunk up the works (i.e. contaminate the solvent). A pressurized capping system would be far more desirable, but this is expensive.

The hexane in the reservoir for the autosampler should also be sparged. This should be done before the AS is turned on since the AS executes a flushing cycle immediately. If any gas bubbles remain in the rinsing or dosing pumps after the automatic 4 flushing cycles, the AS must be flushed again by pressing <Flush> until all bubbles are gone.

The next step is to purge the pump and thereby remove air in the inlet lines and pump:

1) Put on goggles and solvent-proof gloves.
2) Attach a 50 mL syringe to the valve on the front of the pump.
3) Select <Purge>
4) Enter a flow rate ≥ the flow rate in the program to be run.
5) Select the weakest solvent first.
6) Open the valve one complete turn and slowly draw back the plunger until ~25 mL have been withdrawn.

   Notes: You may have to really pull hard on the plunger. If you pull too fast, bubbles will form because of the vacuum you are creating rather than because of gas truly in the line.
7) Switch to sequentially stronger solvents and pull ~25mL of each.

8) Reverse the process until the pump and lines again contain the weakest solvent. Only a few mL's of each solvent need to be drawn for this part. This reverse gradient must be used to prevent bubbles and/or 2-phase systems from abrupt solvent polarity changes. Pump methods starting with the weak solvent can now be run. The pump will not progress from the equilibration step without an INJECT signal from the AS.

The pressure drop should be checked and recorded in the HPLC System Log Book (along with the solvent and flow rate) while the pump is equilibrating. Abnormally low pressure (<1000 psig under the conditions described in the **PROCEDURE** section) may be the result of a leak or a solvent inlet filter which is partially above the solvent. If air has entered the system, the pump will need to be purged again. Abnormally high pressure (>2000 psig under the conditions described in the **PROCEDURE** section) may be because a more viscous solvent than expected is being used, a frit has become plugged, a bubble has formed in the system, the column is degrading, or a more viscous solvent was not completely rinsed out. Bubbles can be cleared from the head of the column (sometimes) by setting flow rate such that pressure is less than 50 psig and then slowly increasing it to the operating flow rate. Reversing flow direction may also work. If residual viscous solvent is the problem, then equilibrating longer at the end of the program may be required.

The fraction collector lines should also be rinsed during equilibration. A collect cycle should be initiated with a waste beaker under the drop former. At least 25 mL of solvent should be run through the line. The drop former contains a polypropylene ferrule which is not a problem during routine operation. When the FC is not in operation, residual solvent in the drop collector can penetrate to the ferrule and extract contaminants. Many peaks were observed on GC chromatographs from the first 22.5 mL of hexane elution when this pre-rinsing was not performed. A hexane rinse is sufficient. GC analysis of subsequent dichloromethane and toluene fractions showed excellent baselines.

The autosampler withdraws sample from the vials by suction. The caps must be loosened or removed from the vials or a vacuum may be created in the vial. This vacuum prevents the complete volume from being sampled. This is especially critical for volumes exceeding 500 μL and when the vial contains little headspace. A 100% Teflon, single-pierce septum (National Scientific, #C4010-10) can be used in place of the bilayer septum.

Summary:
1) Sparge solvents
2) Purge solvents
3) Program pump
4) Equilibrate pump
5) Record pressure
6) Rinse FC collect tubing
7) Remove or loosen vial caps

XII. CALIBRATION AND STANDARDIZATION:

A. HPLC System

Variation has been observed in the elution profiles of Hypercarb PCB columns ([2], Shandon Scientific, pers. commun.), although among-lot variation seems to have decreased since the columns were first marketed [3]. Elution profiles must be confirmed on each column. The column described in the apparatus section has been characterized for elution profiles of individual congeners, Aroclor mixtures, PCNs, PCDFs, PCDDs, and several ECD-active pesticides. The elution profile should be confirmed by triplicate injection of a standard mix of congeners and injections of Aroclor 1254 at loadings of 1, 10, 50, 80 and 100 before each project. The profile should be confirmed weekly or following any sudden changes in back-pressure with either a standard mix of congeners or the PCB extract of a matrix spike.

Recoveries of the nonortho-substituted congeners in standards injected directly on the Hypercarb column range from 80 to 123% (Table 13). Possible interfering congeners (PCB 110, PCB 149, PCB 129, PCB 171) loaded at 35 ng each were completely eluted within 2 min (10 mL) of 100% hexane in the forward direction. No carry-over was detected in the coplanar fraction (See [5], p. 66).

Table 13. Recovery of PCB Congeners Injected on Hypercarb PCB Column.

Congener	Percent Recovery	CV (n=3)
77	79	19%
81	86	15%
126	97	9%
169	114	7%

B. GC-ECD System

Linear range of response for PCB congeners is determined by multiple injections of a PCB standard mix (e.g. see LLW-III, p.78) at concentrations ranging from 5 to 100 pg/ul in 1 ul injections. The concentrations of the internal standard PCB 30 is nominally 200pg/ul in every injection (See Appendix A-1 for explanation of PCB 30 stock concentration). The linear range for a given congener is that range for which the relative response factor (RRF) is within 20% of the mean RRF. A standard mix within the linear range is injected daily. If RRFs for >90% of the congeners are within 20% of the historical mean, then the system is considered to be within control. Control charts are maintained for the area of PCB 30 and the RRFs for selected congeners. Congeners most likely to be out of statistical control are the non-ortho-substituted congeners. They are preferentially retained in the injector by small particles of septa and/or the graphite liner seal. Chromatograms must be monitored for poor peak shape of PCB 77, 81, 126 and 169.

NOTE: If samples are injected by hand, then standards must also be injected by hand. Substantial differences in response (2x) have been observed consistently for late eluting peaks.

XIII. PROCEDURE

The following describes separation and analysis for 3 samples.

A. HYPERCARB PCB HPLC SEPARATION

1. Prepare HPLC system as described above.

2. Use total concentration of PCBs to calculate volume (uL) of PCB extract to load onto HPLC system. Approximately 50 μg of total PCB should be loaded if sufficient extract is available.

3. Check volume of PCB extract in vial against a calibrated vial. Allow approximately 40 μL extra which autosampler (AS) will not be able to withdraw. If volume is close, add up to 3 layers of time tape to bottom of vial to elevate it in the AS.

4. Record volume, g-equivalents and mass of total PCB to be loaded in the project notebook and the HPLC Log.

5. Replace screw top lids with those with Teflon-only septa and place vials in the autosampler (AS).

6. Program fraction collector for 10 custom positions (Method C):

```
EDIT C
TIME     TIME
   Windows? YES
   Programming for:  Rack 1
   Tubes:   custom rack, 10 tubes <Enter>
   Tube position 1 is front left and 10 is
   right rear with 2 tubes per row.
   Collection options?  Peak=Rack 1
                        Non-peak=Valve   <Enter>
   Size:    7:30 min.  <Enter>
PEAK     Windows only:  Window 1   0:00 to 22:30
         Window 2  22:30 to 0:00
         Window 3   <Exit>
DELAY 0:00
RESTART  Momentary, 1 collection, next tube
INIT. WASTE  No  <Back Step>
STORE AS:  C     <Back Step>, <Back Step>
   to READY C
```

7. Place 250 mL flattened round-bottom flasks in the first nine positions in the FC. Label them as fractions 1,2 and 3 of the first extract, then the three fractions of the second extract, etc. Note the shortest path pattern of the FC.

8. Confirm flask positioning by pressing READY C, RUN C, <Run>, <Next Tube>, <Next Tube>, etc. The press <Stop>, <Stop>, RUN C
("Waiting for restart...." should now be displayed)

9. Program AS in the Automatic mode for the three extracts. Everything is cleared from memory by pressing <CE>, <Auto>. Then press <Auto> to begin programming. The keys for the parameters themselves should not be pressed. Entering data or zeroes advances the display to the next parameter. The following example results in 800 μL of the first extract and 650 μL of each of the second and third extracts being sampled. Time E1 at 6 min. switches the column flow to reverse. Time E2 at 30 min. switches the flow back to forward.

Set	First	Last	Vol	Rep	Time A	Time E	Time E	Time E
1	1	1	800	1	30	1,6	2,30	0
2	2	3	650	1	30	1,6	2,30	0
0								

10. Confirm that column flow is in the forward direction by checking the indicator light on the valve controller and the position of the stop peg on the valve itself.

11. Program or recall Method 3 on the pump:

Step	Time	Flow	%A	%B	%C	%D	Curve
0	2.0	5.0	100	0			
1	6.0	5.0	100	0			
2	5.0	5.0	20	80			1
3	16.0	5.0	20	80			
4	3.0	5.0	100	0			1
5	8.0	5.0	100	0			

```
T.E.
   T.E. 1     *20.0
PRES
   MIN 0      MAX 4000
```

If programming, then STORE the method and hit USE to return to ACTIVE mode.

12. Program queue on the pump (from ACTIVE mode): QUE (specify the method that was stored and first and last vials as 1 and 3, respectively), LINK, <Return>, STRT. The pump should start step 0 and display queue notation on the status screen.

13. Confirm flow and pressure for pump and waiting status of FC, then press <Run/Stop> on AS. The ready light on the AS should now blink.

14. If another set is to be run, refill and sparge solvent reservoirs. Purging is not necessary if inlet lines are not removed from the reservoirs. After last set of the day, flush with 100 mL of hexane, remove inlet lines from the solvent reservoirs and cap the reservoirs.

15. Combine fractions 2 and 3 in the flask containing fraction 2. Rinse the fraction 3 flask three times with approximately 0.5 mL hexane each time.

16. Reduce the solvent volume of fraction 1 and fraction 2+3 to 1 mL using a roto-evaporator with the water bath at 34 C.

17. Transfer the flask contents to a screw-top vial and rinse flask three times with approximately 0.3 mL hexane each time.

18. Reduce solvent volume in the vial just to dryness under a gentle stream of purified nitrogen.

19. Add iso-octane to fraction 1 so that the final volume (V_f) is the same volume of PCB extract which was sampled by the AS.

20. Add iso-octane and a solution of internal standard PCB 30 to fraction 2+3 such that PCB 30 is at 200 pg/μL and the nonortho-substituted congeners are within the linear range of the detector.

    Suggestions:
    (a) Dilute PCB 30 stock to obtain a solution with PCB 30 at 200 pg/μL (e.g. [5], p. 82, Standard C82A). Only this standard need be added to the vial.
    (b) Final volume can be estimated from mass of total PCB loaded: V_f(uL) = 5 μL/μg * Mass PCB(μg).
    (c) The minimum V_f is 10 μL.

B. GAS CHROMATOGRAPHY

1. GC description: Perkin-Elmer 8500 gas chromatograph with a ^{63}Ni electron capture detector, split/splitless injector, programmable temperature ramping, and full integration capabilities.

2. Column description: 30 m J&W Scientific DB-5 column with 0.25 mm i.d. and 0.25 μm film thickness.

3. Operating parameters:
   Injector temperature: 230°C
   Injector mode: splitless operation, 4 mL/min septum purge, 45 mL/min split flow.
   Carrier gas: helium at 24 cm/sec
   Detector makeup gas: nitrogen at 59 mL/min
   Detector temperature: 350°C
   Detector saturation level: 0.9% with oven at 70°C.
   Temperature program: 70°C for 1 min then ramp at 10 C/min to 140°C. No hold. Ramp at 2°C/min to 240°C. No hold. Ramp at 10°C/min to 260°C and hold for 5 min. Ramp at 10°C/min to 280°C and hold for 10 min.
   Run time: 75 min.

4. Inject 1.00 μL with a Hamilton 701N 10 μL syringe using sandwich injection technique. Rinse syringe with both hexane and methanol (40 times each) between injections.

5. Save GC integration report in an ASCII file using the Perkin Elmer E8000 software.

XIV. DEMONSTRATION OF STATISTICAL CONTROL

Statistical control of GC performance is demonstrated by daily evaluation of relative response factors for PCB congeners and run-by-run evaluation of internal standard area. The linear range for a given congener is that range for which the relative response factor (RRF) is within 20% of the mean RRF. A standard mix within the linear range is injected daily. If RRFs for >90% of the congeners are within 20% of the historical mean, then the system is considered to be within control. Control charts are maintained for the area of PCB 30 and the RRFs for selected congeners. Congeners most likely to be out of statistical control are the non-ortho-substituted congeners. They are preferentially retained in the injector by small particles of septa and/or the graphite liner seal. Chromatograms must be monitored for poor peak shape of PCB 77, 81, 126 and 169.

Accuracy of the separation step is demonstrated by the recovery of congener standards from the column. Mean recoveries range for standards separated on the Hypercarb column alone range from 79.2 to 114 percent for the four nonortho-substituted congeners (Table 13). Precision of the separation technique is evaluated by comparing duplicate separations and replicate extractions and separations. The replicate analyses reported below included complete extraction from egg tissue, gel permeation chromatography and deactivated alumina/silica gel column clean-up steps and Hypercarb fractionation. The reported relative standard deviations are less than or equal to those reported for analyses of individual congeners without the Hypercarb column separation step; therefore, the separation step appears to contribute very little to the variability observed among replicates of real samples. In fact, the variability in replicate GC injections accounts for nearly all of the variability among replicates.

Table 14. Recovery of PCB Congeners from Chicken Eggs Spiked with 10 ng/g of each congener.

	Total Percent Recovery:					Statistics:		
PCB #	GBR02	GBR03	GBR22	GBR36	GBR45	Mean	CV(%)	N
60	69.8	60.4	82.4	77.8	61.0	70.3	14.0	5
81	54.1	52.1	50.4	50.1	47.0	50.7	5.2	5
77	54.6	49.2	58.1	57.6	51.7	54.2	7.1	5
123	77.6	58.4	82.3	96.3	55.8	74.1	22.9	5
118	85.4	65.4	135	125	71.8	96.4	32.7	5
114	86.3	64.8	83.4	85.7	69.6	78.0	12.9	5
105	65.8	51.2	95.8	81.4	62.0	71.2	24.5	5
158	95.1	70.6	92.7	115	80.1	90.6	18.4	5
126	55.3	50.0	54.9	51.4	50.6	52.4	4.7	5
166	175	127	90.3	112	78.3	116	32.3	5
128	110	70.9	91.9	116	81.0	94.0	20.3	5
167	91.2	61.4	84.3	90.7	83.2	82.2	14.8	5
156	91.0	22.6	93.1	112	69.7	77.6	44.0	5
157	62.9	51.4	71.0	59.7	50.8	59.2	14.3	5
169	59.6	51.3	52.2	49.4	47.9	52.1	8.7	5
170	102	69.3	90.9	114	78.9	91.0	19.6	5
189	100	66.6	87.8	96.5	71.3	84.4	17.6	5

Table 15. Recovery of PCB Congeners in Fractions 2 and 3 (combined) of Chicken Eggs Spiked with 10 ng/g of each congener.

	Percent Recovery:					Statistics		
PCB #	GBR02	GBR03	GBR22	GBR36	GBR45	Mean	CV(%)	N
60	49.1	45.6	38.5	50.1	52.6	47.2	11.6	5
81	51.3	48.4	50.4	50.1	47.0	49.4	3.5	5
77	54.6	49.2	58.1	57.6	51.7	54.2	7.1	5
123	37.5	34.3	29.1	38.3	39.7	35.8	11.8	5
118	49.0	42.5	61.3	67.7	49.0	53.9	19.1	5
114	20.1	20.3	15.3	21.5	22.6	19.9	14.0	5
105	61.7	48.7	72.1	81.4	59.5	64.7	19.3	5
158	0.0	0.0	0.0	0.0	0.0	0.0	---	5
126	55.3	50.0	54.9	51.4	50.6	52.4	4.7	5
166	0.0	0.0	0.0	0.0	0.0	0.0	---	5
128	0.0	0.0	0.0	0.0	0.0	0.0	---	5
167	22.8	19.5	18.1	24.4	24.6	21.9	13.3	5
156	23.9	22.6	20.4	27.0	26.4	24.1	11.3	5
157	53.0	47.1	46.4	53.4	50.8	50.1	6.5	5
169	58.1	49.7	52.2	49.4	47.9	51.5	7.8	5
170	0.0	0.0	0.0	0.0	0.0	0.0	---	5
189	18.4	16.8	13.2	17.8	17.2	16.7	12.2	5

Table 16. Recovery of PCB Congeners in Fraction 1 of Chicken Eggs Spiked with 10 ng/g of each congener.

	Percent Recovery:					Statistics:		
PCB #	GBR02	GBR03	GBR22	GBR36*	GBR45	Mean	CV(%)	N
60	20.7	14.8	43.9	27.7	8.4	23.1	59.1	5
81	2.9	3.7	0.0	46.0	0.0	10.5	190	5
77	0.0	0.0	0.0	94.3	0.0	18.9	224	5
123	40.1	24.1	53.3	58.0	16.1	38.3	47.3	5
118	36.4	22.9	73.6	56.9	22.8	42.5	52.4	5
114	66.2	44.5	68.1	64.3	47.1	58.0	19.5	5
105	4.1	2.5	23.7	0.0	2.5	6.6	148	5
158	95.1	70.6	92.7	115	80.1	90.6	18.4	5
126	0.0	0.0	0.0	0.0	0.0	0.0	---	5
166	175	127	90.3	112	78.3	116	32.3	5
128	110	70.9	91.9	116	81.0	94.0	20.3	5
167	68.4	41.9	66.1	66.3	58.7	60.3	18.1	5
156	67.1	0.0	72.7	84.5	43.4	53.5	62.5	5
157	10.0	4.3	24.7	6.4	0.0	9.1	104	5
169	1.5	1.6	0.0	3.6	0.0	1.4	111	5
170	102	69.3	90.9	114	78.9	91.0	19.6	5
189	81.6	49.8	74.6	78.7	54.1	67.8	21.7	5

*Relative retention times of congeners PCB 77 and PCB 81 were just outside the expected windows.

XV. CALCULATIONS

All congeners are quantitated based on GC peak area relative to the area of PCB 30. A relative response factor (RRF) is calculated from GC injections of congener mix standards (Eq. 1).

$$RRF = \frac{\left(\frac{\text{area of congener}}{\text{pg congener}/\mu\text{L injected}}\right)}{\left(\frac{\text{area of PCB 30}}{\text{pg PCB 30}/\mu\text{L injected}}\right)} \qquad (1)$$

RRFs are compiled in a spreadsheet (e.g. STDAHH.WK3). An historical average of the RRF for each congener within the linear range is calculated using the mean of mean values at different concentration levels.

Concentrations of congeners in final HPLC fraction volumes (V_f) are calculated from the historical mean RRF for each congener (Eq. 2).

$$\frac{\text{pg congener}}{\mu\text{L injected}} = \frac{\left(\frac{\text{area of congener}}{\text{RRF}}\right)}{\left(\frac{\text{area of PCB 30}}{\text{pg PCB 30}/\mu\text{L injected}}\right)} \qquad (2)$$

Concentrations of congeners in the sample are calculated in a series of steps (Eq. 3-6).

$$\text{Dilution factor} = \frac{V_f \text{ of HPLC fraction}}{\text{volume of PCB extract loaded}} \qquad (\textit{same volume units})$$

$$(3)$$

$$\frac{\text{pg congener}}{\mu\text{L PCB extract}} = \left(\frac{\text{pg congener}}{\mu\text{L injected}}\right)(\text{dilution factor}) \qquad (4)$$

$$\frac{\text{g-equivalents sample}}{\text{mL PCB extract}} =$$

$$(\text{sample mass (g)})(5/8 \text{ GPC fraction})(\text{mL PCB extract})$$

$$(5)$$

$$\text{ng congener/g sample} =$$

$$\left(\frac{\text{pg congener}}{\mu\text{L extract}}\right)\left(\frac{\text{mL extract}}{\text{g-equivalents sample}}\right)\left(\frac{1000\mu\text{L}}{\text{mL}}\right)\left(\frac{\text{ng}}{1000\text{pg}}\right) \qquad (6)$$

Congener concentrations should be corrected for recovery (Eq. 7).

$$\text{corrected ng/g} = (\text{ng/g}) / (\text{proportion recovered}) \qquad (7)$$

XVI. ASSIGNMENT OF UNCERTAINTY

Quantitative uncertainty above the limit of quantitation is approximately 20% of the measured value. Most of that variability is from the inherent variability in GC-ECD quantitation in the splitless injection mode. Splitless injection is necessary to achieve the required sensitivity.

A numerical value cannot be assigned to qualitative uncertainty. Compounds which interfere with the nonortho-substituted PCB congeners are only those which are nonpolar, stable through evaporation to dryness and GC injector conditions, relatively planar and not otherwise retained by the Hypercarb column and which exhibit nearly indentical elution on a 30m DB-5 GC column. One PCN isomer may meet these criteria and interfer with PCB 77, but its retention time is slightly different on the DB-5 column and chromatograms from environmental samples have not shown evidence of the expected Halowax pattern of peaks. One PCDD isomer meets the criteria to interfer with PCB 169, but expected concentrations in Great Lakes environmental samples are not significant relative to concentrations of PCB 169.

APPENDIX A-1. INTERNAL STANDARD PCB 30 STOCK CONCENTRATION.

PCB 30 (2,4,6-trichlorobiphenyl) is used as an internal standard for GC injections. A stock made by D.A. Verbrugge on 9-3-91 was assumed to be at 500 μg/mL, based on the manufacturer's description of mass received. The actual concentration is unknown, but the same stock was used in standards and samples from 9-3-91 through at least 3-25-93. Based on response factors of other PCBs and analysis of a stock from another lab, the actual concentration of #30 is approximately 25% of the nominal concentration.

APPENDIX B

STANDARD OPERATING PROCEDURE

ANALYSIS OF ORGANOCHLORINE PESTICIDES and PCBs IN MUSCLE AND EGG TISSUES OF FISH AND BIRDS

Michigan State University
Pesticide Research Center
Aquatic Toxicology Laboratory

Prepared by:
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I. SCOPE

The scope of this method is to determine the concentrations of organochlorine pesticides (OCs) and polychlorinated biphenyls (PCBs) in muscle and egg tissues. Very polar pesticides will not be recovered by this method. The extraction and cleanup procedures are adapted from Schmitt et al. (1985). The method's precision is within 20% and the accuracy >90%. Total PCBs are reported as a mixture of Aroclors 1242, 1248, 1254, and 1260. Chromatograms of standard mixtures are given in the Appendix. The instrument detection limit (IDL), method detection limit (MDL), and method quantitation limit have been determined as described in Taylor (1989).

II. REFERENCES

Schmitt, C.J., J.L. Zajicek, and M.A. Ribick. 1985. National pesticide monitoring program: Residues of organochlorine chemicals in freshwater fish, 1980-81. Arch. Environ. Contam. Toxicol. 14:225-260.

Taylor, J.K. 1989. Quality Assurance of Chemical Measurements. Lewis Publishers, Inc., Chelsea, Michigan, 328 pp.

III. SUMMARY

This method permits the separation of organochlorine pesticides and PCBs from muscle tissues. Ten grams tissue are homogenized with sodium sulfate, extracted with dichloromethane and cleaned up with mixed solvents on a deactivated alumina/silica gel column. The alumina/silica gel fractions are analyzed by gas chromatography with electron capture detector (GC-ECD). OCs and PCBs are confirmed by GC/MS in 10% of the samples.

IV. SIGNIFICANCE AND USE

This method allows the analyst to determine the concentration of organochlorinated pesticides (OCs) and PCBs in a wide variety of species. The method is specific to high lipid muscle and egg tissues. These compound will accumulate in fish and fish eating birds and to large extent is associated with the muscle tissue of the organism. By determining the concentration of OCs and PCBs in the muscle tissue, the total body burden may be directly measured for the individual. This data is integral to the modeling of toxicant distribution and migration through the aquatic environment.

V. INTERFERENCES

There are components in muscle tissues that may produce some interference. This can be avoided by following an adequate cleanup procedure. Interferences will be detected by comparing the environmental samples with periodical runs of "clean" muscle tissue blanks. Prior to initiating the studies the lab facility will be carefully cleaned to reduce contamination risks. Muscle tissue will be used as control and for recovery experiments.

VI. APPARATUS

Gas chromatograph, Perkin Elmer, model 8500, with electron capture detector (ECD) with ^{63}Ni foil at 350°C. Column: DB-5 fused silica capillary column (J & W Scientific), 30 m x 0.25 mm i.d., 0.25μm film thickness. Injector in splitless mode. Septum purge set at 3-5 ml per minute, temperature at 240°C. Carrier gas: helium at 5 psig, flow rate of @ 1 ml per minute. Makeup gas nitrogen at 45 psig. Total flow rate of @ 50 ml/min. Autosampler Perkin Elmer 8300. Data (retention times and area percentages) are transferred directly to a microcomputer.

VII. **REAGENTS AND MATERIALS**

A. Reagents: Dichloromethane ($MeCl_2$), hexane, diethyl ether (peroxide free), petroleum ether, isooctane, benzene, and acetone; Burdick and Jackson, Baxter, Muskegon, Michigan. All solvents used are of high purity or pesticide grade quality.

B. Sodium sulfate, anhydrous, granular and powder forms. Rinse with hexane or methylene chloride in a buchner funnel before use. Let air dry for a while, then dry in the oven at 130^0C for at least 24 hr before use. May keep stored at 130^0C.

C. Glass wool. Soxhlet extract glass wool with methylene chloride or hexane for at least 24 hr before use.

D. Basic alumina (Al_2O_3), activated (Brockmann I), standard grade, ~150 mesh, 58A pore size, surface area 155 m^2/g, pH of aquous supension 9.5 ± 0.5, from Aldrich (Milwaukee, WI). Store in sealed container in cool, dry place. Deactivated by addition of 8% DI water (e.g. 40g alumina plus 3.2 mL DI water) just prior to preparing columns and stirring by hand for five minutes to insure homogeneity.

E. Silica gel 60, 70/230 mesh. Activate at 130^0C for at least 24 hr before use. Store at 130^0C.

F. Glassware. All glassware is washed with liquinox detergent, rinsed with tap and deionized water, then rinsed with acetone and hexane before use.

G. Reference standards.

1. Pesticide matrix spike, (3/90) catalog # 32018, Lot # A000071, Restek Corporation, Bellefonte, PA.
2. PCB matrix spike, IUPAC #14, #65, #166, obtained from Acustandards. Stock and Working solutions were prepared in our lab.
3. Certified reference material, Organochlorinated Pesticides in fish, EPA.
4. Internal standard, PCB 30 & 204, obtained from Acustandards. Stock and working solutions were prepared in our lab.

5. Aroclors 1242, 1248, 1254, and 1260, obtained from Dr. Zabik, Pesticide Research Center, MSU, originally obtained from Monsanto Co. Stock and working solutions were prepared in our lab.
6. Chlorinated hydrocarbon pesticides: Analytical reference standards obtained from U.S. EPA, Quality Assurance Division, Research Triangle Park, NC. Stock and working solutions and mixtures were prepared in our lab.

VIII. HAZARDS AND PRECAUTIONS

Some of the solvents used are flammable and explosive. Solvents should be always used under the hood and away from fire. Use of lab coats and eye protection goggles is important. In case of a spill, skin contact or inhalation problems, follow specifications in material safety data sheets (MSDS). Handling and storage precautions should follow the recommendations described in the respective MSDS. Waste should be collected and disposed properly according to MSU ORCBS indications.

IX. SAMPLING AND SAMPLE PREPARATION

The tissue must be thoroughly ground and homogenized prior to subsampling. Whole fish may be ground using a double blade Hobart food processor. Fillets of fish are easily homogenized in a commercial Blender. Large fish and birds (ie Double Breasted Cormorant) must be ground using a commercial meat grinder located in Anthony Hall (Dr. Aulerich). The intestinal track of birds must be removed in order to prevent stones from damaging the equipment. After homogenization the sample is subdivided into manageable quantities, 50-200 grams. The original sample and sub-samples are stored in baked glass jars with teflon lined lids. The samples may be frozen at -20 °C until analyzed. Repeated thawing and refreezing of the sample should be avoided.

X. PREPARATION OF APPARATUS

Prior to use, the GC instrument performance is determined from previous runs. Confirm settings on instrument status screen of GC. Percent saturation is adjusted to 0.9%. Confirm steady baseline. Column performance should be determined from weekly monitoring of noise, drift and theoretical plates and chromatography in recent runs. The preparation of the GC autosampler system is described in Appendix A.

XI. CALIBRATION AND STANDARDIZATION

Availability and use of appropriate standards: Our pesticide laboratory standards have been evaluated with the use of a certified pesticide matrix spike, catalog # 32018, lot # A000071, Restek Corporation, Bellefonte, PA. The relative response factors obtained for the two sets of standards were within 25%.

The performance of the GC will be monitored daily by measuring the response and retention times of several calibration mixes. The number of theoretical plates will be calculated using two compounds, C20-ATA and 2,4,6-trichlorobiphenyl (IUPAC #30). The ratio of the theoretical plates (#30/C20) will be used to monitor the condition of the column. A record of the retention times, peak responses, theoretical plates, and peak shape will be kept in the GC Log book. If the theoretical plate ratio changes by > ±25% from its mean value, or if serious column deterioration is observed, the column may be replaced if the situation cannot be corrected. If the retention time of any internal standard changes by > 0.5 min from its mean value, the system will be checked and corrected as required.

The linear range of the GC will be established for individual pesticides by using relative response factors (RRF) and for a 1:1:1:1 mix of Aroclors 1242, 1248, 1254 and 1260 by defining a performance relative response factor (PRRF). The PRRF is defined by the equation (ex. for aroclor);

$$PRRF = AR_{\text{total area}} * ISTD_{\text{conc}} / AR_{\text{conc}} * ISTD_{\text{area}}$$

AR= Aroclor Mix   ISTD= Internal Standard
total area= sum of peak areas for the aroclor mix
area= peak area for ISTD
conc= concentration in ng/μl

The PRRF is specific for a 1:1:1:1 mixture of Aroclors, and is used only to monitor instrument performance. The RRF and the PRRF will be constant over the linear range of the detector. Constant is defined as ±3% from the mean value for the respect respose factor. This range will encompass a minimum of 1.5 orders of magnitude using a minimum of 3 concentrations. The target operating linear range will be 5, 2.5, and 0.15ng of Aroclor mix injected, and 0.25, 0.1, and 0.01 ng for OCs. Once the linear range has been established, an individual standard solution for each of the mixtures will be chromatographed. These chromatographs will be used as templates for pesticide mixtures and the Comstar PCB pattern recognition program. The integrity of the template will be checked by daily injection of pesticide mixtures and a 1:1:1:1 Aroclor performance standard. The absolute concentration of the performance standard will be adjusted to the linear range of the instrument. The calculated concentration of the mix should be ±10% of the expected value.

Calibration checks will be run at the beginning and end of a sample set, where a set is approximately 10 samples. If the concentration of the standard mix is outside of the 10% range the template will be rechromatographed prior to further sample analysis. A log of the relative response factors (RRF) for the individual Aroclors will also be maintained as a check of the GC performance over the course of the study. The RRF for Aroclor analysis is defined by the equation:

$$RRF = AR_{total\ area}*ISTD_{conc}/AR_{conc}*ISTD_{area}$$

AR= individual Aroclor
ISTD= internal standard
total area= sum of peak areas for the Aroclor
area= peak area for ISTD
conc= concentration in ng/μl

If the RRF for a given pesticide or aroclor changes by > 10% from its mean value, the instrument will be checked and the appropriate maintenance (ie. bakeout, clean detector, etc.) will be completed before prior continuing with the analyses. The standards should be re-chromatographed and new templates prepared.

XII. PROCEDURE

A. SAMPLE extraction.

1) Transfer 10 g of homogenized tissue to a 500 ml stainless steel homogenizaton cup. Record the exact tissue weight on the sample extraction form.

2) Add Na_2SO_4 at 5 X the sample weight (50g) and blend with the Omni Mixer for about 15 seconds.

3) Remove the mixing cup and blend the mixture by hand, repeat the homogenization with the Omni Mixer.

4) Place the mixing cup in a ice bath approximately 15min until the homogenate is a free flowing powder

when blended by hand. The sample should be stirred periodically during this period.

5) Add the dried mixture to a 22mm id glass column that has been fitted with a plug of glass wool.

6) Elute the column with 150ml of $MeCl_2$ at 3-5 mL/min and collect in a 500ml round bottom flask.

7) Reduce the sample to 1ml by rotoevaporation at 32°C. Quantita-tively transfer the sample to a 15ml centrifuge tube using $MeCl_2$/ hexane (1:1, v/v). Rinse the flask at least 3 times with 1ml of solvent. Dilute the sample to a final volume of 8ml. Note, The centrifuge tube must be calibrated at the 8ml mark.

8) Centrifuge the sample at 2000rpm for 10min. Pipette 80µl of the sample into a tared aluminum weigh boat. Record the weight on the sample extraction sheet. Calculate the number of GPC loops using equation i.

   i) $x = 100 * (W_s - W_t)/0.5$

   x: # GPC loops
   W_t: Tare weight
   W_s: Sample weight

   The sample is split into the number of centrifuge tubes indicated by x.

   ii) $\% \text{ lipid} = 100 * ((W_s - W_t)/80)/(8000/g \text{ sample})$

9) Centrifuge all centrifuge tubes for the sample at 2000rpm for 10min. Continue with gel permeation chromatography (GPC).

B. Gel Permeation Chromatography (GPC)

Refer to the GPC operators guide for information on the preparation of new columns. This equipment should only be operated by trained personnel. The GPC column used for the separation of fish lipid is packed with 60g of SX-3 Bio-Beads Gel, Bio-Rad Co.

1) Prepare the GPC column by flushing for 30min with $MeCl_2$:hexane 1:1. The column should be evenly wet over its entire surface. The pump pressure should be 6-10 psi, if it is not with in this range see the Supervisor.

2) Fill each sample loop with 8 ml of $MeCl_2$:hexane 1:1, and run the GPC with the collect clock set for 2min (dump and wash set to zero) to prepare the apparatus.

3) Load the samples onto the GPC sample loops. The injection valve must be in the load position. Thoroughly rinse the syringe between individual samples. Rinse each sample loop with 8 mL $MeCl_2$:hexane prior to loading.

4) Place the injection valve in the run position. Run the GPC with the following clock settings:

Dump:	34min
Collect:	28min
Wash:	10min

Collect the GPC eluant in 250ml round bottom flasks. If a sample requires two loops both loops may be collected in a single 500ml round bottom flask.

5) Reduce the sample to 1ml by roto-evaporation at 32°, continue with Clean-up.

D. DEACTIVATED ALUMINA/SILICA GEL cleanup and fractionation

1) Prepare columns by placing 1 cm of granular anhydrous Na_2SO_4 on glasswool in a 1 cm x 30 cm i.d. chromatography column fitted with a 250 ml reservoir. Add five grams of silica gel 60 (70/230 mesh), then add 3 g of 8% deactivated alumina and top with another 1 cm layer of sodium sulfate.

2) Wash the column with 20 ml hexane and discard eluent.

3) When hexane reaches the bed level of the alumina, add the GPC eluate (1-2 ml) and allow it to drain to bed level. Rinse flask with three times with 3ml of hexane total, allowing each rinse to drain to the column bed level. Discard eluent.

4) Wash the column with 5 ml of a 0.5:99.5 ratio of benzene:hexane, drain to bed level, then add 35 ml of the solvent. Collect the eluate in a 250 ml round-bottom flask. (This is fraction 1, silica gel.)

5) Elute the column with 40 ml of a 25:75 ratio of diethyl ether:hexane and collect the eluate in a 250

ml round-bottom flask. (This is fraction 2, silica gel).

6) Rotary evaporate both fractions to about 1 ml, then transfer to a centrifuge tube with three rinses of hexane. Use N-evap to evaporate the fractions to approximately 0.5 mL. Add 1 ml of isooctane and N-evap to just less than 1.0 mL. Add 20 μl of PCB #30 (at 10.0 ng/ul) and adjust the volume to exactly 1.0 mL with isooctane. Transfer to 2 ml vial with teflon-lined cap.

7) The sample may or may not need to be diluted prior to GC analysis.
   a) Dilution required: Place 558 μl isooctane, 12 ul of #30 @ 10 ng/ul and 30 ul of the final extract in an autosampler vial. This results in a 1:20 dilution containing #30 at 210 pg/ul.
   b) No dilution required: transfer 250 μl to an autosampler vial.

E. GAS CHROMATOGRAPHY determination.

1. Alumina/Silica Gel 25% fraction. Most pesticides come out in this fraction. use autosampler/GC program 9.

Program 9 conditions: Injector temperature 230 °C, Detector temperature 350 °C. Carrier He at 20 psig (~25cm/s), makeup gas nitrogen at 60 ml/min. Equilibrium time 3 min, total run time 60 min, attenuation 8. See Appendix B-1 for autosampler operation.

Oven temperature program:

	1	2	3	4
Oven temp (°C)	120	150	225	280
Iso time (min)	3	5	10	15
Ramp rate (°C/min)	30	4	20	

2. Alumina/Silica gel 0.5% fraction. PCBs and DDE come out in this fraction. Use autosampler/GC program 6.

Program 6 conditions: Injector and detector temperatures, gas flow rates and other settings remain the same as in program 9, except for the oven temperature program and running time.

Oven temperature program

	1	2	3
Oven temp (°C)	120	260	280
Iso time (mim)	6	0	0
Ramp rate (°C/min)	2	20	

XIII. DEMONSTRATION OF STATISTICAL CONTROL

Statistical control of GC measurements can be demonstrated graphically by the use of control charts (Taylor 1989, p. 129). Initially, a standard of known concentration will be injected for a total of 7 independent measurements. If the range is linear, the mean relative response factor will be used as the central line to maintain statistical control. Standards will be injected every day that a set of samples is run. If the value of the standard is within 1 standard deviation of the mean, the system is considered to be within statistical control. If a known reference standard is used, then the certified concentration value can be used as the central line (Taylor 1989, p. 131). The control limits will be evaluated by the control charts.

In addition, for every set of 10 samples one sample will be run in duplicate. The calculated concentrations will be compared. If the CV (coefficient of variation) is ± 20%, then we can assume that our measurements are within our established method precision. The use of standards of known concentrations will allow construction of standard reference calibration curves against which the sample runs will be compared. If an outlier is suspected, the calculations and data transfers will be rechecked. If the results are still suspect then the samples both before and after suspect and the suspect sample will be reanalyzed. A value will be considered an outlier if there is an assignable cause.

XIV. CALCULATIONS

The concentration of PCBs and OCs will be determined using the internal standard method to eliminate injection variability and the need to maintain the sample at a constant final volume.

A) Organochlorine pesticides: Pesticides will be quantified based on an internal standard (PCB 30) added to the samples after the extraction step. Quantification is carried out by calculating relative response factors based on peak areas.

B) Total PCBs: PCBs will be quantified with the use of COMSTAR (see COMSTAR SOP).

C) PCB Congeners resolved on DB-5 column: PCB peaks in the extract are quantified based on an internal standard (PCB 30) added to the samples after the extraction step. Relative response factors are based on peak areas in a 1:1:1:1 mixture of Aroclors 1242, 1248, 1254 and 1260. Concentrations of congeners in each peak of this standard were determined by calibrating an in-house standard with one analyzed at the National Fisheries Contaminants Research Center (USFWS, Columbia, MO, contact: T.R. Schwartz).

D) Nonortho-substituted PCB congeners: See HYPERC SOP for separation of these congeners from the PCB extract and subsequent quantitation.

XV. CONFIRMATION AND ASSIGNMENT OF UNCERTAINTY

Organochlorine pesticides will be confirmed in approximately 10% of the samples by GC/MS. This confirmation may only be possible for compounds detected at significant concentrations.

Assignment of uncertainty: A range performance chart will be constructed where the relative response factors (RRFs) at low, middle, and high concentrations will be plotted vs concentration. The upper warning limit (UWL) and lower control limit (LCL) will be the 95% CI, and the upper control limit (UCL) the 99.7% CI. Samples with values above the UCL will be diluted and reanalyzed; those with values below the LCL will be tagged as below detection limit.

APPENDIX B-1.
AUTOSAMPLER QUEUES ON THE PE 8500 GC STANDARD OPERATING PROCEDURE

Lisa L. Williams
May 14, 1992

Abbreviations

GC: Perkin Elmer 8500 gas chromatograph
AS: Perkin Elmer 8300 autosampler

Conceptualization

Queues are made up of one or more sets. A queue is a single unattended run session which may consist of multiple samples run with different GC methods and different AS methods. Sets within a queue are run in the sequence of their set numbers (which I don't think can be changed once the set is generated).

A set is a group of samples which are all analyzed with the same GC method, but which may have different AS programs specified for each sample.

A GC method is the specification of instrument conditions for a given run. Items specified include oven programs, injector and detector temperatures, timed events, detector area and baseline sensitivity.

Autosample programs are the specifications for fill, inject, and flush time and the number of injections per vial.

Setting up a queue on GC/AS

1) The AS 8300 must be specified on the Configuration page of the GC.
2) GC Methods used with automation should have equilibration times of at least 2 min.
3) Check % saturation of ECD detector (e.g. 1.0% @ 200 C)
4) Check gas pressures: AS controller, cylinders, GC controllers.
5) Rinse AS with MeOH and hexane.
    a) Select GC Automation Control page, AS USER CONTROL
    b) Increase He pressure on AS gas controller to 15 psig.
    c) FLUSH vial containing MeOH (45 sec)
    d) Return He pressure to 8 psig.
    e) FLUSH vials (2) containing hexane (45 sec each)
6) Modify/Create sets.
    a) select AUTO QUEUE from GC Autom. Control page.
    b) select MODIFY SET or GEN SET
        (i) enter set name, init. vial, # of samples etc.
        (ii) use flush frequency=2, multiple
        (iii) entering sample names:
            (a) type vial number, [Enter]
            (b) [name]
            (c) type in description, [Enter], [Enter/Accept]

    (iv) [page] when finished entering names

7) Check to see that expected sets are status=ON on Automation Queue page. PARK (GC method 3) should be the final set of the queue. Use [SET STATE] to change status.
8) Make sure the vial specified in PARK is NOT included in any of your sets. Modify PARK if necessary.
9) Print queue.
10) Check paper supply in printer.

<u>Setting up a queue on the computer</u> (Communications Guide, p.27+)

1) From DOS: Create subdirectory in which to store run reports (e.g. C:\EGC\MAY14) and check hard drive memory.
2) Load E8000 software (type GC at DOS prompt).
3) Select (9) Automatic data transfer from main menu.
4) Select (2) Generate queue
    a) provide brief description (optional)
    b) transfer method filename: <Enter>
    c) number of runs: (sum of number of samples from all sets in the GC queue)
    d) store raw? N
    e) store report? Y
    f) store DIF? N
    g) reintegrate? N
    h) application file name? <Enter>
    i) accept? Y
5) Select (1) Automation configuration to set *.RPT destination
    a) modify autom. control? Y
    b) data disk options? (3) Winchester
    c) enter path without drive letter (Winchester = C drive)e.g. \EGC\MAY14
    d) modify task? N
6) Select (4) Display queue to check # of runs, etc.

<u>Starting a queue</u> (Communications Guide, p.32+)

1) On GC (Automation Queue page): make sure Output to Screen = NO and Output to Ext. Dev. = YES
2) On GC (Configuration page)
    a) external device = data system
    b) external control = NO
    c) then change external control = YES
    d) go back to Automation Control page
3) On computer (Automation Control page)
    a) select Start Queue and give queue name
    b) recycle? N
    c) you now have 60 sec to press START QUEUE on GC (ignore the rest of the message)
4) On GC (Automation Control page): press START QUEUE

OR

2) On GC (Configuration page)
    a) external device = data system
    b) external control = NO
3) On computer (Automation Control page)
    a) select Start Queue and give queue name
    b) recycle? N
    c) you now have 60 sec to make entries on the GC
        (ignore the rest of the message)
4) On GC (Configuration)
    a) change external control = YES
    b1) go to Automation Control page and press START QUEUE
    or
    b2) go to Instrument page for hand injection