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Deformity Survey and Chemical Residues
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GREEN FROGS IN SOUTHWESTERN MICHIGAN: DEFORMITY SURVEY AND CHEMICAL RESIDUES IN WATER, SEDIMENT, AND TISSUE

By

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ABSTRACT

GREEN FROGS IN SOUTHWESTERN MICHIGAN: DEFORMITY SURVEY AND CHEMICAL RESIDUES IN WATER, SEDIMENT, AND TISSUE

By

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In an attempt to explain the etiology of frog deformities and population declines, many possible causative factors have been examined, including the input of synthetic chemicals into aquatic systems, where frogs spend much of their lives, including their entire developmental stages. This study focused on surveying wetlands in southwestern Michigan that are influenced by agricultural run-off or urban and industrial inputs for deformities in green frogs. Of the 1445 green frogs examined in 1998, only 4 (0.28%) exhibited morphological deformities. Water, sediment and tissue from frog eggs, tadpoles, juveniles, and adults were analyzed for organochlorine (OC) insecticides, polychlorinated biphenyls (PCBs), metals, and triazine herbicides by use of traditional instrumental analysis for most compounds and by Enzyme-Linked Immunosorbent Assay (ELISA) for triazine analysis. Sample extracts were also assayed for their estrogenic and dioxin-like activities using recombinant cell lines. Overall, low concentrations were observed in the frogs and their habitats. No correlation between deformities and chemicals could be made due to the low deformity rate in the region.

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Chapter 1: Survey of Green Frog Deformities in Southwestern Michigan

Introduction

Amphibians belong to a class of about 4,550 species of caecilians, salamanders, toads, and frogs (Stebbins and Cohen, 1995). They occupy a dual role in the ecosystem, serving as predators to many insects and other small animals while acting as prey for a large number of animals, including reptiles, birds, and mammals. Due to the important role of amphibians in the ecosystem, a decline in their populations can have deleterious effects upon many different populations of animals and, thus, upon the many different ecosystems that they inhabit (Stebbins and Cohen, 1995). Further, amphibians serve as excellent sentinels for the effects of chemical stressors during development. This is because they are very sensitive to a variety of factors due to their complex life histories in both the aquatic and terrestrial environments and the permeability of their skin to water and electrolytes (Stebbins and Cohen, 1995). Therefore, a decline in amphibian populations or the expression of developmental abnormalities could be indicative of environmental changes, including exposure to xenobiotics.

Amphibian populations have been declining in various parts of the world for at least two decades (Wake, 1991). In February 1990, a workshop sponsored by the National Research Council's Board on Biology documented declines over the past 20 years in certain amphibian populations around the world. These declines include the almost complete disappearance of boreal toads (*Bufo boreas boreas*) from their traditional breeding range in the West Elk Mountains of Colorado (Carey, 1993), and golden toads

(*Bufo periglenes*) and the harlequin frog (*Atelopus varius*) from Costa Rica's Monteverde Cloud Forest Preserve in the mid-1980s (Pounds and Crump, 1994).

Recently, anatomical deformities of frogs have attracted international attention from both the scientific community and the public. In 1995, a group of Minnesota schoolchildren encountered a large number of northern leopard frogs (*Rana pipiens*) with hypermorphic (multiple digits and/or limbs) and hypomorphic (missing digits and/or limbs) limb abnormalities in a farm wetland in southern Minnesota (Kaiser, 1997). Malformed frogs and other amphibians had been studied previously in different regions; however, this incident brought deformed frogs into the spotlight of many environmental and scientific causes.

Deformed mink frogs (*Rana septentrionalis*) collected from a lake in Minnesota in 1996 and 1997 exhibited numerous limb abnormalities, the most notable being the presence of more than the normal number of hind limbs (Gardiner and Hoppe, 1999). Out of the 23 frogs examined by skeletal analysis, there were a total of 101 hind limbs. The majority of these hind limbs were abnormal due to skeletal dysplasia, characterized by triangles, hypomorphism, split limbs, and supernumary distal elements, and due to skin webbing (inter-limb, inter-segmental, and intra-segmental webbing). The authors speculated that the deformities were caused by exposure to degraded products of the insecticide methoprene that can mimic retinoic acid (Vitamin A), which is known to cause similar deformities under laboratory conditions (LaClair et al., 1998).

Populations of some of the 13 endemic frog and toad species in Michigan have declined in recent years (Michigan Department of Natural Resources (MDNR), 1996), based upon frog call surveys at known and historical breeding locations. The Blanchard's cricket frog (*Acris crepitans blanchardi*) and the boreal chorus frog (*Pseudacris triseriata maculata*) are listed as "special concern" (Michigan Natural Features Inventory (MNFI), 1999). In 1996, the northern leopard frog was observed at only nine percent of its expected breeding sites. Likewise, the green frog (*Rana clamitans*), wood frog (*Rana sylvatica*), and the bullfrog were found at an average of 53, 47, and 4%, respectively, of their expected breeding sites (MDNR, 1996).

Several hypotheses have been proposed to explain these population declines and anatomical malformations. Several possible causative agents have been suggested to cause frog deformities. The proposed mechanisms can be classified as chemical, physical, or biological. Specifically, these hypotheses include the following: 1) chemical contamination in the water (Burkhart et al., 1998), 2) acid precipitation (Gannon, 1997), 3) increased levels of ammonia in the water (Jofre and Karasov, 1999), 4) increased ultraviolet radiation due to the thinning ozone layer (Ankley et al., 1998), and 5) parasites encysting in the developing limb buds (Sessions and Ruth, 1990). The aforementioned factors can also contribute to population declines, as well as general diseases, habitat destruction, climate changes, introduction of predators and competitors, and human consumption (Wake, 1991).

It is possible that the observed effects are caused by not one, but a combination of factors acting in combination (Wake, 1991). Furthermore, while the occurrence of deformities and declines in amphibian populations seem to be global in nature, there is probably no single universal factor to explain all of the population declines and deformities. For example, exposure to synthetic chemicals may be affecting some populations whereas increased UV irradiance may be involved in another region. Most of the proposed hypotheses suggest that humans are contributing to the demise of amphibians and may be indicative of environmental changes. However, most studies of amphibian populations have been conducted relatively recently. Without long-term census data, it will be difficult to determine whether population declines are due to natural fluctuations or anthropogenic factors (Pechmann et al., 1991).

Amphibian deformities have been reported since the 1700s (Van Valen, 1974), which suggests that the deformities currently being observed are a natural phenomenon. Perhaps the increased attention on deformed frogs is leading to the greater number of sightings of frogs with developmental abnormalities. Therefore, more research will be required to fully understand the scope of the amphibian dilemma.

The UV radiation hypothesis has received some support, particularly in regions where the ozone layer has decreased (Gannon, 1997). A less dense ozone layer allows more ultraviolet radiation to reach the earth, where it can cause more damage to life (Berner and Berner, 1996). Deformed frogs have been found in the northern latitudes, where increases in UV-B light have been shown to occur in late spring, early summer, and coinciding with the amphibian breeding season (Ankley et al., 1998). A recent study produced hind-limb deformities in leopard frog embryos by exposing them to UV-B radiation under laboratory conditions for more than 24 days (Renner, 1998). However, this study has also been the center of a great deal of debate, because the laboratory setting for this study does not duplicate actual environmental settings, as far as physical parameters in the water column and the amount of UV-B radiation received by the frog embryos. Another study indicated that treatment of leopard frogs with UV-B light can induce bilateral and often symmetrical ectromelia and ectrodactyly (missing limbs and digits). The authors of this study also admit that these conditions and effects are not representative of those in nature (Ankley et al., 1998).

The biological (parasite) explanation involves one or more types of parasite contributing to the deformities through a variety of mechanisms (Johnson et al., 1999; Sessions and Ruth, 1990). Two major studies have found that naturally occurring trematodes form cysts in amphibian limb buds, which potentially disrupts limb development and leads to a range of deformities. Recently, a fungus known as chytrid skin fungus was found in dead and dying frogs in Arizona (Milius, 1998; Lips, 1999). This fungus has not been known to infect vertebrates, but is the same fungus that is considered responsible for recent amphibian die-offs in Australia and Central America. While this fungus appears to be more responsible for amphibian deaths than deformities, it is still representative of the wide variety of parasites that can infect frogs. One possibility is that the fungal infections are secondary to lesions caused by the chemical or biological etiologies.

Chemicals, primarily pesticides and industrial byproducts, are ubiquitous in the environment. Even remote areas experience some degree of contamination due to atmospheric transport of pollutants, such as polychlorinated biphenyls (PCBs), DDT, and mercury compounds (Wania et al., 1998). Frogs spend the greater part of their developmental stages in the aquatic environment; hence, they can be directly exposed during their critical growth period to any chemicals that may be in the water (Stebbins and Cohen, 1995). Thus, some of these chemicals have the capacity to cause growth anomalies. For instance, a recent study in the Quebec region found more frogs with deformed hind limbs in areas where historically pesticides have been used than from areas that have not been exposed to pesticides (Ouellet et al., 1997). However, no concentrations of residues were measured. Thus, no correlations or gradients of deformities and exposure to chemicals could be established. One pesticide shown to cause life-threatening deformities in leopard frogs is methoprene (Ankley et al., 1998). Degradation products of methoprene have been suggested to affect the retinoid-signaling pathway via interaction with one or more retinoid receptors (Ankley et al., 1998). Many of the compounds currently being studied in relation to the deformity issue have been characterized as environmental endocrine disruptors, compounds that disrupt the normal functioning of the endocrine system (Kendall et al., 1998).

To document deformities in southwestern Michigan, an amphibian deformity survey was initiated in 1998. This deformity survey was a part of a larger study that attempted to link deformities to mechanical disruption and chemical contamination. The objectives of this study were to: 1) assess the incidence of green frog (*Rana clamitans*) deformities in southwestern Michigan; 2) determine concentrations of organochlorine insecticides, PCBs, metals, and triazine herbicides in green frog adult, juvenile, tadpole, and egg tissues from the sampling locations; 3) determine the levels of these compounds in the water and/or sediment inhabited by green frogs; and 4) correlate the incidence of deformities to chemical concentrations in the frogs and/or their environment.

Materials and Methods

The primary study region was southwestern Michigan (Figure 1). This region was chosen because of the variety of habitats available. The sampling locations ranged from agricultural areas, reference locations, and locations with known historical and current PCB contamination (Table 1). Other regions of Michigan are not as likely to have the available green frog habitat amidst these different locations. These seven locations were chosen to give a wide variety of sampling sites with a wide range of potential chemical influences. The reference locations (REF1, REF2, and REF3) are within the Allegan State Game Area in Allegan County, an area of successional forests that is not currently and directly impacted by agriculture or urban activity. The agricultural areas (AGR1, AGR2, and AGR3) are located in St. Joseph and Cass Counties, in a region characterized by cash crop farming of corn and the usage of triazine herbicides, most notably atrazine. The Kalamazoo River passes through urbanized and industrialized areas as well as agricultural areas. The sampling location for this area (KLMZR) is a marsh created by a nearby dam on the river in Kalamazoo County. Specific location ranges are given for the reference and Kalamazoo River sampling locations (Table 1). Specific locations cannot be given for the agricultural sampling locations based on request made by the farmers who own the land.

There were four sampling dates during the summer of 1998 – June 5, June 23, July 22, and August 26. Sampling for tadpoles took place during the daylight hours, while sampling for adults occurred between sunset and sunrise. The sampling dates were chosen based on agricultural applications of triazine herbicides, which would potentially impact the herbicide concentrations of the agricultural locations. The sampling was conducted early in the summer after triazine applications, then later in the summer when less herbicides, if any, were applied (Mehne, personal communication).

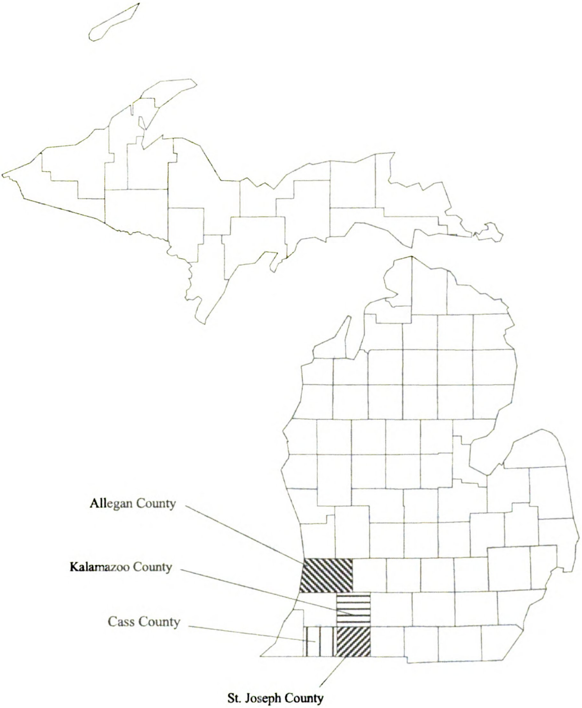

Figure 1. Counties of southwestern Michigan from which green frogs were collected, 1998.

Table 1. Sampling location characteristics.

Site	Characteristics
REF1	118th pond, T.2N. R.14W., section 17. Permanent pond, depth 0-5 ft., various grasses border the pond edge, lily pads, submergent vegetation present. This sampling location is surrounded by mixed hardwood forest.
REF2	Crooked Lake, T.2N. R.15W., section 36. Small lake, depth 0-8 ft., cattails and various grasses border the lake edge, submergent vegetation present. This sampling location is surrounded by mixed hardwood forest.
REF3	Swan Creek, T.2N R.14W., section 20. Marsh in a drainage area, depth of 0-8 ft., various grasses border the marsh edge, submergent vegetation is sparse. This sampling location is surrounded by mixed hardwood forest.
KLMZR	Kalamazoo River, T.2N R.14W., section 10. Marsh in a drainage area, formed from a man-made dam along the Kalamazoo River, depth 0-6 ft., cattails, various grasses, and mixed hardwoods border the marsh edge, submergent vegetation present.
AGR1	Temporary pond, depth 0-5 ft., cattails, saw grass, and other various grasses border the pond edge, submergent vegetation present. Surrounded by corn fields in an agricultural region.
AGR2	Permanent pond, depth 0-3 ft., cattails and various grasses border the pond edge, submergent vegetation present. Surrounded by corn fields in an agricultural region.
AGR3	Drainage ditch, depth 0-3 ft., cattails and various grasses border the ditch edge, no submergent vegetation. This sampling location is located in an agricultural region.

The green frog (*Rana clamitans*) was selected for three reasons. First, the green frog is one of the most abundant frogs in the Great Lakes region (Harding, 1997); thus, these frogs are relatively easy to locate and the study will not be negatively impacting a species with an already small population. Second, green frogs tend to remain in one aquatic location exhibiting less of a tendency to migrate to upland, dryer areas than other frogs (Harding, 1997). Therefore, these frogs are more likely to be affected by chemicals in the body of water where they are caught. Lastly, green frogs are relatively large frogs that eat a variety of prey from plant material to insects to smaller frogs and snakes (Harding, 1997). Since part of their diet places them at a higher trophic level, residues have the potential to bioaccumulate in their bodies, with the possibility of being passed along through the eggs to their offspring (Lu, 1991).

The life stages collected were tadpole, juvenile, and adult. These life stages are all distinct from one another. Tadpoles are found only in the water, and have either no legs or two developing hind limbs, and a tail. Juveniles are found in the transition area between land and water, have both hind limbs and forelimbs, and remnants of a tail or tail stub. Adults lack any form of tail stub and are of breeding size (about three to five inches).

Tadpoles, juveniles, and adults were collected by either dip net or hand. Most adults were collected after sunset. Tadpoles were identified according to Watermolen and Gilbertson (1996). All frogs were examined for anatomical deformities, such as malformations of the tail, eyes, and mouth. Additionally, adults and juveniles were examined for hypomorphic and hypermorphic limb abnormalities. Possible deformities were carefully examined to ensure that the deformity was not simply a wound or scar tissue. For instance, a missing limb may have at first appeared to be a hypomorphic limb abnormality. However, with more careful scrutiny, the skin in the region of where the limb should be may have scar tissue, which would indicate that this "deformity" was actually a wound caused by predation. The majority of frogs captured were immediately released, after examination, back into the same locations from which they were captured. Only the minimal number of frogs necessary for future analyses was kept.

Results and Discussion

A total of 1445 green frog adults, juveniles, and tadpoles were examined from all four sampling dates and all the sampling locations (Table 2). Of these, four were deformed. One adult had six toes on its right hind leg, one juvenile had a shortened forelimb, one juvenile had only three legs, and another juvenile had a malformed rear foot (Table 2).

A greater number of frogs from REF1 were deformed than from the other sampling locations. More frogs were collected from REF1 than from any of the other sampling locations; therefore, the overall percentage of deformed frogs from this site (0.34%) was actually less than the overall percentage of deformed frogs from KLMZR (0.38%) (Table 3). The overall deformity rate among the green frogs collected from southwestern Michigan was 0.28% (Table 3). This percentage is much lower than observed deformity rates from other regions. For instance, 50% of the leopard frogs caught by the school children in the Minnesota wetland in 1995 were deformed (North American Reporting Center for Amphibian Malformations (NARCAM), 2000). Of the 853 anurans observed at the agricultural locations near Quebec, 12% exhibited physical malformations (Ouellet et al., 1997).

It is important for the accuracy of the deformity survey to look at all the life stages of the frogs. For instance, if a tadpole were to have extreme deformities, it may not survive to adulthood. The same is true for juvenile frogs. If it were missing a leg, then it may be preyed upon and not survive to adulthood. Therefore, a more accurate deformity count was conducted by examining frogs of all life stages.

Table 2. Number of green frogs collected, including number of deformities, from southwestern Michigan, 1998.

Location	Adult	Juvenile	Tadpole
	June 5, 1998, N = 154		
REF1	17, 1 deformed[1]	0	20
REF2	0	0	12
REF3	3	0	20
KLMZR	18	0	20
AGR1	0	0	21
AGR2	0	0	22
AGR3	1	0	0
	June 23, 1998, N = 417		
REF1	68	8	202
REF3	7	7	20
KLMZR	11	0	30
AGR1	24	0	0
AGR2	2	0	20
AGR3	14	0	4
	July 22, 1998, N = 636		
REF1	52	145, 1 deformed[2]	230
KLMZR	48	24	29
AGR1	3	0	0
AGR2	3	38	0
AGR3	3	61	0
	August 26, 1998, N = 238		
REF1	8	30, 1 deformed[3]	98
KLMZR	3	47, 1 deformed[4]	35
AGR2	10	4	0
AGR3	2	1	0
	Total Frogs = 1445		

[1] Six toes on right hind leg

[2] Shortened forelimb

[3] Missing hind limb

[4] Malformed rear foot

Table 3. Prevalence of deformities, and total number of green frogs by location, in southwestern Michigan, 1998.

Location	Overall Frog Count	Number Deformed	Percent Deformed
REF1	878	3	0.34%
REF2	12	0	0
REF3	57	0	0
Total REF	947	3	0.32%
KLMZR	265	1	0.38%
AGR1	48	0	0
AGR2	99	0	0
AGR3	86	0	0
Total AGR	233	0	0

The fact that few deformities were observed during this survey may be indicative of a relatively stable green frog population in southwestern Michigan and that the residues measured were either not the causative agent of deformities observed or at least not occurring at sufficient concentrations at these locations. While some Michigan frog populations have been reported to have declined (MDNR, 1996), reports of deformities have been rare. Other reasons for the low deformity rate observed can be due to the possibility that any deformities caused lethality to the frogs earlier in the season or in earlier life stages before they could be recorded.

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Chapter 2: Organochlorine Insecticides and PCBs in Water and Green Frogs from Southwestern Michigan

Introduction

Frog deformities and worldwide amphibian declines have been the focus of a great deal of scientific research and debate over the past 15 years. Several hypotheses have been formulated as to the reasons for these phenomena; however, no definite causes have been found (Burkhart et al., 1998). One possible explanation is exposure to chemical residues. In particular, exposure to chemicals that have the potential to modulate the endocrine system are of current concern (Ankley et al., 1998).

Organochlorine (OC) insecticides, which were introduced in the mid-twentieth century, are a group of pesticides that are chlorinated, persistent, and lipophilic (Bunce, 1994). These compounds include chlordane, which is an insecticide and a fumigant, and lindane (the γ-isomer of hexachlorocyclohexane), which is an insecticide and a scabicide (Verschueren, 1983). 2,2-bis-*p*-chlorophenyl-1,1,1-trichloroethane (DDT), one of the most well known of these compounds, was banned in the United States in 1972 because of its harmful environmental effects (Bunce, 1994). However, it is still in use in some tropical countries and continues to persist, along with its metabolites DDE and DDD, in some parts of the environment in both more and less developed countries (Bunce, 1994).

While there are multiple mechanisms of toxic action depending on the species and compound, most organochlorine compounds are neurotoxic to invertebrates (Wexler, 1998). *p, p*'-DDT has been shown to cause snout deformities in tadpoles of the common

European frog (*Rana temporaria*) (see Osborn et al., 1981). A follow-up study concluded that similar effects could be caused by exposure of tadpoles to the steroid hormone corticosterone (CORT) (Hayes et al., 1997). Hence, it has been hypothesized that DDT can mimic CORT or act as a more general stressor that can lead to an increase in endogenous CORT (Hayes et al., 1997). This increase in CORT may then lead to the observed deformities. Regardless of the mechanism of action, DDT and other organochlorine insecticides have been implicated as a possible cause of deformities in frogs.

Like the OC insecticides, polychlorinated biphenyls (PCBs) are chlorinated hydrocarbons that are lipophilic, persistent, and have the potential to biomagnify in the food chain (Bunce, 1994). In total, there are 209 congeners of PCBs, each with a different number or location of the chlorine substituents. PCBs were first detected in wildlife in the mid-1960s, and were soon discovered to be ubiquitous in the environment. Because of their toxicity to humans and other animals, and their ability to persist in the environment and biomagnify up the food chain, PCB production in North America was banned in 1977. However, due to their recalcitrant properties, great concentrations of PCBs persist in many regions, primarily in aquatic ecosystem (Bunce, 1994; Zakrzewski, 1997).

The toxicity of each PCB congener is determined by its structure. One of the important determinants of biological activity is planarity. The planarity of PCB congeners depends on the number and position of the chlorine atoms. Those congeners with at least two meta- and two para-chlorine atoms are the most toxic (Safe, 1990). Congeners with chlorine atoms in the ortho-positions are considered to be less toxic because they are less planar. Planar PCBs are structurally similar to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). For this reason, planar PCBs elicit similar biological responses as TCDD. These common responses include teratogenicity, carcinogenicity, embryo lethality, and suppression of immune responses (Bunce, 1994). One reason for the extreme toxicity of TCDD is its ability to bind to the aryl-hydrocarbon receptor (Ah-receptor), a cellular receptor that seems to be present in all vertebrates (Safe, 1986). PCBs and dioxins have also been shown to exhibit endocrine disrupting effects (Birnbaum, 1995). While the eggs and tadpoles of some frogs seem to be relatively tolerant to acute exposure of TCDD (Jung and Walker, 1997), TCDD and coplanar PCBs have been shown to offset thyroid hormone status of some vertebrates (Brouwer et al., 1989).

Mixtures of PCBs in the environment differ based on the proportion of congeners in the environmental samples (Bunce, 1994). In order to understand the toxicity of these mixtures, the World Health Organization has derived specific toxic equivalency factors (TEFs) to compare the potential toxicity of environmental PCB mixtures to TCDD (Van den Berg et al., 1998). TEFs indicate an order of magnitude estimate of the toxicity of a compound relative to TCDD, based on *in-vivo* and *in-vitro* toxicity studies. TEF values, in combination with chemical residue data, can be used to calculate toxic equivalent (TEQ) concentrations in various environmental samples, including animal tissues, sediment, and water (Sanderson and Giesy, 1998).

Both DDT and PCBs, along with other synthetic compounds in the environment, have been demonstrated to be estrogen mimics (Kendall et al., 1998). These weak estrogen agonists have been postulated to have the potential to offset the neuro-endocrine system. Specifically, xeno-estrogens have been suggested as possible causative agents of adverse effects, including deformities, which have been observed in wildlife (Villeneuve et al., 1998). Estrogen receptor agonists are compounds that bind to the estrogen receptor (ER), forming a receptor-ligand complex that elicits a biological response. The endogenous estrogen 17B-estradiol binds to the ER with the greatest affinity. The ER binding affinity of other compounds are compared to 17B-estradiol by use of factors called estrogen equivalents (EQs). The response that is elicited is proportional to the EQ (Villeneuve et al., 1998).

The primary objectives of this study were to assess the incidence of green frog deformities in southwestern Michigan, and to correlate those deformities to chemical residues in the frog tissue. No significant deformities were observed among the sampling locations, as discussed in Chapter 1. Therefore, no correlations between the observed residues and deformities could be developed. However, the remaining study objectives remained viable. These objectives involved gathering data on the levels of organochlorine residues in water and in tissues of green frog eggs, tadpoles, juveniles, and adults. Concentrations of *p,p*'-DDT, *p,p*'-DDE, *p,p*'-DDD, the α- and γ-isomers of chlordane, the α-, β-, γ-, and δ-isomers of hexachlorocyclohexane (HCH), and the non-*ortho*-, mono-*ortho*-, and di-*ortho*-substituted PCB congeners were determined in water and tissue. Also, TEQs and EQs of the water and tadpole tissue were determined using in-vitro cell bioassays.

Materials and Methods

Water and Frog Sampling and Preparation

Tadpoles and adults of the green frog (*Rana clamitans*) were collected by dip net and hand from sampling locations in southwestern Michigan (see Chapter 1). All the life stages from all the locations used in the deformity survey were not analyzed for organochlorine residues, based on the availability of frogs at each location and the necessity of those frogs being used in other aspects of the study (Table 4).

Tadpoles and adults were weighed and measured, and the sex of the adults was determined based on physical features. The most common means of sexing green frogs is based on the size of the tympanum. In males, the tympanum is larger than the eyes. Males also may have a yellowish throat, whereas the throat of the females is generally whitish (Harding, 1997). Tadpoles were pooled into samples of approximately 20 g wet wt. by location and date. Pooling of samples was not necessary for the adults. Eggs were collected from the frogs and not from floating egg masses. In each case where frogs contained eggs, the eggs were removed prior to extraction. Most of the eggs were pooled into samples up to a maximum of 20 g by location and date. All frog tissues were stored at –20°C until analysis.

Four liters of water were collected from each sampling location on the first two sampling dates (6/5 and 6/23). Samples were collected into four-liter brown glass solvent bottles by submerging each bottle into the water until full. To ensure that they were clean, bottles were washed with water and Liquinox® (Alconox, New York, NY, USA), rinsed with acetone and hexane, and allowed to dry before use. Water samples were stored at 4°C until analysis.

All glassware, stainless steel homogenizer materials, boiling stones, glass wool, and any other utensils and surfaces that would contact samples during the analysis were cleaned by standard procedures to minimize contamination of the sample. Briefly, analytical items were washed thoroughly with Liquinox® (Alconox, New York, NY, USA) and water, then rinsed with deionized water. Materials were allowed to air dry before being rinsed three times with acetone followed by three rinses with hexane. Solvent-cleaned materials were blown dry with a heat gun and placed on solvent-rinsed aluminum foil. All solvents used for any part of the analysis or cleaning steps were HPLC grade (Burdick and Jackson, Muskegon, MI, USA).

Frog Tissue Extraction and Clean-Up

PCBs and OC pesticides were extracted following methods described elsewhere (Kannan et al., 1995; Khim et al., 1999). The Standard Operating Procedure for the analysis of organochlorines, such as DDT and PCBs, is presented in Appendix C. Each tissue sample was thawed at room temperature for 1 h before being prepared for analysis. Each sample was ground in a stainless steel homogenizer cup by use of an Omni-Mixer (Sorvall®, PRO Scientific Inc., Monroe, CT, USA). Sodium sulfate was slowly added during the blending process (about 150 g anhydrous Na_2SO_4 for a 20 g sample) until the sample was thoroughly dry. Samples were extracted by Soxhlet extraction with a 3:1 ratio of dichloromethane (DCM) to hexane for at least 12 h. Extracts were concentrated to 11 ml and a 1 ml subsample was removed to determine the lipid content of the tissues. Lipid content was measured gravimetrically.

The 10 ml extracts were further concentrated to 3 ml for fractionation. Florisil (60-100 mesh size; Sigma, St. Louis, MO, USA) was activated at 130°C for at least 12 h. For each sample, 10 g of Florisil was suspended in hexane and transferred to a glass chromatography column. The 3 ml extract was placed on top of the Florisil and eluted at a flow rate of approximately 3 ml / min. The first fraction (F1), which contained the least polar analytes, was eluted from the column with 100 ml of hexane. The moderately polar compounds were eluted in the second fraction (F2) with 100 ml of 20% DCM in hexane. The final fraction (F3), which was eluted from the column with 100 ml of 50% methanol in DCM, contained the most hydrophilic compounds.

The volumes of the eluents were reduced by evaporation to 2 ml for F1 and 0.1 ml for F2 and F3. F2 was adjusted to 2 ml with hexane. F3 was taken up to 1 ml with acetonitrile and set aside for latter analyses of triazine herbicides. F1 and F2 eluents were treated with 2 ml aliquots of sulfuric acid to destroy interfering compounds. After the sulfuric acid treatments, the hexane extracts were rinsed with deionized water. The eluents were further concentrated to 1 ml and placed in GC vials for analysis.

Water Extraction and Clean-Up

For each sample, 2 L of water was filtered through glass wool and transferred to a 4 L separatory funnel. The sample in the separatory funnel was shaken for 15 min with 200 ml DCM and allowed to separate for at least 12 h. The DCM fraction, which contained the more hydrophobic compounds, was drained into a flask and the procedure was repeated after a 4 h separation period. The second DCM fraction was added to the same flask as the first and the fractions were concentrated to 0.1 ml. The extracts were adjusted to 1 ml with acetonitrile and placed into GC vials. In order to be injected into the GC/ECD, a 0.2 ml aliquant was later removed, concentrated to 0.1 ml, made up to 0.2 ml with hexane, and placed in GC vial inserts inside GC vials.

Instrumental Analysis

PCBs and OC pesticides in the water and tissue samples were quantified using a gas chromatograph (Perkin Elmer series 600) equipped with ^{63}Ni electron capture detector (GC-ECD). A fused silica capillary column coated with DB-1 (100% dimethylpolysiloxane, 30m x 0.25mm i.d.; J&W Scientific, Folsom, CA, USA) having a film thickness of 0.25 μm was used. The column oven temperature was programmed from 85°C (0.5 min hold) to 110°C at a rate of 10°C/min and then to 260°C at a rate of 2°C /min with a final holding time of 10 min. Injector and detector temperatures were kept at 250°C and 350°C, respectively. Helium was used as the carrier gas and nitrogen was the make up gas. A PCB mixture, containing known concentration of 98 congeners commonly present in aquatic biota, was used as the PCB standard. This mixture was prepared from 98 individual PCB standards which were purchased commercially

(AccuStandard, New Haven, CT, USA). Concentrations of 98 individually resolved peaks were summed to obtain total PCB concentrations. OC pesticides were quantified from individually resolved peak heights based on the peak heights of standards. Detection limits for total PCBs and OC pesticides were 1.0 and 0.01 ng/g, wet wt, respectively. Samples were spiked with PCB and OC pesticide (CLP-023R, CLP-024R, AccuStandard) standards and passed through the entire analytical procedure to test for recoveries (n=3). Recoveries in Florisil F1 were 99±5% and 90±5% for individual PCB congeners and *p, p'*-DDE, respectively (n=3). No other spiked compounds were detected in F1. Recoveries of remaining OC pesticides in F2 were 105±5% (n=3). No other spiked compounds were detected in F2. Recoveries of OCs in SRM were between 90 and 105%. Concentrations reported were not adjusted for recovery.

Cell Bioassay Analysis

The Standard Operating Procedures for the following cell bioassays are presented in Appendix D. MVLN cells are MCF-7 human breast carcinoma cells stably transfected with a luciferase reporter gene under control of estrogen response elements (EREs) of the Xenopus vitellogenin A2 gene (Demirpence et al., 1993). H4IIE-luc cells are rat hepatoma cells which were stably transfected with a luciferase reporter gene under control of dioxin-responsive enhancers (DREs) (Sanderson et al., 1996). All cells were cultured in 100-mm disposable petri plates (Corning, Corning, NY, USA) and incubated in a humidified 95:5 air : CO_2 atmosphere. MVLN and H4IIE-luc were maintained at 37°C. MVLN were grown in Dulbecco's Modified Eagle Medium with Hams F-12 nutrient mixture (Sigma D-2906, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 27.3 I.U. insulin (Sigma I-1882) / L and 1.0 mM sodium pyruvate (Sigma). H4IIE-luc were cultured in Dulbecco's Modified Eagle Medium (Sigma D-2902) supplemented with 10% FBS (Hyclone). Cells were passaged when plates became confluent and new cultures were started from frozen stocks after less than 30 passages.

Detailed methods for the *in vitro* bioassays used in this study have been described elsewhere (MVLN assay (Koistinen et al., 1998); H4IIE-luc assay (Sanderson et al., 1996)). In brief, cells were trypsinized from plates containing 80-100% confluent monolayers, resuspended in media, and diluted to a concentration of approximately 7.5×10^4 cells/ml and seeded into the 60 interior wells of 96-well culture ViewPlates™ (Packard Instruments, Meriden, CT, USA) at 250 μl per well. The 36 exterior wells of each plate were filled with 250 μl culture media. Cells were incubated overnight, then dosed. Test and control wells were dosed with 2.5 μl of the appropriate extract or solvent. Blank wells received no dose. A minimum of three control wells and three blank wells were tested on each plate. For initial screening, extracts were tested in three replicate wells at a single concentration. For dose-response characterization, dilution series consisting of six concentrations of sample, prepared by 3-fold serial dilution, were tested (3 replicate wells per concentration).

All bioassay data was collected electronically and imported into a spreadsheet for data analysis. Relative luminescence units (RLU) were not adjusted for protein. Sample responses, expressed as mean RLU (across 3 replicate wells), were converted to a percentage of the mean maximum response observed for standard curves generated on the same day (%-E2-max. and %-TCDD-max. for 17-β-estradiol and TCDD standards respectively). This was done to normalize responses for day-to-day variability in response magnitude. The mean solvent control response (RLU) was subtracted from both sample and standard responses (RLU) on a plate-by-plate basis, prior to conversion to a percentage, in order to scale values from 0 to 100%-standard-max. Significant responses were defined as those outside of the 95% confidence interval (expressed in %-standard-max.) around the mean solvent control response (0%-standard-max.).

The dose-response relationships obtained from bioassay analysis of sample serial dilutions were generally not complete enough to facilitate formulation of a regression equation. As a result, relative potency estimates were not possible for most of the samples analyzed in this study. In cases where a regression equation could be fit to the dose-response, sample potency (relative to a dioxin or 17-β-estradiol (E2) standard) was calculated as EC-30 of the standard / EC-30 of the sample. In order for such a point estimate of relative potency to be valid, the sample and standard dose-responses must be statistically parallel and have the same maximum achievable response (Putzrath, 1997). This could not be demonstrated for the samples analyzed in this study. Thus, the relative potencies calculated should be regarded as approximations, based on assumptions of equal efficacy and parallel slopes.

Results

Lipids

Egg tissue contained greater lipid content (mean range 9.73 - 11.10%) than did either the adult (mean range 0.50 - 2.63%) or the tadpole tissue (mean range 1.36 - 4.14%) (Table 4).

Table 4. Mass, length, and percent lipid (mean and standard deviation, in parentheses) of green frog adult, tadpole, and egg tissue from southwestern Michigan, 1998.

Sample location and date	Mass (g)	Length (cm)	Percent lipid (%)
Adult			
REF1, 6/5, n=3	37.46 (15.71)	68.20 (10.13)	2.63 (1.03)
REF1, 6/23, n=3	49.46 (0.25)	77.17 (4.80)	0.69 (0.46)
REF1, 7/22, n=3	34.00 (4.00)	71.00 (5.57)	1.56 (0.49)
KLMZR, 6/5, n=3	32.74 (5.90)	65.52 (4.40)	0.54 (0.33)
KLMZR, 6/23, n=2	25.01 (1.52)	58.58 (1.02)	0.84 (0.12)
AGR1, 6/23, n=3	25.52 (3.14)	62.44 (2.26)	0.70 (0.29)
AGR2, 7/22, n=3	24.47 (2.73)	65.67 (2.08)	0.50 (0.28)
AGR3, 7/22, n=2	36.85 (9.69)	70.50 (7.79)	0.87 (0.25)
Juvenile			
KLMZR, 8/26, n=2 pools	NA	NA	1.87 (0.17)
Tadpole			
REF1, 6/5, n=20	2.60 (0.65)	59.28 (5.82)	1.45*
REF1, 6/23, n=25	3.43 (0.42)	66.57 (3.77)	3.49*
REF2, 6/5, n=12	5.57 (1.20)	73.54 (10.76)	1.81*
REF3, 6/5, n=20	4.30 (0.81)	72.80 (6.83)	2.84*
REF3, 6/23, n=20	5.00 (0.82)	73.72 (11.95)	4.14*
KLMZR, 6/5, n=20	3.58 (1.40)	65.44 (10.17)	2.67*
KLMZR, 6/23, n=30	4.37 (1.11)	72.53 (7.60)	2.23*
AGR1, 6/5, n=21	4.38 (1.30)	69.70 (9.10)	1.36*
AGR2, 6/23, n=20	7.84 (2.44)	82.08 (12.49)	3.56*
AGR3, 6/23, n=4	5.52 (1.38)	54.38 (22.62)	2.62*
Egg			
REF1, 6/23, n=2	NA	NA	10.97 (2.06)
KLMZR, 6/23, n=1	NA	NA	9.73*
AGR1, 6/23, n=1	NA	NA	10.94*
AGR3, n=1	NA	NA	11.10*

NA = Not Available

*No standard deviation is recorded for these samples because they were treated as one pooled sample each, each pool being from each location and date.

Organochlorine Insecticides

Concentrations of the isomers of HCH, the isomers of chlordane, and *p, p'*-DDT and its metabolites in all water samples were less than the method detection limit (MDL) of 1.0 ng/L. Raw data of frog tissue concentrations are presented in Appendix A, along with a table depicting the statistical significant differences (α=0.05) between locations and life stages.

For many of the frog samples, there were no significant differences in concentrations between locations. For individual isomers and metabolites of HCH, chlordane, and DDT, concentrations of *p, p'*-DDE and *p, p'*-DDD were greatest in adults (0.61 ng/g, wet wt. for each) (Table 5). Concentrations of β-HCH and α-chlordane were the least (at most 0.02 and 0.01 ng/g, wet wt., respectively) in adult tissues (Table 5). There were no significant differences among sampling locations in concentrations of any of the OCs in adult tissues.

Samples of juveniles contained lesser concentrations of OC insecticides than did the adults. Most concentrations of individual isomers were less than the MDL (0.01 ng/g, wet wt.), with *p, p'*-DDD being the greatest at 0.09 ng/g, wet wt. (Table 5). There were no significant differences in concentrations of OCs in juvenile tissues among locations.

Some differences in concentrations of residues in tadpole tissue among locations were observed. Concentrations ranged from less than 0.01 ng/g, wet wt. for *p, p'*-DDT to 0.29 ng/g, wet wt. for *p, p'*-DDD (Table 5), but were not statistically different among locations. Residue concentrations that differed among location for tadpole tissue included γ-HCH (lindane), α-chlordane, γ-chlordane, and Σchlordane. The greatest concentrations of lindane and γ-chlordane (0.25 and 0.03 ng/g, wet wt., respectively) were observed at the agricultural location AGR1. The greatest concentration of α-chlordane (0.14 ng/g, wet wt.) was observed at agricultural location AGR2 (Tables 6 and 7).

Concentrations of OC insecticides in eggs ranged from less than 0.01 ng/g wet wt. for many of the residues to 5.86 ng/g wet wt. for *p*, *p*'-DDE (Table 5). Concentrations of *p*, *p*'-DDT were significantly different among locations, with the agricultural location AGR1 having the greatest concentration (1.40 ng/g, wet wt.) (Tables 6 and 7).

When all of the tissue concentrations are analyzed statistically, regardless of location, it is evident that the egg tissues have the greatest concentrations of many of the OC residues. These residues are α-chlordane, γ-chlordane, *p*, *p*'-DDE, *p*, *p*'-DDT, ΣDDT, and Σchlordane (Table 8). The concentration of α-HCH was greatest in tadpole tissue (Table 8).

Table 5. Concentrations (ng/g, wet wt., mean and standard deviation, in parentheses) of organochlorine insecticides and total PCBs in green frog tissue from southwestern Michigan, 1998.

Insecticide	Adult[1]	Juvenile[2] (n=2)	Tadpole[2] (n=10)	Egg[3] (n=5)
Values calculated with 0.00 substituted in for the non-detects				
α-HCH	0.02 (0.03)	0.04 (0.04)	0.07 (0.09)	0.00*
β-HCH	0.01 (0.02)	0.00*	0.08 (0.15)	0.00*
γ-HCH	0.06 (0.04)	0.00*	****	0.16 (0.19)
δ-HCH	0.03 (0.07)	0.00*	0.15 (0.27)	0.00*
α-chlordane	0.01 (0.01)	0.00*	****	0.18 (0.30)
γ-chlordane	0.04 (0.04)	0.00*	****	0.10 (0.08)
p, p'-DDT	0.03 (0.07)	0.00*	0.00*	****
p, p'-DDE	0.61 (0.97)	0.01 (0.01)	0.08 (0.14)	5.86 (2.92)
p, p'-DDD	0.61 (0.51)	0.09 (0.01)	0.29 (0.31)	1.71 (2.96)
∑HCH	0.12 (0.12)	0.04 (0.04)	0.33 (0.50)	0.34 (0.37)
∑chlordane	0.05 (0.05)	0.00*	****	0.29 (0.32)
∑DDT	1.24 (1.19)	0.10 (0.01)	0.37 (0.40)	7.91 (5.32)
∑PCBs	19.62 (41.01)	19.12 (5.74)	7.63 (8.35)	79.11 (89.82)
Values calculated with the MDL substituted in for the non-detects				
α-HCH	0.03 (0.03)	0.04 (0.04)	0.08 (0.08)	0.01 (0.00)
β-HCH	0.02 (0.01)	0.01 (0.00)	0.08 (0.14)	0.01 (0.00)
γ-HCH	0.06 (0.04)	0.01 (0.00)	****	0.16 (0.19)
δ-HCH	0.04 (0.07)	0.01 (0.00)	0.16 (0.27)	0.01 (0.00)
α-chlordane	0.01 (0.01)	0.01 (0.00)	****	0.19 (0.30)
γ-chlordane	0.04 (0.04)	0.01 (0.00)	****	0.11 (0.07)
p, p'-DDT	0.03 (0.07)	0.01 (0.00)	0.01 (0.00)	****
p, p'-DDE	0.61 (0.97)	0.02 (0.00)	0.09 (0.14)	5.86 (2.92)
p, p'-DDD	0.61 (0.51)	0.09 (0.00)	0.29 (0.30)	1.71 (2.96)
∑HCH	0.12 (0.12)	0.04 (0.04)	0.34 (0.50)	0.34 (0.37)
∑chlordane	0.05 (0.05)	0.01 (0.00)	****	0.29 (0.32)
∑DDT	1.24 (1.19)	0.10 (0.01)	0.37 (0.39)	7.98 (5.32)
∑PCBs	19.62 (41.01)	19.12 (5.74)	7.63 (8.35)	79.11 (89.82)

[1] n=21 for insecticide analysis and n=22 for PCB analysis

[2] These were pooled samples

[3] Two of the five samples were pooled

* No standard deviations are presented here because the values were below the MDL and none could be calculated.

****At the 95% confidence level, there were significant differences among locations so a total mean could not be calculated.

Table 6. Concentration (ng/g, wet wt., mean and standard deviation, in parentheses) of organochlorine insecticides and total PCBs in green frog tissue from sampling locations that are significantly different at the 95% confidence level in southwestern Michigan, 1998.

	REF1	REF2	REF3	KLMZR	AGR1	AGR2	AGR3
Tadpole							
γ-HCH	0.04 (0.05)	0.01*	0.01 (0.00)	0.01 (0.00)	0.25*	0.01*	0.04*
α-chlordane	0.01 (0.00)	0.04*	0.02 (0.02)	0.01 (0.00)	0.06*	0.14*	0.01*
γ-chlordane	0.01 (0.00)	0.02*	0.01 (0.01)	0.01 (0.00)	0.03*	0.01*	0.01*
Σchlordane	0.01 (0.00)	0.06*	0.02 (0.03)	0.01 (0.00)	0.09*	0.14*	0.01*
Egg							
p, p'-DDT	0.01 (0.00)	NA	NA	0.01 (0.00)	1.40*	NA	0.75*

NA = Not analyzed

*No standard deviation is presented because n=1 pool for these samples.

Table 7. Samples with significant differences in organochlorine residue concentrations among locations. Underlined locations are not significantly different from one another (α=0.05). Locations are arranged in order of increasing concentrations.

Tissue and Residue	Location						
Tadpole							
γ-HCH	REF2	REF3	KLMZR	AGR2	REF1	AGR3	AGR1
	———	———	———	———	———	———	
α-chlordane	REF1	KLMZR	AGR3	REF3	REF2	AGR1	AGR2
	———	———	———	———	———	———	———
	———	———	———	———	———	———	
γ-chlordane	REF1	KLMZR	AGR2	AGR3	REF3	REF2	AGR1
	———	———	———	———	———	———	
			———	———	———	———	———
Σchlordane	REF1	KLMZR	AGR3	REF3	REF2	AGR1	AGR2
	———	———	———	———	———	———	———
	———	———	———	———	———	———	
Egg							
p, p'-DDT	REF1	KLMZR	AGR3	AGR1			

Table 8. Samples with significant differences in organochlorine concentrations between life stages. Underlined life stages are not significantly different ($\alpha=0.05$) from one another. Life stages are arranged in order of increasing concentrations.

Residue	Life Stage			
α-HCH	Adult	Egg	Juvenile	Tadpole
	———	———	———	
		———	———	———
α-chlordane	Adult	Juvenile	Tadpole	Egg
	———	———	———	
		———	———	———
γ-chlordane	Juvenile	Tadpole	Adult	Egg
	———	———	———	
p, p'-DDE	Juvenile	Tadpole	Adult	Egg
	———	———	———	
p, p'-DDT	Juvenile	Tadpole	Adult	Egg
	———	———	———	
ΣDDT	Juvenile	Tadpole	Adult	Egg
	———	———	———	
ΣChlordane	Juvenile	Tadpole	Adult	Egg
	———	———	———	
ΣPCB	Tadpole	Juvenile	Adult	Egg
	———	———	———	

PCBs

Concentrations of PCBs in all water samples were less than the MDL of 1.0 ng/L. PCBs were detected in tissues of adult, juvenile, tadpole, and eggs of the green frogs. There were no significant differences in concentrations of PCBs among locations. The least concentration (7.63 ng/g, wet wt.) was observed in tadpoles while the greatest concentration (79.11 ng/g, wet wt.) was observed in eggs (Table 5). Raw data of frog tissue concentrations of total PCBs as well as concentrations of the di-, mono-, and non-*ortho* PCB congeners are presented in Appendix A.

TCDD-EQs and E2-EQs

Concentrations of TCDD-EQs and E2-EQs were less than the MDL at all locations (Table 9).

Table 9. Cell bioassay detection limits.

Matrix	EQ
Water	E2-EQ < 0.21 pg E2/ml
Water	TCDD-EQ < 0.41 pg TCDD/ml
Tadpole	TCDD-EQ < 41 pg TCDD/ml

Discussion

The differences observed among locations of OC insecticide concentrations for tadpole and egg tissues can be due to a variety of factors. First, general experimental variation can be involved. Second, it is possible that the differences among locations observed for these two life stages are due to the greater lipid content of the eggs and tadpoles. Third, tadpoles may have a greater exposure to residues in the water and sediment, because they feed exclusively on aquatic materials and do not leave the aquatic system. Lastly, perhaps older life stages may have the ability to metabolize and excrete the residues at a faster rate.

When all of the locations were analyzed together, the egg tissue contained significantly greater concentrations of α-chlordane, γ-chlordane, *p, p'*-DDE, *p, p'*-DDT, ΣDDT, and Σchlordane than did tadpoles, juveniles, and adults. This may be due to the greater lipid content of the eggs. OCs tend to accumulate in lipids because of their low polarity and high octanol-water partitioning coefficient (K_{ow}). For instance, the concentrations of ΣDDT in adult, juvenile, tadpole, and egg lipid tissue are 119.23, 5.35, 14.12, and 74.65

ng/g, respectively. Therefore, because the adults have a lower lipid content than the eggs (by an average factor of 10.28), ∑DDT is actually more concentrated in the lipids of the adults than in the lipids of the eggs. A similar pattern is followed with the residues of γ-chlordane, *p, p'*-DDE, *p, p'*-DDT, and ∑chlordane. Adult green frogs consume a variety of prey items, including aquatic and terrestrial insects, other invertebrates, smaller frogs, snakes, and hatchling turtles (Harding, 1997). Therefore, their diet allows them to be comparatively high on the food chain. Thus, they have the capacity to bioaccumulate organochlorine residues. Also, adults, being older than the other life stages analyzed, have had a longer time to accumulate such residues.

Concentrations of OCs in green frogs in the present study were less than those reported in adult spring peepers (*Pseudacris crucifer*) collected from Point Pelee National Park in southern Ontario in April of 1993 (Russell et al., 1995). Historically, DDT had been applied extensively to this park as mosquito control. ∑DDT concentrations reported in this study were as great as 1,188 μg/kg, wet wt. The authors hypothesize that these high concentrations are due primarily to those historical applications of DDT. Half of the amphibian fauna once present at this location has disappeared over the last fifty years. The authors attributed these disappearances to toxic concentrations of pesticides. A later study in neighboring locations of southern Ontario near Point Pelee found lesser concentrations of pesticide residues in adult green frog tissue (Russell et al., 1997). Concentrations of *p,p'*-DDE in green frog tissue ranged from 0.58 to 45.02 μg/kg, wet wt. At these locations, green frogs were the dominant anuran, indicating that at these OC concentrations the green frog population appeared to not be depleted. The lesser concentrations, in that study, are more similar to the concentrations of *p,p*'-DDE in this study.

Concentrations of α-HCH, β-HCH, lindane, *p*, *p*'-DDD, *p*, *p*'-DDE, heptachlor, dieldrin, *o*, *p*'-DDD, *o*, *p*'-DDE, *o*, *p*'-DDT, *p*, *p*'-DDT, heptachlor epoxide, aldrin, and endrin in tissues of heron adults, nestlings, and eggs and in the tissues of their primary prey, frogs (*Rana* spp.) collected in May and June of 1992 and 1993 from wetlands of Thermaikos Gulf, Macedonia, Greece were measured (Albanis et al., 1996). Concentrations of residues in water ranged from <0.01 to 0.21 μg/L. Few residues were detected in adult frogs, with the exception of α-HCH, β-HCH, lindane, *p*, *p*'-DDD, *p*, *p*'-DDE, and heptachlor. Average concentrations of α-HCH, β-HCH, lindane, *p*, *p*'-DDD, and *p*, *p*'-DDE in the frogs were 2.45, 0.56, 3.64, 0.49, and 0.29 μg/kg, dry wt., respectively. Generally, the wet weight to dry weight ratio is five. Therefore, these reported values can be divided by five to compare to the concentrations from the present study. Overall, the concentrations of HCH in the frogs in the study in Greece would still be greater by a factor of about ten on average, while the concentrations of DDD and DDE in Greece would be less by a factor of about eight.

Twenty OC pesticides and 39 PCB congeners were determined in mudpuppies and snapping turtles collected from 1988 to 1992 from the St. Lawrence River in Canada, a river that is greatly impacted by human activities and synthetic chemicals (Bonin et al., 1995). Concentrations of DDD, DDE, DDT, α-HCH, lindane, *trans*-chlordane, and *cis*-chlordane in whole mudpuppies were 1.2 to 24.8, 0.3 to 90.0, ND to 8.3, ND to 6.8, ND to 3.3, ND to 7.1, and 0.3 to 13.9 ng/g, wet wt., respectively. Concentrations of PCBs in whole mudpuppies ranged from 92 to 1082 ng/g, wet wt. These values are slightly greater than the concentrations observed in the present study.

Concentrations of PCBs and OC pesticides were determined in green frogs and leopard frogs collected along the north shores of Lake Erie and Lake Ontario in Ontario, Canada (Gillan et al., 1998). Sediments were also collected in an effort to determine biota-sediment accumulation factors (BSAFs), as well as to determine the cytotoxicity and genotoxicity of the sediments. Concentrations of *p*, *p*'-DDE in green frogs ranged from 19 ng/g lipid wt. in a large pond in a public park area to 754 ng/g lipid wt. from a small pond in an agricultural region. Concentrations of *p*, *p*'-DDT in green frogs ranged from ND in a large pond in a public park area to 60 ng/g lipid wt. in a large marsh in a conservation area. Concentrations of *p*, *p*'-DDE in leopard frogs ranged from 60 ng/g lipid wt. in a shallow creek in a conservation area to 659 ng/g lipid wt. from a large pond in a provincial region. Concentrations of *p*, *p*'-DDT in green frogs ranged from ND in a few sites to 80 ng/g lipid wt. in a small pond in an agricultural area. In the present study, the mean percent lipid content of the adult frogs was 1.04%. Therefore, if concentrations were normalized by lipid weight, the mean concentration of *p*,*p*'-DDE in adult green frogs from southwestern Michigan is 57.86 ng/g, lipid wt., and that of *p*,*p*'-DDT is 2.86 ng/g lipid wt. The value for DDE are within the ranges of the green frogs studied in the Ontario study, and slightly lower than the concentrations in leopard frogs. The value for DDT is within the concentration ranges for both green and leopard frogs in the Ontario study.

PCB concentrations measured in green frogs in regions of southern Ontario were in the low μg/kg levels (Russell et al., 1997). PCBs, polychlorinated dibenzo-*p*-dioxins (PCDDs), and polychlorinated dibenzo-*p*-furans (PCDFs) were measured in northern leopard frogs collected from the lower Fox River and Green Bay regions of Wisconsin in the summers of 1994 and 1995. The concentrations of total PCBs ranged from 3 to 154 ng/g, wet wt. One PCDD and two PCDFs at concentrations of 6 to 8 pg/g were also found in the frogs from one site (Huang et al., 1999). The concentration of total PCBs measured in the present study are comparable to those in Wisconsin. The small concentrations of TEQs in this study were probably due to the fact that concentrations of PCBs were also small.

OC pesticides were determined in fat bodies of breeding male green frogs in 1993 and 1994 from orchard wetlands in rural areas of southern Ontario (Harris et al., 1998). Concentrations of DDD, DDE, and DDT ranged from ND to 0.21 μg/g, 0.05 to 2.81 μg/g, and ND to 0.46 μg/g, respectively. As calculated previously, the mean lipid concentrations of *p,p*'-DDD, *p,p*'-DDE, and *p,p*'-DDT of adult green frogs in the present study were 57.86 ng/g, 57.86 ng/g, and 2.86 ng/g, respectively. Because of the large range of concentrations in the Ontario study, the values for the current study in southwestern Michigan are similar, albeit at the very low end.

EROD activity (hepatic ethoxy-resorufin-*O*-deethylase activity) is a metabolic response that is mediated by the Ah-receptor upon exposure to dioxins and dioxin-like compounds

(Huang et al., 1999). In an aforementioned study (Huang et al., 1999), no significant correlation was found between EROD activity and PCB concentration. This result was consistent with the fact that the frogs from Wisconsin contained relatively small concentrations of PCBs (<152 ng/g wet wt.) compared with what was required for induction in the laboratory (ED50 for EROD is between 700 and 2,300 ng/g wet wt.). Concentrations of PCBs in green frog tissue from the present study in southwestern Michigan were lesser than those observed in frog tissue from Wisconsin (<20 ng/g wet wt. in adults); hence, it would be unlikely that significant EROD induction would be observed in these tissues.

The toxicities of some insecticides to amphibians have been determined. Static bioassays to determine the relative acute toxicities of pesticides to western chorus frog (*Pseudacris triseriata*) and Fowler's toad (*Bufo fowleri*) tadpoles found that endrin was the most toxic OC insecticide to the chorus frog tadpoles and the second most toxic insecticide to the Fowler's toad tadpoles (Sanders, 1970). Lindane was the least toxic insecticide to both frog and toad 4 and 5-wk old tadpoles, with a 96-h TL50 value of 4.4 mg/L for toads and a 96-h TL50 value of 2.7 mg/L for frogs. Median tolerance limit (TL50 or LC50) values for toad tadpoles decreased with age (Sanders, 1970). For example, the 96-h TL50 value for 1-wk old Fowler's toad tadpoles exposed to waterborne exposures of DDT was measured at 0.75 mg/L, while the 96-h TL50 for 7-wk old tadpoles was measured at 0.03 mg/L. This indicates that DDT might be most toxic to advanced tadpole developmental stages (Sanders, 1970). Dieldrin was observed to have approximately the same 96-h TL50 values as DDD in Fowler's toad tadpoles (0.15 mg/L) but was found to be eight times more toxic than DDT to western chorus frog tadpoles (0.10 mg/L versus 0.80 mg/L) (Sanders, 1970). These values are much greater than those concentrations measured in the water samples in the present study. Exposure of tadpoles to dieldrin resulted in structural abnormalities, lesser rates of development, and changes in behavior (Cooke, 1972).

DDT has been shown to adversely impact the development and behavior of common European frogs (Cooke, 1970). Uncoordinated activity, weight loss, and restricted development were observed in tadpoles following 1-h exposures to 0.1, 1.0, and 10.0 mg/L aqueous concentrations of DDT. After treatment, tissue concentrations of DDT ranged from 140 to 180 μg/g (Cooke, 1970). Tadpoles exposed for 24 and 48 h to aqueous concentrations of DDT (ranging from 0 to 0.5 mg/L) have exhibited hyperactive behavior either just before or just after developing limb buds (Cooke, 1972). Tissue concentrations of whole tadpoles with this behavior ranged from 2,000 to 4,000 μg/kg (Cooke, 1972). Exposures to aqueous DDT at 0.1 mg/L for 2 d produced histological changes in mandibles of some tadpoles (Osborn et al., 1981). This has been attributed to the disruptive effect DDT has on the development of skin glands in the region above the upper mandible and to the hyperactivity that DDT causes in tadpoles (Osborn et al., 1981). Effects of DDT exposure were more severe for acute exposures than for chronic exposures even though the two types of exposure resulted in the same concentrations in tadpoles (Cooke, 1973). The degree of crowding in laboratory populations was positively related to the severity of sub-lethal effects, including hyperactivity and mandibular deformities, due to exposure to DDT (Cooke, 1979). Hyperactivity caused by sub-lethal exposure to DDT in tadpoles has been postulated to potentially result in greater risk from predation (Cooke, 1971). None of these previously observed abnormalities were observed in the tadpoles of the present study in southwestern Michigan, as would be expected due to the lower water concentrations of DDT.

Conclusion

The study presented here attempted to link deformities in green frog populations of southwestern Michigan to organochlorine insecticide and PCB residues in their environment and tissues. However, only 0.28% of green frogs exhibited obvious physical abnormalities. Concentrations of DDT and its metabolites, chlordane, hexachlorocyclohexane, and PCBs were relatively low, in comparison to similar studies, in the water and in the tissues of eggs, tadpoles, juveniles, and adults. There were few significant differences in organochlorine concentrations between the agricultural, Kalamazoo River, and reference locations. In other studies, frogs with similar concentrations of organochlorine residues exhibited no physical deformities. Overall, the low levels of organochlorine residues in this study can be used as reference background levels, from which it can be assumed that at or below these measured concentrations, no significant frog deformities should be observed.

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Chapter 3: Metals in Sediment and Green Frogs from Southwestern Michigan

Introduction

Frog deformities and worldwide amphibian declines have been the focus of scientific research and debate over the past 15 years. Several hypotheses to explain these phenomena have been proposed; however, no definite causes have been found (Burkhart et al., 1998). One possible explanation is exposure to chemical residues. In particular, exposure to chemicals that have the potential to modulate the endocrine system are of current concern (Ankley et al., 1998).

Metals of environmental interest include elements which are macro-nutrients or micro-nutrients in the biosphere and others which have no known biological function and are generally regarded as toxic (Bunce, 1994). Elements that are biologically essential include: calcium (Ca), cobalt (Co), copper (Cu), chromium (Cr), iron (Fe), magnesium (Mg), manganese (Mn), potassium (K), and zinc (Zn). Metals with no established biological functions include: aluminum (Al), arsenic (As), barium (Ba), cadmium (Cd), lead (Pb), mercury (Hg), nickel (Ni), scandium (Sc), strontium (Sr), and titanium (Ti) (Jacobs, 1996). It is important to note that, as Paracelsus states, "No substance is a poison by itself. It is the dose that determines the poison" (Lu, 1991). Hence, even the generally non-toxic metals can be toxic at high enough concentrations in the body, while the toxic metals may elicit no effects due to extremely low concentrations.

Macrominerals are distinguished from the microminerals by their occurrence in the body. Macrominerals are generally believed to constitute at least 0.01% of total body weight

(Hunt and Groff, 1990). Calcium is a structural component of bones and plays an integral role in intracellular and hormonal secretion regulation, muscle contraction, blood clotting, and the activation of some enzyme systems. Like calcium, magnesium is also a constituent of bone structure. Magnesium is important in nerve impulse transmission, protein synthesis, and enzyme activation. Another macromineral, potassium, functions as an electrolyte in the body.

Microminerals constitute less of the total body weight than do macrominerals; however, there is discrepancy in their actual definition (Hunt and Groff, 1990). Chromium is involved in the normal use of blood glucose and the function of insulin, and cobalt is necessary for the synthesis of Vitamin B12. Copper is required for the proper use of iron in the body, and is involved in the synthesis of cytochrome oxidase and the development of connective tissue and blood vessels. Iron is a necessary component of hemoglobin and myoglobin for oxygen transport and cellular use, as well as being a facilitator in the transfer of electrons in the electron transport chain. Manganese is needed for normal brain function, as well as for its role in enzyme systems, collagen formation, bone growth, urea formation, fatty acid and cholesterol synthesis, and the digestion of proteins. Zinc is important for energy metabolism and protein synthesis.

While much of the literature lists arsenic and nickel as possessing no biological function, they may indeed be microminerals (Hunt and Groff, 1990). Arsenic is present throughout the earth's crust and is present in varying concentrations in soils. Fallout sources such as pesticides, smelters, and coal-fired power plants can increase the concentrations of arsenic in certain regions. Arsenic is believed to be necessary for normal growth and for the usage of iron in many animals. Nickel occurs in ores, and smelting and industrial uses tend to increase its levels in the environment. While it is known to be a human carcinogen as well to cause dermal hypersensitivity (Lu, 1991), nickel is possibly involved in hormonal membrane or enzyme activity.

As mentioned above, there are several metals with no known biological function. Among these is aluminum, which comprises about 8% of the earth's crust (Gerhardsson and Skerfving, 1996). It is widely used as a building material and industrially as an abrasive and chemical catalyst (Cory-Slechta, 1996). Aluminum has been thought to be a neurotoxin for over 100 years. In 1898, Dollken described the studies of Siem in 1886, in which aluminum was shown to elicit nerve and muscle paralysis in frogs, cats, dogs, and rabbits (summarized by Cory-Slechta, 1996). Another toxic metal, cadmium, is increasingly used in industry in battery manufacture, metal coating by electroplating, as well as being a pigment in paints and a stabilizer in plastics (Waalkes and Misra, 1996). The primary sources of cadmium in the environment include mining, refining, and smelting operations. Cadmium, a known carcinogen, has a similar chemical nature as zinc, and as such, displaces zinc in many biological processes. Cadmium can also affect calcium homeostasis (Waalkes and Misra, 1996).

While mercury constitutes very little of the earth's crust (about 0.5 ppm) (Cory-Slechta, 1996), it's toxicity is well studied. Mercury, a component of many batteries, is used in the manufacture of organomercurials, which are used in agriculture as fungicides. Its electrical applications include mercury as a component of fluorescent and germicidal lamps in addition to its use as a sealant to exclude air from tungsten light bulb filaments. (Bunce, 1994). Mercury is more toxic and lipophilic in its methylated forms. Because of its increased lipophilicity, it can bioconcentrate and cross the blood-brain barrier, which leads to its neurotoxic effects (Bunce, 1994).

Like mercury, lead occurs in nature primarily as the sulfide (Bunce, 1994). It has been used as a metal for centuries, most currently its uses include the addition of organolead compounds to gasoline, the lead-acid storage battery, and as a component of solder. The similarity in ionic structures between lead and calcium leads to the replacement of calcium in the body, particularly in bones, with lead. Lead, like mercury, is a neurotoxin which causes more damage in its organic forms (Bunce, 1994).

Other potentially toxic metals of interest include barium, which occurs in ores of barite, and is used in a variety of applications, such as alloys in vacuum tubes and spark plugs, and as a radioactive tracer (Lewis, 1993). It is toxic in its salt form (Lu, 1991). Scandium has no major industrial use, but has been used as an artificial radioactive isotope in tracer studies and leak detection (Lewis, 1993). It has been implicated as a potential carcinogen (Waalkes and Misra, 1996). Strontium is used in alloys (Lewis, 1993). Titanium is used in alloys and as structural constituent in a variety of materials, including aircraft, missiles, textile machinery, and chemical equipment (Lewis, 1993). Like scandium, it has been implicated as a carcinogen (Waalkes and Misra, 1996).

Some metals have been postulated to be endocrine disruptors, and as such, have the potential to disrupt the normal development of frogs and other amphibians. The best known of these is tributyltin (TBT), a suspected androgen (Villeneuve et al., 1998). TBT, an organotin component of antifouling paints on ships, is a known endocrine disruptor which causes imposex in gastropods (Fent, 1996). Triphenyltin (TPT) is an organotin compound that is commonly used in agriculture as a fungicide and can reach the environment through leaching, runoff, and volatilization. Within the past decade, the use of organotins has been restricted due to their toxicity to aquatic organisms, and the effects they have on animals of higher trophic levels. Increased concentrations of TPT have been shown to decrease survival and growth rates and increase time to metamorphosis of two tadpole species (Fioramonti et al., 1997). Further, short-term exposure to TPT has been shown to affect the swimming and feeding behavior of tadpoles of the European frog (*Rana esculenta*) (see Semlitsch et al., 1995). Alterations in these behaviors can prove harmful to the growth, development, and survival of amphibians.

The primary objectives of this study were to assess the incidence of green frog deformities in southwestern Michigan, and to correlate those deformities to chemical residues in the frog tissue. No significant deformities were observed among the sampling locations, as discussed in Chapter 1. Therefore, no correlations between the observed residues and deformities could be developed. However, the remaining study objectives remained viable. These objectives involved gathering data on the levels of metals in sediment and in tissues of green frog tadpoles, juveniles, and adults. Concentrations of

Al, As, Ba, Ca, Cd, Co, Cr, Cu, Fe, Hg, K, Mg, Mn, Ni, Pb, Sc, Sr, Ti, and Zn were determined. These metals were all analyzed and expressed as total metals, even though they may be in various physical or chemical forms in tissue (Jacobs, 1996). Many of these metals, as previously mentioned, are not commonly considered toxic and are actually necessary to the survival of organisms. However, as a general monitoring study, the data gathered here may be useful in knowing background metal concentrations in sediment and green frogs from southwestern Michigan for future reference.

Materials and Methods

Tadpoles and adults of the green frog (*Rana clamitans*) were collected by dip net and hand from seven sampling locations in southwestern Michigan (see Chapter 1). Tadpoles and adults were weighed and measured, and the sex of the adults was determined based on physical features. The most common means of sexing green frogs is based on the size of the tympanum. In males, the tympanum is larger than the eyes. Males also may have a yellowish throat, whereas the throat of the females is generally whitish (Harding, 1997). Tadpoles were pooled into samples of approximately 20 g wet wt. by location and date. Pooling of samples was not necessary for the adults. The frogs used for this analysis were not the same frogs as those used in the organochlorine analysis (Chapter 2). The livers were the only tissues of adult frogs analyzed for metals. They were removed from the adults using acid-washed teflon forceps and spatulas. The primary reason for choosing livers is because the liver possesses a larger capacity than most other organs to bind chemicals such as metals (Lu, 1991). The same is true of the kidneys; however, the liver is easier to locate and remove from frogs. All frog tissue was stored in acid-washed plastic jars at –20°C until analysis.

Sediment cores were collected from the following sampling locations – REF1, REF3, KLMZR, AGR2, and AGR3. The upper 10 cm was separated and designated the upper layer while the next 10 cm layer was designated the lower layer. Each layer was air dried for 48 h. Air drying, rather than drying in an oven, was necessary in order to minimize Hg volatilization. Dried sediment samples were then ground using pestle and mortar. All sediment samples were stored in acid-washed plastic jars at –20°C until analysis.

Before a digestion was begun, all vessels and utensils were cleaned. All teflon materials, including liners, lids, spatulas, and autosampler bottles, were acid-washed with nitric acid and soaked for at least 24 h in a distilled-deionized water (DDW) bath.

Each sample of sediment or frog tissue was weighed (about 0.500 g dry wt.) into a teflon digestion vessel liner that contained 10 ml of nitric acid. The vessels were sealed and placed in a microwave oven for digestion. Digestion lasted 15 min, followed by at least a 20 min cooling period. After cooling, 90 ml of DDW was added to each sample vessel. Each sample leachate was filtered through a 0.4 µm Nuclepore filter into Nalgene™ bottles. Half of the leachate was set aside for the full metal scan, while the other half was prepared for mercury analysis. This involved adding gold chloride (10 µL of $HAuCl_4$ / 1.0 mL sample) (Aldrich Chemical Company, Inc., Milwaukee, WI, USA) to each sample to keep mercury in solution (Gill and Fitzgerald, 1987).

For analysis, samples were diluted in order to scan most of the metals in range of the detector. Sediment samples were diluted 100-fold, and tissue samples were diluted 5-fold. The analysis was done with a Platform ICP-MS (Micromass, Inc., Manchester, England). Samples were introduced with a concentric nebulizer that was connected to a CETAC ASX-500 auto-sampler.

Results

Raw data of sediment and frog tissue metal concentrations are tabulated in Appendix B, along with a table depicting statistical p-values for significant differences ($\alpha=0.05$) between locations and life stages. There were no significant differences between the upper and lower layers of sediments. Therefore, sediment layers were analyzed as individual samples.

Concentrations of five of the 20 elements analyzed (Al, Ca, Co, Mg, Sr) in sediments differed significantly among locations when the MDL was substituted for non-detectable concentrations (Table 10). For those elements that did not differ significantly among locations, the least concentrated were Cd (≤0.21 µg/g dry wt.), Hg (≤0.22 µg/g dry wt.), Ni (≤2.0 µg/g dry wt.), Sc (≤2.4 µg/g dry wt), and As (≤3.8 µg/g dry wt). The most concentrated elements in sediment that did not differ significantly among locations were Fe (9.5×10^2 µg/g dry wt.), Mn (3.6×10^2 µg/g dry wt.), K (2.3×10^2 µg/g dry wt.), Ba (1.0×10^2 µg/g dry wt.), and Cr (42 µg/g dry wt.) (Table 10).

Of the metals for which concentrations differed significantly among locations, Al was most concentrated at the agricultural location AGR2 ($9.9\text{x}10^3$ µg/g dry wt.) (Tables 9 and 10). This site also exhibited the greatest Co concentration in sediment (6.4 µg/g dry wt.) (Tables 9 and 10). The greatest concentrations of Ca, Mg, and Sr were observed at the Kalamazoo River site, KLMZR. These concentrations were $1.7\text{x}10^5$, $4.6\text{x}10^3$, and 96 µg/g dry wt., respectively (Tables 11 and 12).

Concentrations of four of the elements analyzed (Ba, Ca, Mg, and Ti) in adult livers differed significantly among locations when the MDL was substituted for non-detectable concentrations (Table 10). Concentrations of elements that did not differ significantly among location included Cd (≤0.08 µg/g dry wt.), As (≤0.10 µg/g dry wt.), Co (≤0.13 µg/g dry wt), and Sc (≤0.23 µg/g dry wt) at the low end to K ($3.3\text{x}10^3$ µg/g dry wt.), Fe ($1.1\text{x}10^3$ µg/g dry wt.), Cu (65 µg/g dry wt.), Al (58 µg/g dry wt.), and Zn (27 µg/g dry wt.) (Table 10).

In adult livers that differed significantly among location, the metals Ba, Ca, Mg, and Ti were most concentrated at the reference location REF3. The concentrations were 38, $8.6\text{x}10^2$, $4.8\text{x}10^2$, and 4.1 µg/g dry wt., respectively (Tables 11 and 12). The location REF3 had a significantly higher level of Mg in adult livers than all the other locations (Table 12).

Juvenile tissues were most concentrated with Ca ($2.8\text{x}10^4$ µg/g dry wt.), Fe ($1.9\text{x}10^4$ µg/g dry wt.), K ($4.2\text{x}10^3$ µg/g dry wt.), Mg ($1.0\text{x}10^3$ µg/g dry wt), and Al ($3.7\text{x}10^2$ µg/g dry wt). Metals that were least concentrated in juvenile tissues were Cd (≤0.03 µg/g dry wt.), Hg (≤0.17 μg/g dry wt.), Co (≤0.20 μg/g dry wt.), and As (≤0.30 μg/g dry wt.) (Table 10).

Tadpole tissues contained the greatest concentrations of Fe (6.5×10^4 μg/g dry wt.), Ca (2.3×10^4 μg/g dry wt.), K (5.8×10^3 μg/g dry wt.), Mg (1.0×10^3 μg/g dry wt), and Al (5.9×10^2 μg/g dry wt). Metals that were least concentrated in tadpole tissues were Cd (≤0.23 μg/g dry wt.), Hg (≤0.24 μg/g dry wt.), Co (≤0.41 μg/g dry wt.), and Sc (≤0.80 μg/g dry wt.) (Table 10).

Table 10. Metal concentrations (µg/g, dry wt., mean and standard deviation, in parentheses) in sediment and green frog tissue from southwestern Michigan, 1998.

Metal	Adult[1]	Juvenile[2]	Tadpole[3]	Sediment[4]
Values with 0.0 substituted for non-detects				
Al	57 (1.2x10^2)	3.7x10^2 (3.3x10^2)	5.9x10^2 (2.1x10^2)	****
As	0*	0*	0*	0*
Ba	****	30 (27)	35 (36)	66 (85)
Ca	2.4x10^2 (3.3x10^2)	2.8x10^4 (1.1x10^4)	2.0x10^4 (1.4x10^4)	2.0x10^4 (2.2x10^4)
Cd	0*	0*	0*	0.18 (0.28)
Co	0*	0*	0*	0*
Cr	0*	0*	0*	0*
Cu	65 (30)	1.4 (2.5)	1.8 (3.7)	2.1 (7.3)
Fe	1.1x10^3 (2.3x10^3)	1.9x10^4 (2.7x10^4)	6.5x10^4 (5.8x10^4)	6.8x10^2 (1.6x10^3)
Hg	0*	0*	0*	0*
K	****	4.3x10^3 (6.5x10$^{2)}$	5.5x10^3 (3.6x10^3)	1.5x10^2 (5.0x10^2)
Mg	****	1.0x10^3 (4.5x10^3)	9.2x10^2 (6.5x10^2)	****
Mn	0*	50 (86)	2.8x10^2 (4.6x10^2)	3.6x10^2 (7.0x10^2)
Ni	0.61 (2.5)	0*	0*	2.0 (5.6)
Pb	0*	3.9 (3.6)	0*	20 (14)
Sc	0*	0*	0*	0*
Sr	0*	5.8 (10)	4.0 (11)	****
Ti	1.3 (1.5)	17 (8.2)	22 (8.0)	8.2 (14)
Zn	23 (14)	49 (25)	30 (32)	0*
Values with MDL** substituted for non-detects				
Al	58 (1.2x10^2)	3.7x10^2 (3.3x10^2)	5.9x10^2 (2.1x10^2)	****
As	0.10 (0.14)	0.30 (0.25)	1.3 (0.82)	3.8 (3.1)
Ba	****	34 (21)	45 (28)	1.0x10^2 (59)
Ca	****	2.8x10^4 (8.0x10^2)	2.3x10^4 (2.0x10^3)	****
Cd	0.08 (0.11)	0.03 (0.04)	0.23 (0.37)	0.21 (0.27)
Co	0.13 (0.17)	0.20 (0.21)	0.41 (0.27)	****
Cr	8.3 (18)	8.8 (5.6)	9.5 (8.4)	41 (61)
Cu	65 (30)	4.0 (2.3)	4.2 (2.7)	13 (7.2)
Fe	1.1x10^3 (2.3x10^3)	1.9x10^4 (2.7x10^4)	6.5x10^4 (5.8x10^4)	9.5x10^2 (1.5x10^3)
Hg	0.43 (0.79)	0.17 (0.12)	0.24 (0.17)	0.22 (0.34)
K	3.3x10^3 (7.2x10^2)	4.3x10^3 (6.5x10^2)	5.8x10^3 (3.1x10^3)	2.3x10^2 (5.6x10^2)
Mg	****	1.0x10^3 (4.5x10^2)	1.1x10^3 (5.1x10^2)	****
Mn	2.9 (4.8)	73 (73)	3.2x10^2 (4.4x10^2)	3.6x10^2 (7.0x10^2)
Ni	0.9 (2.6)	2.9 (0.01)	2.9 (0.01)	2.0 (5.6)
Pb	0.76 (0.01)	3.9 (3.6)	1.6 (1.5)	24 (9.9)
Sc	0.23 (0.18)	0.53 (0.23)	0.80 (0.39)	2.4 (0.88)
Sr	0.31 (0.69)	11 (6.4)	13 (8.5)	****
Ti	****	17 (8.2)	22 (8.2)	19 (11)
Zn	27 (7.9)	49 (25)	46 (20)	24 (18)

[1] n=16 livers

[2] n=3 pooled samples

[3] n=7 pooled samples

[4] n=12 samples of top and bottom layers

* No standard deviations are presented here because the values were below the MDL and none could be calculated.

**The MDL is a function of sample mass and recovery, and varies from sample to sample. The range of concentrations is presented in this table by substituting in both 0.0 and the MDLs for non-detected concentrations.

****At the 95% confidence level, there were significant differences among locations so a total mean could not be calculated.

Table 11. Metal concentrations (µg/g dry wt., mean and standard deviation, in parentheses)[1] in sediment and green frog tissue from sampling locations that are significantly different at the 95% confidence level in southwestern Michigan, 1998.

	REF1	REF3	KLMZR	AGR1	AGR2	AGR3
			Adult			
Ba	12 (17)	37*	4.2 (5.11)	1.8 (2.7)	16 (6.7)	NS
Ca	$1.8x10^{2}$ (30)	$8.6x10^{2}$*	$1.1x10^{2}$ (32)	$3.7x10^{2}$ (9.9)	$6.0x10^{2}$ ($3.1x10^{2}$)	NS
Mg	$2.5x10^{2}$ (55)	$4.8x10^{2}$*	$2.6x10^{2}$ (26)	$3.0x10^{2}$ (28)	$2.2x10^{2}$ (25)	NS
Ti	3.3 (0.74)	4.1*	2.8 (0.28)	3.2 (0.38)	2.2 (0.63)	NS
			Sediment			
Al	$3.3x10^{3}$ ($1.2x10^{3}$)	$3.1x10^{3}$ ($1.4x10^{3}$)	$3.7x10^{3}$ ($6.9x10^{2}$)	NS	$9.9x10^{3}$ ($2.7x10^{3}$)	$3.9x10^{3}$ ($3.0x10^{2}$)
Ca	$1.3x10^{4}$ ($3.5x10^{3}$)	$1.3x10^{4}$ ($1.8x10^{2}$)	$1.7x10^{5}$ ($2.8x10^{4}$)	NS	$3.7x10^{3}$ ($2.0x10^{3}$)	$6.5x10^{3}$ ($1.5x10^{3}$)
Co	0.09 (0.04)	1.9 (0.42)	2.5 (1.0)	NS	6.4 (2.4)	1.5 (0.06)
Mg	$1.2x10^{3}$ ($2.4x10^{2}$)	$1.9x10^{3}$ ($1.8x10^{2}$)	$4.6x10^{3}$ ($7.3x10^{2}$)	NS	$1.3x10^{3}$ ($6.2x10^{2}$)	$1.1x10^{3}$ ($1.8x10^{2}$)
Sr	21 (8.9)	28 (12)	96 (21)	NS	13 (1.3)	9.5 (0.78)

[1] Values using the MDL for non-detects

NS = Not sampled

*No standard deviations could be calculated for these because n=1.

Table 12. Samples for which metal concentrations varied significantly among locations. Underlined locations are not significantly different ($\alpha=0.05$). Locations are arranged in order of increasing concentrations, from left to right.

Sample, Metal	Location				
Adult, Ba	AGR1	KLMZR	REF1	AGR2	REF3
	———	———	———	———	
			———	———	———
Adult, Ca	KLMZR	REF1	AGR1	AGR2	REF3
	———	———	———	———	———
Adult, Mg	AGR2	REF1	KLMZR	AGR1	REF3
	———	———	———	———	
Adult, Ti	AGR2	KLMZR	AGR1	REF1	REF3
	———	———	———	———	
		———	———	———	———
Sediment, Al	REF3	REF1	KLMZR	AGR3	AGR2
	———	———	———	———	
Sediment, Ca	AGR2	AGR3	REF1	REF3	KLMZR
	———	———	———	———	
Sediment, Co	REF1	AGR3	REF3	KLMZR	AGR2
	———	———	———	———	
Sediment, Mg	AGR3	REF1	AGR2	REF3	KLMZR
	———	———	———	———	
Sediment, Sr	AGR3	AGR2	REF1	REF3	KLMZR
	———	———	———	———	

Discussion

Many of the metals found at relatively low concentrations in sediments, such as Cd, Hg, Ni, Sc, As, and Sr, are usually not present in most sediments and are generally considered to be toxic, at high enough concentrations (Bunce, 1994). However, because most were below the limit of detection, and may even be bound in the soil, which reduces their bioavailability, they are not considered to be a potential threat to the wildlife, such as frogs, of the aquatic system. The more concentrated metals, such as Al, Ca, Mg, Fe, Mn, and K are components of biological systems and/or sediments; therefore, the high concentrations are not alarming. As stated above, the greatest concentrations of Al and Co were observed in the sediment at AGR2. This may be due to natural variation, because these concentrations were within acceptable levels. The same is true for the increased concentrations of Ca, Mg, and Sr at KLMZR.

A similar pattern of metal concentrations is observed in frog tissues. Concentrations of toxic metals, such as Pb, Cd, As, Co, Hg, Cr, Sc, and Sr, in adult livers were relatively low. Essential metals, such as Ca, Mg, Al, Fe, and K exhibited the greatest concentrations in adult livers. There appears to be no pattern of accumulation within locations based on concentrations in each matrix. For instance, in livers that differed among locations, the location where the greatest concentrations of Ca and Mg were observed was REF3, whereas the location where the greatest concentrations of those elements were observed in sediment was KLMZR. The significantly greatest concentrations of Ba, Ca, Mg, and Ti were observed at REF3; however, this data is based on only one frog liver, and as such, may not be representative of the entire population.

As with the adult livers, essential metals were most concentrated in the tissues of juveniles and tadpoles. Ca, Fe, K, Mg, and Al exhibited the greatest concentrations in each, while Ni, Cd, Hg, Co, As, Sc, Cr, and Sc were relatively low in each. Pb was much less concentrated in tadpole tissues and adult livers than in juvenile tissues.

Mercury was determined in mudpuppies and snapping turtles collected from 1988 to 1992 from the St. Lawrence River in Canada, a river that is greatly impacted by human activities and synthetic chemicals (Bonin et al., 1995). Concentrations ranged from <20 to 45 ng/g, wet wt. in mudpuppies and 50 to 180 ng/g, wet wt., in turtle eggs (Bonin et al., 1995). Unlike the present study, concentrations in this study were not specifically measured in liver tissue. Generally, the wet weight to dry weight ratio is five. Therefore, these reported values can be multiplied by five to compare to the concentrations in dry weight from the present study. The mercury concentration reported in frog livers in the present study (≤430 ng/g, dry wt.) is comparable to the values of those in the St. Lawrence River study. Even though these species are different from the green frog, these data indicate that a variety of aquatic organisms are capable of absorbing mercury.

Concentrations of Cd, Pb, Zn, and Cu in soil and wildlife within 30 km of a zinc smelter site in eastern Pennsylvania were determined seven years after smelting was terminated in 1980 (Storm et al., 1994). Concentrations of Cd, Pb, Zn, and Cu in green frog tadpole whole body tissue ranged from 0.3 to 1.5, 2.3 to 5.0, 23.1 to 117.0, and 0.3 to 0.8 mg/kg, wet wt., respectively. When calculated on a dry weight basis, the concentrations of Cd and Pb are higher from the smelter study by at least a factor of about 7 each when compared to the values for tadpole tissue in the present study. Concentrations of Zn and Cd are more comparable between the two studies, with Zn being at least 2.5 times more concentrated at the smelter site and Cd levels being similar. Overall, these metal concentrations were similar for adult red-backed salamander and eastern newt tissue (Storm et al., 1994).

Conclusion

The study presented here attempted to link deformities in green frog populations of southwestern Michigan to metals in their environment and tissues. However, only 0.28% of green frogs exhibited obvious physical abnormalities. Concentrations of essential metals and concentrations of metals that possess no known biological functions were measured in sediment and tissues of tadpoles, juveniles, and adults. There were few significant differences in metal concentrations between the agricultural, Kalamazoo River, and reference locations. Overall, the low levels of the known toxic metals can be used as reference background levels, from which it can be assumed that at or below these measured concentrations, no significant frog deformities should be observed.

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Chapter 4: Triazine Herbicide Concentrations in Water and Green Frogs from Southwestern Michigan

Introduction

Frog deformities and worldwide amphibian declines have been the focus of a great deal of scientific research and debate over the past 15 years. Several hypotheses have been formulated as to the reasons for these phenomena; however, no definite causes have been found (Burkhart et al., 1998). One possible explanation is exposure to chemical residues. In particular, exposure to chemicals that have the potential to modulate the endocrine system are of current concern (Ankley et al., 1998).

A class of chemicals that belong to those being scrutinized are the triazine herbicides, most notably atrazine. Atrazine is primarily used for the control of certain annual broadleaf and grass plants in regions of corn farming, as well as in other agricultural locations (Solomon et al., 1996). Atrazine is found in many surface and ground waters in North America, and aquatic ecological effects are a concern (Solomon et al., 1996). Recently, atrazine has been found to have the potential to be an endocrine disruptor (Sanderson et al., 2000).

Many analytical techniques for triazine herbicides in water and tissue exist. A traditional means of analysis involves instrumental analysis by gas chromatography (GC) coupled mass spectrometer detection (MS) (Thurman et al., 1992; Pereira and Rostad, 1990). This method is sensitive, selective, and quantitative. However, due to the amount of labor and time involved in this procedure, the use of GC-MS is not always practical. Another method for the analysis of triazine herbicides has been developed, known as the Enzyme Linked

Immunosorbent Assay (ELISA). This method has proven useful for analyzing this class of herbicides (Huber, 1985; Thurman et al., 1990), even though it is often not as selective or specific as instrumental techniques (Rubio et al., 1991). Comparisons of the two methods by analyzing atrazine in water and soil have indicated that results from ELISA tests are comparable to those obtained from GC-MS techniques (Lydy, 1996; Amistadi, 1997).

In this study, concentrations of triazine herbicides in water and green frog tadpole and juvenile tissue from southwestern Michigan were determined. ELISA analysis was used for water and GC-MS and ELISA analysis were used for frog tissue. A new method for the determination of triazines in extracted tissue by ELISA was developed and compared with the GC-MS method. Because no green frog deformities were observed in the study area, it is unlikely that the range of concentrations of triazine were sufficient to cause such effects in southwestern Michigan (Chapter 1).

Materials and Methods

Water and Tadpole Sampling and Preparation

Four liters of water were collected from each sampling location on the first two sampling dates -- 6/5 and 6/23 of 1998. Samples were collected into four-liter brown glass solvent bottles by submerging each bottle into the water until full. To ensure that they were clean, bottles were washed with water and Liquinox® (Alconox, New York, NY, USA), rinsed with acetone and hexane, and allowed to dry before use. Water samples were stored at 4°C until analysis.

Tadpoles of the green frog (*Rana clamitans*) were collected by methods discussed by dip net and by hand (see Chapter 1). Tadpoles were weighed, measured, and pooled into samples of approximately 20 g wet wt. by location and date. Tissues were stored at –20°C until analysis.

All glassware, stainless steel homogenizer materials, boiling stones, glass wool, and any other utensils and surfaces that would contact samples during the analysis were cleaned by standard procedures to minimize contamination of the sample. Briefly, analytical items were washed thoroughly with Liquinox® (Alconox, New York, NY, USA) and water, then rinsed with deionized water. Materials were allowed to air dry before being rinsed three times with acetone followed by three rinses with hexane. Solvent-cleaned materials were blown dry with a heat gun and placed on solvent-rinsed aluminum foil. All solvents used for any part of the analysis or cleaning steps were HPLC grade (Burdick and Jackson, Muskegon, MI, USA).

Water Analysis with ELISA

Prior to analysis, water samples were allowed to heat up to ambient temperature and were shaken and filtered through glass wool to remove organic particles. The ELISA assay was a commercial ELISA kit designed specifically for the analysis of water samples directly from the field (EnviroGard™ Triazine Plate Kit, Strategic Diagnostics Incorporated, Newark, DE, USA). The instructions given with the kit were followed with no changes. A spectrophotometer (Cayman Chemical Plate Scanning Spectrophotometer and Autoreader, Ann Arbor, MI, USA) was used to measure the mass of triazines in the wells of the plate. Absorbance results were normalized based on the blanks and a calibration graph was made for the standards. Mean normalized sample absorbance results were compared to the standard calibration graph to obtain the value for the concentration of triazines in each water sample.

Tadpole Tissue Analysis with ELISA

Extraction methods were similar to those for OCs (Kannan *et al.,* 1995, Khim *et al.,* 1999). Each tissue sample was thawed at room temperature for 1 h before being prepared for analysis. Each sample was ground in a stainless steel homogenizer cup by use of an Omni-Mixer (Sorvall®, PRO Scientific Inc., Monroe, CT, USA). Sodium sulfate was slowly added during the blending process (150 g anhydrous Na_2SO_4 for a 20 g sample) until the sample was thoroughly dry. Samples were extracted by Soxhlet extraction with a 3:1 ratio of dichloromethane (DCM) to hexane for at least 12 h. Extracts were concentrated to 11 ml and a 1 ml subsample was removed to determine the lipid content of the tissues. Lipid content was measured gravimetrically.

The 10 ml extracts were further concentrated to 3 ml for fractionation. Florisil (60-100 mesh size; Sigma, St. Louis, MO, USA) was activated at 130°C for at least 12 h. For each sample, 10 g of Florisil was suspended in hexane and transferred to a glass chromatography column. The 3 ml extract was placed on top of the Florisil and eluted at a flow rate of approximately 3 ml / min. The first fraction (F1), which contained the least polar analytes, was eluted from the column with 100 ml hexane. The moderately polar compounds were eluted in the second fraction (F2) with 100 ml of 20% DCM in hexane.

The final fraction (F3), which was eluted from the column with 100 ml of 50% methanol in DCM, contained the most hydrophilic compounds.

The volumes of the eluents were reduced by evaporation to 2 ml for F1 and 0.1 ml for F2 and F3. F2 was adjusted to 2 ml with hexane. F2 and F3 were the fractions considered for this study. It was determined by use of a standard that 100% of the atrazine in the samples would elute in F3. Therefore, F1 and F2 were set aside for OC analysis. F3 was diluted to 1 ml with acetonitrile.

In order to ensure that the solvent (acetonitrile) would not adversely impact the results of the ELISA assay, several series of atrazine standards were prepared in varying ratios of acetonitrile to water. The calibration regression of atrazine in 5% acetonitrile in water yielded an R^2-value of 0.9938. Therefore, aliquots of tadpole extracts were diluted to 5% acetonitrile in water before being dosed into the ELISA plate wells. The same ELISA kit as that used in the water analysis was used and the methods were the same.

Gas Chromatographic Analysis of Tadpole Tissue

Undiluted extracts were used to verify ELISA results. Samples were injected into a Hewlett Packard Model 5890 Series II Plus Gas Chromatograph Model 5972 with mass spectrometer (Hewlett Packard) by a Model 7673 autosampler automatic injector (Hewlett Packard).

Results

Triazine herbicide applications in the study region are composed primarily of atrazine (Summer, personal communication). Therefore, while values are reported as triazine concentrations due to the non-specificity of the ELISA test, it can be assumed that the majority of the residues are atrazine.

Table 13. Triazine herbicide concentrations in water and green frog tadpole and juvenile tissues from southwestern Michigan, 1998.

Location	Water Concentration, μg/L (n=2)	Tadpole Concentration, ng/g wet wt.*	Juvenile Concentration, ng/g wet wt.**
REF1	0.063	0.411	0.195
REF2	0.057	0.629	NS
REF3	0.040	0.230	NS
KLMZR	0.034	0.339	0.201
AGR1	0.056	0.442	NS
AGR2	0.214	0.347	NS
AGR3	0.308	0.347	NS

*n=2 pools for REF1, REF3, KLMZR, and AGR2
n=1 pool for REF2, AGR1, and AGR3
**n=1 pool for REF1 and n=2 pools for KLMZR
NS = Not Sampled

The greatest triazine concentrations in water were observed at the agricultural locations AGR2 and AGR3 (0.214 and 0.308 μg/L), while the least concentrations were found at the location on the Kalamazoo River, KLMZR, and one of the reference locations, REF3 (0.034 and 0.040 μg/L, respectively) (Table 13). The other agricultural location, AGR1, had the third least triazine concentrations, 0.056 μg/L (Table 13).

As analyzed with the ELISA test, the least triazine concentrations in tadpole tissue were observed in tadpole were from REF3 (0.230 ng/g, wet wt.) and KLMZR (0.339 ng/g, wet wt.), similar to the results from the water analysis (Table 13). The greatest concentrations in tissues were from the reference location REF2 (0.629 ng/g, wet wt.) and AGR1 (0.442 ng/g, wet wt.) (Table 13). The triazine concentrations in tissues of juveniles were similar to each other, and both locations exhibited juvenile tissues that contained less triazines than the least tadpole tissue concentrations (Table 13).

The method detection limit (MDL) for the tissue samples analyzed by GC-MS ranged from 2.15 to 2.83 ng/g, wet wt. Therefore, these detection limits were too high to compare exactly to the results from the ELISA test. However, these results do not refute the results from the ELISA test either.

Discussion

The ELISA kit used in this study has been shown to accurately measure concentrations of triazines in non-extracted water (Strategic Diagnostics, personal communication). Therefore, it can be assumed that the ELISA kit was able to accurately measure the triazine concentrations in water. It is logical that the greatest water herbicide concentration was observed at two of the three agricultural locations due to the known usage of atrazine in the region. However, the third agricultural location did have one of the lesser triazine concentrations. This location is known to have a buffer zone built around the sampling pond to minimize run-off (Mehne, personal communication). Therefore, this buffer zone may be the reason for the relatively small concentrations in water at this location.

Based on the results of the ELISA analysis, greater triazine concentrations were observed in the tissue of the tadpoles than in juvenile tissue. This may be due to the greater potential of the tadpoles to be exposed to the herbicides in the water than the juveniles since the tadpoles live solely in the water and receive all their nourishment from the aquatic food chain.

Concentrations of triazines in tissue were not well correlated with water concentrations. The tadpoles from the agricultural locations contained approximately the same concentrations as those from the reference locations. These results could represent the actual concentrations, or they could be a result of error from use of the ELISA, especially from possible interactions with the solvent in the extracts. More research is needed in order to be able to justify the use of ELISA tests for tissue extracts. Overall, however, it is possible that the ELISA is correct, because the results are comparable to those from the GC-MS in terms of being within the range of possible concentrations.

Conclusion

The study presented here attempted to link deformities in green frog populations of southwestern Michigan to triazine herbicide residues in their environment and tissues. However, only 0.28% of green frogs exhibited obvious physical abnormalities. Atrazine, a triazine herbicide believed to be an endocrine disruptor (Sanderson et al., 2000), is present in many North American waterways. Concentrations of triazine herbicides, including atrazine, were measured in water and green frog tadpole and juvenile tissue using both enzyme-linked immunosorbent assay (ELISA) and gas chromatography with mass spectrometer detection (GC-MS). A method for the determination of triazines in tissue by ELISA was developed. The ELISA method is a standard method used for the determination of triazines in water. More testing of this method is necessary before it can be used as a standard analysis tool for the determination of triazines in tissues. Slightly higher levels of triazines in water were observed at the agricultural locations. Because no deformed green frogs were observed at these locations, it can be assumed that the levels of triazines observed in water do not cause deformities.

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APPENDICES

APPENDIX A

ORGANOCHLORINE PESTICIDES AND PCBs: RAW DATA AND STATISTICAL SIGNIFICANCE TABLES

Table A.1. Organochlorine insecticide concentrations (ng/g, wet wt., mean and range, in parentheses) in adult green frog tissue from southwestern Michigan, 1998.

Pesticide	REF1, 6/5 (n=2)	REF1, 6/23 (n=3)	REF1, 7/22 (n=3)	AGR1, 6/23 (n=3)	AGR2, 7/22 (n=3)	AGR3, 7/22 (n=2)	KLMZR, 6/5 (n=3)	KLMZR, 6/23 (n=2)
α-HCH	0.04 (<0.01-0.08)	<0.01*	<0.01*	0.04 (0.02-0.08)	<0.01*	<0.01*	0.02 (<0.01-0.07)	0.05 (<0.01-0.09)
β-HCH	0.03 (<0.01-0.05)	0.02 (<0.01-0.05)	0.01 (<0.01-0.04)	<0.01*	<0.01*	<0.01*	0.01* (<0.01-0.03)	<0.01*
γ-HCH	0.07 (0.05-0.09)	0.02 (<0.01-0.05)	0.05 (0.04-0.06)	0.11 (0.08-0.14)	0.03 (<0.01-0.06)	0.03 (0.03-0.04)	0.07 (0.03-0.12)	0.11 (0.07-0.15)
δ-HCH	0.16 (0.08-0.25)	<0.01*	<0.01*	<0.01*	<0.01*	<0.01*	0.10 (<0.01-0.19)	<0.01*
α-chlordane	<0.01*	<0.01*	0.01* (<0.01-0.04)	<0.01*	<0.01*	<0.01*	<0.01*	<0.01*
γ-chlordane	0.06 (0.03-0.10)	0.02 (<0.01-0.03)	0.08 (0.08-0.17)	0.04 (0.02-0.05)	0.04 (0.02-0.05)	0.02 (<0.01-0.02)	0.06 (0.02-0.10)	0.04 (0.02-0.06)
p,p'-DDT	<0.01*	<0.01*	<0.01*	<0.01*	<0.01*	<0.01*	<0.01*	<0.01*
p,p'-DDE	1.50 (0.87-2.22)	0.19 (0.03-0.28)	2.22 (0.97-4.05)	0.09 (0.06-0.12)	0.20 (0.04-0.39)	0.14 (0.11-0.18)	0.18 (0.06-0.27)	0.40 (0.51-0.29)
p,p'-DDD	0.82 (0.65-0.99)	0.40 (<0.01-0.86)	0.88 (0.66-1.19)	0.24 (0.11-0.38)	0.62 (0.07-1.36)	0.19 (0.17-0.22)	1.18 (0.31-1.92)	0.36 (0.25-0.47)

* No ranges are presented here because the values were all below the MDL.

Table A.2. Organochlorine insecticide concentrations (ng/g, wet wt., mean and range[3]) in green frog tadpole and juvenile tissue from southwestern Michigan, 1998.

Pesticide	REF1, 6/5	REF1, 6/23	REF3, 6/5	REF3, 6/23	REF2, 6/5	KLMZR, 6/5	KLMZR, 6/23	KLMZR[1], 8/26	AGR1, 6/5	AGR2, 6/23	AGR3, 6/23
α-HCH	<0.01	<0.01	0.08	0.23	0.09	<0.01	0.20	0.04 (0.01-0.06)	0.12	<0.01	<0.01
β-HCH	<0.01	0.19	0.09	<0.01	<0.01	0.03	<0.01	<0.01[2]	0.45	<0.01	<0.01
γ-HCH	<0.01	0.07	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01[2]	0.25	<0.01	0.04
δ-HCH	<0.01	0.42	<0.01	<0.01	0.26	<0.01	<0.01	<0.01[2]	0.80	<0.01	0.02
α-chlordane	<0.01	<0.01	<0.01	0.03	0.04	<0.01	<0.01	<0.01[2]	0.06	0.14	<0.01
γ-chlordane	<0.01	<0.01	<0.01	0.01	0.02	<0.01	<0.01	<0.01[2]	0.03	<0.01	<0.01
p,p'-DDT	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01[2]	<0.01	<0.01	<0.01
p,p'-DDE	0.02	<0.01	0.09	0.42	0.05	<0.01	<0.01	0.01 (<0.01-0.02)	<0.01	<0.01	0.20
p,p'-DDD	0.11	0.47	0.66	0.86	0.13	0.44	0.20	0.09 (0.08-0.10)	<0.01	<0.01	<0.01

[1] This sample represents 2 pools of juvenile green frogs.

[2] No ranges are presented because the values were below the MDL.

[3] No ranges are presented for the tadpole tissues because n=1 pool for each.

Table A.3. Organochlorine insecticide concentrations (ng/g, wet wt., mean and range[2], in parentheses) in green frog egg tissue from southwestern Michigan, 1998.

Pesticide	REF1, 6/23 (n=2)	KLMZR, 6/23 (n=1)	AGR1, 6/23 (n=1)	AGR3, 6/23 (n=1)
α-HCH	<0.01[1]	<0.01	<0.01	<0.01
β-HCH	<0.01[1]	<0.01	<0.01	<0.01
γ-HCH	0.30 (0.09-0.50)	0.08	0.11	0.02
δ-HCH	<0.01[1]	<0.01	<0.01	<0.01
α-chlordane	0.10 (<0.01-0.20)	0.70	<0.01	<0.01
γ-chlordane	0.09 (0.06-0.13)	0.13	0.20	<0.01
p,p'-DDT	<0.01[1]	<0.01	1.40	0.75
p,p'-DDE	3.86 (2.63-5.10)	9.43	3.83	8.34
p,p'-DDD	0.38 (0.13-0.62)	6.99	0.26	0.54

[1] No ranges are presented here because values were below the MDL.

[2] No ranges are presented for KLMZR, AGR1, and AGR2 because n=1 pool.

Table A.4. Significant differences of OC concentrations (α=0.05) between life stages[1] and between locations[2] and interactions. Values marked with an asterisk (*) indicate significance.

Residue	P-Value (0.00 Substituted for Non-Detects)			P-Value (0.01 Substituted for MDLs)		
	Life Stage (LS)	Location (LOC)	LS x LOC	Life Stage (LS)	Location (LOC)	LS x LOC
α-HCH	0.0284*	0.0548	0.7268	0.0281*	0.0714	0.7199
β-HCH	0.0004*	0.0014*	<0.0001*	0.0002*	0.0006*	<0.0001*
γ-HCH	0.0186*	0.2860	0.0381*	0.0253*	0.3013	0.0401*
δ-HCH	0.0085*	0.0221*	0.0002*	0.0075*	0.0185*	0.0001*
α-chlordane	<0.0001*	0.0002*	<0.0001*	<0.0001*	<0.0001*	<0.0001*
γ-chlordane	0.0007*	0.4419	0.1871	0.0011*	0.5111	0.2091
p,p'-DDT	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*
p,p'-DDE	<0.0001*	0.4696	0.0005*	<0.0001*	0.4716	0.0005*
p,p'-DDD	<0.0001*	0.0002*	<0.0001*	<0.0001*	0.0002*	<0.0001*
ΣHCH	0.0098*	0.0308*	<0.0001*	0.0091*	0.0300*	<0.0001*
Σchlordane	<0.0001*	0.0018*	<0.0001*	<0.0001*	0.0018*	<0.0001*
ΣDDT	<0.0001*	0.0552*	<0.0001*	<0.0001*	0.0556	<0.0001*
ΣPCBs	0.0208*	0.2205	0.0823	0.0208*	0.2205	0.0823

n = 39 observations

[1] Life stages are Adult, Juvenile, Tadpole, and Egg.

[2] Locations are REF1, REF2, REF3, KLMZR, AGR1, AGR2, AGR3.

Table A.5. Concentrations (ng/g, wet wt., mean and range, in parentheses*) of total PCBs and of di-, mono-, and non-*ortho* PCBs in adult green frog tissue from southwestern Michigan, 1998.

Site	REF1, 6/5, (n=3)	REF1, 6/23, (n=3)	REF1, 7/22, (n=3)	KLMZR, 6/5, (n=3)	KLMZR, 6/23, (n=2)	AGR1, 6/23, (n=3)	AGR2, 7/22, (n=3)	AGR3, 7/22, (n=2)
ΣPCBs	5.47	2.74	54.14	58.85	12.02	5.82	7.83	1.48
	(4.46-6.57)	(2.39-2.94)	(6.11-149.94)	(17.09-140.55)	(10.78-13.26)	(3.25-8.35)	(1.65-15.98)	(1.01-1.95)
Di-*ortho*								
138	0.17	0.06	1.68	1.62	0.34	0.59	0.32	0.08
	(0.13-0.22)	(0.04-0.08)	(0.14-4.73)	(0.44-3.95)	(0.26-0.41)	(0.10-1.36)	(0.06-0.72)	(0.06-0.10)
153	1.62	0.60	21.26	19.61	4.29	1.42	2.30	0.71
	(0.91-2.26)	(0.25-0.88)	(1.59-60.03)	(4.79-47.68)	(3.53-5.04)	(0.84-2.19)	(0.27-5.72)	(0.48-0.94)
170	0.06	0.02	1.10	0.61	0.22	0.16	0.39	0.03
	(0.04-0.08)	(<0.01-0.03)	(0.11-3.03)	(0.16-1.44)	(0.20-0.24)	(0.05-0.28)	0.09-0.85)	(0.02-0.03)
180	0.21	0.09	2.68	1.40	0.49	0.28	0.56	0.08
	(0.20-0.23)	(0.07-0.10)	(0.19-7.62)	(0.38-3.40)	(0.48-0.50)	(0.16-0.44)	(0.12-1.24)	(0.04-0.11)
194	0.02	<0.01	0.04	0.11	0.05	0.04	0.09	0.02
	(<0.01-0.03)		(0.03-0.06)	(0.04-0.25)	(0.04-0.05)	(0.02-0.06)	(<0.01-0.19)	(0.01-0.03)
Mono-*ortho*								
105	<0.01	<0.01	4.05	0.66	0.08	<0.01	0.84	<0.01
			(<0.01-11.59)	(<0.01-1.36)	(<0.01-0.15)		(<0.01-2.11)	
118	0.71	0.33	10.23	10.53	1.72	1.52	1.61	0.33
	(0.59-0.87)	(0.22-0.44)	(0.33-29.67)	(2.78-24.25)	(1.19-2.24)	(2.81-0.73)	(0.34-3.26)	(0.26-0.40)
156	0.16	0.05	0.13	0.13	0.05	0.07	0.13	<0.01
	(<0.01-0.25)	(<0.01-0.08)	(<0.01-0.39)	(<0.01-0.38)	(<0.01-0.10)	(0.06-0.07)	(<0.01-0.38)	
Non-*ortho*								
77	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
126	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
169	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

*Ranges are not presented for those values below the MDL.

Table A.6. Concentrations (ng/g, wet wt., mean and range[2], in parentheses) of total PCBs and of di-, mono-, and non-*ortho* PCBs in green frog tadpole and juvenile tissue from southwestern Michigan, 1998.

Site	REF1, 6/5	REF1, 6/23	REF3, 6/5	REF3, 6/23	REF2, 6/5	KLMZR, 6/5	KLMZR, 6/23	KLMZR[1], 8/26	AGR1, 6/5	AGR2, 6/23	AGR3, 6/23
ΣPCBs	3.89	<1.00	9.84	8.19	2.81	28.22	13.98	19.12 (15.06-23.17)	2.83	4.06	2.50
Di-ortho											
138	0.13	<0.01	0.20	0.01	0.09	0.97	0.39	0.97 (0.47-1.46)	0.20	0.09	0.17
153	0.26	<0.01	0.69	0.70	0.21	2.28	1.19	2.81 (1.30-4.32)	0.46	0.38	0.45
170	0.04	<0.01	0.04	0.08	0.02	0.33	0.09	0.39 (0.10-0.68)	0.09	0.02	0.04
180	0.18	<0.01	0.08	0.20	0.18	0.84	0.26	0.76 (0.31-1.21)	0.26	0.12	0.14
194	<0.01	<0.01	0.27	0.03	<0.01	<0.01	<0.01	0.07 (<0.01-0.13)	0.39	<0.01	<0.01
Mono-ortho											
105	<0.01	<0.01	0.17	0.16	<0.01	1.12	0.67	0.40 (<0.01-0.79)	<0.01	<0.01	0.04
118	<0.01	<0.01	0.31	0.31	0.05	0.05	1.00	2.54 (1.10-3.97)	0.30	<0.01	0.16
156	<0.01	<0.01	0.44	0.58	<0.01	0.17	0.05	0.16 (0.04-0.27)	0.03	<0.01	0.03
Non-ortho											
77	<0.01	<0.01	<0.01	<0.01	<0.01	0.29	0.06	<0.01	<0.01	<0.01	<0.01
126	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
169	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

[1] This sample represents 2 pools of juveniles.

[2] No ranges are presented for tadpoles because n=1 pool for each sample.

Table A.7. Concentrations (ng/g, wet wt., mean and range[2], in parentheses) of total PCBs and of di-, mono-, and non-*ortho* PCBs in green frog egg tissue from southwestern Michigan, 1998.

Site	REF1, 6/23, (n=2)	KLMZR, 6/23, (n=1)	AGR1, 6/23, (n=1)	AGR3, 6/23, (n=1)
ΣPCBs	25.50 (16.4-34.59)	236.93	63.02	44.61
Di-*ortho*				
138	0.80 (0.65-0.94)	6.38	3.04	1.88
153	10.4 (6.5-14.3)	112.69	26.51	25.33
170	0.54 (0.40-0.68)	3.29	1.73	0.72
180	0.72 (0.64-0.8)	6.94	2.90	1.45
194	0.12 (0.05-0.19)	<0.01	0.45	0.16
Mono-*ortho*				
105	0.17 (<0.01-0.34)	3.02	3.82	3.24
118	4.73 (4.26-5.20)	24.24	13.88	8.32
156	<0.01[1]	0.88	0.50	<0.01
Non-*ortho*				
77	<0.01[1]	<0.01	<0.01	<0.01
126	<0.01[1]	<0.01	<0.01	<0.01
169	<0.01[1]	<0.01	<0.01	<0.01

[1] No ranges are presented here because values were below the MDL.

[2] No ranges are presented for KLMZR, AGR1, and AGR2 because n=1 pool.

APPENDIX B:

METAL RAW DATA AND STATISTICAL SIGNIFICANCE TABLES

Table B.1. Metal concentrations (µg/g, dry wt., mean and range, in parentheses) in sediments from southwestern Michigan, 1998.

Metal	REF1*	REF1**	REF3*	REF3**	KLMZR*	KLMZR**	AGR2*	AGR2**	AGR3*	AGR3**
Al	2.5x10^{3}	4.1x10^{3}	2.2x10^{3}	4.1x10^{3}	3.6x10^{3} (3.5x10^{3}-3.6x10^{3})	3.8x10^{3} (2.9x10^{3}-4.6x10^{3})	1.2x10^{4}	7.9x10^{3}	4.0x10^{3}	3.6x10^{3}
As	1.7	1.9	4.9	3.7	5.6 (1.6-9.8)	6.4 (2.5-10)	4.4	3.0	1.2	1.2
Ba	20	76	2.2x10^{2}	79	1.2x10^{2} (77-1.7x10^{2})	1.3x10^{2} (1.2x10^{2}-1.4x10^{2})	1.4x10^{4}	82	45	38
Ca	1.0x10^{4}	1.5x10^{4}	1.3x10^{4}	1.3x10^{4}	1.6x10^{5} (1.5x10^{5}-1.7x10^{5})	1.8x10^{5} (1.5x10^{5}-2.1x10^{5})	5.1x10^{3}	2.2x10^{3}	7.5x10^{3}	5.4x10^{3}
Cd	0.32	0.70	0.71	0.16	0.10 (<0.01-0.19)	<0.01	0.39	<0.01	0.10	0.05
Co	0.11	0.10	1.6	2.2	2.4 (1.2-3.6)	2.6 (2.2-3.1)	8.1	4.7	1.5	1.4
Cr	14	77	2.3x10^{2}	13	24 (20-28)	31 (27-36)	23	24	7.0	5.0
Cu	8.3	8.4	5.1	26	21 (17-25)	11 (9.9-13)	16	14	6.0	5.4
Fe	2.2x10^{2}	8.0x10^{2}	1.0x10^{2}	5.1x10^{2}	2.5x10^{3} (1.1x10^{2}-4.9x10^{3})	1.9x10^{3} (5.3x10^{2}-3.3x10^{3})	27	2.3x10^{2}	2.0x10^{2}	5.4x10^{2}
Hg	0.13	0.29	1.3	0.09	0.12 (0.11-0.13)	0.12 (0.11-0.12)	0.09	0.12	0.07	0.07
K	<50	<50	<50	<50	5.2x10^{2} (0.00-1.0x10^{3})	8.8x10^{2} (0.00-1.8x10^{3})	<50	<50	<50	<50
Mg	9.8x10^{2}	1.3x10^{3}	1.8x10^{3}	2.0x10^{3}	4.3x10^{3} (3.5x10^{3}-5.0x10^{3})	4.8x10^{3} (4.6x10^{3}-5.1x10^{3})	1.7x10^{3}	8.5x10^{2}	1.2x10^{3}	9.8x10^{2}
Mn	64	1.4x10^{2}	3.2x10^{2}	1.3x10^{2}	1.0x10^{3} (1.6x10^{2}-1.9x10^{3})	9.4x10^{2} (2.5x10^{2}-1.6x10^{2})	8.0x10^{2}	5.6x10^{2}	1.1x10^{2}	80
Ni	<1.2	<1.2	<1.2	<1.2	2.4 (0.00-4.7)	9.6 (0.00-19)	<1.2	<1.2	<1.2	<1.2
Pb	31	40	7.2	19	20 (17-23)	25 (20-30)	40	23	18	15
Sc	1.4	3.5	4.6	1.9	2.3 (2.0-2.6)	2.5 (2.4-2.6)	2.5	2.6	1.6	1.6
Sr	15	28	36	20	92 (66-1.2x10^{2})	99 (98-101)	14	12	10	8.9
Ti	20	25	11	15	18 (14-22)	26 (8.2-43)	26	29	7.9	4.3
Zn	29	18	26	34	19 (5.3-32)	14 (10-18)	70	1.0	25	20

*Represents top 5 cm of sediment

**Represents next 5 cm of sediment

n=1 for all samples except for KLMZR, where two samples of each layer were analyzed. For those samples where n=1, no ranges are presented.

Table B.2. Metal concentrations (µg/g, dry wt., mean and range, in parentheses) in adult green frog tissue from southwestern Michigan, 1998.

Metal	REF1, n=3	REF3[1], n=1	KLMZR, n=5	AGR1, n=3	AGR2, n=4
Al	26 (0.00-62)	<10	1.0 x10^2 (0.00-4.6 x10^2)	85 (0.00-2.0 x10^2)	26 (0.00-1.0 x10^2)
As	0.13 (0.04-0.30)	0.35	0.04 (0.00-0.15)	0.15 (0.00-0.40)	0.05 (0.00-0.20)
Ba	12 (0.79-31)	37	4.2 (0.24-13)	1.8 (0.00-5.0)	16 (7.2-23)
Ca	1.8 x10^2 (1.5 x10^2-2.1 x10^2)	8.6 x10^2	1.1 x10^2 (88-1.7 x10^2)	3.7 x10^2 (3.6 x10^2-3.8 x10^2)	6.1 x10^2 (2.1 x10^2-8.9 x10^2)
Cd	0.16 (0.05-0.38)	0.20	0.10 (0.03-0.23)	0.04 (0.00-0.07)	<0.04[2]
Co	0.10 (0.00-0.28)	0.16	0.02 (0.00-0.06)	0.13 (0.24-0.06)	0.27 (0.07-0.65)
Cr	11 (0.95-28)	2.0	1.5 (0.93-2.10)	25 (1.5-73)	3.7 (1.2-8.5)
Cu	40 (1.9-92)	92	64 (30-98)	84 (70-95)	64 (40-1.1 x10^2)
Fe	4.3x10^2 (1.4x10^2-9.8x10^2)	2.6x10^3	2.3x10^2 (1.2x10^2-3.9x10^2)	3.6x10^3 (3.8x10^2-9.6x10^3)	5.6x10^2 (4.5x10^2-8.2x10^2)
Hg	1.3 (0.18-3.4)	0.25	0.17 (0.12-0.21)	0.20 (0.11-0.35)	0.35 (0.23-0.44)
K	2.8 x10^3 (2.4 x10^3-3.6 x10^3)	5.1 x10^3	3.5 x10^3 (3.2 x10^3-3.8 x10^3)	3.6 x10^3 (3.0 x10^3-3.9 x10^3)	3.0 x10^3 (2.1 x10^3-3.2 x10^3)
Mg	2.5 x10^2 (2.2 x10^2-3.1 x10^2)	4.8 x10^2	2.6 x10^2 (2.4 x10^2-3.0 x10^2)	3.0 x10^2 (2.7 x10^2-3.3 x10^2)	2.2 x10^2 (2.0 x10^2-2.6 x10^2)
Mn	8.0 (1.6-20)	6.2	0.75 (0.27-1.5)	3.0 (2.1-3.9)	1.1 (0.00-2.3)
Ni	<2.9[2]	<2.9	<2.9[2]	3.3 (0.00-9.8)	<2.9[2]
Pb	<0.76[2]	<0.76	<0.76[2]	<0.76[2]	<0.76[2]
Sc	0.34 (0.09-0.81)	0.25	0.15 (0.12-0.19)	0.16 (0.09-0.21)	0.32 (0.20-0.39)
Sr	0.66 (0.00-2.0)	1.2	<0.31[2]	<0.31[2]	0.46 (0.00-1.8)
Ti	3.3 (2.8-4.2)	4.1	2.8 (2.5-3.2)	3.2 (2.8-3.6)	2.2 (1.3-2.7)
Zn	26 (18-37)	43	25 (22-28)	30 (27-34)	26 (15-41)

[1] No ranges are presented for REF3 because n=1.

[2] No ranges are reported for these values because they are below the MDL.

Table B.3. Metal concentrations (µg/g, dry wt., mean and range, in parentheses) in green frog tadpole and juvenile tissue from southwestern Michigan, 1998.

Metal	REF3, n=2	KLMZR, n=2	AGR1[1], n=1	AGR2, n=2	REF1*[1], n=1	KLMZR*, n=2
Al	3.7x10^{2} (3.1x10^{2}-4.3x10^{2})	6.4 x10^{2} (4.1 x10^{2}-8.8 x10^{2})	7.8 x10^{2}	6.5 x10^{2} (5.8 x10^{2}-7.1 x10^{2})	5.1 x10^{2}	3.1 x10^{2} (0.00-6.1 x10^{2})
As	1.4 (1.0-1.9)	1.5 (0.5-2.6)	1.9	0.69 (0.52-0.86)	0.39	0.26 (0.02-0.50)
Ba	34 (26-42)	60 (19-1.0 x10^{2})	23	52 (46-59)	39	31 (10-51)
Ca	1.4x10^{4} (6.4x10^{3}-2.2x10^{4})	2.8x10^{4} (1.3x10^{4}-4.2 x10^{4})	3.4 x10^{4}	2.3 x10^{4} (2.2 x10^{4}-2.4 x10^{4})	3.5 x10^{4}	2.5 x10^{4} (1.6 x10^{4}-3.4 x10^{4})
Cd	0.23 (0.04-0.41)	0.02 (0.00-0.04)	0.12	0.51 (0.00-1.01)	0.01	0.04 (0.01-0.07)
Co	0.20 (0.19-0.20)	0.49 (0.20-0.78)	0.20	0.64 (0.54-0.73)	0.13	0.24 (0.04-0.44)
Cr	7.9 (4.7-11)	9.1 (2.4-16)	25	3.8 (2.8-4.8)	14	6.1 (2.9-9.3)
Cu	3.6 (3.2-4.1)	3.3 (1.7-4.9)	1.7	6.8 (3.8-9.7)	6.2	2.9 (1.6-4.3)
Fe	2.4x10^{4} (2.1x10^{4}-2.8x10^{4})	4.6x10^{4} (1.1x10^{4}-8.1x10^{4})	1.4x10^{5}	8.6x10^{4} (2.7x10^{4}-1.5x10^{5})	1.6x10^{3}	2.9x10^{4} (5.4x10^{3}-5.0x10^{4})
Hg	0.16 (0.09-0.23)	0.16 (0.13-0.19)	0.60	0.24 (0.16-0.31)	0.31	0.10 (0.09-0.11)
K	4.9x10^{3} (2.4x10^{3}-7.4x10^{3})	5.5x10^{3} (7.7x10^{3}-3.2x10^{3})	2.0x10^{3}	8.8 x10^{3} (8.2 x10^{3}-9.5 x10^{3})	4.5 x10^{3}	4.2 x10^{3} (3.5 x10^{3}-4.8 x10^{3})
Mg	7.0x10^{2} (3.8x10^{2}-1.0 x10^{3})	1.4 x10^{3} (6.9 x10^{2}-2.0 x10^{3})	1.2 x10^{3}	1.2 x10^{3} (1.1 x10^{3}-1.3 x10^{3})	1.2 x10^{3}	1.0 x10^{3} (5.6 x10^{2}-1.4 x10^{3})
Mn	1.1 x10^{2} (84-1.4 x10^{2})	6.7 x10^{2} (45-1.3 x10^{3})	1.4 x10^{2}	2.6 x10^{2} (1.9 x10^{2}-3.4 x10^{2})	67	77 (3.6-1.5 x10^{2})
Ni	<2.9[2]	<2.9[2]	<2.9	<2.9[2]	<2.9	<2.9[2]
Pb	0.73 (0.71-0.75)	3.0 (1.8-4.3)	0.40	1.7 (0.50-2.9)	7.1	2.3 (0.02-4.5)
Sc	0.53 (0.30-0.76)	0.72 (0.43-1.0)	1.5	0.83 (0.70-0.95)	0.73	0.43 (0.28-0.58)
Sr	7.8 (3.6-12)	17 (6.0-28)	20	10 (9.3-11)	12	11 (4.6-17)
Ti	15 (9.2-21)	21 (13-29)	29	27 (26-28)	25	14 (8.8-18)
Zn	42 (19-65)	42 (19-65)	40	56 (48-63)	77	35 (31-38)

* These samples represent pools of juvenile frogs.

[1] Where n=1 pool, no range can be presented.

[2] No ranges are reported for these values because they are below the MDL.

Table B.4. Significant differences of metal concentrations (α=0.05) between life stages[1] and between locations[2] and interactions. Values marked with an asterisk (*) indicate significance.

Metals	P-Value (0.00 Substituted for Non-Detects)			P-Value With MDLs for Non-Detects		
	Life Stage (LS)	Location (LOC)	LS x LOC	Life Stage (LS)	Location (LOC)	LS x LOC
Al	<0.0001*	0.0004*	<0.0001*	<0.0001*	0.0004*	<0.0001*
As	NC	NC	NC	<0.0001*	0.2023	0.6006
Ba	0.0383*	0.2767	0.5915	<0.0001*	0.0562	0.2827
Ca	0.0002*	0.0633	0.0892	<0.0001*	<0.0001*	<0.0001*
Cd	0.0111*	0.0829	0.0871	0.2387*	0.2555	0.2615
Co	NC	NC	NC	<0.0001*	0.0002*	<0.0001*
Cr	NC	NC	NC	0.1097	0.2241	0.5526
Cu	<0.0001*	0.4767	0.8086	<0.0001*	0.3969	0.8472
Fe	<0.0001*	0.2269	0.0739	<0.0001*	0.2280	0.0736
Hg	NC	NC	NC	0.7418	0.4475	0.6541
K	<0.0001*	0.2385	0.0015	<0.0001*	0.5263	0.0085*
Mg	<0.0001*	<0.0001	<0.0001	<0.0001*	<0.0001*	<0.0001*
Mn	0.1891	0.2754	0.6730	0.0169*	0.1823	0.5462
Ni	0.6494	0.5655	0.7056	0.6852	0.6487	0.6866
Pb	<0.0001*	0.1003	0.4748	<0.0001*	0.0033*	0.0217*
Sc	NC	NC	NC	<0.0001*	0.2130	0.6760
Sr	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*	<0.0001*
Ti	0.0004*	0.5259	0.5389	<0.0001*	0.0355*	0.6720
Zn	0.0004*	0.6787	0.3131	0.0188*	0.6439	0.5823

n = 38

[1] Life stages are Adult, Juvenile, Tadpole, and Sediment.

[2] Locations are REF1, REF3, KLMZR, AGR1, AGR2, AGR3.

NC = Not Calculated.

APPENDIX C:

STANDARD OPERATING PROCEDURE FOR ORGANOCHLORINE EXTRACTION AND ANALYSIS

Michigan State University
Aquatic Toxicology Laboratory
National Food Safety and Toxicology Center

STANDARD OPERATING PROCEDURE

ANALYSIS OF DDT AND ITS METABOLITES IN BIOLOGICAL AND ENVIRONMENTAL MATRICES

K. Kannan and J.P. Giesy

1.0 SCOPE AND APPLICATION

This SOP describes the method to determine the concentrations of DDT and it metabolites in extracts from biota and sediments/soils using fused-silica capillary columns with electron capture detectors (ECD). Following are the list of target compounds that can be analyzed by the method described herein. 2,4'-DDD and 2,4'-DDE can be analyzed by this method, but they fractionate in both the fractions of the silica gel column, and coelute with some PCN congeners. Since these two compounds account for only a minor portion of the total DDT concentrations, they are not quantified by this method. However, 2,4'-DDT is quantified because it is a constituent of the technical DDT preparation accounting for 20% of the total DDTs. Measurement of 2,4'-DDT can shed light on the chronology of exposure to DDTs. This method is a modification of the U.S. EPA method 8081A.

Compound	CAS Registry No.
4,4'-DDD	72-54-8
4,4'-DDE	72-55-9
4,4'-DDT	50-29-3
2,4'-DDT	789-02-6

1.2 This Method can also be used for the determination of PCBs. Therefore, this method is suitable for those samples that need both PCBs and DDT analysis done in the same extraction procedure.

1.3 The analyst must select columns, detectors and calibration procedures most appropriate for the specific analytes of interest in a study. Matrix-specific performance data must be established and the stability of the analytical system and instrument calibration must be established for each analytical matrix.

1.4 This method is restricted to use by, or under the supervision of, analysts experienced in the use of gas chromatographs (GC) and skilled in the interpretation of gas chromatograms. Each analyst must demonstrate the ability to generate acceptable results with this method.

2.0 SUMMARY OF METHOD

2.1 A measured amount of sample (20 g wet tissue for biota or dry wt for soil or sediments) is extracted using the appropriate matrix-specific sample extraction technique.

2.2 Solid samples are homogenized with anhydrous sodium sulfate and extracted with methylene chloride-hexane (3:1) using a Soxhlet extractor (Method 3540 of the USEPA).

2.3 A variety of cleanup steps may be applied to the extract, depending on the nature of the matrix interferences and the target analytes. Suggested cleanups include Florisil (Method 3620 of the USEPA), silica gel (Method 3630 of the USEPA) and sulfur (Method 3660 of the USEPA).

2.4 After cleanup, the extract is analyzed by injecting a 1-L sample into a gas chromatograph with a fused silica capillary column and electron capture detector (GC/ECD) or a mass selective detector (GC/MSD).

3.0 INTERFERENCES

3.1 Sources of interference in this method can be grouped into three broad categories.

3.1.1 Contaminated solvents, reagents, or sample processing hardware.

3.1.2 Contaminated GC carrier gas, parts, column surfaces, or detector surfaces.

3.1.3 Compounds extracted from the sample matrix to which the detector will respond.

3.1.4 Interferences co-extracted from the samples will vary considerably from waste to waste. While general cleanup techniques are referenced or provided as part of this method, unique samples may require additional cleanup approaches to achieve desired degrees of discrimination and quantitation.

3.2 Interferences by phthalate esters introduced during sample preparation can pose a major problem in pesticide determinations.

3.2.1 These materials may be removed prior to analysis using Silica Gel Cleanup.

3.2.2 Common flexible plastics contain varying amounts of phthalate esters which are easily extracted or leached from such materials during laboratory operations.

3.2.3 Cross-contamination of clean glassware routinely occurs when plastics are handled during extraction steps, especially when solvent-wetted surfaces are handled.

3.2.4 Interferences from phthalate esters can best be minimized by avoiding contact with any plastic materials and checking all solvents and reagents for phthalate contamination. Exhaustive cleanup of solvents, reagents and glassware may be required to eliminate background phthalate ester contamination.

3.3 Glassware must be scrupulously cleaned. Clean all glassware as soon as possible after use by rinsing with the last solvent used. This should be followed by detergent washing with hot water, and rinses with tap water and organic-free reagent water. Drain the glassware and dry it in an oven at 130 C for several hours, or rinse with acetone and hexane prior to use. Store dry glassware in a clean environment.

3.4 The presence of elemental sulfur will result in broad peaks that interfere with the detection of early-eluting organochlorine pesticides. Sulfur contamination should be expected with sediment samples.

3.5 Waxes, lipids, and other high molecular weight materials can be removed by acidic silica gel column chromatography or sulfuric acid treatment.

3.6 Other halogenated pesticides or industrial chemicals may interfere with the analysis of DDTs. Co-eluting chlorophenols may be eliminated by using silica gel column chromatography. Polychlorinated biphenyls (PCBs) also may interfere with the analysis of the organochlorine pesticides. Silica gel column fraction techniques will be used to separate DDTs from the PCBs. However, p,p'-DDE would appear in PCB fraction and therefore adequate caution should be exercised in interpreting p,p'-DDE concentrations.

4.0 APPARATUS AND MATERIALS

4.1 Gas chromatograph: an analytical system complete with gas chromatograph suitable for on-column and split-splitless injection and all required accessories including syringes, analytical columns, gases, electron capture detectors (ECD), and recorder/integrator or data system.

4.2 GC columns

Any one of the following columns will be used. When necessary, confimation of the target analytes will be made by injecting samples in a column of different polarity.

4.2.1 A DB-5 MS (5%-phenyl)-methylpolysiloxane) 30 m x 0.25 mm i.d. fused silica capillary column chemically bonded with at 0.25 μm film thickness (nonpolar column).

4.2.2 A DB-1701 (30 m x 0.25 mm ID) fused silica capillary column chemically bonded with 14%cyanoporpyl phenyl methylpolysiloxane at 0.25 μm film thickness (mid polar).

4.2.3 A DB-1 (30 m x 0.25 mm ID) fused silica capillary column chemically bonded with 100% dimethylpolysiloxane at 0.25 μm film thickness (polar).

5.0 REAGENTS

5.1 Reagent grade or pesticide grade chemicals shall be used in all tests. Unless otherwise indicated, it is intended that all reagents shall conform to specifications of the Committee on Analytical Reagents of the American Chemical Society, where such specifications are available. Other grades may be used, provided it is first ascertained that the reagent is of sufficiently high purity to permit its use without lessening the accuracy of the determination.

NOTE: Store the standard solutions (stock, composite, calibration, internal, and surrogate) at 4 C in polytetrafluoroethylene (PTFE)-sealed containers in the dark. When a lot of standards is prepared, it is recommended that aliquots of that lot be stored in individual small vials. All stock standard solutions must be replaced after one year or sooner if routine QC tests indicate a problem.

5.2 Solvents used in the extraction and cleanup procedures include n-hexane, dichloromethane, acetone, and must be exchanged to hexane or isooctane prior to analysis. Acetone or toluene may be required for the preparation of some standard solutions. All solvents should be pesticide quality or equivalent, and each lot of solvent should be determined to be phthalate free.

5.3 Organic-free reagent water - All references to water in this method refer to organic-free reagent water.

5.4 Stock standard solutions (1000 mg/L) - May be prepared from pure standard materials or can be purchased as certified solutions.

5.4.1 Prepare stock standard solutions by accurately weighing about 0.0100 g of pure compound. Dissolve the compound in isooctane or hexane and dilute to volume in a 10-mL volumetric flask. If compound purity is 96 percent or greater, the weight can be used without correction to calculate the concentration of the stock standard solution. Commercially prepared stock standard solutions can be used at any concentration if they are certified by the manufacturer or by an independent source.

5.5 Calibration standards should be prepared at a minimum of five different concentrations by dilution of the composite stock standard with isooctane or hexane. The concentrations should correspond to the expected range of concentrations found in real samples and should bracket the linear range of the detector.

5.6 Surrogate standards

The performance of the method should be monitored using surrogate compounds. Surrogate standards are added to all samples, method blanks, matrix spikes, and calibration standards. The following compounds are recommended as possible surrogates.

5.6.1 Tetrachloro-m-xylene has been found to be a useful surrogate for both the single-column and dual-column configurations.

6.0 PROCEDURE

6.1 Sample extraction

Solid samples (20 g) are homogenized with anhydrous sodium sulfate and extracted with methylene chloride-hexane (3:1) using a Soxhlet apparatus. Sodium sulfate should be cleaned with methylene chloride or baked at 450°C overnight prior to use. The amount sodium sulfate added to samples varies with the moisture content of the samples. Generally, for tissues such as fish muscle or liver, sample to sodium sulfate ratio is 1:6. After weighing 20 g of homogenized sample, sodium sulfate is weighed and added to sample in a pestle and mortar or in a homogenizer cup. Samples are homogenized into fine powder and then transferred into a Soxhlet apparatus. All the glassware and homogenizer parts that come in contact with samples will be rinsed with alconox detergent, nonapure water, acetone and hexane prior to use. Homogenizer parts will be rinsed with detegent, nonapure water, acetone and hexane between each sample. Surrogate standard (TCMX) will be spiked at the amount of 50 ng final mass. For matrix spike and matrix spike duplicates, appropriate standards will be spiked at 50 ng final mass of individual DDT compounds. Samples will be extracted for 16 h and the Soxhlet cycle should be set at 5 cycles/hour (Rheostat set at 5). Condenser should be cleaned with acetone and hexane and cool water should be turned on prior to use. Extreme care should be taken to avoid contamination of samples while handling the sample-weighing and homogenizing. Watch for the first two cycles to complete after turning the Soxhlet on.

6.2. Concentration and Lipid content estimation

After 16 h, turn the Soxhlet off. Let the condenser water flow until the system gets cooled down. Rotary evaporate the solvent at 40°C to approximately 5 to 10 mL. Transfer the extract into a clean graduated test tube. Rinse the round-bottom flask three times with hexane and transfer the contents into the test tube. Make up the volume of the solvent to 11 mL. Transfer 1 mL into a pre-weighed aluminum weighing disk (W1). Dry the solvent and place it in n oven at 80°C for 1 h. Cool it down in a dessicator for 30 min and take the final weight (W2). Don't touch the aluminum weighing dishes by hand. Oil from the fingers can affect the results of fat content. Use clean forceps to handle the weighing dish.

$$\text{Lipid content } (\%) = W2\text{-}W1 \;*\; 10/\text{sample wt (g)} \;*\; 100$$

Lipid content is measured only for biological samples and for soil samples it is not performed. However, for soil/sediments, the volume is made to 10 mL instead of 11 mL and sulfur is removed with activated copper treatment. Activated copper is prepared by treating/immersing copper granules with concentrated hydrochloric acid for 30 min with occasional stirring. Acid is decanted safely and copper is rinsed with nonapure water followed by acetone and hexane rinsing. After activation, copper granules should be shining. Lack of metallic luster means that the activation is not proper. Transfer active copper granules into the test tube in which soil/sediment sample extracts are in. Add copper until it stops to turn black. The amount of copper varied from samples to samples depending on the sulfur content.

6.3 Extract cleanup

The specific cleanup procedure used will depend on the nature of the sample to be analyzed and the data quality objectives for the measurements. For samples of biological origin multilayer silica gel column chromatography is performed. Silica gel is packed in a glass column (10 mm i.d.) mounted vertically in the following order: 2 g silica gel (100-200 mesh, Aldrich), 2 g 40% acidic-silica and 2 g silica. A thin layer of sodium sulfate is added at the top. Prior to the transfer of samples, silica gel should cleaned by passing 100 mL of hexane through the column. Discard the eluate. Then transfer 10 mL of the sample extract and elute the first fraction with 150 mL of hexane. This fraction contains PCBs and p,p'-DDE and a portion of p,p'-DDT. Separation tests need to conducted with every lot of silica gel to find out the volume of hexane needed to elute all the PCBs in fraction 1. Batch to batch variation in the composition of the silica gel or Florisil or overloading the column may cause a change in the elution patterns of the DDTs and PCBs. When compounds are found in two fractions, add the concentrations found in the fractions, and correct for any additional dilution. This volume can vary from 120 to 160 mL. The second fraction is eluted with 20% dichloromethane and hexane (100 mL) contains p,p'-DDT, p,p'-DDD. Both the fractions are collected separately in a flat bottom flask and rotary evaporated to approximately 1 mL. The extract is transferred to a test tube and volume is made up to 1 mL accurately. Additional clean up with sulfuric acid may be needed for lipid rich samples. The 1 mL of the final extract is transferred into a GC vial for instrumental analysis.

6.4 Instrumental analysis

This method allows the analyst to choose between a single-column or a dual-column configuration in the injector port.

6.4.1 Single-column analysis

A narrow-bore (0.25 mm ID) DB-5 or DB-1701 column is used to separate DDT compounds with greater chromatographic resolution. Use of narrow-bore columns is suitable for relatively clean samples or for extracts that have been prepared with one or more of the clean-up options referenced in the method.

Operating conditions vary depending on the sample. Generally, column oven temperature is programmed from 80°C (1 min hold) to 120°C at a rate of 10°C/min and then to 260°C at a rate of 2°C/min, with a final holding time of 10 min. Injector and detector temperatures are kept at 250 and 350°C, respectively. Helium is the carrier gas and nitrogen is the make-up gas. Once established, the same operating conditions must be used for both calibrations and sample analyses throughout the project. Always run an acceptable blank prior to running any standards or samples. Calibration standards will be injected periodically to check for the instrument calibration status.

6.4.2. Calibration

The procedure for external calibration is used. In most cases, external standard calibration is preferred because of the sensitivity of the electron capture detector and the probability of the internal standard being affected by interferences. For calibration verification (each 12-hour shift) all target analytes required in the project plan must be injected.

<u>NOTE</u>: Because of the sensitivity of the electron capture detector, the injection port and column should always be cleaned prior to performing the initial calibration.

6.4.3 A 1 to 2 μL injection volume of each calibration standard is recommended. Other injection volumes may be employed, provided that the analyst can demonstrate adequate sensitivity for the compounds of interest.

6.4.4 Calibration factors

When external standard calibration is employed, calculate the calibration factor for each analyte at each concentration, the mean calibration factor, and the relative standard deviation (RSD) of the calibration factors, using the formulae below.

6.4.4.1 Calculate the calibration factor for each analyte at each concentration as:

$$\mathrm{CF} = \frac{\text{Peak Area (or Height) of the Compound in the Standard}}{\text{Mass of the Compound Injected (in nanograms)}}$$

6.4.5. Calculate the mean calibration factor for each analyte as:

$$\text{mean CF} = \overline{\mathrm{CF}} = \frac{\sum_{i=1}^{n} \mathrm{CF}_i}{n}$$

where n is the number of standards analyzed.

6.4.5.1 Calculate the standard deviation (SD) and the RSD of the calibration factors for each analyte as:

$$\mathrm{SD} = \sqrt{\frac{\sum_{i=1}^{n} (\mathrm{CF}_i - \overline{\mathrm{CF}})^2}{n-1}} \qquad \mathrm{RSD} = \frac{\mathrm{SD}}{\overline{\mathrm{CF}}} \times 100$$

If the RSD for each analyte is 20%, then the response of the instrument is considered linear and the mean calibration factor can be used to quantitate sample results. If the RSD is greater than 20%, then linearity through the origin cannot be assumed. The analyst must use a calibration curve or a non-linear calibration model (e.g., a polynomial equation) for quantitation.

6.4.6 Retention time windows

Absolute retention times are used for compound identification. Retention time windows are crucial to the identification of target compounds. A drift in retention time could be possible depending on the cleanliness of the sample. The experience of the analyst should weigh heavily in the interpretation of the chromatograms.

7.0 GAS CHROMATOGRAPHIC ANALYSIS OF SAMPLE EXTRACTS

7.1.1 The same GC operating conditions used for the initial calibration must be employed for samples analyses.

7.1.2 Verify calibration each 12-hour shift by injecting calibration verification standards prior to conducting any sample analyses. Analysts should alternate the use of high and low concentration mixtures of single-component analytes and multi-component analytes for calibration verification. A calibration standard must also be injected at intervals of not less than once every twenty samples (after every 10 samples is *recommended* to minimize the number of samples requiring re-injection when QC limits are exceeded) and at the end of the analysis sequence.

7.1.2.1 If the calibration does not meet the ±15% limit (either on the basis of each compound or the average across all compounds), check the instrument operating conditions, and if necessary, restore them to the original settings, and inject another aliquot of the calibration verification standard. If the response for the analyte is still not within ±15%, then a new initial calibration must be prepared.

7.1.3 Compare the retention time of each analyte in the calibration standard with the absolute retention time. Each analyte in each standard must fall within its respective retention time window. If not, the gas chromatographic system must either be adjusted so that a second analysis of the standard does result in all analytes falling within their retention time windows, or a new initial calibration must be performed and new retention time windows established.

7.1.4 Inject a 1 or 2-μL aliquot of the concentrated sample extract. Record the volume injected to the nearest 0.05 μL and the resulting peak size in area units.

7.1.5 Tentative identification of an analyte occurs when a peak from a sample extract falls within the absolute retention time window. Each tentative identification may be confirmed using either a second GC column of dissimilar stationary phase or using another technique such as GC/MS.

When results are confirmed using a second GC column of dissimilar stationary phase, the analyst should check the agreement between the quantitative results on both columns once the identification has been confirmed. Unless otherwise specified in an approved project plan, the higher result should be reported, as this is a conservative approach relative to protection of the environment. If the relative percent difference of the results exceeds 40%, steps may be taken to address the discrepancy.

7.1.6 When using the external calibration procedure, the quantity of each component peak in the sample chromatogram, which corresponds to the compounds used for calibration purposes, is determined as follows. Proper quantitation requires the appropriate selection of a baseline from which the peak area or height can be determined.

$$\text{Concentration } (\mu g/kg) = \frac{(A_x)(V_t)(D)}{(\overline{CF})(V_i)(W_s)}$$

where,

A_x = Area (or height) of the peak for the analyte in the sample.

V_t = Total volume of the concentrated extract (L).

D = Dilution factor, if the sample or extract was diluted prior to analysis. If no dilution was made, D = 1. The dilution factor is always dimensionless.

$\overline{CF}$= Mean calibration factor from the initial calibration (area/ng).

V_i = Volume of the extract injected (L). The injection volume for samples and calibration standards must be the same. For purge-and-trap analysis, V_i is not applicable and therefore is set at 1.

W_s= Weight of sample extracted (g). The wet weight or dry weight may be used, depending upon the specific application of the data. If units of kilograms are used for this term, multiply the results by 1000.

Using the units specified here for these terms will result in a concentration in units of ng/g, which is equivalent to μg/kg.

7.1.6.1 If the responses exceed the calibration range of the system, dilute the extract and reanalyze.

7.1.6.2 Generally for DDT compounds, there are no coeluting peaks. If partially overlapping or coeluting peaks are found, change GC columns or try GC/MS quantitation.

7.1.6.3 Each sample analysis must be bracketed with an acceptable initial calibration, calibration verification standard(s) (each 12-hour analytical shift), or calibration standards interspersed within the samples.

When a calibration verification standard fails to meet the QC criteria, all samples that will be reinjected after the last standard that last met the QC criteria must be evaluated to prevent mis-quantitations and possible false negative results, and re-injection of the sample extracts may be required. More frequent analyses of standards will minimize the number of sample extracts that would have to be reinjected if the QC limits are violated for the standard analysis.

However, if the standard analyzed <u>after</u> a group of samples exhibits a response for an analyte that is <u>above</u> the acceptance limit, i.e., >15%, and the analyte was <u>not</u> detected in the specific samples analyzed during the analytical shift, then the extracts for those samples do not need to be reanalyzed, as the verification standard has demonstrated that the analyte would have been detected were it present. In contrast, if an analyte above the QC limits <u>was</u> detected in a sample extract, then re-injection is necessary to ensure accurate quantitation. If an analyte was not detected in the sample and the standard response is more than 15% below the initial calibration response, then re-injection is necessary to ensure that the detector response has not deteriorated to the point that the analyte would not have been detected even though it was present (i.e., a false negative result).

7.1.7 If the peak response is less than 2.5 times the baseline noise level, the validity of the quantitative result may be questionable. The analyst should consult with the source of the sample to determine whether further concentration of the sample is warranted.

7.2 Validation of GC system qualitative performance

7.2.1 Use the calibration standards analyzed during the sequence to evaluate retention time stability.

7.2.2 Each subsequent injection of a standard during the 12-hour analytical shift (i.e., those standards injected every 20 samples, or more frequently) must be checked against the retention time windows. If any of these subsequent standards fall outside their absolute retention time windows, the GC system is out of control. Determine the cause of the problem and correct it. If the problem cannot be corrected, a new initial calibration must be performed.

7.3 GC/MS confirmation may be used in conjunction with either single-column or dual-column analysis if the concentration is sufficient for detection by GC/MS.

7.4 Suggested chromatographic system maintenance - When system performance does not meet the established QC requirements, corrective action is required, and may include one or more of the following.

> GC injector ports can be of critical concern, especially in the analysis of DDT. Injectors that are contaminated, chemically active, or too hot can cause the degradation ("breakdown") of the analytes. DDT breakdown to DDD, or DDE. When such breakdown is observed, clean and deactivate the injector port, break off at least 30 cm of the column and remount it. Check the injector temperature and lower it to 205°C, if required.

8.0 QUALITY CONTROL and METHOD PERFORMANCE

The detection limits for individual DDT compounds through the analytical procedure is 1 ng/g, wet wt for biota and 1 ng/g, dry wt, for sediments. Recoveries of DDT compounds spiked on to sediments and fish tissues and passed through the whole analytical procedure ranged from 90-103%. A standard reference material will be run along with every 20 samples and the observed values will be compared with those of certified values. In addition, a method blank, a matrix spike, a duplicate will be done. The laboratory must evaluate surrogate recovery data from individual samples versus the surrogate control limits developed by the laboratory. This method has been applied in a variety of commercial laboratories for environmental and waste matrices.

9.0 REFERENCES

1. USEPA. Method 8081A. Analysis of organochlorine pesticides by gas chromatography. U.S. Environmental protection Agency, Wahington, DC.

APPENDIX D:

STANDARD OPERATING PROCEDURES FOR *IN-VITRO* CELL BIOASSAYS

Michigan State University
National Food Safety and Toxicology Center
Aquatic Toxicology Laboratory

STANDARD OPERATING PROCEDURE

H4IIE-luc Bioassay For The Detection Of Ah Receptor Agonists

Version 1 September 14, 1998

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DEFINITIONS AND ACRONYMS

AhR	Aryl hydrocarbon receptor
ATL	Aquatic Toxicology Laboratory (Michigan Sate University)
DCCFBS	Fetal bovine serum that has been charcoal-stripped
EC50	conentration of test agent that causes 50% of maximal response
FBS	Fetal bovine serum
H4IIE-luc	rat hepatoma cells stably transfected with an AhR-controlled luciferase reporter gene construct
PBS	Phosphate-buffered saline
RLU	Relative luminescent units
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin

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1.0. PURPOSE

The H4IIE-luciferase induction assay is an *in vitro* technique for the identification of aryl hydrocarbon (Ah) receptor-active compounds. The technique uses rat hepatoma cells (H4IIE-luc) stably transfected with an AhR-controlled luciferase reporter gene construct were developed at Michigan State University by Dr. Jac Aarts (Univ. of Wageningen, The Netherlands; Sanderson et al., 1996). The assay is also referred to as the chemical activated luciferase gene expression (CALUX) system (Murk et al., 1996). These cells express firefly luciferase in response to Ah receptor agonists. Luciferase activity is measured conveniently and with high sensitivity as light emission using a plate-scanning luminometer. Luciferase induction potential is assessed by comparison of the response to that of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the most potent agonist for the mammalian Ah receptor.

2.0 SCOPE AND APPLICATION

Ah receptor agonists include polyhalogenated aromatic hydrocarbons (PHAHs), such as polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) which are persistent environmental contaminants found in all parts of the world. A number of these compounds cause a variety of adverse effects in laboratory studies on rats and mice (reviewed by Poland and Knutson 1982). These include hepatotoxicity, certain types of cancer, thymic atrophy and other immunotoxicities, a wasting syndrome, reproductive toxicities, terata and the induction of enzymes and porphyrins. A number of these toxicities have also been observed in wildlife in areas with elevated levels of PHAHs, particularly in fish-eating birds in the Great Lakes (reviewed by Gilbertson *et al.* 1991; Giesy *et al.* 1994a; 1994b).

Interest exists in assessing the risk posed by these PHAHs to fish and wildlife, which may also reflect the risk to humans. One aspect of risk assessment is the use of bioanalytical assays to detect and determine the toxicity of complex mixtures of these chemicals in extracts of environmental compartments such as soil, water and biota. Quantitative instrumental analysis of complex mixtures of these compounds is a difficult and expensive task. Furthermore, demonstrating the presence of one or many of these compounds in samples provides only limited information on their biological potency, particularly when present in a complex mixture with many potential interactions.

In order to develop a suitable bioassay, an understanding of the mechanism of action for the compounds is required. In the case of a number of PHAHs, considerable knowledge of the mechanism by which they cause their toxicities has been acquired. As previously mentioned, PHAHs are persistent agonists for the Ah receptor (Poland and Knutson 1982). Binding of agonist to receptor results in an activated receptor-ligand complex that translocates to the nucleus. Here it interacts with specific sequences on the DNA, termed dioxin-responsive elements (DREs;

also called XREs or AhREs), in order to alter gene transcription (reviewed by Whitlock 1990; Okey *et al.* 1994). A rapid and sensitive response that is under direct regulation of the Ah receptor is the induction of cytochrome P-450 1A isoenzymes (CYP1A1 and 1A2) and their associated ethoxyresorufin O-deethylase (EROD) activity (Nebert and Gonzalez 1987). Good correlations exist between the Ah receptor-binding affinity of persistent PHAHs and their EROD-inducing potency *in vitro*, and, dependent on the endpoint, their toxic potential *in vivo* (Safe 1986; 1990). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), in particular, binds tightly to the Ah receptor, and is a potent inducer of EROD activity and highly toxic. For the above reasons, the capacity of single compounds or complex environmental mixtures to induce EROD activity is considered to be a reasonable measure of their toxic potential. This mechanistic knowledge has been applied *in vivo* in biological monitoring of fish and birds, and *in vitro* in the use of bioassays for the screening of environmental extracts for Ah-active components. The H4IIE rat hepatoma cell bioassay (Tillitt *et al.* 1991), is widely used for this purpose. In this assay, EROD-inducing potencies (ED_{50} values) of single compounds and environmental samples are determined from complete dose-response curves and compared to that of TCDD in order to express the biological potency of the tested samples in TCDD-equivalents (TCDD-EQs). The bioassay integrates potential non-additive interactions among Ah receptor agonist and between Ah receptor agonists and other compounds by measuring a final receptor-mediated response (Giesy *et al.* 1994a).

For the same purpose as the currently used wild-type H4IIE bioassay (H4IIE-wt), a recombinant H4IIE cell line (H4IIE-luc) has been developed that exhibits Ah receptor-mediated luciferase expression (Aarts *et al.* 1995). This cell line has been stably transfected with a luciferase reporter gene under transcriptional control of several DREs from mouse (Aarts *et al.* 1993; Denison *et al.* 1993). These DRE sequences are highly conserved among species, unlike the Ah receptor which can exhibit greatly different ligand-binding properties among species. A preliminary report using luciferase-transfected mouse Hepa1c1c7 hepatoma cells indicated that these cells are more sensitive to Ah receptor agonists than the wild-type cells (Aarts *et al.* 1993; Sanderson *et al.*, 1996). It has been suggested that luciferase-transfected cell lines would have more favorable properties than their respective wild-types, such as greater selectivity, sensitivity and dynamic range. This has been postulated because the Ah receptor-mediated expression of luciferase, being foreign to the cell, is probably not affected by post-transcriptional and -translational events which influence CYP1A1 expression. Furthermore, the recombinant cells would not be dependent on a functional CYP1A1 gene or protein for responsiveness, although the Ah receptor-mediated pathway would still need to be present. Another theoretical consideration is that luciferase is assayed on the basis of light production for which extremely sensitive detectors exist; also, the turnover number or molecular activity of luciferase is so high that it allows the detection of very few molecules of the enzyme, relative to CYP1A1. Further, recombinant cells are readily amenable to further improvements in responsiveness by genetic engineering of the reporter gene construct.

The threshold dose (*i.e.*, detection limit) and ED_{50} (*i.e.*, effective dose to elicit 50% of the maximal response) for luciferase induction in H4IIE-luc cells were approximately 0.1 and 1.2 pg/well, respectively, as determined from 41 separate standard TCDD curves analyzed in 1997 (Figure 1). Coefficients of variation (standard deviation/mean x 100) for the assay were under 10% at all concentrations tested. For a sample size of 20 g tissue and a final extract volume of 0.25 ml, the H4IIE-luc assay will detect 1 part per trillion (ppt; pg/g, wet weight) TCDD-equivalents. With a sample size of 5 g tissue, 4 pg/g (wet weight) TCDD-equivalents will be detected.

3.0. SAFETY CONSIDERATIONS

TCDD and many related compounds have been found to be carcinogenic. In addition, the ethidium homodimer used in the cell viability assay is a powerful mutagen. Care should be taken to minimize exposure. According to institutional guidelines (refer to the Safety Manual for the Aquatic Toxicology Laboratory at Michigan State University), medium should be collected in a liquid trap for disposal as hazardous waste.

4.0. EQUIPMENT, MATERIALS, AND REAGENTS

4.1. Maintenance, preparation and use of H4IIE-luc cells

The assay uses a stably transfected cell line developed by Dr. Jac Aarts at Michigan State University (Sanderson et al., 1996). Briefly, rat hepatoma cells [American Type Culture Collection (ATCC) catalog #CRL 1548)] were stably-transfected with an inducible reporter plasmid, pGudLuc1.1. This plasmid contains the firefly luciferase gene under PHAH-inducible control of four DREs. Exposure of these cells to Ah receptor-active chemicals results in induction of luciferase activity in a time-, dose-, and AhR-dependent manner.

4.1.1. Maintain adherant cells in continuous culture in 100 mm tissue culture plates (Corning #25020-100, Cambridge, MA; 1-800-492-1110), 75 cm^2 flasks (Corning #25113-75) or any appropriate vessel at a maximum density of 80-90% confluence in 10% Full Medium (See **Media Preparation**). Incubator conditions are 37 C, 5% CO_2 humidified atmosphere.

4.1.2. Subculture cells 1:6 every week (depending upon density) maintaining a minimum cell density of 15-25%.

4.2. Instruments

4.2.1. Dynatech ML3000 Luminometer
(Dynatech Technical Support: (800) 336-4543; Chantilly, VA)

4.2.2. Cytofluor 2300/2350 Fluorescence Measurement System
(Millipore Technical Support: (800) 645-5476; Bedford, MA)

NOTE: *All users of the Dynatech ML3000 Luminometer and the Cytofluor 2300 must read and be familiar with the Operators Manual before using the instrument. A working knowledge of Microsoft Windows is also necessary. For both the luminometer and fluorescence measurement system, instrument use is recorded in a log book (located next to the instrument). For each use, the person, date, number of samples, type of samples, and results of proficiency standards are recorded. In addition, any abnormal operation of the instrument is recorded. Calibration is performed by analyzing a positive control or sample with known activity each day that the assay is run. If the positive control exceeds ± 20% of the on-going average (as determined by a comparison to a proficiency curve maintained in the instrument log book), the positive control will be rerun. If exceedance is confirmed with the second analysis, the manufacturer will be contacted as a corrective action so that the instrument can be recalibrated.*

4.2.3. Pipets

a. Eppendorf Repeat Pipetter (Brinkmann Instruments #22 26 000-6; Westbury, NY) with sterile, Biopur 12.5 ml combitips (Brinkmann Instruments #22 49 520-8) for dispensing cells into the 96-well plate and for changing media. Calibration checked weekly by weight of water check and the results are entered in calibration log book. Adjustments made by MSU Biochemistry Instrument Shop when accuracy exceeds manufacturer's specifications of ± 0.3%.

b. Rainin Pipetman Pipets (0.5 - 10 μl capacity, Rainin #P-10; 20 - 200 μl, Rainin #P-200 ; 100 - 1000 μl, Rainin #P-1000; Woburn, MA) for making sample dilutions, dosing cells, etc. Calibration checked weekly by weight of water check and the results are entered in calibration log book. Adjustments made by MSU Biochemistry Instrument Shop when accuracy exceeds manufacturer's specifications of ± 2.5% for P-10, 1.0 % for P-200, and 3.0% for P-1000.

c. Brinkmann Eppendorf Multichannel Pipetter 30-300 μl (Brinkmann Instruments #22 45 120-1) for washing cells and adding reagents for viability and Luclite reagents. Calibration checked weekly by weight of water check and the results are entered in calibration log book. Adjustments made by MSU

Biochemistry Instrument Shop when accuracy exceeds manufacturer's specifications of ± 1.5%.

d. Drummond Pipet-Aid Filler/Dispenser (Drummond #4-000-111-TC, available from Fisher #13-681-15D) for dispensing volumes greater than 1 ml, such as for changing media on cells.

***NOTE:** For all pipettors, except the Drummond Pipet-Aid, calibration test results are recorded weekly, along with the name of the person and any abnormal operation of the instrument.*

4.3. Supplies and Biochemicals

4.3.1. 96-Well ViewPlates™ (Packard Instruments #6005181; Meriden, CT); white 96-well plates, sterile, tissue culture treated, with lids and self-adhesive sticker for bottom of plates

Cost: $297/50 plates;
Ordering: (800) 856-0734
Technical support: (800) 323-1891

4.3.2. Cell viability assay reagents; sold either as a kit from Molecular Probes (#L-3224; Eugene, OR) or as individual components:

Calcein AM (Molecular Probes #C-3100);
MW = 994.87; made up as 4000x (2 mM) stock (50 μg/12.56 μl DMSO)

Ethidium homodimer I (Molecular Probes #E 1169)
MW = 857; made up as 2000x (1 mM) stock in DMSO

Ordering: (800) 438-2209
Technical support: (541) 465-8353

4.3.3. LucLite™ Kit (Packard Instruments # 6016911 - 1000 assay kit; Meriden, CT). Make fresh on same day as assay. Dissolve one bottle of lyophilized reagent with 10ml buffer (supplied) for every 133 assays (individual wells) to be analyzed.

Cost: $420/1333 assays using 75 μl/assay (or 1000 assays if using the manufacturer's suggested volume of 100 μl/assay. Preliminary studies showed equivalent responses at both 75 μl and 100 μl).
Ordering: (800) 856-0734
Technical support: (800) 323-1891

4.3.4. 10x Trypsin-EDTA solution (Sigma #T-4174, St. Louis, MO) for dissociation of cells from plates. From a 10x concentrated solution, a 1x working solution is prepared using sterile PBS as the diluent.

4.3.5. Dulbecco's phosphate-buffered saline (PBS) with Ca^{2+} and Mg^{2+}

First, make up 10 L of Ca^{2+}-free and Mg^{2+}- free Dulbecco's PBS:

2.0 g	KCl
2.0 g	KH_2PO_4
80.0 g	NaCl
11.5 g	Na_2HPO_4
10.0 L	H_2O[a]

[a]distilled/deionized or Nanopure biological grade

To each 1 L of PBS, add 0.1 g anhydrous $CaCl_2$ and 0.1 g $MgCl_2 \bullet 6H_20$ Keep at room temperature (good for at least 2 - 4 weeks).

4.4. Media Preparation

4.4.1. 10% Full Medium

Dulbecco's Modified Eagle's Medium (Sigma #D-2902) **without phenol red** (known to be estrogenic and with unknown influence on H4IIE cells), and sodium bicarbonate. Prepare as instructed by the manufacturer, adjust the pH to ~7.3, and then add :

10% fetal bovine serum (Hyclone defined FBS # SH30070.03;
Logan, UT; 1-800-492-5663)

4.4.2. 10% DCCFBS Medium

Prepared as for the full medium, except the fetal bovine serum is replaced with dextran/charcoal-stripped fetal bovine serum (available from Hyclone #SH30068.03).

5.0 METHOD, PROCEDURES, AND REQUIREMENTS

5.1. Sample Preparation

Two types of samples may be assayed: pure compounds and mixtures derived from environmental or tissue samples. For pure compounds, sample preparation consists of dissolving the material in an appropriate solvent. The solvent of choice is isooctane (because of its low toxicity and for direct comparison to TCDD, which is dissolved in isooctane). However, if the material is insoluble in isooctane, the following solvents can be tried:, ethanol, acetone, *p*-dioxane, acetonitrile, and methanol. A stock solution should be prepared at 5 mM for compounds of unknown activity and stored in amber glass vials at -20^{0}C. However, it may be necessary to test concentrations near the limit of solubility of the compound. Ideally, a volume of 1-2 ml at the highest possible concentration should be obtained for standards and samples. This volume is sufficient for splitting samples for instrumental analysis and for preparation of serial dilutions.

Samples derived from environmental matrices such as tissue, water, or sediment should be extracted and concentrated according to appropriate protocols (in accordance with SOP #211 - Extraction and Analysis of PCBs and Non-ortho Coplanar PCBs in Biological Matrices). The volume of sample should be recorded before re-dissolution in the assay solvent so that a dilution or concentration factor can be calculated. To prepare dilutions, 0.5 ml of isooctane (assuming that this is the solvent of choice) is added to five appropriately labeled 2 ml amber GC glass vials with teflon lined caps. Then 0.5 ml of the 1x extract (original extract that is undiluted) is added to the first vial (labeled 0.5x), the lid enclosure tightened, and then the sample is vortexed well. Then 0.5 ml of the 0.5x stock is added to the next vial (labeled 0.25x), the lid enclosure tightened, and then the sample is vortexed well. These steps are repeated until all dilutions are prepared. The sample dilutions will be 100, 50, 25, 12.5, 6, and 3% of the extract. If less than 1 ml of original 1x extract is available, the above mentioned volumes should be proportionately reduced. After dissolution in the assay solvent, the samples should be stored at -20^{0}C.

5.2. Standards Preparation

A large range of standards should be prepared to deliver a final concentration of **TCDD** between 0.03 - 100 pg/well in a volume of 1.25 µl (*i.e.*, make 200x stock solutions). Generally, 6 concentrations will achieve a full dose response curve (final = 0.1, 0.3, 1, 3, 10, 30 pg/well) for TCDD. Ideally, standards should be dissolved in the same solvent as the samples, but this is not always possible. In this case, be sure to conduct assays with both solvent controls and compare them to blanks (see Dosing Cells). A stock of TCDD in isooctane is maintained in Room 181, URCF, in a 1 liter volumetric flask. The concentration is written on the flask and is tested for purity and accuracy by GC/MS and GC/ECD by comparison to commercially available certified standards. To make dilutions, prepare the following stock concentrations (assuming a stock concentration of 10000 ng/ml):

final mass. in wells (pg/1.25 ul)	stock concentration (ng/ml)
12500	10000
10000	8000
1000	800
100	80
The following six concentrations are used for the bioassay:	
30	24
10	8
3	2.4
1	0.8
0.3	0.24
0.1	0.08

***NOTE**: Adherence to these procedures will insure consistent response of cells. Timing and cell density are critical. An assay may be completed in 5 days.*

5.3. Plating Cells (Day 1)

Prior to confluence, write down the passage number of the cells, aspirate media from cell culture dishes, wash the cells with sterile PBS without Ca^{2+} and Mg^{2+}, and trypsinize the cells with 3 ml of 1x sterile trysin-EDTA solution for 5 minutes at 37^{0}C. Add trysinized cell suspension to 27 ml of "10% Full Medium" and determine the number of cells/ml with a hemacytometer (for more information on cell counting, refer to "Cell Culture: A Manual of Basic Techniques", by Dr. Ian Freshney, 1996) . Dilute the cell solution to a concentration of 60,000 cells/ml with media. Add 0.25 ml of cell suspension to each well (15,000 cells) of a 96-well ViewPlate™ using an Eppendorf™ repeat pipettor. Care must be taken that the cell suspension is uniform each time that the pipettor is refilled. This is done by gently inverting the tube or bottle of cell suspension end-over-end several times immediately prior to refilling the pipettor. If the outer 36 wells are not being used for the experiment (recommended), fill them with either sterile media or PBS to maintain humidity consistently across the plate. Use of the outer wells is not recommended because of an edge effect caused by inconsistent growth of cells in these wells.

***NOTE**: cell number per well is one of the largest contributors to variation in the data - so take plenty of time to do this step properly. Cell passage number should be noted to monitor long-term changes in the responsiveness and growth characteristics of the cells.*

5.4. Dosing Cells (Day 2)

Dose cells 24 hours after plating and continue exposures for 3 days. First, examine cells to ensure consistent plating. Aspirate media (attach p-10 tips to the suction line to minimize cell scraping), and replace with 0.25 ml of DCCFBS media that is prewarmed to 37^0C.

***NOTE**: the use of cold media should be avoided because it may "shock" or stress the cells and it may cause precipitation of test agents that have poor water solubility).*

A typical plate design is shown in Figure 2. Use at least three replicates per treatment. Note that with TCDD, only two replicates are used for a typical plate design. Each control and TCDD concentration are averaged for all plates within a given experiment. Use a negative control with no treatment (blank) and a solvent control treated with pure solvent only. Use at least five concentrations of each compound or extracts tested. Prepare 200x chemical stocks in the appropriate solvent (see Sample Preparation). In general, it is best to dose cells directly in the well; however, for water miscible solvents, the test agent can be added to a sterile glass vial containing 2 ml of media, mixed, and then added to each well. Each well shall receive 1.25 µl of sample or standard or solvent control as noted in the plate design below.

Earlier versions of this assay determined that cross-talk can occur when high activity samples are directly adjacent to low activity samples. Therefore, as a corrective action, the plate design shown in Figure 2 was developed. Note that two blank columns border samples, so that there is no cross-talking between samples and TCDD and between samples and controls.

Row/ Col	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		0.1 pg TCDD	0.1 pg TCDD			C1 Conc. 1	C1 Conc. 1	C1 Conc. 1			solvent control	
C		0.3 pg TCDD	0.3 pg TCDD			C1 Conc. 2	C1 Conc. 2	C1 Conc. 2			solvent control	
D		1 pg TCDD	1 pg TCDD			C1 Conc. 3	C1 Conc. 3	C1 Conc. 3			solvent control	
E		3 pg TCDD	3 pg TCDD			C1 Conc. 4	C1 Conc. 4	C1 Conc. 4			blank	
F		10 pg TCDD	10 pg TCDD			C1 Conc. 5	C1 Conc. 5	C1 Conc. 5			blank	
G		30 pg TCDD	30 pg TCDD			C1 Conc. 6	C1 Conc. 6	C1 Conc. 6			blank	
H												

Figure 2. A typical plate design for determination of Ah receptor agonist activity in H4IIE-luc cells. Note that blanks, solvent controls, and a standard curve for TCDD can be analyzed on the same plate with one test sample (labeled C1, at six different concentrations). Two blank columns are recommended between samples and either TCDD or controls to prevent any possible cross-contamination.

5.5. Conducting the Bioassay (Day 5)

5.5.1. Preparation steps (prior to assay)

a. Inspect plates visually with and without microscope - check degree of confluence, homogeneity from well-to-well, and any signs of cytotoxicity or altered morphology

b. Set up cytofluor for viability assay:

set 1: excitation = B emission = B sensitivity = 3
set 2: excitation = C emission = E sensitivity = 4

***NOTE**: sensitivity can be adjusted if values are too high (9999) or low (not different from blanks)*

c. Preparation of viability assay reagent (refer to Supplies and Biochemicals Section):

Each plate will need 1.8 ml or 3 ml, depending on whether 36 or 60 wells are used per plate, respectively plus a little extra (2 ml). Dilute the appropriate amounts of calcein and ethidium with the appropriate volume of media without FBS as shown below:

Number of plates	Volume of viability assay reagent needed [total volume (ml); calcein stock (µl); Ethidium stock (µl)]	
	using 36 wells	using 60 wells
1	3.8 ml; 0.95 µl; 1.9 µl	5 ml; 1.25 µl; 2.5 µl
2	5.6 ml; 1.4 µl; 2.8 µl	8 ml; 2 µl; 4 µl
3	7.4 ml; 1.85 µl; 3.7 µl	11 ml; 2.75 µl; 5.5 µl
4	9.2 ml; 2.3 µl; 4.6 µl	14 ml; 3.5 µl; 7 µl
5	11 ml; 2.75 µl; 5.5 µl	17 ml; 4.25 µl; 8.5 µl
6	12.8 ml; 3.2 µl; 6.4 µl	20 ml; 5 µl; 10 µl
7	14.6 ml; 3.65 µl; 7.3 µl	23 ml; 5.75 µl; 11.5 µl
8	16.4 ml; 4.1 µl; 8.2 µl	26 ml; 6.5 µl; 13 µl

***CAUTION**: ethidium homodimer is a powerful mutagen - handle with care and throw contaminated tips, etc., into biohazard bags*

d. Set up luminometer:
Mode = Cycle;
Data = All;
Gain = High;
Cycle = 1-3;
Pause = 2 sec;
Mix = Off;
Temp. = 30^{0}C;
A/D reads = 20 (number of times that each well is analyzed per cycle)

e. Set up vacuum aspirator (attach p-10 tips to each suction line to minimize scraping surface area and only use the plate washer to aspirate and **not** to dispense PBS)

f. Prepare LucLite substrate solution and luciferase positive control (must be used on the same day as prepared)

1) Reconstitute lyophilized substrate solution by adding 10 ml of Assay buffer solution A (provided with kit) to one vial of lyophilized substrate (each vial is enough for 133 assays if 75 µl is used). Agitate gently until a homogeneous solution is formed (a slight turbidity is acceptable). Equilibrate to room temperature before use.

***NOTE**: if more than one vial is reconstituted, combine and mix to prevent any variation from the substrate between plates.*

2) Reconstitute the lyophilized luciferase positive control with 200 µl of distilled (or nanopure water). Each vial contains sufficient luciferase for 20 controls.

5.5.2. Cell Viability Assay Procedure (process one plate at a time)

a. remove plate from incubator and aspirate media, then rinse 1 time with PBS

b. Add 50 µl of PBS with Ca^{2+} and Mg^{2+} to all wells using a 8-channel pipet

c. Add 50 µl of viability assay reagent to all wells using a 8-channel pipet

d. Incubate at room temperature for 10 minutes and then scan plate in the Cytofluor instrument

e. Export/print data (check that values are appropriate, otherwise adjust sensitivity and rescan). Password protect data files with a project-specific password. Data analysis are discussed in Section E along with an example raw data file and a Microsoft Excel spreadsheet version of a sample data analysis.

f. Aspirate viability reagent/PBS, and rinse 1 time with PBS using vacuum aspirator

g. Seal the bottom of the ViewPlates with self-adhesive TopSeal (or tape very well). This increases the signal detection in the luminometer.

h. Add 75 µl of PBS with Ca^{2+} and Mg^{2+} (or media without serum or phenol red) to all wells using an 8-channel pipet

i. Working under SUBDUED light conditions, add 75 µl/well of reconstituted LucLite substrate solution, cover with foil, and agitate gently.

j. Wait twenty minutes before counting the plate to allow full signal generation.

k. Measure luminescence on the Dynatech ML3000 luminometer

l. Save, print, and copy data to disk. Password protect data files with a project-specific password. Data analysis are discussed in Section E along with an example raw data file and a Microsoft Excel spreadsheet version of a sample data analysis.

5.6. Protein Determination

Protein determination is readily accomplished in the same plates with same cell lysates after measuring luminescence. A fluorescamine-based protein assay (Sanderson *et al.*, 1996) or Micro-BCA Assay (available from Pierce (800) 874-3723) can be used. Degree of confluency of wells was verified microscopically, and in most cases this made normalization to protein unnecessary; therefore, luciferase activity is reported as either relative light units (RLU) or percent of solvent control.

5.7. Data Analysis

5.7.1. Viability Data

Average the three measurements for calcein AM and ethidium. Divide the average calcein AM fluorescence for each sample by its ethidium homodimer fluorescence to obtain a live to dead ratio. Graph the average calcein AM fluorescence and standard deviation for the negative control, solvent control, and each concentration tested. Examine the calcein AM data visually. If the blank has greater viability than the other treatments, the solvent may be toxic to the cells. If viability decreases with increasing concentration of the test substance, the test substance may be toxic to the cells. In either of these cases, the luciferase data must be regarded with great suspicion. If the solvent is toxic, try a different solvent or a lower concentration of solvent. If the test substance is toxic, try extracting the toxic component (*e.g.*, removing sulfur compounds from sediment extracts), or conclude that cytotoxicity is likely to preclude any dioxin-like effects.

5.7.2. Luciferase Data

Calculate averages and standard deviations for each treatment. Graph the change in response and its standard deviation against concentration. Relative potencies should be calculated through conversion of the data to probit values or any other appropriate transformation that linearizes the sigmoidal dose-response curve. For a complete description and decision tree for data analysis methods, refer to the Standard Operating Procedures entitled, "Estimation of Relative Potencies Based on In Vitro Bioassay Results". To convert data to probit values, the concentration producing maximal induction is set at 100% and the responses of all other concentrations are converted to a percentage of this maximal level. A lookup table function can be used in a spreadsheet program such as Excel or Lotus 1,2,3 to convert percentages to probit values (contact the Aquatic Toxicology Laboratory for a copy of this table on disk).

6.0. RECORDS, DOCUMENTATION, AND QC REQUIREMENTS

6.1 Records and Documentation

The primary analyst shall document any anomalies and/or deviation from the specified method in a bound, serially numbered, laboratory notebook with tear-out carbon copies. All electronic files and hardcopies will be kept at the Aquatic Toxicology Laboratory at Michigan State University and a duplicate copy will be kept in the Archive Room of Dr. John Giesy (Dept. of Zoology, Michigan State University). This information will also be recorded in the data package and listed in the Case Narrative Form (in accordance with SOP 802). The primary analyst will sign and date any forms as the analyst.

The technical reviewer will record any problems noted during the technical review. The technical reviewer will return the items to the analyst for corrections prior to inclusion into the data package. The technical reviewer will sign and date all forms as the reviewer.

6.2 QC Requirements and Data Quality Objectives

The threshold dose and EC_{50} for luciferase induction in H4IIE-luc cells were approximately 0.1 and 1.2 pg/well, respectively, as determined from 41 separate standard TCDD curves analyzed in 1997 (Figure 1). Coefficients of variation (standard deviation/mean x 100) for the assay were under 10% at all concentrations tested for any single day of experiments. A proficiency curve is maintained for the EC_{50} and threshold doses for TCDD standard curves (every plate has a standard curve). If for a particular plate, the EC_{50} or threshold for the TCDD standard exceeds $\pm$ 20 % of the proficiency curve, then the sample on the plate in question will be reanalyzed. For a sample size of 20 g tissue and a final extract volume of 0.25 ml, the H4IIE-luc assay will detect 1 part per trillion (ppt; pg/g, wet weight) TCDD-equivalents. With a sample size of 5 g tissue, 4 pg/g (wet weight) TCDD-equivalents will be detected.

7.0. RESPONSIBILITIES

The primary analyst will complete the analysis as specified in this SOP and provide documentation of raw data and any anomalies and provide data to the data analyst who will perform data calculations in accordance with SOP 202.

The technical reviewer will determine if data quality objectives were met, notify the analyst if any problems were found.

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Standard Operating Procedure

MCF-7 ERE-Luciferase Cell Bioassay For The Detection Of Estogen Receptor Agonists And Antagonists

Version 2.0 July, 1997

Alan Blankenship, Dan Villeneuve,
Vincent J. Kramer, and John P. Giesy

Supported through:

National Food Safety and Toxicology Center,
Institute for Environmental Toxicology,
Department of Fisheries and Wildlife, and the Department of Zoology

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I. Introduction

The luciferase induction assay is an *in vitro* technique for the identification of estrogenic and anti-estrogenic compounds. The technique uses human breast carcinoma MCF-7 cells stably transfected with an estrogen receptor-controlled luciferase reporter gene construct (MCF-7 ERE-Luc; obtained from Dr. Michel Pons, Institut National de la Sante et de la Recherche Medicale, 60 Rue de Navacelles, 34090 Montpelier, Phone: 67.04.37.00; Pons *et al.*, 1990). These cells express firefly luciferase in response to estrogen agonists. Luciferase activity is measured conveniently and with high sensitivity as light emission using a plate-scanning luminometer. Estrogen antagonists can be detected by measuring a decrease in luciferase production (decrease in light) after administration of the test compound in the presence or absence of 17-β-estradiol (E_2). The technique has been modified from the published procedure to run in 96-well plates, allowing the analysis of a large number of samples. Luciferase induction potential is assessed by comparison of the response to that of standard compounds: E_2 for agonists and ICI 182,780 for antagonists.

II. Materials

A. Preparation and use of MCF-7 ERE-Luc cells

The assay uses a stably transfected cell line donated by Dr. Michel Pons. Briefly, human breast carcinoma MCF-7 cells [American Type Culture Collection (ATCC) catalog #HTB-22) were stably-transfected with a luciferase reporter gene plasmid consisting of the X. laevis vitellogenin promoter region containing 4 estrogen responsive elements and the herpes simplex thymidine kinase promoter upstream of the firefly luciferase reporter gene (Pons, *et al.* 1990). The cells produce luciferase in response to treatment with E_2.

1. Maintain adherant cells in continuous culture in 100 mm tissue culture plates (Corning #25020-100, Cambridge, MA; 1-800-492-1110), 75 cm^2 flasks (Corning #25113-75) or any appropriate vessel at a maximum density of 80-90% confluence in 10% Full Medium (See **Media Preparation**). Incubator conditions are 37 C, 5% CO_2 humidified atmosphere.

NOTE: MCF-7 cells are a transformed human cell line that must be handled in a laminar flow hood, preferably NSF Type II B2 containment. All items contacting the cells should be considered infectious material and sterilized before final disposal. Personnel should follow all institutional safety precautions including safety glasses, gloves and lab coat.

2. Subculture cells 1:2 every 3 to 4 days (depending upon density) maintaining a minimum cell density of 20-30%.

B. Instruments

1. Dynatech ML3000 Luminometer
(Dynatech Technical Support: (800) 336-4543; Chantilly, VA)

2. Cytofluor 2300/2350 Fluorescence Measurement System
(Millipore Technical Support: (800) 645-5476; Bedford, MA)

NOTE: All users of the Dynatech ML3000 Luminometer and the Cytofluor 2300 must read and be familiar with the Operators Manual before using the instrument. A working knowledge of Microsoft Windows is also necessary.

C. Supplies and Biochemicals

1. 96-Well ViewPlates™ (Packard Instruments #6005181; Meriden, CT); white 96-well plates, sterile, tissue culture treated, with lids and self-adhesivesticker for bottom of plates
Cost: $297/50 plates;
Ordering: (800) 856-0734
Technical support: (800) 323-1891

2. Cell viability assay reagents; sold either as a kit from Molecular Probes (#L-3224; Eugene, OR) or as individual components:

Calcein AM (Molecular Probes #C-3100);
MW = 994.87; made up as 4000x (2 mM) stock
(50 µg/12.56 µl DMSO)

Ethidium homodimer I (Molecular Probes #E 1169)
MW = 857; made up as 2000x (1 mM) stock in DMSO

Ordering: (800) 438-2209
Technical support: (541) 465-8353

3. LucLite™ Kit (Packard Instruments # 6016911 - 1000 assay kit; Meriden, CT) Make fresh on same day as assay. Dissolve one bottle of lyophilized reagent with 10 ml buffer (supplied) for every 133 assays (individual wells) to be analyzed.

Cost: $420/1333 assays using 75 µl/assay (or 1000 assays if using the manufacturer's suggested volume of 100 µl/assay. Preliminary studies showed equivalent responses at both 75 µl and 100 µl).
Ordering: (800) 856-0734
Technical support: (800) 323-1891

4. Dulbecco's phosphate-buffered saline (PBS) with Ca^{2+} and Mg^{2+}

First, make up 10 L of Ca^{2+}-free and Mg^{2+}- free Dulbecco's PBS:

2.0 g	KCl
2.0 g	KH_2PO_4
80.0 g	NaCl
11.5 g	Na_2HPO_4
10.0 L	H_2O[a]

[a] distilled/deionized or Nanopure biological grade

1 L of PBS, add 0.1 g anhydrous $CaCl_2$ and 0.1 g $MgCl_2 \bullet 6H_20$
Keep at room temperature (good for at least 2 - 4 weeks).

D. Media Preparation

1. 10% Full Medium

Dulbecco's Modified Eagle's Medium with Ham's F-12 Nutrient Mixure (1:1 mix; Sigma #D-2906) **without phenol red**, sodium bicarbonate, and with 15 mM HEPES buffer and L-glutamine. Prepare as instructed by the manufacturer, adjust the pH to ~7.3, and then add :

10% fetal bovine serum (Hyclone defined FBS # SH30070.03; Logan, UT; 1-800-492-5663)
1 mM sodium pyruvate
1 mg/ml bovine insulin (Sigma #I-1882)

2. 10% DCCFBS Medium

Prepared as for the full medium, except the fetal bovine serum is replaced with dextran/charcoal-stripped fetal bovine serum (available from Hyclone #SH30068.03).

III. Hazards and Precautions

E_2 and many other estrogenic compounds have been found to be carcinogenic. In addition, the ethidium homodimer used in the cell viability assay is a powerful mutagen. Care should be taken to minimize exposure. According to institutional guidelines, medium should be collected in a liquid trap for disposal as hazardous waste.

IV. Sample and Standards Preparation

A. *Sample Preparation*

Two types of samples may be assayed: pure compounds and mixtures derived from environmental or tissue samples. For pure compounds, sample preparation consists of dissolving the material in an appropriate solvent. The solvent of choice is ethanol (because of its water miscibility and low toxicity). However, if the material is insoluble in ethanol, the following solvents can be tried: isooctane, acetone, *p*-dioxane, acetonitrile, and methanol. A stock solution should be prepared at 5 mM for compounds of unknown activity and stored in amber glass vials at -20^{0}C. However, it may be necessary to test concentrations near the limit of solubility of the compound.

Samples derived from environmental matrices such as tissue, water, or sediment should be extracted and concentrated according to appropriate protocols. The volume of sample should be recorded before re-dissolution in the assay solvent so that a dilution or concentration factor can be calculated. After dissolution in the assay solvent, the sample should be stored at -20^{0}C.

B. *Standards Preparation*

A large range of standards should be prepared to deliver a final concentration of **17-β-estradiol (E_2)** between 0.15 - 500 pM (200x stocks in ethanol between 30 pM - 100 nM). Generally, 8 concentrations will achieve a full dose response curve (final = 0.15, 0.5, 1.5, 5, 15, 50, 150, 500 pM) for E_2. Ideally, standards should be dissolved in the same solvent as the samples, but this is not always possible. In this case, be sure to conduct assays with both solvent controls and compare them to blanks (see Dosing Cells).

ICI 182,370 is a potent anti-estrogen in this cell line (Wakeling and Bowler, 1992). Construct an 8 point concentration range to cover the entire response range of these cells as follows: 10 μM, 1 μM, 100 nM, 10 nM, 1 nM, 0.1 nM, 0.01 nM, 0.001 nM.

V. Procedure

> **NOTE**: Adherence to these procedures will insure consistent response of cells. Timing and cell density are critical. An assay may be completed in a 5 day week.

A. Plating Cells (Day 1)

Prior to confluence, trypsinize the cells and determine the number of cells/ml. Dilute the cell solution to a concentration of 60,000 cells/ml with media. Add 0.25 ml of cell suspension to each well (15,000 cells) of a 96-well ViewPlate™ using an Eppendorf™ repeat pipettor. Care must be taken that the cell suspension is uniform each time that the pipettor is refilled. This is done by gently inverting the tube or bottle of cell suspension end-over-end several times immediately prior to refilling the pipettor. If the outer 36 wells are not being used for the experiment (recommended), fill them with either sterile media or PBS to maintain humidity consistently across the plate. Use of the outer wells is not recommended because of an edge effect caused by inconsistent growth of cells in these wells.

NOTE: cell number per well is one of the largest contributors to variation in the data - so take plenty of time to do this step properly.

B. Dosing Cells (Day 2)

Dose the cells 24 hours after plating (exposures continue for 3 days). First, examine the cells to ensure consistent plating. Aspirate the media (I attach p-10 pipet tips to the suction line to minimize cell scraping), and replace with 0.25 ml of 10% DCCFBS media that has been prewarmed to 37^0C.

NOTE: the use of cold media should be avoided because it may "shock" or stress the cells and it may cause precipitation of test agents that have poor water solubility).

A typical plate design is shown in Figure 1. Use at least three replicates per treatment. Use a negative control with no treatment (blank) and a solvent control treated with pure solvent only. Use at least five concentrations of each compound or extracts tested. Prepare 200x chemical stocks in the appropriate solvent (see Sample Preparation). In general, it is best to dose the cells directly in the well; however, for water miscible solvents, the test agent can be added to a sterile glass vial containing 2 ml of media, mixed, and then added to each well.

Row/ Col	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		0.5 pM E_2	0.5 pM E_2	0.5 pM E_2	C1 Conc. 1	C1 Conc. 1	C1 Conc. 1	C2 Conc. 1	C2 Conc. 1	C2 Conc. 1	solvent control	
C		1.5 pM E_2	1.5 pM E_2	1.5 pM E_2	C1 Conc. 2	C1 Conc. 2	C1 Conc. 2	C2 Conc. 2	C2 Conc. 2	C2 Conc. 2	solvent control	
D		5 pM E_2	5 pM E_2	5 pM E_2	C1 Conc. 3	C1 Conc. 3	C1 Conc. 3	C2 Conc. 3	C2 Conc. 3	C2 Conc. 3	solvent control	
E		15 pM E_2	15 pM E_2	15 pM E_2	C1 Conc. 4	C1 Conc. 4	C1 Conc. 4	C2 Conc. 4	C2 Conc. 4	C2 Conc. 4	blank	
F		50 pM E_2	50 pM E_2	50 pM E_2	C1 Conc.5	C1 Conc.5	C1 Conc.5	C2 Conc.5	C2 Conc.5	C2 Conc. 5	blank	
G		150 pM E_2	150 pM E_2	150 pM E_2	C1 Conc. 6	C1 Conc. 6	C1 Conc. 6	C2 Conc. 6	C2 Conc. 6	C2 Conc. 6	blank	
H												

Figure 1. A typical plate design for determination of estrogen agonist activity in MCF-7 ERE-Luc cells. Note that blanks, solvent controls, and a standard curve for E_2 can be analyzed on the same plate with two test samples (labeled C1 and C2, both at six different concentrations).

C. Conducting the Bioassay (Day 5)

1. Preparation steps (prior to assay)

a. Inspect plates visually with and without microscope - check degree of confluence, homogeneity from well-to-well, and any signs of cytotoxicity or altered morphology

b. Set up cytofluor for viability assay:
set 1: excitation = B emission = B sensitivity = 3
set 2: excitation = C emission = E sensitivity = 4

NOTE: sensitivities can be adjusted if values are too high (9999) or too low (not different from blanks)

c. Prepare viability assay reagent:

CAUTION: ethidium homodimer is a powerful mutagen - handle with care and throw contaminated tips, etc., into biohazard bags

Each plate will need 3 ml or 5 ml, depending on whether 60 or 96 wells are used per plate, respectively plus a little extra (2 ml)
Dilute the appropriate amounts of calcein and ethidium with the appropriate volume of media without FBS as shown below:

Number of plates	Volume of viability assay reagent needed [total volume (ml); calcein stock (μl); Ethidium stock (μl)]	
	using 60 wells	using 96 wells
1	5 ml; 1.25 μl; 2.5 μl	7 ml; 1.75 μl; 3.5 μl
2	8 ml; 2 μl; 4 μl	12 ml; 3 μl; 6 μl
3	11 ml; 2.75 μl; 5.5 μl	17 ml; 4.25 μl; 8.5 μl
4	14 ml; 3.5 μl; 7 μl	22 ml; 5.5 μl; 11 μl
5	17 ml; 4.25 μl; 8.5 μl	27 ml; 6.75 μl; 13.5 μl
6	20 ml; 5 μl; 10 μl	32 ml; 8 μl; 16 μl
7	23 ml; 5.75 μl; 11.5 μl	37 ml; 9.25 μl; 18.5 μl
8	26 ml; 6.5 μl; 13 μl	42 ml; 10.5 μl; 21 μl

d. Set up luminometer:

Mode = Cycle;
Data = All;
Gain = High;
Cycle = 1-3;
Pause = 2 sec;
Mix = Off;
Temp. = 30^{0}C;
A/D reads = 20 (number of times that each well is analyzed per cycle)

e. Set up vacuum aspirator (attach p-10 tips to each suction line to minimize scraping surface area and only use the plate washer to aspirate and **not** to dispense PBS)

f. Prepare LucLite substrate solution and luciferase positive control (must be used on the same day as prepared)

1) Reconstitute lyophilized substrate solution by adding 10 ml of Assay buffer solution A (provided with kit) to one vial of lyophilized substrate (each vial is enough for 133 assays if 75 μl is used). Agitate gently until a homogeneous solution is formed (a slight turbidity is acceptable). Equilibrate to room temperature before use.

   NOTE: if more than one vial is reconstituted, combine and mix to prevent any variation from the substrate between plates.

2) Reconstitute the lyophilized luciferase positive control with 200 μl of distilled (or nanopure water). Each vial contains sufficient luciferase for 20 controls.

2. Cell Viability Assay Procedure (process one plate at a time)

a. remove plate from incubator and aspirate media, then rinse 1 time with PBS

b. Add 50 µl of PBS with Ca^{2+} and Mg^{2+} to all wells using a 8-channel pipet

c. Add 50 µl of viability assay reagent to all wells using a 8-channel pipet

d. Incubate at room temperature for 10 minutes and then scan plate in the Cytofluor instrument

e. Export/ print data (check that values are appropriate, otherwise adjust sensitivity and rescan)

f. Aspirate viability reagent/PBS, and rinse 1 time with PBS using vacuum
aspirator

g. Seal the bottom of the ViewPlates with self-adhesive TopSeal (or tape very well). This increases the signal detection in the luminometer.

h. Add 75 µl of PBS with Ca^{2+} and Mg^{2+} (or media without serum or phenol red) to all wells using an 8-channel pipet

i. Working under SUBDUED light conditions, add 75 µl/well of reconstituted substrate solution and agitate gently.

j. Wait five minutes before counting the plate to allow full signal generation.

k. Measure luminescence on the Dynatech ML3000 luminometer

l. Save, print, and copy data to disk

D. Protein Determination

Protein determination is readily accomplished in the same plates with same cell lysates after measuring luminescence. A fluorescamine-based protein assay (Sanderson *et al.*, 1996) or Micro-BCA Assay (available from Pierce (800) 874-3723) can be used. It is our experience, however, that normalizing to protein concentration prevents proper analysis of the dose-response luminescent data because estrogen-mediated luciferase induction and estrogen-induced protein synthesis cocorrelate together. Degree of confluency of wells was verified microscopically, which was found to make normalization to protein unnecessary; therefore, luciferase activity is reported as either relative light units (RLU) or percent of solvent control.

E. Data Analysis

Viability Data - Average the three measurements for and ethidium. Divide the average ethidium homodimer fluorescence for each sample by its calcein AM fluorescence to obtain a dead to live ratio. Graph the average calcein AM fluorescence and standard deviation for the negative control, solvent control, and each concentration tested. Examine the calcein AM data visually. If the blank has greater viability than the other treatments, the solvent may be toxic to the cells. If viability decreases with increasing concentration of the test substance, the test substance may be toxic to the cells. In either of these cases, the luciferase data must be regarded with great suspicion. If the solvent is toxic, try a different solvent or a lower concentration of solvent. If the test substance is toxic, try extracting the toxic component (*e.g.*, removing sulfur compounds from sediment extracts), or conclude that cytotoxicity is likely to preclude any estrogenic/antiestrogenic effects.

Luciferase Data - Calculate averages and standard deviations for each treatment. Graph the change in response and its standard deviation against concentration. Relative potencies should be calculated through conversion of the data to probit values or any other appropriate transformation that linearizes the sigmoidal dose-response curve. To convert data to probit values, the concentration producing maximal induction is set at 100% and the responses of all other concentrations are converted to a percentage of this maximal level. A lookup table function can be used in a spreadsheet program such as Excel or Lotus 1,2,3 to convert percentages to probit values (contact the Aquatic Toxicology Laboratory for a copy of this table on disk).

Alternatively, the dose-response data may be transformed in the same way as receptor-binding data to produce analogies of Woolf and Scatchard plots. Due to the limitations of the underlying assumptions of these analyses, care should be taken to include only data points that are on the linear rising part of the dose-response curves, when plotted on a log-linear scale. - ***At low concentrations of ligand the assumption that free ligand concentration is equal to total concetration does not hold. At high concentrations of ligand, the response starts to level off and decrease (receptor saturation is assumed). The ideal range is probably from the ED_{20}-ED_{80}.*** - The ED_{50} and maximum response ($EROD_{max}$) values generated using Scatchard/Woolf plots are always compared with the visually determined values for confirmation. It is not necessary to have the complete dose-response curve in order to determine ED_{50} and $EROD_{max}$ values, although the determinations become less reliable. Do not accept ED_{50} values which are higher than the highest tested dose. If a full dose-response curve cannot be attained, increase the number of concentrations on the linear rising part of the curve in order to have sufficient data points to perform a reliable Scatchard/Woolf analysis. A preferred approach is to concentrate the sample.

VII. References

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