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The present thesis is a partial fulfilment of the requirements for an Master of Science Degree in Veterinary Medicine for International Students at the Swedish University of Agricultural Sciences (SLU), in the field of Toxicology.

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To my parents and sister

“All meaningful and lasting change starts first in your imagination and then works its way out. Imagination is more important than knowledge.”

-------Albert Einstein

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ABSTRACT

Polyhalogenated organic pollutants are xenobiotics, which are believed to cause considerable environmental pollution and human health problems as a result of their persistence, toxicity, and transformation into hazardous metabolites. These chemicals are persistent and are biomagnificated in the ecosystem. Amphibians (frogs) are considered as reliable indicators of environmental health because they form an important link in the food chain between insects and vertebrates. Furthermore, they are inhabitants of both aquatic and terrestrial environments and occupy different positions in the food web at different stages of their life cycle. The different stages in the life cycle of frogs are tadpole, juvenile and adult stages. The tadpole is transformed to a juvenile frog by metamorphosis. Disruption of this life cycle by xenobiotics can have disastrous effects on the amphibian populations. Polyhalogenated organic compounds have been known to cause endocrine disruption, effects on gene expression and induction of cytochrome P 450 enzymes in frogs. The present study was conducted to compare the tissue disposition of a polybrominated compound, 2, 2', 4, 4', 5-pentabromodiphenyl ether (BDE 99) in tadpole and juvenile stages of *Xenopus tropicalis* (West African clawed frog). The study was performed using whole-body autoradiography and scintillation counting at different time intervals after dietary exposures of ^{14}C-BDE 99 at stage 54 (tadpoles) and stage 66 (juvenile frogs). From the results of the study it was concluded that there was a significant difference between the uptake and retention of the chemical between tadpoles and frogs. Juvenile frogs had higher uptake and retention of the radio labelled chemical than tadpoles. The substance was retained in adipose tissue over 64 days in both stages. However, the localisation of substance was similar in both stages.

Key words: 2, 2', 4, 4', 5-pentabromodiphenyl ether; BDE 99; PBDE; tissue disposition; frogs; *Xenopus tropicalis;* polyhalogenated organic pollutants

BACKGROUND

CHAPTER 1: POLYHALOGENATED ORGANIC POLLUTANTS

The rapid growth in human population and urbanization has caused concern that pollution may reduce biodiversity in ecosystems. Man-made chemicals such as pesticides, refrigerants, fire retardants and paints cause considerable environmental pollution and human health problems as a result of their persistence, toxicity, and transformation into hazardous metabolites. Many xenobiotics (chemical substances that are foreign to the biological system), introduced for industrial use, are halogenated, and halogenation is often implicated as a reason for persistence (Neilson *et al*., 1985).

Halogens (from the Greek hals, "salt," and gennan, "to form or generate") are the salt forming elements from the Group VIIA of the modern periodic table and include elements Fluorine, Chlorine, Bromine and Iodine. Therefore, polyhalogenated organic pollutants include Polyfluorinated, Polychlorinated, Polybrominated and Polyiodinated compounds.

Polyfluorinated Organic Pollutants:

Fluorine is one of the most reactive elements in ionic form and one of the most stable in bound form. Hence, the fully fluorinated hydrocarbons are very stable and the stability of these chemicals thus renders them practically non-biodegradable and thus, very persistent in the environment (Key *et al*., 1997, 1998; Prescher *et al*., 1985). Taves (1968) first reported the presence of organic fluorine in humans and the organic form was originally suggested to be perfluorooctanoate (PFOA) or, possibly, perfluorooctane sulfonate (PFOS) (Taves *et al*., 1976).

PFOS and PFOA are found in industrial and consumer applications ranging from stain resistant coatings for clothing fabrics, leather, upholstery, and carpets, to oil-resistant coatings for paper products approved for food contact, electroplating, and electronic etching bath surfactants, photographic emulsifier, aviation hydraulic fluids, fire-fighting foams, floor polishes, and insecticide formulations (Renner, 2001; Seacat *et al*., 2002).

PFOS and PFOA have very low volatility and vapour pressure. Certain sulphonamide intermediary compounds derived from PFOS, such as alkyl amides, alkyl alcohols, and alkyl acids, may sublime (3M, 2003). However, little is known about the transport and fate of these fluorochemicals in the environment.

Toxicity of PFOS in monkeys, rats, fish, and humans have been summarized in recent reviews (OECD, 2002). Sub chronic exposure to PFOS led to significant

weight loss accompanied by hepatotoxicity and reductions of serum cholesterol and thyroid hormones. Teratogenic effects have been recorded in rat, rabbit, and mouse with PFOS (potassium and lithium salts) (Case *et al*., 2001; Christian, *et al*., 1999; Gortner, 1980; Henwood *et al*., 1994; Thibodeaux *et al*., 2003; Wetzel, 1983).

Polychlorinated organic pollutants:

Chlorine was first isolated in 1774 by the Swedish chemist, C W Scheele. Since then, a lot of chlorinated compounds have been synthesized and used by man. The Stockholm Convention of the United Nations on Persistent Organic Pollutants identified 12 most persistent polychlorinated organic pollutants as "Dirty dozen". These include aldrin, chlordane, dichloro-diphenyl-trichloroethane (DDT), dieldrin, dioxins, furans, endrin, heptachlor, hexachlorobenzene, mirex, polychlorinated biphenyls (PCBs) and toxophene. The use of this set of chemicals has been banned or restricted in most countries.

Insecticides aldrin, dieldrin, chlordane, endrin and toxophane have been mainly used to kill termites, grasshoppers and other insect pests. These chemicals have been reported to be persistent in the environment in one form or other and all have been reported to be toxic in fish and other aquatic animals (IPCS, 1995; Jorgensson, 2001). The most infamous of persistent organic pollutants has been the insecticide DDT. It was widely used during World War Two to protect soldiers and civilians from malaria, typhus and other diseases spread by insects. Further it was used in many countries to combat malaria. Long-term exposure has been associated with chronic ailments in humans. Its best-known toxic effect is to thin the shells of birds' eggs (IPCS, 1995). Heptachlor, which has been mostly used to kill soil insects and termites, is believed to be responsible for the decline of many wild bird populations, including Canada geese and American kestrels in the Columbia River basin of the United States by means of adult mortality and lowered reproduction (Blus *et al*., 1984). Mirex is used to combat fire ants and has also been used as a fire retardant in plastics, rubber and electrical goods (Merck, 1989). Heptachlor and Mirex have been classified in IARC Group 2B as possible human carcinogens based on evidence in animal experiments (IARC, 1991).

A weedicide, hexachlorobenzene (HCB) was introduced in 1945 to kill fungi that affect food crops (Edwards *et al*., 1991). 3000-4000 people in eastern Turkey ate HCB-treated seed grain between 1954 and 1959; they developed a variety of symptoms including colic and metabolic disorder called porphyria turcica and 14 percent died (Peters *et al*., 1986). Hexachlorobenzene is reasonably anticipated to be a human carcinogen based on sufficient evidence of carcinogenicity in experimental animals (IARC 1982, 1987).

Polychlorinated Biphenyls (PCBs) which have been most studied amongst organic pollutants are a family of 209 related compounds that are used in industry as heat exchange fluids, in electric transformers and as additives in paint and plastics.

Health effects due to exposure to PCBs include acne-like skin conditions in adults and neurobehavioral and immunological changes in children. PCBs are known to be carcinogenic in animals (ATSDR, 2001).

Dioxins and furans are produced unintentionally due to incomplete combustion of hospital waste, municipal waste and hazardous waste. They are also released during manufacture of pesticides and other chlorinated substances. Dioxins have 75 positional isomers while furans have 135. These have been linked to a number of adverse effects in humans, including immune and enzyme disorders. The most toxic member of the dioxin family is 2,3,7,8-tetrachlorodibenzodioxin (TCDD) and it is mostly from studies on this compound that we know about the mechanism of the other chemicals in the same group (Silbergeld & Gasiewicz, 1989). TCDD has been reported to have toxic effects on all organ systems and has also been reported to possess carcinogenicity (Goldstein & Safe, 1989; Silbergeld & Mattison, 1987; Silbergeld & Gasiewicz, 1989). Furans are similar to dioxins and produce many of the same toxic effects.

Polyiodinated organic pollutants:

Amongst the polyiodinated organic pollutants iodinated X ray contrast media Iopromide contributes to the burden of absorbable organic halogens in the sewage systems. Steger-Hartmann *et al*. (1999) conducted short-term toxicity tests of Iopromide in bacteria, algae, crustaceans and fish and found no toxic effects even at concentrations 100 times the predicted environmental concentrations.

Polybrominated Organic Pollutants:

Discovered by Balard in 1826, bromine is the only liquid non-metallic element. Bromine is one of the few chemical elements with fire-resistance properties and is hence used to manufacture compounds termed as the Brominated flame-retardants (BFRs). These include about 75 substances that are used to prevent fire in electronic appliances, fabric coverings and foam filled furniture (BSEF, 2001). The presence of BFRs in high volumes in environment has posed a potential health risk to organisms exposed to these environmental contaminants (de Wit, 2002; McDonald, 2002).

BFRs of particular environmental relevance can be classified as polybrominated biphenyls (PBBs), hexabromocyclododecane (HBCD), tetrabromobisphenol-A (TBBPA) and polybrominated diphenyl ethers (PBDEs) (de Wit, 2002).

Polybrominated biphenyls (PBBs):

PBBs have a molecular formula of $C_{12}H_{(10-x-y)}Br_{(x+y)}$ where both x and y = 1 to 5. Theoretically 209 congeners are possible. PBBs, manufactured for commercial

use, consist of mainly hexa-, octa-, nona-, and decabromobiphenyls (WHO/IPCS, 1994a). The PBBs were introduced as the commercial mixture Fire Master in the early 1970s. In 1973, livestock on certain farms in Michigan, USA were exposed to Fire Master FF-1 (a commercial product) after it was mistaken as a feed supplement and was mixed with feed. This PBB contamination of animal feed resulted in massive loss of livestock after which this product was discontinued from the market in USA (Dunckel, 1975).

PBBs remain in the environment for a long time. Apart from a slight photo degradation by ultraviolet light, no degradation of PBBs has been reported either from water, soil or plants (Chou *et al*., 1978, Jacobs *et al*., 1976, Jacobs *et al*., 1978). PBBs have been reported to bioaccumulate in fish (Zitko, 1977) and ducks (Hesse & Powers, 1978).

Damstra *et al*., (1982) have suggested hepatotoxicity, neuromuscular weakness, immunotoxicity and adverse reproductive effects of PBBs on the basis of animal experiments. High doses of PBB exposure have shown to cause liver cancers in rats and mice (Kimbrough, 1978).

Hexabromocyclododecane (HBCD):

HBCD mixtures are obtained by bromination of cyclododeca-1,5,9-triene (CDT) isomers. Bromination of CDT results in six stereo centers at positions 1, 2, 5, 6, 9, and 10. From all 4 possible CDT isomers, 16 HBCD stereoisomers may be formed. They are used as additive flame-retardants in insulation panels and blocks for building construction. When these products are incinerated recycled or dumped at waste sites HBCDs are released in the environment. In the environment they are transferred from sediment to invertebrates and then to predatory fish and further to fish-eating predators such as seals and birds (Morris *et al*., 2004, Leonards *et al*, 2004).

HBCDs have been suggested to inhibit hepatic enzyme CYP1A in juvenile rainbow trout and feral eelpout. (Ronisz *et al*, 2001). Darnerud (2003) has suggested thyroid disrupting ability of HBCDs. Furthermore Helleday *et al*. (1999) have indicated that they may cause cancer by nonmutagenic mechanism.

Tetrabromobisphenol-A (TBBPA):

TBBPA is used as a reactive flame retardant. The phenol hydroxyl group of this chemical react covalently, resulting in incorporation of this chemical into the polymer. TBBPA is used as a reactive flame retardant in laminates for printed wiring boards, thus adding to the safety of electronic equipment and installations. In addition, TBBPA is used as an additive flame retardant in plastics. .

TBBPA has neither shown inhalation or dermal toxicity nor any teratogenecity (WHO, 1995). Rodent studies with single dose oral exposure of TBBPA showed LD_{50} to be approximately >5 g/kg in rats and >4g/kg in mice, suggesting it to have

no acute toxicity (WHO, 1995). *In vitro* studies showed major adverse effects in primary hepatocytes with inhibition of CYP2C9 (Boecker *et al*., 2001), immunotoxicity by its ability to specifically inhibiting the expression of interleukin-2 receptor alpha chain (CD25) (Pullen *et al*., 2003). In addition, neurotoxicity in cerebral granule cells and rat brain synaptosomes by inhibition of plasma membrane uptake of the neurotransmitters dopamine, glutamate and gamma-amino-n-butyric acid (GABA) and generation of free radicals have been shown by Mariussen & Fonnum, 2002. TBBPA had minor estrogenic effect in comparison to lower brominated bisphenols (Meerts *et al*, 2001), TBBPA has been suggested as a potential thyroid disruptor because of its ability to competitively bind to the serum transport protein transthyretin (Kitamura *et al*., 2002; Haddow *et al*., 1999; Meerts *et al.*, 1999; Meerts *et al.*, 2001).

Polybrominated diphenyl ethers (PBDEs):

PBDEs are high volume chemicals used as flame-retardants in electric and electronic equipments, textiles and paint. As they are added to polymers without forming covalent bonds, they can leach into the environment during production, use and disposal of the products (Sjodin *et al*., 2001). Since the early 1980s, PBDEs have been detected in all compartments of the environment worldwide, making them global and ubiquitous contaminants.

PBDEs can have 209 possible congeners because of large number of positions where the bromine atom/s can bind into two phenyl rings. The number of bromine atoms classifies these 209 congeners: for example, pentabromodiphenyl ethers (penta-BDEs) have five bromine atoms, octa-BDEs have eight, deca-BDEs have ten. Six out of the 209 PBDE congeners are typically found within three commercial mixtures; therefore researchers have conducted a significant amount of research regarding these six congeners (BDE-47, BDE-99, BDE-100, BDE-143, BDE-154 and BDE-209), of these BDE-47, BDE-99, BDE-100 are penta or tetra compounds, while BDE-143, BDE-154 and BDE-209 are hexa or deca compounds. The lower brominated compounds such as tetra and penta are believed to bioaccumulate in the body fat of animals more readily than higher brominated compounds such as deca, octa, and hexa (Darnerud *et al*., 2001; 2003).

PBDEs have several toxic effects. Nneurobehavioral effects of PBDEs include primarily impairment in motor behavior, reduced learning and reduction in memory process (Branchi *et al*, 2003). Reproductive effects e.g. delay in onset of puberty, decrease in the sperm count and reduction in weight of gonads have been seen in male rats. (Birnbaum and Staskal, 2004). PBDEs closely resemble thyroid hormones (T3 & T4) and bind competitively to thyroid hormone transfer proteins thus acting as endocrine disrupters (McDonald, 2002). Chronic exposure to deca-BDE resulted in hepatic and pancreatic adenomas in rats, whereas, a combined incidence of hepatocellular adenomas and carcinomas was seen in mice (NTP, 1986).

CHAPTER 2: ROLE OF FROGS IN STUDYING POLYHALOGENATED ORGANIC POLLUTANTS

Amphibians:

Amphibians, the name derived from the Greek word *amphibios* meaning "living a double life," reflects this dual life strategy: tadpole stage and adult stage. All the living amphibians throughout the world have been grouped in 3 orders, namely Apoda (Caecilians), Salientia (Frogs and Toads) and Caudata (Salamander and Newts). Of the three orders, Salientia comprising of anurans are most wide spread. Frogs are found in all continents except in Antarctica (Encyclopaedia Britannica, 2005).

Frogs have two stages in life: larval/tadpole stage and adult stage. The larvae or tadpoles transform to the adult stage via metamorphosis. This occurs two weeks to three years after hatching, depending upon the species (Porter, 1972). Metamorphosis involves radical structural changes of the body: the gills disappear, lungs and limbs develop rapidly (hind limbs first), the tail is absorbed and the digestive tract shortens. Eventually the adult frog becomes an air breather (Romer, 1959). Respiration is accomplished via internal lungs as well as via the skin. The length of the metamorphic period is also species dependent. Several amphibian species are non-metamorphosing and retain both larval characteristics and their aquatic lifestyle (genus Necturus) (Porter, 1972).

Adult anurans, unlike the larvae, are carnivorous, consuming almost any moving prey of the right size. This includes worms, insects, other amphibians, reptiles and occasionally mammals. Throughout its life cycle an anuran can occupy several different positions within the food web (Porter, 1972). Interruptions of this life cycle by toxic contaminants can have disastrous effects on amphibian populations (Paulov, 1977). Recent amphibian conservation summit that took place in Washington DC, USA in September, 2005 observed that nearly one-third of the world's amphibian species have been classified as threatened with extinction. Decline in the frog populations all over the world has been suggested to be caused by change in climate, habitat reduction, increased UV radiation and increased exposure to xenobiotics like organ halogens (Wake, 1991). To evaluate if amphibians can be employed as useful indicators of environmental quality, toxicity of contaminants to amphibians in lab bioassays and field experiments as well as their general effects on populations will have to be studied.

Effects of polyhalogenated organic pollutants on frogs:

Several factors are responsible for the concerns regarding organic contaminants in frogs. These contaminants are persistent in the environment and can

biomagnificate. Frogs can serve as vehicle for these contaminants in the food chain as well as between aquatic and terrestrial environments (Sparling *et al*., 2000).

Toxic effects observed due to polyhalogenated pollutants in frogs include effects on endocrine disruption (especially thyroid and sex hormones), effect on gene expression and induction of cytochrome P 450 enzymes.

Several researchers have studied different effects of these contaminants on amphibians. Ankley *et al*. (2004) studied the effects of PFOS on survival and development of the northern leopard frog (Rana pipiens) from early embryogenesis through complete metamorphosis and observed delay in metamorphosis suggesting thyroid disruption activity. A chemical of the PCB group (3, 3', 4, 4'- tetrachlorobiphenyl) was chronically exposed to metamorphosing tadpoles of northern leopard frog (*Rana pipens*) to observe the effects on behaviour, morphology, competitive performance and corticosteron content since all these factors play a major role in tadpole development. This chemical is known to have endocrine, developmental and toxic effects in other animals. A study revealed that the activity, competitive performance and corticosteron levels were decreased although the morphology of mouthparts and body proportions remained unaffected (Glennemeier & Denver, 2002). Qin *et al* (2003) studied the effect of PCBs in gonadal differentiation in *X. laevis* tadpoles through the complete metamorphosis and reported significant feminization effects.

Reeder *et al*. (2005) studied gonads of cricket frogs (*Acris crepitans*) from museum collections in Illinois to compare the number of intersex frogs in 5 different time periods based on the era of use of different organochlorines. The gonads of frogs were examined *in situ* with a dissecting microscope to identify sex. The researchers concluded that endocrine disruption due to antiestrogenics like PCBs, DDT and other heavy metals contributed to the decline in frog population in Illinois.

Jelaso *et al*. (2005) exposed metamorphosing *X. laevis* tadpoles to PCB mixture Arclor 1254 to measure changes in gene expression for six genes that play important role in development and physiology. The results showed that low dose exposure to A 1254 results in increased gene expression of nerve growth factor and proopiomelanocortin while high dose exposure results in increased gene expression of cytochrome P 450 1A1. A field study in the Fox River and Green Bay, Wisconsin, USA was conducted to test the hypothesis that cytochrome P 450 activity is more induced in metamorphs than tadpoles of *X. laevis* frogs. The study ratified the hypothesis suggested by Jung *et al*. (2004).

There have been many studies on accumulation of these chemicals in adult frogs of different species but unfortunately the information on bioconcentration and effects is very limited (Sparling *et al*., 2000).

CHAPTER 3: *XENOPUS* AS LABORATORY SPECIES

Brief History:

Genus *Xenopus* belongs to the family Pipidae that is a group of tongue less aquatic African frogs. *Xenopus*, which literally means: "strange foot" has small black claws on the inner three toes of the hind limbs (Encyclopaedia Britanica, 2005). *Xenopus laevis* was once widely used for tests for human pregnancy because researchers found that young female-clawed frogs would lay eggs when injected with minute quantities of a human hormone found in the urine of pregnant women (Hogben *et al*, 1931). *Xenopus* was found to be very useful for research related to embryogenesis due to several factors, including the ability to stimulate production of eggs year round, the external development of embryos, and the large number of eggs per spawning. Further, it was found that the size of the eggs and embryos is large enough to readily permit microsurgical manipulation and injection, and *Xenopus* embryos show remarkable powers of healing subsequent to these procedures (Gurdon & Hoopwood, 2000). Nieuwkoop & Faber (1956) published the first Normal Table of *X.laevis,* which described all stages of development from the fertilized egg till the end of metamorphosis.

In the 1970's, it was discovered by John Gurdon (Gurdon *et al*., 1970) that injection of haemoglobin mRNA into *Xenopus* oocytes causes them to synthesize the foreign proteins (Lindsay, 2005). His most important discovery, however, was showing that a frog skin cell nucleus, if put in place of the egg nucleus, was able to program full development of the egg into a frog. This was a very novel discovery at the time, because it was the first experiment that showed the genes in all cells were essentially the same. In the early 1980s, neurobiologists discovered that *Xenopus* oocytes, if injected with mRNA or genes encoding ion channels from other species, produce these channels and place them in their plasma membrane (Lindsay, 2005).

At the same time, scientists realized that they could use *X.laevis* as a powerful system for easily studying vertebrate development, which led to the development of the Frog Embryo Teratogenesis Assay *Xenopus* (FETAX) assay (Gurdon & Hoopwood, 2000). Lastly, other characteristics of *Xenopus* eggs make them extremely useful to cell biologists because one *Xenopus* egg is 1000 times bigger than a somatic cell, and contains huge stockpiles of all the components required to make mature cells (Kay *et al*., 1991).

Overall, the discovery that *Xenopus* could be used as a model organism for neuroscience, developmental biology, and cell biology research has led to many important discoveries in science.

Xenopus tropicalis

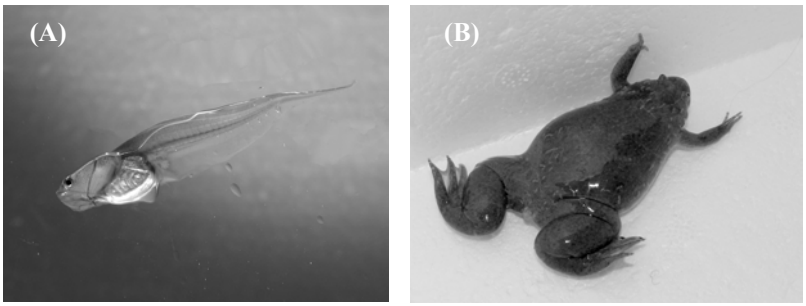

Figure 1 *Xenopus tropicalis*: (A) tadpole and (B) adult female.

Xenopus tropicalis is a close native of the traditionally used *Xenopus* species *X.laevis*. It is half the size and has half the number of chromosomes (20) in comparison to *X.laevis*, yet it has all the advantages of *X.laevis* as a laboratory species. *X.tropicalis* can be maintained easily and produces 1000-3000 eggs per spawning and has a shorter generation time (<5 months) in comparison to *X.laevis*. Several researchers are trying to explore the possibility of *X.tropicalis* in the assays and methods standardized for *X. laevis* (Amya *et al*., 1991).

Use of Xenopus in toxicity testing models

Frog Embryo Teratogenesis Assay *Xenopus* (FETAX)

FETAX is conducted at the embryonic stage of development. This stage of development has demonstrated sensitivity and may provide information that is useful in estimating the chronic toxicity of a test material. FETAX uses dual endpoints, mortality and malformation of embryos to assess developmental toxicity. Validation tests of FETAX have indicated close correlation with other bioassays used for toxicity assessment of single compounds, as well as mixtures (ASTM, 1998). Fort *et al*. (2004) compared the sensitivity of *X.tropicalis* and X.laevis in the FETAX model and concluded that *X.tropicalis* could be used effectively for FETAX thereby reducing the time of study to 48h from 96h.

Xenopus metamorphosis assay (XEMA)

In the XEMA test, exposure of *X. laevis* tadpoles is initiated at stages 48 to 50 and continued for 28 d. Development and growth of tadpoles are assessed by endpoints as developmental stage and whole body length (Opitz *et al*. 2005). Mitsui *et al*. (2005) have tested this assay in *X. tropicalis* after exposures to

propylthiouracil and thyroxin and concluded that *X. tropicalis* is equally sensitive as *X. laevis* and thus a suitable replacement for this assay.

Xenopus Tail Resorption Assay

This Assay is an endocrine (thyroid) disruption assay using advanced *Xenopus* larvae to screen materials that may disrupt thyroid function (Fort *et al*., 2000). In this assay, tadpoles are exposed for approximately 14 days from developmental stages between 58 and 60 through stage 66. Tadpoles at the "just bud" stage are exposed to varying concentrations of the test material. Photographic images of the test organisms are taken at different time intervals and tail length determined.

Xenopus Vitellogenin Assay

Another endocrine disruption assay involving *Xenopus* is based on the detection of vitellogenin in the blood of treated males. One of the most important and sensitive responses to estrogen is the upregulation of protein production. A particularly well-known estrogenic response in all oviparous and ovoviviparous vertebrates is the induction of the lipoprotein vitellogenin in liver cells. Vitellogenin may prove useful as a biomarker in *Xenopus* for identifying xenobiotics with estrogenic activity (Palmer and Palmer, 1995).

Evaluation of Reproductive Toxicity using *Xenopus*

Fort *et al*. (2001) have evaluated the utility of *X. laevis* for assessing reproductive toxicity. The investigators concluded that this model appears to be a useful tool in the initial assessment and prioritization of potential reproductive toxicants for further testing.

Xenopus Limb Bud Assay

The *Xenopus* Limb Bud Assay is a test method for exploring limb mal-development, including possible mechanisms of action. This assay is proposed as a model for screening materials that may cause limb deformities in the workplace or the environment. The assay uses blastula stage *Xenopus* embryos raised to about Developmental. Stage 58 to 59. The first four days of the test are similar to the standard FETAX test where as at the end of the 96-hour exposure period, the developing embryos are transferred to larger containers and at the end of the exposure period, the incidence of malformations, survival, and total organism counts are determined. This methodology has been used to identify agents associated with limb mal-development in pond water and sediment collected in Minnesota, U.S. to evaluate the developmental toxicity of thalidomide (Fort *et al*.,

2000), and to evaluate the effects of two sulfonylurea herbicides, sulfometuron methyl and nicosulfan, on limb development (Fort *et al*., 2001).

GENERAL AIM OF THE THESIS

The aim of the present study was to compare the tissue disposition of a polybrominated compound, 2, 2', 4, 4', 5-pentabromodiphenyl ether (BDE 99) in tadpole and juvenile stages of *Xenopus tropicalis* (West African clawed frog). Whole-body autoradiography and liquid scintillation counting were used to follow the disposition after dietary exposure of the radiolabelled substance ^{14}C-BDE at different development stages, stage 54 (tadpoles) and stage 66 (juvenile frogs).

MAJOR CONCLUSIONS

- Retention of ^{14}C-BDE 99 is mainly seen in fat tissue and bone marrow.
- The chemical did not show any covalent binding.
- Juvenile frogs have a greater uptake than tadpoles.
- Tadpoles have faster elimination than juveniles.
- The substance has affinity to melanin.
- The substance is excreted via bile and probably urine.

REFERENCES

3M, 2003. Environmental and Health Assessment of Perfluorooctane Sulfonate and its Salts. Available on US EPA Administrative Record AR-226.

Amaya, E., 1998. Frog genetics: Xenopus tropicalis jumps into the future. Trends Genet, 14: 253–254.

American Society for Testing and Materials, 1998. Standard guide for conducting the frog embryo teratogenesis assay — Xenopus (FETAX). In Annual Book of ASTM Standards, vol 11.05: Philadelphia, PA; 826–836.

amphibian. Encyclopaedia Britannica. 2005. From Encyclopaedia Britannica Online. <http://search.eb.com/eb/article-9110233> [Accessed September 26, 2005].

Ankley, G.T., Kuehl, D.W., Kahl, M.D., Jensen, K.M., Butterworth, B.C. and Nichols, J.W., 2004. Partial life-cycle toxicity and bioconcentration modeling of perfluorooctanesulfonate in the northern leopard frog (Rana pipiens). Environ Toxicol Chem, 23(11):2745-55.

ATSDR, 2001. ToxFAQs™ for Polychlorinated Biphenyls (PCBs), Agency for Toxic Substances and Disease Registry Information Centre: http://www.atsdr.cdc.gov/tfacts17.html

Birnbaum, L. S., and Staskal, D. F., 2004. Brominated flame-retardants: cause for concern? Environ Health Perspect, 112(1):9-17.

Blus, L.J., C.J. Henny, D.J. Lenhart and T.E. Kaiser, 1984. Effects of heptachlor- and lindane-treated seed on Canada geese. Journal of Wildlife Management. 48: 1097-1111.

Boecker, R.H., Schwind, B., Kraus, V., Pullen, S., Tiegs, G.. 2001. Cellular disturbances by various brominated flame retardants [Abstract]. Presented at the Second International Workshop on Brominated Flame Retardants, 14–16 May 2001, Stockholm, Sweden.

Branchi, I., Capone, F., Alleva, E., and Costa, L.G., 2003. Polybrominated diphenyl ethers: neurobehavioral effects following developmental exposure. Neurotoxicology. 2003, (3):449-62.

BSEF, Bromine Science and Environmental Forum, 2001. Bromine: frequently asked questions. Available on www.bsef.com. Accessed on June 20, 2005.

Case, M.T., York, R.G., Christian, M.S., 2001. Rat and rabbit oral developmental toxicology studies with two perfluorinated compounds. Int. J. Toxicol. 20, 101–109.

Chou, S. F., Jacobs, L.W., Penner, D. and Tiedje, J.M., 1978. Absence of plant uptake and translocation of polybrominated biphenyls (PBBs). Environ. Health Perspect. 23: 9-12.

Christian, M.S., Hoberman, A.M., York, R.G., 1999. Combined Oral (gavage) Fertility, Developmental and Perinatal/Postnatal Reproduction Toxicity Study of PFOS in Rats. Argus Research Laboratories, Inc., Horsham, PA. US EPA Docket 8EHQ-0200-00374.

Damstra. T., Jurgelski, W. Jr., Posner, H.S., Vouk, V.B., Bernheim, N.J., Guthrie, J., Luster, M., and Falk, H.L., 1982. Toxicity of polybrominated biphenyls (PBBs) in Domestic and laboratory animals. Environ Health Perspect, 44:175-88.

Darnerud, P.O., Eriksen, G.S., Johannesson, T., Larsen, P.B. and Viluksela, M., 2001. Polybrominated diphenyl ethers: occurrence, dietary exposure, and toxicology. Environ Health Perspect. 109(1): 49-68.

Darnerud, P. O., 2003. Toxic Effects of Brominated Flame Retardants in Man and in Wildlife. Environ. Int. , 29, 841-853.

de Wit CA, 2002. An overview of brominated flame retardants in the environment. Chemosphere, 46:583– 624.

Dunckel, A.E.,. 1975. An updating on the polybrominated biphenyl disaster in Michigan. J Am Vet Med Assoc 167:838–841.

Edwards, I.R., D.G. Ferry and W.A. Temple. 1991. Fungicides and related compounds. In: W.J.Hayes Jr. and E.R. Laws Jr. (eds.), Handbook of Pesticide Toxicology: Volume 3, Classes of Pesticides. Academic Press, Inc., Toronto, pp.1409-1470.

Fort, D.J., Rogers, R.L., Morgan, L.A., Miller, M.F., Clark, P.A., White, J.A., Paul, R.R., Stover, E.L., 2000. Preliminary validation of a short-term morphological assay to evaluate adverse effects on amphibian metamorphosis and thyroid function using Xenopus laevis. J Appl Toxicol, 20(5):419-25.

Fort, D.J., Rogers, R.L., Paul, R.R., Miller, M.F., Clark, P., Stover, E.L., Yoshioko, J., Quimby, F., Sower, S.A., Reed, K.L., Babbitt, K.J., Rolland, R., 2001. Effects of pond water, sediment and sediment extract samples from New Hampshire, USA on early Xenopus development and metamorphosis: comparison to native species. J Appl Toxicol 21(3):199-209.

Fort, D.J., Stover, E.L., Bantle, J.A., Dumont, J.N., Finch, R.A., 2001. Evaluation of a reproductive toxicity assay using Xenopus laevis: boric acid, cadmium and ethylene glycol monomethyl ether. J Appl Toxico, 21(1):41-52.

Fort, D.J., Rogers, R.L., Thomas, J.H., Buzzard, B.O., Noll, A.M. and Spaulding, C.D., 2004. Comparative Sensitivity of Xenopus tropicalis and Xenopus laevis as Test Species for the FETAX Model. J. Appl. Toxicol. 24, 443–457.

Glennemeier, K.A., Denver, R.J., 2001. Sublethal Effects of Chronic Exposure to an Organochlorine Compound on Northern Leopard Frog (Rana pipiens) Tadpoles. Effects of PCBs on Tadpole Biology, 2001 by John Wiley & Sons, Inc.

Glennemeier, K.A., Denver, R.J.., 2002. Small changes in whole-body corticosterone content affect larval Rana pipiens fitness components. Gen Comp Endocrinol, 27(1):16-25.

Goldstein, J.A. and Safe, S., 1989. Mechanism of action and structure-activity relationships for the chlorinated dibenzo-p-dioxins and related compounds. In: Kimbrough RD, Jensen AA, eds. Halogenated biphenyls, naphthalenes, dibenzodioxins and related compounds, 2nd ed. Amsterdam, Elsevier Science Publishers B.V., 239-293.

Gortner, E.G., 1980. Oral Teratology Study of FC-95 in Rats Riker Laboratories, Inc. Experiment Number: 0680TR0008, December, 1980. US EPA AR-226 226-0016.

Gurdon, J.B., and Hopwood, N., 2000. The introduction of Xenopus laevis into developmental biology: of empire, pregnancy testing and ribosomal genes. Int. J. Dev. Biol. 44: 43-50.

Gurdon, J. B., Lane, C. D., Woodland, H. R. & Marbaix, G., 1971. Use of Frog Eggs and Oocytes for the Study of Messenger RNA and its Translation in Living Cells. Nature 233, 177-182.

Haddow, J.E., Palomaki, G.E., Allan, W,C., Williams, J.R., Knight, G.J., Gagnon, J., 1999. Maternal thyroid deficiency during pregnancy and subsequent neuropsychological development of the child. N Engl J Med, 341:549–555.

Hall, A. J., Kalantzi, O. I., Thomas, G. O., 2003. Polybrominated Diphenyl Ethers (PBDEs) in Grey Seals During Their First Year of Life—Are They Thyroid Hormone Endocrine Disrupters? Environ. Pollut., 126, 29–37.

Helleday, T., Tuominen, K.L., Bergman, A., Jenssen, D.., 1999 Brominated Flame Retardants Induce Intragenic Recombination in Mammalian Cells. Mutat. Res., 439, 137–147.

Henwood, S.M., McKee-Pesick, P., Costello, A.C., Osmitz, T.G., 1994. Developmental toxicity study with lithium perfluorooctane sulfonate in rats. Teratology 49, 398.

Hesse, J.H. and Powers, R.A., 1978. Polybrominated biphenyls (PBB) contamination in the Pine River, Gratiot and Midland Counties, Michigan. Environ. Health Perspect. 23: 19-25.

Hogben, L., Charles, E. and Slome, D. (1931). Studies on the pituitary. VIII.The relation of the pituitary gland to calcium metabolism and ovarian function in Xenopus. J. Exp. Biol. 8: 345-354.

IARC. 1979a. Toxaphene. In: IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, Volume 20: Some Halogenated Hydrocarbons. International Association for Research on Cancer, Lyon, France, pp. 327-348.

IARC. 1979b. Mirex. In: IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, Volume 20: Some Halogenated Hydrocarbons. International Association for Research on Cancer, Lyon, France, pp.283-302.

IARC. 1982. Chemicals, Industrial Processes and Industries Associated with Cancer in Humans. IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans, Supplement 4. Lyon, France: International Agency for Research on Cancer. 292 pp.

IARC. 1987. Overall Evaluations of Carcinogenicity. IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans, Supplement 7. Lyon, France: International Agency for Research on Cancer. 440 pp.

IARC. 1991. Chlordane and heptachlor. In: IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Volume 53. Occupational exposures in insecticide application, and some pesticides. International Association for Research on Cancer, Lyon, France. pp. 115-178.

IPCS, 1995. An Assessment Report on: DDT-Aldrin-Dieldrin-Endrin-Chlordane-Heptachlor-Hexachlorobenzene-Mirex-Toxaphene-Polychlorinated, Biphenyls-Dioxins and Furans. The International Programme on Chemical Safety (IPCS) within the framework of the Inter-Organization Programme for the Sound Management of Chemicals (IOMC).

Jacobs, L.W., Chou, S. F. and Tiedje, J.M. 1976 Fate of polybrominated biphenyls (PBBs) in soils. Persistence and plant uptake. J. Agr. Food Chem. 24:1198-1201.

Jacobs, L.W., Chou, S. F. and Tiedje, J.M., 1978. Field concentrations and persistence of polybrominated biphenyls in soils and solubility of PBB in natural waters. Environ. Health Perspect. 23: 1-8.

Jelaso, A,M., DeLong, C., Means, J. and Ide, C.F., 2005. Dietary exposure to Aroclor 1254 alters gene expression in Xenopus laevis frogs. Environ Res, 98(1):64-72.

Jorgenson, J.L., 2001. Aldrin and Dieldrin: A Review of Research on Their Production, Environmental Deposition and Fate, Bioaccumulation, Toxicology, and Epidemiology in the United States. Environ Health Perspect. 109 (1):113-39.

Jung, R.E., Karasov, W.H., Melancon, M.J., 2004. Cytochrome P450 activity in green frogs (Rana clamitans melanota) exposed to water and sediments in the Fox River and Green Bay, Wisconsin, USA. Bull Environ Contam Toxicol, 73(6): 955-62.

Kay, B. K. and Benjamin H. Peng. (Eds.). (1991). Methods in Cell Biology, Vol. 36. Xenopus laevis: Practical Uses in Cell and Molecular Biology. San Diego: Academic Press.

Key, B.D., Howell, R.D., Criddle, C.S., 1997. Fluorinated organics in the biosphere. Environ. Sci. Technol. 31, 2445–2454.

Key, B.D., Howell, R.D., Criddle, C.S., 1998. Defluorination of organofluorine sulfur compounds by Pseudomonas sp. strain D2. Environ. Sci. Technol. 32, 2283–2287.

Kimbrough, R.D., Burse, V.W., Liddle, J.A., 1978. Persistent liver lesions in rats after a single oral dose of polybrominated biphenyls (firemaster FF-1) and concomitant PBB tissue levels. Environ Health Perspect, 23:265-73.

Kitamura, S., Jinno, N., Ohta, S., Kuroki, H., Fujimoto, N., 2002. Thyroid hormonal activity of the flame retardants tetrabromobisphenol A and tetrachlorobisphenol A. Biochem Biophys Res Commun 293:554–559.

Kudo, N., Kawashima, Y., 2003. Toxicity and toxicokinetics of perfluorooctanoic acid in humans and animals. J. Toxicol. Sci. 28, 49– 57.

Leonards, P.; Vethaak, D.B., Brandsma, S.A., Kwadijk, C.A., Micic, D.A., Jol, J.B., Schout, P.B., de Boer, J.A., 2004. Species-specific accumulation and biotransformation of Polybrominated Diphenyl Ethers and Hexabromocyclododecane in Two Dutch Food Chains. In Proceedings of the Third International Workshop on Brominated Flame Retardants, BFR 2004, Toronto, Canada, 2004; pp 283–286.

Lindsay, C., 2005. Frogs Leap to the Forefront in Biological Research. Organism of the Quarter: Xenopus laevis. Frogs Leap to the Forefront in Biological Research Saltman Quarterly, Volume 2, Issue 1.

Lyman, W.J., 1990. Handbook of Chemical Property Estimation Methods. Washington, DC:American Chemical Society. Petreas M, She J, Brown R, Winkler J, Winham G, Rogers E, 46:107–112.

Mariussen, E., Fonnum, F., 2002. The effect of pentabromodiphenyl ether, hexabromocyclododecane and tetrabromobisphenol-A on dopamine uptake into rat brain synaptosomes. Organohalogen Compounds 57:395–399.

McDonald, T.A., 2002. A perspective on the potential health risks of PBDEs. Chemosphere, 46:745– 55.

Meerts, I.A., Assink, Y., Cenihn, P., Weijers, B., van den Berg, H., Koeman, J., 1999. Distribution of the flame retardant tetrabromobisphenol A in pregnant and fetal rats and effect on thyroid hormone homeostasis. Organohalogen Compounds 40:375–379.

Meerts, I.A., Letcher, R.J., Hoving, S., Marsh, G., Bergman, A., Lemmen, J.G., 2001. *In vitro* estrogenicity of polybrominated diphenyl ethers, hydroxylated PDBEs, and polybrominated bisphenol A compounds. Environ Health Perspect 109:399–407.

Merck, 1989. The Merck index: An encyclopedia of chemicals, drugs, and biologicals. 11th ed.,Budavari S, O'Neil MJ, Smith A, *et al*. eds. Rahway, NJ: Merck and Co., Inc., 321, 977.

Mitsui, N., Fujii, T., Miyahara, M., Oka, T., Kashiwagi, A., Kashiwagi, K., Hanada, H., Urushitani, H., Santo, N., Tooi, O., Iguchi, T., 2005. Development of metamorphosis assay using Silurana tropicalis for the detection of thyroid system-disrupting chemicals. Ecotoxicol Environ Saf. In-press.

Morris, S., Allchin, C.R., Zegers, B.N., Haftka, J.J., Boon, J.P., Belpaire, C., Leonards, P.E., Van Leeuwen, S.P. and De Boer, J. 2004. Distribution and fate of HBCD and TBBPA brominated flame retardants in North Sea estuaries and aquatic food webs. Environ Sci Technol. 1;38(21):5497-504.

Neilson, A. H., A. -S. Allard, and M. Remberger. 1985. Biodegradation and transformation of recalcitrant compounds, p.29-86. In 0. Hutzinger, (ed.) The handbook of environmental chemistry, vol. 2, part C. Springer-Verlag KG, Berlin.

Nieuwkoop, P.D. and Faber, J. (Eds.) (1956). Normal Table of Xenopus laevis (Daudin): A systematical and chronological survey of the development from the fertilized egg till the end of metamorphosis. North-Holland Publ. Co., Amsterdam.

NTP, 1986. Toxicology and Carcinogenesis Studies of Decabromodiphenyl Oxide (CAS No 1163-19-5) in R344/N rats and B6C3F1 mice (feed studies). NTP Technical Report Series no 309. Research Triangle Park, NC:National Toxicology Program.

OECD link Unclassified ENV/JM/RD(2002)17/FINAL. www.oecd.org. Accessed on July 17, 2005.

Opitz, R., Braunbeck, T., Bogi, C., Pickford, D.B., Nentwig, G., Oehlmann, J., Tooi, O., Lutz, I., Kloas, W., 2005. Description and initial evaluation of a Xenopus metamorphosis assay for detection of thyroid system-disrupting activities of environmental compounds. Environ Toxicol Chem, 24(3):653-64.

Palmer, B.D., Palmer, S.K., 1995. Vitellogenin induction by xenobiotic estrogens in the red-eared turtle and African clawed frog.Environ Health Perspect, 103 (4): 19-25.

Paulov, S., 1977. The effects of N-Phenyl-N'-Methyl Urea (defenuron) on the development of amphibia (Rana temporaria L). Biologia (Bratislava), 32: 607-611.

Peters, H.A., D.J. Cripps, R. W. Lambrecht, E. Ertiirk, C.R. Morris and G. T. Bryan. 1986. History and geography of hexachlorobenzene poisoning in southeastern Turkey. In: C.R. Morris and J.R.P. Cabral, eds. Hexachlorobenzene: Proceedings olan International Symposium.International Agency for Research on Cancer, IARC Scientific Publications No.77, Lyon, France.pp. 131-132.

Porter, K.R., 1972. Herpetology, WB Sanders Co., Philadelphia, PA, USA.

Prescher, D., Gross, U., Wotzka, J., Txcheu-Schlueter, M., Starke, W., 1985. Environmental behavior of fluoro surfactants: Part 2. Study on biochemical degradability. Acta Hydrochim. Hydrobiol. 13, 17– 24.

Pullen, S., Boecker, R., Tiegs, G., 2003. The flame retardants tetrabromobisphenol A and tetrabromobisphenol A-bisallylether suppress the induction of interleukin-2 receptor alpha chain (CD25) in murine splenocytes. Toxicology 184:11–22.

Qin, Z.F., Zhou, J.M., Chu, S.G. and Xu, X.B., 2003 Effects of Chinese domestic polychlorinated biphenyls (PCBs) on gonadal differentiation in Xenopus laevis. Environ Health Perspect, 111(4):553-6.

Reeder, A.L., Ruiz, M.O., Pessier, A., Brown, L.E., Levengood, J.M., Phillips, C.A., Wheeler, M.B., Warner, R.E. and Beasley, V.R., 2005. Intersexuality and the cricket frog decline: historic and geographic trends. Environ Health Perspect, 113(3):261-5.

Reistad, T., Mariussen, E., Fonnum, F., 2002. The effect of brominated flame retardants on cell death and free radical formation in cerebellar granule cells. Organohalogen Compounds 57:391–394.

Renner, R., 2001. Growing concern over perfluorinated chemicals. Environ. Sci. Technol. 35, 154A– 160A.

Romer, A.S., 1959, The Vertebrate Story (4th edition), University of Chicago Press, Chicago, USA.

Ronisz, D., Finne, E.F., Karlsson, H., Forlin, L., 2001. Sublethal Effects of the Flame Retardants Hexabromocyclododecane (HBCDD) and Tetrabromobisphenol A (TBBPA) in Juvenile Rainbow Trout (Oncorhynchus mykiss). Aquat. Toxicol. 69, 229–245.

Seacat, A.M., Thomford,P.J., Hansen, K.J., Olsen,G.W., Case,M.T., Butenhoff, J.L., 2002. Subchronic toxicity studies on perfluoroctanesulfonate potassium salt in Cynomolgus monkeys. Toxicol. Sci. 68, 249– 264.

Silbergeld, E., Mattison, D., 1987. Experimental and clinical studies on the reproductive toxicology of 2,3,7,8-tetrachlorodibenzo-p-dioxin. Am J Ind Med 11:131–144.

Silbergeld, E.K., and Gasiewicz, T.A., 1989. Commentary: Dioxins and the Ah receptor. Amer. J. of Industrial Med. 16:455-474.

Sjödin, A., Carlsson, H., Thuresson, K., Sjölin, S., Bergman, A., Ostman, C., 2001. Flame retardants in indoor air at an electronics recycling plant and at other work environments. Environ. Sci. Technol. 35, 448–454.

Sparling, D. W., Linder,G., Bishop, C. A., Eds.; 2000. Ecotoxicology of AmphibiansandReptiles. Society of Environmental Contamination and Chemistry (SETAC): Pensacola, FL, USA. pp. 1-14.

Sparling, D. W., Linder,G., Bishop, C. A., Eds.; 2000. Ecotoxicology of AmphibiansandReptiles. Society of Environmental Contamination and Chemistry (SETAC): Pensacola, FL, USA. pp. 461-494.

Steger-Hartmann, T., Länge, R. and Schweinfurth, H., 1999. Environmental Risk Assessment for the Widely Used Iodinated X-Ray Contrast Agent Iopromide (Ultravist). Ecotoxicology and Environmental Safety 42, 274–281.

Taves, D., Guy, W., Brey, W., 1976. Organic fluorocarbons in human plasma: prevalence and characterization. In: Filler, R. (Ed.), Biochemistry Involving Carbon– Fluorine Bonds. American Chemical Society, Washington, DC, pp. 117–134.

Taves, D.R., 1968. Evidence that there are two forms of fluoride in human serum. Nature 16, 1050– 1051.

Ter Schure, A.F., Larsson, P., Merila, J. and Jonsson, K.I., 2002. Latitudinal fractionation of polybrominated diphenyl ethers and polychlorinated biphenyls in frogs (Rana temporaria), 2002. Environ Sci Techno, 36(23):5057-61.

Thibodeaux, J.R., Hanson, R.G., Rogers, J.M., Grey, B.E., Barbee, B.D., Richards, J.H., Butenhoff, J.L., Stevenson, L.A., Lau, C., 2003. Exposure to perfluorooctane sulfonate during pregnancy in rat and mouse: I. Maternal and prenatal evaluations. Toxicol. Sci. 74, 369– 381.

Wake, D. B., 1991. Declining Amphibian Populations. Science, 253: 860.

Wetzel, L.T., 1983. Rat teratology study, T-3351, final report. Hazelton Laboratories America, Inc. Project Number: 154–160, December 19, 1983. US EPA AR-226 226-0014.

WHO, 1984d. Camphechlor. Environmental Health Criteria 45. World Health Organization,Geneva.

WHO. 1992a. Endrin. Environmental Health Criteria 130. World Health Organization, Geneva.

WHO. 1995. Tetrabromobisphenol A and Derivatives. Environmental Health Criteria 172. Geneva:World Health Organization.

WHO/ICPS, 1994a. Environmental Health Criteria 152: Polybrominated Biphenyls. World Health Organization, Geneva.

WHO/ICPS, 1994b. Environmental Health Criteria 162: Brominated Diphenyl Ethers. World Health Organization, Geneva..

Zitko, V, 1977. The accumulation of polybrominated biphenyls by fish. Bull. Environ. Contam. Toxicol. 17: 285-292.

RESEARCH PAPER

Disposition of ^{14}C-BDE 99 in *Xenopus tropicalis* after dietary exposure to tadpoles and juveniles.

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ABSTRACT

The present study was conducted to compare the disposition of ^{14}C labeled 2, 2', 4, 4', 5-pentabromodiphenyl ether (^{14}C-BDE 99) in tadpoles and juveniles of *Xenopus tropicalis*. The study was performed using whole-body autoradiography with freeze-dried or solvent extracted tissue sections and scintillation counting at different time intervals (6h, 12h, 1d, 2d, 4d, 8d, 16d, 32d and 64d) after dietary exposures of ^{14}C-BDE 99 at stage 54 (tadpoles) and stage 66 (juvenile frogs). The localization of labelled substance was similar in tadpoles and juvenile frogs. A high accumulation of radioactivity in adipose tissue and bone marrow was observed. Affinity to eye melanin was also prominent. In addition, labelled substance was seen in considerable amounts in liver, kidney and gall bladder. However, no covalent binding was observed in any tissue. Observation of labelling in the gall bladder and kidney indicates that BDE 99 is eliminated via bile and urine in frogs. There were significantly higher levels of radioactivity in juvenile frogs than in tadpoles. Furthermore, it was a significant decline of the total radioactivity in tadpoles after one day post exposure. In juvenile frogs there was no marked decline of the total radioactivity after the highest levels. Thus, the retention of the substance was higher in juveniles than in tadpoles. The retention of the radioactivity was predominantly confined to adipose tissue both in tadpoles and juvenile frogs. Lower levels of adipose tissue in the early stages in tadpoles may be one reason for the difference in retention and elimination of BDE 99 in frogs pre- and post metamorphosis.

INTRODUCTION

Polybrominated diphenylethers (PBDEs) belong to the class of brominated flame-retardants. These are compounds used in textiles, plastics, electronics and other consumer articles to prevent fire. PBDEs can have theoretically 209 congeners (based on the number and position of bromination) divided into congener groups from mono to deca BDEs. Recent reviews (Birnbaum & Staskal, 2004; Domingo, 2004; Gill *et al*., 2004; Martin *et al*., 2004) have raised concerns about these chemicals as major environmental contaminants. These concerns have prompted governments all over the world to initiate studies for generating more information regarding their significance in order to take necessary regulatory actions (Kemmlein *et al*., 2003).

Major toxic effects of PBDEs include neurobehavioral effects, effects on reproduction, endocrine disruption and carcinogenicity. Neurobehavioral effects are primarily impairment in motor behavior, reduced learning and reduction in memory process (Eriksson *et al*, 2001). Reproductive effects comprise of delay in onset of puberty, decrease in the sperm count and reduction in weight of gonads (Birnbaum & Staskal, 2004). PBDEs have a chemical structure that resembles thyroid hormones (T3 & T4). It has been shown that some PBDEs competitively bind to thyroid hormone transfer proteins, thus acting as endocrine disrupters (McDonald, 2002). Chronic exposure to deca-BDE resulted in hepatic and pancreatic adenomas in rats, whereas, a combined incidence of hepatocellular adenomas and carcinomas was seen in mice (NTP, 1986).

Several scientists have studied the fate and toxicokinetics of these chemicals in various animal models. Different tissue distribution studies on various tetra and penta BDEs in rats and mice conclude that these chemicals have highest affinity to adipose tissue followed by liver, lung, kidney and brain (Örn & Klasson-Wehler, 1998; Hakk *et al*., 2002; Staskal *et al*., 2005; Darnerud & Risberg, 2005). The studies with Penta-BDE showed that this substance had a long half-life (~23 days), suggesting potential bioaccumulation (Staskal *et al*., 2005). Studies on deca-BDE by Mörck *et al*.(2003) and Sandholm *et al*.(2003) in rats show highest concentrations of decabromodiphenyl ether (BDE 209) in plasma and other blood rich tissues like liver, adrenals, kidneys and heart whereas the concentration in adipose tissue was comparatively lower; also, deca-BDE was rapidly excreted (~3days). Faeces were the major route of excretion in all the above studies.

Total concentrations of all PBDEs have increased exponentially in the past years in the aquatic biota with predominance of tetra and penta congeners (Ikonomou *et al*., 2002). Haglund *et al*. (1997) quantified these compounds in human adipose tissue, commercial fish oils and various aquatic species of Baltic sea and concluded that these chemicals bioconcentrate in the lipid-rich tissues. Burreau *et al*. (2000) conducted tissue distribution studies of ^{14}C-BDE 47 in pike (*Esox lucius*) using whole-body autoradiography and observed the presence of

radioactivity for a considerably long duration (>65 days) with most of it stored in lipid rich tissues.

Ter Schure *et al*. (2002) studied the presence of six Polychlorinated biphenyls (PCBs) and two PBDEs (BDE 47 and BDE 99) in Common frog (*Rana temporaria*) from the Scandinavian Peninsula and reported significant levels of both PCBs and PBDEs. They suggested the possible role of these xenobiotics in the decline of amphibian populations. The decline in frog populations can be alarming because frogs form important link in the food chain between insect and vertebrate populations Further, they are inhabitants of both aquatic and terrestrial environments. Therefore, they are considered as reliable indicators of environmental health (Sparling *et al*., 2000). Decline in the frog populations all over the world has been suggested to be caused by change in climate, habitat reduction, increased UV radiation and increased exposure to xenobiotics like organohalogens (Wake, 1991).

The aim of the present study was to compare the disposition of ^{14}C labelled 2,2',4,4',5-pentabromodiphenyl ether (^{14}C-BDE 99) in metamorphosing tadpoles and juvenile frogs. Whole-body autoradiography with freeze-dried or solvent extracted tissue sections and liquid scintillation counting were used to follow the disposition of the substance in *Xenopus tropicalis* (West African clawed frog). ^{14}C-BDE 99 was administered to the frogs by dietary exposure at different development stages, stage 54 (tadpoles) and stage 66 (juvenile frogs) according to Nieuwkoop & Faber, (1994).

MATERIALS AND METHODS

Chemicals

^{14}C labeled 2, 2', 4, 4', 5-pentabromodiphenyl ether (^{14}C-BDE-99) with specific radioactivity 49 Ci (1813 GBq)/mol was kindly provided by Prof. Åke Bergman, Department of Environmental Chemistry, Stockholm University, Stockholm, Sweden. Human chorionic gonadotropin (hCG) was obtained from Sigma Aldrich Sweden AB; tricane methane sulphonate (MS 222) was obtained from Apoteket (Sweden). Other chemicals used in the study were of analytical grade and were obtained from regular commercial sources.

Animals

Xenopus tropicalis breeding was induced using human hCG. Mating was carried out by sub cutaneously administering adult male and female a primer dose of 20 IU of hCG, 48 hours prior to mating. This was followed by a dose of 100 IU immediately before introducing them to breeding chambers. Injections were made into the dorsal lymph sac by inserting the needle just beneath the skin between the dorsal lateral line stripes. Eggs obtained were maintained in 50% charcoal-filtered tap water and 50% deionized water till they hatched. Larvae (n = 500) were

randomly collected (using a plastic pipette which was cut on the tip so as to avoid injury to the larvae) and raised for the study. All animals were raised in 10 L aquaria with a density of 25 animals per aquaria. The water used was a mixture of 25% charcoal-filtered tap water with 75% deionised water. All animals were maintained at 26-27 °C and 12h day/night cycle during the entire experiment. Among the larvae that showed optimum growth, 144 were selected for the study. Seventy-two larvae were randomly selected for exposure at stage 54 the remaining 72 were further raised for later exposure at stage 66 (Nieuwkoop & Faber, 1994).

Dosing Solutions

^{14}C-BDE-99 was dissolved in 99.5% (v/v) ethanol and mixed with feed for exposure of the tadpoles. Standard tadpole feed Sera Micron (Sera, Germany) and Salmon feed (Aller 514, Aller aqua, Sweden) was used for stage 54 tadpole and stage 66 juvenile frogs, respectively. For exposure at stage 54, feed (6g) was added to 6µCi of chemical dissolved in ethanol after which ethanol was evaporated and the mixture was suspended in water to make 8ml (total volume) of feed preparation. The tadpoles were given 374 µl of the feed preparation per gram body weight. For exposure at stage 66, 15µCi of the chemical was dissolved in 300µl of ethanol. Each juvenile frog was exposed to one ^{14}C-BDE99 loaded pellet of Salmon feed. The pellet adsorbed with 6.25 µl of the ethanol solution per gram body weight.

Experimental Design

Two separate experiments were performed. In the first experiment the frogs were exposed at stage 54 and in the second at stage 66. All animals were kept individually in 400 ml water in plastic jars during the experiments. ^{14}C BDE-99 mixed with food was added in the water in each jar. The nominal exposure for each individual was 0.3 µCi (612nmol)/g (bw). Exposure was continued for 12 h until all individuals had eaten most of the food where after the water in the aquaria was changed. For exposure at stage 66 one pellet adsorbed with the chemical was dropped in each aquaria and the refusals were removed after 48h. Tadpoles were fed three times a day with Sera Micron, whereas, juvenile frogs were fed with Salmon feed once a day during the entire study. Water in all jars changed on alternate days.

For both experiments individuals (seven per sampling occasion) were sampled at 6h, 12h, 24h, 2d, 4d, 8d, 16d and 32d after exposure. At day 64 five individuals were sampled. Individuals were euthanized by transferring them to a beaker with tricane methane sulphonate (MS 222) dissolved in tap water. Wet weights of all individuals were recorded. Five individuals were dissected and eye, brain, filter apparatus (in tadpoles)/lungs (juvenile frogs), gall bladder, liver, kidney, intestine, skin, muscle and fat along with the carcass were weighed and collected for scintillation counting. In the tadpoles exposed at stage 54 lungs were sampled from day 16, gonad fat was sampled from 8d. The filter apparatus could not be detected on last three sampling occasions. Two individuals were embedded in carboxymethylcellulose (CMC) for whole-body autoradiography. At 64 days

survival time, only sampling for scintillation counting was performed. Remaining individuals were euthenised using MS 222.

Whole- body autoradiography

Tape section whole-body autoradiography was carried out as described by Ullberg (1982). Sagittal sections were taken from ventral to dorsal side of the two individuals embedded in each carboxymethylcellulose block. To investigate the distribution of nonextractable radioactivity a few sections of each sampling occasion were extracted successively with ethanol 99.5% (2 min), heptane (0.5 min, twice), ethanol 99.5% (1 min), ethanol 50% (1 min) and tap water (10 min) (Brandt & Brittebo, 1989). Sections were exposed to X-ray films (Structurix; Agfa-Gevaert N.V., Belgium) for approximately 2 months. Sections were handled at –20°C during the entire procedure until development, except for the extracted sections.

Sample Analysis

Tissues for scintillation counting were digested in 1-2 ml of Soluene-350 (Packard-Canberra) at 45°C for 24-72 hours. Thereafter 10 ml liquid scintillation cocktail (Hionic-Flour, Packard-Canberra) was added. The radioactivity was determined by liquid scintillation spectroscopy in a liquid scintillation counter (Tri-Carb 1900CA, Packard, Canberra).. Coloured samples were treated with 0.3-0.5ml of 35% hydrogen peroxide for bleaching (Packard Manual).

Statistical Analysis

Comparison between the scintillation results of the two experiments was done by Mann – Whitneys U test. Kruskal - Wallis test was used to see if there was significant difference between observations at different survival times within one experiment. The significance level was set at 95% ($p < 0.005$).

RESULTS

A depiction of the body weights and respective stages of development is shown in Table 1. The body weights for tadpoles exposed at stage 54 increased steadily and the individuals weighed approximately three times more at 32 days after the exposure than at the time of exposure. On day 8 of the experiment the tadpoles had developed from stage 54 to 56, on day 16 they were at stage 59. Between day 16 and 32 from the start of the experiment they were fully metamorphosed to frogs (stage 66). Body weights for the frogs exposed at stage 66 doubled (approximately) from the time of exposure to the end of the experiments. Thus the growth of the tadpoles and frogs was satisfactory and neither the chemical nor the radioactivity affected the normal metamorphosis and development of the individuals in the experiment.

Table 1 Body weights and stage in life cycle at different time intervals after exposure to ^{14}C-BDE 99.

Time after exposure	Exposure at Stage 54		Exposure at Stage 66	
	Body weight (g)	Stage	Body weight (g)	Stage
0 (At exposure)	0.267 ± 0.056	54	0.480 ± 0.080	66
6h	0.255 ± 0.055	54	0.447 ± 0.106	66
12h	0.232 ± 0.018	54	0.426 ± 0.069	66
1d	0.210 ± 0.041	54	0.424 ± 0.041	66
2d	0.211 ± 0.050	54	0.526 ± 0.096	66
4d	0.314 ± 0.042	55	0.484 ± 0.034	66
8d	0.456 ± 0.125	56	0.623 ± 0.119	66
16d	0.568 ± 0.120	59	0.794 ± 0.150	66
32d	0.330 ± 0.026	66	0.850 ± 0.196	66
64d	0.712 ± 0.102	66	0.868 ± 0.220	66

Body weights: Mean ± S.D, n = 5.

Whole-body autoradiography

Tadpoles exposed at stage 54

In autoradiograms of freeze-dried tissue sections of tadpoles killed 6 hours after the exposure a high level of radioactivity was seen in the anterior part of the intestines and the liver (Fig 1A). The middle region of the intestines showed a heterogonous labelling, whereas the distal part of the intestines showed lack of labelling. A moderate labelling was present in the gall bladder whereas lower labelling was seen in the heart-blood and blood vessels of the filter apparatus. In

the tail there was a very low heterogeneous labelling probably corresponding to some pigmented structures and blood capillaries.

Twelve hours and 1day after the exposure the autoradiograms showed high levels of radioactivity in the liver. Somewhat lower labelling was observed in the contents of the intestines, kidney and the melanin lining of the eye. Moderate labelling was present in the heart and central nervous system (CNS). Filter apparatus and tail muscles showed very lower levels of labelling.

Autoradiography of tadpoles, 2 days after exposure showed high levels of radioactivity in the eye melanin and liver (Figure 1B). Lower levels were present in the gall bladder, kidneys and contents of the intestines, which were heterogeneously labelled. Moderate levels of radioactivity were seen in tail muscles, filter apparatus and skin melanin.

Whole-body autoradiograms of tadpoles killed 4 days after of exposure showed high labelling in melanin of the eye and abdominal fat. Low levels were seen in the liver, gall bladder and kidneys whereas the skin and tail muscles showed weak labelling.

Eight days after exposure the pictures showed high levels of radioactivity in the abdominal fat and eye melanin (Figure 1C). Comparatively lower level of labelling was present in the kidney and liver and an even lower level of radioactivity was present in the rest of the body

In autoradiograms of tadpoles killed 16 days after exposure the predominant labelling was seen in the abdominal fat, the level of radioactivity in the eye melanin was lower. No radioactivity was seen in any of the other organs.

Pictures from tadpoles killed 32 days after exposure showed marked localization of radioactivity only in the abdominal fat and there was no detectable labelling in other tissues.

Whole-body autoradiograms of tadpoles killed at all survival intervals, with tissues sections extracted with organic solvents, showed no detectable labelling.

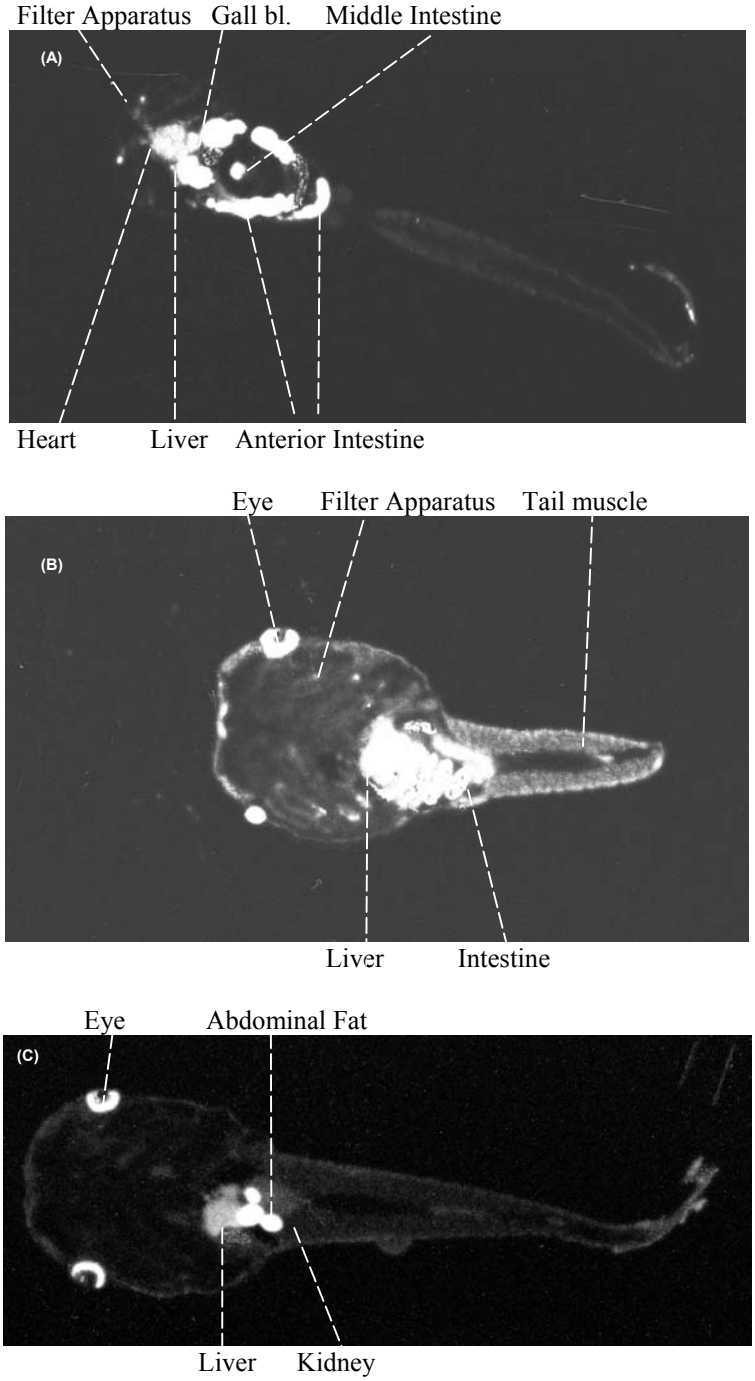

Figure 1: Whole-body autoradiograms of freez-dried sections of tadpole exposed with 0.3 µ Ci/g (bw)^{14}C-BDE 99 and killed (A) 6 h, (B) 2 d and (C) 8 d after exposure.

Frogs exposed at stage 66

Whole-Body autoradiograms of freeze-dried tissue sections of frogs killed 6 hours after exposure showed high levels of radioactivity in the abdominal fat and intestine (Fig 2A). A somewhat lower degree of labelling was seen in the bone marrow. Moderate labelling was present in the liver and the kidney. In addition a low degree of labelling was present in the gallbladder and some structures in the body probably corresponding to blood capillaries and some pigmented structures.

Autoradiography of frogs 12 hours after exposure showed high levels of radioactivity in adipose tissue e.g. in the abdomen and gonad fat. A high labelling was also seen in the contents of the intestines. A lower labelling was present in the liver and kidney. The contents of the gall bladder showed a moderate labelling. In addition a low degree of labelling was present in eye melanin, CNS and the lungs. A faint labelling was seen in muscles and skin.

In pictures of frogs 1 day after exposure the most highly labelled structures were adipose tissue in the abdomen and in gonads. The contents of the gall bladder showed also a high labelling. In addition there were considerable levels of radioactivity in the liver and kidney. In the eye melanin and the contents of the intestines there was a lower labelling. Moderate labelling was seen in the lungs and an even lower labelling in CNS and in muscles and skin.

In frogs killed 2 days after exposure the greatest labelling was seen in the abdominal fat and in the bone marrow (Figure 2B). There was also a heterogeneous labelling in the contents of the intestines. In addition a considerable labelling was seen in the eye melanin and the kidney. Lower levels of radioactivity were detected in the olfactory mucosa, the liver and the contents of the gall bladder. Some other structures in the body showed faint labelling

In autoradiograms of frogs 4 days after exposure was the highest levels of radioactivity present in the abdominal fat and bone marrow. Eye melanin showed a marked labelling. The contents of the gall bladder and the kidney had a lower labelling. A moderate labelling was seen in the liver and skin melanin. In addition there was a faint labelling present in the lungs, muscles and skin.

Autoradiograms of frogs killed 8, 16, and 32 days after exposure showed similar pictures (Figure 2C). The predominant labelling was localized to the abdominal fat and bone marrow. A marked labelling was also seen in the eye melanin. There was a faint labelling in the liver, the contents of the gall bladder and the kidney. In other tissues of the body there was no detectable labelling.

Whole-body autoradiograms of juvenile frogs killed at all survival intervals, with tissues sections extracted with organic solvents, showed no detectable labelling.

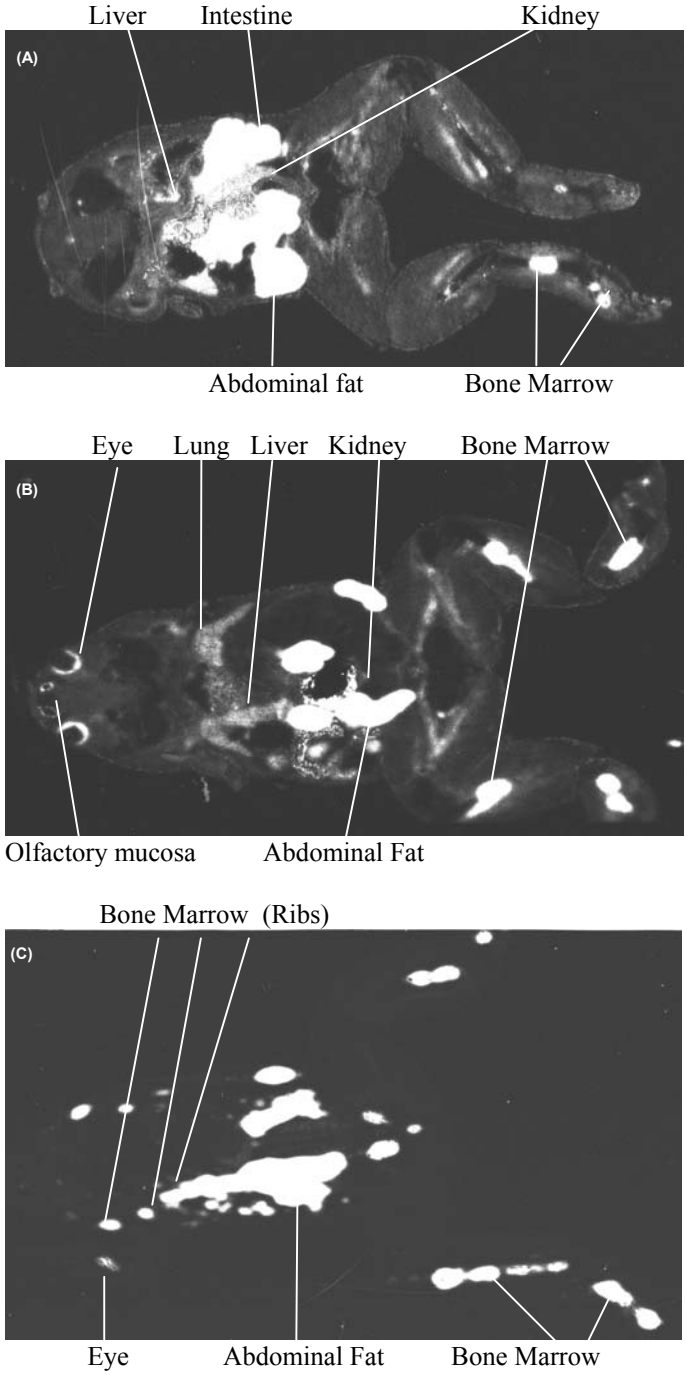

Figure 2: Whole-body autoradiograms of freez-dried sections juvenile frogs of exposed with 0.3 µ Ci/g (bw) ^{14}C-BDE 99 and killed (A) 6 h, (B) 2 d and (C) 32 d after exposure.

Table 2 Levels of radioactivity equivalents (expressed as pmol/g tissue) in various tissues observed at different survival time in *X.tropicalis* tadpoles after oral exposure of 612nmol/g (bw) of ^{14}C - BDE 99 at stage 54. Mean ± S.D, n = 5.

Tissues	Time after exposure								
	6h	**12h**	**1d**	**2d**	**4d**	**8d**	**16d**	**32d**	**64d**
Eye	1624 ± 1288	2863 ± 1356	9664 ± 3248	9558 ± 7638	4842 ± 2408	2505 ± 512	997 ± 456	610 ± 355	76 ± 68
Brain	1223 ± 847	2132 ± 1462	4727 ± 1716	1763 ± 1017	1189 ± 862	171 ± 192	143 ± 98	81 ± 105	30 ± 23
Filter apparatus	854 ± 386	1118 ± 711	2044 ± 732	902 ± 748	299 ± 218	123 ± 48	N.D.	N.D.	N.D.
Lungs	N.M.	N.M.	N.M.	N.M.	N.M.	N.M.	37 ± 26	109 ± 92	15 ± 11
Gall bladder	1742 ± 764	5367 ± 3076	4945 ± 3077	5407 ± 3411	1760 ± 1449	649 ± 339	405 ± 564	934 ± 989	88 ± 81
Liver	19586 ± 14475	23692 ± 18443	24706 ± 15123	13190 ± 11583	2739 ± 1954	1710 ± 514	324 ± 155	537 ± 846	87 ± 85
Kidney	3278 ± 2176	7148 ± 5672	11454 ± 11318	5084 ± 5285	4296 ± 3077	2775 ± 4945	454 ± 437	396 ± 415	90 ± 72
Intestine	10407 ± 7095	7583 ± 4515	11623 ± 5242	4647 ± 4351	1920 ± 1477	330 ± 127	113 ± 51	103 ± 111	34 ± 31
Muscle	786 ± 579	1039 ± 577	2142 ± 608	1157 ± 1212	743 ± 581	139 ± 59	55 ± 30	105 ± 128	41 ± 43
Skin	888 ± 670	1169 ± 557	1987 ± 1394	1839 ± 2103	522 ± 465	195 ± 87	67 ± 68	74 ± 86	8 ± 6
Gonad Fat	N.M.	N.M.	N.M.	N.M.	N.M.	40622 ± 62236	28602 ± 23304	7971 ± 9157	1745 ± 1747
Carcass	891 ± 459	957 ± 623	1953 ± 884	1148 ± 889	484 ± 352	177 ± 67	81 ± 49	87 ± 110	25 ± 22

N.D.: Not detected; N.M.: Not Measured

Table 3 Levels of radioactivity equivalents (expressed as pmol/g tissue) in various tissues observed at different survival time in *X.tropicalis* juvenile frogs after oral exposure of 612nmol/g (bw) of ^{14}C - BDE 99 at stage 66. Mean ± S.D, n = 5.

Tissues	Time after exposure								
	6h	**12h**	**1d**	**2d**	**4d**	**8d**	**16d**	**32d**	**64d**
Eye	3012 ± 4509	2122 ± 1350	4035 ± 962	2831 ± 1999	2575 ± 920	1507 ± 195	988 ± 454	1022 ± 330	2205 ± 549
Brain	734 ± 194	1475 ± 909	1894 ± 513	1557 ± 1201	1243 ± 509	356 ± 92	238 ± 122	232 ± 177	466 ± 228
Lungs	1216 ± 579	2361 ± 2034	4060 ± 3878	1803 ± 513	3826 ± 3909	1380 ± 1800	422 ± 214	1113 ± 1035	630 ± 281
Gall bladder	2276 ± 1225	8161 ± 8328	19014 ± 9347	6679 ± 5069	13547 ± 7981	1874 ± 822	3117 ± 2304	2369 ± 481	8366 ± 2323
Liver	5335 ± 4307	13001 ± 10782	10193 ± 7099	5042 ± 3285	6896 ± 6239	1567 ± 672	1989 ± 1660	1817 ± 1943	5829 ± 7297
Kidney	4507 ± 4265	11650 ± 11337	14322 ± 2770	10064 ± 7271	11714 ± 3615	6173 ± 3472	3723 ± 1924	3382 ± 1471	3494 ± 1152
Intestine	33631 ± 11310	28860 ± 28419	9536 ± 2952	1838 ± 1002	1638 ± 773	289 ± 155	328 ± 187	385 ± 112	938 ± 334
Muscle	1662 ± 799	1683 ± 1465	2363 ± 756	1471 ± 1383	1972 ± 641	921 ± 325	933 ± 568	1076 ± 405	701 ± 353
Skin	1084 ± 241	1687 ± 1123	2312 ± 770	1015 ± 714	1170 ± 431	310 ± 105	219 ± 135	191 ± 82	425 ± 196
Gonad Fat	35713 ± 24283	69595 ± 40078	270040 ± 115792	118146 ± 103913	272138 ± 113009	88615 ± 26854	67715 ± 42512	42555 ± 13076	37163 ± 25543
Carcass	813 ± 188	1544 ± 1106	2032 ± 578	1229 ± 895	1406 ± 376	589 ± 208	676 ± 414	795 ± 182	761 ± 146

Radioactivity measurements

The concentration of radioactivity in different tissues of tadpoles exposed at stage 54 is shown in Table 2. At 6 hours after the exposure the highest level was found in the liver followed by intestine and kidneys, whereas it was comparatively low in other tissues. Twelve hours after the exposure the pattern of distribution remained the same even though the concentration of radioactivity had increased approximately twice in all tissues except intestine where it showed lower levels than that observed at 6 hours. On day 1, liver had highest concentrations followed by intestine and kidney and eye, amongst other organs brain had comparatively high levels of radioactivity. After 2 days from the beginning of the experiment the highest concentrations were found in liver followed by eye. Gonad fat that was detectible and measured eight days after exposure was found to have very high concentrations of radioactivity on day 8, day 16, day 32 and day 64. Radioactivity equivalents in other tissues were reduced to trace amounts by day 64 after exposure. Concentration of radioactivity observed in the abdominal fat was 15-20 times higher than that observed in other tissues even at 64 days after exposure.

Measurements of concentrations of radioactivity in various tissues after exposure at stage 66 demonstrated the presence of the highest concentrations in the gonad fat at all time intervals after exposure (Table 3). Among the other tissues high concentrations where found in the intestines, gall bladder, kidney and liver. At 6 hours and 12 hours after exposure the intestines showed higher concentrations than the liver and the kidneys. Gall bladder, kidney and liver reached the highest concentrations one day after exposure. One day after of beginning of experiment kidney, liver and intestines showed slightly lower levels than the gall bladder. The eye showed moderate concentrations of radioactivity that remained rather constant over the whole experiment.

Percentage dose of radioactivity

In the experiment with tadpoles exposed at stage 54 the total radioactivity increased from six hours to one day with the highest levels (~28% of given dose) seen on day 1 after exposure (Figure 3). The radioactivity content in the animals was significantly ($p = 0.0005$) lowered from day 2 to day 64 after exposure.

The highest levels (~ 49% of given dose) of total radioactivity was observed in juvenile frogs 12 hours after the exposure (Figure 3). It was no significant decline in the levels of radioactivity after 12 hours although a tendency was seen ($p = 0.0885$).

Statistical analysis comparing the absorbed dose of ^{14}C-BDE in the individuals at different survival times after exposures at stage 54 and stage 66 revealed that there were significantly higher levels of radioactivity ($p < 0.0001$) in the frogs exposed at stage 66 than in the tadpoles exposed at stage 54 (Figure 3).

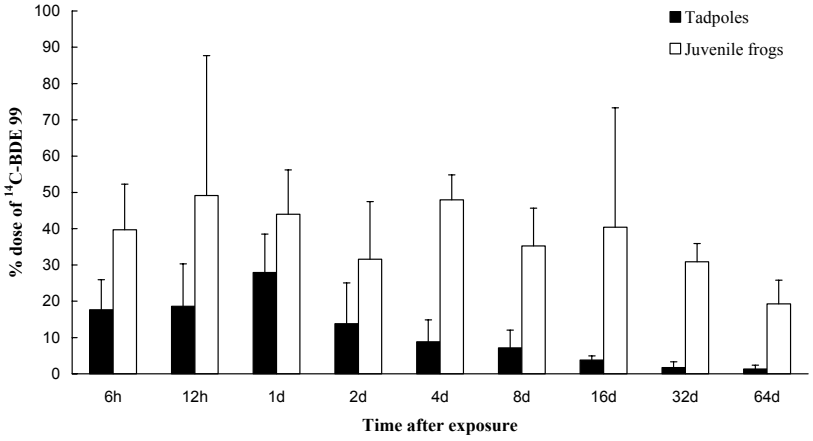

Figure 3: Percentage of radioactivity from whole individuals obtained at different time intervals after oral exposure of ^{14}C-BDE 99.
Mean ± S.D., n = 5.

DISCUSSION

The results from this study suggest that there is a significantly difference in uptake and retention of BDE 99 between the exposures at stage 54 and 66. The highest levels of radioactivity were approximately 28% and 49 % of given dose at stages 54 and 66, respectively, concluding that the juvenile frogs had a higher uptake of the chemical than the tadpoles. The reason for difference in uptake cannot be explained by the observations of the present study. However, the foraging behaviour as well as physiology and morphology of the feeding and gastrointestinal systems differ totally between the two stages (Paula, 2000). Conceivably this may cause the observed difference in uptake between the different stages.

The retention of this lipophilic chemical is probably related to the fat content in the body. The highest levels of radioactivity were present in the adipose tissue in both tadpoles and juvenile frogs. It was noted that reduction of the radioactivity in the whole body of the tadpoles from 4 days to 8 days after exposure at stage 54 was considerably lower than the reduction in all other tissues, except for fat and eye melanin. In *Xenopus* tadpoles the gonads possess rudimentary fat bodies that start accumulating adipose substance at stage 51 and these are provided with adipose cells at stage 57 (Neiuwkoop & Faber, 1994). Further, the secretion of thyroxin, which activates lipogenesis in liver and other tissues (Paula, 2000),

begins at stage 54 (Shi *et al*., 1996) and the thyroid gland is completely developed by stage 56 (Neiuwkoop and Faber, 1994). During the first days after exposure tadpoles were remaining in stage 54, therefore, they had low amounts of adipose tissue for redistribution and accumulation of the chemical. The affinity of this chemical to lipid rich tissues (Staskal *et al*., 2005; Darnerud & Risberg, 2005) and the fact that adipose tissues increases in amounts at later stages in developing tadpoles support the observation of more rapid elimination in tadpoles than in juvenile frogs. In the juvenile frogs, fat tissue and bone marrow showed the highest levels of

radioactivity. This was evident even 64 days after exposure. The presence of radioactivity in the bone marrow is most likely due to the presence of fat in the bone marrow (Cotran *et al*., 1994). In addition, the bone marrow develops as the limb develops gets calcified after metamorphosis (Carey & Bryant, 1995) suggesting the reason for higher retention in juvenile frogs than tadpoles.

High levels of radioactivity were seen in liver after exposures both at stage 54 as well as 66. This may be explained by a high blood flow transporting the substance to the liver leading to high concentrations in this tissue. Tadpole liver had higher (approximately two to four times) radioactivity than juvenile frog liver at initial survival times. It was also observed that the radioactivity from liver of tadpoles was eliminated more rapidly than from liver of juvenile frogs. Therefore, it can be inferred that due to lower availability of adipose tissue in tadpoles high proportion of the chemical was transported to liver, metabolized and excreted rapidly. The possibility of passive partitioning of BDE 47 in liver due to the lipid content has been discussed by Burreau *et al*. (2000), however the authors concluded that active hepatic accumulation followed by metabolism was responsible for uptake and excretion of the chemical in liver.

Radioactivity in considerable amounts was observed in bile and kidneys at different survival intervals after exposure in both tadpoles and juvenile frogs. Örn & Klasson-Wehler (1998) compared the disposition and excretion of BDE 47 between rats and mice and reported that for rat the major excretory pathway was faeces whereas for mice it was urine. Hakk *et al*. (2002) observed that BDE 99 in rat was excreted in higher amounts via faeces and bile than via urine. In the present study no substantial conclusion can be made with regard to excretion because of the inability to collect urine and practical difficulty in collecting faeces in this aquatic species. However, the presence of radioactivity in bile and kidneys in this study indicates that BDE 99 is excreted both via the urine and bile in frogs.

Whole body autoradiography revealed the presence of high levels of radioactivity in the melanin of eye capsule and skin. Further, it was observed from the extracted sections that the chemical did not bind irreversibly to tissue melanin. A similar observation was made by Burreau *et al*. (2000) where the tissue distribution of BDE 47 was studied in pike using whole body autoradiography. In that study the author suggested the binding to be by electrostatic and van der Waal's forces. A

similar possibility can be suggested for the present study. Larsson *et al*. (1988) first hypothesised melanin binding of hydrophobic xenobiotics by this mechanism.

The presence of radioactivity in other tissues may be due to affinity of the chemical to lipids or pigmented structures in tissues. The observation of radioactivity in the olfactory mucosa cannot be explained from this study. However, a localization of radioactivity in the nasal mucosa after intravenous injection of ^{14}C- BDE 47 has been reported in mice (Darnerud & Risberg 2005).

There was no covalent binding observed from autoradiograms of extracted tissue sections. From this it can be suggested that there is no covalent binding of BDE 99 or its metabolites in these frogs. The bioaccumulation of the substance in different tissues is probably due to the fat solubility only. Burreau *et al.* (2000) observed very low amount of irreversible binding of BDE 47 in pike but the authors were doubtful about it.

From the present study it can be concluded that BDE 99 is eliminated rapidly from metamorphosing tadpoles whereas it was retained for a longer period in the metamorphosed frogs. An explanation for this is the lower levels of adipose tissue in the tadpoles. The activation of lipogenesis and storage of energy in the form of fat is thyroxin dependent in frogs (Paula., 2000) and thus the fat tissue appears at later stages of development. In addition, BDE 99 has been suggested to interfere with thyroid hormone homeostasis by competitively binding to the transthyretin (Hakk *et al*., 2002). All the above factors suggest that if tadpoles were exposed to concentrations that can disrupt thyroxin function there would be a delay in metamorphosis. On the other hand it appears that the bioaccumulation would be prominent after metamorphosis. Further studies on dose dependent effect of BDE99 on frogs are suggested. This might help to understand the risk posed by this chemical to the amphibian population.

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REFERENCES

Birnbaum, L. S. and Staskal, D. F., 2004. Brominated flame-retardants: cause for concern? Environ Health Perspect, 112(1): 9-17.

Brandt, I., Brittebo, E.B., 1989. The use of autoradiography as a tool to study enobiotic metabolism. In: Hutson, D.H.,Caldwell, J., Paulson, G.D. (Eds.), Intermediary Xenobiotic Metabolism in Animals. Methodology, Mechanisms and SigniÆcance. Taylor & Francis, London New York, Philadelphia, pp. 295-314.

Burreau, S., Broman, D., Orn, U., 2000. Tissue distribution of 2,2',4,4'-tetrabromo[14C]diphenyl ether ([14C]-PBDE 47) in pike (Esox lucius) after dietary exposure--a time series study using whole body autoradiography. Chemosphere, 40(9-11): 977-85.

Carey, C. and Bryant, C.J., 1995. Possible interrelations among environmental toxicants, amphibian development, and decline of amphibian populations. Environ Health Perspect, 103(4): 13-7.

Cotran, R.S., Kumar, V., Robbins, S.L., 1994. Robbins Pathologic Basis of Disease. 5th edition. Philadelphia, W.B. Saunders, pp. 585-586.

Darnerud, P. O. and Risberg, S., 2005. Tissue localisation of tetra- and pentabromodiphenyl ether congeners (BDE-47, -85 and -99) in perinatal and adult C57BL mice. Chemosphere, In-press.

Darnerud, P. O.,2001. Toxic effects of brominated flame retardants in man and in wildlife. Environ Int, 29(6): 841-53.

Domingo, J.L, 2004. Human exposure to polybrominated diphenyl ethers through the diet. J Chromatogr A, 1054(1-2): 321-26.

Eriksson, P., Viberg, H., Jakobsson, E., Örn, U., Fredriksson, A., 2002. A brominated flame retardant, 2,2',4,4',5-pentabromodiphenyl ether: uptake, retention, and induction of neurobehavioral alterations in mice during a critical phase of neonatal brain development. Toxicol Sci., 67(1): 98-103.

Gill, U., Chu, I., Ryan, J. J., Feeley, M., 2004. Polybrominated diphenyl ethers: human tissue levels and toxicology. Rev Environ Contam Toxicol, 183: 55-97.

Haglund, P.S., Zook, D.R., Buser H-S, Hu J., 1997. Identification and quantification of polybrominated diphenyl ethers and methoxy-polybrominated diphenyl ethers in Baltic biota. Environ Sci Technol, 31: 3281-3287.

Hakk., H, Larsen, G., Klasson-Wehler, E., 2002. Tissue disposition, excretion and metabolism of 2,2',4,4',5-pentabromodiphenyl ether (BDE-99) in the male Sprague-Dawley rat. Xenobiotica, 32(5): 369-82.

Ikonomou, M. G., Rayne, S., Addison, R.F.. 2002. Exponential increases of the brominated flame retardants, polybrominated diphenyl ethers, in the Canadian Arctic from 1981 to 2000. Environ Sci Technol 36(9): 1886-92.

Kemmlein, S., Herzke, D., Law, R. J., 2003. BFR-governmental testing programme. Environ Int. 2003, 29(6): 781-92.

Larsson, P., Larsson, B.S., Tjalve, H., 1988. Binding of aflatoxin B1 to melanin.Food Chem Toxicol, 26(7): 579-86.

Martin, M., Lam, P. K., Richardson, B. J., 2004. An Asian quandary: where have all of the PBDEs gone? Mar Pollut Bull, 49(5-6): 375-82.

McDonald, T. A., 2002. A perspective on the potential health risks of PBDEs. Chemosphere, 46(5): 745-55.

Morck, A., Hakk, H., Orn, U., Klasson Wehler, E., 2003. Decabromodiphenyl ether in the rat: absorption, distribution, metabolism, and excretion. Drug Metab Dispos, 31(7): 900-7.

Nieuwkoop, P.D., Faber, J., 1994. Normal Table of Xenopus laevis (Daudin), third ed. Garland, New York.

Nieuwkoop, P.D., Faber, J., 1994a. Normal Table of Xenopus laevis (Daudin), third ed. Garland, New York. pp. 137-139.

Nieuwkoop, P.D., Faber, J., 1994b. Normal Table of Xenopus laevis (Daudin), third ed. Garland, New York. pp. 144.

NTP, 1986. Toxicology and Carcinogenesis Studies of Decabromodiphenyl Oxide (CAS No 1163-19-5) in R344/N rats and B6C3F1 mice (feed studies). NTP Technical Report Series no 309. Research Triangle Park, NC:National Toxicology Program.

Paula, F.P. Henry, 2000. Aspects of Amphibian Anatomy and Physiology. In Sparling, D. W., Linder,G., Bishop, C. A., (Eds.) Ecotoxicology of AmphibiansandReptiles. Society of Environmental Contamination and Chemistry (SETAC): Pensacola, FL, USA. pp. 71-110.

Sandholm, A., Emanuelsson, B. M., Wehler, E. K., 2003. Bioavailability and half-life of decabromodiphenyl ether (BDE-209) in rat. Xenobiotica, 33(11):1149-58.

Shi, Y.B., Wong, J., Puzianowska-Kuznicka, M., Stolow, M.A., 1996. Tadpole competence and tissue-specific temporal regulation of amphibian metamorphosis: roles of thyroid hormone and its receptors. Bioessays, 18(5): 391-9.

Sparling, D. W., Linder,G., Bishop, C. A., Eds.; 2000. Ecotoxicology of AmphibiansandReptiles. Society of Environmental Contamination and Chemistry (SETAC): Pensacola, FL, USA. pp. 1-14.

Staskal, D. F., Diliberto, J. J., DeVito, M. J., Birnbaum, L. S., 2005. Toxicokinetics of BDE 47 in female mice: effect of dose, route of exposure, and time.Toxicol Sci, 83(2): 215-23.

Ter Schure, A. F., Larsson, P., Merila, J., Jonsson, K. I., 2002. Latitudinal fractionation of polybrominated diphenyl ethers and polychlorinated biphenyls in frogs (Rana temporaria). Environ Sci Technol, 36(23): 5057-61.

Ullberg, S., Larsson, B., Tjälve, H., 1982. Autoradiography. In Biologic Application of Radiotracers (H.J. Glenn, ed.), pp. 56-108. CRC Press, Inc., Boca Raton, Florida USA.

Wake, D. B., 1991. Declining Amphibian Populations. Science, 253: 860.

Örn, U. and Klasson-Wehler, E., 1998. Metabolism of 2,2',4,4'-tetrabromodiphenyl ether in rat. Xenobiotica, 28(2): 199-211.

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