Title

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Journal

Environmental Health Perspectives, 104(7)

ISSN

1542-4359

Authors

Villalobos, SA
Anderson, MJ
Denison, MS
et al.

Publication Date

1996-07-01

DOI

10.1289/ehp.96104734

Peer reviewed

Dioxinlike Properties of a Trichloroethylene Combustion-Generated Aerosol

S. Alex Villalobos,[1] Michael J. Anderson,[1] Michael S. Denison,[2] David E. Hinton,[1] Katherine Tullis,[2] Ian M. Kennedy,[3] A. Daniel Jones,[6] Daniel P. Y. Chang,[5] GoSu Yang,[3] and Peter Kelly[4]

[1]Department of Anatomy, Physiology and Cell Biology, School of Veterinary Medicine, [2]Department of Environmental Toxicology, [3]Department of Mechanical and Aeronautical Engineering, [4]Department of Chemistry, [5]Department of Civil and Environmental Engineering, and [6]Facility for Advanced Instrumentation, University of California, Davis, CA 95616 USA.

Conventional chemical analyses of incineration by-products identify compounds of known toxicity but often fail to indicate the presence of other chemicals that may pose health risks. In a previous report, extracts from soot aerosols formed during incomplete combustion of trichloroethylene (TCE) and pyrolysis of plastics exhibited a dioxinlike response when subjected to a keratinocyte assay. To verify this dioxinlike effect, the complete extract, its polar and nonpolar fractions, some containing primarily halogenated aromatic hydrocarbons, were evaluated for toxicity using an embryo assay, for antiestrogenicity using primary liver cell cultures, and for the ability to transform the aryl hydrocarbon receptor into its DNA binding form using liver cytosol in a gel retardation assay. Each of these assays detect dioxinlike effects. Medaka (*Oryzias latipes*) embryos and primary liver cell cultures of rainbow trout (*Oncorhynchus mykiss*) were exposed to concentrations of extract ranging from 0.05 to 45 µg/l. Cardiotoxicity with pericardial, yolk sac, and adjacent peritoneal edema occurred after exposure of embryos to concentrations of 7 µg/l or greater. These same exposure levels were associated with abnormal embryo development and, at the higher concentrations, death. Some of the fractions were toxic but none was as toxic as the whole extract. In liver cells, total cellular protein and cellular lactate dehydrogenase activity were not altered by *in vitro* exposure to whole extract (0.05–25 µg/l). However, induction of cytochrome P4501A1 protein and ethoxyresorufin *O*-deethylase activity occurred. In the presence of whole extract, estradiol-dependent vitellogenin synthesis was reduced. Of the fractions, only fraction 1 (nonpolar) showed a similar trend, although vitellogenin synthesis inhibition was not significant. The soot extract and fractions bound to the Ah receptor and showed a significantly positive result in the gel retardation/DNA binding test. Chemical analyses using GC-MS with detection limits for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and dibenzofuran in the picomole range did not show presence of these compounds. Our results indicate that other chemicals associated with TCE combustion and not originally targeted for analysis may also pose health risks through dioxinlike mechanisms. *Key words*: Ah receptor, antiestrogen, complex mixture, dioxinlike toxicity, dioxin-response element binding, embryo/cardiovascular toxicity, incomplete combustion by-products, liver, trichloroethylene, vitellogenin. *Environ Health Perspect* 104:734–743 (1996)

Incineration has been widely used as a means for disposal of municipal, hospital, and industrial hazardous wastes. Its use has been curtailed in recent years because of concern about the emission of toxic by-products associated with the soot particles, especially chlorinated phenols, aromatic hydrocarbons, polychlorinated dibenzodioxins, and dibenzofurans (*1–3*). These emissions arise from improper operation of incinerators or from transients (*4–6*) in operation during which inadequate temperature and mixing conditions in the combustion zone may lead to incomplete combustion. These transient discharges, also known as puffs, are characterized by large transient emissions of soot and toxic volatile organic hydrocarbons (*4–7*). Although they are relatively rare during incinerator operation, puffs contribute a major fraction of the toxic compounds in incinerator effluent. For example, Wendt (*3*) demonstrated in a toluene-fed kiln that puffs can emit approximately 10,000 ppm of hydrocarbons for a period of about 20 seconds. Depending on the precursor chemistry, additional reactions downstream of the high temperature regions may lead to the formation of dioxins (*8*). Atmospheric transport of incinerator emissions may result in wide-spread dispersal and subsequent deposition of these particles in various environmental matrices (*9*) including soil, water, and vegetation (*10*).

Dioxin and dioxinlike compounds constitute a diverse and important group of contaminants widely spread in the environment, where they persist as complex mixtures (*7,11,12*). One particular compound, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), has been the subject of considerable concern with regard to incinerator emissions. TCDD and related halogenated aromatic hydrocarbons, including 2,3,7,8-tetrachlorodibenzofuran (TCDF), produce a wide variety of species- and tissue-specific toxic and biological effects, such as teratogenesis, immunotoxicity, hepatotoxicity, tumor promotion, and induction of numerous enzymes, including microsomal cytochrome P4501A1 (CYP1A1) (*7,13*).

Many hazardous waste sites contain chlorinated solvents, including trichloroethylene (TCE). For example, the McClellan Air Force Base in Sacramento (California) contains soil that is heavily contaminated by TCE; it was used as a cleaning agent on aircraft. Earlier experiments by Blankenship et al. (*14*) found that extracts from soot aerosols formed during the combustion of TCE exhibited a dioxinlike response when subjected to a keratinocyte bioassay. These experiments showed that all of the hazardous material was associated with the aerosol and that little was found in the gas phase of the flames. Chemical analyses of the soot extracts indicated that, at picomole levels, TCDD/TCDF were not detected, suggesting that chlorinated fulvalenes, among other chlorinated hydrocarbons, were major components of the mixture and that these may have been responsible for the toxic response. Because of its environmental importance and in view of the previous experience with toxic TCE aerosols, TCE was chosen as the model waste for this study.

Although conventional chemical analyses of incineration by-products identify compounds of known toxicity, they often fail to indicate the presence of other chemicals which may also pose health risks. The purpose of the present investigation was to verify whether materials with dioxinlike properties were present in the chemically

Address correspondence to I.M. Kennedy, Department of Mechanical and Aeronautical Engineering, University of California, Davis, Davis, CA 95616 USA.

Trout-specific antibodies and standards were gifts from Michael Miller, West Virginia University, and Ray Simon, formerly of the U.S. Fish Health Center, Kearneysville, West Virginia. Anti-scup CYP1A1 (Mab 1-12-3) was a gift of John Stegeman, Woods Hole Oceanographic Institution. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin was obtained from S. Safe, Texas A&M University. A.V. thanks Miguel González-Doncel and Swee Teh for their assistance in the histological preparations and evaluations. This research was supported by the NIEHS Superfund Basic Research Program (P42ESO4699), by the Ecotoxicology Program of the University of California Toxic Substances Research and Teaching Program, and by the US EPA-UC Davis Center for Ecological Health Research (R819658).
Received 29 March 1996; accepted 10 April 1996.

complex TCE soot mixture and its fractions. Dioxinlike effects (e.g., cardiotoxicity and yolk sac edema) have been investigated in medaka (*Oryzias latipes*) embryos. Biological potency has been demonstrated *in vitro* both by measuring interference of compounds from the mixture with estrogen receptor using rainbow trout (*Oncorhynchus mykiss*) liver cells and by monitoring the mixture's binding affinity to the Ah receptor and further ability to convert it into its DNA binding form.

Methods

Flame Conditions and Chemical Analysis

Unmixed or poorly mixed combustion can be modeled in a well-defined laboratory experiment with a laminar diffusion flame. Poor mixing with relatively long residence times in an incinerator is then modeled by increasing the flame length beyond the point at which soot breaks through the flame tip. The nature of the compounds that are emitted from these flames is typical of the material that could be found in puffs from incinerators.

A mixture of TCE and methane (CH_4) was burned in a laminar diffusion flame. TCE vapor was generated by passing CH_4 through an impinger containing liquid TCE that was maintained at a constant temperature. The mole fraction of TCE in the methane was 0.51; the flow rates were 696 ml/min of CH_4 and 734 ml/min of TCE. This mixture was supplied to an axisymmetric laminar diffusion flame burner. The co-flow burner assembly consisted of a circular Plexiglas chamber with a 67-mm inside diameter. The round nozzle was made of thin-walled stainless steel tubing with a 6-mm outside diameter. Soot was collected from the post-flame gases with a 47-mm PTFE-coated glass fiber filter in line with a sorbent tube.

The sorbent tube was prepared by packing 100 mm lengths of Pyrex glass tubing (12 mm O.D.) with 3.5 g of Carbotrap C. Glass wool plugs were inserted into both ends. The filters were Soxhlet extracted for 16 hr with 250 ml of dichloromethane (CH_2Cl_2) using anhydrous sodium carbonate (Na_2CO_3) to neutralize adsorbed acids. CH_2Cl_2 extracts were roto-evaporated to a volume of 10 ml, divided into 10 aliquots, and stored at - 20°C. Each aliquot was dry-evaporated under a stream of nitrogen at 25°C and reconstituted in 1 ml of analytical grade dimethylsulfoxide (DMSO) for bioassays.

An individual aliquot was applied to a silica gel column and four fractions were eluted with different solvents including fraction 1 (nonpolar compounds) with *n*-hexane, fraction 2 (primarily PAHs and chlorinated PAHs) with *n*-hexane/CH_2Cl_2 (3:2 v:v), fraction 3 (intermediate polarity) with CH_2Cl_2, and fraction 4 (polar compounds) with methanol. Control fractions, prepared by Soxhlet extractions of blank cellulose extraction thimbles, were obtained using identical laboratory procedures.

Analyses were performed on extracts and fractions using a VG Trio-2 mass spectrometer coupled to a Hewlett Packard 5890 gas chromatograph. Separations were performed using a 30-m DB-17 capillary column with helium as carrier gas. Electron ionization (70 eV) mass spectra were obtained; compounds were quantified based upon average molar response factors obtained for a series of PAHs and chlorinated aromatic standards.

Embryo Toxicity Assay

Egg collection and broodstock maintenance followed the procedure described by Marty et al. (*15*). Medaka female broodstock, maintained at 25°C under a 16 hr light:8 hr dark photoperiod stimulating continuous egg production, were individually netted and eggs <5 hr old were carefully removed from extruded clusters. Filaments that attached adjacent eggs were broken by gently rolling clusters between moistened finger tips. Individual (blastula stage) eggs were kept in continuously aerated embryo rearing medium (ERM) (*16*).

Embryo exposures were repeated until the whole TCE soot extract and individual fractions were tested. Each exposure was conducted as a completely randomized design (*17*) which consisted in pooling eggs and distributing them (n = 8) by stratified random assortment to individual 20 ml borosilicate vials (Fisher Scientific, Pittsburgh, Pennsylvania) in each of four replicates. Each vial contained 2 ml of solution and 18 ml of air space. A double layer of teflon tape (Scientific Instruments, Randallstown, Maryland) and screw-type lid were used to hermetically seal each vial. For each experiment, vials were coded for blind study except for one additional ERM replicate (known control, not included in statistical analysis), which served as a reference for time of normal development. Due to the hazardous nature of this complex mixture and to the blind randomized experimental design, embryos were maintained in vials under static (non-renewal) conditions for duration of embryonic development (8 days). After exposure and rinsing in clean ERM, embryos were transferred to clean vials and allowed to complete their development. Static non-renewal conditions have been used when testing dioxin, dioxin-like compounds, and other complex mixtures (*17–20*). Oxygen requirements during medaka development in a closed system (no access to free air), are approximately 23 ml of ERM/egg (*21*). Since dissolved oxygen in air is 25–30 times greater than in ERM, sufficient aeration was provided given the eggs:ERM/eggs:air ratio.

For exposure, soot whole extract (WE) and fraction stock solutions were dissolved in ERM (pH 7 ± 0.2) using DMSO (WE) or DMSO/fraction as vehicle solvents. All vehicle concentrations were restricted to 500 µl/l (0.05% v/v). This concentration has shown in pilot tests to produce no embryonic toxicity. Estimated maximum concentration of incomplete combustion by-products was 0.09 µg/µl of vehicle (i.e, 500 µl/l × 0.09 µg/µl = 45 µg/l). The range of interest in these pilot studies was determined between a stock solution of 500 µl carrier (containing WE soot) in 1 liter ERM, and a respective dilution of 1:100 (1 ml of stock in 100 ml ERM). The intermediate concentrations were chosen so that there could be 5 equidistant intervals in a log scale, based on the absolute difference (2.0) between log 45 µg/l, and log 0.45 µg/l. This conversion resulted in intervals of 0.4 log units, which when reconverted (antilog) to a linear scale gave concentrations of 45, 18, 7.2, 2.7, 0.9, and 0.45 µg/l. Controls consisted of embryos exposed to vehicle or ERM alone.

Embryos were observed daily under a dissecting microscope for normal and abnormal development. Mortality and sublethal endpoints including pericardial and peritoneal edema, eye and/or subdermal edema, hemostasis, yolk resorption, cephalic and spinal deformities, and hatching success were observed. The transparent chorion of medaka embryonated eggs permits direct visualization of heart beat. Cardiac activity was monitored by averaging heart rate (in beats per minute ± SD) of at least three embryos per vial. This monitoring was done daily until hatching. Evaluation was continued through the first 4–5 days after hatching. Development, including swim (or air) bladder inflation and swimming activity, was monitored. A hatchling was considered normal if it swam vigorously, and had normal gross morphology and an inflated swim bladder. Medaka hatchlings inflate swim bladders within 24 hr (*15*). To confirm and extend observations with the dissecting microscope, a limited number of normal and abnormal embryos/larvae were fixed in 10% buffered formalin, dehydrated in a graded ethanol series and embedded in complete glycolmethacrylate monomer (*22*). Sections (4 µm thickness) were cut on an LKB Historange microtome, mounted to glass slides, and stained with hematoxylin and eosin (H&E) or toluidine blue.

Serial sectioning was performed to validate locations within a given embryo/larva.

For statistical purposes, all embryos that failed to hatch were considered abnormal. Differences from the controls were identified with Wilcoxon's sign-rank test ($p<0.05$), using the JMP statistical software package (SAS Institute, Cary, North Carolina). The additional ERM replicate was excluded from statistical calculations.

Liver Cell Assays

Sexually immature male and female rainbow trout (400–600 g mean weight) from Mt. Lassen trout farm (Red Bluff, California) were housed in a large (4 × 1.7 × 1 m) concrete tank at the Institute of Ecology aquaculture facility at UC-Davis. Gonadosomatic indices (gonad weight/body weight × 100) ranged between 0.25 and 0.75%. Fish were held under natural photoperiod in constant flow (Lake Berryessa, California) water at temperatures between 14 and 15°C and fed Silver Cup trout pellets at approximately 1% body weight/day. Fish were acclimated to the above holding conditions at least 2 weeks before experimentation.

Medium 199, L-glutamine, antibiotic–antimycotic solution, buffer salts, anti-rabbit IgG alkaline phosphatase conjugated antibodies, *p*-nitrophenyl phosphate (PNPP), pyruvate, NADH, and NADPH were purchased from Sigma (St. Louis, Missouri). 17β-Estradiol was purchased from Steraloids (Wilton, New Hampshire). Anti-mouse IgG horseradish peroxidase-conjugated antibody was purchased from Amersham (Arlington Heights, Illinois). Tween 20, enzyme immunoassay grade nonfat dry milk, and 3,3′,5,5′-tetramethylbenzidine (TMB) solution were purchased from Bio-Rad (Burlingame, California). Diethanolamine was purchased from Aldrich (Milwaukee, Wisconsin), collagenase (269 U/mg) from Worthington Biochemicals (Newark, New Jersey), and 7-ethoxyresorufin and resorufin from Molecular Probes (Eugene, Oregon). All other chemicals were of analytical grade.

Cells were isolated following a two-step perfusion technique (*23*) with the following modifications: no heparin was injected into the animals and the perfusion medium was a calcium-free HEPES buffered Hank's salt solution, pH 7.6 (*24*). Following liver digestion and tissue disassociation, cells were washed two times and resuspended in medium 199 (see below). Viability was assessed by phase microscopy and trypan blue dye exclusion. Typically 90% or more of the cells were viable.

Cell cultures followed procedures of Pesonen and Andersson (*25*) with one exception: HEPES buffered medium 199 at pH 7.6 contained no additional Na_2HPO_4 because high concentrations caused precipitation and interfered with the ELISA assays. Cells were plated on 60- or 100-mm diameter Falcon polystyrene tissue culture dishes (Beckton Dickinson, Oxnard, California) at a concentration of approximately 1.65×10^5 cells/cm^2 and placed in a humidified Ambi-Hi-Low incubator (Baxter, McGaw Park, Illinois) at 15°C in air atmosphere.

Cells were allowed to attach to tissue culture dishes and acclimate to culture conditions for 24 hr before the first media change and dosing. Cells were then treated with fresh medium 199 containing either DMSO alone (control), WE (0.6–25 µg/l), or each of the fractions (in DMSO). Due to the use of 4 fractions and testing of each with cells from a single trout, a single concentration (11.25 µg/l) was used. The total concentration of DMSO in the media was maintained at 0.05% (v/v) as described above. Simultaneously, 1 µM 17β-estradiol or an equivalent volume of ethanol (carrier control) was added to the medium. Cells from control and treatment groups were always obtained from the same fish.

After 48 hr of exposure, cells were gently scraped off the dishes with a teflon rod and placed in individual centrifuge tubes. Tubes were centrifuged at 150*g* for 2 min at 4°C to separate media from cells. Resultant cell pellet was resuspended in 1 ml of 0.1 M phosphate buffer, pH 7.5 (80mM Na_2HPO_4, 20 mM NaH_2PO_4) with 20% glycerol and sonicated for 5 sec on ice. Cell homogenates and media were immediately frozen on dry ice and stored at -80°C until assays were performed.

Determinations of vitellogenin (Vg) and albumin (Alb) released into the cell culture media and cellular CYP1A1 content were estimated by indirect ELISA as described (*26,27*) using monoclonal (MAb) anti-trout Vg (MAb SD6C) (*28*), polyclonal rabbit anti-trout Alb, and anti-scup CYP1A1 (MAb

Table 1. List of the major incomplete combustion by-products present in trichloroethylene whole extract

Retention time (min)	Monoisotopic m/z	Tentative identification	% of TIC area[a]
17.17	248	C_6HCl_5, pentachlorobenzene	5.1
20.37	282	C_6Cl_6, hexachlorofulvene	1.0
20.75	282	C_6Cl_6, hexachlorobenzene	12.3
21.32	272	C_8HCl_5	0.5
21.62	296	$C_7H_2Cl_6$, heptachlorobicyclo-[2.2.1]hepta-2,5-diene	0.5
21.85	310	$C_8H_4Cl_6$	1.6
22.15	296	$C_7H_2Cl_6$, heptachlorobicyclo-[2.2.1]hepta-2,5-diene	0.7
23.08	342	C_8HCl_7	0.9
24.13	306	C_8Cl_6	1.0
24.23	342	C_8HCl_7	1.0
24.55	330/264	$C_7HCl_7/C_{10}H_4Cl_4$	0.9
25.62	330	C_7HCl_7	1.4
25.85	322	$C_9H_4Cl_6$	0.6
26.20	376	C_8Cl_8	3.6
27.08	300/376	$C_{10}H_5Cl_5/C_8Cl_8$	1.2
27.23	298	$C_{10}H_3Cl_5$	2.1
27.55	300	$C_{10}H_5Cl_5$	0.8
27.67	300	$C_{10}H_5Cl_5$	1.0
27.90	298	$C_{10}H_3Cl_5$	0.9
28.17	298	$C_{10}H_3Cl_5$	0.6
29.58	298	$C_{10}H_3Cl_5$	0.9
30.92	334	$C_{10}H_4Cl_6$	3.2
31.05	334	$C_{10}H_4Cl_6$	2.3
32.07	332	$C_{10}H_2Cl_6$	1.4
33.02	332	$C_{10}H_2Cl_6$	1.0
33.40	332	$C_{10}H_2Cl_6$	1.6
36.23	366	$C_{10}HCl_7$	5.5
37.02	366	$C_{10}HCl_7$	3.7
43.30	400	$C_{10}Cl_8$	5.2
45.15	390	$C_{12}HCl_7$	0.8

[a]Listed peaks account for 63% of area of total ion chromatogram (TIC), with $C_{10}H_xCl_{8-x}$ compounds representing ~30%. The remaining area is distributed among at least 200 smaller peaks.

1-12-3). Dilutions of media or cell extracts (10–100-fold) in phosphate buffered saline, pH 7.5 (PBS: 80mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl) were used.

Ethoxyresorufin *O*-deethylase (EROD) activity of whole cell homogenates followed method of Burke et al. (*29*) adapted for microplate format (Cambridge microtiter plate fluorometer, model 7620). Briefly, fluorescence (excitation 530 nm and emission 585 nm) in 80–100 µg of whole cell homogenates, incubated in 100 mM potassium phosphate buffer, pH 8.0 (90 mM K_2HPO_4, 10 mM KH_2PO_4, 0.25 µM ethoxyresorufin, and 0.5 mM NADPH) to a final reaction volume of 0.2 ml, were recorded at 30–40 sec intervals over 5 min at 24°C. Determinations of cellular lactic dehydrogenase (LDH) activity were made following the method of Bergmeyer and Berndt (*30*). Protein concentrations of cell homogenates were determined using the Bio-Rad DC protein assay kit, with bovine serum albumin (BSA) as the standard.

Each exposure group of liver cells consisted of three to four dishes per treatment, with duplicate determinations per dish. Significant differences between means of various treatment groups were determined by ANOVA ($p<0.05$) and means were contrasted using Dunnett with control group and Tukey-Kramer methods. All statistical analyses were performed using the JMP procedure of SAS software (SAS Institute).

Gel Retardation/DNA Binding Assay

Based on the ability of Ah receptor (AhR) ligands to convert this receptor to its DNA binding form, a gel retardation assay was used to measure the amount of inducible protein [^{32}P]DNA-complex. This provided an indirect way to detect dioxinlike chemical(s). Guinea pig hepatic cytosol was used as the source for the receptor, based on previous determinations which indicated that this species is the most optimal for the transformation and DNA binding analyses of ligand:AhR complexes (*31*).

In the assay, hepatic cytosol prepared from male Hartley guinea pigs (250–300 g; Michigan Department of Public Health, Lansing, MI), was suspended in ice-cold HEDG buffer (25 mM HEPES, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 10% (v/v) glycerol) and aliquots were stored at -80°C as previously described (*32,33*). Protein concentrations were measured by the method of Bradford (*34*) using BSA as the standard. For gel retardation analysis, 125 µl cytosol (16 mg of protein/ml) was incubated with DMSO (20 µl/ml), 15 nM TCDD in DMSO or an aliquot (2.5 µl) of the soot WE or fractions (in DMSO) for 2 hr at 20°C. Gel retardation analysis of the samples was carried out using [^{32}P]-labeled dioxin-responsive element (DRE)-containing DNA oligonucleotide as described by Helferich and Denison (*33*) and the resulting protein–DNA complexes were detected following autoradiography of dried gels. Quantitation of the inducible protein–DNA complex was carried out as described by Denison and Yao (*32*). The 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was obtained from S. Safe (Texas A&M University) and [γ-^{32}P] ATP (6,000 Ci/mmol) from New England Nuclear. Molecular biological reagents were obtained from New England Biolabs.

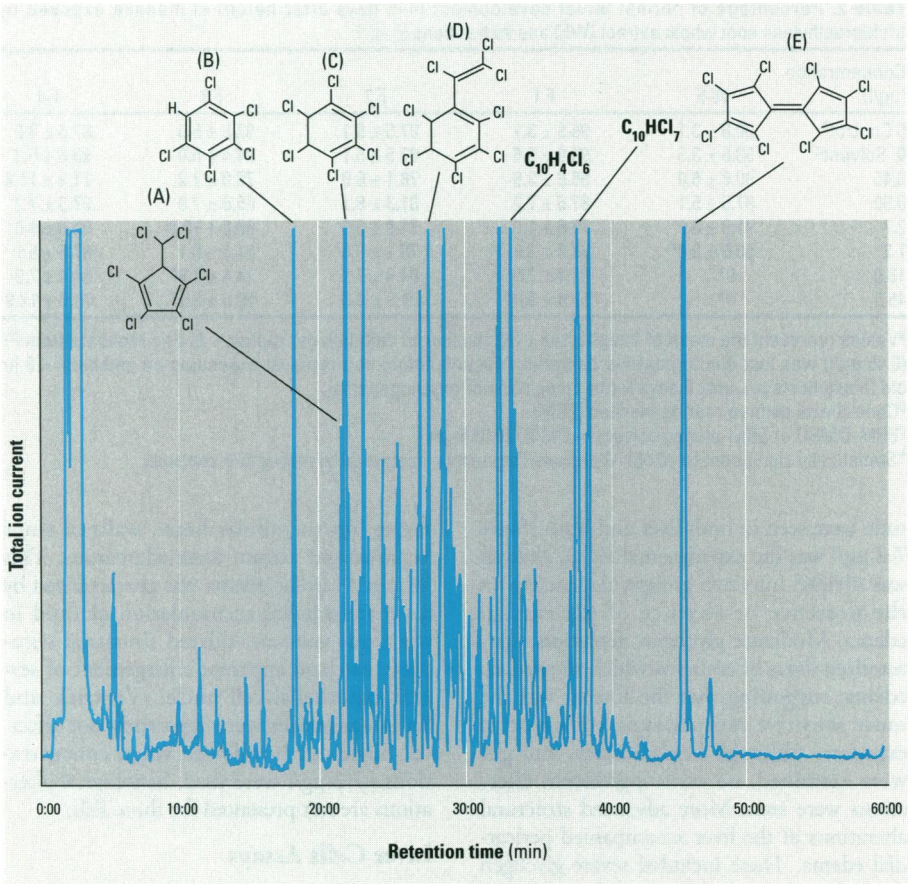

Figure 1. Total ion chromatogram of trichloroethylene soot whole extract. More than 250 incomplete combustion by-products were formed during pyrolysis. (A) Hexachlorofulvene, (B) pentachlorobenzene, (C) hexachlorobenzene, (D) octachlorostyrene, (E) octachlorofulvalene.

Results

Analytical Chemistry

Combustion of the TCE/CH_4 mixture produced a flame characterized by heavy soot production, approximately 100 mg/g of fuel burned. CH_2Cl_2 extracts of the soot had a dark blue color, which may be attributed to the presence of significant amounts of chlorinated fulvalenes ($C_{10}H_xCl_{8-x}$, depending on number of H and Cl substitutions, or $C_{10}Cl_8$ = octachlorofulvalene), structural isomers of naphthalenes (*14*). $C_{10}H_xCl_{8-x}$ compounds represented ~30% of the total ion chromatogram (Table 1). GC/MS analysis of the WE indicated that over 250 organics (Fig. 1) were formed during TCE pyrolysis. Nearly all were chlorinated mono- and polyunsaturated aliphatics, cyclic polyenes 1-, 2-, and 3-ring aromatics, phenols, fulvenes (structural isomers of benzene), and the above mentioned fulvalenes. With a limit of detection of about 1 pM in the extract, no polychlorinated biphenyls (2,3,7,8-TCDD or TCDF) were detected.

Embryo Toxicity Assay

The combined percentage of normal development for all controls was above 90 (Table 2). Greatest toxicity was seen after exposure to WE (Table 2 and Fig. 2). Based on the nominal concentrations previously estimated, the observed WE concentration in which 50% of larvae (EC_{50}) showed signs of abnormality was 7.2 µg/l, while at 2.7 µg/l, no effect was observed. The calculated EC_{50} was ~4.3 µg/l (y = -51.64 log x + 136.75, r^2 = 0.92). The two higher WE concentrations proved lethal to most embryos. The few embryos that hatched were extremely weak and did not inflate swim bladders. Statistical analyses (Wilcoxon's sign-rank test) showed

significant differences at and above 7.2 µg/l. The toxicity trend observed after exposure to the individual fractions indicated that fractions 3 and 1 were the most toxic, while fractions 2 and 4 had no significant effects. However, none of the toxic fractions were as toxic as WE. For fraction 1, approximately 40% of embryos exposed to 45 µg/l developed abnormally, while lower concentrations showed variable effects (Tables 2 and 3; Fig. 2). Results with fractions 2 and 4 were similar: no more than 29% of the exposed embryos developed abnormally regardless of concentration (Table 2). Fraction 3 was slightly more toxic than WE over the range of 0.45–2.7 µg/l, and became less toxic at higher concentrations (approximately 65% and 50% of embryos exposed to 18 and 45 µg/l were abnormal) (Tables 2 and 3; Fig. 2).

The predominant embryonic defect was edema, pronounced in pericardial cavity but also present in the peritoneal cavity and yolk sac (Table 3 and Fig. 3). Embryonic mortality was rarely seen. Within the first 6 days of exposure, 13 (1%) out of a combined total of 1280 embryos died. Of these, only 6 (4 deaths in 48 hr or less and 2 delayed hatchings) were observed in controls. During these first 6 days, no symptoms of cardiovascular toxicity (i.e., bradycardia or tachycardia) that would indicate formation of edema were apparent (data not shown). Two to four days later, depending on concentration, mild pericardial edema appeared and progressed rapidly, often leading to death before hatching (Fig. 3). In these severely affected embryos, the process of heart chamber formation observed as a shunt of blood from left to right was apparently terminated, and a pulsatile single tube had appeared in individual fish who had earlier shown evidence of more developed heart formation. Other lesions included hemostasis, a severe darkening over brain, and larger than normal yolk sac. Cephalic/spinal abnormalities were rare (<0.5%). The highest concentration of WE compatible with control hatch frequency was 7.2 µg/l. Fifty percent of hatchlings exposed to this concentration showed normal structure and were able to inflate swim bladders and move about. The remainder could not inflate swim bladders; edemas became more severe, often extending from pericardial and peritoneal/yolk sac areas to the eyes (Fig. 4). Finally, these hatchlings could not swim or maintain equilibrium.

In embryos showing no evidence of gross alterations, light microscopy revealed additional lesions. The lower concentrations of WE (0.45 and 0.90 µg/l) caused no apparent lesions, but 2.7 µg/l was associated with mild hepatocyte glycogen depletion in liver hepatocytes. At concentrations of 7.2 µg/l and above, changes of greater magnitude were seen in both liver and heart. Since 7.2 µg/l was the experimental EC_{50}, analysis was divided into two groups, depending on the presence or absence of pericardial edema. Moderate glycogen depletion characterized livers of embryos which showed no edema, suggesting that the former was the more sensitive morphologic indicator of exposure. Although heart, kidney, and gut were examined, no other significant alterations were seen. More advanced structural alterations of the liver accompanied pericardial edema. These included severe glycogen depletion, mild lipidosis, and occasional enlarged hepatocytes. In embryos which developed pericardial edema but showed no regression to tubular heart, walls of sinus venosus and atrium were edematous. This localized cardiac edema was characterized by a subendothelial accumulation of fluid in the sinus venosus, dilated sinoatrial compartment, and apparent enlargement of several endothelial cell nuclei. Ventricle and bulbus arteriosus were apparently not affected. Since death followed when concentrations >7.2 µg/l were used, histological alterations are not presented for those fish.

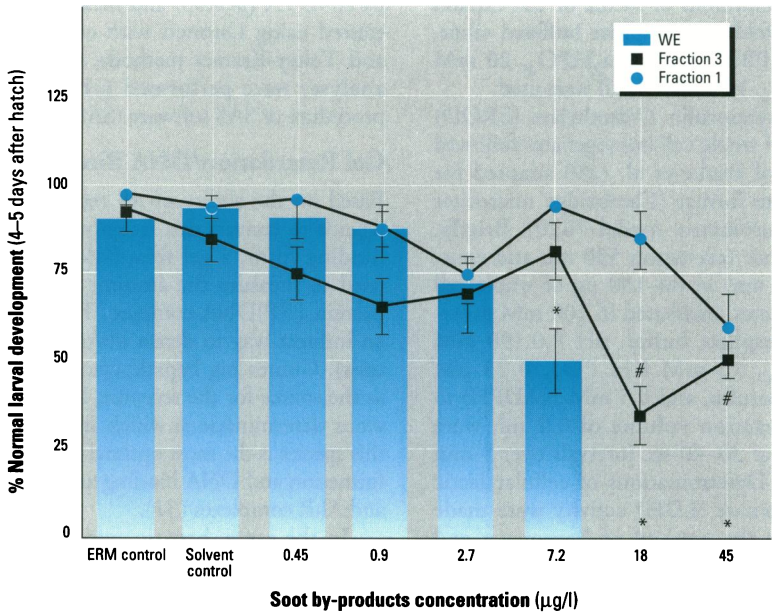

Figure 2. Effect of incomplete combustion by-products from trichloroethylene soot whole extract (bars) and fractions 1 and 3 on the development of medaka after static non-renewal exposures at embryonic stages. Each point represents the mean of four replicates ± SE, eight embryos per replica. In fraction 1 (0.45 µg/l), one replicate was lost due to bacterial infection. Significant ($p<0.05$, Wilcoxon's sign-rank test) abnormalities compared to controls were seen at concentrations ≥7.2 µg/l for whole extract (*), 45 µg/l for F1 (+), and >18 µg/l for F3 (#).

Table 2. Percentage of normal larval development (4–5 days after hatch) of medaka exposed to trichloroethylene soot whole extract (WE) and its fractions (1–4)[a]

Concentration (µg/l)	WE	F1	F2	F3	F4
0 Control[b]	90.6 ± 3.1	96.9 ± 3.1	87.5 ± 5.1	92.5 ± 5.6	87.5 ± 7.2
0 Solvent[c]	93.8 ± 3.5	93.8 ± 3.6	87.5 ± 5.1	84.4 ± 6.0	93.8 ± 6.3
0.45	90.6 ± 6.0	95.8 ± 3.6	78.1 ± 6.0	75.0 ± 7.2	71.9 ± 11.8
0.90	87.5 ± 5.1	87.5 ± 7.2	81.3 ± 8.1	65.6 ± 7.9	87.5 ± 7.2
2.7	71.9 ± 6.0	75.0 ± 1.0	71.9 ± 6.0	68.8 ± 10.8	90.6 ± 6.0
7.2	50.0 ± 9.0*	93.8 ± 3.6	78.1 ± 6.0	81.3 ± 8.1	87.5 ± 5.1
18.0	0*	84.4 ± 7.9	84.4 ± 7.9	34.4 ± 7.9*	84.4 ± 7.9
45.0	0*	59.4 ± 9.4*	71.9 ± 6.0	50.0 ± 5.1*	71.9 ± 12.9

[a]Values represent the mean of 4 replicates ± SE; number of embryos per replica = 8. One replicate from F1 (0.45 µg/l) was lost due to possible bacterial infection. Static non-renewal exposures on embryos ~10 hr old (blastula stage), until 8 days (after completion of organogenesis).
[b]Control was embryo rearing medium (ERM).
[c]ERM-DMSO or ERM-eluting solvent in DMSO (0.05% v/v).
*Statistically significant ($p<0.05$), Wilcoxon's sign-rank, compared to respective controls.

Liver Cells Assays

At concentrations between 0.05 and 1.2 µg/l and in the absence of 17β-estradiol in the culture media, WE induced EROD

Table 3. Percent (%) of medaka embryos/larvae with selected abnormalities after continuous exposure to trichloroethylene soot whole extract (WE) and fractions 1 and 3 (F1, F3)

Concentration (µg/l)	Pericardial/other edema	Abnormal larval activity	Death resulting from edema	Delayed/ incomplete hatch
WE				
Control[a]	0	0	0	3
Solvent[b]	3	3	3	3
0.45	6	0	6	3
0.90	0	9	0	3
2.70	0	18	0	0
7.20	50	44	50	0
18.0	100	6[c]	88	6
45.0	100	3[c]	88	9
F1				
Control[a]	0	3	0	0
Solvent[b]	0	3	0	0
0.45	3	0	3	0
0.90	0	9	0	3
2.70	0	12	0	0
7.20	0	3	0	0
18.0	3	9	0	3
45.0	12	34	9	0
F3				
Control[a]	0	6	0	0
Solvent[b]	0	9	0	3
0.45	0	25	0	0
0.90	3	28	3	0
2.70	9	22	0	0
7.20	0	19	0	0
18.0	9	38	6	0
45.0	6	22	6	3

[a]Control was embryo rearing medium (ERM).
[b]ERM-DMSO or ERM-eluting solvent in DMSO (0.05% v/v). Values represent mean of nearest whole number from four replicates, except for 0.45 µg/l of F1 (loss of 1 replicate).
[c]Edemas produced the bulk of late embryonic mortality.

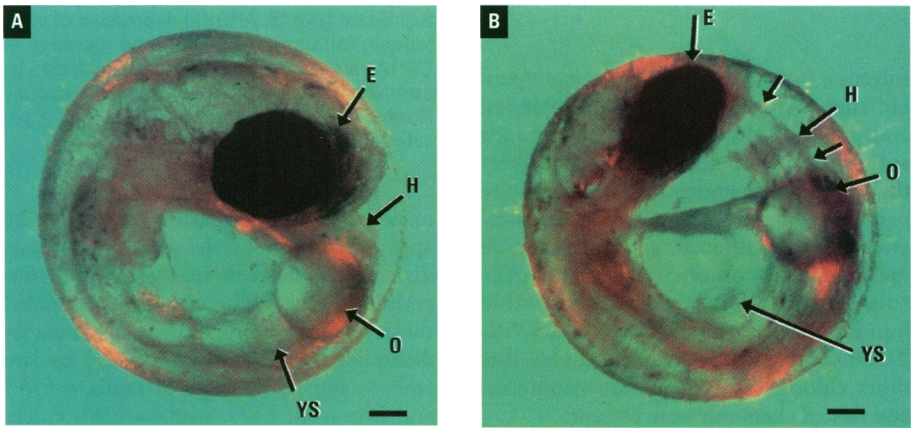

Figure 3. Normal (A) and abnormal (B) late-stage (216 hr) medaka embryos after control and trichloroethylene whole extract treatments. Note how pericardial edema (small arrows) results in separation of embryo proper from yolk sac. E, Eye; H, heart; O, oil droplet; YS, yolk sac. Bar = 100 µm.

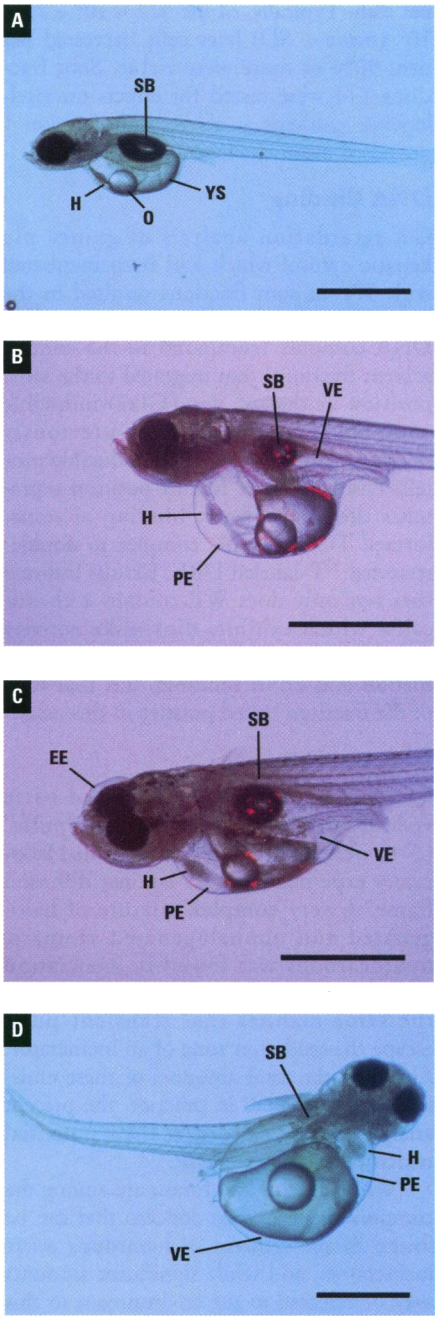

Figure 4. Normal (A), and abnormal (B–D) medaka larvae after control and trichloroethylene whole extract treatments. Note in B and C pericardial (PE), peritoneal/visceral (VE), and eye (EE) edema in larvae that managed to inflate swim bladder (SB) and all of the above plus no swim bladder inflation in D. H, Heart; O, oil droplet; YS, yolk sac. Severe edema preceded death. Bar = 1 mm.

activity. This induction was maximal at 0.6 µg/l. CYP1A1 protein synthesis was significantly increased at the three higher concentrations (Fig. 5). More WE was required to cause a detectable rise in CYP1A1 protein than for a rise in EROD activity. Only fraction 1 showed a significant increase in CYP1A1 protein level and EROD activity (29 and 45%, respectively).

All concentrations from 0.6 to 25 µg/l of WE depressed trout liver cell response to 17β-estradiol relative to the 17β-estradiol-only positive control. Inversely, CYP1A1 protein was induced in a concentration dependent manner with increasing concentrations of extract, with CYP1A1 protein synthesis maximal at 25 µg/l (Fig. 6). However, EROD activity at all concentrations tested was not significantly different from carrier or positive (17β-estradiol-only) controls (data not shown). Mean CYP1A1 protein level was higher, but not significantly, at the 3.95 µg/l WE in the absence of 17β-estradiol (Fig. 6). Significant depression of albumin synthesis (20–30%) was seen only at the higher concentrations (3.95–25 µg/l) of WE. However, the viability of cells exposed to all concentrations of WE was confirmed by phase contrast microscopy, cellular protein, and cellular LDH activity

per dish. Typically, of the $322 \times 10^6 \pm 73 \times 10^6$ (mean ± SD) liver cells harvested per fish, 90% or more were viable. Soot fractions 1–4 were tested for effects on vitellogenin synthesis as above; only fraction 1 depressed mean vitellogenesis (30%).

DNA Binding

Gel retardation analysis of guinea pig hepatic cytosol which had been incubated with WE or soot fractions resulted in the formation of a soot-inducible protein-^{32}P-DNA complex (compared to the control solvent fractions) that migrated to the same position as that of the TCDD-inducible complex (Fig. 7). We have previously shown (*32*) that the TCDD-inducible protein-DNA complex in this position represents the high affinity binding of transformed TCDD-(AhR) complex to double-stranded ^{32}P-labeled DRE. Results indicate that not only does WE contain a chemical(s) which exhibits dioxinlike activity (i.e., it binds to AhR activating its transformation and DNA binding), but that each of the fractions tested positive in this assay.

Discussion

Transient emissions of soot and toxic volatile organic hydrocarbons or "puffs" (*4–6*) were modeled in a well-defined laboratory experiment with a laminar diffusion flame. A very complex mixture of halogenated and nonhalogenated aromatic hydrocarbons was found in association with the aerosol that escaped the flame in the same manner that transient puffs escape the oxidation zone of an incinerator. Although the total amounts of these emissions may be small in practice, the present analysis has revealed that their potential toxicity may be significant.

While dioxins and furans are among the compounds of greatest concern that can be found in the effluent of hazardous waste incinerators, and while significant amounts may be released to the environment in this way (*35*), attention should not be exclusively directed toward these compounds. Harris et al. (*19,20*) found that certain PCB congeners and dioxin, extracted from Lake Ontario rainbow trout skeletal muscle, were toxic to medaka embryos. These compounds are present in Great Lakes biota at concentrations ranging from parts per trillion to parts per billion. It has been proposed that these non-*ortho*-substituted PCBs may contribute more to the overall toxicity than dioxins, which are present at lower orders of magnitude.

Nearly all chemical species in the mixture studied herein were heavily chlorinated (4-, 5-, 6-Cl) and sometimes perchlorinated. They included benzenes, styrenes, fulvenes, butadienes, fulvalenes, cyclopentadienes, naphthalenes, acenaphthylenes, and phenols. Although many compounds still remain unidentified, it is very likely that these as yet unidentified organics were configurational isomers of the main compounds just mentioned, given the possible mathematical combinations of chlorine substitutions across the many double bonds. Despite the absence of 2,3,7,8-TCDD and -TCDF, it is conceivable that other chlorinated dioxins, dibenzofurans, and related chemicals were present.

Results of this study confirm and extend previous work showing the presence of dioxinlike compounds (*14*) in this complex soot mixture and demonstrate that the WE and fractions 3 and 1 (products of mixed polarity and no polarity, respectively) caused toxicity and exhibited biological activity. The major developmental toxicity endpoint of this study was edema of pericardial cavity with extension to peritoneal cavity and yolk sac. Severe pericardial edema was accompanied by an uncoiling of the fused endocardial tube. This defect resulted in a reversal of initial chamber formation to that of a single, pulsatile tube. The latter, normally seen at an earlier stage of development, was also accompanied by apparent rupture of the posterior pericardial membrane with release of fluid into peritoneal cavity. These changes resembled those reported after exposure to dioxin or dioxinlike compounds (*12,38–41*) by late embryo and larval stages of rainbow (*36*) and lake trout (*37*), medaka (*18*), chick, fish-eating birds (terns, herons, double crested cormorants, and herring gulls), and rodents. Furthermore, the generation of toxicity in medaka embryos exposed to TCE soot resembled that of TCDD, where early development proceeded normally and was followed by a gradual progression of cardiotoxicity.

Histopathological studies have suggested that edema of endothelial cells and myocardial interstitium was an important early stage in cardiotoxicity (*37*). Interestingly, juvenile yellow perch (*Perca flavescens*), respond more aggressively with myocyte necrosis, hypertrophy, and hyperplasia of pericardial mesothelium as well as fibrinous

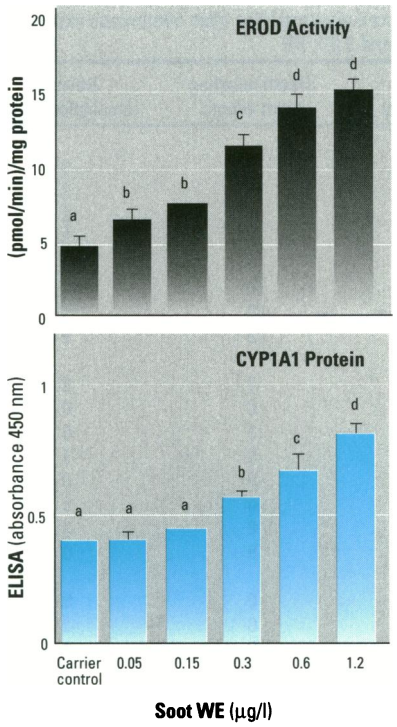

Figure 5. Soot whole extract (WE) induces ethoxyresorufin *O*-deethylase (EROD) activity and CYP1A1 protein in rainbow trout liver cells. Error bars = standard deviation. Means with the same letter are not significantly different ($p<0.05$, ANOVA). Number of dishes per treatment = 3–4, with duplicate determinations per dish.

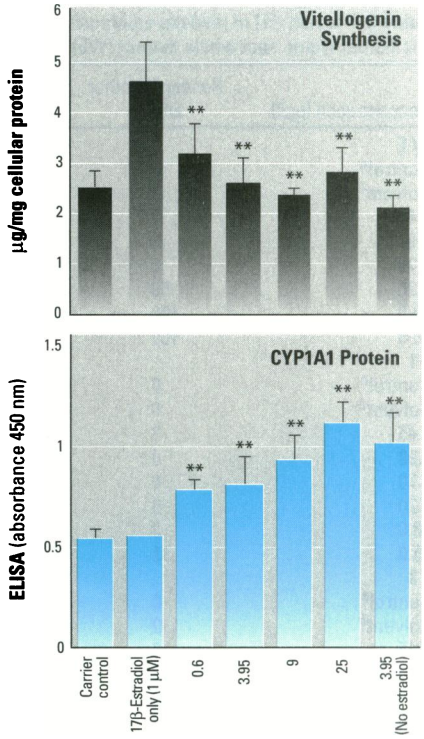

Figure 6. Effect of various concentrations of whole extract (WE) on vitellogenin and CYP1A1 protein levels in rainbow trout liver cells simultaneously exposed to 1 µM 17β-estradiol or carrier control. Error bars = standard deviation. Significant ($p<0.05$, ANOVA) depression of vitellogenin (all concentrations) and increase in CYP1A1 protein (all concentrations), indicated by asterisks, is relative to 17β-estradiol-only control. Number of dishes per treatment = 3–4, with duplicate determinations per dish.

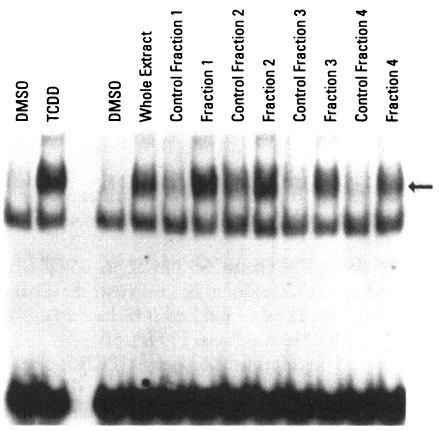

Figure 7. Soot and soot fractions stimulate arylhydrocarbon receptor (AhR) transformation and DNA binding. Guinea pig cytosol, incubated in the presence of 15 nM TCDD, whole extract, various control solvents or soot solvent fractions, was mixed with ^{32}P-labeled DRE oligonucleotide and specific protein–DNA complexes resolved by gel retardation as described in Methods. The arrow indicates the position of the inducible AhR:DNA complex. The following are densitometry readings (%) of the chemically induced bound complexes relative to that obtained with TCDD. Control readings were subtracted as background. TCDD (100%); whole extract (55%), F1 (69%), F2 (52%), F3 (62%), F4 (34%).

pericarditis (*42*). Although our initial histologic analyses have not revealed altered endothelial morphology, it is possible that fluid loss occurred through this tissue into pericardial and peritoneal cavities. The occurrence of edema in mammals, birds, and fish by TCDD and related compounds (*37*) suggests a common mechanism related to endothelial dysfunction (*43*). Immunohistochemical studies have localized CYP1A1 to endothelium of heart in scup (*Stenotomus chrysops*) (*44*) and salmonids, and embryonic induction occurs commonly in endothelial cells (*45*). It is possible that CYP1A induction (mediated through AhR activation) in our study could have led to oxidative injury and loss of endothelial integrity. Octachlorofulvalene appears to be a potent inhibitor and substrate of certain glutathione *S*-transferases (GSTs) (*46*), as are many extensively chlorinated compounds. Perhaps some embryo toxicity may be related to changes in cellular redox status resulting from depletion of reduced glutathione or GST inactivation.

Embryonic chick edema after TCDD or toxic PCB congener exposure suggested that increased prostaglandin synthesis, as a sequel to AhR activation, could mediate CYP1A induction and cardiotoxicity. Such a relationship was suggested by the ability of benoxaprofen, an anti-inflammatory drug, to reduce toxicity in 3,4,3′,4′-tetrachlorobyphenyl-treated embryos without affecting CYP1A induction, supporting a role for arachidonic acid metabolites (prostaglandins, leukotrienes, etc.) as mediators in toxicity, rather than induction itself (*38*). Wisk and Cooper (*47*) exposed medaka embryos to dioxin (≥10 ng/l) or beta-naphthoflavone (BNF; 50 µg/l) and found increased activity of benzo(*a*)pyrene hydroxylase. Induction of these CYP1A-associated enzymes over a period of days suggests that embryos have an intact AhR-mediated activation pathway. However, while benzo(*a*)pyrene hydroxylase induction, hemorrhage, and edema were seen in some medaka after dioxin treatment, others showed similar induction but no vascular changes at nontoxic levels of BNF. This suggests that CYP1A induction is not a prerequisite of cardiotoxicity. Nevertheless, the importance of AhR mediated events in embryonic cardiovascular toxicity needs further study.

While we are not aware of these types of studies in fish, investigations in other animal models have shown interaction between the CYP1A-AhR system and other CYP isoforms. These linkages involve metabolic alterations of endogenous substrates through biochemical pathways, which include antioxidant enzymes, metallothioneins, heat shock proteins, steroid receptors, oncogenes, tumor suppresor genes, glutathione, and GSTs (*45*). Possible involvement of rodent CYP1B1 in edematous lesions and overall dioxinlike toxicity, depending on tissue-specificities, is being investigated. Although highly inducible by TCDD/PAHs (via AhR) and involved in PAH metabolism (*48*), the presence of CYP1B1 in fish remains to be demonstrated.

Edematous spaces, devoid of cells, were observed in heart, peritoneum, and skin of medaka embryos, a condition similar to that of chickens exposed to TCDD and toxic PCBs (*38,39*). We cannot state whether the developing medaka has white blood cells capable of emigration into extravascular spaces, and we cannot rule out a compound-induced cytopenia. Other possible mechanisms underlying edema continue to be investigated. *In situ* nuclear magnetic resonance analyses from our laboratory suggest that transient depression of certain energy phosphate metabolite levels (mainly ATP) may lead to deficient ion translocation and consequent edema (Villalobos, in preparation). We are also focusing attention on the relative abundance of basement membrane components in control versus treated medaka embryos.

While WE adversely affected normal development in a concentration dependent manner, various concentrations of fractions 3 or 1 did not exhibit such a relationship. This finding may be related to solubility but has persisted over repeated assays. Perhaps combustion by-products of nonpolar and/or intermediate polarity act synergistically in the WE to produce effects whose impact was not apparent when a single fraction was assayed. However, synergism has not been specifically tested. Moreover, direct comparisons of the toxicity of combined fractions with WE are complicated by losses of volatile compounds or the reactivity of constituents like the chlorinated fulvenes and fulvalenes. Thus, evaluation of the toxicity of individual fractions should be viewed as a qualitative guide indicative of the polarity of the most toxic components of WE.

In vitro observations revealed no direct cellular toxicity but vitellogenin in medium was reduced. Fish liver cells are sensitive indicators of exposure to aquatic pollutants that have dioxinlike activity (*49–52*). Hepatocytes and biliary epithelial, and endothelial cells contain the readily inducible enzyme CYP1A1 (*45*). The liver plays a key role in reproduction in fish, being a component of the hypothalamic, pituitary, gonadal, and liver reproductive axis (*53*). In these oviparous vertebrates, the egg yolk precursor protein vitellogenin is synthesized in the liver and transported by the circulatory system to the developing oocytes. Vitellogenesis is under direct control of estrogens (*54*), and since CYP1A1-inducing compounds such as dioxin are known antiestrogens in mammals (*55*), the possibility exists that vitellogenesis and gonadal maturation could be disrupted in exposed fish.

At the concentrations tested, WE was not overtly toxic to liver cells but induced dioxinlike effects. EROD activity and the amount of CYP1A1 protein increased in a concentration-dependent manner, confirming the dioxinlike activity of component(s) of the extract. The EROD activity assay proved more sensitive in detecting significant changes in CYP1A1 expression at low WE concentrations than the CYP1A1 ELISA assay. 17β-Estradiol may have had an inhibitory or antagonistic effect upon EROD and CYP1A1 protein induction in cultured liver cells, as has been previously demonstrated *in vivo* with feminized brook trout (*56*) and in mouse fetal cell cultures (*57*). Higher concentrations of WE significantly increased CYP1A1 protein, but EROD activity remained unchanged. At concentrations above 0.6 µg/l, components of the WE may have competitively inhibited binding of ethoxyresorufin to CYP1A1. Substrate inhibition by PCBs in fish liver cell EROD assays has been demonstrated *in vivo* and *in*

vitro (*58,59*). These effects underscore the importance of conducting direct measurements of enzyme concentration in addition to enzyme activity.

Trout liver cells exposed simultaneously to noncytotoxic concentrations of 17β-estradiol and WE showed much less vitellogenin in medium than did similar cells exposed to 17β-estradiol alone. Vitellogenin levels and CYP1A1 protein appeared to be negatively correlated. Higher concentrations (3.95–25 µg/l) of the extract may affect the secretory capacity of liver cells; however, even at the 0.6 µg/l concentration (where albumin synthesis was not depressed) vitellogenin production was still compromised. From the fractions, only fraction 1 showed an effect on CYP1A1 protein or EROD activity (both increased), or vitellogenin (reduced). CYP1A1 inducing compounds may suppress vitellogenin production in fish liver cells by an antiestrogenic mechanism mediated through the AhR, similar to that described in mammals (*60*). We investigated whether this mechanism might apply to teleost liver, since AhR has been identified in this organ (*45*).

Numerous studies have revealed that most of the critical and sensitive toxic and biological responses to TCDD and related compounds are mediated by its soluble AhR, to which these chemicals bind with high affinity (*7,13,61*). After ligand binding, the halogenated aromatic hydrocarbon:AhR complex undergoes transformation into its DNA binding form and translocates into the nucleus (*62,63*). The transformed complex associates with a specific DNA sequence, the dioxin responsive element (DRE), resulting in transcriptional activation of adjacent responsive genes (*63–66*). Since previous studies have demonstrated a high correlation between binding of a chemical to the AhR and its degree of toxicity, the relative biological/toxicological potency of complex mixtures of chemicals can be estimated by measuring the ability of an unknown chemical/mixture to activate the AhR or an AhR-dependent response (*61,67*). Previously, we have utilized a gel retardation DNA binding assay to demonstrate that transformed TCDD:AhR complexes, formed *in vitro*, can bind to a DRE oligonucleotide specifically and with high affinity, mimicking that which occurs *in vivo* (*32,65,66*). Since there appears to be an excellent correlation between the ability of a given chemical to stimulate AhR transformation/DNA binding and its ability to activate gene expression, this technique has been utilized as a sensitive bioassay for the detection of dioxinlike chemicals (*33*).

The gel retardation assay results indicated that WE contains dioxinlike chemicals which not only bind to the AhR but also induce its transformation and DNA binding. The formation of inducible protein–DNA complexes by each soot fraction implies that the soot must contain numerous AhR ligands. Given the correlation between the ability of a given chemical to stimulate AhR transformation/DNA binding and its ability to activate gene expression, our results suggest that WE and fractions might also alter gene expression in mammals. In addition, given the role of the AhR in mediating toxicity of these chemicals (*7,13,61*), it is very likely that some of the toxicity produced by these compounds was AhR-mediated. Fractions 2 and 4 were not associated with developmental cardiotoxicity but did bind to the AhR inducing its transformation and DNA binding. While these processes may lead to cardiotoxicity, mediating factors are not known and need investigation.

In summary, CH_2Cl_2 extracts of TCE combustion aerosol proved toxic/bioactive using a battery of bioassays. The pattern of toxicity was identical to that previously reported for dioxin. Chemical analyses performed herein documented the presence of at least 250 chlorinated incomplete combustion by-products in the whole soot extract, but the obvious target compounds, TCDD and TCDF, were not present at detectable (picomole) levels. These results indicate that an array of toxic effects may arise from substances other than those targeted by conventional chemical analyses. They also suggest a need for bioassay-directed assessments of toxicity/biological potency in complex mixtures.

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