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DDA, A Water-Soluble DDT Metabolite, for Human Biomonitoring and Wildlife Exposure Surveillance

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

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in

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by

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ABSTRACT OF THE DISSERTATION

DDA, A Water-Soluble DDT Metabolite, for Human Biomonitoring and Wildlife Exposure Surveillance

by

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Doctor of Philosophy, Graduate Program in Environmental Toxicology
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The fate and distribution of DDA [2,2-bis(4-chlorophenyl)acetic acid, CAS No.:83-05-6], a water-soluble DDT [1,1,1-trichloro-2,2-di(4-chlorophenyl) ethane, CAS No.: 50-29-3] metabolite and its potential role in environmental surveillance and biomonitoring was investigated.

An analytical method for urinary DDA detection using pentafluorobenzyl bromide derivatization in human urine was developed (LOQ 10 ppb). Repeat of a 1946 human oral DDT study showed rapid DDA excretion in urine. Urine biomonitoring of Indoor Residual Spraying (IRS) program applicators demonstrated significantly higher DDA levels during spraying season (59 μg/L) than 1-month post-season (11 μg/L) and indicated low DDT exposure. DDA levels of applicators were similar to those of general U. S. population during earlier periods of DDT use. DDE does not form DDA. DDA analysis is sensitive, specific and technically simple and adaptable for measurement of low level DDT exposures in applicators or residents where DDT is used in IRS.

DDA is a fecal chemical biomarker of DDT exposure in chickens based upon DDT feeding studies (10 to 3000 ppm) in White Leghorn and ISA Brown hens. . Dose-dependent, rapid DDA excretion was observed. Blood and egg yolk DDTs

(DDT/DDE/DDD) reflected body burden. Chlortetracycline HCl (~20 mg/kg drinking water) showed gut microflora contributed to DDA formation. Chicken feces from previous IRS treated areas indicated low background levels of DDTs and DDA. Chickens may be used as a sentinel species in monitoring current environmental DDT exposure.

DDTs are present in the sediments of Southern California Bight (SCB). Only ~10% of total DDTs discharged into the SCB are accounted for using available monitoring data (sediment, water, and biota). DDE is the dominant contaminant and analysis of white croakers and gull and brown pelican feces yielded no evidence of current DDT exposure based upon DDA residues. DDA was detected in SCB sediment (up to 76 μg/kg dry weight). DDT and DDD levels at the primary wastewater outfall indicated substantial potential for DDT transformation to DDA. Still culture of SCB sediment revealed rapid DDA formation following DDT fortification. DDA formation may be important in the natural recovery of SCB and provide important insight into resolution of the DDT mass balance.

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ABBREVIATIONS AND DEFINITIONS

ATSDR: Agency for Toxic Substances and Disease Registry

Biomonitoring: direct measurement of people's exposure to toxic substances in the environment by measuring the substances or their metabolites in human specimens, such as blood or urine

CAS: Chemical Abstract Services

CDC: Centers for Disease Control and Prevention

DBP: 4, 4-Dichlorobenzophenone

DDA: 2, 2-bis (4-chlorophenyl) acetic acid

DDD: (1, 1-dichloro-2, 2-bis(p-chlorophenyl)ethane

DDE: 1, 1-bis (4-chlorophenyl)-2, 2-dichloroethene

DDT: 1, 1, 1-trichloro-2, 2-di (4-chlorophenyl) ethane

DIPEA: N, N-Diisopropylethylamine

FAO: Food and Agriculture Organization of the United Nations

IRS: Indoor Residual Spraying

GC-ECD: Gas Chromatography-Electron Capture Detector

GC-MS: Gas chromatography-Mass Spectrometry

JWPCP: Joint Water Pollution Control Plant

LACSD: Sanitation Districts of Los Angeles County

LD50: the dose that kills half (50%) of the animals tested (LD = "lethal dose")

LOD: Limit of Detection

LOQ: Limit of Quantification

MRM: Multiple Reaction Monitoring

NPDES: National Pollutant Discharge Elimination System

PFBBr: Pentafluorobenzyl bromide

POPs: Persistent Organic Pollutants

RSD: Relative Standard Deviation

RTECS: Registry of Toxic Effects of Chemical Substances

SCCWRP: Southern California Coastal Water Research Project

SCB: Southern California Bight

SIM: Selective Ion Monitoring

USAID: United States Agency for International Development

USEPA: United Sates, Environmental Protection Agency

WHO: World Health Organization

CHAPTER 1

DDT: History and Current Status

"To only a few chemicals does man owe as great a debt as to DDT. In little more than two decades DDT has prevented 500 million human deaths due to malaria that would have otherwise have been inevitable..."

--- *The National Academy of Sciences* (1970)

However, the chemical compound that has saved more human lives than any other in history, DDT, was banned by order of William Ruckelshaus, head of the newly formed Environmental Protection Agency in 1972 (EPA, 1975; Edwards, 2004). The ban answered the policy question of whether DDT should be used in the U.S.; it did not, however, answer the scientific question of whether DDT use was safe for humans or the environment. Huge political and scientific debate concerning the DDT ban and health consequences continues even now (Conis, 2010).

DDT was a very effective insecticide and was widely used in agriculture and control of vector-borne diseases during 1940s to 1970s before the developed countries banned it in the early 1970s (Metcalf, 1973). DDT saved millions of people's lives, but extensive use of DDT caused great concerns related to DDT accumulation, persistence, and potential health effects in humans and the environment. Today DDT is one of the twelve persistent organic pollutants (POPs) listed by the Stockholm Convention with the goal of global elimination or restriction of production and use (2001).

Currently DDT is sanctioned by the World Health Organization for restricted use in Indoor Residual Spraying (IRS) in malaria control (WHO, 2007). Today, one child from Africa is killed by malaria every 30 seconds (Clark, 2003). In 2009, malaria is estimated to cause about 225 million illnesses and 781,000 deaths annually (USAID, 2011). DDT application in IRS has been the most effective and

controversial strategy in battling malaria and other vector-borne diseases since its concerns in environmental contamination, human exposure and possible health effects (Roberts, 2010; WHO, 2011).

Bouwman et al. (2011) recently summarized the large body of evidence concerning the controversial use of DDT in anti-malaria campaigns. The viewpoints on DDT use in IRS were divided into three groups (Bouwman et al., 2011). The anti-DDT viewpoint wants to eliminate any production and use of DDT because of environmental and health concerns (Lewis, 2008). The centrist-DDT point of view pragmatically accepts the current need for DDT to combat malaria transmission using IRS but meanwhile recognizes the risks of DDT exposure in the immediate residential environment of millions of people (Steiner, 2009). The pro-DDT viewpoint considers DDT safe to use in IRS when applied correctly and promotes DDT to be used as IRS in malaria control where it is still effective. Even if eventually human health effects are found to be caused by DDT, these effects would be far less than those caused by malaria (Africa Fighting Malaria, 2010; Roberts et al., 1997). A most recent WHO evaluation, *DDT in indoor residual spraying: Human Health Aspects* (WHO, 2011), concluded that in general, levels of DDT exposure reported in studies were below levels of concern for health in IRS areas. The controversial status of DDT as a pesticide and environmental contaminant will continue to limit the availability of this insecticide in antimalaria campaigns. Management of control programs and public health policy may be as important as any effects of DDT itself (Personal observation, Chapter 5).

When DDT is used, it works on the malaria vector *Anopheles* through three chemical actions: spatial repellence, contact irritancy, and toxicity (Grieco et al.,

2007). Grieco et al. (2007) found low mortality for DDT at the highest concentration of 250 nmoles/cm^2 (only 15% mortality after 24 hrs) and DDT was considered to be a very poor killing agent. The effectiveness of vector disease control was attributable mostly to the spatial repellent action of DDT on house walls (Roberts and Alecrim, 1991; Roberts et al., 2000). Resistance of mosquito to DDT toxicity was reported in many studies (Hemingway et al., 2002; Hargreaves et al., 2003). However, toxicity is not the only chemical action of DDT so that resistance could not completely eliminate its usefulness (Grieco et al., 2007).

Once DDT is used, DDT residue is found in soil, water, and air, accumulated in fatty tissues of living organisms and deposited in soil and sediment with up to 10 years of half-life (ATSDR, 2002). DDT, DDE, and DDD persist in the soil for a very long time. DDT breaks down slowly into DDE, DDD and DDA, generally by the action of microorganisms. In surface water, DDT binds to particles in the water, settles, and is deposited in sediments. DDT is taken up by small organisms and fish in the water and accumulates to higher levels in fish and marine mammals (such as seal and whale fat), reaching levels many thousands of times higher than those in water (ATSDR, 2002). This process of "bioaccumulation" is frequently demonstrated, but not often directly associated with adverse effects (toxicity). DDT and its more persistent metabolite DDE have long been associated with egg shell thinning in some bird species though the mechanism has never been fully established (Cooke, 1973; EPA, 1975; Anderson et al., 1975; Lundholm, 1997). DDTs (both o, p'- and p, p'-DDT, DDD, and DDE), especially o, p'-DDT which binds to the estrogen receptor and acts as an estrogen mimic, were associated with potential endocrine disrupting effects in wildlife (Tyler et al., 1998). Exposure of

juvenile guppies (*Lebistes reticulaus*) to environmental contaminants (the fungicide vinclozolin and the persistent DDT metabolite p, p'-DDE) induced demasculinisation and reduced sperm count in adult males (Bayley et al., 2002).

Human DDT exposure (mainly from food) has been associated with many diseases, but none of them was confirmed as causation (ATSDR, 2002; WHO, 2008, 2011). The most recent review of potential adverse health effects of DDT has been published by WHO (2011). It includes discussion of evidence of immunotoxicity, diabetes, liver cancer, breast cancer, testicular germ cell tumors, thyroid hormones, fertility, menstrual cycle alterations, fetal loss in women, change in gestational age and rates of preterm birth, reduced childhood growth, and neurocognitive effects. The reviewers concluded that in terms of relevant exposure scenarios for the general population (specifically in countries using IRS), evidence to date does not point to concern about levels of exposure for any of the end-points that were assessed (WHO, 2011).

The persistence of DDT continues to promote study of health and environmental effects of DDT. In the present study its disposition in humans, animals, and the environment is a predominant theme. DDT metabolism in humans mainly goes to two end products, namely DDE and DDA (Figure 1-1). DDT is less persistent than its most important breakdown product DDE (ATSDR, 2002). On the other hand, DDT is more persistent than its water-soluble derivative DDA which is excreted very rapidly (Neal et al., 1946; Roan et al., 1971; Chen et al., 2009). Both DDE and DDA are less toxic and lack the neurotoxicity of DDT (Neal et al., 1946; Judah, 1949; Perry and Hoskins, 1950).

DDA isolated from rabbit urine was reported as the first DDT metabolite in

early DDT toxicity studies (Stohlman and Smith, 1945; White and Sweeney, 1945). DDA was demonstrated to be a non-toxic, detoxifying process since it is rapidly excreted and acted as a reduction pathway of DDT. However, soon DDA research was overlooked since the start of huge concerns of DDT in diet. Telford and Guthrie (1945) reported that administration of high levels of DDT to rats and goats produced milk lethal to animals that consumed it. The persistence of DDT and its lipophilic derivatives DDE and DDD as contaminants of the food supply and their occurrence in human adipose (Howell, 1948) and biota became centrally important public health and regulatory concerns. Furthermore, invention of gas chromatography- electron capture detector in the late 1950s advanced the sensitive and specific analysis after simple extraction of these chlorinated lipophilics to reveal the widespread environmental distribution of trace levels of DDTs (Goodwin et al., 1961). DDA, the water-soluble degradate, was largely neglected as a DDT metabolite (Heberer and Dünnbier, 1999).

Later DDT metabolism studies showed DDA formation is via conversion of DDT to DDD (Figure 1-1), a lipophilic metabolite and precursor of DDA following DDT exposure (Wedemeyer, 1967; Roan et al., 1971; Gold and Brunk, 1984). DDA was observed to be excreted in human urine within 24 h of DDT exposure (Neal et al., 1946; Roan et al., 1971; Chen et al., 2009). The rapid DDA excretion in urine following DDT exposure may be a potentially useful DDT exposure biomarker (Roan et al., 1971; Chen et al., 2009). Recently, DDA has been applied in a pilot human DDT urine biomonitoring study in South Africa and demonstrated some usefulness in estimating DDT exposure from IRS (Chen et al., 2009). Application of DDA in humans and other sentinel species may be a useful tool in monitoring IRS related

human and environmental DDT exposures.

Furthermore, formation of DDA following DDT exposure represents important DDT metabolism and detoxification. Detection of DDA in urine of occupationally exposed workers and general population demonstrated this important excretion pathway following DDT exposure in humans (Ortelee, 1958; Durham et al., 1965; Chen et al., 2009). DDA was also detected in environmental samples such as sediment and water in some recent studies (Heberer and Dünnbier, 1999; Schwarzbauer et al., 2003). Occurrence of DDA in both humans and the environment indicates a potential important role of DDA in natural recovery of DDT through DDA.

DDE is a dominant metabolite in living organisms and the environment from historical DDT exposures. DDE is a more stable than DDT and generally recalcitrant to further degradation (Stull et al., 1996; ATSDR, 2002; Jaga and Dharmani, 2003; CDC, 2009; Ventakesan et al., 2010). Although DDT and DDE are always grouped together as DDTs to represent DDT related health concerns, DDT and DDE don't share the same toxicology. DDT is an insecticide and is neurotoxic (Smith et al., 1946). DDE is a persistent DDT metabolite and represents a detoxifying process (Perry and Hoskins, 1950). DDE is at least one magnitude more effective than DDT as an androgen receptor antagonist (Kelce et al., 1995). DDT is shown to be a full estrogenic agonist while DDE only act partially as an estrogenic agonist (Soto et al., 1997). DDE appears to have been a more potent inducer of eggshell thinning than DDT (Cooke, 1973; EPA, 1975; Anderson et al., 1975; Lundholm, 1997). All these toxicological differences demonstrate a misleading conduct to group these two compounds together. Rats and human studies

demonstrated that DDE was incapable of forming DDA when DDE was fed in the diet (Peterson and Robison, 1964; Roan et al., 1971). Therefore, DDA could be used as an indicator to distinguish DDT and DDE exposure. Exposure assessment applying DDA as a biomarker could reveal current DDT exposures rather than convey the uncertainty of DDTs (DDT/DDE/DDD) exposure.

The one end of DDT story (DDE) has been extensively studied, but the other end (DDA) deserves more attention. Research on DDA in human and environment exposure scenarios may provide a better tool for DDT exposure monitoring and better understanding of the environmental fate of DDT.

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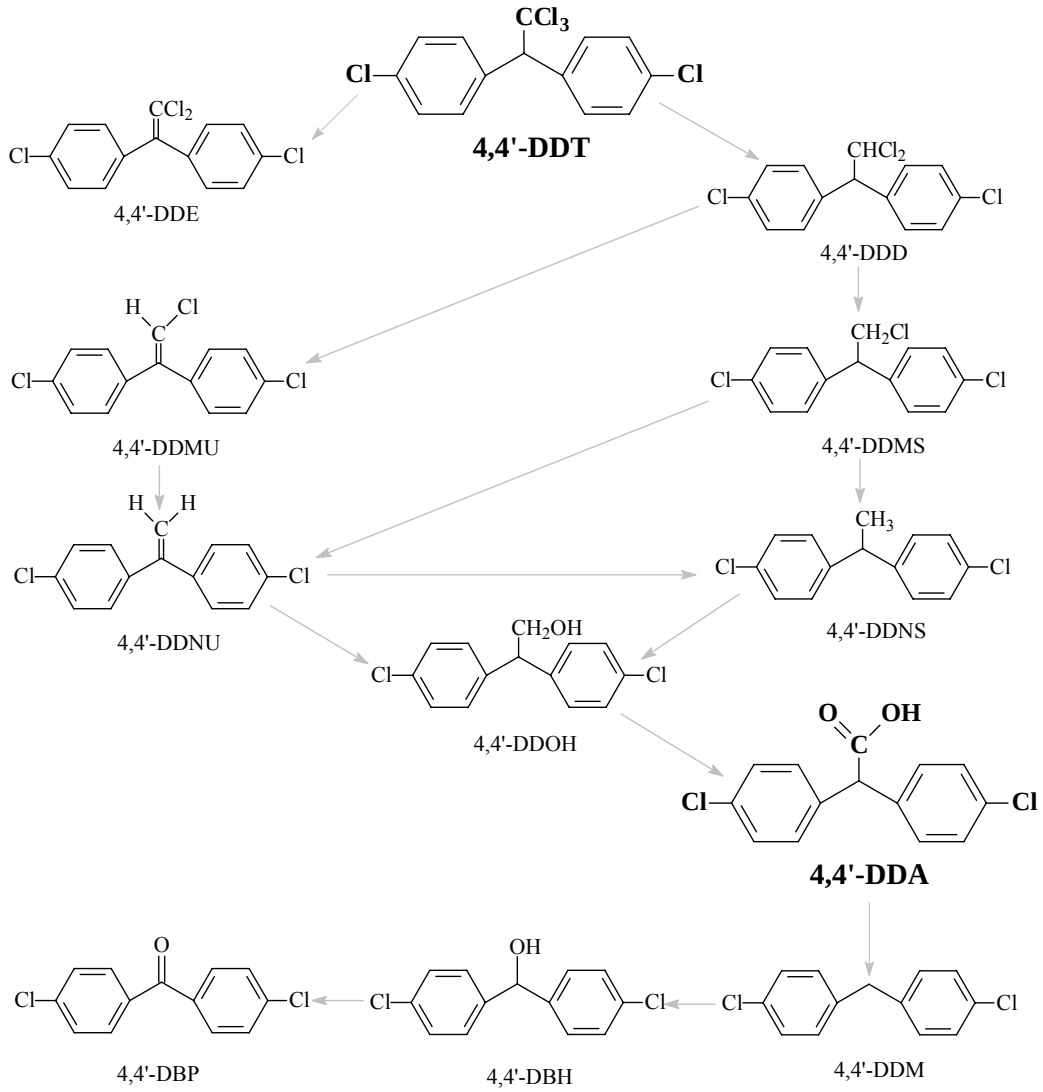

Fig. 1-1 Pathway of DDT to DDA
(Adapted from Bumpus and Aust, 1982; Gold and Brunk, 1984)

CHAPTER 2

Overview of the Fate and Distribution of DDA, A Water-Soluble DDT Metabolite and Its Role in Environmental Surveillance and Biomonitoring

Introduction

DDA [(bis(4-chlorophenyl)acetic acid] is a water-soluble DDT metabolite formed and excreted following DDT exposure that has recently been used as a biomarker of DDT exposure in applicators in Indoor Residential Spraying (IRS) in malaria vector control (Chen et al., 2009). DDA was first discovered and isolated from rabbit urine in early DDT metabolism studies in 1945 (Stohlman and Smith, 1945; White and Sweeney, 1945). Later DDA was characterized as a water-soluble DDT detoxification product in human urine (Neal et al., 1946). DDT metabolism in living organisms (Figure 1-1) includes formation of a reductive dechlorination product DDD. DDD is further degraded and readily excreted as DDA (Wedemeyer, 1967; Roan et al., 1971; Gold and Brunk, 1984). DDA has also been reported as a metabolite of DDD in mammals (Bowery et al. 1965) and insects (Plapp et al., 1965). DDE cannot be converted to DDA in living organisms (Peterson and Robison, 1964). DDA toxicity results from renal effects at very high dosages (LD50 740 mg/kg) relative to those that occur as a DDT metabolite (Koschier et al., 1980). However, DDA is generally considered non-toxic due to its rapid excretion at low urine levels (Neal et al., 1946; Roan et al., 1971; Miller, 1977; Clark, 1977; Chen et al., 2009).

DDA has been identified as a DDT metabolite in various species including humans (Neal et al., 1946; Roan et al., 1971; Chen et al., 2009), monkeys (Durham et al., 1963; Miller, 1977; Clark, 1977), rabbits (Stohlman and Smith, 1945; White and Sweeney, 1945), rats (Dale et al., 1962; Peterson and Robison, 1964), mice (Gingell, 1976; Gold and Brunk, 1982), hamsters (Gingell, 1976), dogs (Woodard et al., 1948), houseflies (Sternburg and Kearns, 1950), cockroaches (Robbins and Dahm, 1955), body lice (Perry et al., 1963), plants (Zimmer and Klein, 1972; Arjmand and

Sandermann, 1985) and microorganisms (Wedemeyer, 1967; Pfaender and Alexander, 1972) and inferred in some bird species (Abou-Donia and Menzer 1968; Ahmed and Walker, 1979; Sidra and Walker, 1980) and fish (Pritchard et al., 1973; Addison and Willis, 1978). Identification of DDA in multiple species indicates that DDA may be a common metabolite following DDT exposure (Ware et al., 1980; Heberer and Dünnbier, 1999).

DDT was detected in both human and environmental specimens following use of DDT in agriculture and vector-borne disease control (Rogan and Chen, 2005). Studies of occupationally exposed persons and the general population revealed DDA in urine at elevated and background DDT exposure levels (Hayes et al., 1956; Cueto, et al. 1956; Ortelee, 1958; Durham et al., 1965; Hayes et al., 1971). Roan et al. (1971) reported that DDT (or DDD) exposure, but not DDE, was linked with rapid DDA excretion in volunteers receiving technical DDT, DDD, DDE, and DDA. Roan et al. (1971) also observed that during DDA feeding, DDA returned to pre-dose levels within 2 to 3 days at the end of the feeding period. On the other hand, DDA returned to pre-dose levels during a prolonged period of about 4 months following termination of DDT or DDD administration to individual volunteers. This characteristic of DDT metabolism makes DDA excretion an especially valuable tool for monitoring current DDT exposure and bioavailability. Later experimental feeding studies with Rhesus monkeys (n=3) established blood levels of 470 to 850 ppb DDT and urine excretion of 500 to 1000 µg DDA/d during a 224 d, 100 ppm DDT diets feeding (Miller, 1977; Clark, 1977). DDA excretion ranged from 800 to 1400 µg/d at the end of the feeding period, but within 35 d urine excretion of DDA dropped to 50 to 150 µg/d. Because DDA derives only from DDT (or DDD) but not from DDE, urine

biomonitoring represents a potentially powerful means to assess current DDT exposure (Chen et al., 2009).

DDA has been reported in few environmental studies. DDA was quantified in surface and ground water and sediment of Teltow Canal in Berlin, Germany, a highly contaminated site (up to 9,700 μg DDT/kg dry weight sediment) where a previous chemical production plant was located (Dünnbier et al., 1997; Heberer and Dünnbier, 1999; Schwarzbauer et al., 2003; Frische et al., 2010). Up to 0.76 μg/L DDA in surface water (Heberer and Dünnbier, 1999) and 190 μg/L DDA in ground water (Frische et al., 2010) in Teltow Canal was detected. DDA was indicated as the main DDT metabolite in the ground water. Sediment DDA was reported to be the source of water contamination in the area (Dünnbier et al., 1997). Bound sediment of Teltow Canal contained up to 91,000 μg DDA/kg dry weight following alkaline hydrolysis of pre-extracted sediment (Schwarzbauer et al., 2003). Although DDA was classified as a persistent environmental contaminant in the area (Heberer and Dünnbier, 1999), the source and relative persistence of DDA compared to its lipophilic precursors has not been determined. DDA was also found to account for 52 to 93% of the total DDT residues in water but was detected rarely in the sediments of Bohai Bay and its adjacent Haihe Basin, China (Wan et al., 2005).

While DDA may be a common water-soluble DDT metabolite in plants and animals, most studies concerning the fate and transport of DDT utilize lipophilic DDTs (DDT + DDE + DDD) as a measure of DDT contamination. Easy extraction and invention and widespread availability of gas chromatography with electron capture detection (GC-ECD) in the late 1950s facilitated simple and relatively easy analysis of lipophilic DDTs (Goodwin et al., 1961). DDA in the water-soluble

portion of samples was simply “missing” in most studies. However, detection of DDA in human and environmental samples may represent an important role of DDA in the metabolism and environmental fate of DDT. The following review represents an extensive literature search (1945-2011) of words “DDA and DDT” within SciFinder, Medline, and Toxline computer databases concerning DDA occurrence in humans, environment, other living things, DDA toxicity, and DDA human and environmental applications.

Chemical identification

Chemical name: Acetic acid, bis(p-chlorophenyl)-

Synonyms: Bis(p-chlorophenyl)acetic acid; Bis(4-chlorophenyl)acetic acid; 2,2-Bis(p-chlorophenyl)acetic acid; Bis(p-chlorphenyl)essigsaeure; Benzeneacetic acid, 4-chloro-alpha-(4-chlorophenyl)- 4-Chloro-alpha-(4-chlorophenyl)benzeneacetic acid; p, p’-DDA; DDA (degradation product); Dichlorodiphenylacetic acid; p, p’-Dichlorodiphenylacetic acid; Di(p-chlorophenyl)acetic acid

CAS registry No.: 83-05-6

RTECS Number: AF5475000

Physical properties:

Physical appearance: Colorless

Molecular weight: 281.14

Molecular formula: C14H10Cl2O2

Molecular structure: $(C6H4Cl)_2$-CH-COOH

Density: 1.373 g/cm^3 (Predicted)

M.P.:167-168 ºC

F. P.: 202.6 ± 25.9 °C (Predicted)

pKa: 3.6

Water solubility: soluble when pH > 2.0

Bioconcentration factor (25 ºC): 1380-1090 (pH 1-3); 370 (pH 4) (Predicted)

Koc (25 ºC): 6160-4840 (pH 1-3); 1650 (pH 4) (Predicted)

Toxicity:

The TDLo - Lowest published toxic dose for rat is 250 mg/kg (Reproductive - Specific Developmental Abnormalities - urogenital system)

LD50-740 mg/kg (male rat, oral), 600 mg/kg (female rat, oral)

Occurrence of DDA as a DDT derivative in the environment

DDA is rarely detected in the environmental samples. As a water-soluble compound, acidification and derivatization have to be done to extract and analyze samples containing DDA (Heberer and Dünnbier, 1999). In contrast, easy processing and detection of lipophilic DDTs (DDT/DDD/DDE) reveal the existence of these compounds in the environment (Goodwin et al., 1961; ATSDR, 2002). Persistence, bioaccumulation, and potential toxicity of lipophilic DDTs draw virtually all the attention concerning DDT residues and DDA is largely neglected as a potentially important environmental DDT degradate and contaminant (Heberer and Dünnbier, 1999; ATSDR, 2002).

Only recently has DDA been quantified in water and sediment. DDA was first detected in surface and ground waters downstream from a previous DDT manufacturing plant in Teltow Canal in Berlin, Germany (Dünnbier et al., 1997; Heberer and Dünnbier, 1999). Up to 9,700 μg DDT/kg dry weight was found in the

sediment of Teltow Canal (Schwarzbauer et al., 2003). Analyses of surface water samples taken from the canal showed that DDA could leach by bank filtration through the subsoil into the ground water aquifers (Dünnbier et al., 1997). DDA level in the surface water was up to 0.76 μg/L in Teltow Canal (Heberer and Dünnbier, 1999). A more recent study revealed levels as high as 190 μg/L DDA in ground water in the same area and indicated DDA as the main DDT metabolite in ground water (Frische et al., 2010). Since DDA accounted for more than 60% of total DDT residues in the water of Teltow Canal, the author indicated other DDT-contaminated superfund sites should also contain DDA in their systems (Heberer and Dünnbier, 1999). Sediment DDA was reported to be the source of water contamination in Teltow Canal (Dünnbier et al., 1997). Following alkaline hydrolysis of pre-extracted sediment, significant amount of DDA was found to be bound to sediment of Teltow Canal at levels up to 91,000 μg/kg dry weight (Schwarzbauer et al., 2003). A significant proportion of DDA was bound to the macromolecular organic matter by ester bonds based upon enhanced release of DDA after alkaline hydrolysis. The alkaline hydrolysis step seems to be crucial to release DDA residue in the bound, non-extractable sediment (Schwarzbauer et al., 2003). Unfortunately alkaline hydrolysis is not usually performed in DDT residue extraction of sediments (Schiff, 2000; Wan et al., 2005; Yu et al., 2011) and information on DDA occurrence is not available as a consequence. Significant amount of dichlorobenzophenone (DBP) (up to 42,000 μg/kg), a generally considered terminal DDT metabolite (Figure 1-1), was also detected in the sediment of Teltow Canal (Schwarzbauer et al., 2003). Detection of DBP can be considered as further evidence of DDA formation since DDA is on the pathway to form DBP as suggested in other research (Wedemeyer,

1967; Pfaender and Alexander, 1972).

Microbial biodegradation may contribute to the formation of DDA in sediment since significant amounts of DDD (up to 130,000 μg/kg), a known precursor of DDA (Wedemeyer, 1967; Roan et al., 1971; Gold and Brunk, 1984), were also found in the sediment (Schwarzbauer et al., 2003). In a separate study, DDA was found to account for 52 to 93% of the total DDT residues in water, but it was detected rarely in the sediments of Bohai Bay and its adjacent Haihe Basin in China (Wan et al., 2005). Addition of zero-valent iron has enhanced DDT degradation in contaminated lake sediment but had little effect on production of DDA (Eggen and Majcherczyk, 2006).

Degradation of DDT in soil usually proceeds by two routes depending on the existing environmental conditions. Under anaerobic conditions the first and major biotransformation product of DDT is DDD. Guenzi and Beard {1967) incubated ^{14}C-DDT with soil and reported that DDT was dechlorinated to DDD in anaerobic soil cultures. DDD can be further degraded to some polar metabolites including DDA. DDA is usually formed in a small amount and can be further degraded to DBP, the generally recognized terminal DDT metabolite (Guenzi and Beard 1967; Mitra and Raghu 1988; Xu et al., 1994; Boul, 1996). In contrast, DDT is dehydrochlorinated to produce predominantly DDE under aerobic soil conditions (Boul, 1996).

Occurrence of DDA in living things

DDA is identified in a variety of living organism studies as an important breakdown product of DDT metabolism. DDA was found in microorganisms, higher animals, and its formation has been inferred in birds and fish.

Microorganism

Study of DDT metabolism in microorganisms was driven by mainly two forces. Earlier research of DDT metabolism in rats demonstrated that DDD, a precursor of DDA, was formed in the intestine due to gut microflora metabolism instead of liver microsomal enzyme systems (Mendel and Walton, 1966). Further breakdown products including DDA may be formed in the intestine by microflora. Since DDT persists in the soil and sediment for long time, it is also important to know which microorganisms could metabolize DDT for bioremediation purposes (Wedemeyer, 1967).

Wedemeyer (1967) for the first time demonstrated a complete pathway of DDT metabolism from DDT → DDD→ DDMU→ DDMS →DDNU→ DDA, or DDT→ DDE under anaerobic conditions in *Aerobacter aerogenes*. DDA was later confirmed in some other microorganism species as an important DDT metabolite including *Bacillus spp.* and *E. coli* (Longlois et al., 1970), *Hydrogenomonas* (Pfaender and Alexander, 1972), and *Trichoderma viride* (Patil et al., 1970).

Recent reports on DDA detection in water (Heberer and Dünnbier, 1999) and sediment (Schwarzbauer et al., 2003) may indicate that microorganisms have played an important role in DDT biodegradation and indicate DDA is a more important degradation product of DDT in the environment than has been appreciated. Although DDA was confirmed in microorganism and environmental studies, DDA was not included in many soil and sediment DDT analysis largely due to its high water-solubility, requirement of acidification for successful liquid-liquid extraction, and binding to the particulate matter in the soil or sediment that could not be extracted using the regular extracting tool i.e. Soxhlet extraction. Sediment samples

containing DDA must be hydrolyzed to release DDA as a free, extractable fraction (Schwarzbauer et al., 2003). On this basis the extent of DDA availability in the environment may be underestimated (Heberer and Dünnbier, 1999) amongst reports concerning the lipophilic derivatives of DDT that dominate the literature.

Higher animals

DDA was the first DDT metabolite identified in early DDT metabolism studies. It was isolated in rabbit urine and identified as a water-soluble metabolite of DDT (Stohlman and Smith, 1945; White and Sweeney, 1945). Tolerance of intravenous dosages of 100 mg/kg b.w. DDA in rats demonstrated that DDA is a relatively non-toxic compound (Judah, 1949).

DDT metabolism in rabbits (Stohlman and Smith, 1945; White and Sweeney, 1945), rats (Judah, 1949), mice and hamster (Gingell, 1976) revealed DDA as a principle water-soluble DDT metabolite. Peterson and Robison (1964) recovered the following metabolites from rats given DDT orally and postulated the metabolic pathway to be: DDT → DDD→ DDMU→ DDMS→ DDNU→ DDOH→ DDA. DDE was proven not be able to convert to DDA in rats. Wallcave et al. (1974) reported urinary DDA in mice and hamster was excreted as a base labile glucuronide, and as more stable glycine and alanine conjugates. Gradually increased DDA excretion in starved rats was observed even though the DDT intake was reduced to half (Dale et al., 1962) and DDA excretion may be used to demonstrate DDT storage in body fat. Rhesus monkeys excreted DDA in their urine at 500 to 1000 μg DDA/d during a 224 d 100 ppm DDT diets feeding (Miller, 1977; Clark, 1977). DDA excretion ranged from 800 to 1400 μg/d at the end of the feeding period. Within 35

d urine excretion of DDA dropped to 50 to 150µg/d. The relatively rapid dropping of DDA in urine in response to the termination of DDT feeding indicated a potential application of DDA as a urinary biomarker of DDT exposure (Chen et al., 2009). Since DDA accounted for >99% of DDT extracted from the urine, the percent of the daily intake of DDT excreted in the urine was estimated at 2-6% (Clark, 1977) at a dosage of 5 mg/kg-d in feed. Rhesus monkeys exposed to 200 ppm DDT excreted 1.2% as DDA (Durham et al., 1963).

Levels of DDTs in general population have fallen significantly since 1970s. Total DDT concentration in breast milk fat was 2.9 µg/g in 1972 at the time DDT was banned and 0.3 µg/g in 1992 in Sweden (Rogan and Chen, 2005). In a study of people from northern Texas, the concentrations of DDT and DDE in adipose tissues decreased from 7,950 ppb in 1970 to 5,150 ppb in 1974, and then to 1,670 ppb in 1983 (ATSDR, 2002). Lipid adjusted DDE level in serum was declining from 260 ng/g in 1999-2000 to 238 ng/g in 2003-2004 in the U. S. general population (CDC, 2009).

Detection of DDA in higher animals in these experimental studies demonstrated DDT is not solely persistent. These studies showed the importance of DDA excretion in relatively short-term exposure of occupational and other highly exposed persons. The role of DDA excretion that may have in the reduction of DDTs body burden is not known. The slow decline in DDT levels in general population and in the environment may be partly from DDT degradation and excretion as DDA at levels that far below the analytical limits for detection with readily available methods (ca. 10 ppb).

Birds

Even though the effects of DDT in birds have been the subject of conjecture and extensive study knowledge of the metabolism of DDT in avian species is limited. DDA was identified in droppings of Japanese quail following an intraperitoneal injection of ^{14}C-labeled DDT at a rate of 13.4 mg/kg body wt (Ahmed and Walker, 1979). Twenty-four percent of injected DDT was present as DDA in droppings after 56 days and DDA was the major excretion product. DDA was identified in acid-released droppings in feral pigeon (*Columba livia*) following intraperitoneal injection of ^{14}C-labeled DDT at a dose rate of 1.5-2.2 mg/kg (Sidra and Walker, 1980). The rate of excretion of ^{14}C in droppings of feral pigeon was low in comparison to that was found in the Japanese quail. Of the ^{14}C in droppings, 40-47% was present as DDT, DDE, and DDD, compared with 30% in a similar study upon the Japanese quail (Ahmed and Walker, 1979). DDA formation in chickens is limited to study of feces and livers of chicks fed 100 ppm DDT (Abou-Donia and Menzer 1968). In later chicken research, high-producing laying hens fed 0, 0.1, 0.5, and 1 mg/kg DDT in diet for 16 weeks (Kan and Jonker-den Rooyn, 1978) excreted 53.5% of ingested DDT in eggs (50%) and feces (3.5%) as DDT and DDE during the steady-state period. Since 46.5% daily intake was not detected in the feces and eggs, the author suspected the rest of daily intake must have been metabolized or excreted by other routes. DDA was not included in the analysis feces in that study. As demonstrated in other species, DDA might be formed in chickens and can be an important DDT excretion pathway.

Others

DDA was also detected in some insect and plant species and inferred in fish. Study of houseflies (Sternburg and Kearns, 1950) ruled out the possibility that DDA may be an intermediate transitory product in the metabolism of DDT by susceptible flies. DDA was first demonstrated as an insect DDT metabolite in a body lice study and DDA was shown to be further metabolized to DBP (Perry et al., 1963). Less than 10% of radio-labeled DDT was excreted in feces of American cockroach as DDT, DDE, or DDA (Robbins and Dahm, 1955). Two dimensional chromatography demonstrated a compound cochromatographing with DDA and accounted for 60-90% of the polar metabolites. A second compound behaved similar to DBP when flounders were injected with 100 μg/kg radio-labeled DDT (Pritchard et al., 1973). Injection of ^{14}C-labeled DDT in rainbow trout also revealed DDA as a polar DDT metabolite (Addison and Willis, 1978).

Topical application of ^{14}C-labeled DDT to spinach and cabbage permitted the isolation of the polar metabolite DDA and an unidentified DDA-conjugate (Zimmer and Klein, 1972). Incubation of ^{14}C-labeled DDT with cell suspension cultures of wheat and soybean demonstrated formation of 1-2.5% of polar metabolites. DDA was identified as a major polar DDT metabolite of both soybean and wheat (Arjmand and Sandermann, 1985).

DDA toxicity studies

As a principle polar DDT metabolite in animals and humans, DDA was suspected to be responsible for DDT toxicity when DDT exposure occurred in the early DDT toxicity studies (Stohlman and Smith, 1945; White and Sweeney, 1945).

Soon DDA was found to be relatively non-toxic that was rapidly excreted after its formation (Neal et al., 1946; Judah, 1949; Chen et al., 2009). DDA is much less toxic than DDT and appears to contribute little to the acute toxic hazard of the parent compound (Durham et al., 1965). The acute oral LD50 of DDA is 740 mg/kg in male rats and 600 mg/kg in female rats and is in the low toxicity grade (Gaines, 1960).

DDT and DDD were demonstrated to alter gene expression in human uterine cell lines through estrogen receptor-independent mechanisms (Frigo et al., 2002). However, DDA was shown to have no effect on activator protein-1 activity (Frigo et al., 2002) and no any affinity to bind to and transcriptionally activate the human estrogen receptor (Chen et al., 1997). Potential renal toxicity of DDA was studied since DDA can be transported by the isolated renal tubules of the winter flounder, and to undergo net tubular secretion by the kidney of the winter flounder and goosefish (Pritchard et al., 1977). DDA could be potentially accumulated to toxic levels if it was concentrated to high intracellular levels (Pritchard, 1976). High concentrations of DDA could produce numerous effects on renal functions at ~10 mg DDA exposure level. However, since DDA is present at extremely low levels in man and animals, no acute effect of DDA on renal function is expected (Koschier et al., 1980). Detection of DDA in the surface and ground water in Teltow Canal in Berlin, Germany raised concerns of DDA as a potential persistent environmental contaminant (Heberer and Dünnbier, 1999; Frische et al., 2010). At this trace level in water, it is unlikely to have any human adverse effects but may cause some issues for aquatic species with continuous DDA exposure.

Knowledge of DDA disposition in humans

DDA was first reported in urine of a human volunteer who orally took a single dosage of 11 mg/kg b.w. DDT (Neal et al., 1946). Rapid excretion of DDA in urine within 24 h of DDT exposure was demonstrated (Neal et al., 1946; Chen et al., 2009). Potential application of DDA as a urinary biomarker was indicated (Roan et al., 1971; Chen et al., 2009).

DDA was measured in DDT exposed workers and the general population at the time of widely DDT use before its ban in early 1970s. Early colorimetric (Schechter and Haller, 1944) and ion exchange methods (Cueto et al., 1956) lack the sensitivity and specificity of later gas chromatographic methods (Cranmer et al., 1969; Cranmer and Copeland, 1973). An ELISA method of limited application has also been published (Banerjee, 1987). Recently a simple and sensitive method was developed using pentafluorobenzyl bromide as derivatizing agent that can detect as low as 0.1 μg/L DDA in urine (Chen et al., 2009). A survey of the status of the public's DDT exposures included DDA measurement using the ion exchange method of Cueto et al. (1956) with limit of quantification (LOQ) of about 0.02 ppm DDA. Persons who ate an average diet and lacked known occupational exposure had urine levels that ranged from ≤0.02 to 0.35 ppm. Seventy-four percent of the 79 samples analyzed were below the LOQ (Durham et al, 1965). Ortelee (1958) estimated that background levels of DDA in the general population were 0.08 ppm at about the same time. These studies demonstrated the widespread occurrence of low levels of DDA in humans exposed to DDT in the general population.

DDA in urine correlated reasonably well with DDT storage in body fat (Durham et al., 1965). Under medical supervision, pesticide applicators (Durham et

al., 1965), formulating plant workers (Ortelee, 1958), and volunteers given daily dosages of 0, 3.5, and 35 mg per person for up to 25 months (Hayes et al., 1956; 1971) excreted DDA in urine consistent with estimated levels of exposure. Hayes et al. (1956) also reported urinary DDA excretion accounted for an average of 19.0% (ranged 13.6-27.4%) of the entire ingested DDT dose in the volunteers. Excretion of DDA was maximal (up to 476 ppb in urine) within 14 h after exposure and appeared to be inhibited by the increasing levels of DDE in the blood following a single intensive exposure to DDT water-wettable powder under industrial conditions (Edmundson et al., 1969).

Roan et al. (1971) studied the temporal relationship between DDT exposure and DDA excretion in volunteers receiving technical DDT, DDD, DDE, and DDA. They clearly established that current DDT (or DDD) exposure, but not DDE, was linked with DDA excretion. DDA returned to pre-dose levels within 2 to 3 days of DDA feeding, but returned to pre-dose levels following DDT or DDD over 4 months following termination of DDT or DDD administration to individual volunteers. These characteristics of DDT metabolism make DDA excretion an especially valuable tool for monitoring current DDT exposure and bioavailability. Because DDA derives only from DDT (or DDD) to the exclusion of DDE, urine biomonitoring represents a potentially powerful means to assess ongoing DDT exposure.

Durham et al. (1965) stated that it was much easier to obtain urine samples for DDA analysis than to procure surgically the fatty tissue required to measure DDT and DDE storage levels. Measure of DDA in urine offers an obvious opportunity to assess prior human exposure to DDT, or DDD, or both (Roan et al., 1971). Recently, urinary DDA as a DDT exposure biomarker was applied in a pilot DDT applicator

urine biomonitoring in South Africa. DDA levels were higher during the spraying season than one month post season and represented low exposures relative to no adverse effect levels determined in the U. S. during earlier periods of active DDT use in the 1960s (Chen et al., 2009).

Since DDT is sanctioned for use in indoor residual spraying (IRS) in malaria endemic areas (WHO, 2007), assessment of low level DDT exposure in local applicators and residents are critical to support continuing use of DDT. DDE to total DDT ratio is currently applied by the WHO (2011) to estimate DDT exposure. Ratios of 0.8 or above in blood or fat represent old DDT exposure. In contrast to this traditional method, detection of DDA in urine provides a much simpler and more accurate means to reflect current DDT exposure (Chen et al., 2009).

Discussion

DDA appears to be a common DDT metabolite following DDT exposure in humans, other living species, and in the environment (Ware et al., 1980; Heberer and Dünnbier, 1999). Detection of DDA as a polar metabolite represents a DDT detoxifying process (Neal et al., 1946; Judah, 1949; Chen et al., 2009). DDA has been shown to be relatively non-toxic and exposure would raise little health concern at the apparent environmental levels. Extent of DDA formation in reducing environmental DDT levels is still unknown. Since most available studies didn't include DDA as a regular analyte, it is hard to predict how much DDA contributes to the overall DDT degradation in both living organisms and the environment. Detection of DDA in various species and the environment indeed demonstrates DDA as an important water-soluble DDT metabolite, though DDA has been largely

neglected as a DDT metabolite and potential environmental contaminant (Heberer and Dünnbier, 1999). More research needs to be done to fully reveal DDA's contribution to the natural reduction of DDT levels in living systems and the environment.

Since DDA is rapidly excreted following active DDT exposure, DDA could be a very useful biomarker of DDT exposure in humans and other species. DDA detection in excreta could reflect direct DDT exposure (Roan et al., 1971; Chen et al., 2009). DDA is available in non-invasive samples such as urine and feces (Neal et al., 1946; Roan et al., 1971; Chen et al., 2009). DDA is relatively stable and available in pure form (Ware et al., 1980; Heberer and Dünnbier, 1999). DDA can be detected in low biologically and environmentally relevant amounts (Heberer and Dünnbier, 1999; Schwarzbauer et al., 2003). Application of DDA in urine biomonitroing of applicators and residents exposed to DDT in IRS could be a useful tool in conducting overall DDT exposure assessment in anti-malaria campaign. Furthermore, detection of DDA in some sentinel species may reflect the status of environmental DDT contamination in IRS area and other areas with legacy DDT issues.

It is important to separate DDT and DDE in terms of their dissimilar behaviors in toxicology. Since DDE cannot be converted to DDA in living things (Peterson and Robison, 1964), DDA could be used to distinguish DDT and DDE exposure in scenarios that attribute adverse effects to DDT.

Research objectives

The objectives of this research were to investigate possible use of DDA as a chemical biomarker of DDT exposure and evaluate the occurrence of DDA as an environmental contaminant. The following studies were done to investigate

important role of DDA played in DDT reduction and biomonitoring:

1) **DDA as a chemical biomarker of human DDT exposure: method development and application in human urine biomonitoring in malaria Indoor Residual Spraying;**
2) **DDA as a chemical biomarker in chicken feces of DDT exposure: laboratory DDT chicken feeding studies to evaluate chickens as a sentinel species for study of environmental fate and transport of DDT;**
3) **Measurement of fecal DDA in chicken feces as a surveillance tool to assess current DDT exposure potential;**
4) **Occurrence of DDA with legacy DDTs in sediments and wildlife DDT-contaminated areas of Southern California Bight and Long Island, NY.**

In the first objective, a new method for the derivatization of low levels of DDA in human urine was developed. The method was applied to repeat a human oral DDT exposure study (Neal et al., 1946) with a 2 mg/kg b.w. DDT single oral dosage in a volunteer. Authentic human urine specimens from IRS area in South Africa were collected and analyzed using the method we developed to evaluate current DDT exposure status in DDT applicators in IRS (Chen et al., 2009).

In the second objective, a series of controlled chicken DDT feeding studies were conducted to investigate feasibility of using fecal DDA as a chemical biomarker of DDT exposure in chickens. Chicken feces, eggs, and blood were analyzed to fully explore chicken as a sentinel species for environmental DDT exposure. Role of gut microflora in DDA formation was evaluated in an antibiotic feeding study.

In the third objective, chicken feces from IRS areas in South Africa were collected and analyzed for DDTs and DDA. Environmental DDT exposure status was evaluated in both DDT sprayed areas and non-spray area to investigate if DDT was released from DDT IRS. Very low levels of DDT, DDE, and DDA were measured in chicken feces documenting the persistence of the chlorohydrocarbon, but the research failed to provide evidence of the source of the exposure since the scheduled 2010 applications of DDT did not occur.

In the last objective, DDA occurrence in Southern California Bight was measured for the first time. Sediment, wildlife feces and fish samples in the area were analyzed. Pilot sediment samples from Long Island, NY were analyzed and DDA occurrence in the area was confirmed.

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Chapter 3

DDA, a Water-Soluble Chemical Biomarker of DDT Exposure in Human Urine

Introduction

When humans absorb DDT [(1, 1, 1-trichloro-2, 2-bis(4-chlorophenyl)ethane], some is rapidly transformed and excreted in urine as a water-soluble metabolite DDA [2, 2-bis(4-chlorophenyl)acetic acid]. In 1945 before the extensive use of DDT, persistence and environmental dispersal of DDT and its residues were documented; DDA was isolated from urine following oral administration of DDT to rabbits (Stohlman and Smith, 1945; White and Sweeney, 1945). DDA was later characterized as a water-soluble DDT detoxification product in humans (Neal et al., 1946). DDT and DDD, (1, 1-dichloro-2, 2-bis(4-chlorophenyl)ethane) DDT's reductive dechlorinated derivative, are both insecticidal and potential sources of DDA. At about the same time, Telford and Guthrie reported that administration of high levels of DDT to rats and goats produced milk lethal to animals that consumed it (Telford and Guthrie, 1945). The persistence of DDT and its lipophilic derivatives DDE (1, 1-bis (4-chlorophenyl)-2, 2-dichloroethene) and DDD as contaminants of the food supply and their occurrence in human adipose (Howell, 1948) and biota became centrally important public health and regulatory concerns.

DDA in urine correlated reasonably well with DDT storage in body fat (Durham et al., 1965). Under medical supervision, pesticide applicators (Durham et al., 1965), formulating plant workers (Ortelee, 1958), and volunteers given daily dosages of 0, 3.5, and 35 mg per person for up to 25 months (Hayes et al., 1956 and 1971) excreted DDA in urine consistent with estimated levels of exposure. A survey of the status of the public's DDT exposures included DDA measurement using the ion exchange method of Cueto, et al. (1956) with limit of quantification (LOQ) about 0.02 ppm DDA. Persons who ate an average diet and lacked known occupational

exposure had urine levels that ranged from ≤0.02 to 0.35 ppm. Seventy-four percent of the 79 samples analyzed were below the LOQ (Durham et al., 1965). Ortelee (1958) estimated that background levels of DDA in the general population were 0.08 ppm at about the same time. These studies demonstrated the widespread occurrence of low levels of DDA in humans exposed to DDT in the general population.

Roan et al. (1971) studied the temporal relationship between DDT exposure and DDA excretion in volunteers receiving technical DDT, DDD, DDE, and DDA. They clearly established that current DDT (or DDD) exposure, but not DDE, was linked with DDA excretion. This characteristic of DDT metabolism makes DDA excretion an especially valuable tool for monitoring current DDT exposure and bioavailability. Rhesus monkeys (n=3) were fed 100 ppm DDT diets for 224 d (Miller, 1977; Clark, 1977). At the end of that period blood levels ranged from 470 to 850 ppb DDT. During the same period urinary DDA averaged 500 to 1000 µg/d. At the end of the feeding period, DDA excretion ranged from 800 to 1400 µg/d. Within 35 d urine excretion of DDA dropped to 50 to 150 µg/d. Roan et al. (1971) observed that DDA returned to pre-dose levels within 2 to 3 days of feeding DDA, but returned to pre-dose levels following DDT or DDD over 4 months following termination of DDT or DDD administration to individual volunteers. Because DDA derives only from DDT (or DDD) to the exclusion of DDE, urine biomonitoring represents a potentially powerful means to assess ongoing DDT exposure.

Early methods for measuring DDA in biological samples have varied. The Schechter and Haller (1944) colorimetric tests were used extensively in early DDT research that included DDA. An ion exchange procedure (Cueto et al., 1956), gas liquid chromatography with microcoulometric (Roan et al., 1971) or electron capture

detection following formation of methyl (Cranmer et al., 1969) or chloroethyl (Cranmer and Copeland, 1973) esters, are also available analytical procedures. Early colorimetric and ion exchange methods lack the sensitivity and specificity of later gas chromatographic methods. An ELISA method of limited application has also been published (Banerjee, 1987).

Here we report a new method for the derivatization of low levels of DDA in human urine for use in DDT exposure assessment. After mild acid hydrolysis, DDA is derivatized with pentafluorobenzyl bromide and diisopropylethyl amine for GC-MS analysis. Additionally the initial human study of Neal et al. (1946) has been replicated. A sensitive new procedure has been developed and validated using authentic urine specimens from backpack DDT applicators in Swaziland and South Africa. The derivatization procedure is sensitive and specific and can be easily performed with readily available reagents and under simple laboratory conditions. DDA analysis provides a rapid means to assess DDT exposure and bioavailability. Theses analytical procedures can also be applied to forensic and ecologic applications where DDT exposure occurs.

Materials and Methods

Human subjects research

A human subject study protocol was reviewed and approved by the Institutional Review Board, University of California, Riverside, for the conduct of this research (Appendix 1). Public health officials in Swaziland and South Africa obtained local permissions for subject participation and assurance of participant anonymity.

Chemicals

4, 4-DDA: 98.0% (Sigma-Aldrich); 4, 4-DDT: 98.6% (Supelco); 4, 4-DDE: 99.2% (Supelco); 4, 4-DDD: 97.9% (Supelco); PFBBr: 99% (Sigma-Aldrich); DIPEA: 99% (Sigma-Aldrich); n-hexane: 99.9% (Fisher Scientific); ethyl acetate: 99.9% (Fisher Scientific); acetone: 99.9% (Fisher Scientific); DDT technical used in Africa: DDT 75% WP (Avima, South Africa).

Pilot oral DDT metabolism study

A human oral DDT study (Neal et al., 1946) was repeated in a single adult male (170 kg) at 2 mg DDT/kg. After 6 pre-administration complete 24 h urine collections, DDT was ingested with a morning meal of fried potatoes and whole milk. Urine collection continued for 2 weeks. Specimens were stored frozen and analyzed for DDT and DDA as described below.

Sample collection and handling

Acid-washed 250-mL Nalgene bottles were used to collect urine specimens from applicators during DDT spray season and post season in Swaziland and South Africa. The maximum urine volume was about 230 ml to avoid overfilling for freezer storage. Urine samples were stored in coolers with blue ice for FedEx transport to the U. S. The samples were ice-cold when received and were in good condition. Samples were weighed and their condition recorded. Sample weight was made up to 200 g with deionized water if the original weight was less than 200 g.

Each urine specimen was divided into eight 25 ml subsamples and a 5 ml aliquot was taken for creatinine measurement. One subsample was analyzed for

DDA on the day the urine specimens were received and processed. All the other subsamples were stored frozen until further analysis.

DDT/DDD/DDE and DDA extraction

DDT/DDD/DDE: The pH of an 8 ml aliquot of urine was adjusted to above pH 10 by addition of 5 N KOH. Sodium chloride was added to help minimize emulsification. The aqueous phase was extracted 3 times with 8 ml n-hexane and the organic extract dried over anhydrous sodium sulfate. The organic extract was concentrated under nitrogen to less than 5 ml and transferred to an 7-mL vial. The organic extract was evaporated to dryness under nitrogen and redissolved in 0.4 ml n-hexane for GC analysis.

DDA: The pH of the resulting aqueous phase from above was adjusted to less than pH 2 using 6 N HCl. The aqueous phase was extracted 3 times with 8 ml n-hexane and the organic extract dried over anhydrous sodium sulfate. The organic extract was concentrated under nitrogen to less than 5 ml and transferred to an 7-mL vial. The organic extract was evaporated to dryness under nitrogen and prepared for DDA derivatization.

DDA derivatization

Because of the thermal instability and the low volatility of organic acids, compounds like DDA must be derivatized for GC analysis (Boucharat et al., 1998). In our method, 400 µl PFBBr (2% in n-hexane v/v) and 200 µl DIPEA (2% in n-hexane v/v) were added to the dried n-hexane extracts of acidified urine. After 1 h at room temperature, the reactants were evaporated to dryness under nitrogen and

redissolved in 0.4 ml ethyl acetate for GC analysis.

GC-ECD analysis of DDT/DDD/DDE and DDA PFB-ester

GC-ECD analysis was done using a Hewlett-Packard HP 5890 gas chromatograph and a split/splitless injector operating in the splitless mode. The operating temperature of the injector was 250ºC. Chromatographic separation was performed on a HP-5ms capillary column (30 m × 0.32 mm i.d. × 0.25 μm film; Agilent Technologies, Inc. USA). Helium was used as carrier gas and nitrogen as the make-up gas. The initial column temperature of 50ºC was raised at a rate of 30ºC/min to 180ºC and then increased at a rate of 5ºC/min to 220ºC. Finally the temperature was raised by a rate of 1ºC/min to 250ºC. The detector temperature was 280ºC. The injection volume was 1 μl.

GC-MS analysis for DDA PFB-ester

GC-MS analysis was done using an HP 6890 gas chromatograph with a Hewlett-Packard HP MSD 5973 mass spectrometer in electron impact ionization (EI) mode. EI mass spectra were obtained at ionization energy of 70 eV. The MS transfer line temperature was maintained at 280ºC. Injector temperature was 250ºC. Injection (1 μl) was done in the pulsed splitless mode at a pressure of 45 psi. The pulse time was 1.5 min. Helium was used as carrier gas with constant flow of 1.0 ml/min. Chromatographic separation was performed on a HP-5ms capillary column (30 m × 0.25 mm i.d. × 0.25 μm film; Agilent Technologies, Inc. USA). The initial column temperature of 50ºC was increased at 15ºC/min to 300ºC and held constant for 10 min. For quantification of DDA PFB-ester, the GC-MS was operated in a

selective ion monitoring (SIM) mode. The characteristic ion m/z 460 $[M]^+$ was used as quantitative ion, m/z 235 and 237 were used as qualitative ions.

Results

Method development

The DDA derivatization scheme is shown in Figure 3-1. This differs from a previously published procedure in the use of DIPEA instead of triethylamine as catalyst and by a 1 h reaction period at room temperature instead of 110ºC for 1 h (Heberer and Dünnbier 1999). Under the conditions used here, recovery studies were done by spiking 10, 50 and 100 ppb DDA in 8 ml control human urine specimens. The mean recovery ranged from 76 to 84% with a relative standard deviation (RSD) of 11 to 13% (Table 3-1).

The limit of detection (LOD) for DDA was in the range of 0.1 µg/L urine by GC-ECD and 2 µg/L urine by GC-MS. For reproducibility of the GC-MS method, 5 aliquots from the same urine sample were analyzed and yielded a RSD of 12%. The GC-MS procedure was adopted for routine analysis since it gave greater specificity and was not compromised by trace impurities.

DDT oral repeat study

DDA was identified as the most important urine metabolite of DDT in 1945 (Stohlman and Smith, 1945; White and Sweeney, 1945). After developing the DDA PFBBr derivatization procedure, we confirmed the 1946 human oral study of Neal et al. (1946). In the previous case 11 mg/kg DDT was administered to a volunteer and urine was collected and analyzed using the relatively nonspecific Schechter-Haller

method for DDA (1944). In spite of the extensive use of DDT and literally ubiquitous human exposure to measurable residues of DDT and its degradates, very limited contemporary studies of human DDT metabolism employing specific determination of DDA have been published (Neal et al., 1946; Roan et al., 1971). The volunteer in our present study was administered with 2 mg/kg b.w. DDT. The results are shown in Figure 3-2. Peak excretions appeared after 1 or 2 days.

Neal et al. (1946) utilized the colorimetric procedures for analysis of urine and demonstrated maximum excretion (4 mg DDA) on Day 2 following administration of DDT. Approximately 2% (mole %) of the dose was recovered as DDA. In the present study at a lower dosage, 0.4% of the 340 mg DDT dose was recovered as DDA and peak excretion occurred during the first 24 h following administration. The specificity of the analysis probably contributed to the sharper excretion profile. Prolonged, low-level DDA excretion was measured during the 14 d post administration period. No other analytes were detected in urine. In both cases, DDA excretion appeared within 24 h of exposure, consistent with the reports of Roan et al. (1971) who studied persons with continuing chlorinated hydrocarbon exposure. Rapid reduction of DDA excretion following termination of DDT exposure is a feature of DDT metabolism that is extremely important support for the use of DDA excretion in occupational and residential biomonitoring.

DDT applicator urine surveillance study

The urine levels of DDA and DDT in occupationally exposed persons are summarized in Table 3-2. Complete results of DDA and DDT residue in urine are shown in Appendix 2. Four separate sets of urine specimens were collected.

Swaziland-1 was collected from less experienced DDT applicators and Swaziland-2 from applicators with multiple years of experience. Both sets of urine specimens were obtained during the spray season. The mean DDA excretion of less experienced applicators was significantly higher than that of more experienced applicators ($p < 0.05$). This observation warrants further study and may contribute to the usefulness of biomonitoring during applicator training.

An additional set of urine specimens provided by active DDT applicators was designated KwaZulu-Natal-3 in Table 3-2. The mean DDA excretion of applicators during the spray season (collections 1, 2 and 3) and the mean excretion of a separate group, post season (KwaZulu-Natal-4) were 59.1 µg/L (range 3.6-407 µg/L, median 30 µg/L) and 10.6 µg/L (range 0.5-44 µg/L, median 4.7 µg/L), respectively. A two-sample t-test was applied and the seasonal difference in DDA excretion was statistically significant ($p < .05$). The urine DDT excretion in these urine specimens was relatively stable at a very low level (0.24-2.78 µg/L, median 0.52 µg/L). The urine levels must be compared very cautiously since they represent different groups of applicators in each case.

The DDA/DDT mole ratio can be used to represent the relationship between the water-soluble product and its lipophilic precursor. When DDT metabolism occurs, the portion that results in DDA represents a product that is rapidly eliminated and a putative biomarker of DDT exposure. During the spray season when DDT exposures occurred, the corresponding DDA/DDT ratio was 143. About one month after the spray season (in other workers) the ratio was reduced to 31. The lower level of DDA excretion represents lower DDT availability after the spray period.

Discussion

DDT metabolism in humans forms DDA, a stable, water-soluble metabolite that is a useful urine biomarker of active DDT exposure. The characteristics of DDA as a biomarker seem to make it ideal for DDT exposure monitoring and surveillance. The determination of DDA in human urine has been demonstrated under laboratory conditions. The field tests of the procedures have provided useful evidence of its application in low-level DDT exposure scenarios as encountered among applicators in anti-malaria programs. The procedures may be useful in the assessment of contemporary exposures as well.

DDA derivatization optimization

The influence of various reaction temperatures such as -20 ºC, 4 ºC, room temperature, and 60 ºC on the DDA-PFB ester recovery were investigated. There was no significant difference among different temperatures ($p > 0.05$). This turns out to be an advantage of the method in that the derivatization can be done at room temperature without using special heating or cooling facilities. The procedures may be adopted for routine DDA analysis with gas-liquid chromatography in academic, public health, and commercial laboratories.

After 1 h derivatizing reaction, the DDA-PFB ester may still be formed during frozen storage as there are trace PFBBr and DIPEA residues left in the final solution. We recommend that formation of DDA PFB-ester from both DDA standard and sample should be done at the same time so that fresh DDA PFB-ester standard can be applied in sample analysis.

DDT oral volunteer study

A 2 mg/kg b.w. DDT oral human volunteer study was done in 2007 to repeat the study of Neal et al. (1946). DDA excretion appeared within 24 h of exposure in both studies, consistent with the reports of Roan et al. (1971) who studied persons with continuing chlorinated hydrocarbon exposure. DDA excretion level maintained above pre-dose level during the 14 d post-administration period. No other analytes were detected in urine. DDA recovery of 0.4% (mole %) in the present study and approximately 2% in Neal et al. (1946) study indicated DDA formation is not a major metabolic pathway in initial stage of DDT exposure. DDA detection in urine demonstrated its potential application in urine biomonitoring since it is rapid to excrete, simple to collect and specific to analyze.

DDA detection in DDT applicator urine

Using authentic urine specimens of DDT applicators, DDA was found at higher levels during the spray season than the levels measured one month after spraying had concluded. These results must be interpreted cautiously since different groups of workers provided urine specimens in each case. The routine work practice includes 8 h/d, 5 d/wk for about half a year, therefore representing sub-chronic occupational exposure. From the greater than 2-order of magnitude person-to-person difference in daily DDA excretion a large worker-to-worker exposure variability is inferred. When detailed work practice information becomes available, it is likely that exposure reduction measures could be developed. Existing data represent low exposures relative to no adverse effect levels determined in the U. S. during earlier periods of active DDT use in the 1960s (Durham et al., 1965). The

excretion of DDA in urine after active DDT exposure can therefore be used as a promising biomarker to detect present DDT exposure in scenarios where DDT exposure happens.

These pilot studies show the feasibility of sensitive analysis of urinary DDA from low level DDT-exposed persons. The results may be useful for training and guidance for public health officials concerned about the extent and duration of DDT exposure in occupationally exposed persons and the public. DDA analysis can be an important adjunct to future DDT exposure assessment studies (WHO, 2007).

Outlook of applying urinary DDA for DDT exposure assessment in IRS

Indoor residual spray is a primary intervention for malaria control in the WHO Global Malaria Program (WHO, 2007). The Stockholm convention on persistent organic pollutants has given an exemption for the production and public health use of DDT for indoor applications to vector-borne diseases, mainly because of the absence of equally effective and efficient alternatives. It is expected that there will be a continued role for DDT in malaria control until equally cost-efficient alternatives are developed (WHO, 2007). The precise temporal relationship of the DDA/DDT mole ratio will be evaluated as a rapid and readily available indicator of DDT availability and DDA metabolism in workers and residents in future studies.

A joint FAO/WHO Meeting on Pesticide Residues (2000) undertook a reevaluation of DDT and its primary metabolites. It included storage of DDT and its lipophilic metabolites in human body fat; the presence of those residues in human milk and potential carcinogenicity; and biochemical and toxicological information including hormone-modulating effects. The role of DDA as a hydrophilic excretion

product for the purpose of biomonitoring DDT exposure was not included in this review (FAO/WHO, 2000). Likewise, the current Concise International Chemical Assessment document undergoing peer review at this time (WHO, 2007) does not include the possibility that DDT exposure may be monitored using urinary DDA excretion.

The WHO program calls for DDT use to be closely monitored (WHO, 2007). To avoid undue exposure of householders and spray operators, standard operating procedures must be in place and strictly followed. The methods and techniques for monitoring operator exposure outlined here provide a tool for monitoring DDT exposure that is relatively simple, sensitive, and proven to measure DDT exposure under conditions of use. The results reported here document the sensitivity and specificity of DDA analysis in authentic urine specimens of applicators.

Conclusions

DDT metabolism in humans yields DDA as principal urinary metabolite and potential exposure biomarker. A method for DDA analysis in human urine was developed using PFBBr and DIPEA. The limit of detection for DDA was 0.1 µg/L urine by GC-ECD and 2 µg/L urine by GC-MS; relative standard deviation of 12%.

A 2 mg/kg b.w. DDT oral human volunteer study was done in 2007 to repeat the study of Neal et al. (1946). DDA excretion appeared within 24 h of exposure in both studies, consistent with the reports of Roan et al. (1971) who studied persons with continuing chlorinated hydrocarbon exposure. DDA in urine maintained above pre-dose level during the 14 d post-administration period. No other analytes were detected in urine. DDA recovery of 0.4% (mole %) in the present study and

approximately 2%in Neal et al. (1946) study indicated DDA formation is not a major metabolic pathway in initial stage of DDT exposure. DDA detection in urine demonstrated its potential application in urine biomonitoring since it is rapid to excrete, simple to collect and specific to analyze.

Urine specimens from DDT applicators in Swaziland and South Africa were analyzed to evaluate the method. The mean DDA levels during the spray season and post-season were 59 and 11 μg/L, respectively. These results must be interpreted cautiously since different groups of workers provided urine specimens in each case. The DDA urinalysis may be a feasible monitoring strategy for low-level occupational and residential DDT exposure assessment in antimalaria campaigns.

Rapid reduction of DDA excretion following termination of DDT exposure is a feature of DDT metabolism that is extremely important to support the use of DDA excretion in occupational and residential biomonitoring.

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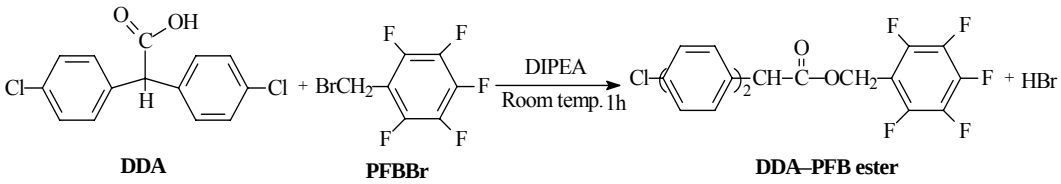
Table 3-1. Results of recovery experiments in human urines

Spike level (μg/L)	Recovery % (Mean ± SD)	Relative Standard Deviation % (n = 4)
10	81.8 ± 9.3	11.5
50	83.8 ± 10.5	12.6
100	76.4 ± 8.5	11.2

Table 3-2. Summary of DDA and DDT urine excretion in African applicators

Collection	Number of specimens	DDA (µg/L) mean ± SD	DDT (µg/L) mean ± SD	DDA/DDT mole ratio
Swaziland-1 [a]	8	28 ± 9.1	1.3 ± 0.66	33
Swaziland-2 [a]	9	14 ± 6.4	0.99 ± 0.30	19
KwaZulu-Natal-3 [b]	20	92 ± 99	0.56 ± 0.48	243
KwaZulu-Natal-4 [b]	19	11 ± 12	0.41 ± 0.15	31

[a] Swaziland-1 (less experienced applicators) and Swaziland-2 (more experienced applicators) were obtained from the same time and area during the spray season. [b] KwaZulu-Natal-3 specimens were obtained during the spray season and KwaZulu-Natal-4 specimens were obtained one month post-season from different applicators.



DDA **PFBBr** **DDA–PFB ester**

Fig. 3-1 Reaction formula of DDA derivatization

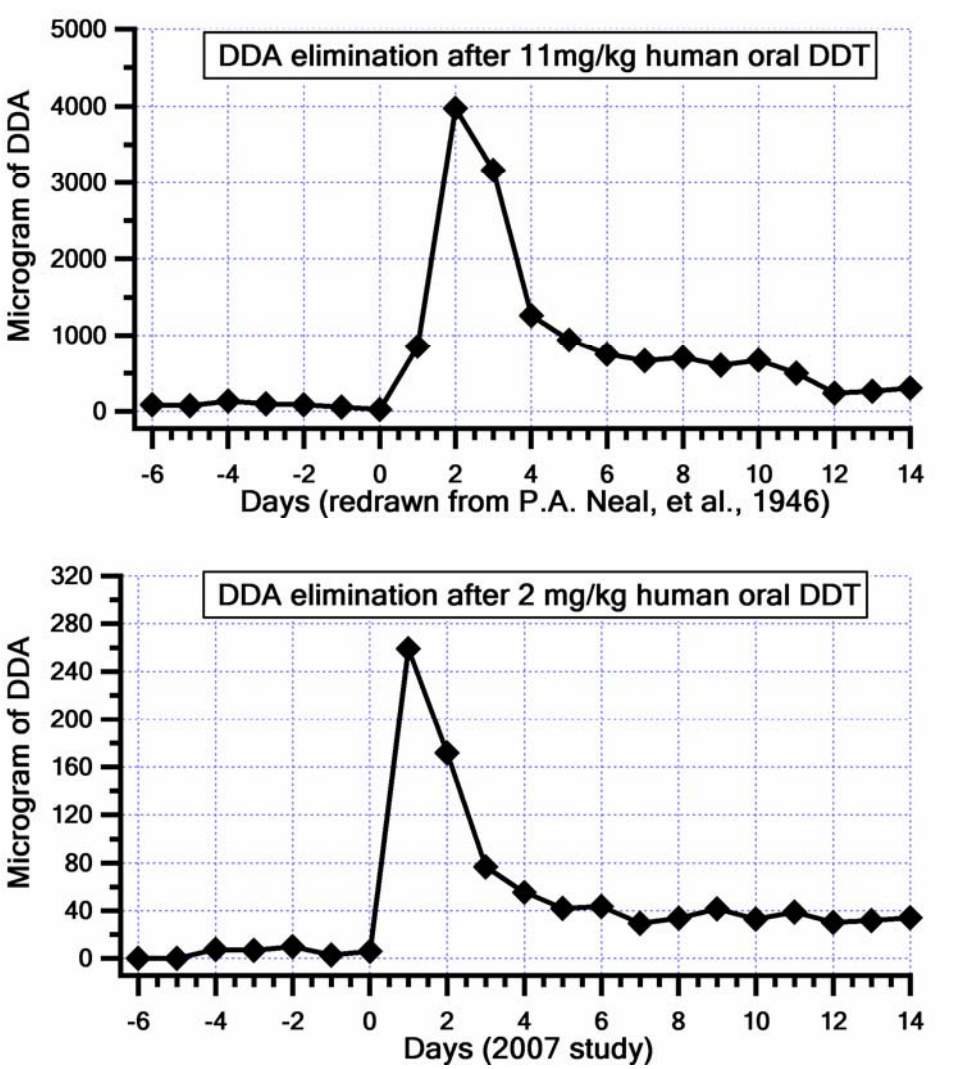


Fig. 3-2 DDA excretion in urine following oral administration of DDT in original 1946 study and in present confirmatory research.

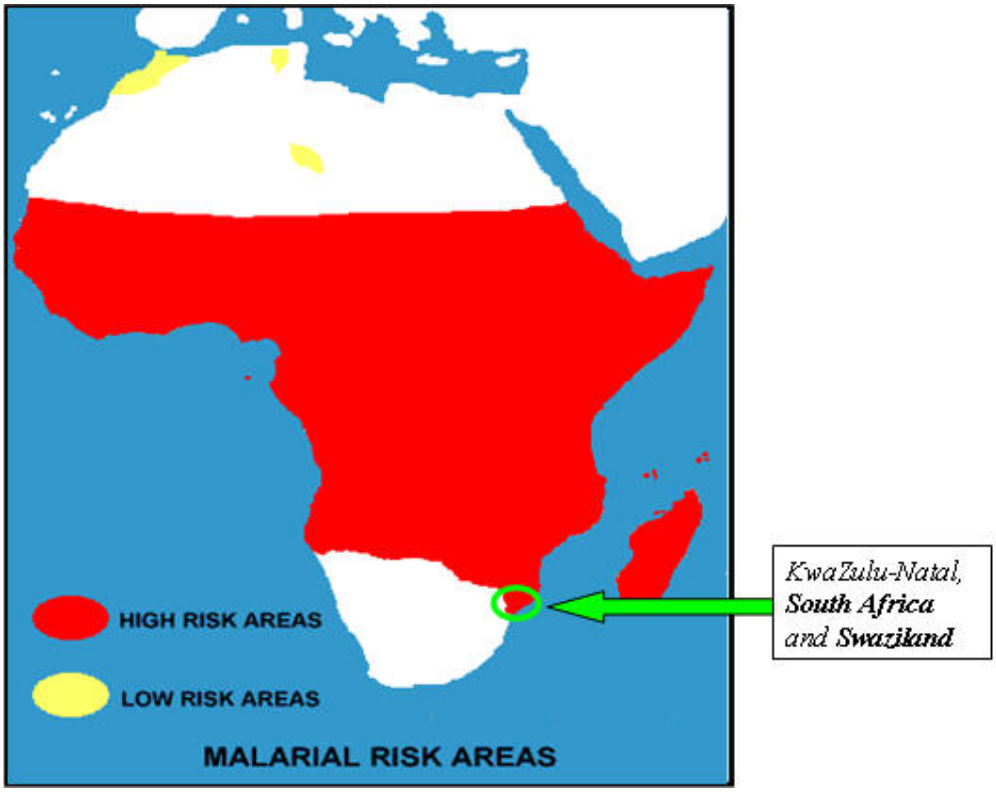


Fig. 3-3 Distribution of malaria in Africa (the arrow shows our sampling sites)

CHAPTER 4

Fecal DDA As a Biomarker of DDT Exposure in Chickens

Introduction

A specific and sensitive analytical method for DDA analysis in urine has been applied in biomonitoring of DDT applicators in a malaria control program in South Africa (Chen et al., 2009). Measurement of DDA in biological specimens may provide a simple, rapid, and useful tool to estimate current human and environmental DDT exposures. Although DDA was the first DDT metabolite discovered and isolated in 1945 (Stohlman and Smith, 1945; White and Sweeney, 1945), this water-soluble DDT detoxification product has received little consideration in fate and transport studies. DDT and its lipophilic residues DDD and DDE occur together and are frequently reported as DDTs, the sum of DDT + DDD+ DDE. DDD may be a precursor of DDE and DDA (Peterson and Robison, 1964). DDA excretion indicates current DDT exposure since it is not formed from DDE. This makes urine excretion of DDA a potentially useful biomarker of DDT exposure for occupational, residential, and environmental surveillance where there is the possibility of DDTs exposure.

As a DDT metabolite, DDA is rapidly excreted following DDT ingestion in humans (Neal et al., 1946; Roan et al., 1971; Chen et al., 2009) and Rhesus monkeys (Miller, 1977; Clark, 1977).

DDA excretion in human urine is dose-dependent (Durham et al., 1965). Rapid excretion of DDA during DDT exposure and quick decline after termination of exposure in humans and monkeys indicate that DDA can be a useful biomarker for current DDT exposure (Miller, 1977; Clark, 1977; Chen et al., 2009).

DDT can be converted to DDA in both chemical and biological processes. Conversion of DDT to DDA by pure chemical reaction under sunlight may be important to determine overall environmental fate of DDT and DDA in aqueous

systems (Ware et al., 1980). Studies of DDA distribution in living things and the environment is very limited compared to its persistent lipophilic counterparts. As a device for evaluating DDT exposure, DDA measurement may well prove simpler and more reliable than measurement of serum DDT concentrations for human exposure (Roan et al., 1971). The contribution of DDA to overall DDT degradation is uncertain. The importance of DDA as a DDT metabolite has been neglected and remains largely unknown (Heberer and Dünnbier, 1999).

The metabolic capability to form DDA from DDT has been demonstrated in humans (Neal et al., 1946; Roan et al., 1971), rabbits (Stohlman et al., 1945), rats (Peterson and Robison, 1964), monkey (Miller, 1977; Clark, 1977), and bacteria (Wedemeyer, 1967) and inferred in bird and fish (Addison and Willis, 1978; Ahmed and Walker, 1979). DDA formation in chickens has been documented in residue studies of feces and livers of chicks fed 100 ppm ^{14}C-DDT (Abou-Donia and Menzer 1968). As an important domestic fowl and a food source, chickens contaminated with DDT draw great regulatory and health concerns in DDT-sprayed areas in the anti-malaria program (Van Dyk et al., 2010).

In an early chicken research, high-producing laying hens were fed 0, 0.1, 0.5, and 1 mg/kg DDT in diet for 16 weeks (Kan and Jonker-den Rooyn, 1978). DDT residues in chicken fat and fat of eggs were dose-dependent. Egg fat level correlated well with abdominal fat level ($r>0.85$) and represented DDT body burden. Total DDT levels in fat increased as feeding continued and dropped slowly after feeding. Balance between DDT uptake and excretion was estimated by measuring DDT residues in eggs and feces after residues reached a plateau (steady-state) after about 4 weeks of feeding. Most (53.5%) of the ingested DDT was excreted in eggs (50%)

and feces (3.5%) as DDT and DDE during the steady-state period. Since 46.5% daily intake was not accounted for in the feces and eggs, the author suspected the rest of daily intake must have been metabolized or excreted by other routes (Kan and Jonker-den Rooyn, 1978). DDA was not included in the analysis. As was demonstrated here and in other species (see below), DDA might be formed in chickens and can be an important DDT excretion pathway.

Metabolism studies in other bird species have revealed DDA as a DDT metabolite. DDA was isolated in droppings of Japanese quail following an intraperitoneal injection of ^{14}C-labeled DDT at a rate of 13.4 mg/kg body wt (Ahmed and Walker, 1979). Twenty-four percent of injected DDT was converted as DDA in droppings after 56 days and DDA was the major excretion product. DDA was isolated from acid-released droppings in feral pigeon (*Columba livia*) following intraperitoneal injection of ^{14}C-labeled DDT at dose rate of 1.5-2.2 mg/kg (Sidra and Walker, 1980). The rate of removal of ^{14}C in droppings of feral pigeon was low in comparison to that found in the Japanese quail.

Roan et al. (1971) hypothesized an important fraction of biodegradation by the pathway of DDT to DDA is enteric in location by introducing DDT directly into the gut since the drop in DDA excretion was not associated with any corresponding decline in serum DDT levels. Lack of DDD in tissues of Japanese quail after death indicated substantial quantities present in droppings were produced by microorganisms within gut or within the dropping themselves (Ahmed and Walker, 1979). Gut microflora may play an important role in conversion of DDT to DDA in birds and other animals (Peterson and Robison, 1964; Barker et al., 1965; Mendel and Walton, 1966; Wedemeyer, 1967; Braunberg et al., 1968; Roan et al., 1971; Ahmed

and Walker, 1979). It is interesting to investigate possible contribution of gut microflora in DDA formation in chickens and the study may help to understand DDA formation in other species i. e. humans.

The aim of this chapter is to study the potential DDA formation and excretion in chickens following a series of dietary DDT exposures. Chicken feces, blood, and eggs will be analyzed for DDT and its selected derivatives DDA, DDD, and DDE. Feasibility of applying DDA as a DDT exposure biomarker in chickens will be investigated.

Materials and methods

Animal use

An animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee, University of California, Riverside (Appendix 3).

Housing

Adult White Leghorn (1.4 ± 0.1 kg, Appendix 4) and ISA Brown (1.9 ± 0.2 kg, Appendix 4) hens (2 years old) were maintained in a secured, shaded chicken house. Chickens were housed in individual cages (55 cm × 30 cm × 45 cm) that permitted separate daily collection of feces with minimum contamination by spilled feed. Automatic lighting maintained a 16:8 light–dark cycle. Low and high temperatures were recorded daily and observed levels in January and July, 2010, are reported here. The average low and high temperatures were 7.2 ºC (5.5 to11.1) and 17.2 (11.1 to24.4) in January. The average low and high temperatures in July were 22.2 ºC (18.9 to 23.3) and 38.9 ºC (30 to 46.7). All chickens had access to fresh standard laying

mash (100 or 150 g/d, seasonal; Kruse Perfection Brand, Kruse Grain & Milling, Goshen & Ontario, CA) and water ad libitum. An automatic mister system was operated in the summer to provide cooling for the hens when ambient temperatures exceeded 30°C.

Chemicals

Chemicals included p, p'-DDT, 98.6% (Supelco, Bellefonte, PA); p, p'-DDA, 98.0% (Sigma- Aldrich, St Louis, MO); p, p'-DDE, 99.2% (Supelco); p, p'-DDD, 97.9% (Supelco); p, p'-dichlorobenzophenone (DBP), 99.0% (Sigma- Aldrich, St Louis, MO); pentafluorobenzyl bromide (PFBBr), 99% (Sigma-Aldrich); diisopropylethyl amine (DIPEA), 99% (Sigma-Aldrich); n-hexane, 99.9% (Fisher Scientific); ethyl acetate, 99.9% (Fisher Scientific, Hampton, NH); acetone, 99.9% (Fisher Scientific), 6N HCl, 10 N KOH, 97% acetic acid; Duramycin® 10 Chlortetracycline HCl soluble powder 6.4oz (Durvet, Blue Springs, MO).

Preparation of diets and feeding

Feed containing 10, 100, 300, 1000, and 3000 ppm DDT was prepared for feeding studies. Briefly, p, p'-DDT was dissolved in 200 ml acetone and thoroughly mixed with 400 g feed using a stirring bar to make a wet mixture. The mixture was dried overnight in a hood and another 5600 g feed was added and mixed thoroughly by hand. The chickens each received 100 or 150 g of the DDT feed daily for 8 consecutive days. Fresh drinking water was provided daily. DDT and its selected derivatives (DDD, DDE, and DDA) were measured in the feces, blood, and eggs.

Study design for laboratory DDT feeding studies

A series of DDT feeding studies were conducted to investigate DDA formation in chickens following DDT exposure. Feasibility of using DDA as a chicken DDT exposure biomarker was explored using sets of 4 White Leghorn hens (Studies 1-4) or 5 ISA Brown hens (Studies 5 and antibiotic studies 1-2). DDT was administered during each study as specified in Table 4-1.

Sampling and analysis of feces

Two to 4 control fecal samples were collected before the respective DDT feeding periods. Chicken feces were collected daily on steel pans (Wilton 12" x 16" with nonstick coating) positioned beneath each cage. Feces (300-900 g/d) from the sets of 4 or 5 chickens were stored frozen in 1000-mL Nalgene bottles before further treatment. Collections from individual hens ranged from 50 to 200 g/d. Sample size was dependent upon the amount available to eliminate visible contamination by feed.

Frozen feces were thawed and blended with deionized (DI) water to make a 0.5 g/mL homogenate. A 50 g aliquot was transferred to a 250-mL Nalgene bottle and further diluted with 100 ml water. Ten ml 6N HCl was added, the bottle was closed and placed into double Ziploc bags and transferred to a water bath at 90 ºC for 1 hour. After acid hydrolysis the samples were cooled and prepared for acid-base extraction.

The pH of the mixture was adjusted to above 10.0 using 3 to 4 ml 10 N KOH. The mixture was extracted 3 times with 70 ml n-hexane. The organic layer was transferred to a 250-mL Nalgene bottle. Centrifugation was applied if separation of

the phases was not complete. Anhydrous Na_2SO_4 was added to dry the n-hexane. The n-hexane was evaporated to dryness and redissolved in 1 ml n-hexane for gas chromatography with electron capture detector (GC-ECD) analysis. Recoveries of DDT/DDD/DDE in feces were in a range of 70.3% to 84.1% (Table 4-2).

The pH of the aqueous portion was adjusted to less than pH 2.0 using 2 to 3 ml 6 N HCl. The solution was extracted 3 times with 70 ml n-hexane and the organic layer was transferred to a 250-mL Nalgene bottle. Centrifugation was applied if separation of the phases was not complete. Anhydrous Na_2SO_4 was added to dry the extract. The organic layer was evaporated to dryness and derivatized as previously described (Chen et al, 2009). In brief, 0.4 ml of 2% DIPEA in n-hexane and 0.8 ml of 2% PFBBr in n-hexane was added and reaction was maintained at room temperature for 1 h. After the reacting solution was reduced to dryness under nitrogen, the DDA derivative was redissolved in 1 ml ethyl acetate for gas chromatography with mass spectrometry (GC-MS) analysis. Recoveries of DDA in feces (n=3 for each spike level) was 70.6% at 0.005 μg/g feces and 71.3% at 0.05 μg/g feces (Table 4-2).

Sampling and analysis of blood

Whole blood (ca. 3 ml) was collected from the brachial vein on day 8 of each feeding period in chicken studies 4-5 and antibiotic studies 1-2. The specimens were refrigerated and stored in vials containing 1.5 g 4% sodium citrate anticoagulant solution.

The analysis of blood for DDT, DDE and DDD was adapted from Waliszewski et al. (1991). Briefly, 1 g of whole blood containing anticoagulant was

transferred to a 20-mL vial containing 2 ml 97% acetic acid. After 30 min to hydrolyze and liberate DDT from complexes with endogenous substances of the blood, the blood was extracted 3 times with 5 ml n-hexane. The n-hexane extract was cleaned-up by vigorously vortexing for 1 min with 1 ml conc. H_2SO_4 to deplete lipid contents and organic hydrocarbons. After removal of the H_2SO_4, the n-hexane extract was washed with 2 % Na_2SO_4 solution. Anhydrous Na_2SO_4 was added to dry the n-hexane. The n-hexane extract was evaporated to dryness and redissolved in 1 ml n-hexane for GC-ECD analysis. Recoveries of DDT/DDD/DDE in blood were in a range of 62.0% to 96.9% (Table 4-2).

DDA extraction and analysis: DDA was obtained by heating 1 g of blood and 5 ml 0.1N HCl mixture in a tightly sealed vial at 100 ºC for 1h and then extracted 3-times with 5 ml n-hexane, and derivatized as above. Recoveries of DDA in blood were in a range of 75.9% to 78.9% (Table 4-2).

Sampling and analysis of eggs

Eggs were collected daily and stored in refrigerator prior to analysis (Appendix 7).

Method for egg analysis was adopted from An et al. (2002). Egg yolk was separated from the white by hand and 1 g egg yolk was transferred to a 20-mL vial. The yolk was vortexed 3 times with 5 ml n-hexane. The extracts were cleaned-up by vigorously vortexing1 min with 2 ml conc. H_2SO_4 to delete fat content. After removal of the concentrated sulfuric acid layer, the n-hexane extract was washed with 2% sodium sulfate solution. Anhydrous Na_2SO_4 was added to dry the n-hexane. The n-hexane extract was evaporated to dryness and redissolved in 1 ml n-hexane for

GC-ECD analysis. The egg white was treated the same way except no H_2SO_4 cleanup step was needed. Recoveries of DDT/DDD/DDE in egg white and yolk were in a range of 64.6% to 86.3% (Table 4-2).

DDA was analyzed in egg yolk or egg white extracts. The samples (1 g) were vortexed with 5 ml 0.1N HCl, extracted 3 times with 5 ml n-hexane, and derivatized as described above. Recoveries of spiked DDA (in acetone) in eggs were in a range of 69.6% to 78.8% (Table 4-2).

GC-ECD analysis for DDT and selected derivatives DDD/DDE/DBP

Lipophilic DDT derivatives were analyzed using an HP 5890 gas chromatograph with a ^{63}Ni electron capture detector. Injector temperature was 250 ºC. Injection volume was 1 µl. Nitrogen was used as carrier gas with a flow rate of 1.0 ml/min. Chromatographic separation was performed on a HP-5ms capillary column (30 m × 0.25 mm i.d. × 0.25 µm film; Agilent Technologies, Inc. USA). The initial column temperature of 50 ºC was maintained for 1 min, increased at 30 ºC/min to 180 ºC, and then increased at 5 ºC/min to 240 ºC and held constant for 10 min.

GC-MS analysis of DDA

DDA derivative was analyzed using a HP 6890 gas chromatograph with a HP MSD 5973 in electron impact ionization (EI) mode at ionization energy of 70 eV. The MS transfer line temperature was 280 ºC. Injector temperature was 250 ºC. Injection (1 µl) was done in the pulsed splitless mode at a pressure of 45 psi. The pulse time was 1.5 min. Helium was used as carrier gas with constant flow of 1.0 ml/min. Chromatographic separation was performed on a HP-5ms capillary column

(30 m × 0.25 mm i.d. × 0.25 μm film; Agilent Technologies, Inc. USA). The initial column temperature of 50 ºC was increased at 15 ºC/min to 300 ºC and held constant for 10 min. For quantification of DDA PFB-ester, the GC-MS was operated in a selective ion monitoring (SIM) mode. The characteristic ion m/z 460 $[M]^+$ was used as quantitative ion, m/z 235 and 237 were used as qualitative ions.

Quality control

The recoveries of DDT derivatives (DDA, DDT, DDD, DDE, and DBP) were evaluated in chicken feces, blood and eggs to which known amounts of respective DDT derivatives were added as liquid spikes prior to sample preparation and extraction. The recoveries of DDT and selected derivatives are reported in Table 4-2. The overall recoveries ranged from 62.0% to 96.9%. Instrument detection limits (IDL) for DDT, DDD, DDE, and DBP were 0.01μg/mL on GC-ECD and 0.1μg/mL for DDA derivative on GC-MS.

DDA excretion in feces of antibiotic-treated chickens

Chlortetracycline HCl, a broad-spectrum antibiotic for poultry and livestock to control a wide range of gram-positive and gram-negative bacteria, atypical organisms such as chlamydiae, mycoplasmas, and rickettsiae, and protozoan parasites (Chopra et al., 2001), was mixed in drinking water to make a 600 mg chlortetracycline HCl /gallon drinking water solution (ca. 160 mg/L). Each chicken was provided 500 ml antibiotic contained water during the antibiotic treatment period. Water was changed daily and water intake was recorded. Control water (500 ml) was kept in the chicken house to track loss of water from evaporation daily. An averaged 259 ml daily water

intake from the studied chickens was recorded and chickens were given the antibiotic at a dosage of approximately 22-23 mg/kg b.w. (average body weight was 1.9 kg).

Two antibiotic feeding studies were performed. The first feeding study (Antibiotic study 1) was done by feeding chickens 100 ppm DDT diet for 8 days with normal drinking water, followed by an 8-day, 300 ppm DDT diet feeding with chlortetracycline HCl in the drinking water. The second feeding study (Antibiotic study 2) was done using chickens fed 300 ppm DDT diet for 8 days with normal drinking water and followed by a 8-day, 300 ppm DDT diet feeding with Chlortetracycline HCl in the drinking water. Feces were collected daily.

Paired t-test was applied to compare DDA excretion behaviors before and after antibiotic treatment. The *p*-value less than 0.05 was considered significant.

At the end of each study period, feces from antibiotics treated chickens and control chickens were sent to California Animal Health and Food Safety Laboratory (CAHFS laboratory, San Bernardino) for semiquantitative bacterial aerobic culture analysis.

Results and discussion

Fecal DDA excretion in chickens following DDT feeding

When DDT was fed in a series of controlled DDT studies rapid DDA excretion in feces was demonstrated in chickens exposed to 10 to 3000 ppm DDT in diet. DDA was found in chicken feces at the lowest level of 10 ppm DDT (Table 4-3). DDA excretion levels ranged from 0.02 to 19.4 μg DDA/ g fresh feces. DDA excretion in chicken feces was dose-dependent (Figure 4-1). DDA excretion levels increased as DDT feeding level increased. The estimated dosages in chickens were

between 0.6 and 186 mg/kg b.w. After the final feeding period, DDA levels declined slowly in about one month as was shown in chicken study 5 (Figure 4-2). DDA was not detected in either blood or eggs. DDA was shown to be excreted in chicken feces during DDT exposure.

Complete results of DDA and lipophilic DDT residues in chicken feces from each study are presented in Appendix 5. White Leghorn hens were used in studies 1 to 4. ISA Brown hens were used in chicken study 5 and antibiotic studies 1 and 2. DDA was found in 24 h after DDT feeding started in study 1. DDA excretion decreased rapidly in about 10 days post feeding (Figure 4-3a). No significant DDA increase was observed when 10 ppm DDT was fed following 100 ppm DDT feeding in study 2 (Figure 4-3b, c, d). DDT exposure level has to be high enough to show an increased DDA excretion in chicken feces. Study 3 and 4 demonstrated that DDA excretion in chickens is dose dependent. DDA level increased immediately as higher DDT exposure occurred (Figure 4-3e, f and Figure 4-3g, h). A prolonged DDT exposure in study 4 did not lead to an increased DDA excretion during DDT feeding compared to study 3. DDA level declined within several days after DDT feeding stopped in each study. Direct DDT dosing is the source of DDA formation during feeding period.

A more complete dose-dependent relationship between DDA excretion and DDT dose was established in study 5 (Figure 4-2) using ISA Brown hens. Fecal DDA level and DDT dose positively correlates to each other during DDT feeding periods, the Pearson's correlation coefficient (r) is 0.994, the *p*-value equals to 0.001. DDA levels in chicken feces declined to 1/10 of peak excretion in about a month range post DDT feeding. DDT feeding levels were not toxic up to 1000 ppm as was

shown in study 5. Neurotoxic effect was observed and death of 1 chicken was reported at 3000 ppm feeding level.

It is noted that in birds the ureters open into the cloaca, and the urine is stored in the cloaca or intestine until defecation of a semisolid mixture of urine and feces from the cloaca (Skadhauge, 1968). DDT residues detected in chicken feces may be from either urine or feces or both. The source of DDT residues will not be investigated in the present study since chicken urine was not separated from feces. DDT residues will always be reported as being found in feces.

The DDA excretion pattern in chicken feces was similar to DDA urine excretion in human and other animals (Stohlman et al., 1945; Neal et al., 1946; Miller, 1977; Clark, 1977; Chen et al., 2009). Detection of DDA in 24 h following DDT feeding in the present study is consistent with findings of rapid DDA excretion in humans and monkeys following active DDT exposures (Neal et al., 1946; Miller, 1977; Clark, 1977; Chen et al., 2009). Rapid DDA excretion was reported in human urine in 24 h following oral DDT exposure in volunteer of Neal et al. (1946) and a repeated oral study in Chen et al. (2009). Approximately 2% (mole %) DDT dose in Neal et al. (1946) at 11 mg/kg b.w. and 0.4 % DDT dose in Chen et al. (2009) at 2 mg/kg b.w. was excreted as DDA in urine. Similar DDA excretion percentage was found in the present chicken studies. Approximately 0.1% to 1.1% of DDT was excreted in the chicken feces at different feeding levels. The DDA excretion percentage decreased as DDT dose in chickens increased from 10 ppm (1.1%) to 3000 ppm (0.1%). DDA excretion in chickens is dose-dependent. Fecal DDA levels increased as DDT feeding level increased. Similar finding was observed in humans exposed to 3.9, 7.7, and 15.4 mg of technical DDT for up to 183 days (Roan et al.,

1971). DDA levels declined to 1/10 of peak DDA level after DDT feeding stopped in a month but still above pre-dose level. DDA levels continued significantly above pre-dose levels over four months following termination of DDT dose in a human oral DDT ingestion study (Roan et al., 1971). Rapid DDA formation and excretion demonstrated that fecal DDA in chickens may be used as a useful DDT exposure biomarker. Chickens may be a useful sentinel species of environmental DDT exposure (Chapter 5).

Lipophilic DDT residues in feces

Feces contained DDT (<0.1 to 5134.5 μg/kg), DDD (<0.1 to 2686.8 μg/kg), and DDE (<0.1 to 333.4 μg/kg) during the feeding periods. Levels of all three compounds increased as DDT dose increased reflecting increased DDT body burden. Detection of DDD and DDE was early evidence of DDT metabolism in chickens. In our present study, DDA was the dominant DDT derivative in chicken feces. DDA to DDTs (Sum of DDT, DDD, and DDE) mole ratio was up to 53 during DDT feeding periods. DDA was shown to be the major metabolite of DDT excretion in feces.

Blood levels of DDT

Blood total DDT is an important parameter in estimating DDT exposure in humans and animals. Blood total DDT could be used to reflect DDT body burden and indicate stage of DDT exposure (Radomski et al., 1971; Bergonzi et al., 2009).

Chicken whole blood contained significant levels of DDT (47-16738 μg/kg) and DDE (9-5349 μg/kg) in study 5. DDD levels were relatively low (1-132 μg/kg). Complete results of whole blood DDT residues are shown in Appendix 6. DDA was

analyzed but not detected in any of the blood samples (method LOD was 0.1 mg/L). Whole blood DDT was shown to be dose-dependent during DDT feeding period after logarithmic transformation (Figure 4-4). The Pearson's correlation coefficient (r) was 0.9508 (p-value < 0.001). The total DDT levels in whole blood declined after DDT feeding period (Figure 4-5). The depletion half-life of total DDT in blood was approximately 2 weeks. A conversion factor of 1.18 for whole blood to plasma or serum could be applied if plasma or serum concentration is needed (D'Orazio et al., 2006). The fecal DDA and whole blood total DDT levels positively correlated to each other during and post feeding, the Pearson's correlation coefficient (r) was 0.848 and 0.938 during and post feeding, respectively. Circulating DDT in blood has been indicated as a source of DDA formation in humans, the DDA producing enzyme systems appeared to respond to rapidly changing concentration of circulating DDT (Edmundson et al., 1970). DDT circulated in blood reflects DDT body burden and may be a possible source for DDA formation in chickens. Besides, continuing biliary excretion of DDT after dosing may deliver an adequate amount of DDT to the gut to account for the continuing above pre-dose DDA levels (Roan et al., 1971).

DDT residues in blood have been applied to indicate DDT body burden in humans with DDT exposure in anti-malaria campaign. Limited data are available for DDT levels in human blood in IRS area. Higher levels of DDT were reported in occupationally exposed IRS workers than in residents of IRS treated homes and the general population living in areas where IRS was used extensively (WHO, 2011). The DDT applicators had mean lipid adjusted blood serum levels of 77.8 ng total DDT /g (8.7-241.1). The population living in areas where IRS was used extensively had mean lipid adjusted blood serum levels of 9.8 ng total DDT /g (1.09-21.8) and

persons from areas where IRS is not practiced had blood serum levels of 5.0 ng/g (0.38-26.1) attributable to general environmental exposure (WHO, 2011). Analysis of chicken blood in the DDT sprayed area may indicate chicken body burden and stage of environmental DDT exposure.

DDE to total DDT ratio was widely used to evaluate DDT exposure status in various scenarios to indicate if recent DDT exposure occurs (WHO, 2011). The DDE to total DDT ratio obtained in study 5 is shown in Figure 4-6. The ratio was relatively stable during DDT feeding period (ranged from 0.13 to 0.19). The ratio was significantly increased (p-value < 0.001) post DDT feeding (ranged from 0.23 to 0.37). There was a time-dependent increase in the DDE to total DDT ratio once DDT exposure ended. The internal DDT was continuously converted to DDE as a common DDT metabolic pathway. The conversion of DDT to DDA also contributed to the increased DDE to total DDT ratio since less DDT was available. Ratios of DDE/DDTs of 0.8 and above suggest no recent exposure to the parent compound (WHO, 2011). Lower DDE to total DDT ratios during DDT feeding and higher ratios post feeding demonstrated that blood total DDT levels in chickens can be a useful tool to estimate DDT exposure status. The blood DDT analysis confirmed the effectiveness of chickens to absorb DDT from diet and contributed to the understanding of DDT disposition in chickens.

DDT excretion in chicken eggs

Chicken eggs were analyzed during DDT feeding and post DDT feeding periods in studies 3, 4, and 5 to investigate DDT absorption, distribution, and excretion in chicken eggs in support of using fecal DDA as a biomarker. The results

were reported as μg/kg yolk. Eggs from each feeding period were randomly selected and analyzed for DDT, DDD, DDE, and DDA. DDA was analyzed but not found in any egg samples.

Complete results of egg analyses are shown in Appendix 7. Egg yolk contained DDT (5-6785 ng/g), DDD (<600 ng/g), and DDE (1-4044 ng/g) in study 5 (Figure 4-7). DDE to total DDT ratios (DDT and its derivatives) typically rise with time following exposure to DDT. The average DDE to total DDT ratio in chicken study 5 increased from 0.09 at the beginning of DDT feeding to 0.26 one month later. Ratios of 0.8 and above suggest no recent exposure to the parent compound (WHO, 2011). Average DDE to total DDT ratio during each feeding period in study 5 is expressed in Figure 4-8. DDE to total DDT ratios were low during the DDT feeding periods (averaged 0.10). The ratio increased as DDT feeding ended (averaged 0.26). This time-related feature is important because the increased ratio indicated a continued DDT metabolism to DDE and DDA in chickens. Ratio of DDE to total DDT in eggs may reflect the status of DDT exposure and metabolism in chickens.

Distribution of DDT in egg yolk and egg white

Since yolk contains more fatty materials, lipophilic DDT and derivatives are inclined to accumulate in egg yolk (Siddiqui and Saxena, 1983; Furusawa, 2002). DDT in egg yolk was measured in many studies to reflect DDT levels in egg (Smith et al., 1970; Gilbert et al., 1976). Up to 30% of total DDT egg residues were found in egg white and average 85% of total DDT residues were stored in egg yolk in the present study (Table 4-4). There were 6.3 times more total DDT residues in egg yolk than in egg white. Therefore, the lipophilic DDT residues were accumulated

mainly in the egg yolk. This confirmed the work of Siddiqui and Saxena (1983) who found that total DDT residues in the egg yolk of the poultry-farm and domestic hens were 3 to 5 times higher than those in the egg white. Since egg yolk contains most of DDT residues, only yolk was analyzed in later studies. An estimated whole egg DDT residue levels can be obtained by dividing a conversion factor[1] of 1.9 to convert egg yolk DDT level to whole egg DDT levels.

Influence of yolk formation in DDT excretion in chicken eggs

DDT excretion in chicken eggs is dose-dependent (Kan and Tuinstra, 1976). However, an immediate increase in DDT egg excretion level was not observed when DDT feeding levels increased in the present study. The physiology of chicken egg formation begins with the yolk formation in the ovary by continuous or discrete layer deposition of yolk materials. Egg yolk formation usually takes 7-11 days for the majority of yolk to deposit before ovulation. The egg is then laid in 24-27 h after the start of ovulation (Gilbert, 1971). Low DDT levels in eggs were expected at the beginning stage of DDT feeding since the yolk was formed before the start of DDT feeding. Egg DDT levels were significantly higher post feeding than during feeding as were shown in study 3 (Appendix 7). Whole egg total DDT levels were 3-fold and 6-fold higher in post feeding period than during feeding in study 3-1 (10 ppm DDT diet) and 3-2 (100 ppm DDT diet), respectively. The increased post feeding total DDT levels indicated there may be an interval between DDT ingestion, egg yolk formation, and excretion in chicken eggs. As DDT feeding continued and DDT dose increased, every 10-11 days a significant increase in total DDT levels in eggs was

[1]Yolk to whole egg concentration ratio=$C_{yolk} \times m_{whole\ egg} / (C_{yolk} \times m_{yolk} + C_{white} \times m_{white})$. The ratio of 1.9 was an average ratio from results of 12 eggs.

found in study 5 (Appendix 7). A 10-11 day yolk formation period in the studied chickens was estimated. The peak total DDT excretion level (9781-13755 μg/kg) in egg yolk was observed 19 to 26 days post DDT feeding. Total DDT levels in egg yolk declined slowly and maintained relatively high (3269.3 μg/kg) when post feeding ended. The depletion half-life of total DDT in yolk was calculated as about 6 weeks after 16-week feeding of 0.1-1 ppm DDT in diets in high producing laying hens (Kan and Jonker-den Rooyen, 1978). A 5 to 6 week depletion half-life can be expected from the present study since the total DDT levels were still high at the end of sampling more than one month after DDT feeding.

The present study was also consistent with another study in which highest levels of DDT and DDE in yolk were observed on the fifth and sixth days in laying hens treated orally with a single dose of DDT at 1 mg/kg body wt (Furusawa and Morita, 2001). There is an interval between ingestion and excretion of DDT in chicken eggs. The content of DDT residues in the egg depends largely on the physiology of egg yolk formation (Furusawa and Morita, 2001).

Few eggs were collected in study 4 since the studied chickens were molting at the time of study. Total DDT levels in egg yolk in study 3 were relatively high compared to similar feeding levels in study 5 (Appendix 7). However, since only few eggs were available for analysis, no conclusions can be formed about DDT levels and egg yolk formation in this case. Egg production could be highly reduced when hens molt and the hens could deplete large amount of DDT via the egg yolk. However, at this time egg production was very low and the overall rate of depletion was probably not increased greatly (Smith et al., 1970).

Egg DDT indicates if DDA is available in feces

Egg total DDTs level relatively well correlated with fecal DDA during DDT feeding. The correlation coefficient r is 0.8198 (*p*-value=0.089). No correlation relationship was established between egg DDT and fecal DDA levels post feeding. Generally when egg total DDTs levels are high, excretion of DDA in feces is likely. DDA was detected far above pre-dose level after one month of DDT feeding while egg total DDTs levels were still high (total DDTs >2232 μg/kg yolk). Egg DDTs reflects body burden and also may indicate current DDT exposure if DDA is present and prominent in feces. Fecal DDA is a more specific biomarker of DDT exposure since detection of DDA reveals recent DDT exposure while DDT in eggs may represent a previous exposure.

As DDT was found in chicken eggs in areas where DDT was used for malaria control, it would be of interest and important to clarify the source of exposure to investigate DDA as a biomarker of chicken exposure to DDT.

Excretion of DDA in feces as an important depletion process in chickens with DDT exposure

Excretion of DDT and its derivatives in feces and eggs are the two processes that chickens could reduce the DDT body burden. Excretion of DDT through eggs (total DDT+DDD+DDE) and feces (total DDT+DDD+DDE+DDA) in study 5 were shown in Table 4-5. Amount of DDT excreted as fecal DDA was more than combined fecal and egg total DDTs during feeding in study 5. The mole ratio between fecal DDA and total DDTs was 4.35 (ranged from 0.53-14.24) during DDT feeding period and 1.97 (ranged from 0.35-7.45) post DDT feeding. Fecal DDA was

shown to be a major depletion process for DDT exposure. In a previous high-producing laying hens study, 3.5% of ingested DDT in feces and 50% in eggs (0.5 ppm DDT diet for 16 weeks) was reported to be excreted after DDTs residue reached plateau. This early mass balance calculation using DDT excreted in feces and eggs can only account for 53.5% of total DDT administered in the study. The author concluded that the rest of missing DDT might be excreted in other routes or metabolized into other unknown metabolites (Kan and Jonker-den Rooyen, 1978). The finding of fecal DDA excretion in the present study likely accounts for the "missing DDT" in the work of Kan and Jonker-den Rooyen (1978) as DDA was not included in their analysis. Assuming that all the rest of 46.5% daily DDT intake was excreted as DDA in feces during the steady-state at 0.5 ppm DDT diet level in the Kan and Jonker-den Rooyen (1978) study, the hypothetical mole ratio between fecal DDA and total DDT is between 0.88 and 1.10 when the residue is represented all as either DDE or DDT (Assuming daily feed consumption was 110 g as the paper mentioned). The mole ratio in our work and Kan and Jonker-den Rooyen's study are similar and indicates fecal DDA may probably contribute to depletion of DDT in Kan and Jonker-den Rooyen (1978) study although DDA was not included in the analysis.

It is noticed that the fecal DDA to total DDT ratio during the present feeding study decreased from above 10 at 10 ppm dose level to 1 at 3000 ppm dose level (Table 4-5). It seemed that the capability of metabolic enzymes to convert DDT into DDA was not as efficient as DDT dose increased and more DDT was excreted in eggs and feces in the form of intact DDT and its lipophilic metabolites DDE and DDD at higher DDT dose.

Role of gut microflora in DDA formation in chickens

Gut microflora contain a wide variety of metabolizing enzymes with differing levels of activity toward metabolizing endogenous and exogenous compounds (Scheline, 1973; Ilett et al., 1990). Microflora have been shown to play an important role in metabolizing organochlorine pesticides in rats (Traber et al., 1988). Earlier researches showed that various microorganism species isolated from gut microflora could degrade DDT into its dechlorination product DDD, a well established precursor of DDA (Peterson and Robison, 1964; Barker et al., 1965, Braunberg et al., 1968). The normal gut microflora were pointed out to be the major agent for formation of DDD in intact rats fed DDT (Mendel and Walton, 1966). DDA was confirmed in *Aerobacter aerogenes* as an important metabolite on the DDT degradation pathway (Wedemeyer, 1967). So far the location of DDA formation in higher animals is still not clearly established. Since its precursor DDD was reported to be formed by microflora, DDA may be formed in intestine in humans (Roan et al, 1971) and probably in other organisms.

No information was available about gut microflora effect on DDT metabolism in chickens. The hypotheses that gut microflora may contribute to DDA formation in chickens was tested with two antibiotic treatment studies utilizing Chlortetracycline HCl. A 2- to 3- fold decrease of DDA excretion in chickens was observed after chickens received Chlortetracycline HCl at 22-23 mg/kg b.w. in their drinking water. Complete results of DDT residues detected during the antibiotic treatment studies are presented in Appendix 5. Comparisons of DDA excretion before and after antibiotics treatment are shown in Figure 4-9.

The average daily difference between each 100 ppm feeding in the original

and present study and the average daily difference between each 300 ppm feeding before and after antibiotic treatment in antibiotic study 1 (Figure 4-9a) were significantly different (p-value< 0.001) based on paired t-test comparison. The antibiotic treatment in 300 ppm feeding led to a 3-fold decrease in DDA excretion compared to DDA excretion at the original 300 ppm DDT feeding level without antibiotic treatment. The average DDA excretion level in the normal 300 ppm feeding and antibiotic treated 300 ppm feeding was compared using a paired t-test in antibiotic study 2 (Figure 4-9b). The antibiotic treatment led to a 2-fold significant decrease in DDA excretion (p-value< 0.01). Gut microflora were shown to be involved in DDA formation in chickens in the present study. The mechanism of DDA formation is not known, however gut microflora seem to contribute to DDA formation in chickens. This finding may also help to interpret role of gut microflora in DDT metabolism in other organisms.

Feces from antibiotic treated chickens and control chicken feces were sent to the CAHFS laboratory for bacterial aerobic culture analysis. Results of semiquantitative fecal bacteria culture analysis were obtained (Appendix 8). Reduction of microflora was observed in some feces of antibiotic treated chickens compared to those of controls. However, this evidence was not strong enough to confirm the effectiveness of antibiotic treatment in chickens.

Conclusions

The results of current study demonstrated fecal DDA as a chemical biomarker of DDT exposure in chickens. DDA excretion in feces was dose-dependent. Rapid DDA excretion was found in chicken feces following DDT exposure in diet. DDA

excretion levels declined in several days after DDT feeding. Fecal DDA can be a simple and useful DDT exposure biomarker that may be useful to distinguish DDT and DDE exposure in environmental studies.

Chicken blood and egg DDT can reflect DDT body burden and be indicators of fecal DDA. Fecal DDA excretion was the major depletion process for reduction of body burden of DDT in chickens when DDT exposure occurred. The antibiotic treatment indicated an important role of gut microflora in the metabolism of DDT to DDA in chickens and potentially in other organisms.

Chicken or birds may be used as a sentinel species to estimate dietary and environmental DDT contaminations. Demonstration of DDT exposure using DDA as a biomarker may represent a useful tool to clarify some public health and regulatory concerns related to the occurrence and toxicology of these persistent chlorinated hydrocarbons in the environment.

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Table 4-1. Study design for chicken feeding studies

Study I.D.	DDT Dose (ppm)	Dosage (mg/kg b.w.)	Pre-feeding				During feeding				Post feeding			
			Duration (day)	Monitoring			Duration (day)	Monitoring			Duration (day)	Monitoring		
				Feces	Eggs	Blood		Feces	Eggs	Blood		Feces	Eggs	Blood
1	1000	56.8	2	+[a]			4	+			9	+		
2-1	100	6.1	2	+			4	+			10	+		
2-2	100	6.1	2	+			4	+			10	+		
2-3	10	0.6	2	+			4	+			10	+		
3-1	10	0.6	2	+	+		4	+	+		8	+	+	
3-2	100	6.1	2	+	+		4	+	+		8	+	+	
4-1	10	0.6	3	+	+		8	+	+		8	+	+	
4-2	100	5.6	3	+	+		8	+	+	+	8	+	+	+
5-1	10	0.6	2	+	+	+	8	+	+	+	N/A[b]	+	+	+
5-2	100	6.2	N/A	+	+		8	+	+	+	N/A	+	+	+
5-3	300	18.6	N/A	+	+		8	+	+	+	N/A	+	+	+
5-4	1000	62	N/A	+	+		8	+	+	+	N/A	+	+	+
5-5	3000	186	N/A	+	+		6	+	+	+	N/A	+	+	+
5-6	0	0	N/A	+	+		N/A	+	+	+	36	+	+	+
A1-1[c]	100	6.3	N/A	+	+	+	8	+	+	+	N/A	+	+	
A1-2	300	18.8	N/A	+	+		8	+	+	+	N/A	+	+	
A2-1	300	19.4	N/A	+	+	+	8	+	+	+	N/A	+	+	
A2-2	300	19.4	N/A	+	+		8	+	+	+	N/A	+	+	

[a] "+" represents the specific sample was collected at the time. Sample without collection was left in blank. [b] "N/A" designates dates with no activity. [c] Antibiotic study was represented as "A".

Table 4-2. Recovery of DDT and selected derivatives in fortified chicken specimens

Sample [a]	Spike level [b]	DDA % (Mean ± S.D.)	DDT % (Mean ± S.D.)	DDD % Mean ± S.D.)	DDE % (Mean ± S.D.)	DBP % (Mean ± S.D.)
Feces	0.005	70.6 ± 8.2	84.1 ± 15.3	70.6 ± 18.8	70.3 ±12.8	70.0 ± 1.2
(µg/g)	0.05	71.3 ± 7.1	77.7 ± 22.3	77.0 ± 7.3	74.7 ± 9.9	72.3 ± 3.7
Blood	0.1	75.9 ± 3.6	96.9 ± 11.1	68.8 ± 6.9	77.6 ± 12.9	N/A[c]
(µg/L)	1	78.9 ± 3.5	66.9 ± 16.0	62.0 ± 5.6	77.4 ± 9.9	N/A
Egg yolk	0.1	69.9 ± 1.2	67.4 ± 3.2	67.4 ± 5.9	64.6 ± 3.1	N/A
(µg/g)	1	69.6 ± 1.3	72.1 ± 6.2	76.3 ± 9.2	68.1 ± 6.5	N/A
Egg white	0.1	78.8 ± 2.0	86.3 ± 3.0	73.8 ± 2.1	81.0 ± 1.6	N/A
(µg/g)	1	76.8 ± 4.0	76.3 ± 4.0	79.5 ± 6.2	72.9 ± 4.0	N/A

[a] Instrument detection limits (IDL) for DDT, DDD, DDE, and DBP were 0.01µg/ml on GC-ECD and 0.1µg/ml for DDA derivative on GC-MS.
[b] Number of replicates was 3 for each spike level. [c] DBP analysis was not included in blood and egg samples.

Table 4-3. Summary of DDT residue levels in chicken feces.

Diet	Dosage [a] (mg/kg-d)	Day [b]	DDT derivatives (µg/kg fresh feces)				DDE/ total DDT [c]	DDA/ total DDT
			DDA	DDE	DDD	DDT		
Study 1 [d] White Leghorn-4d, 1000ppm DDT	56.8	Mean ± SD (pre- feeding)	0	0	0	0	N/A	N/A
		Mean ± SD (during feeding)	3026.8±674.7	46.7±38.7	344.3±131.7	1640.3±217	0.02±0.01	1.5±0.5
		Mean ± SD (post feeding)	1026.3±682.6	11.2±2.5	91.1±191.3	65.5±47.7	0.13±0.06	9.9±4.5
Study 2-1 White Leghorn-4d, 100ppm DDT	6.1	Mean ± SD (pre- feeding)	0	0	0	0	N/A	N/A
		Mean ± SD (during feeding)	659. 5±237.7	3.7±1.0	25.9±16.7	105.3±50.9	0.04±0.01	6.1±3.3
		Mean ± SD (post feeding)	104.7±47.8	3.7±0.9	3.7±2.9	37.1±34.1	0.14±0.12	3.5±2.8
Study 2-2 White Leghorn-4d, 100ppm DDT	6.1	Mean ± SD (pre- feeding)	47.6±10.4	1.1±0.1	0	18.8±21.2	0.1±0.1	4.9±4.7
		Mean ± SD (during feeding)	208.9±40.0	3.6±1.4	9.3±6.7	48.4±13.6	0.06±0.03	3.6±1.1
		Mean ± SD (post feeding)	97.5±48.0	3.7±1.4	3.8±3.2	39.4±27.9	0.10±0.06	2.7±2.2
Study 2-3 White Leghorn-4d, 10ppm DDT	0.6	Mean ± SD (pre- feeding)	108.4±26.4	1.4±0.1	1.1±0.1	4.6±2.3	0.21±0.08	15.5±1.2
		Mean ± SD (during feeding)	107.8±61.7	2.8±1.5	2.6±1.6	10.9±4.4	0.18±0.07	6.5±2.6
		Mean ± SD (post feeding)	129.1±57.8	4.2±3.6	3.9±3.9	20.6±23.4	0.25±0.24	8.2±5.71
Study 3-1 White Leghorn-4d, 10ppm DDT	0.6	Mean ± SD (pre- feeding)	0	0	0	0	N/A	N/A
		Mean ± SD (during feeding)	79.5±15.0	1.0±0.1	11.9±3.2	10.7±2.4	0.04±0.01	3.6±1.4
		Mean ± SD (post feeding)	25.8±23.0	1.0±0.5	1.8±2.7	5.3±4.2	0.19±0.07	3.7±3.6
Study 3-2 White Leghorn-4d, 100ppm DDT	6.1	Mean ± SD (pre- feeding)	17.3±6.9	0.5±0.7	0	11.3±4.1	0.11±0.08	1.5±0.2
		Mean ± SD (during feeding)	526.6±165.7	2.3±0.6	27.7±8.4	48.6±14.3	0.03±0.00	6.6±1.1
		Mean ± SD (post feeding)	187.7±92.9	1.0±0.00	8.6±4.4	21.6±5.0	0.03±0.01	5.8±2.3
Study 4-1 White Leghorn-8d, 10ppm DDT	0.6	Mean ± SD (pre- feeding)	0	0	0	0	N/A	N/A
		Mean ± SD (during feeding)	538.9±115.4	2.3±2.9	18.8±10.2	28.6±11.1	0.06±0.04	12.5±6.4
		Mean ± SD (post feeding)	229.1±134.3	4.9±1.5	7.3±2.5	17.2±7.8	0.18±0.08	7.7±2.9

Table 4-3. Summary of DDT residue levels in chicken feces. (Continued.)

Diet	Dosage (mg/kg-d)	Day	DDT derivatives (µg/kg fresh feces)				DDE/ total DDT	DDA/ total DDT
			DDA	DDE	DDD	DDT		
Study 4-2 White Leghorn-8d, 100ppm DDT	5.6	Mean ± SD (pre- feeding)	187.5±62.3	4.5±0.6	6.8±1.3	10.7±0.9	0.21±0.03	8.5±2.6
		Mean ± SD (during feeding)	1522.1±310.9	6.6±1.8	25.1±14.2	90.1±70.0	0.12±0.13	25.9±26.9
		Mean ± SD (post feeding)	679.6±157.4	6.5±0.9	12.9±4.0	65.4±21.1	0.08±0.03	8.5±3.0
Study 5-1 ISA Brown-8d, 10ppm DDT	0.6	Mean ± SD (pre- feeding)	0	0	0	0	N/A	N/A
		Mean ± SD (during feeding)	62.4±36.4	0.7±1.2	1.3±0.9	5.0±4.7	0.07±0.05	14.7±12.3
Study 5-2 ISA Brown-8d, 100ppm DDT	6.2	Mean ± SD (during feeding)	445.6±96.9	0.7±0.2	8.4±2.1	9.7±3.9	0.04±0.01	24.5±6.6
Study 5-3 ISA Brown-8d, 300ppm DDT	18.6	Mean ± SD (during feeding)	1279.6±323.1	4.0±2.1	31.1±14.1	97.9±47.7	0.03±0.00	11.0±3.8
Study 5-4 ISA Brown-8d, 1000ppm DDT	62	Mean ± SD (during feeding)	3117.1±842.3	22.9±5.3	246.2±59.7	734.5±342.6	0.02±0.01	3.4±1.4
Study 5-5 ISA Brown-6d, 3000ppm DDT	186	Mean ± SD (during feeding)	13536±4131	190.2±92.5	1836.2±622.8	4762.5±339	0.03±0.01	2.0±0.6
Study 5-6 ISA Brown	No DDT	Mean ± SD (post feeding)	2935.1±2651.1	40.5±15.9	94.9±68.3	421.1±464.4	0.09±0.03	9.4±16.9

Table 4-3. Summary of DDT residue levels in chicken feces. (Continued.)

Diet	Dosage (mg/kg-d)	Day	DDT derivatives (µg/kg fresh feces)				DDE/ total DDT	DDA/ total DDT
			DDA	DDE	DDD	DDT		
Antibiotic Study 1-1								
ISA Brown-8d 100ppm DDT	6.3	Mean ± SD (1b-8b)	236.3±53.4	4.8±2.7	54.3±63.2	48.7±17.4	0.05±0.01	2.9±1.2
Antibiotic Study 1-2[e]								
ISA Brown-8d 300ppm DDT	18.8	Mean ± SD (9b-16b)	372.3±117.1	11.4±6.0	108.4±104.3	158.5±97.3	0.05±0.02	2.1±1.7
Antibiotic Study 2-1								
ISA Brown-8d 300ppm DDT	19.4	Mean ± SD (1b-8b)	653.9±216.7	11.5±6.1	116.7±42.6	434.3±422.8	0.02±0.01	1.9±1.5
Antibiotic Study 2-2 [e]								
ISA Brown-8d 300ppm DDT	19.4	Mean ± SD (9b-16b)	351.2±89.8	8.6±3.8	82.3±47.0	287.4±271.8	0.03±0.01	1.3±0.8

[a] Daily feed consumption for White Leghorn hens was approximately 80g (out of 100g) and for ISA Brown hens was approximately 120g (out of 150g). Average chicken weight for each study was listed in Appendix 4. [b] Each DDT feeding level included three stages: a pre-DDT control feeding period, a DDT feeding period and a post DDT control feeding period. [c] Total DDT = sum of (DDT+DDD+DDE). [d] Each study number designated a set of chickens fed with various levels of DDT. Chickens were killed at the end of each study. [e] Antibiotics were administered in drinking water during the feeding period.

Table 4-4. Comparison of total DDT residues in yolk, white and whole egg

Egg #	Yolk (μg/kg)	White (μg/kg)	Whole egg (μg/kg)	Yolk:White	Yolk:Whole egg
1	1532	289	792	5.3	1.9
2	1099	349	663	3.1	1.7
3	990	612	769	1.6	1.3
4	6727	644	2970	10.4	2.3
5	7554	714	3218	10.6	2.3
6	5513	1298	3015	4.2	1.8
7	3669	951	1961	3.9	1.9
8	3582	1713	2375	2.1	1.5
9	5666	989	2811	5.7	2.0
10	3095	1191	1924	2.6	1.6
11	12280	927	5066	13.2	2.4
12	15343	1192	6017	12.9	2.6
Average				6.3	1.9
SD				4.3	0.4

Table 4-5. Excretion of DDT residues via eggs and feces

Days	Fecal DDA (nmol)	Fecal total DDT (nmol) [a]	Egg total DDT (nmol)	DDA/DDT mole ratio
6	10	18	1	0.53
8	30	1	2	14.24
11	313	30	3	9.67
13	258	31	12	5.97
18	364	38	22	6.03
20	503	62	17	6.34
22	826	72	75	5.63
24	714	99	101	3.58
26	580	173	99	2.13
28	706	574	75	1.09
30	1560	586	106	2.25
33	1113	902	175	1.03
36	5862	3782	131	1.50
38	4350	4195	177	0.99
Average				4.35
41	2162	523	408	2.32
43	819	152	429	1.41
44	952	199	595	1.20
45	3886	232	479	5.46
47 [b]	3579	191	290	7.45
50	2587	589	253	3.07
55	776	227	184	1.89
59	50	238	805	0.49
63	528	202	1009	0.44
66	568	498	1137	0.35
69	584	272	294	1.03
72	742	286	496	0.95
74	397	288	292	0.69
76	492	226	271	0.99
Average				1.97

[a] Fecal DDT level was adjusted by a factor of 7.1-fold since we observed a feed contamination of feces during the feeding of chickens.

[b.] Since Feces were analyzed every other day post feeding, some days (47, 55, 59, 63, and 69) only egg DDT was analyzed. Feces DDA and total DDT data on an adjacent day were used to compare to the egg DDT.

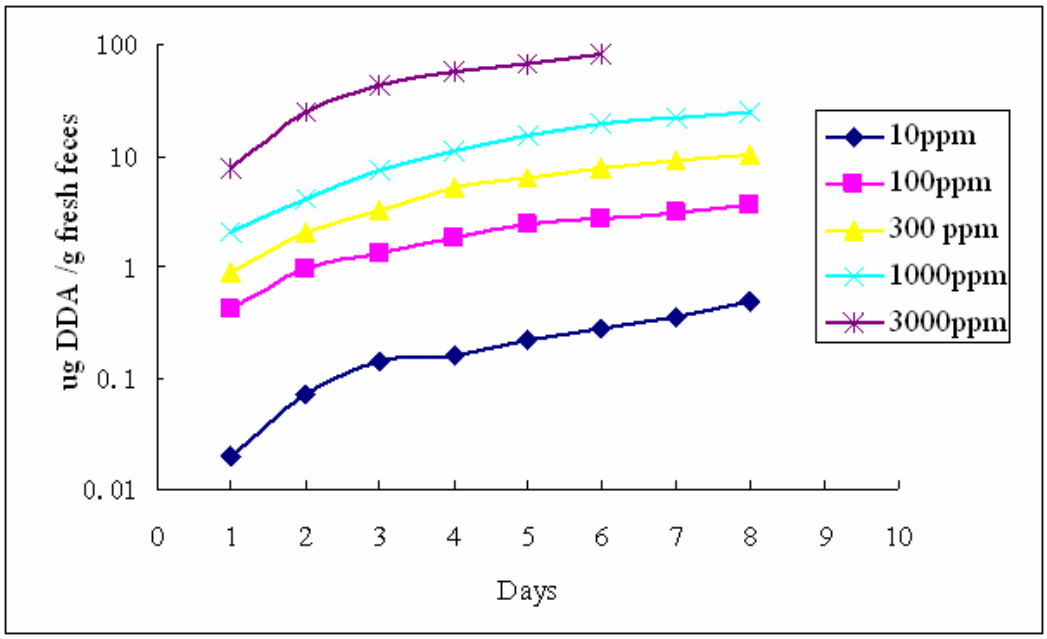

Fig. 4-1 Cumulative DDA excretion in chicken feces following DDT diets. DDA excretion is immediate and dose-dependent. Collection of feces in 3000 ppm feeding study stopped on day 6 due to neurotoxicity.

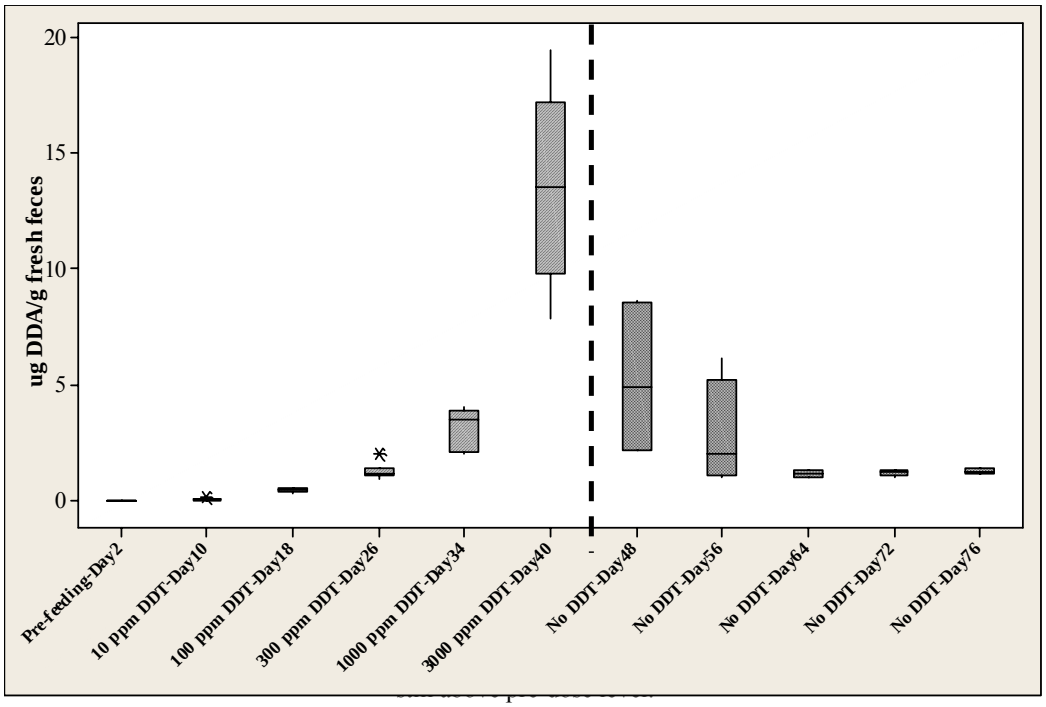

Fig. 4-2 DDA excretion in chicken feces was rapid and increased as DDT dose increased. DDA levels in every 4 to 8 days post feeding were expressed. DDA level slowly declined after feeding period but still above pre-dose level. Collection of feces in 3000 ppm feeding study stopped on day 6 due to neurotoxicity.

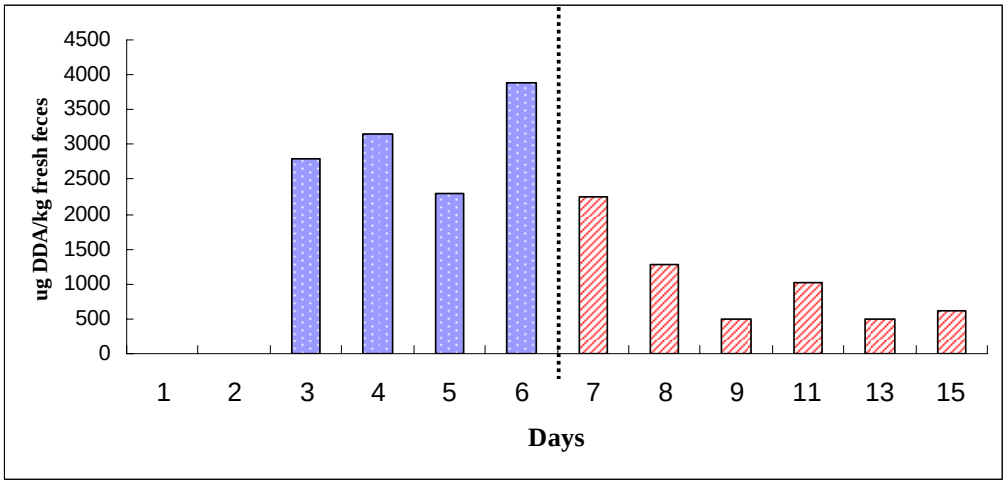

Fig. 4-3a Fecal DDA excretion following 1000 ppm DDT dietary exposure (Chicken study 1)

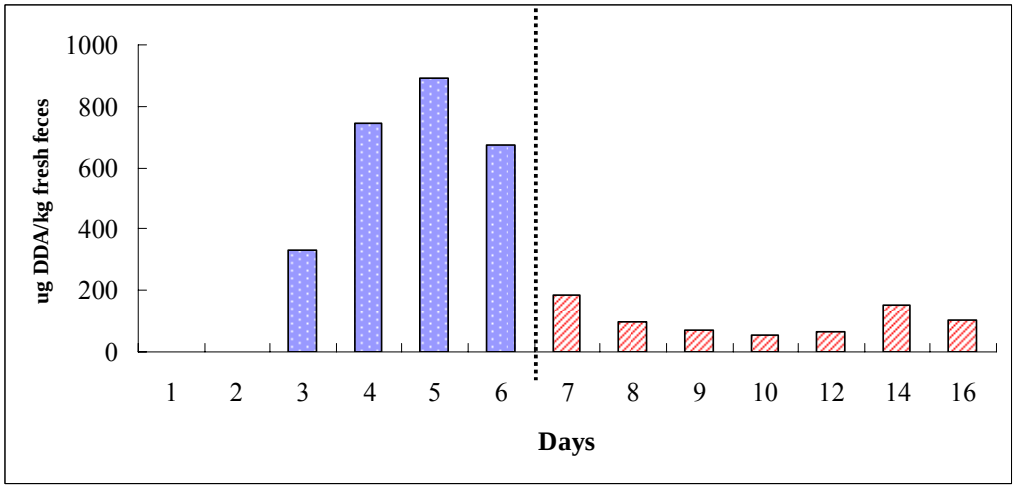

Fig. 4-3b DDA excretion following 100ppm DDT diet exposure (Chicken study 2)

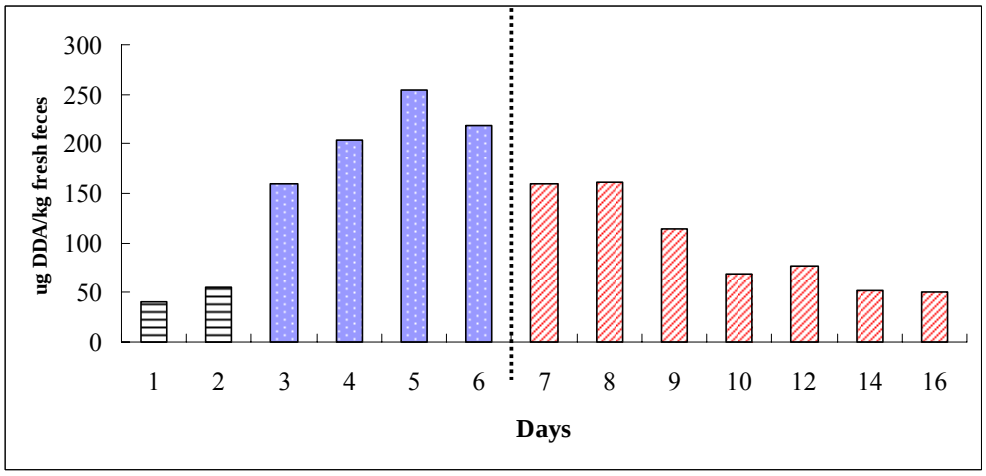

Fig. 4-3c DDA excretion following 100ppm DDT diet exposure (Chicken study 2)

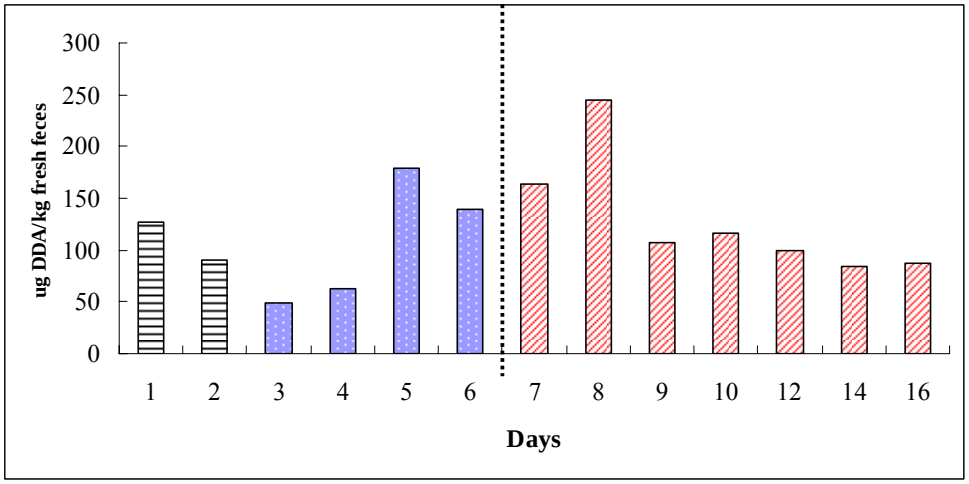

Fig. 4-3d DDA excretion following 10ppm DDT diet exposure (Chicken study 2)

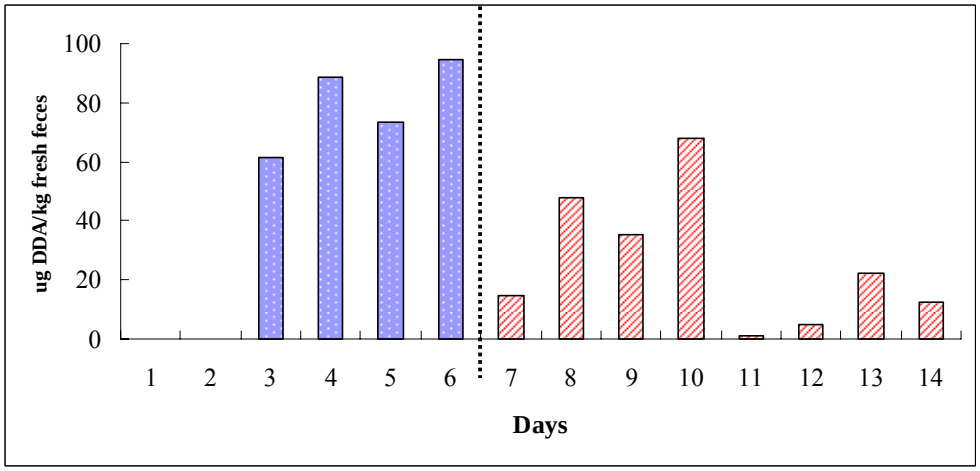

Fig. 4-3e DDA excretion following 10ppm DDT diet exposure (Chicken study 3)

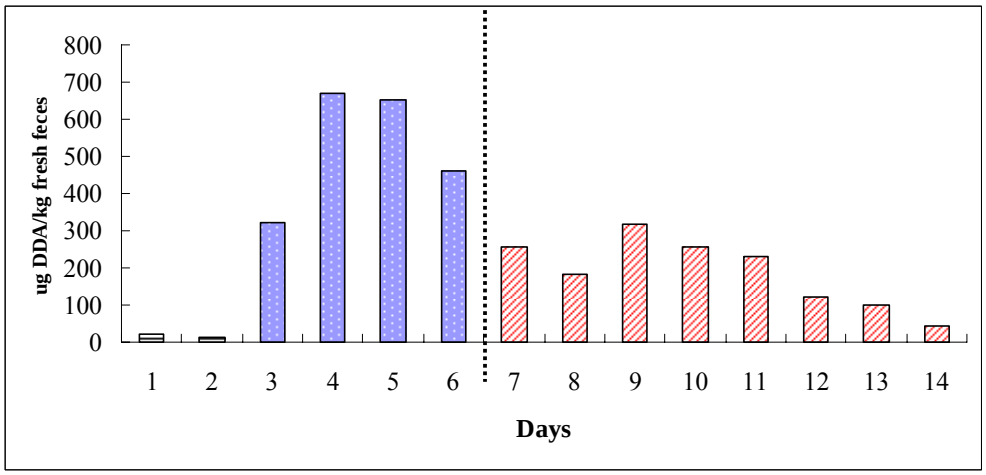

Fig. 4-3f DDA excretion following 100ppm DDT diet exposure (Chicken study 3)

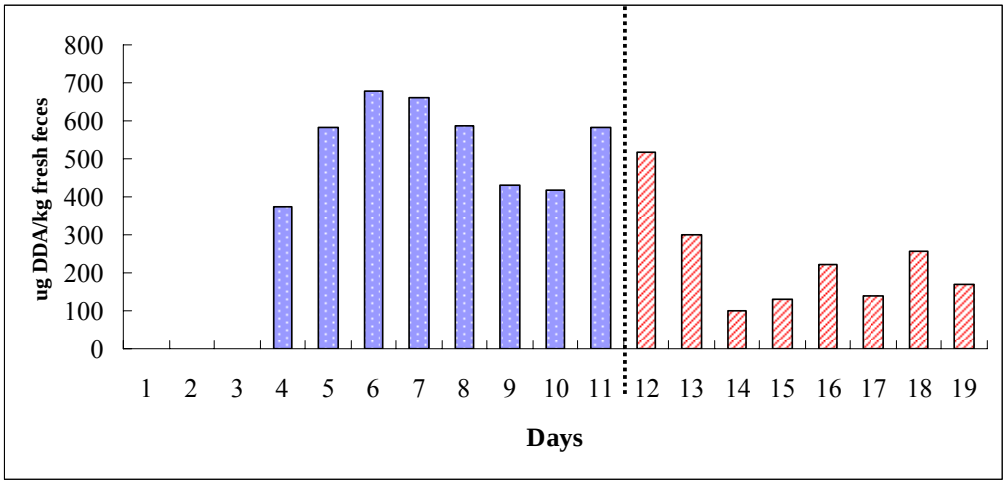

Fig. 4-3g DDA excretion following 10ppm DDT diet exposure (Chicken study 4)

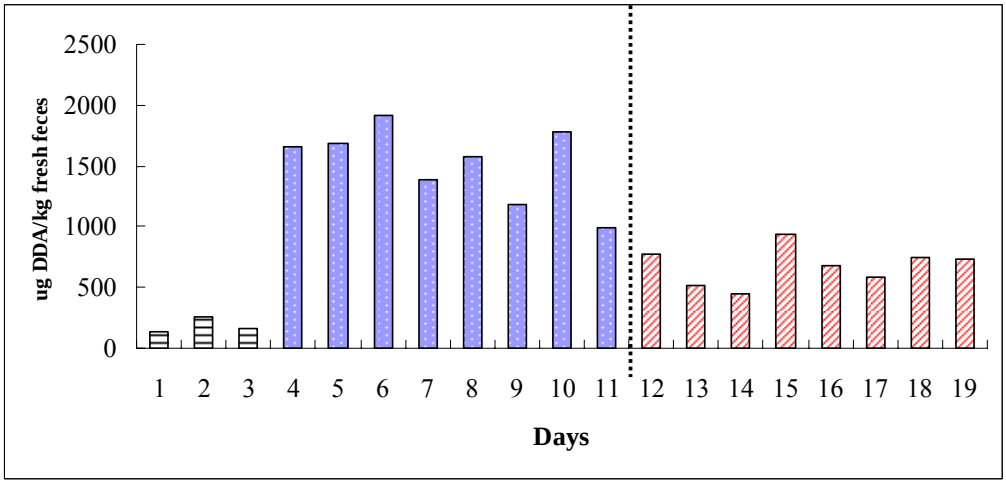

Fig. 4-3h DDA excretion following 100ppm DDT diet exposure (Chicken study 4)

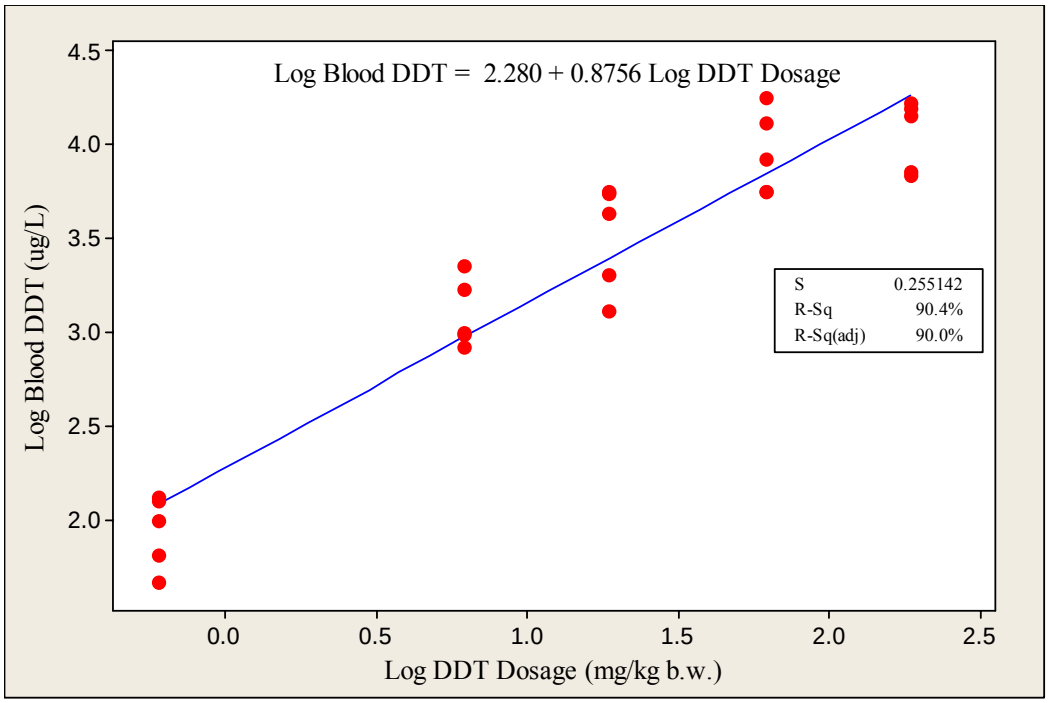

Fig. 4-4 Blood DDT levels increased as DDT dosage increased (all the log scale). Blood DDT may be a source of DDA formation in chickens.

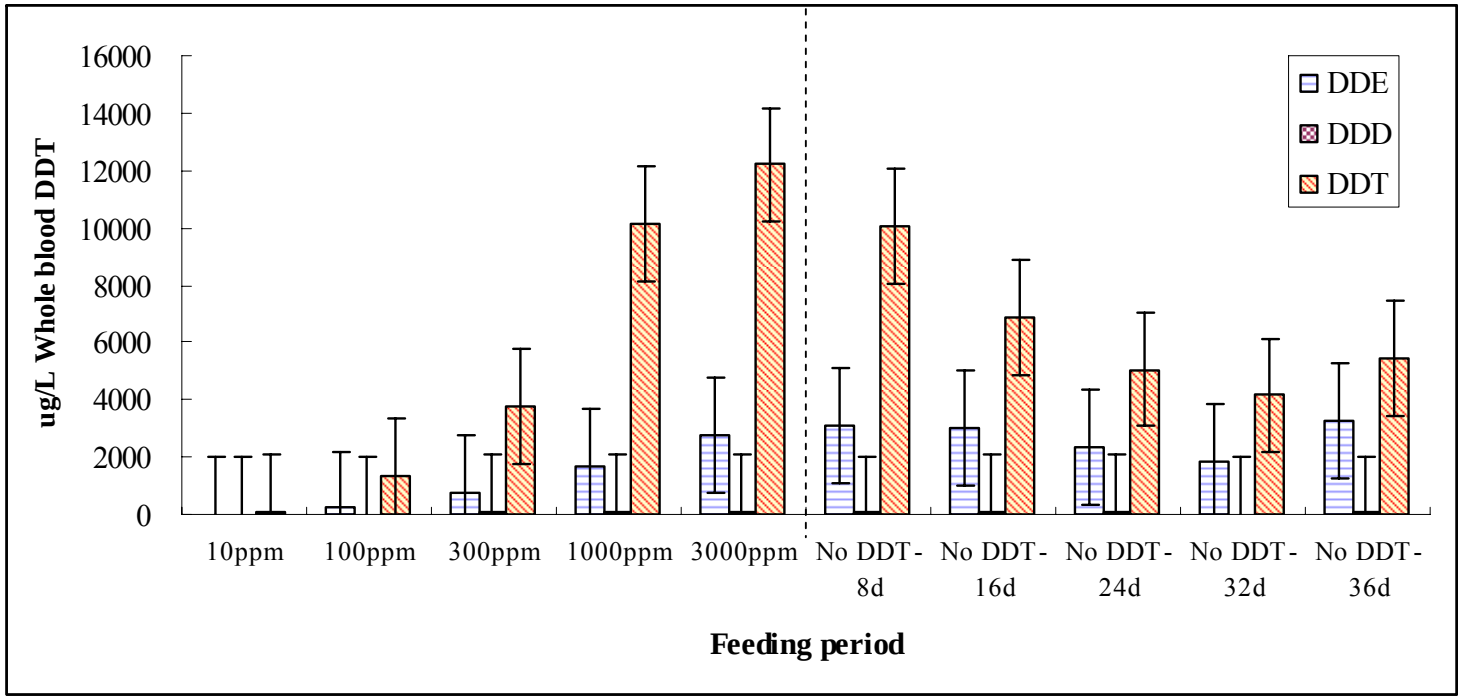

Fig. 4-5 DDT/DDD/DDE whole blood levels during chicken feeding study 5. DDT level in the whole blood is high during feeding period and decreased with a depletion half-life of about 2 week post feeding.

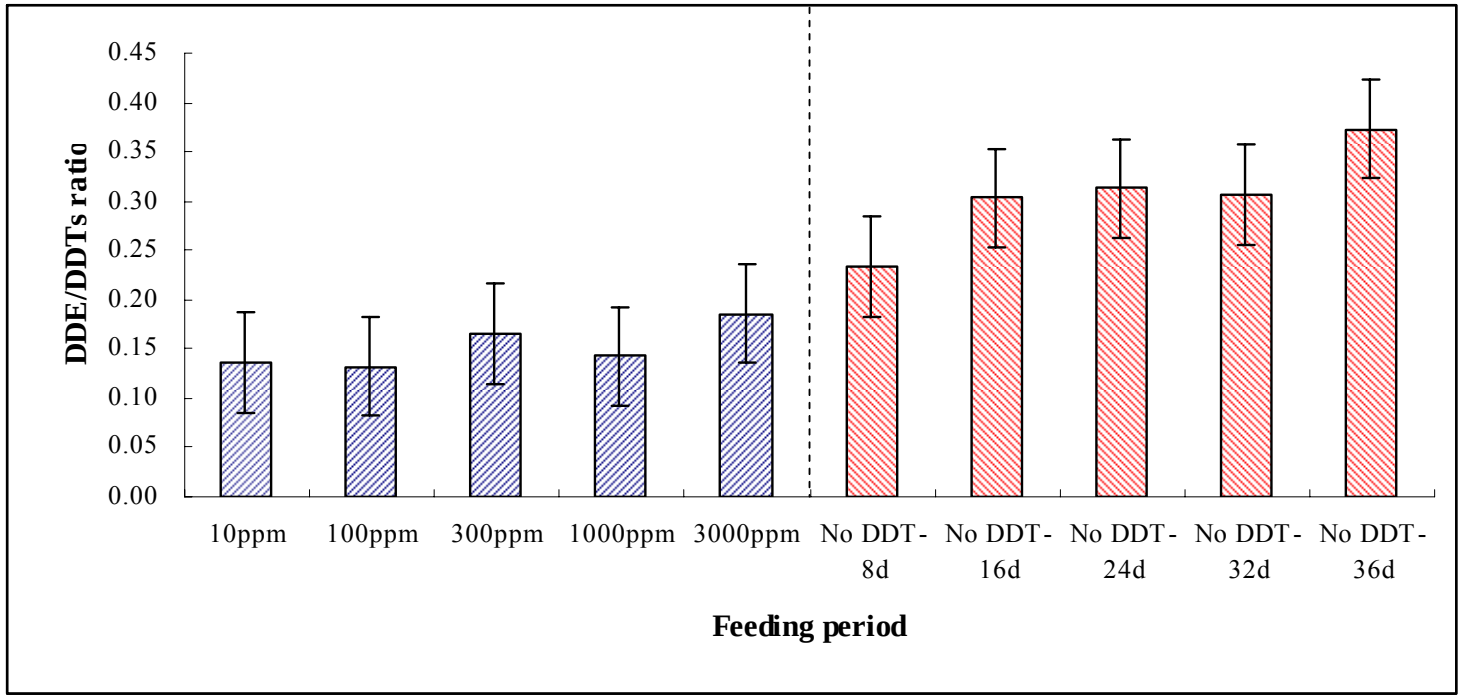

Fig. 4-6 DDE to total DDT ratio in whole blood during chicken feeding study 5. Average DDE to total DDT ratio during feeding (every 8 days) and every 4 or 8 days post feeding was expressed. DDE to total DDT ratio post feeding was significantly higher than during feeding. Internal transformation of DDT to DDE was indicated as ratio continued to increase post feeding.

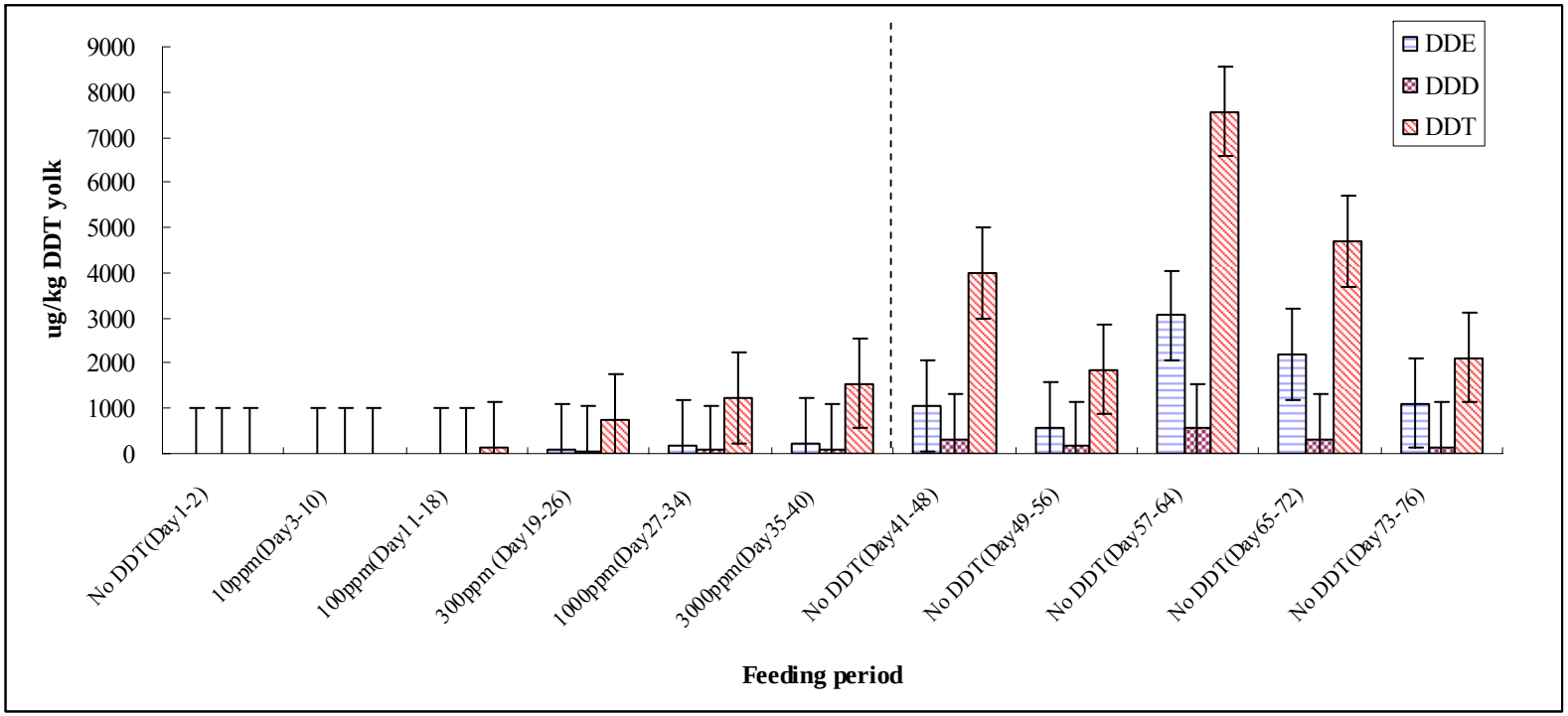

Fig. 4-7 DDT/DDD/DDE egg yolk excretion during chicken study 5

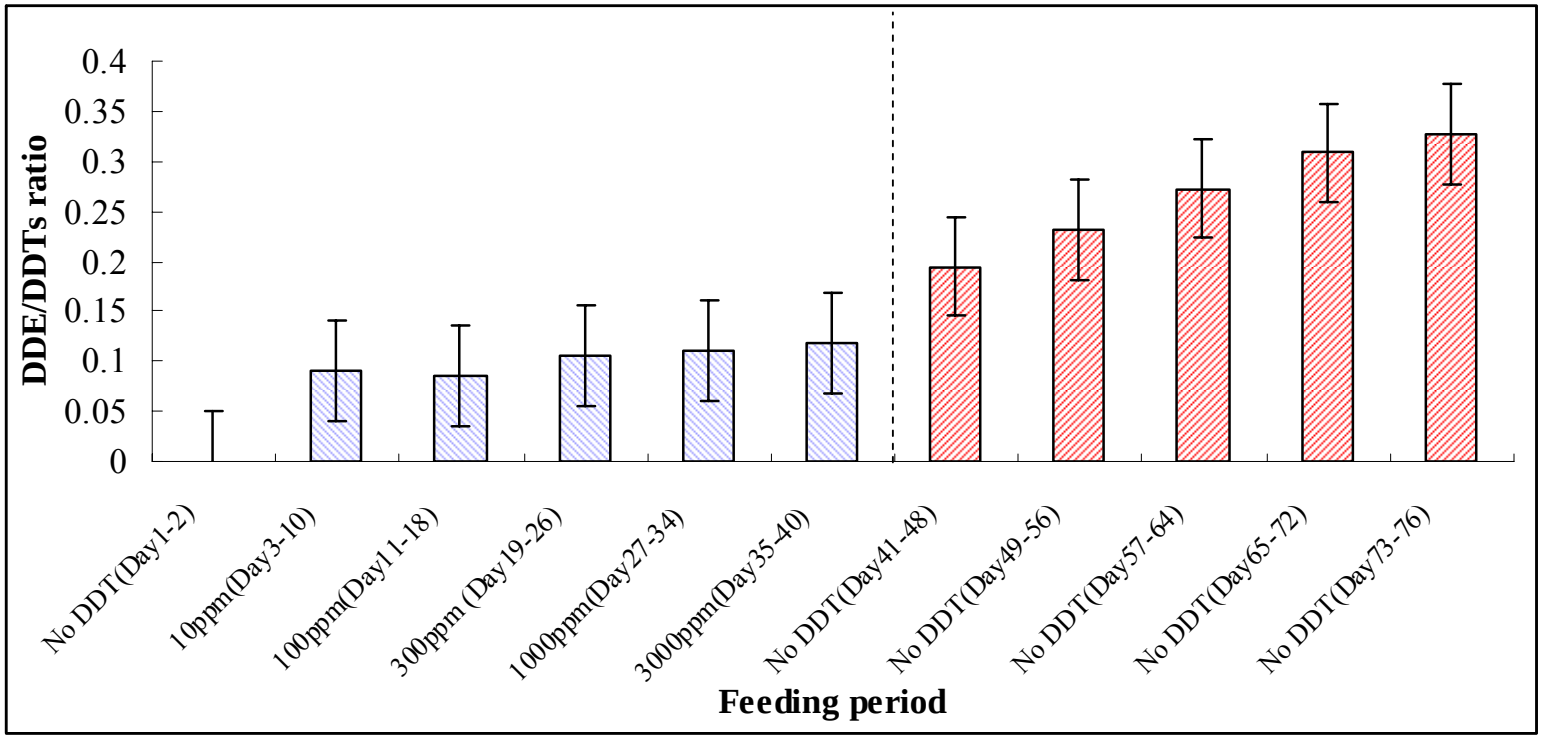

Fig. 4-8 DDE to total DDT ratios in egg yolk during chicken feeding study 5. Average DDE to total DDT ratio during feeding (every 8 days) and every 4 or 8 days post feeding was expressed. DDE to total DDT ratio post feeding was significantly higher than during feeding. Internal transformation of DDT to DDE was indicated as ratio continued to increase post feeding.

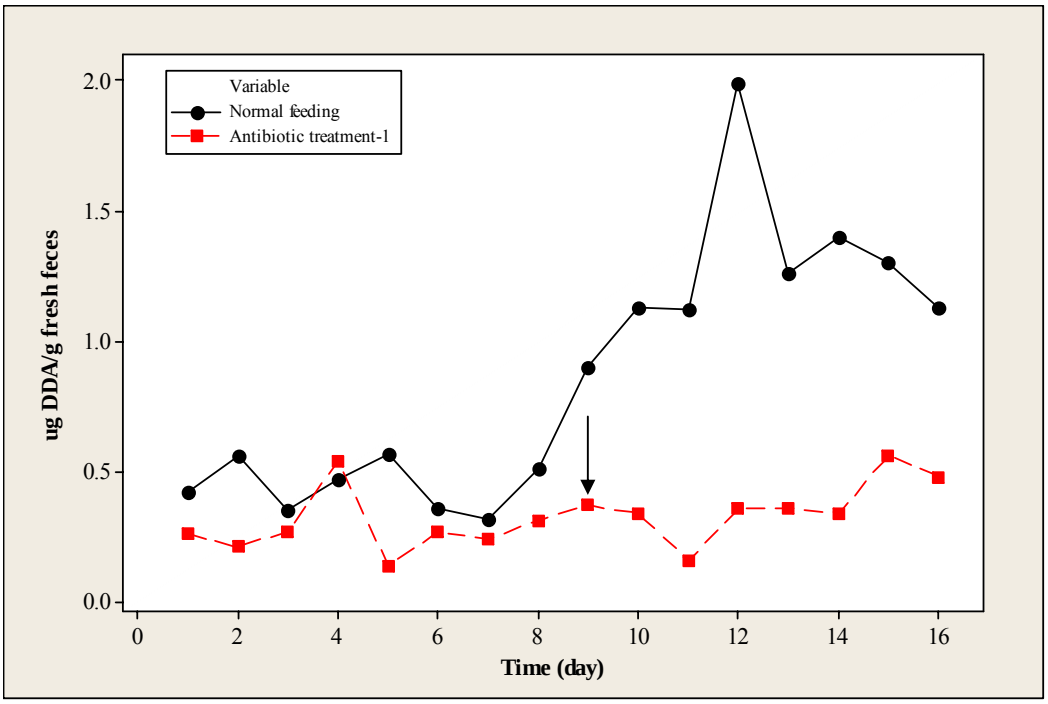

Fig. 4-9a Antibiotic study 1. Fecal DDA excretion was less than normal when antibiotic was administered. The arrow represents the start of antibiotic dosing. Chlortetracycline HCl (estimated 22 mg/kg) in drinking water produced no adverse effects.

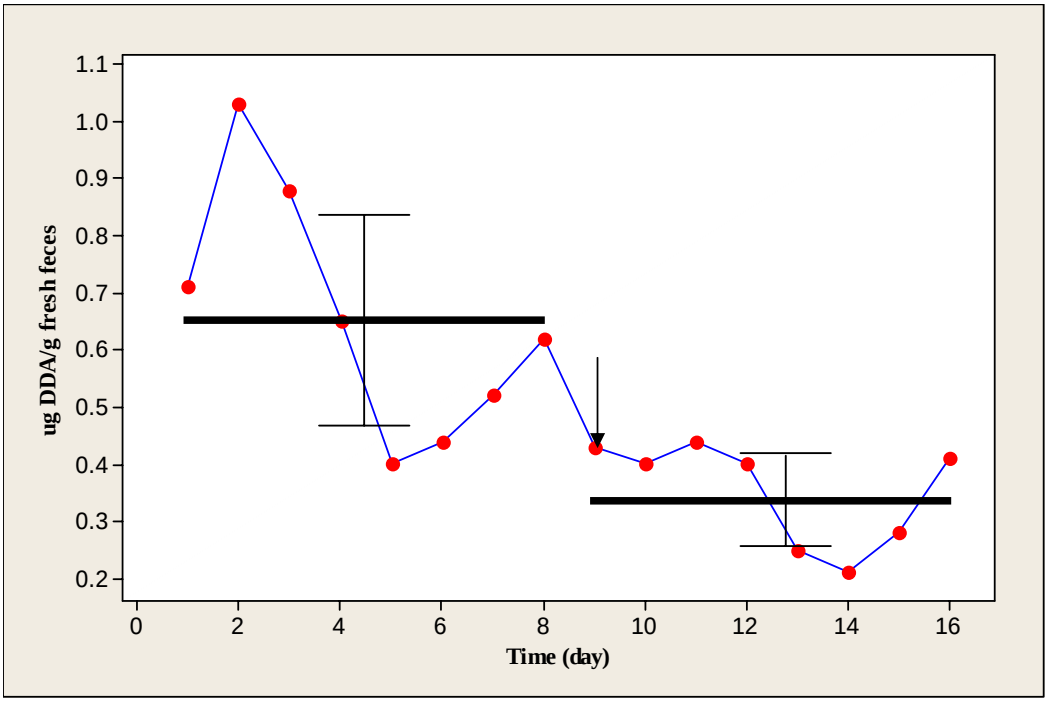

Fig 4-9b Antibiotic study 2. Mean fecal excretion of DDA was 0.66± 0.22µg/g fresh feces during an 8-day feeding of 300 ppm DDT followed by an 8-day feeding of 300 ppm DDT + Chlortetracycline HCl (estimated 23 mg/kg) when mean fecal DDA was 0.35±0.09µg/g fresh feces. The arrow represents the start of antibiotic dosing. Chlortetracycline HCl in drinking water produced no adverse effects.

CHAPTER 5

Pilot Surveillance of DDT Exposure Using Fecal DDA As a Biomarker Following IRS of DDT in Anti-Malaria Program

Introduction

The use of DDT in Indoor Residual Spraying (IRS) for malaria control was demonstrated as early as 1943. Successful reductions of malaria morbidity and mortality in Italy, United States, Guyana, South Africa, and Taiwan where IRS was used are described by Roberts (2010). Today DDT is listed in Annex B of the Stockholm Convention and its use is restricted to government-authorized disease vector management in accord with World Health Assembly Resolution 50.13 (1997).

Use of DDT in IRS requires a report on the quantities and conditions of use in the malaria management program in accord with Annex B, Part II of the Stockholm Convention (WHO, 2007). The national plan must confine DDT use to disease vector control, implement alternatives to DDT, and take measures to strengthen health care. Implementation of alternatives requires knowledge of human health risks and environmental implications. DDT exposure surveillance and monitoring associated with IRS are a means to provide current assessments that are important for public health and policy formulation.

IRS has been associated with environmental contamination and human exposure of DDT (Serada et al., 2009; Van Dyk et al., 2010). However, the levels of DDT exposure reported in studies were below levels of concern for health in general. To ensure that all exposures are below levels of concern, best application measures must be strictly followed to protect both residents and workers (WHO, 2011). Studies are needed to apportion the contribution of DDT used in IRS to other possible sources of exposure to contribute to understanding the extent of human exposure and to expand knowledge of the extent of environmental contamination associated with IRS.

Knowledge of fate and transport of DDT directly associated with IRS is very

limited. Water, sediment, fish, domestic chickens and wild birds from DDT sprayed areas in Limpopo Province were analyzed for DDT and its metabolites DDD and DDE (Barnhoon et al., 2009). The levels of DDT, DDD, and DDE were less than 2 ppb in water of both sprayed and non-sprayed areas. Up to 63 mg/kg DDE and 8.5 mg/kg DDD and 6.5 mg/kg DDT were detected in fish fats from DDT sprayed area. Domestic chicken contained up to 12 mg/kg DDE, 21 mg/kg DDD, and 12 mg/kg DDT in the fat in DDT sprayed villages. In another study conducted in Limpopo Province of South Africa, high levels of p, p'-DDT and o, p'-DDT, the components of the DDT wettable powder applied in IRS were found in indoor air and floor dust in the sprayed village. Low levels of total DDT were found in soil, water and vegetables with pre-dominance of p, p'-DDE in those samples indicating metabolism of the applied DDT spray (Van Dyk et al., 2010). Human blood serum collected from the exposed village showed mean total DDT and p, p'-DDE concentrations of 7.3 and 5.9 μg/g lipid, respectively (Van Dyk et al., 2010).

As a major local food source, chickens contained high DDT residues in DDT sprayed villages (Barnhoon et al., 2009; Van Dyk et al., 2010). Chicken muscle and liver contained measurable DDT. Mean level of 240 mg/kg DDT was detected in chicken fat in the sprayed village compared with 540 μg/kg in the control village (Van Dyk et al., 2010). The DDT contamination in chickens was likely related to the use of DDT for malaria control and chickens should be further evaluated as possible animal biomarker for human IRS exposures (Barnhoon et al., 2009). However, pre-dominant DDE in all samples may indicate an earlier DDT exposure caused the body burden instead of current DDT use in IRS (WHO, 2011).

In a recent personal communication (Bornman, 2010), chicken eggs obtained from Limpopo Province were analyzed for DDT residues. Eggs from untreated and

DDT-IRS treated homes were included. DDT and its lipophilic derivatives DDE and DDD in ppb to ppm levels were found in whole chicken eggs (Table 5-1). DDT use in IRS was considered to be the source of DDT exposure in the chickens. The DDE to total DDT ratio in chicken eggs in the DDT sprayed area ranged from 0.44 to 0.67 (mean ratio was 0.58). The use of DDE/total DDT to indicate the origin of the DDT exposure may be a useful feature of measurements of egg residues. Larger amounts of DDT relative to lower levels of DDE may represent DDT exposures in more recently sprayed areas.

It is difficult to define exposure pathways that can yield reported body burdens from IRS deposition of 2 g/m^2 on walls and ceilings of residences. Low soil levels don't seem to support DDT contamination in the environment (Van Dyk et al., 2010). Chickens that live within villages where IRS may be used are an important food. The occurrence of residues in adipose and meat and eggs at high levels relative to those of chickens in villages where DDT was not used supported this study of chickens as a potential sentinel organism. If IRS was directly resulting in the elevated DDT levels previously observed (Barnhoon et al., 2009; Bornman, 2009; Van Dyk et al., 2010), the exposure would promptly produce DDA excretion in chickens.

To evaluate the feasibility of using DDA as an environmental DDT biomarker for chickens, a baseline DDT disposition study was done (Chapter 4). Rapid DDA excretion was shown in White Leghorn and ISA Brown hen feces following active DDT feeding (10 to 3000 ppm). DDA excretion in feces was dose-dependent during the DDT feeding periods. DDA level declined when DDT feeding stopped. Neurotoxicity and one death occurred at the high dose (between 1000 and 3000 ppm feeding level). DDA detection in chicken feces may be used as a biomarker of current environmental DDT exposure. Since living species as sentinels of

environmental pollution has been applied in other research (Burger and Gochfeld, 2004; Castilla, 1996; Pricharda et al., 1997), chickens could possibly be used as environmental sentinels in surveillance and monitoring of DDT following IRS.

Chicken feces obtained from IRS village and control village in South Africa were analyzed in this study to evaluate fecal DDA as chemical biomarker of DDT exposure. Potential environmental DDT exposure from DDT use in IRS was investigated.

Materials and methods

Chemicals

Chemicals included p, p'-DDT, 98.6% (Supelco, Bellefonte, PA); p, p'-DDA, 98.0% (Sigma- Aldrich, St Louis, MO); p, p'-DDE, 99.2% (Supelco); p, p'-DDD, 97.9% (Supelco); p, p'-dichlorobenzophenone (p, p'-DBP), 99.0% (Sigma- Aldrich, St Louis, MO); pentafluorobenzyl bromide (PFBBr), 99% (Sigma-Aldrich); diisopropylethyl amine (DIPEA), 99% (Sigma-Aldrich); n-hexane, 99.9% (Fisher Scientific); ethyl acetate, 99.9% (Fisher Scientific, Hampton, NH); acetone, 99.9% (Fisher Scientific), 6N HCl, 10N KOH.

Study sites

Lufule 1 is a 'control no-spray area' where IRS has not been performed. Tshikhudini and Lufule 2 were reported IRS DDT sprayed areas. Control samples were collected in the Lufule1 area prior to the start of 2010-2011 IRS DDT spray season and 10-week post DDT spray. Pre-spray, 2-week, 5-week, and 10-week post DDT spray samples were collected from the Tshikhudini and Lufule 2 areas as well.

Chicken feces sampling and treatment

Chicken feces (5 g to 21 g) were collected in the field and stored frozen until processing prior to shipment to the United States. As required by a U. S. Department of Agriculture permit for importation and transportation of fecal specimens of avian origin (Appendix 8), the feces were heated at 100°C for 25 min in equal volumes (10 to 25 ml) of 0.1 N HCl.

During the acid-heat treatment the sample bottles were covered with a special cap that included a 1-inch diameter glass marble ball in a ca. 7/8-inch diameter hole in the center of the cap to avoid pressurizing the system. The bottle was held in a wire rack in water bath and heated at 100 ºC for 25 min. The cooled bottles were capped and frozen for shipment in insulated containers. The chilled samples were frozen for shipping by international express freight, refrozen upon receipt, and thawed prior to analysis.

Sample integrity during international transport was investigated by treating 6 chicken feces homogenate samples from previous DDT feeding studies with acid and heat treatment described above. The samples were kept in the dark at room temperature for 5 days and then analyzed for DDA in the sample. Paired t-test was applied to evaluate the short period fecal DDA stability under room temperature.

Chicken feces analysis

The acid and heat treated feces were thawed and 50 ml D. I. water was added. The pH of the mixture was adjusted to above 10.0 using 3 to 4 ml 10 N KOH. The mixture was extracted 3 times with 70 ml n-hexane. The organic layer was transferred to a 250-mL Nalgene bottle. Centrifugation was applied if separation of the phases was not complete. Anhydrous Na_2SO_4 was added to dry the n-hexane.

The n-hexane was evaporated to dryness and redissolved in 1 ml n-hexane for gas chromatography with electron capture detector (GC-ECD) analysis. Recoveries of DDT/DDD/DDE/DBP in feces were in a range of 70.3% to 84.1%. The method detection limits for DDT, DDD, DDE, and DBP were 1 μg/kg dry feces.

The pH of the aqueous portion was adjusted to less than pH 2.0 using 5 ml 6 N HCl. The solution was extracted 3 times with 70 ml n-hexane and the organic layer was transferred to a 250-mL Nalgene bottle. Centrifugation was applied if separation of the phases was not complete. Anhydrous Na_2SO_4 was added to dry the extract.

The organic layer was evaporated to dryness and derivatized following the method of Chen et al. (2009). In brief, 0.4 ml of 2% DIPEA in n-hexane and 0.8 ml of 2% PFBBr in n-hexane was added and reaction was maintained at room temperature for 1 h. After the reacting solution was reduced to dryness under nitrogen, the DDA derivative was redissolved in 1 ml ethyl acetate for gas chromatography with mass spectrometry (GC-MS) analysis. Recoveries of DDA in feces were in a range of 70.6% to 71.3%. The method detection limit for DDA was 1 μg/kg dry feces.

GC-ECD analysis for DDT/DDD/DDE/DBP

Lipophilic DDT derivatives were analyzed using an HP 5890 gas chromatograph with a ^{63}Ni electron capture detector. Injector temperature was 250 ºC. Injection volume was 1 μl. Nitrogen was used as carrier gas with a flow rate of 1.0 ml/min. Chromatographic separation was performed on a HP-5ms capillary column (30 m × 0.25 mm i.d. × 0.25 μm film; Agilent Technologies, Inc. USA). The initial column temperature of 50 ºC was maintained for 1 min, increased at 30 ºC/min

to 180 ºC, and then increased at 5 ºC/min to 240 ºC and held constant for 10 min.

GC-MS Analysis for DDA-PFB-ester

DDA-PFB-ester was analyzed using a HP 6890 gas chromatograph with a HP MSD 5973 in electron impact ionization (EI) mode at ionization energy of 70 eV. The MS transfer line temperature was 280 ºC. Injector temperature was 250 ºC. Injection (1 µl) was done in the pulsed splitless mode at a pressure of 45 psi. The pulse time was 1.5 min. Helium was used as carrier gas with constant flow of 1.0 ml/min. Chromatographic separation was performed on a HP-5ms capillary column (30 m × 0.25 mm i.d. × 0.25 µm film; Agilent Technologies, Inc. USA). The initial column temperature of 50 ºC was increased at 15 ºC/min to 300 ºC and held constant for 10 min. For quantification of DDA-PFB-ester, the GC-MS was operated in a selective ion monitoring (SIM) mode. The characteristic ion m/z 460 $[M]^+$ was used as quantitative ion, m/z 235 and 237 were used as qualitative ions.

GC-MS/MS confirmation for DDT derivatives

The DDT and its selected derivatives DDD, DDE, and DDA in feces were confirmed using a Varian 3800 GC (Varian Instruments, Sunnyvale, CA) coupled with a Varian 1200 triple-quadrupole mass spectrometer. Injector temperature was 250 ºC. Injection volume was 2 µl. Chromatographic separation was performed on a HP-5ms capillary column (30 m × 0.25 mm i.d. × 0.25 µm film; Agilent Technologies, Inc. USA). The initial column temperature of 80 ºC was kept for 1 min and increased at 20 ºC/min to 190 ºC, then increased at 10 ºC/min to 250 ºC and held for 5 min, finally increased at 30 ºC/min to 300 ºC and held constant for 5 min.

The tandem quadrupole instrument was operated in electron ionization (EI)

mode. The MS/MS detector interface temperature was set at 200 °C, source temperature at 170 °C and extended dynamic range (EDR) maximum. The filament was switched on after 7.0 min, approximately 1 min before the elution of the first peak of interest. The MS/MS conditions in the multiple reaction monitoring (MRM) mode. Helium (99.997% purity) at a flow-rate of 1 ml min^{-1} was used as carrier and argon (137 kPa) as the collision gas.

Stability of DDA in feces

The stability of DDA in feces under natural environmental conditions could limit environmental surveillance and monitoring. A surrogate study evaluated the stability of DDA in feces collected following feeding 100 ppm DDT diet for 5 days. The feces were thoroughly mixed and divided into twenty-five 50 g samples in petri dishes covered with perforated polyvinylidene chloride stretch wrap (SC Johnson, Racine, WI) to avoid losses to the wind and birds. The samples were kept in partial shade to permit direct sunlight but avoid rain on the UCR campus, November 2009-April 2010. A random number generator was used to draw samples in sets of 5 on days 0, 7, 30, 90, and 180 for DDA analysis. The samples were analyzed as described in the *chicken feces analysis* section. Results were expressed in Figure 5-1.

Results and discussion

DDA excretion in chickens

DDA has been applied as a human urine biomarker in a pilot study for DDT exposure of the DDT IRS applicators (Chen et al., 2009). Detection of DDT in chickens in IRS village supported chicken as a potential sentinel species to signal

environmental DDT exposures following DDT IRS. Controlled DDT feeding studies were conducted prompted by findings of DDT-contaminated chickens in previous IRS programs (Barnhoon et al., 2009; personal communication, Bornman, 2010; Van Dyk et al., 2010).

Rapid DDA excretion occurred in White Leghorn and ISA Brown chickens following 10 to 3000 ppm DDT dietary exposure. DDA was detected within 24 h of DDT feeding. This is consistent with findings of rapid DDA excretion in humans and monkeys following active DDT exposures (Neal et al., 1946; Miller, 1977; Clark, 1977; Chen et al., 2009). DDA excretion was dose-dependent in chickens. Fecal DDA levels increased as DDT feeding level increased. Similar finding was observed in humans exposed to 3.9, 7.7, and 15.4 mg of technical DDT for up to 183 days (Roan et al. 1971). DDA levels declined to 1/10 of peak DDA level after DDT feeding stopped in a month but remained above pre-dose levels. Fecal DDA was shown to be a useful chicken DDT exposure biomarker. Chickens could be a useful sentinel species of environmental DDT exposure.

DDA stability under natural conditions

Fecal DDA stability under natural conditions was evaluated. Results of remained DDA in feces during a 180-day study period are presented in Figure 5-1. DDA in chicken feces follows a first order decay under natural conditions. About 20% DDA was left after 180 day. DDA could be converted to some unidentified products in microorganisms (Subba-Rao and Alexander, 1985) and slowly photo-decomposed under sunlight (Ware et al., 1980). Photodecomposition of DDA in aqueous solution under sunlight led to rapid formation of DBP, a seemingly terminal metabolite of DDT. DBP can be easily lost by volatilization from the

surface of the reaction mixture (Ware et al., 1980) and further degradation into hydroxylated DBP or dichlorobenzhydrol (Xiao et al., 2010; Subba-Rao and Alexander, 1985). No detectable DBP was found in this study. Since significant amount of DDA was still available after 180 days, DDA is relatively stable under natural conditions and therefore seems suitable to be used in environmental surveillance and monitoring of current DDT exposure.

Surveillance of DDT exposure in chickens following DDT IRS in South Africa

Homogenized feces samples containing DDA were acid and heat treated and held at room temperature for 5 days to simulate worst-case international transport conditions. No losses of DDA were found (Table 5-2; *p*-value=0.913) during the study period.

Pilot chicken feces environmental monitoring study was performed during 2010-2011 DDT IRS season in South Africa. Chicken feces collected from areas with reported IRS DDT use and from a control area were analyzed for DDT and its selected derivatives (DDT, DDD, DDE, DBP, and DDA).

Detection of DDT and derivatives at very low levels in chicken feces from reported IRS areas indicated some DDT contamination of chickens based upon experimental feeding studies. The terminal DDT metabolite DBP was analyzed but not detected in any of feces samples (all below method detection limit of 1 μg/kg dry feces).

Chicken feces from Lufule 1 in the control non-IRS area and Tshikhudini and Lufule 2, the two areas where DDT IRS was initially reported to have occurred, consistently revealed only trace levels of contamination. They were unchanged during monitoring. Chicken feces from the three villages contained DDA (below

LOD to 46 μg/kg), DDT (below LOD to 92 μg/kg), DDD (below LOD to 38 μg/kg), and DDE (below LOD to 95 μg/kg) as shown in Table 5-3. The measured levels apparently represent low level background contamination.

Two sets of pre-DDT spray samples from Tshikhudini and Lufule 2 areas contained higher residue levels of DDT derivatives. DDA (308-647 μg/kg dry feces) was confirmed with both characteristic ion m/z 235 and 460 on GC-MS. These pre-spray samples also contained DDT (120-208 μg/kg), DDE (54-156 μg/kg), and DDD (44-129 μg/kg). Detection of DDA and DDTs in these unsprayed areas indicated DDT contamination in the area at the time of sampling. The source of the DDT exposure was unknown. It was not evident in later samples from the same area (inconsistent with the expected disposition of DDT in chickens, Chapter 4). However, DDT release from current IRS did not occur since the area had not been sprayed since 2008 and was not sprayed with DDT as scheduled in 2010 (Bornman, personal communication, 2011).

Since DDA levels in most samples were very close to the method detection limit (1 μg/kg dry feces), only characteristic ion m/z 235 on GC-MS could be identified, the most characteristic ion m/z 460 could not be identified. Those low levels of DDA in selected chicken feces were later confirmed by GC-MS/MS at limit of detection of about 0.1 μg/kg dry feces. "Non-detected" was used for DDA level below 1 μg/kg dry feces in Table 5-3.

A lower ratio of DDE to DDTs is generally held to indicate recent exposure to DDT (WHO, 2011). Measurements of most human populations with no recent exposure suggest that the ratio is 0.8 plus. In the experimental chicken feeding studies DDE/DDTs ranged from 0.01 to 0.15 in feces when chickens were fed 10 to 3000 ppm DDT diets. DDE exposures may result from environmental and dietary

sources as well as DDT metabolism in chickens. The DDE to DDTs ratio in feces of present study was close to 0.5 at background levels of exposure (Table 5-4). The source and time of DDT in those villages is unknown, however, the last recorded use of DDT in IRS occurred in December 2008 (Bornman, personal communication, 2011). No DDT IRS occurred in the three monitored villages in 2010 as scheduled (Personal communication, Bornman, 2011). The measured levels apparently represent low level background DDT, DDE, DDD, and DDA contamination.

Total DDT found in chicken samples in Van Dyk et al. (2010) study didn't provide a direct link of DDT exposure from IRS. DDE dominance in muscle, fat, and liver of chickens indicated a previous use instead of current DDT exposure in chickens. The mean DDE to DDTs ratios were between 0.58 and 0.79 which was close to 0.8 (WHO, 2011) and may indicate an older DDT exposure instead of current exposure.

The only legal use of DDT in the area was the IRS program. However, no plausible route of DDT exposure in chickens was postulated or demonstrated in the present study.[2] Illegal use of DDT for the purpose of other than vector control may also contribute to DDT contamination in local environment (Van Dyk et al., 2010).

Conclusions

Fecal DDA was used as a biomarker of environmental DDT exposure of chickens in a pilot environmental surveillance study in a region where IRS was to be performed in an anti-malaria campaign in 2010. DDT IRS did not occur as scheduled. Very low background levels of DDA, DDE, DDD, and DDT were detected in chicken feces in three areas that were monitored (Table 5-3). DDA was

[2] It was established July, 2011 that a pyrethroid (Fendona) was substituted for DDT in the IRS program that was monitored. Bornman, personal communication, 2011.

present in substantially higher levels (up to 647 μg/kg) in two sets of pre-spray fecal samples from unknown activity (but not IRS DDT spraying). The source of the DDT exposure at the low levels observed is unknown, but the finding may represent successful use of fecal DDA in DDT surveillance as proposed here.

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Table 5-1. Chicken eggs from areas of DDT IRS-treated and untreated homes in Limpopo, South Africa (Bornman, personal communication, 2011)

Sample	*o, p'*-DDE	*o, p'*-DDD	*o, p'*-DDT	*p, p'*-DDE	*p, p'*-DDD	*p, p'*-DDT	DDE / DDTs [b]
IRS-DDT Treated							
DE 1	<50[a]	<50	<50	2599	6	2611	0.44
DE 2	<50	<50	<50	2702	<5.0	3108	0.47
DE 3	<50	<50	448	30619	<5.0	17320	0.49
DE 4	<50	<50	157	6398	17	7992	0.50
DE 6	<50	<50	131	4967	<5.0	5133	0.56
DE 7	<50	<50	78	7624	<5.0	3821	0.58
DE 8	<50	<50	89	7361	<5.0	3753	0.59
DE 9	<50	<50	78	8477	5	4095	0.63
DE 10	<50	<50	66	7836	<5.0	3748	0.66
DE 11	<50	<50	79	8215	<5.0	5612	0.66
DE 12	75	68	103	4911	<5.0	3359	0.67
DE 13	<50	<50	69	6208	5	4774	0.67
Average							0.58
No IRS							
TE 3	<50	<50	<50	107	6	78	0.56
TE 4	<50	<50	<50	<50	<5.0	<50	N/A
TE 5	<50	<50	<50	<50	<5.0	<50	N/A
TE 6	<50	<50	<50	<50	8	<50	N/A

[a] Concentration in whole egg ng/g, lipid not determined. Limit of quantification for each DDT derivative was 50 ng/g. [b] Residue levels below method detection limit were not included in the calculation of total DDT.

Table 5-2. Stability of DDA in a 5-day study to simulate international transport

Sample # [a]	No heat/0.1 N HCl (µg DDA/g Feces)	100 ºC for 25 min and 0.1 N HCl[b] (µg DDA/g Feces)
1	2.84	2.97
2	2.03	1.42
3	2.17	1.55
4	1.68	2.70
5	1.80	1.88
6	1.46	1.29
Mean ± SD	2.00 ± 0.48	1.97 ± 0.70

[a] Homogenized feces samples were randomly picked from previous chicken study 5 and antibiotic study 2 of DDT chicken feeding studies in 2010. [b] The heated and acidified samples were stored at room temperature in a dark box for 5 days to simulate transport between South Africa and the USA.

Table 5-3. Results of South African feces analysis

Spray record	Sampling site	Label #	DDA (µg/kg dry)	DDT (µg/kg dry)	DDD (µg/kg dry)	DDE (µg/kg dry)
Control no-spray area (14-15 Dec 2010)	Lufule 1[b]	11	19	44	ND	62
		12	46	37	38	93
		13	23	26	18	38
		14	11	39	NDa	56
		15	13	13	17	11
Pre-spray (14-15 Dec 2010)	Tshikhudini[c]	16	308	120	44	91
		17	505	208	76	156
		18	393	184	129	79
	Lufule 2[d]	19	491	156	84	98
		20	647	176	78	54
2-week Post spray (4-5 Jan 2011)	Tshikhudini	21	3	ND	ND	5
		22	ND	8	ND	2
		23	7	15	2	13
	Lufule 2	24	15	6	ND	30
		25	11	26	ND	10
		26	17	21	ND	25
		27	15	25	ND	27
		28	14	15	ND	23
		29	6	ND	ND	13
		30	32	13	11	95
5-week Post spray (24 Jan 2011)	Tshikhudini	31	19	16	ND	22
		32	15	14	ND	16
		33	26	6	ND	20
	Lufule 2	34	16	13	2	70
		35	13	13	ND	10
		36	13	25	ND	9
		37	16	18	ND	19
		38	15	15	ND	8
		39	14	13	ND	7
		40	9	9	1	40
HCl Control (SA)	Reagent blank	41	ND	ND	ND	ND
		42	ND	ND	ND	ND
		43	ND	ND	ND	ND
		44	ND	ND	ND	ND
		45	ND	ND	ND	ND
HCl Control (UCR)	Reagent blank	C-1	ND	ND	ND	ND
		C-2	ND	ND	ND	ND
		C-3	ND	ND	ND	ND

Table 5-3. Results of South African feces analysis (Continued.)

Spray record	Sampling site	Label #	DDA (µg/kg dry)	DDT (µg/kg dry)	DDD (µg/kg dry)	DDE (µg/kg dry)
10-week post spray (8-9 Mar 2011)	Tshikhudini	46	44	46	ND	72
		47	17	25	ND	19
		48	12	20	ND	20
		49	6	0	ND	24
		50	6	16	ND	12
		51	41	92	ND	32
		52	5	15	ND	19
		53	ND	ND	ND	9
		54	7	15	ND	14
		55	23	13	ND	12
10-week post spray (7 and 9 Mar 2011)	Lufule 2	56	6	14	ND	11
		57	4	8	ND	3
		58	ND	ND	ND	1
		59	10	11	ND	17
		60	28	26	ND	23
		61	10	17	ND	25
		62	12	18	ND	7
		63	ND	9	ND	9
		64	ND	14	ND	13
		65	6	10	ND	5
10-week post spray (7-8 Mar 2011)	Lufule 1 (Control no-spray area)	66	ND	ND	ND	ND
		67	ND	ND	ND	ND
		68	ND	ND	ND	ND
		69	ND	ND	ND	ND
		70	ND	ND	ND	ND
		71	ND	ND	ND	ND
		72	ND	12	ND	11
		73	ND	ND	ND	2
		74	ND	ND	ND	ND
		75	ND	ND	ND	ND
HCl Control (UCR)	Reagent blank	C-4	ND	ND	ND	ND
		C-5	ND	ND	ND	ND

[a] "ND" Non-detected were used to express levels of DDT and its selected derivatives in samples that were below method detection limits of 1 µg/kg dry feces. [b] Lufule 1 was a 'Control no-spray area' with no DDT IRS that was sampled at the time of Pre-spray sampling and at the 10 week sampling period [c] Tshikhudini and [d] Lufule 2 received DDT IRS and were sampled Pre-spray and after 2, 5, and 10 weeks.

Table 5-4. Average DDE/DDTs in South African chicken feces

	Tshikhudini	Lufule 2	Lufule 1
Pre-spray	0.30	0.39	0.49±0.13
2-week post spray	0.51±0.30	0.68±0.18	N/A[a]
5-week post spray	0.62±0.16	0.45±0.22	N/A
10-week post spray	0.56±0.22	0.44±0.12	0.57±0.14

[a] No samples were collected at week 2 and 5 for Lufule 1 area.

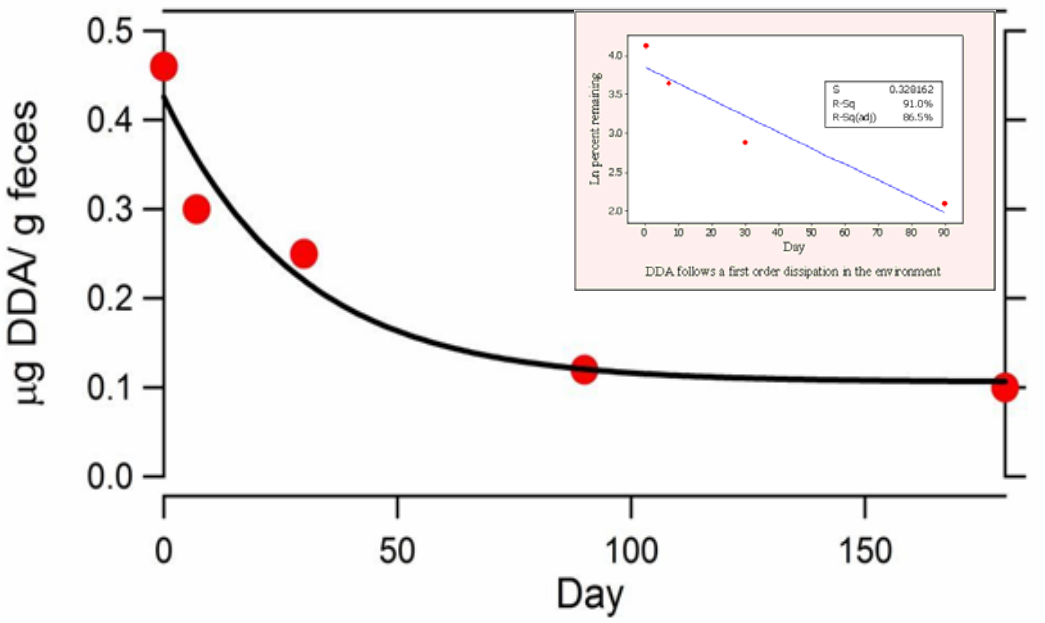

Fig. 5-1 DDA is relatively stable under natural California conditions.

Chapter 6

Occurrence of DDA in Southern California Bight Sediment

Introduction

DDT wastes in process water were discharged into the waste water system from Montrose Chemical Corporation and ultimately into the Southern California Bight through the Whites Point outfalls from the Joint Water Pollution Control Plant (JWPCP) operated by Los Angeles County Sanitation Districts (LACSD) during 1940-70s. Over 2400 metric tons of DDT was discharged in the SCB during DDT manufacturing period. This caused significant contamination of DDT in the Southern California Bight (SCB) (Ferré, et al., 2010). Although DDT introduced from JWPCP dropped to below the reporting limit (LACSD, 2004) decades after termination of DDT manufacturing, a significant quantity of DDT residues remain detectable in sediment, water column, and biota in SCB. It was reported that approximately 71% of sediment in SCB were still contaminated with DDT (Schiff et al., 2006). The water column contained measurable DDT residues which suggested SCB as a continuous, significant source of DDT contamination to the global oceans (Zeng, et al., 2005). Marine biota, i.e. California sea lions and the Pacific sanddab (*Citharichthys sordidus*), also accumulate measurable DDT residues (Kannan et. al. 2004; Schiff and Allen, 2000).

DDT mass balance calculation revealed that approximate 11% of discharged DDT can be recovered in SCB by summing up total DDTs (p, p'-DDT, p, p'-DDD, p, p'-DDE, o, p'-DDT, o, p'-DDD, o, p'-DDE) in sediment (266 metric tons), water column (10 metric tons) and marine biota (~25 kg) (Gully et al., 2008). The remaining DDT, almost 90% of that discharged, is simply "missing". Possible reasons for the apparent DDT loss includes overestimated DDT discharge, deposition of DDT in the deep basins near the LA margin, deposition of DDT in the sediment out

of LA Margin, biodegradation of DDT to some unmeasured compounds, accumulation of DDT in other unmeasured biological compartments, transport of DDT out of SCB.

Among all these possibilities, the role of microbial biodegradation of DDT in sediment in SCB as a natural means of reducing environmental contamination has received little attention. DDT has been shown to be degraded into less persistent and more polar metabolites in contaminated German soil and sediments (White and Herndon, 1995; Schwarzbauer et al., 2003). Therefore, the loss of total DDT may be due to formation of more water-soluble and less persistent metabolites like DDA and DBP, which are not included in the SCB DDT monitoring program so far.

DDT metabolism includes formation of DDA, a water-soluble degradation product and potential chemical biomarker of exposure (Figure 1-1; Stohlman and Smith, 1945; Neal et al., 1946; Wedemeyer, 1967; Roan et al., 1971; Chen et al., 2009). DDA has been identified in a variety of species following DDT exposure. It is found in mammals (Stohlman and Smith, 1945; Neal et al., 1946; Roan et al., 1971), microorganisms (Wedemeyer, 1967) and inferred in birds and fish (Addison and Willis, 1978; Ahmed and Walker, 1979). Although DDA was discovered in early DDT metabolism study and seemed to be a universal DDT metabolite (Stohlman et al., 1945; Heberer and Dünnbier, 1999), it has been neglected both as an important DDT metabolite and potential environmental contaminant (Heberer and Dünnbier, 1999). The invention of gas chromatography-electron capture detector (GC-ECD) in late 1950s facilitated easier detection of the lipophilics following simple extraction techniques (Goodwin et al., 1961). DDA in the water-soluble portion of samples was not usually included in the routine analysis and was simply missing (Heberer and

Dünnbier, 1999). Therefore, the role of DDA formation in the DDT metabolism and reduction in living systems is relatively unexplored.

Lipophilic DDTs (DDT/DDD/DDE) are generally measured and reported in samples contaminated with DDT to reflect DDT body burden and exposure status. Since DDT use has been banned or severely restricted for more than 40 years in many countries, current DDT residues are dominated by DDE, the most persistent metabolite in both humans and in the environment (ATSDR, 2002; CDC, 2005).

Although DDT and DDE are usually grouped together as DDTs to represent DDT in discussions of health concerns, DDT and DDE do not share the same toxicology. DDT is an insecticide and works as a neurotoxin (Smith et al., 1946; 2010). DDE is a persistent DDT metabolite and represents a detoxifying process (Perry and Hoskins, 1950). DDE is at least one magnitude more effective than DDT as an androgen receptor antagonist (Kelce et al., 1995). DDT is shown to be a full estrogenic agonist while DDE only act partially as an estrogenic agonist (Soto et al., 1997). DDE appears to have been a more potent inducer of eggshell thinning than DDT (Cooke, 1973b; EPA, 1975; Anderson et al., 1975; Lundholm, 1997). All these toxicological differences demonstrate experimental differences between DDT and DDE in addition to the major difference associated with their biological and environmental stability that complicates the development of dose-response relationships.

DDE is more stable than DDT. Rats and human studies have demonstrated that DDE was incapable of forming DDA when DDE was fed in the diet (Peterson and Robison, 1964; Roan et al, 1971). Therefore, DDA could be a perfect indicator to distinguish DDT and DDE exposure. Exposure assessment applying DDA as a

biomarker could reveal current DDT exposure status which provides proper judgment on hazard identification based on what is really exposed. Application of DDA may provide a valuable tool for DDT related forensic and regulatory issues.

Few studies have emphasized on the contribution of DDA to DDT metabolism and environmental fate. DDA was first quantified in surface and ground water in Berlin, Germany, originated from canal sediment contaminated by DDT residues where a previous chemical production plant located (Dünnbier et al., 1997). Concentration of DDA in surface water was up to 0.76 μg/L in Teltow Canal (Heberer and Dünnbier, 1999). A recent study revealed as high as 190 μg/L DDA levels in ground water samples in the same area and indicated DDA as the main DDT metabolite in ground water (Frische et al., 2010). Sediment DDA was reported to be the source of water contamination in the area (Dünnbier et al., 1997). DDA was found to be bound to sediment of Teltow Canal to levels up to 91,000 μg/kg dry sediment weight following alkaline hydrolysis of pre-extracted sediment. Microbial biodegradation may probably contribute to the formation of DDA in sediment since significant amount of DDD (up to 130,000 μg/kg), a known precursor of DDA, was found in the sediment (Schwarzbauer et al., 2003). In a separate study, DDA was found to account for 52 to 93% of the total DDT residues in water but was detected rarely in the sediments of Bohai Bay and its adjacent Haihe Basin in China (Wan et al., 2005).

While most studies focus on lipophilic DDTs as a measure of DDT contamination, detection of DDA in sediment and water may indicate an important role of DDA in DDT natural recovery in the environment that is not fully investigated. Occurrence of DDA in environmental and wildlife specimens may indicate a more

important role in understanding disappearance of DDT in the living systems.

The aim of these studies was to clarify the nature and availability of DDT residues in the extensive DDTs (DDT/DDD/DDE) contamination of sediments, fish, and wildlife in the SCB. Since DDA is formed from DDT and DDD but not DDE, the occurrence of DDA as a chemical biomarker would represent the available DDT/DDD residues rather than the more persistent DDE.

Therefore the occurrence of DDTs and p, p'-DDA was measured in contaminated sediments, fish collected in waters posted with warnings of DDT contamination, and bird feces (primarily seagulls and California brown pelicans) from areas of interest. In addition, DDA formation in sediment was evaluated by DDT fortification contaminated sediment in still culture. Sediment samples from Long Island, NY, a previous DDT contaminated area (Gammon et al., 2002), were also measured to evaluate overall DDA occurrence in the environment.

Materials and methods

Total of 18 archived sediment samples from LACSD 2009 monitoring program and 12 pilot environmental sediment samples from Long Island were analyzed for both lipophilic p, p'-DDT residues and p, p'-DDA. Pilot wildlife (white croaker and wildlife feces) specimens were collected and analyzed along the coastal line of SCB to evaluate p, p'-DDT exposure status in SCB using p, p'-DDA as an indicator.

Southern California Bight Sediment sampling and analysis

Surface sediment samples were obtained from archived LACSD 2009

National Pollutant Discharge Elimination System (NPDES) permit required monitoring program. Total of 18 frozen samples were received (0C, 3C, 6 A-D, 7 A-D, 8 A-D, 9 A-D). A map of sampling sites is presented in Figure 6-1. Samples that were analyzed in this study are circled with dots.

Chemicals

Chemicals included p, p'-DDT, 98.6% (Supelco, Bellefonte, PA); p, p'-DDA, 98.0% (Sigma- Aldrich, St Louis, MO); p, p'-DDE, 99.2% (Supelco); p, p'-DDD, 97.9% (Supelco); p, p'-dichlorobenzophenone (p, p'-DBP), 99.0% (Sigma- Aldrich, St Louis, MO); pentafluorobenzyl bromide (PFBBr), 99% (Sigma-Aldrich); diisopropylethyl amine (DIPEA), 99% (Sigma-Aldrich); n-hexane, 99.9% (Fisher Scientific); ethyl acetate, 99.9% (Fisher Scientific, Hampton, NH); acetone, 99.9% (Fisher Scientific), dichloromethane (DCM) 99.9% (Fisher Scientific), methanol, 99.9% (Fisher Scientific), acetate acid, 6N HCl, 10N KOH, active copper (Fisher Scientific), MP alumina N32-63, active (EcoChrom^{TM}, Eschwege, Germany), silica gel for col. Chromatography, 40-60μm, 150 Å (New Jersey, USA).

Analytical procedure for DDT and derivatives in sediment

Extractable DDT residues were obtained following Eganhouse et al. (2000). Specifically, frozen sediments were thawed and homogenized and a 30 g aliquot was transferred to a pre-extracted cellulose thimble. The sediments were Soxhlet extracted in methanol followed by dichloromethane (DCM) for 12 h each. Methanol was back extracted 3 times with 50 ml DCM. D. I. water (50 ml) and 6 N HCl (0.5 ml) were added in the methanol extract before DCM extraction. After back

extraction of the methanol, the DCM extracts were combined. Water and elemental sulfur were removed respectively by adding excess anhydrous sodium sulfate and activated copper. The DCM extracts were concentrated to about 1 ml by rotary evaporator.

Sediment extracts were separated into three fractions by column chromatography [2g, 1:2 (v/v) alumina/silica gel column, both 3% deactivated with H_2O]. Fraction 1: 10 ml n-hexane; Fraction 2: 20 ml of 26% DCM in n-hexane; Fraction 3: 20 ml methanol with 2% acetic acid. The Fraction 1 and Fraction 2 were concentrated by rotary evaporator and analyzed for DDT/DDD/DDE/DBP using GC-MS. The PCB congener 7 (2, 4-Dichlorobiphenyl, characteristic ion 222, 224 m/z) in acetone were used as internal standard and added prior to analysis. The Fraction 3 was dried and derivatized using Chen et al. (2009) described below and analyzed for the DDA derivative using GC-MS.

DDA in dried Fraction 3 was derivatized using 0.4 ml N, N-diisopropylethyl amine (DIPEA) and 0.8 ml pentafluorobenzyl bromide (PFBBr) in a 7-mL vial for 1 h at room temperature. The reacting solution was made to dryness under nitrogen stream and redissolved in 1 ml acetone with PCB congener 7 and analyzed using GC-MS.

Bound DDT residues were obtained from a modified method of Schwarzbauer et al (2003). The pre-extracted sediment sample was placed in a sealed sample bottle and 10 ml 10 N KOH and 40 ml methanol were added. Subsequently the bottles were heated at 95 ºC for 24 h alkaline hydrolysis. After cooling, the solution was transferred to a 150-mL centrifuge tube and centrifuged at 5000 g for 3 min. Water was added and the solution was transferred to a 250-mL separatory

funnel. The pH was adjusted to less than 2 by addition of 6 N HCl. Subsequently the solution was extracted 3 times with 50 ml DCM. The DCM layer was dried with anhydrous sodium sulfate and concentrated to about 1 ml.

The crude extracts were separated into two fractions by column chromatography (2 g silica gel) and eluted using 20 ml DCM (Fraction 1) and 20 ml methanol solution with 2% acetic acid (Fraction 2). Fraction 1 was made to dryness with nitrogen evaporator and redissolved in 1 ml acetone for DDT/DDD/DDE/DBP analysis. The PCB congener 7 was added as internal standard prior to analysis. Fraction 2 was made to dryness with nitrogen evaporator and derivatized for DDA analysis using Chen et al. (2009) described above.

Water analysis

Sea water in the sediment DDA formation test was processed following the method of Chen et al (2009). Briefly, the pH of sea water was adjusted to above 10 by addition of 1 ml 10 N KOH and extracted 3 times with 50 ml n-hexane. The organic extract was dried over anhydrous sodium sulfate and evaporated to dryness. The organic extract was redissolved in 1 ml acetone for GC-ECD analysis. The pH of the above solution was then adjusted to less than 2 using 3 ml 6N HCl and extracted 3 times with 50 ml n-hexane. The organic extract was dried over anhydrous sodium sulfate and evaporated to dryness. DDA was derivatized as described above and analyzed using GC-MS.

Sediment DDA formation experiment

In order to investigate DDA formation in sediment of SCB, a controlled 5-day

laboratory still culture study was conducted by adding known amount of DDT in the fresh DDT contaminated sediments in SCB.

Fresh sediment samples were obtained from LACSD benthic sediment sampling fieldtrip in July, 2011. Totally 3 replicates (each about 100 g) from 3 sampling sites in LACSD DDT monitoring program were received. One replicate from each site was randomly selected and combined to make a 300 g composite sample and thoroughly mixed. The composite was sub-divided into five 50 g sub-samples. Instant ocean sea water (United Pet Group, Inc. Cincinnati, OH) was prepared and 50 ml was added into each sub-sample. Use of self prepared sea water could eliminate the factor that bacteria in real ocean sea water may be able to metabolize DDT.

One sub-sample from each set of control samples was analyzed on day 0 to obtain existing DDT and DDA levels in the sediment and day 5 to be used as a positive control. Then 5000 µg DDT was dissolved in acetone and mixed with the sea water and sediment mixture thoroughly. The sample jar was closed tightly and stored in refrigerator at 8 ºC. One sub-sample containing DDT from each set of DDT samples was analyzed on Day 0 and the others were analyzed on Days 2 and 5.

Sediment and sea water was separated by centrifugation prior to analysis and selected DDT derivatives (DDT/DDD/DDE/DBP/DDA) were measured in the sediment and water.

Southern California Bight wildlife sampling and analysis

Total of 9 white croaker samples were obtained from archives of SCCWRP's SCB regional monitoring program and anglers at Cabrillo Beach pier, San Pedro CA.

A total of 53 pelagic bird species have been documented in the SCB (McGrath and Feenstra, 2005). Wildlife bird feces samples were collected from Cabrillo Beach breakwater, San Pedro, CA and Guadalupe Beach at Santa Maria, CA. Western gull, California gull and brown pelican were the main bird species resting on the break water of Cabrillo Beach (Alps, personal communication, 2011). All fish and feces samples were stored frozen prior to analysis.

Fish tissue analysis

Five croakers from each sampling site of SCCWRP monitoring program and individual croakers from Cabrillo Beach pier were each homogenized using blender and an aliquot of 50 g homogenate was analyzed for DDT residues. After addition of 50 ml D. I. water, the homogenate was hydrolyzed with 10 ml 6 N HCl in waterbath for 1 h. Lipophilic DDT residues were extracted using 100 ml n-hexane for 3 times in blender after pH of the homogenate was adjusted to above 10. Necessary centrifugation was applied when layer separation was not complete. The extract was cleaned up with 10 ml concentrated sulfuric acid and evaporated to dryness. The final solution was in 1 ml n-hexane for GC-ECD analysis. DDA was extracted from the above alkaline homogenate using 100 ml n-hexane for 3 times in blender after pH of the homogenate was adjusted to less than 2 using 6N HCl. The n-hexane extract was dried and derivatized following the method described above for GC-MS analysis.

Wildlife feces analysis

The wildlife feces sample was thawed and homogenized with equal weight of

D. I. water in blender and an aliquot of 50 g homogenate was analyzed for DDT residues. After dilution with 100 ml D. I. water, the homogenate was hydrolyzed with 10 ml 6 N HCl in waterbath for 1 h. Lipophilic DDT residues were extracted using 100 ml n-hexane for 3 times after pH of the homogenate was adjusted to above 10. The n-hexane extract was evaporated to dryness and redissolved in 1 ml n-hexane for GC-ECD analysis. DDA was extracted from the above alkaline homogenate using 100 ml n-hexane for 3 times after pH of the homogenate was adjusted to less than 2. The n-hexane extract was evaporated to dryness and derivatized following the method described above for GC-MS analysis.

GC-ECD analysis

GC-ECD analysis was done using an HP 5890 gas chromatograph with an electron capture detector. Injector temperature was 250 ºC. Injection volume was1 µl. Nitrogen was used as carrier gas with a flow rate of 1.0 ml/min. Chromatographic separation was performed on a HP-5ms capillary column (30 m × 0.25 mm i.d. × 0.25 µm film; Agilent Technologies, Inc. USA). The initial column temperature of 50 ºC was maintained for 1 min, increased at 30 ºC/min to 180 ºC, and then increased at 5 ºC/min to 240 ºC and held constant for 10 min.

GC-MS analysis

GC-MS analysis was done using an HP 6890 gas chromatograph with a HP 5973 MSD in electron impact (EI) ionization mode at ionization energy of 70 eV. The MS transfer line temperature was 280 ºC. Injector temperature was 250 ºC. Injection (1 µl) was done in the pulsed splitless mode at a pressure of 45 psi. The

pulse time was 1.5 min. Helium was used as carrier gas with constant flow of 1.0 ml/min. Chromatographic separation was performed on a DB-1701 capillary column (30 m × 0.25 mm i. d. × 0.25 um film; Agilent Technologies, Inc. USA). The initial column temperature of 50 ºC was increased at 15 ºC/min to 300 ºC and held constant for 10 min. For quantification of DDT residues, the GC-MS was operated in a selective ion monitoring (SIM) mode. The quantitative ions (m/z) were listed in Table 6-1.

GC-MS/MS confirmation for DDT derivatives

The DDT and its selected derivatives DDD, DDE, and DDA-PFB-ester in sediment were confirmed using a Varian 3800 GC (Varian Instruments, Sunnyvale, CA) coupled with a Varian 1200 triple-quadrupole mass spectrometer. Injector temperature was 250 ºC. Injection volume was 2 µl. Chromatographic separation was performed on a HP-5ms capillary column (30 m × 0.25 mm i.d. × 0.25 µm film; Agilent Technologies, Inc. USA). The initial column temperature of 80 ºC was kept for 1 min and increased at 20 ºC/min to 190 ºC, increased at 10 ºC/min to 250 ºC and held for 5 min, finally increased at 30 ºC/min to 300 ºC and held constant for 5 min.

The tandem quadrupole instrument was operated in electron ionization (EI) mode. The MS/MS detector interface temperature was set at 200 °C, source temperature at 170 °C and EDR maximum. The filament was switched on after 7.0 min, approximately 1 min before the elution of the first peak of interest. The MS/MS conditions in the multiple reaction monitoring (MRM) mode. Helium (99.997% purity) at a flow-rate of 1 ml min^{-1} was used as carrier and argon (137 kPa) as the collision gas.

Quality control

The recovery rates of DDT residues in sediment, fish and bird feces were not adjusted since recoveries of most compounds were in the acceptable ranges of 70–120%. Instrument limit of detections (LOD) of DDT/DDD/DDE/DBP were 0.01 mg/L on GC-ECD and 0.1 mg/L for DDA on GC-MS.

Results and discussion

DDA occurrence in sediment of SCB

DDA was detected and confirmed for the first time in both extractable and bound extracts of sediment samples from the Sanitation Districts of Los Angeles County 2009 NPDES permit required monitoring program. As a water-soluble DDT residue, DDA was not included in previous, traditional residue analysis. DDA represents about 0.3 % of the surface DDT residue at the highest level (or 0.03 % of total DDTs) in our present study. DDA was detected from below the limit of quantification (0.5 μg/kg) to 76 μg/kg dry sediment weight in sediment samples (DDA levels in extractable and bound residue analysis were combined for each sample). Further GC-MS/MS analysis (limit of detection ~0.05 μg/kg dry sediment) confirmed DDA existence even in samples with no detection by GC-MS analysis. DDA occurrence in SCB is a common feature of DDTs contamination and it may represent a water soluble derivative that is important in the natural recovery of SCB. There is a concentration dependent distribution of DDA detection in sediment samples. At site 8C where DDT level was highest, DDA concentration were also high compared to other sites with lower DDT residues. A terminal DDT metabolite DBP was also detected (<0.5 to 67 μg/kg) in most samples. Detection of DBP is further evidence

of DDA formation since DDA is on the pathway to form DBP in biological systems (Wedemeyer, 1967).

DDT breakdown on GC column was observed after start of analysis and introduced some uncertainty into the analysis. An EPA monitoring method for GC-derived breakdown of DDT was subsequently applied (Foreman et al., 1997). A performance evaluation standard (PES) containing DDT, but not DDD or DDE was injected at regular intervals throughout the GC analytical sequence to monitor the breakdown as calculated using peak areas as follows:

$$\%\ \text{p,p'-DDT breakdown} = \frac{\text{area p,p'-DDD} + \text{area p,p'-DDE}}{\text{area p,p'-DDT} + \text{area p,p'-DDD} + \text{area p,p'-DDE}} \times 100$$

About 3 to 24 % breakdown of DDT occurred during the analysis. As a result reported DDD and DDE levels may be increased 23 % and 1 % relative to the true levels in sediment extracts. Injection port liner was changed when DDT breakdown exceeded 20 % as indicated by injection of the PES.

Complete DDT residue results are summarized in Table 6-2 for extractable residues and Table 6-3 for bound DDT residues. DDE was dominant in most samples with a range of 127-81,437 μg/kg dry sediment weight. DDD levels varied between 11 and 185,743 μg/kg dry sediment weight. DDT levels ranged from 17 to 25,114 μg/kg dry sediment weight. The bound DDT/DDD/DDE residues were negligible compared to the residues found in the Soxhlet extractable part. Site 8C contained the highest residue levels of every DDT residue.

DDD is a known precursor of DDA (Gold et al., 1984). As one of the lipophilic DDT contaminants DDD is likely to biodegrade and disappear more rapidly than DDT and DDE since the DDD level is low to non-detectable in aged human and

environmental samples with previous DDT contamination (CDC, 2005). DDD levels in most sediment samples were low. DDD was associated with further degradation to more polar metabolites such as DDA and DBP compared to DDE. For example, the DDD level in site 0C was 11 μg/kg and DDE was 238μg/kg (Table 6-2). Potential DDT natural recovery through degradation of DDD and formation of DDA in site 8C seems to be substantial. Measurements of DDA may demonstrate a continuous natural recovery of DDT in the SCB. DDA may play an important role in DDT metabolism and reduction in sediment of SCB.

DDA formation may be a key to answer mass balance questions concerning the total DDT discharged to the SCB and the amount of DDTs accounted for in biological and environmental monitoring (Gully et al., 2008).

The extent of DDA formation in the sediment is unknown, but the finding of DDA in the sediment of SCB provides an additional new consideration for the regional DDT monitoring program. Although DDT and its more persistent metabolite, DDE, have very long half-life in the environment (ATSDR, 2002), these chemicals are further degraded into more polar, less persistent forms i. e. DDA, DBP as shown in our study. More complete accounting of DDT related residues in addition to the lipophilic DDTs (o, p'- and p, p'-DDT/DDD/DDE) should be included in the regional DDT monitoring to help to address mass balance questions in the SCB.

Sediment DDA formation test

The mechanism of formation of DDA in sediment is uncertain. Rapid DDA formation was observed within 2h of DDT addition in the fresh sediment collected in July 2011. Complete results are shown in Table 6-4. DDA levels were

significantly higher in the DDT treated samples than the control samples (p-value < 0.001). DDT fortified samples (5000 μg/sample) of sediment contained up to 1500 μg/kg dry wt when DDA residues in the Soxhlet (12.6%) and alkaline hydrolyzed extracts (87.4%) were combined**.** DDA levels (up to 40 μg/kg) in the control samples on Days 0 and 5 were relatively low, similar to previously reported levels (up to 76 μg/kg, Table 6-2 and Table 6-3), and unchanged during the study. DDA levels in the DDT treated sediments were not significantly different over the 5-day study period (p-value > 0.1). No time-dependent increase in DDA quantity in DDT treated sediment was observed in the present study. DDA was only detected on day 5 in sea water at level of 3.5 μg/L representing very slow release of DDA from sediment into the water system in still culture. The DDD level on Day 5 was higher than the DDD levels on Day 0 and Day 2 and all were comparable with the control range (Day 0 and Day 5). DDE levels in either control or DDT treated sediment were relatively unchanged and indicated an anaerobic degradation in this case (Guenzi and Beard, 1967).

The steady sediment DDA level and negligible release in water during the 5-day study indicated that DDA was mostly formed at the initial stage of DDT exposure on day 0. The mechanism of rapid biotic or abiotic DDA formation is unknown. The observed formation of DDA which was not time dependent may have been limited by one or more of the following considerations:

1. Limited availability by rapid binding of DDT to sediment materials;
2. Lack of cofactor due to rapid exhaustion of an essential factor for degradation;
3. Limited binding or catalytic sites;

4. Lack of oxygen since rapid change to anaerobic conditions would occur in still culture.

Increased DBP levels following addition of DDT to the sediment were also noted during analysis of the extracts. DBP levels were less than 9 μg/kg in the control and up to 163 μg/kg in the DDT treated sediment. Detection of DBP in the sediment may also represent further DDA biotic or abiotic degradation.

It is noted that DDA residues in the alkaline hydrolyzed extracts were 6 to 10 times higher than in the regular Soxhlet extracts. The Soxhlet extracts contained up to 144 μg DDA/kg dry wt and the alkaline hydrolyzed extracts held 523 to 1357 μg DDA/kg. This finding demonstrated strong binding of DDA to the non-extractable particulate matter in the sediment as previously indicated by Schwarzbauer et al. (2003) in the authentic environmental samples. It is therefore crucial to include alkaline hydrolysis in order to obtain DDA residue in the sediment. Unfortunately, alkaline hydrolysis is not usually performed in DDT residue extraction in sediments (Schiff, 2000; Wan et al., 2005; Yu et al., 2011) and information on DDA occurrence is not available as a consequence.

DDA determination in Long Island sediment

An additional set of DDT contaminated sediments collected by USGS were analyzed to determine residual DDA. DDT was widely used on Long Island, NY, primarily for control of mosquitos and gypsy moths before its ban in the United States in 1972 (Gammon et al., 2002). DDT accumulation in human body and high breast cancer rates in local communities raised huge concerns about the legacy DDT in both humans and the local environment (Gammon et al., 2002). Detectable total DDTs

were reported in sediment, mussels, and shellfish (Foehrenbach, 1972; Turgeon and O'Connor, 1991). However, DDA was not included in sediment sample analysis in the region.

DDA was confirmed in a pilot Long Island sediment analysis study following alkaline hydrolysis of pre-Soxhlet extracted sediments. Complete results are shown in Table 6-5. DDA was found in most of the sediments and represented up to 7.5% of total DDT (DDT+DDD+DDE) residues. DBP was also detected and was a sign of further degradation of DDA. Higher DDA levels were also observed in sediments with higher DDD levels. Since DDD is a known DDA precursor, it is expected that the DDD residues can be converted to DDA (Gold et al., 1984). The relatively high DDD levels in these sediments indicated anaerobic degradation of DDT (Pereira et al., 1996; Huang et al, 2001).

Occurrence of DDA in both SCB and Long Island and detection of DDA in sediment and water of Teltow Canal in Berlin (Heberer and Dünnbier, 1999; Schwarzbauer et al., 2003) provide evidence that DDA may be a generally important DDT metabolite in the environment. Since DDA is more water-soluble and relatively stable in water, DDA may become relatively persistent when bound to sediments. Alternatively, the slow release of this water soluble DDT derivative may represent an important pathway for the natural reduction of environmental levels of DDT and associated DDA precursors. Each possibility warrants further study given the attention that continues to be assigned to environmental contamination by DDT and its persistent derivatives.

Fish and wildlife bird feces DDT surveillance

Pilot wildlife DDT surveillance showed that DDE was dominant in most of the marine samples in SCB. DDA was not detected in either croaker or bird feces samples. DDE dominated in the wildlife bird feces at levels up to 6.7 μg/kg fresh feces (Table 6-6). No recent DDT exposure was indicated in the feces. The DDE/DDTs ratio is 1 since only DDE is identified. DDT residue levels in white croakers are shown in Table 6-7. DDE accounted for 48 to 100 % of total DDT residues in the white croakers. DDD was relative low in each sample (<17 μg/kg). DDT was low in most croaker samples except for two samples from SCCWRP monitoring program with relatively high levels of DDT (134 μg/kg in SCCWRP-2 and 73μg/kg in SCCWRP-5). Dominance of DDE and lack of DDA in wildlife samples indicated no recent DDT exposure and its limited availability in SCB**.**

Conclusions

Analysis of archived sediment samples from SCB revealed DDA existence in DDT contaminated sediments. DDA formation may represent an important unexplored DDT degradation pathway in the contaminated area. The transformation of DDT may represent a natural recovery process that deserves consideration in discussion of means to mitigate the impact of DDT on SCB.

DDE, a terminal persistent residue, is dominant in most sediment samples indicating no recent DDT exposure occurs in most parts of SCB. However, the site 8C, the primary waste water outfall, represents some current DDT exposure from residues held within sediment since DDD and DDT levels in 8C were high according to the present results (Table 6-2). Detection of DDD in relatively high level in Long

Island sediment indicated some anaerobic degradation of DDT. More DDA formation was expected as a result of further degradation of DDD and DDT in the region.

Analysis of archived sediment samples from Long Island also revealed DDA existence in DDT contaminated sediments. These findings support the suggestion that DDA may be generally important as a water-soluble DDT derivative in sediments.

Whether DDA formation is biotic or abiotic in sediments is uncertain. DDA was formed in the sediment of SCB fortified with DDT in still culture. DDA was rapidly formed at the initial stage of the test and no further degradation occurred during the 5 d observation period. DDA was bound to the non-extractable particulate matter in the sediment and alkaline hydrolysis was required to release DDA from sediment for analysis. In this respect the newly formed DDA behaved similarly to the bound residues that were present in the archived SCB samples.

DDE dominance and lack of DDA detection in wildlife bird feces and white croakers in SCB reflected no recent DDT exposure occurred in SCB. Detection of DDA in biological system could be used as an indicator of recent DDT exposure.

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Table 6-1. DDT and its selected derivatives analyzed in sediment

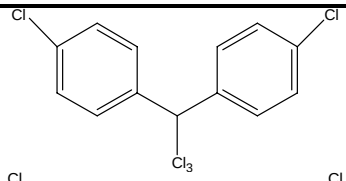
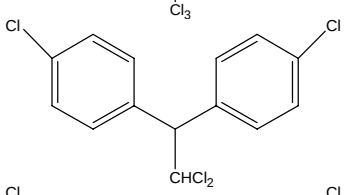
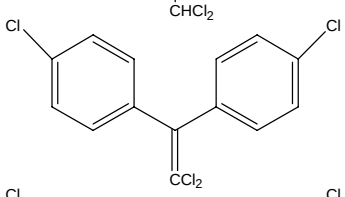
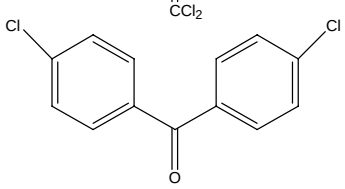
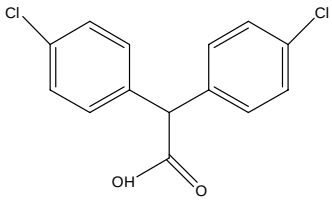
Compound Name	Chemical Structure	Quantification Ions (m/z)
p, p'-DDT		235, 237
p, p'-DDD		235, 237
p, p'-DDE		246, 248
p, p'-DBP		139, 141
p, p'-DDA		235, 237, 460

Table 6-2. Extractable DDT residues of surface sediments from Southern California Bight, 2009 (μg/kg dry sediment weight)

LACSD Sample ID [a]	Dry weight %	p, p'-DDA	p, p'-DBP	p, p'-DDE	p, p'-DDD	p, p'-DDT	Sum of (DDT+ DDD+DDE)
0C-1	63.7	<0.5 [b]	11	238	11	17	266
3C-1	60.7	<0.5	4	492	26	19	537
6A-1	51.0	<0.5	11	653	75	93	821
6B-1	45.4	<0.5	19	1515	121	55	1691
6C-1	51.1	<0.5	16	1001	73	34	1108
6D-1	73.3	<0.5	5	141	10	48	199
7A-1	52.3	2	<0.5	980	40	30	1050
7B-1	54.8	<0.5	5	1475	225	63	1763
7C-1	66.9	<0.5	<0.5	2257	80	33	2370
7D-1	66.5	<0.5	<0.5	237	9	18	264
8A-1	44.7	<0.5	<0.5	3076	59	40	3175
8B-1	47.2	<0.5	<0.5	1890	172	77	2139
8C-1	51.7	33	<0.5	81437	185743	25114	292294
8D-1	70.7	<0.5	<0.5	220	22	78	320
9A-1	49.9	<0.5	26	661	925	432	2018
9B-1	55.5	<0.5	10	850	54	56	960
9C-1	63.9	<0.5	15	453	43	55	551
9D-1	72.5	<0.5	<0.5	127	13	35	175

[a] All analyzed surface sediment samples (2 cm) are from the Sanitation Districts of Los Angeles County (LACSD) 2009 monitoring program. [b] Method limit of detection (LOD) was used for the non-detectable level.

Table 6-3. DDT residues in pre-extracted sediments following alkaline hydrolysis of surface sediment extracts (μg/kg dry sediment weight)

LACSD Sample ID [a]	p, p'-DDA	p, p'-DBP	p, p'-DDE	p, p'-DDD	p, p'-DDT	Sum of (DDT+ DDD+DDE) [c]
0C-1	<0.5 [b]	<0.5	<0.5	<0.5	<0.5	1
3C-1	2	7	<0.5	<0.5	<0.5	1
6A-1	2	11	12	<0.5	9	21
6B-1	3	23	1	2	<0.5	3
6C-1	<0.5	8	9	25	6	40
6D-1	<0.5	<0.5	1	<0.5	<0.5	2
7A-1	1	<0.5	9	<0.5	<0.5	10
7B-1	2	38	12	37	5	54
7C-1	6	16	5	2	<0.5	7
7D-1	4	8	2	4	<0.5	6
8A-1	7	28	6	3	<0.5	9
8B-1	11	35	9	5	<0.5	14
8C-1	43	67	12	<0.5	<0.5	13
8D-1	3	6	4	2	5	11
9A-1	<0.5	<0.5	<0.5	<0.5	<0.5	1
9B-1	1	16	<0.5	<0.5	<0.5	1
9C-1	2	3	<0.5	<0.5	<0.5	1
9D-1	3	<0.5	1	3	<0.5	4

[a] All analyzed surface sediment samples (2 cm) are from the Sanitation Districts of Los Angeles County (LACSD) 2009 monitoring program. [b] Method limit of detection (LODs) were used for the non-detectable levels. [c] LOD/2 for those non-detectable levels were used in the sum of (DDT+DDD+DDE) as for statistical significance.

Table 6-4. Results of sediment DDA formation test

Sample I.D.	Dry weight %	DDA (μg)	DDA conc. (ppb)	DBP (μg)	DBP conc. (ppb)	DDE (μg)	DDE conc. (ppb)	DDD (μg)	DDD conc. (ppb)	DDT (μg)	DDT conc. (ppb)	total DDTs (μg)	total conc. (ppb)
Sample1-1(control[a]	52.3	0.1	3	N/A	N/A	363	13888	194	7405	12	446	568	21739
Sample2-1(control)	52.4	1	40	0.2	7	243	9282	261	9953	17	655	521	1988
Sample3-1(control)	51.8	1	40	0.2	9	515	19889	284	10947	109	4225	908	35061
Sample1-2(DDT)	52.3	22	839	4	163	563	21520	1066	40747	3376	129086	5004	191353
Sample2-2(DDT)	52.4	19	732	3	131	555	21197	1127	43001	5050	192728	6732	256925
Sample3-2(DDT)	51.8	25	970	0	0	380	14685	801	30915	2567	99098	3748	144698
Sample1-3(DDT)	52.3	16	621	3	117	223	8534	596	22807	1144	43756	1964	75096
Sample2-3(DDT)	52.4	21	799	2	62	425	16209	1415	54003	2570	98076	4409	168288
Sample3-3(DDT)	51.8	39	1501	1	38	629	24282	1550	59855	3063	118257	5242	202397
Sample1-4(DDT)[b]	52.3	27	1039	2	86	504	19289	1150	43966	2673	102236	4328	165491
Sample2-4(DDT)[b]	52.4	20	778	1	28	291	11112	1046	39916	2365	90265	3702	141293
Sample3-4(DDT)[b]	51.8	19	743	2	87	711	27432	2178	84084	3136	121098	6025	232613
Sample1-5(control)	52.3	1	39	0	0	493	18840	624	23855	24	911	1140	43606
Sample2-5(control)	52.4	1	25	0.1	3	261	9977	609	23258	43	1624	913	34858
Sample3-5(control)	51.8	1	34	0	0	778	30029	930	35886	19	734	1726	66649

[a] The hydrolysis sample of 1-1 was lost due to damage of container. No results of DDA and DBP were obtained from this part.
[b] No HCl was added before extraction of soxhlet-treated samples, there was some loss for DDA level in these three samples.

Table 6-5. DDT residues in Long Island sediments (μg/kg dry sediment weight)

	DDA	DBP	DDE	DDD	DDT
LIM19	54	10	392	2411	477
LIM20	2	13	86	99	298
LIM22	15	3	333	1313	198
LIM24	<0.5	0	45	69	116
LIM25	19	0	44	152	37
LIM26	<0.5	11	111	302	89
LIM28	63	14	384	1658	1433
LIM29	13	15	178	1034	1454
LIM30	<0.5	28	84	122	27
LIM31	<0.5	17	184	68	236
LIM33	11	12	524	1474	577
LIM34	5	8	222	290	1303

Table 6-6. DDT surveillance analysis in wildlife bird feces (DDT residue level in wildlife feces is expressed as μg/kg)

Sample I.D.	p, p'-DDA	p, p'-DDT	p, p'-DDD	p, p'-DDE	p, p'-DBP
CBB-1[a]	< 1[c]	< 0.5	< 0.5	1.6	< 0.5
CBB -2	<1	<0.5	<0.5	2.1	<0.5
CBB -3	<1	<0.5	<0.5	6.7	<0.5
CBB -4	<1	<0.5	<0.5	5.2	<0.5
CBB -5	<1	<0.5	<0.5	4.9	<0.5
CBB -6	<1	<0.5	<0.5	3.0	<0.5
CBB -7	<1	<0.5	<0.5	3.6	<0.5
Santa Maria[b]	<1	<0.5	<0.5	<0.5	<0.5

[a] Cabrillo beach breakwater (CBB) bird feces samples were collected with paper towels wiping feces on the breakwater rocks. Every 30-50 g feces was collected in a Ziploc bag as one sample. [b] Santa Maria bird feces sample was collected and considered as a control since this location is far from DDT contaminated areas. [c] Method limit of detection (LOD) was used for the non-detectable level.

Table 6-7. Pilot DDT surveillance in white croakers in Southern California Bight (DDT residue level in white croaker is expressed as μg/kg)

Sample I.D.	p, p'-DDE	p, p'-DDD	p, p'-DDT	p, p'-DDA
SCCWRP-1[a]	152	6	3	<1[c]
SCCWRP-2	138	17	134	<1
SCCWRP-3	284	8	6	<1
SCCWRP-4	312	3	4	<1
SCCWRP-5	92	6	73	<1
Cabrillo pier-1[b]	114	3	2	<1
Cabrillo pier-2	94	5	2	<1
Cabrillo pier-3	13	<0.5	1	<1
Cabrillo pier-4	11	<0.5	<0.5	<1

[a] SCCWRP: Southern California Costal Water Research Project. Five white croaker samples were obtained from SCCWRP SCB monitoring program. [b] White croakers were also obtained from anglers at Cabrillo beach pier. [c] Method limit of detection (LOD) was used for the non-detectable level.

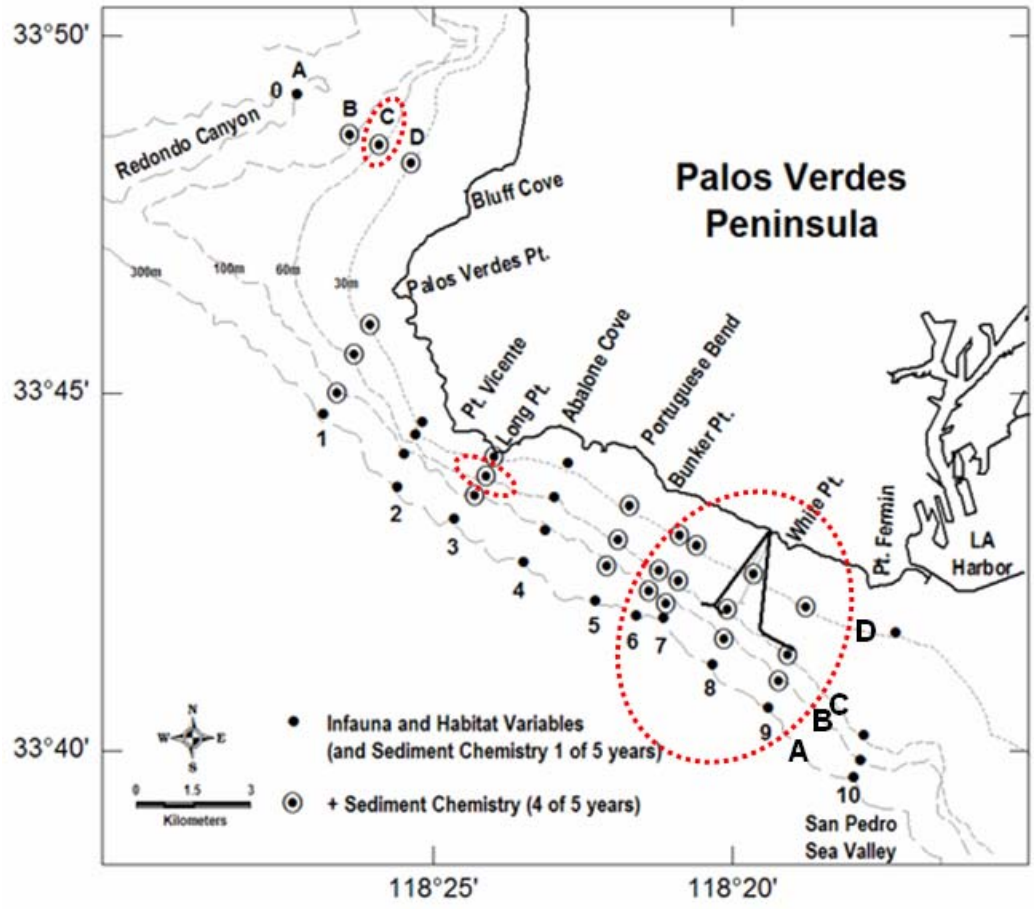


Fig. 6-1 Map of LACSD sediment sampling sites. The stations are arranged along 11 transects (Transects 0 through 10), and four isobaths. D stations are positioned at 30 m (the inner shelf), C stations at 61 m (mid-shelf), B stations at 152 m (the outer shelf), and A stations at 305 m (the upper slope). Analyses are performed on all 44 sites every five years. A sub set of 24 stations are sampled and analyzed annually. Samples analyzed in UCR study are circled with dots. (● Sampling site monitored every 5 years by LACSD; ◉ Annual LACSD monitoring site; ⬭ Sampling site processed by UCR-PCEP)

CHAPTER 7

Summary

The possible use of DDA as a chemical biomarker of DDT exposure in human and the environment has been investigated and the occurrence of DDA as an environmental contaminant has been evaluated.

DDA as a chemical biomarker of human DDT exposure: method development and application in human urine biomonitoring in malaria Indoor Residual Spraying

DDT metabolism in humans yields DDA as principal urinary metabolite and potential exposure biomarker. A method for DDA analysis in human urine was developed using PFBBr and DIPEA (Chen et al., 2009). The limit of detection for DDA was 0.1 µg/L urine by GC-ECD and 2 µg/L urine by GC-MS.

A 2 mg/kg b.w. DDT oral human volunteer study was done to repeat the study of Neal et al. (1946). DDA excretion appeared within 24 h of exposure in both studies, consistent with the reports of Roan et al. (1971) who studied persons with continuing chlorinated hydrocarbon exposure. DDA maintained above pre-dose level during the 14 d post-administration period. No other analytes were detected in urine. DDA recovery of 0.4% (mole %) in the present study and approximately 2%in Neal et al. (1946) study indicated DDA formation is not a major metabolic pathway in initial stage of DDT exposure. DDA detection in urine demonstrated its potential application in urine biomonitoring since it is rapid to excrete, simple to collect and specific to analyze.

Urine specimens from DDT applicators in Swaziland and South Africa were analyzed to evaluate the method. The mean DDA levels during the spray season and post-season were 59 and 11 µg/L, respectively. These results must be interpreted

cautiously since different groups of workers provided urine specimens in each case. The DDA urinalysis may be a feasible monitoring strategy for low-level occupational and residential DDT exposure assessment in anti-malaria campaigns.

Rapid reduction of DDA excretion following termination of DDT exposure is a feature of DDT metabolism that is extremely important support for the use of DDA excretion in occupational and residential biomonitoring.

DDA as a chemical biomarker in chicken feces of DDT exposure: laboratory DDT chicken feeding studies to evaluate chickens as a sentinel species for study of environmental fate and transport of DDT

The results of current study demonstrated fecal DDA as a chemical biomarker of DDT exposure in chickens. DDA excretion in feces was dose-dependent. Rapid DDA excretion was found in chicken feces following DDT exposure in diet. DDA excretion levels declined in several days after DDT feeding. Fecal DDA can be a simple and useful DDT exposure biomarker that may be useful to distinguish DDT and DDE exposure in environmental studies.

Chicken blood and egg DDT can reflect DDT body burden and be indicators of fecal DDA. Fecal DDA excretion was the major depletion process for reduction of body burden of DDT in chickens when DDT exposure occurred. The antibiotic treatment indicated an important role of gut microflora in the metabolism of DDT to DDA in chickens and potentially in other organisms.

Chicken or birds may be used as a sentinel species to estimate dietary and environmental DDT contaminations. Demonstration of DDT exposure using DDA as a biomarker may represent a useful tool to clarify some public health and

regulatory concerns related to the occurrence and toxicology of these persistent chlorinated hydrocarbons in the environment.

Measurement of fecal DDA in chicken feces as a surveillance tool to assess current DDT exposure potential

Fecal DDA was used as a biomarker of environmental DDT exposure of chickens in a pilot environmental surveillance study in a region where IRS was to be performed in an anti-malaria campaign in 2010. DDT IRS did not occur as scheduled. Very low background levels of DDA, DDE, DDD, and DDT were detected in chicken feces in three areas that were monitored (Table 5-3). DDA was present in substantially higher levels (up to 647 μg/kg) in two sets of pre-spray fecal samples from unknown activity (but not IRS DDT spraying). The source of the DDT exposure at the low levels observed is unknown, but the finding may represent successful use of fecal DDA in DDT surveillance as proposed here.

Occurrence of DDA with legacy DDTs in sediments and wildlife DDT-contaminated areas of Southern California Bight and Long Island, NY.

Analysis of archived sediment samples from SCB revealed DDA existence in DDT contaminated sediments. DDA formation may represent an important unexplored DDT degradation pathway in the contaminated area. The transformation of DDT may represent a natural recovery process that deserves consideration in discussion of means to mitigate the impact of DDT on SCB.

DDE, a terminal persistent residue, is dominant in most sediment samples indicating no recent DDT exposure occurs in most parts of SCB. However, the site

8C, the primary waste water outfall, represents some current DDT exposure from residues held within sediment since DDD and DDT levels in 8C were high according to the present results (Table 6-2). Detection of DDD in relatively high level in Long Island sediment indicated some anaerobic degradation of DDT. More DDA formation was expected as a result of further degradation of DDD and DDT in the region.

Analysis of archived sediment samples from Long Island also revealed DDA existence in DDT contaminated sediments. These findings support the suggestion that DDA may be generally important as a water-soluble DDT derivative in sediments.

Whether DDA formation is biotic or abiotic in sediments is uncertain. DDA was formed in the sediment of SCB fortified with DDT in still culture. DDA was rapidly formed at the initial stage of the test and no further degradation occurred during the 5 d observation period. DDA was bound to the non-extractable particulate matter in the sediment and alkaline hydrolysis was required to release DDA from sediment for analysis. In this respect the newly formed DDA behaved similarly to the bound residues that were present in the archived SCB samples.

DDE dominance and lack of DDA detection in wildlife bird feces and white croakers in SCB reflected no recent DDT exposure occurred in SCB. Detection of DDA in biological system could be used as an indicator of recent DDT exposure.

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Appendices

Appendix 1. Approved human subject study protocol

UNIVERSITY OF CALIFORNIA, RIVERSIDE
Institutional Review Board
Office of Research Integrity

Date: August 31, 2007

TO: Krieger, Robert
Entomology

FM: Monica Wicker, Senior IRB Analyst
Institutional Review Board

RE: Human Subjects Protocol No. HS-07-072
"DDA Analysis in Urine of Pesticide Applicators"

The UCR Institutional Review Board has approved your above referenced protocol, good 8/30/2007 to 8/30/2012. The term of an approved IRB is five years, however annual IRB renewal IS required. The initial approval of this protocol will be valid for one year. Each year thereafter (up to 4 times), you will be mailed the 'Continuing Review of the Approved Human Subjects Protocol' form which will allow you to indicate whether you wish to keep the protocol active or not. This form is automatically sent out 2 months prior to the expiration of the initial approval date. For your information, according to a policy adopted by the Institutional Review Board on April 16, 1987:

"An investigator may be granted up to four one-year extensions on each individual human subjects protocol. If the investigator wishes to continue using the protocol in question after expiration of the fourth extension, s/he will be required to submit a new protocol application for Committee review."

Should you have any questions, please do not hesitate to contact me at (951) 827-4861. Thank you.

Cc: Margaret Fehn
Department Chair, cover & approval notice
ORA file
Faculty Advisor(s): N/A N/A

UNIVERSITY OF CALIFORNIA - (Letterhead for interdepartmental use)

HS approval is effective from date of this notice and good for one year. Annual reviews are required to keep project active.

UNIVERSITY OF CALIFORNIA, RIVERSIDE
Institutional Review Board
Office of Research Affairs
August 30, 2007

APPROVAL NOTICE

1. **INVESTIGATOR:** Krieger, Robert
2. **ACADEMIC UNIT:** Entomology
3. **PROJECT TITLE:** "DDA Analysis in Urine of Pesticide Applicators"

4. **I.R.B. NUMBER:** **HS - 07-072** **5. PROJECT PERIOD:** 2007 - 2008
6. **FUNDING SOURCE:** Various Donors
7. **SPECIAL POPULATION:** None **45 CFR 46 Section #:** 46.101(b)(4)
8. **REVIEW CATEGORY:** Exempt 46.101(b)(4)
9. **SPECIAL CONDITIONS:** **None**

THE INSTITUTIONAL REVIEW BOARD HAS REVIEWED THE PROPOSED USE OF HUMAN SUBJECTS IN THE PROJECT REFERENCED AND HAS DETERMINED:

1. X HUMAN SUBJECTS ARE AT MINIMAL RISK.
2. _____ HUMAN SUBJECTS ARE AT MORE THAN MINIMAL RISK (Informed consent required).
3. _____ INFORMED CONSENT / ASSENT PROCESS APPROVED.
4. X INFORMED CONSENT NOT NECESSARY (Existing archival data).
5. _____ INFORMED CONSENT NOT NEEDED / WAIVED.

CONDUCT OF THIS ACTIVITY IS SUBJECT TO CONTINUING REVIEW AS FOLLOWS (Please note: All projects must be reviewed either semi annually, annually or other from date of approval) :

_____	SEMI ANNUAL Review Required	Date of Review	_____
X	ANNUAL Review Required	Date of Review	August 30, 2008
_____	Other Review Required	Date of Review	_____

THE INVESTIGATOR SHALL REPORT PROMPTLY ANY (1) CHANGES or (2) UNANTICIPATED PROBLEMS INVOLVING RISK TO SUBJECTS OR OTHERS, INCLUDING ANY ADVERSE REACTIONS TO BIOLOGICAL, DRUGS, RADIOISOTOPE-LABELED DRUGS, OR TO MEDICAL DEVICES:

1. - To IRB: Changes - Submit proposal requesting review.
2. - To PHS, if PHS SPONSORED - Provide the IRB with documentation of this action.
3. - To FDA, Significant Adverse Drug Reaction - Report on Form FD1639

DATE APPROVED **8/30/2007**

Dr. Robert Hanneman

DR. ROBERT A. HANNEMAN, CHAIR
UCR INSTITUTIONAL REVIEW BOARD

CATEGORIES OF EXEMPTION: EXEMPT

APPENDIX A

Research activities in which the only involvement of human subjects will be in one or more of the following categories may be exempt from full committee review. The final determination of status will be made by a subcommittee.

1. Research conducted in established or commonly accepted educational settings, involving normal educational practices, such as: (I) research on regular and special education instructional strategies, or (II) research on the effectiveness of or the comparison among instructional techniques, curricula, or classroom management methods.

2. Research involving the use of educational tests (cognitive, diagnostic, aptitude, achievement), survey procedures, interview procedures or observation of public behavior unless: (i) Information obtained is recorded in such a manner that human subjects can be identified, directly or through identifiers linked to the subjects; and (ii) any disclosure of the human subjects responses outside the research could reasonably place the subjects at risk of criminal or civil liability or be damaging to the subjects' financial standing, employability, or reputation.

3. Research involving the use of educational tests (cognitive, diagnostic, aptitude, achievement), survey procedures, interview procedures, or observation of public behavior that is not otherwise exempt if: (i) The human subjects are elected or appointed public officials or candidates for public office; or (ii) federal statue (s) require (s) without exception that the confidentiality of the personally identifiable information will be maintained throughout the research and thereafter.

4. Research, involving the collection or study of existing data, documents, records, pathological specimens, or diagnostic specimens, if these sources are publicly available or if the information is recorded by the investigator in such a manner that subjects cannot be identified, directly or through identifiers linked to the subjects.

5. Research and demonstration projects which are conducted by or subject to the approval of department or agency heads, and which are designed to study, evaluate, or otherwise examine: (i) Public benefit or service programs; (ii) procedures for obtaining benefits or services under those programs; (iii) possible changes in or alternatives to those programs or procedures; or (IV) possible changes in methods or levels of payment for benefits or services under those programs.

6. Taste and food quality evaluation and consumer acceptance studies, (i) if wholesome foods without additives are consumed or (ii) if a food is consumed that contains a food ingredient at or below the level and for a use found to be safe, or agricultural chemical or environmental contaminant at or below the level found to be safe, by the Food and Drug Administration or approved by the Environmental Protection Agency or the Food Safety and Inspection Service of the U.S. Department of Agriculture.

HS-07-072

Last Update: 11/07/2005

APPLICATION FOR APPROVAL TO USE HUMAN PARTICIPANTS

(Application must be typed or Word processed - handwritten forms will not be accepted.)

Investigator: Robert Krieger
Phone: (951) 827 - 3724
Email: bob krieger@ucr.edu
Alternate email:
(If Applicable)
Project Title:
DDA Analysis in Urine of Pesticide Applicators

Title: CE Specialist
Department: Entomology
Dept Chair: R. Carde
Faculty Advisor:
(If Applicable)

Project Period: 2007-2008

Funding Source: Various Donors

I will conduct the study identified above in the manner described on the attached narrative. If I decide to make any changes in the procedure, or if a participant is injured, or if any problems occur which involve risk or the possibility of risk to participants or others, I will immediately report such occurrences or contemplated changes to the UCR Institutional Review Board, Office of Research Integrity, 200 University Office Building, Rms. 209/211, (951) 827-4810/4811.

Bob Krieger 8/24/07 — 8/24/07

Investigator Signature Date Department Chair Signature Date

IF THE INVESTIGATOR IS A STUDENT, A FACULTY ADVISOR/DEPT CHAIR/DEAN/DIRECTOR MUST SIGN BELOW: I have read and approve of this protocol. I believe this is research as defined by DHHS (i.e., a systematic investigation designed to develop or contribute to generalizable knowledge) and that the student is competent to conduct the activity as described herein.

Faculty Advisor Signature Date

Investigator's suggested category of review: ☒ Exempt ☐ Expedited ☐ Full Committee Review
(Select one)

(Please see Appendices A and B, Categories of Exemption and Expedition. Research procedures not described on these lists may require full Committee review. If you have questions, call the UCR Institutional Review Board, ext. 2-4810/4811.)

APPROVED

****

NOTICE OF COMMITTEE ACTION

Date Human Subjects Tutorial Taken 12/26/07 RK (Main Investigator's only, add other to Section 10.)
Tutorial web-site: **http://www.ora.ucr.edu/appTutorial/TutorialClient/Introduction.asp**

The UCR Institutional Review Board has reviewed the proposed use of human participants in the project identified above and has determined its review category to be:

#4 45CFR46.101(b)(4)
Exempt ____ Expedited ____ Full Committee Review

Human Participants at Minimal Risk ✓ Human Participants at More Than Minimal Risk ___

Signed Consent Form ____
Consent Statement (written) ____
Consent Statement (oral) ____
No Consent Needed ✓ analyzing samples only

Next Review Date 8/30/08

8/30/07
Date Approved

Approved – Dr. Robert Hanneman, Chair
Dr. Jan Blacher, Vice Chair
Institutional Review Board

 Application No. _____

INSTRUCTIONS: Please submit one (1) typewritten copy of this application and one copy of the complete research proposal to the UCR IRB in the Office of Research Integrity, University Office Building, Rms. 209/211. For information about Committee review dates or help with preparing the application itself, please contact the UCR Institutional Review Board, ext. 2-4810/4811)

1. **PURPOSE:** Specific aims of the research project.

Analyze DDA, a water soluble DDT metabolite in human urine, in specimens anonymously and voluntarily contributed by persons who apply DDT in anti-malaria programs in Africa. Specimens will be numbered and used to proof a new method of DDA urinalysis being developed in the UC Riverside, Department of Entomology, Personal Chemical Exposure Program, for later use as a biomarker in human DDT exposure studies.

2. **PARTICIPANTS:** How many participants will be involved? Describe who the participants will be and how they will be obtained. Are the participants under the age of 18? Is English their dominant language? Will the participant population include any special populations such as language impaired or handicapped persons, the mentally disabled, prisoners, or institutionalized persons? (Please see Appendix D, "Advertising for Study Participants," and Appendix E, "Payment to Participants.")

Applicators who are regularly employed in DDT anti-malaria treatment programs will be asked to anonymously contribute urine specimens under supervision of a local public health official. The role of the public health official is to certify that all participants contributed urine specimens free of any coercion or fear of reprisal. No demographic data will be collected except occupation--"Applicator" and "Date."

The sole requirement for voluntary participation is employment as a DDT applicator in the anti-malaria program.

3. **PROCEDURES:** Describe how participants will be involved in the study. For example, how often will the participants be involved? For what period of time will they be involved? Where will the study take place? What data will be recorded and how? Who will assist the investigator? Will machines, equipment, and/or instruments be used? If so, please list and describe their use.

The urine specimens will be collected during a 10 day period in which the goal is preparation of 10 to 20 convenience specimens. Urine specimens will be consecutively numbered. No personal identifiers will be included with any of the specimens. Urine collection cups and vials to contain 25 to 90 ml urine for transport of the specimens will be provided in an insulated container containg "blue ice" suitable for expedited, overseas delivery by DHL or FedEx

Collection will be facilitated by a local, volunteer public health official who will oversee collection of the urine specimens and arrange for prepaid shipping.

4. **DECEPTION:** Will deception be necessary? ☒ No ☐ Yes If yes, please explain.

5. **RISKS:** Determine what risks, if any, there might be to participants. Assess the likelihood of the seriousness of risks such as physical, psychological, social, financial, legal or political. Describe procedures that will be used to minimize potential risks to participants.

There is no knowledge of the extent of worker exposure to DDT during anti-malaria spray programs. The levels are expected to be low relative to toxic amounts, but there are many people who regard any exposure to DDT as a harmful effect. This viewpoint is held by many people in spite of lack of scientific foundation. Knowledge of exposure must be accompanied by understanding of dose-response relationships to avoid alarming workers.

A this stage of the work potential risk to participants is nil, since our focus is on analytical issues rather than on exposure assessment. We are being as sensitive to the existing concerns about DDT exposure as possible, even though the concern is not well grounded and a major objective of our overall program is to clarify health issues related to DDT use in anti-malaria programs.

If the risk of this work is not "0", it is as close as you can get to that unattainable mark, I think.

6. **RISK-BENEFIT RATIO:** Activities involving human participants can be approved only if expected benefits outweigh potential risks. **Describe possible benefits** to the participants, a class of participants, society in general, or the advancement of science. State your reasons for believing that the benefits of your proposed activity outweigh potential risks. (This question **MUST** be answered).

If the availability of authentic urine specimens speeds development of a DDT biomarker analysis of the water-soluble part of DDT metabolism, the results could have profound effects on society's willingness to accept DDT as an acceptable tool against malaria. At this time there is understandable, but ill-founded, resistance to DDT use, with many people and jurisdictions holding that use and exposure are virtually eternal. This viewpoint is contrary to earlier (1972 and forward at UC Davis) studies with subhuman primates and a body of human work of others virtually ignored research that was pushed aside when gas-liquid chromatography and electron capture detectors became readily available about the same time Silent Spring was published (1962).

7. **CONFIDENTIALITY:** Describe procedures to be used to maintain confidentiality. Who will have access to any identifying information? Where will data be stored? When will the data be destroyed? If findings are published or made public, how will the participants' identities be masked?

Workers will provide a urine specimen to a public health officer in Africa. No demographic data or individual identifiers will be obtained. No results will be reported regarding worker exposure. The PCEP staff will use the specimens to make judgements about the feasibility of their new analytical method for DDA analysis. The Africa samples will be designated "Authentic DDT Applicator Urine Specimens."

8. **CONSENT:** **Please provide a Consent Statement** even if this consent is to be presented to the participants verbally. Consent Writer Web Application: http://oi.ucr.edu:8101/consent/
Our public health cooperator to be named will collect 10 to 20 urine specimens from volunteer DDT applicators. All participants will remain anonymous. No health benefit will be associated with providing a urine specimen.

9. **DEBRIEFING:** Please provide a summary of any explanation of the purposes of this study that will be given to the participants after their participation. This is only necessary if deception is involved.

The research concerns the new method of analysis for DDA. There will be no communication with volunteers except personal thanks conveyed by the cooperating public health official at the time of specimen collection.

6 Application No. _____

10. **PROJECT ROSTER:** Please provide the names of all the individuals who will work on this project. This page will not be made available to the public. Give either the University Employee ID # **or** a valid UC Riverside email address so that we can document training for regulatory agencies. Include all investigators, student employees, post-doctoral researchers, staff research associates, post-graduate researchers and technicians who will actually work experimentally.

Federal regulations require that all UCR personnel participating in human participants' research complete the UCR Human Subjects Tutorial before initiating research activities. The tutorial can be found at: http://www.ora.ucr.edu/appTutorial/TutorialClient/Introduction.asp and the person MUST register, complete the tutorial, print out the certification page and send it to the Office of Research Integrity / Office of Research. Enrolling in the UCR Human Subjects Tutorial is required and protocols cannot be approved without completion of it.

The principal investigator is responsible for keeping this roster current. You must amend the protocol when staff are added or subtracted from this project. Submit protocol amendments electronically to Monica Wicker (monica.wicker@ucr.edu), Research Integrity Office, Office of Research, 211 UOB.

Last Name	First Name	Date Tutorial completed	UC ID Number or SSN	Email Address
Krieger	Robert	12/26/00		bob.krieger@ucr.edu
Vega	Helen	11/4/04		helen.vega@ucr.edu

11. **ATTACHMENTS:** List all supplementary material to be considered a part of this protocol. Collate and staple these materials to this application. Following are the kinds of attachments that are necessary to complete many applications:

- Informed consent statement (See Appendix F). (http://or.ucr.edu:8101/consent/)
- Parent information letter to be used when minor children are involved. Please note that parents must be informed and their permission obtained.
- Child assent (if necessary).
- Sample instruments including cover letters of introduction or sample dialogue.
- Authorization or letters of access from cooperating institutions, such as public schools, restricted housing, or businesses.
- Approval from another institution (another UC campus, a hospital, a school) assisting in the study when that institution is required to carry out an independent review of the use of human participants.

Appendix 2. Results of DDA and DDT in urine of African applicators.

Collection [a]	Subject No.	DDA (μg/l)	DDT (μg/l)	DDA/DDT mole ratio
Swaziland-1	1	45.8	1.42	41
	2	28.3	0.86	42
	3	24.3	1.13	27
	4	17.9	1.51	15
	5	31.9	0.83	48
	6	33.2	0.95	44
	7	23.9	0.81	37
	8	18.9	2.78	9
	Mean ± S.D.	28.0 ± 9.1	1.29 ± 0.66	33
Swaziland-2	9	22.7	0.90	32
	10	13.8	0.77	23
	11	12.6	0.56	28
	12	12.6	1.04	15
	13	8.6	1.03	11
	14	10.4	0.80	16
	15	13.9	1.56	11
	16	26.8	1.31	26
	17	7.5	0.94	10
	Mean ± S.D.	14.3 ± 6.4	0.99 ± 0.30	19
KwaZulu-Natal-3	18	30.4	0.51	75
	19	53.7	2.54	27
	20	96.8	0.61	200
	21	43.0	0.54	100
	22	29.8	0.62	61
	23	28.0	0.31	114
	24	26.5	0.31	108
	25	51.9	0.37	177
	26	39.2	0.26	190
	27	186.9	0.36	655
	28	407.1	0.52	988
	29	194.1	0.69	355
	30	152.3	0.50	384
	31	3.6	0.38	12
	32	235	0.42	706
	33	84.6	0.67	159
	34	46.1	0.43	135
	35	21.2	0.40	67
	36	67.2	0.39	217
	37	34.4	0.35	124
	Mean ± S.D.	91.6 ± 99.0	0.56 ± 0.48	243

Appendix 2. Results of DDA and DDT in urine of African applicators (Continued.)

	Subject No.	DDA (μg/l)	DDT (μg/l)	DDA/DDT mole ratio
KwaZulu-Natal-4	38	15.6	0.30	66
	39	18.1	0.30	76
	40	44.0	0.87	64
	41	2.4	0.33	9
	42	3.8	0.31	15
	43	4.4	0.42	13
	44	2.5	0.24	13
	45	32.0	0.34	119
	46	5.5	0.33	21
	47	2.6	0.49	7
	48	4.7	0.31	19
	49	0.5	0.25	3
	50	11.3	0.31	46
	51	11.5	0.57	25
	52	4.0	0.43	12
	53	1.0	0.44	3
	54	22	0.55	50
	55	10.9	0.52	26
	56	4.6	0.53	11
	Mean ± S.D.	10.6 ± 11.6	0.41 ± 0.15	31

[a] Swaziland-1 (less experienced applicators) and Swaziland-2 (more experienced applicators) were obtained from the same time and area during the spray season. KwaZulu-Natal-3 specimens were obtained during the spray season and KwaZulu-Natal-4 specimens were obtained one month post-season from different applicators.

Appendix 3. Approved animal use protocol.

UC Riverside Institutional Animal Care and Use Committee (IACUC)
Animal Use Protocol for Experiments Only (AUP) Application

For Breeding procedures use AUP-B and AUP-BE

Revised: 7/28/08

For assistance please contact the Compliance Office at 951-827-4809 or the Office of the Campus Veterinarian at 951-827-6332.

The term of an approved AUP is three years. However, annual IACUC renewal is required.

Federal regulations require that all UC Riverside personnel participating in animal research, teaching and care complete the UCR Animal Care and Use Training, Health and Education program before initiating animal research activities (http://vet.ucr.edu/Training.htm).

Enrolling in the UC Riverside Animal Occupational Health Program is a component of the training program.

New protocol applications cannot be approved without completion of the training, health and education program.

UC Riverside may be required by law to release a copy of this application to the public.

Please e-mail completed forms to iacuc@ucr.edu, Office of Research Affairs, Compliance Office, 207 UOB, for IACUC review.

UC Riverside Animal Care and Use Protocol Application

E-mail to: iacuc@ucr.edu
Please use a minimum font size of 10
Handwritten forms are not accepted

Protocol:
Submitted:
Revised:
Amended:
Approved:
Expires:

1.0

Investigator		Contact	
Last Name:	Krieger	Last Name:	Krieger
First:	Robert	First:	Robert
Middle:	I	Middle:	I
email:	Bob.krieger@ucr.edu	email:	Bob.krieger@ucr.edu
Department:	Entomology	Department:	Entomology
Phone / Fax:	951-827-3724 951-827-5803	Phone:	951-827-3724
After hrs. #:	951-827-3724	After hrs. #:	951-827-3724

1.1 Project Title	Metabolic Disposition of DDT: Dichlorodiphenyltrichloroethane in chickens		
Previously approved AUP	[ ] Yes [x] No	If yes, previous protocol #	
1.2 Funding source		Is the project funded?	[x] Yes [ ] No UCR various donors

Is the protocol for newly funded NIH research? Yes [ ] No [x]

**If this protocol is submitted for a newly funded NIH grant, please attach the relevant animal-related pages from section *D. Experimental Design and Methods* and section *F. Vertebrate Animals* that will allow a direct comparison between this protocol and the animal work proposed in your grant. This comparison of NIH grants and Animal Use and Care protocols is required by PHS policy and only applies to newly funded NIH grants. Please contact the IACUC staff if you have questions associated with this requirement.

1.3 Project Type

[x] Research Project [ ] Teaching Project [ ] Student Project [ ] Field Study

1.4 Procedures: Provide a brief, one to two sentence layman's description of the procedures employed on the animals in this project.

Chickens will be fed non-toxic levels of DDT and its contaminants. DDA and other metabolites will be analyzed in feces and eggs

(DDT: Dichlorodiphenyltrichloroethane insecticide; DDA: Dichlorodiphenylacetic acid)

1.5 Species (common name)	Total # for AUP	Source
Chicken (White leghorn hens)	28	Local commercial

1.6 Animal Husbandry Requirements: Describe general requirements, include any special husbandry conditions; food, water, temperature, humidity, light cycles, caging type, and bedding requirements..

Chickens will be provided laying mash (100 g/d/chicken) and water ad lib in individual wire cages in natural day/light in shaded locked enclosure equipped with evaporative cooling in Middgeville, Ag Ops, UC Riverside.

1.7 Animal Housing:

Proposed housing location:	Midgeville: Ag Ops	Day use only:	NA

Campus Vivarium Space Allocation (Contact appropriate administrator.) [ ] Approved [ ] Pending [x] Not Applicable

Animals will be maintained by: [ ] Vivarium [X] Investigator (*attach husbandry SOP*) [ ] Other (*attach husbandry SOP*)

Note: Feed (about 100 g/day/hen) and water will be provided ad lib daily

1.8 Environmental Enrichment:

Federal regulations require environmental enrichment for all laboratory animals, e.g., co-housing, tubes, hide boxes, foraging supplements, etc. However, the IACUC is aware that environmental enrichment may not be suitable for some research protocols.

If environmental enrichment is <u>not suitable</u> for your research protocol please justify below. Otherwise leave blank.

Laying hens will not be provided environmental enrichment. Cages are kept in shaded area with good ventilation. Evaporative cooling is provided when temperatures are high. Feces and eggs will be removed daily

1.9 Instructions for animal care staff: Check applicable entries.

Sick Animals	Dead Animals	Pest Control
[x] Call Investigator	[x] Call Investigator	[x] Call Investigator
[ ] Clinician to treat	[ ] Save for Investigator	[ ] OK to use pesticides
[ ] Terminate	[ ] Bag for disposal	[x] No Pesticides in animal area
[ ] Necropsy	[ ] Necropsy	

1.10 What Veterinarian or veterinary clinic will provide care for your animals? (check one)

[x] Office of the Campus Veterinarian [ ] Other Veterinarian*

If you checked "Other Veterinarian", please provide:

Veterinarian:	NA	Address:	NA
Day phone:	NA	Fax:	NA
Emergency phone:	NA	Email:	NA

**Please contact the Office of the Campus Veterinarian, 787-6332 for current information about training and record keeping requirements.*

2.0 Hazardous Materials and Reagents: Please provide the following information about the use of hazardous materials and reagents in your AUP. Complete section *9.0* in addition to this section, if hazardous materials and reagents are used in the AUP. Contact Environmental Health and Safety (951-787-5528) for assistance in completing sections 2.0 and 12.0.

Infectious Agent? (e.g. Viruses, Bacteria, Prions) or Recombinant DNA?	[ ] Yes [x] No	Agent(s):		[ ] Lab [ ] Vivarium
Ionizing Radiation? (e.g., Cs^{137}, P^{32}, H^3, S^{35})?	[ ] Yes [x] No	Radiation:		[ ] Lab [ ] Vivarium
High-Field Intensity Non-Ionizing Radiation?	[ ] Yes [x] No	Radiation:		[ ] Lab [ ] Vivarium
Anesthetic Gas?	[ ] Yes [x] No	Gas:		[ ] Lab [ ] Vivarium
Chemical Carcinogen? (Calif. Prop 65 List)*?	[x] Yes [ ] No	Chemical:	DDT up to 1000 ppm in diet (Prop 65)	[x] Lab [ ] Vivarium
Toxic Chemical or Select Agent** (LD_{50} mg/kg)?	[x] Yes [ ] No	Reagent(s):	DDT up to 1000 ppm in diet	[x] Lab [ ] Vivarium

			FAO 1965 rat oral 150-420 mg/kg body weight. Chicken >1,300 mg/kg bw	
Flammable Material?	[ ] Yes [x] No	Specify:		[ ] Lab [ ] Vivarium
Other?	[ ] Yes [x] No	Specify:		[ ] Lab [ ] Vivarium

* See web site www.oehha.ca.gov/prop65/prop65_list/newlist.html. ** See web site www.cdc.gov/od/chs/firsat.htm.

2.1 Minimum Personal Protective Equipment: Check applicable entries.

[x] Protective outer clothing, e.g., lab coat, disposal gown or surgical scrubs.
[ ] Safety glasses or disposable face shield.
[x] Disposable gloves
[ ] Non-slip shoe covers or disposable booties
[ ] Face mask of filtering face piece (N-95)
[ ] Other (please specify);

3. Objective and Significance:

Please provide a brief description of the objectives and significance of the study, bearing in mind your target audience may be a faculty member or private citizen from an unrelated discipline.

Objective:

DDT is a valuable insecticide for anti-malaria management and a persistent organic pollutant that is globally distributed in miniscule amounts in air, water, soil and animal adipose, including humans. DDA is a poorly studied, non-toxic, water-soluble breakdown product of DDT. We want to know if it is formed in chickens and other birds as an excretion product that would result in lower body burden. Feces and eggs will be analyzed.

Significance: Please provide a statement of relevance to human or animal health, the advancement of knowledge, or the good of society.

DDT toxicity may be influenced by the ability of animals to form DDA which acts to clear DDT from their bodies. At levels that do not produce signs of neurotoxicity, birds like chickens may eliminate DDT in feces and eggs. DDA is an important water-soluble DDT metabolite that may represent an important detoxification pathway for some species. We will determine the qualitative and quantitative importance of DDA formation in chickens by measuring DDA excretion in feces (a means to eliminate water soluble metabolites in birds) and eggs (a pathway usually operating with more fat-soluble products like DDT, DDE, and DDD; best known as DDTs in pesticide science). DDA excretion in bird feces or eggs has not been considered as a disposition pathway to our knowledge. The significance of the pathway will be determined by the relationship of DDT:DDA in feces and eggs. Development of an excretion model will be considered if warranted by the results of this research.

4.2 Experimental Procedures:

Describe the use of animals in your project in detail. Use terminology that will be understood by individuals outside your field of expertise. Please write a detailed description of all animal procedures in a logical progression, beginning with receipt of the animals and ending with euthanasia or the study endpoint. List each study group and describe all the specific procedures that will be performed on each animal in each study group.

Please provide a complete description of the surgical procedure(s) including Anesthesia, Analgesia, and/or Neuromuscular blocking agents.

SOP: If the procedure(s) will be performed by vivarium or veterinary staff with an established, IACUC-approved Standard Operating Procedure (SOP), please identify SOP title. If not, please provide a detailed copy of the procedures.

Field Studies: If animals in the wild will be used, describe how they will be observed, any interactions with the animals, whether the animals will be disturbed or affected, and any special procedures anticipated. Indicate if Federal or State permits are required and whether they have been obtained.

(Note: This cell will expand to whatever length you require. You may make this section as long as you wish, but try to be concise. Some projects may require one or two pages.)

1. Chickens will be fed diets containing technical and purified DDT.
   a. The basic diet will be commercial laying mash (100 g/chicken/d).
   b. The technical DDT contains DDT and DDD; both precursors of DDA (the water-soluble product excreted in feces of chickens and human urine).
   c. DDT in high purity will be obtained from chemical supply houses.
2. Feces and eggs will be collected during a 2-day control period, a 4-day DDT feeding period, and during an 8-day clearance period.
3. 24-Hour feces samples will be collected from flat pans and stored frozen in Nalgene bottles and eggs will be refrigerated until processing for GC/MS analysis for DDT, DDA, and DDE.
4. Feeding levels will include 10, 100, and 1000 ppm DDT.
5. At the end of the 8-day clearance period, the chickens will be euthanized using CO_2 and the carcasses will be double bagged in heavy plastic bags and stored frozen. Disposal will be as directed by Ms. Leslie Karpinski at 2-5912.

4.2a Study Groups and Numbers: Define, in the form of a table, the numbers of animals to be used in each experimental group described above. The table may be presented on a separate page as an attachment to this protocol if you prefer. The format should be four columns: Study Group, Procedures and Drugs, Number of Animals, Category of Pain and Distress. The number of rows should follow from the number of study groups; you may add as many rows as you require. The chart must fully account for the number of animals you intend to use under this protocol. Assign each group to a pain and distress category according to the chart below.

Study Group	Procedures and Drugs	Number of Animals	Pain and Distress Category (1-4) *
1	DDT tech 1000 ppm feeding	4	1
2	DDT tech 100 ppm (2 times)	2 sets of 4	1
3	DDT tech 10 ppm (2 times)	2 sets of 4	1
4	DDT pure 10 ppm(2 times)	2 sets of 4	1
		TOTAL 28	

* Pain and Distress Categories

Category	Description
1	Little discomfort or stress.
2	Minor stress or pain of short duration.
3	Moderate to severe distress.
4	Severe pain near, at or above the pain tolerance threshold. **Requires annual institutional USDA report.**

Further descriptions of these categories are included in the instructions following this document.

4.3 Surgery: complete this section if surgery was noted in 4.0. [x] Not Applicable

Where will the surgery be conducted?

Building: NA Room: NA

Who will be the surgeon? NA

4.4 This project will involve Multiple Major Surgical Procedures [ ] Yes [ ] No [x] Not Applicable

Please provide scientific justification for multiple major surgical procedures:

4.5 Anesthesia monitoring: Please complete the following: [x] Not Applicable

Please identify the physiologic parameters monitored during the procedure to assess adequacy of anesthesia and when additional anesthesia will be administered.

4.6 Post-surgical monitoring: please complete the following: [x] Not Applicable

Please identify the physiologic parameters monitored, and interval(s) and for what duration of monitoring.

When will analgesics be administered and at what interval(s)?

If post-operative analgesics cannot be given, please provide scientific justification.

4.7 Drugs to be used (except for euthanasia); anesthetics, analgesics, tranquilizers, neuromuscular blocking agents or antibiotics: *Post-procedural analgesics should be given whenever there is possibility of pain or discomfort that is more than slight or momentary.* [ ] Not Applicable

Provide the following information about any of these drugs that you intend to use in this project

Species	Drug	Dose (mg/kg)	Route	When and how often will it be given?
CHICKEN	DDT	10, 100, 1000 PPM IN DIET (ABSORBED DOSE UNKNOWN, BUT WILL BE MUCH LESS THAN NOMINAL 1-100 MG)	FEED	Even at a dietary level of 1000 ppm, this level of DDT in diet is neither pharmacologic or toxicologic. I think there is every reason not to list it as a drug. It will be poorly absorbed, the chickens will keep laying eggs (that will contain a residue of unknown amount), and there will be no signs of adverse effects at these levels of exposure (4 days @ ppm levels).

4.8 Neuromuscular blocking agents can conceal inadequate anesthesia and therefore require special justification. If you are using a neuromuscular blocking agent, please complete the following: [x] Not Applicable

Why do you need to use a neuromuscular blocking agent?

What physiologic parameters are monitored during the procedure to assess adequacy of anesthesia?

Under what circumstances will incremental doses of anesthetics-analgesics be administered?

5.0 Adverse effects:

Describe **all significant** adverse effects that may be encountered during the study (such as pain, discomfort; reduced growth, fever, anemia, neurological deficits; behavioral abnormalities or other clinical symptoms of acute or chronic distress or nutritional deficiency). If genetically-altered animals are used, please describe any potential adverse effects that could be associated with the desired genotype, if known.

None are expected at these dosages. Anorexia and ataxia may be observed as signs of poor condition or distress. We will be alert to these conditions during daily cage-side observation.

Describe criteria for monitoring the well-being of animals on study and criteria for terminating/modifying the procedure(s) if adverse effects are observed.

Day to day observation. The levels of dietary exposure are below toxicity thresholds.

How will the signs listed above be ameliorated or alleviated? Please provide scientific justification if these signs cannot be alleviated or ameliorated.

Anorexia and/or ataxia are conditions that would require veterinary evaluation and guidance. We will contact the Campus Veterinarian if either condition is observed in any of the chickens. A complete description of any unanticipated findings will be submitted to the IACUC and the Campus Veterinarian.

***Note:** If any significant adverse effects not described above occur during the course of the study, a complete description of these unanticipated findings and the steps taken to alleviate them must be submitted to the IACUC as an amendment to this protocol.*

6.0 Disposition of animals: At what point in the study, if any, will the animals be euthanized?

At end of the feeding period and subsequent 8-day holding period the chickens will be euthanized using CO_2.

6.1 Is death an endpoint in your experimental procedure? [ ] Yes [x] No

"Death as an endpoint" refers to acute toxicity testing, assessment of virulence of pathogens, neutralization tests for toxins, and other studies in which animals are not euthanized, but die as a direct result of the experimental manipulation. If death is an endpoint, explain why it is not possible to euthanize the animals at an earlier point in the study. If you can euthanize the animals at an earlier point, describe the clinical signs that will dictate euthanasia.

NA

6.2 Methods of euthanasia: Even if your study does not involve killing the animals, you should show a method that you would use in the event of unanticipated injury or illness. If anesthetic overdose is the method, show the agent, dose, and route.

Species	Method	Drug	Dose (mg/kg)	route
Chicken	Narcosis	CO_2	-	inhalation

6.3 Surplus animals: What will you do with any animals not euthanized at the conclusion of the project?

All chickens used in these studies will be euthanized with CO_2.

7.0 Literature search for alternatives and unnecessary duplication:

Federal law specifically requires this section. You are required to conduct a literature search to determine that either 1) there are no alternative methodologies by which to conduct this class/lab, or 2) there are alternative methodologies, but these are not appropriate for your particular class/lab. "Alternative methodologies" refers to reduction, replacement, and refinement (the three R's) of animal use, not just animal replacement. You must also show that this use of animals is not ***unnecessarily*** *duplicative of other studies.*

UC Davis and Johns Hopkins University provide on-line access to a number of databases that can be used to search for alternatives. Visit http://www.vetmed.ucdavis.edu/Animal_Alternatives/main.htm, or http://altweb.jhsph.edu. Additional links can be found at the UCR, campus veterinarian, website; http://www.ora.ucr.edu/vet/OffCampusLinks.htm.

What was the date on which you conducted this search? June 2, 2009

7.1 List the databases searched or other sources consulted. List a **minimum** of two databases searched and/or other sources consulted. Include the years covered by the search. *The literature search must have been performed within the last six months.*

Database Name	Years Covered	Keywords / Search Strategy
Medline	1977 to present	DDT, DDA, Bird, Chicken
Toxline	1900-present (hits 1980-1988)	DDT, DDA, Bird, Chicken

7.2 Result of search for alternatives: Please comment on the application(s) of any identified alternatives, including how these alternatives may be or may not be incorporated to modify a procedure to either lessen or eliminate potential pain and distress.

None, the work is chemical specific and the chicken is known to tolerate conveniently large amounts of DDT without adverse effects.

7.3 Animal numbers justification: Please describe the consideration given to reducing the number of animals required for this study; this could include any *in vitro* studies performed prior to the proposed animal studies. Please also provide information on how you arrived at the number of animals required. If preliminary data is available and if relevant, please provide a power analysis or other statistical method used to determine the number of animals necessary. For studies where a statistical method such as a power analysis is not appropriate (such as pilot studies, tissue collection), please provide a brief narrative describing how the requested animal numbers were determined to be necessary.

Minimal numbers of animals are being used to determine the extent of DDA formation in chickens. Ingestion rates and body fat will vary in chickens of about the same age and weight obtained from commercial sources. We expect small amounts of DDT and DDA to be eliminated in feces and eggs. We are using DDT and DDD (DDT technical) since large amounts are available from a supply in the original Pesticide Residue laboratory here at UCR. Both DDT and DDD are DDA precursors and the use of that product should maximize our opportunity to measure DDA excretion. With that information in hand, the more fundamental uncertainty of the extent of DDT metabolism and excretion of DDA will then be addressed.

It is hoped that analyses of samples from individual birds can be analyzed, but if this is not the case, use of up to 4 pooled specimens will be feasible. In our work with human urine, the method sensitivity is about 1 ppb (parts per billion). It will likely be less sensitive in chicken feces and egg samples (and additional clean-up may be required). Being able to pool samples provides analytical flexibility.

These are pilot studies. The ultimate scope of our research with chickens will be determined by the predictive value of findings in these studies. It remains important to demonstrate dose-dependent DDA excretion in feces and eggs. With this basic knowledge concerning DDA excretion in hand, additional studies may be developed to model body burden and excretion in chickens and wildlife that feed on contaminated diets (for example, birds that consume fish from the LA Bight).

7.4 Species rationale: Please provide the rationale for the species chosen, and any consideration given to the use of non-mammalian or invertebrate species, or the use of non-animal systems (e.g., cell or tissue culture, computerized models).

We are ultimately interested in potential of DDA formation as a detoxication pathway in birds. It is well-known that some gallinules are not sensitive to the egg-shell thinning effect of DDT exposure whilst raptors seem much more sensitive. Whether this is qualitative or quantitative deserves careful study. It is remarkable that DDA formation has received such little attention since its discovery as a detoxication product in 1945.

7.5 Has this study been previously conducted? [ ] Yes [x] No

If the study has been conducted previously, explain why it is scientifically necessary to replicate the experiment.

Although there has been much research on DDT and avian species, the emphasis has been on reproduction as a response and "DDTs" as stimulus (actually DDT, DDD and DDE). In this work the "water solubles", represented by DDA, have been ignored since they lack neurotoxicity, reproductive effects, and do not occur as food residues.

There is no doubt that DDA has been present in many, many previous studies since its discovery in 1945 (!), but its role as a detoxication product that is rapidly excreted has not been evaluated in birds (including chickens) and has received remarkably little attention as a measure of exposure in general.

We have recently submitted a manuscript on human excretion of DDA related to DDT use in antimalaria campaigns in Africa.

8.0 Project Roster: Please provide the names of all the individuals who will work with animals on this project. *This page is not subject to FOIA requests.* Please provide either the University Employee ID number OR a valid UC Riverside e-mail address, in order for the IACUC to confirm that the requirements of training and occupational health for regulatory agencies have been met. Include all investigators, student employees, post-doctoral fellows, staff research associates, post-graduate researchers, and laboratory assistants who will actually work with the animals. You do not need to include the vivarium staff in which your animals will be housed, unless they are an active participant in the proposed research plan, or staff members that are only working with tissues or animals post-euthanasia. **This roster is specifically for individuals working with live vertebrate animals.**

Occupational Health Program: Supervisors must enroll their employees in the campus Animal Users Occupational Health Program. For further information, visit our web site at, http://vet.ucr.edu/Training.htm/.

Training: Federal regulations require that all UCR personnel participating in animal research, teaching and care, including the protocol Principal Investigator (PI) complete the UCR Animal Care and Use Training and Education program before initiating animal research activities (http://vet.ucr.edu/Training.htm). Enrolling in the UCR Occupational Health Program is a component of the training program. New protocols cannot be approved without completion of the training, health and education program. Training is not required for individuals that only handle animal tissue and blood products.

Supervisors are responsible for insuring that their employees are adequately trained both in the specifics of their job and in the requirements of the Federal Animal Welfare Act.

The principal investigator is responsible for keeping this roster current. You must amend the protocol when staff are added or subtracted from this project. To add a person (after they have completed the required training and forms), submit a Verification Form indicating that you would like to "add the person to your protocol" to the Office of the Campus Veterinarian, Room 216 University Office Building. If you are subtracting a person from your protocol, contact iacuc@ucr.edu, Compliance Office, Office of Research Affairs, 207 UOB.

Last Name	First Name	Middle Initial	Title/Degree
Krieger	Anasthasia	D.	Diploma

UCR ID Number OR E-mail address: gioklie.krieger@gmail.com

Describe training and experience relevant to the procedures described in this protocol:

Several years experience with flock of backyard chickens. Meticulous in care of home and animals. Day-to-day care, collection of feces and eggs. Completed UCR Animal Care and Use Training and Education program.

Last Name	First Name	Middle Initial	Title/Degree
Chen	Zhenshan		M.S. Chemistry

UCR ID Number OR e-mail address: Zhenshan.chen@email.ucr.edu

Describe training and experience relevant to the procedures described in this protocol:

Advanced to candidacy in environmental toxicology. Preparation of diets, day-to-day care, collection of feces and eggs, and analysis of feces and eggs. Completed UCR Animal Care and Use Training and Education program.

Developed analytical method for analysis of ppb levels of DDA in urine. Applied to human specimens of DDT applicators from Africa. Prepared manuscript that is currently in review.

Last Name	First Name	Middle Initial	Title/Degree
Krieger	Robert	I.	Cooperative Extension Specialist

UCR ID Number OR e-mail address: bob.krieger@ucr.edu

Describe training and experience relevant to the procedures described in this protocol: Professor of Veterinary and Comparative Toxicology, Washington-Oregon-Idaho Regional Veterinary Medical Education Progam, U. Idaho/WSU; Veterinary Toxicologist, Washington Animal Disease Diagnostic Laboratory (1981-86); Assoc Prof UC Davis 5y NIEHS studies of biochemical responses of Rhesus monkeys to dietary (non-toxic) DDT.

Note: I believe that "NA" is the proper entry for 4.7 above, but I do not want to see the work held up if there is some regulatory reason to respond otherwise. It is important to our work to use "non-toxic dosages of DDT." I believe that is just what we will do (if this protocol is approved).

Part of my experience is having administered DDT to monkeys at UC Davis for more than 5 years at doses up to 500 ppm for extended periods of time during the continual monitoring of sensitive biochemical parameters. Two doctoral dissertations were written from the work. The monkeys all survived cyclical periods of DDT exposure, metabolism and DDT clearance. The animals were sold to other biomedical investigators after 6 years as experimental animals in this research.

Currently a manuscript on DDA urine excretion in humans has been submitted and is in review for publication in the International Journal of Toxicology.

9.0 Materials Transfer Agreement.

Does this AUP require the transfer of research material into or out of the UC Regents system? [ ] Yes [x] No

Does the transfer of research material require a Materials Transfer Agreement? [ ] Yes [x] No

If the transfer of research material requires a Materials Transfer Agreement, please supply a copy of all pending or approved agreements.

Questions regarding Material Transfer Agreements should be addressed to Nora Hackett (nora.hackett@ucr.edu), Manager of Intellectual Property Services, Office of Research Affairs, 217 UOB.

10.0 Assurances for the Humane Care and Use of Vertebrate Animals:

Principal Investigator's Statement:

I have read and agree to abide by the policies outlined in the ***UC Riverside's Institutional Animal Care and Use Committee*** training manual. This project will be conducted in accordance with all applicable laws, policies, and regulations governing the use of animals including: the provisions of the *PHS/NIH Guide for the Care and Use of Laboratory Animals* in research and instruction; the ***ILAR Guide for the Care and Use of Laboratory Animals;*** and the UC Riverside Animal Welfare Assurance on file with the US Public Health Service.

These proposed research activities do not unnecessarily duplicate previous experiments.

I will advise the Animal Use and Care Administrative Advisory Committee in writing of any significant changes in the procedures or personnel involved in this project.

Principal Investigator	*Rank / Title*	*Submission Date*
Robert Krieger	*CE Specialist/ Toxicology*	*June 2, 2009*

AUP Approval for Dr. Krieger, A-20090010

From: Trish <patricia@ucr.edu>
To: <bob.krieger@ucr.edu>
CC: <kathrine.fruge@ucr.edu> <cassandra.hadnot@ucr.edu>
Date: Jul 06 2009 - 2:52pm

IACUC Policy for Monitoring Numbers of Research Animals Used.pdf - 172k
krieger chicken husbandrySOP sub 6-2-09 app 7-2-09.doc - 27k
krieger 20090010 sub 06-2-09 app 7-2-09.doc - 228k

Please note: This email serves as notification of approval. You will not be receiving a paper copy of this notice or the amendment in the mail. If you would like the Office of Research Integrity to provide you with a printed copy, please contact iacuc@ucr.edu

TO: Bob Krieger

Entomology

FM: Patricia Steen, IACUC Analyst

Institutional Animal Care and Use Committee

Office of Research Affairs

RE: Animal Use Protocol No. A-20090010

"Metabolic Disposition of DDT, Dichlorodiphenyltrichlorotrichloroethane in chickens"

The Institutional Animal Care and Use Committee has approved your above referenced protocol on 7/2/09. A copy of the approved protocol is attached for your files. Investigators may be granted two one-year extensions on each individual laboratory animal protocol. Your renewal date is 7/10. I will send a reminder prior to that date.

In light of the recently implemented policy for "Monitoring Numbers of Research Animals Used", you should know that your 80% number is 40 and the 100% number is 50 (see attached policy). Should you have any questions or comments, please do not hesitate to contact me or Bill Schmechel, the Director of Research Integrity at x24810 or 24809. Thank you.

Special Conditions: none

UNIVERSITY OF CALIFORNIA

Appendix 4. Chicken body weight (gram)

Study #	Breed	Number	Weight (gram) Mean ± S.D.
1	White Leghorn	4	1408±17
2	White Leghorn	4	1311±67
3	White Leghorn	4	1313±54
4	White Leghorn	4	1421±122
5	ISA Brown	5	1936±339
Antibiotic 1	ISA Brown	4	1914±135
Antibiotic 2	ISA Brown	5	1852±80

Appendix 5. Fecal DDT and derivatives analysis during DDT feeding of chickens

Diet	Dosage [a] (mg/kg-d)	Day [b]	DDT derivatives (µg/kg fresh feces)				DDE/ total DDT [c]	DDA/ total DDT
			DDA	DDE	DDD	DDT		
Study 1 [e] White Leghorn-4d, 1000ppm DDT	56.8	1a	0	0	0	0	N/A [d]	N/A
		2a	0	0	0	0	N/A	N/A
		3b	2785.3	48.8	289.9	1804	0.02	1.3
		4b	3146.6	30.7	185.4	1443.4	0.02	1.9
		5b	2285.9	99.1	474.2	1850.4	0.04	0.9
		6b	3889.4	8.2	427.5	1463.4	0.01	2.0
		7c	2260.6	14.6	481.5	159.3	0.02	3.4
		8c	1280.4	11.7	19.0	67.6	0.12	13
		9c	486.4	9.2	7.6	45	0.15	7.9
		11c	1017.4	10.1	15.5	49.2	0.14	13.6
		13c	489.2	13.6	17	43.7	0.18	6.6
		15c	623.9	8.2	6.2	28.2	0.19	14.6
		Mean ± SD (pre- feeding)	0	0	0	0	N/A	N/A
		Mean ± SD (during feeding)	3026.8±674.7	46.7±38.7	344.3±131.7	1640.3±217	0.02±0.01	1.5±0.5
		Mean ± SD (post feeding)	1026.3±682.6	11.2±2.5	91.1±191.3	65.5±47.7	0.13±0.06	9.9±4.5

Appendix 5. Fecal DDT and derivatives analysis during DDT feeding of chickens (Continued)

Diet	Dosage (mg/kg-d)	Day	DDT derivatives (µg/kg fresh feces)				DDE/ total DDT	DDA/ total DDT
			DDA	DDE	DDD	DDT		
Study 2-1 White Leghorn-4d, 100ppm DDT	6.1	1a	0	0	0	0	N/A	N/A
		2a	0	0	0	0	N/A	N/A
		3b	329.7	4.1	50.9	175.7	0.02	1.4
		4b	746.5	3.1	17.8	102.6	0.03	6
		5b	889.8	5	16.2	87.5	0.05	8.2
		6b	671.9	2.7	18.8	55.3	0.04	8.7
		7c	182.8	4.6	9.1	83.3	0.05	1.9
		8c	100.2	4.6	6.4	36.9	0.10	2.1
		9c	72.4	2.3	2.2	15.3	0.12	3.7
		10c	54.3	3.6	2.8	17.3	0.15	2.3
		12c	64.0	2.7	2.3	14.8	0.14	3.2
		14c	153.3	3.5	2.4	86.8	0.04	1.7
		16c	105.9	4.4	1	5.6	0.40	9.6
		Mean ± SD (pre- feeding)	0	0	0	0	N/A	N/A
		Mean ± SD (during feeding)	659. 5±237.7	3.7±1.0	25.9±16.7	105.3±50.9	0.04±0.01	6.1±3.3
		Mean ± SD (post feeding)	104.7±47.8	3.7±0.9	3.7±2.9	37.1±34.1	0.14±0.12	3.5±2.8

Appendix 5. Fecal DDT and derivatives analysis during DDT feeding of chickens (Continued)

Diet	Dosage (mg/kg-d)	Day	DDT derivatives (µg/kg fresh feces)				DDE/ total DDT	DDA/ total DDT
			DDA	DDE	DDD	DDT		
Study 2-2 White Leghorn-4d, 100ppm DDT	6.1	1a	40.2	1.1	0	3.8	0.22	8.2
		2a	54.9	1	0	33.8	0.03	1.6
		3b	159.1	3.4	4.9	31.4	0.09	4
		4b	203.7	2.3	7.3	60.4	0.03	2.9
		5b	255.1	3	5.7	43.6	0.06	4.9
		6b	217.7	5.5	19.3	58.2	0.07	2.6
		7c	159.2	4.5	8.2	40.2	0.09	3
		8c	162	4.8	1.5	15.9	0.22	7.3
		9c	113.9	5.9	8.3	97.3	0.05	1
		10c	67.7	1.8	2.7	21.6	0.07	2.6
		12c	77.3	2.8	3.1	41.1	0.06	1.6
		14c	51.8	2.8	1.5	18.3	0.12	2.3
		16c	50.8	3.6	1	41.2	0.08	1.1
		Mean ± SD (pre- feeding)	47.6±10.4	1.1±0.1	0	18.8±21.2	0.1±0.1	4.9±4.7
		Mean ± SD (during feeding)	208.9±40.0	3.6±1.4	9.3±6.7	48.4±13.6	0.06±0.03	3.6±1.1
		Mean ± SD (post feeding)	97.5±48.0	3.7±1.4	3.8±3.2	39.4±27.9	0.10±0.06	2.7±2.2

Appendix 5. Fecal DDT and derivatives analysis during DDT feeding of chickens (Continued)

Diet	Dosage (mg/kg-d)	Day	DDT derivatives (µg/kg fresh feces)				DDE/ total DDT	DDA/ total DDT
			DDA	DDE	DDD	DDT		
Study 2-3 White Leghorn-4d, 10ppm DDT	0.6	1a	127.1	1.3	1.2	6.2	0.15	14.6
		2a	89.7	1.5	1	3	0.27	16.3
		3b	49.7	2.4	1.6	7.5	0.21	4.3
		4b	62.8	3.1	1.8	8.1	0.24	4.8
		5b	178.5	4.7	4.9	17.1	0.18	6.7
		6b	140	1	1.9	10.9	0.07	10.1
		7c	164.3	1	2.2	6.7	0.1	16.6
		8c	245.2	4.6	10.6	59.6	0.06	3.3
		9c	107.6	1.5	1.7	10	0.11	8.2
		10c	115.7	2.7	8.2	48.3	0.05	2
		12c	99.7	2.3	1.1	4.1	0.31	13.3
		14c	84.7	11.4	2.1	14.4	0.41	3
		16c	86.5	5.6	1.1	1.2	0.71	10.9
		Mean ± SD (pre- feeding)	108.4±26.4	1.4±0.1	1.1±0.1	4.6±2.3	0.21±0.08	15.5±1.2
		Mean ± SD (during feeding)	107.8±61.7	2.8±1.5	2.6±1.6	10.9±4.4	0.18±0.07	6.5±2.6
		Mean ± SD (post feeding)	129.1±57.8	4.2±3.6	3.9±3.9	20.6±23.4	0.25±0.24	8.2±5.71

Appendix 5. Fecal DDT and derivatives analysis during DDT feeding of chickens (Continued)

Diet	Dosage (mg/kg-d)	Day	DDT derivatives (µg/kg fresh feces)				DDE/ total DDT	DDA/ total DDT
			DDA	DDE	DDD	DDT		
Study 3-1 White Leghorn-4d, 10ppm DDT	0.6	1a	0	0	0	0	N/A	N/A
		2a	0	0	0	0	N/A	N/A
		3b	61.2	1	15.9	13.1	0.03	2
		4b	88.4	1	8.5	11.3	0.05	4.3
		5b	73.6	1.1	13	11	0.04	2.9
		6b	94.6	1	10.2	7.3	0.05	5.1
		7c	14.6	1	1.5	2.7	0.19	2.8
		8c	47.8	1	1	2.7	0.21	10.2
		9c	35.4	1	1	2.4	0.23	8
		10c	67.7	2	8.2	6.1	0.12	4.2
		11c	1	1	2.5	3.8	0.14	0.1
		12c	5.1	1	0	2.2	0.31	1.6
		13c	22.1	0	0	14.2	N/A	1.6
		14c	12.4	1	0	8.4	0.11	1.3
		Mean ± SD (pre-feeding)	0	0	0	0	N/A	N/A
		Mean ± SD (during feeding)	79.5±15.0	1.0±0.1	11.9±3.2	10.7±2.4	0.04±0.01	3.6±1.4
		Mean ± SD (post feeding)	25.8±23.0	1.0±0.5	1.8±2.7	5.3±4.2	0.19±0.07	3.7±3.6

Appendix 5. Fecal DDT and derivatives analysis during DDT feeding of chickens (Continued)

Diet	Dosage (mg/kg-d)	Day	DDT derivatives (µg/kg fresh feces)				DDE/ total DDT	DDA/ total DDT
			DDA	DDE	DDD	DDT		
Study 3-2 White Leghorn-4d, 100ppm DDT	6.1	1a	22.1	0	0	14.2	N/A	1.6
		2a	12.4	1	0	8.4	0.11	1.3
		3b	322.4	1.7	30.8	32.2	0.03	5
		4b	669.0	3.1	38.2	56.2	0.03	6.9
		5b	653.6	2.4	20.5	64.1	0.03	7.5
		6b	461.5	2.1	21.4	41.7	0.03	7.1
		7c	257.3	1	14.6	19.4	0.03	7.4
		8c	181.2	1	7.4	23	0.03	5.8
		9c	316.3	1	11.8	18.8	0.03	10
		10c	254.4	1	11.4	25	0.03	6.8
		11c	228.3	1	11.7	28.5	0.02	5.5
		12c	120.2	1	5.6	26.2	0.03	3.7
		13c	100.6	1	3.8	18.8	0.04	4.3
		14c	43.5	1	2.6	13.3	0.06	2.6
		Mean ± SD (pre- feeding)	17.3±6.9	0.5±0.7	0	11.3±4.1	0.11±0.08	1.5±0.2
		Mean ± SD (during feeding)	526.6±165.7	2.3±0.6	27.7±8.4	48.6±14.3	0.03±0.00	6.6±1.1
		Mean ± SD (post feeding)	187.7±92.9	1.0±0.00	8.6±4.4	21.6±5.0	0.03±0.01	5.8±2.3

Appendix 5. Fecal DDT and derivatives analysis during DDT feeding of chickens (Continued)

Diet	Dosage (mg/kg-d)	Day	DDT derivatives (µg/kg fresh feces)				DDE/ total DDT	DDA/ total DDT
			DDA	DDE	DDD	DDT		
Study 4-1 White Leghorn-8d, 10ppm DDT	0.6	1a	0	0	0	0	N/A	N/A
		2a	0	0	0	0	N/A	N/A
		3a	0	0	0	0	N/A	N/A
		4b	376	1.3	14.8	8.5	0.05	15.3
		5b	582.4	0	14.7	38.4	N/A	11
		6b	676.9	0	14.5	41.2	N/A	12.2
		7b	661.8	0	4.4	20.2	N/A	26.9
		8b	584.8	2.2	12.3	38.3	0.04	11.1
		9b	431.3	3.4	35.8	30.7	0.05	6.2
		10b	415.7	2.5	24.1	23.4	0.05	8.3
		11b	581.9	8.6	29.5	28	0.13	8.8
		12c	516.4	6.2	12.4	28.6	0.13	10.9
		13c	298.7	4.3	8.1	25.2	0.11	7.9
		14c	102.1	3.7	4.1	23.5	0.12	3.3
		15c	130.2	3.4	6.8	18.7	0.12	4.5
		16c	222.7	7.9	6.5	9.6	0.33	9.3
		17c	137.3	3.9	6.2	11.7	0.18	6.3
		18c	257.2	4.5	8.3	10	0.2	11.3
		19c	167.9	5	5.8	10.4	0.24	7.9
		Mean ± SD (pre)	0	0	0	0	N/A	N/A
		Mean ± SD (during)	538.9±115.4	2.3±2.9	18.8±10.2	28.6±11.1	0.06±0.04	12.5±6.4
		Mean ± SD (post)	229.1±134.3	4.9±1.5	7.3±2.5	17.2±7.8	0.18±0.08	7.7±2.9

Appendix 5. Fecal DDT and derivatives analysis during DDT feeding of chickens (Continued)

Diet	Dosage (mg/kg-d)	Day	DDT derivatives (µg/kg fresh feces)				DDE/ total DDT	DDA/ total DDT
			DDA	DDE	DDD	DDT		
Study 4-2 White Leghorn-8d, 100ppm DDT	5.6	1a	137.3	3.9	6.2	11.7	0.18	6.3
		2a	257.2	4.5	8.3	10	0.2	11.3
		3a	167.9	5	5.8	10.4	0.24	7.9
		4b	1660.6	7.2	6.1	10	0.31	71.3
		5b	1679.5	9	5.7	11.4	0.34	64.3
		6b	1913.7	5.7	23.4	39.5	0.08	27.9
		7b	1386.7	5.7	32.5	54	0.06	15
		8b	1582.7	9	44.3	186.4	0.04	6.6
		9b	1184.5	7.3	40.3	131.6	0.04	6.6
		10b	1775.3	4.7	27	167.3	0.02	8.9
		11b	993.7	4.4	21.5	120.2	0.03	6.8
		12c	776.5	6	12	109.5	0.05	6.1
		13c	519.5	5.4	16	73.3	0.06	5.5
		14c	446.1	6.5	19	65.1	0.07	4.9
		15c	936.5	5.9	17.4	68.4	0.06	10.2
		16c	685.2	6.3	8.9	50.6	0.1	10.4
		17c	583.5	8.3	11.3	65.3	0.1	6.9
		18c	754	6.2	9.1	51.2	0.09	11.3
		19c	735.4	7.2	9.6	39.6	0.13	13
		Mean ± SD (pre)	187.5±62.3	4.5±0.6	6.8±1.3	10.7±0.9	0.21±0.03	8.5±2.6
		Mean ± SD(during)	1522.1±310.9	6.6±1.8	25.1±14.2	90.1±70.0	0.12±0.13	25.9±26.9
		Mean ± SD (post)	679.6±157.4	6.5±0.9	12.9±4.0	65.4±21.1	0.08±0.03	8.5±3.0

Appendix 5. Fecal DDT and derivatives analysis during DDT feeding of chickens (Continued)

Diet	Dosage (mg/kg-d)	Day	DDT derivatives (µg/kg fresh feces)				DDE/ total DDT	DDA/ total DDT
			DDA	DDE	DDD	DDT		
Study 5-1 ISA Brown-8d, 10ppm DDT	0.6	1a	0	0	0	0	N/A	N/A
		2a	0	0	0	0	N/A	N/A
		3b	22	0.2	0.9	1.8	0.07	7.6
		4b	54	0.3	0.9	4.1	0.06	10.2
		5b	67	0.1	1.4	1.9	0.03	19.7
		6b	19	3.6	1.4	15.8	0.17	0.9
		7b	64	0.5	1	3.6	0.1	12.5
		8b	63	0	0	1.5	N/A	42
		9b	73	0.3	1.7	5.9	0.04	9.2
		10b	137	0.4	3	5.4	0.05	15.6
		Mean ± SD (pre- feeding)	0	0	0	0	N/A	N/A
		Mean ± SD (during feeding)	62.4±36.4	0.7±1.2	1.3±0.9	5.0±4.7	0.07±0.05	14.7±12.3

Appendix 5. Fecal DDT and derivatives analysis during DDT feeding of chickens (Continued)

Diet	Dosage [a] (mg/kg-d)	Day [b]	DDT derivatives (µg/kg fresh feces)				DDE/ total DDT [c]	DDA/ total DDT
			DDA	DDE	DDD	DDT		
Study 5-2 ISA Brown-8d, 100ppm DDT	6.2	11b	418	0.4	8	4.7	0.03	31.9
		12b	564	1	7.4	12.7	0.05	26.7
		13b	354	0.6	7.1	15.5	0.03	15.3
		14b	472	0.7	8	10.3	0.04	24.8
		15b	565	0.7	5.9	12.9	0.04	29
		16b	357	0.6	8	6.1	0.04	24.3
		17b	322	1.1	12.2	9.7	0.05	14
		18b	513	0.7	10.8	5.8	0.04	29.7
		Mean ± SD (during feeding)	445.6±96.9	0.7±0.2	8.4±2.1	9.7±3.9	0.04±0.01	24.5±6.6

Appendix 5. Fecal DDT and derivatives analysis during DDT feeding of chickens (Continued)

Diet	Dosage (mg/kg-d)	Day	DDT derivatives (μg/kg fresh feces)				DDE/ total DDT	DDA/ total DDT
			DDA	DDE	DDD	DDT		
Study 5-3 ISA Brown-8d, 300ppm DDT	18.6	19b	902	1.8	16.8	52.7	0.03	12.7
		20b	1134	2.3	19.2	66	0.03	13
		21b	1116	2.6	26.5	63	0.03	12.1
		22b	1990	3.2	23.4	99.7	0.03	15.8
		23b	1265	4.1	27.6	102.1	0.03	9.5
		24b	1395	3.8	31.6	66.6	0.04	13.7
		25b	1302	6.2	58.9	141.7	0.03	6.3
		26b	1133	8	44.6	191.2	0.03	4.6
		Mean ± SD (during feeding)	1279.6±323.1	4.0±2.1	31.1±14.1	97.9±47.7	0.03±0.00	11.0±3.8

Appendix 5. Fecal DDT and derivatives analysis during DDT feeding of chickens (Continued)

Diet	Dosage (mg/kg-d)	Day	DDT derivatives (µg/kg fresh feces)				DDE/ total DDT	DDA/ total DDT
			DDA	DDE	DDD	DDT		
Study 5-4 ISA Brown-8d, 1000ppm DDT	62	27b	2045	14.9	182	523.8	0.02	2.8
		28b	2032	29.6	176.1	1340.3	0.02	1.3
		29b	3484	26.6	237.5	1144.5	0.02	2.5
		30b	3462	17.2	213	413.7	0.03	5.4
		31b	3967	25.7	226.4	842.4	0.02	3.6
		32b	4082	18.2	281.7	452.6	0.02	5.4
		33b	2356	25.8	336	573.8	0.03	2.5
		34b	3509	24.9	317.2	584.8	0.03	3.8
		Mean ± SD (during feeding)	3117.1±842.3	22.9±5.3	246.2±59.7	734.5±342.6	0.02±0.01	3.4±1.4

Appendix 5. Fecal DDT and derivatives analysis during DDT feeding of chickens (Continued)

Diet	Dosage (mg/kg-d)	Day	DDT derivatives (µg/kg fresh feces)				DDE/ total DDT	DDA/ total DDT
			DDA	DDE	DDD	DDT		
Study 5-5 ISA Brown-6d, 3000ppm DDT	186	35b	7816	67.6	859.2	4235.1	0.01	1.5
		36b	16429	181.4	1657.9	4512	0.03	2.6
		37b	19434	124.6	1575	4890.3	0.02	2.9
		38b	13320	189.9	2093.8	5043.2	0.03	1.8
		39b	10479	244.1	2144.7	4759.8	0.03	1.5
		40b	13740	333.4	2686.8	5134.5	0.04	1.7
		Mean ± SD (during feeding)	13536±4131	190.2±92.5	1836.2±622.8	4762.5±339	0.03±0.01	2.0±0.6

Appendix 5. Fecal DDT and derivatives analysis during DDT feeding of chickens (Continued)

Diet	Dosage (mg/kg-d)	Day	DDT derivatives (μg/kg fresh feces)				DDE/ total DDT	DDA/ total DDT
			DDA	DDE	DDD	DDT		
Study 5-6 ISA Brown	No DDT	41c	7739	43.3	186.7	2381.1	0.02	3
		42c	4248	32.3	98.9	413.1	0.06	7.8
		43c	2202	17.9	63.8	273.8	0.05	6.2
		44c	2149	21.9	67.7	301.8	0.06	5.5
		45c	4863	19.2	40.6	398.3	0.04	10.6
		46c	8583	16.4	45.8	63.3	0.13	68.4
		48c	8660	26.1	49.2	100.4	0.15	49.3
		50c	6114	42.5	327.5	204.6	0.07	10.6
		52c	2538	40.2	192.4	191.9	0.09	6
		54c	1560	39.3	64.2	205.1	0.13	5.1
		56c	979	37.1	61.2	232.2	0.11	3
		58c	1134	57.6	65.6	381.3	0.11	2.2
		60c	1202	53.3	75.6	460.2	0.09	2
		62c	1003	45.2	57.3	329.4	0.1	2.3
		64c	1340	47.5	64.2	490.2	0.08	2.2
		66c	1029	74.4	145.2	445.1	0.11	1.5
		68c	1228	37.2	81.1	385	0.07	2.4
		70c	1210	36.4	54.6	408.2	0.07	2.4
		72c	1325	44.6	54.4	363.8	0.1	2.9
		74c	1154	73.9	114.4	388.3	0.13	2
		76c	1378	43.9	82.1	425.6	0.08	2.5
		Mean ± SD (post feeding)	2935.1±2651.1	40.5±15.9	94.9±68.3	421.1±464.4	0.09±0.03	9.4±16.9

Appendix 5. Fecal DDT and derivatives analysis during DDT feeding of chickens (Continued)

Diet	Dosage (mg/kg-d)	Day	DDT derivatives (μg/kg fresh feces)				DDE/ total DDT	DDA/ total DDT
			DDA	DDE	DDD	DDT		
Antibiotic Study 1-1 ISA Brown-8d 100ppm DDT	6.3	1b	261.3	2.7	27.6	57.4	0.03	3.0
		2b	211.6	2.9	23.7	37.6	0.05	3.3
		3b	273.2	2.5	22.2	34.8	0.04	4.6
		4b	198.1	10.6	207.2	83	0.04	0.7
		5b	136.4	4.9	28.3	38.2	0.07	1.9
		6b	267.2	5.2	42	50.8	0.05	2.7
		7b	236.1	3.3	22.4	30	0.06	4.2
		8b	306.7	6.6	60.7	57.9	0.05	2.5
		Mean ± SD (1b-8b)	236.3±53.4	4.8±2.7	54.3±63.2	48.7±17.4	0.05±0.01	2.9±1.2
Antibiotic Study 1-2 ISA Brown-8d 300ppm DDT	18.8	9b-antibiotic [f]	374.1	2.5	25.2	71.2	0.03	3.8
		10b-antibiotic	344.9	8.9	54.4	111	0.05	2.0
		11b-antibiotic	156	14.5	102.9	300.9	0.03	0.4
		12b-antibiotic	358.8	19.1	332.6	238.2	0.03	0.6
		13b-antibiotic	361.9	17.1	68.6	98.7	0.09	2.0
		14b-antibiotic	342.5	12.6	48.5	111.5	0.07	2.0
		15b-antibiotic	562	3.3	44.1	59.2	0.03	5.3
		16b-antibiotic	478.5	12.8	190.7	277.4	0.03	1.0
		Mean ± SD (9b-16b)	372.3±117.1	11.4±6.0	108.4±104.3	158.5±97.3	0.05±0.02	2.1±1.7

Appendix 5. Fecal DDT and derivatives analysis during DDT feeding of chickens (Continued)

Diet	Dosage (mg/kg-d)	Day	DDT derivatives (µg/kg fresh feces)				DDE/ total DDT	DDA/ total DDT
			DDA	DDE	DDD	DDT		
Antibiotic Study 2-1 ISA Brown-8d 300ppm DDT	19.4	1b	706.9	5.1	104.4	176.7	0.02	2.5
		2b	1031.1	7.2	74.8	126	0.03	5.0
		3b	878.9	8.6	82.1	196.3	0.03	3.1
		4b	645.3	21.6	211.8	1313.6	0.01	0.4
		5b	397.8	10	107.2	190.3	0.03	1.3
		6b	436.5	9.5	100.5	201.5	0.03	1.4
		7b	517.3	20.3	126.6	454	0.03	0.9
		8b	617.5	9.6	126.4	816.3	0.01	0.7
		Mean ± SD (1b-8b)	653.9±216.7	11.5±6.1	116.7±42.6	434.3±422.8	0.02±0.01	1.9±1.5
Antibiotic Study 2-2 ISA Brown-8d 300ppm DDT	19.4	9b-antibiotic	425.2	5	67.6	101.7	0.03	2.4
		10b-antibiotic	395.4	5.2	49.3	126.6	0.03	2.2
		11b-antibiotic	441.5	7.9	109.2	109.7	0.03	2.0
		12b-antibiotic	397.4	14.6	176	609.7	0.02	0.5
		13b-antibiotic	254.4	11.1	97.3	159.4	0.04	1.0
		14b-antibiotic	207	5.4	26.5	168.6	0.03	1.0
		15b-antibiotic	279.2	6.9	46	203	0.03	1.1
		16b-antibiotic	409.2	12.9	86.6	820.2	0.01	0.4
		Mean ± SD (9b-16b)	351.2±89.8	8.6±3.8	82.3±47.0	287.4±271.8	0.03±0.01	1.3±0.8

[a] Daily feed consumption for White Leghorn hens was approximately 80g (out of 100g) and for ISA Brown hens was approximately 120g (out of 150g). Average chicken weight for each study was listed in Appendix 4. [b] Each DDT feeding period included three stages: "a" represents pre-DDT control feeding day; "b" represents DDT feeding day; "c" represents post DDT control feeding day. [c] Total DDT = sum of (DDT+DDD+DDE). [d] Ratio was not available when numerator is 0. [e] Each study number designated a set of chickens fed with various levels of DDT. Chickens were killed at the end of each study. [f] Antibiotics were administered in drinking water during the feeding period.

Appendix 6. Chicken feeding study whole blood analysis

Diet	Dosage [a] (mg/kg-d)	Chicken #	Whole blood (µg/L)			Total DDT [b]	DDE/total DDT
			DDE	DDD	DDT		
Study 4-2 White Leghorn-8d 100ppm DDT	5.6	1	2.6	15.3	103.6	121.5	0.02
		2	4	24.2	205.3	233.5	0.02
		3	1.9	13.1	81.1	96.1	0.02
		4	1.2	11.6	71.2	84	0.01
		Mean ± SD	2.4±1.2	16±5.6	115±62	113.8±68.3	0.02±0.01

Appendix 6. Chicken feeding study whole blood analysis (Continued)

Diet	Dosage (mg/kg-d)	Chicken #	Whole blood (µg/L)			Total DDT	DDE/total DDT
			DDE	DDD	DDT		
Study 5-1 ISA Brown-8d, 10ppm DDT	0.6	1	9	2	47	56	0.16
		2	24	1	126	150	0.16
		3	14	1	99	113	0.12
		4	10	1	66	76	0.13
		5	18	1	132	150	0.12
		Mean ± SD	15±6.2	1.2±0.4	94±37	109±43	0.14±0.02
Study 5-2 ISA Brown-8d, 100ppm DDT	6.2	1	340	56	1722	2118	0.16
		2	342	53	2269	2664	0.13
		3	138	28	992	1158	0.12
		4	120	21	836	978	0.12
		5	119	13	976	1108	0.11
		Mean ± SD	212±118	34±19	1359±615	1605±746	0.13±0.02
Study 5-3 ISA Brown-8d, 300ppm DDT	18.6	1	1273	124	5525	6622	0.18
		2	1048	104	5681	6834	0.15
		3	250	85	1303	1638	0.15
		4	391	34	2041	2465	0.16
		5	869	132	4286	5286	0.16
		Mean ± SD	766±434	96±39	3767±2005	4569±2392	0.16±0.01

Appendix 6. Chicken feeding study whole blood analysis (Continued)

Diet	Dosage (mg/kg-d)	Chicken #	Whole blood (µg/L)			Total DDT	DDE/total DDT
			DDE	DDD	DDT		
Study 5-4 ISA Brown-8d, 1000ppm DDT	62	1	1414	66	5657	7138	0.2
		2	1599	61	8468	10128	0.16
		3	969	87	5646	6702	0.14
		4	1853	81	12986	14920	0.12
		5	2670	122	17972	20764	0.13
		Mean ± SD	1701±631	83±24	10146±5304	11930±5927	0.15±0.03
Study 5-5 ISA Brown-6d, 3000ppm DDT	186	1	5349	107	15748	21204	0.25
		2	2988	78	16738	19804	0.15
		3	1704	54	6850	8608	0.2
		4	1549	40	7191	8780	0.18
		5	2377	64	14446	16887	0.14
		Mean ± SD	2793±1539	69±26	12195±4794	15057±6014	0.18±0.14
Study 5-6 ISA Brown-8d	No DDT (Day 8)	1	2310	39	7187	9536	0.24
		2	1740	34	4100	5874	0.3
		3	2953	51	9095	12099	0.24
		4	5302	72	19791	25165	0.21
		Mean ± SD	3076±1564	49±17	10043±6817	13169±8396	0.25±0.04

Appendix 6. Chicken feeding study whole blood analysis (Continued)

Diet	Dosage (mg/kg-d)	Chicken #	Whole blood (µg/L)			Total DDT	DDE/total DDT
			DDE	DDD	DDT		
Study 5-6 ISA Brown-8d	No DDT (Day 16)	1	2634	61	6099	8794	0.3
		2	3096	107	6044	9247	0.33
		3	2806	79	6049	8934	0.31
		4	3569	115	9291	12975	0.28
		Mean ± SD	3027±409	91±25	6871±1614	9988±2001	0.31±0.02
	No DDT (Day 24)	1	2404	65	5084	7553	0.32
		2	1471	45	2545	4061	0.36
		3	2160	43	4375	6578	0.33
		4	3313	74	8260	11647	0.28
		Mean ± SD	2337±761	57±15	5066±2383	7460±3156	0.32±0.03
	No DDT (Day 32)	1	2036	40	4747	6823	0.30
		2	1523	35	2920	4478	0.34
		3	2024	43	4371	6438	0.31
		4	1821	32	4574	6427	0.28
		Mean ± SD	1851±240	38±5	4153±836	6042±1058	0.31±0.03
	No DDT (Day 36)	1	3757	65	6481	10303	0.36
		2	3217	54	4761	8032	0.40
		3	1516	21	2097	3634	0.42
		4	4533	54	8382	12969	0.35
		Mean ± SD	3256±1279	49±19	5430±2669	8735±3954	0.38±0.03

Appendix 6. Chicken feeding study whole blood analysis (Continued)

Diet	Dosage (mg/kg-d)	Chicken #	Whole blood (μg/L)			Total DDT	DDE/total DDT
			DDE	DDD	DDT		
Antibiotic Study 1-1 ISA Brown-8d, 100ppm DDT	6.3 (Day4)	1	57	11	249	317	0.18
		2	90	13	414	517	0.17
		3	51	13	200	264	0.19
		4	101	24	628	753	0.13
		Mean ± SD	75±25	15±6	373±193	463±222	0.17±0.03
	6.3 (Day8)	1	250	52	1159	1461	0.17
		2	294	54	1491	1839	0.16
		3	119	22	584	725	0.16
		4	233	50	1490	1773	0.13
		Mean ± SD	224±75	45±15	1181±428	1450±510	0.16±0.02
Antibiotic Study 1-2 ISA Brown-8d, 300ppm DDT	18.8 (Day12)	1	292	85	1579	1956	0.15
		2	826	306	4805	5937	0.14
		3	293	72	1752	2117	0.14
		4	349	93	2449	2891	0.12
		Mean ± SD	440±259	139±112	2646±1488	3225±1853	0.14±0.01
	18.8 (Day16)	1	704	102	4205	5011	0.14
		2	1304	203	6504	8011	0.16
		3	804	94	4502	5400	0.15
		4	956	106	5464	6526	0.15
		Mean ± SD	942±263	126±51	5169±1040	6237±1346	0.15±0.01

Appendix 6. Chicken feeding study whole blood analysis (Continued)

Diet	Dosage (mg/kg-d)	Chicken #	Whole blood (μg/L)			Total DDT	DDE/total DDT
			DDE	DDD	DDT		
Antibiotic Study 2-1 ISA Brown-8d, 300ppm DDT	19.4 (Day4)	1	228	139	1989	2357	0.1
		2	60	50	371	481	0.12
		3	79	67	855	1002	0.08
		4	99	75	918	1092	0.09
		5	110	84	1091	1286	0.09
		Mean ± SD	115±66	83±34	1045±592	1244±690	0.10±0.02
	19.4 (Day8)	1	279	182	2736	3197	0.09
		2	116	73	1062	1252	0.09
		3	171	167	2012	2351	0.07
		4	107	106	1123	1335	0.08
		5	169	123	1353	1645	0.1
		Mean ± SD	168±69	130±45	1657±711	1956±818	0.09±0.01

Appendix 6. Chicken feeding study whole blood analysis (Continued)

Diet	Dosage (mg/kg-d)	Chicken #	Whole blood (μg/L)			Total DDT	DDE/total DDT
			DDE	DDD	DDT		
Antibiotic Study 2-2 ISA Brown-8d, 300ppm DDT	19.4 (Day12)	1	249	126	2473	2849	0.09
		2	356	175	3452	3983	0.09
		3	62	64	620	746	0.08
		4	194	85	1887	2167	0.09
		5	582	353	3960	4894	0.12
		Mean ± SD	289±195	161±116	2478.4±1318	2928±1607	0.09±0.02
	19.4 (Day16)	1	221	248	2506	2975	0.07
		2	87	134	837	1059	0.08
		3	390	427	4795	5613	0.07
		4	347	463	4008	4818	0.07
		5	216	195	3792	4203	0.05
		Mean ± SD	252±120	293±145	3187±1550	3734±1779	0.07±0.01

Appendix 7. Egg yolk DDT and derivatives levels in DDT feeding of chickens

Diet	Dosage (mg/kg-d) [a]	Date	Egg #	Egg yolk (μg/kg)			Total DDT [b]	DDE/Total DDT
				DDE	DDD	DDT		
Study 3-1 White Leghorn-4d, 10ppm DDT (Whole egg) [c]	0.6	pre- feeding (1a-2a) [d]	5	0	0	0.7	0.7	0
		during feeding (3b-6b)	4	0.1	0	1.8	1.9	0.05
		post feeding (7c-14c)	3	0.5	0	6	6.5	0.08
Study 3-2 White Leghorn-4d, 100ppm DDT (Whole egg)	6.1	pre- feeding (1a-2a)	4	4.3	1.6	30.7	36.6	0.12
		during feeding (3b-6b)	5	8.6	6.5	93.5	108.6	0.08
		post feeding1 (7c-10c)	4	51.9	37.5	563.2	652.6	0.08
		post feeding2 (11c-14c)	4	89.8	44.6	633.9	768.3	0.12
Study 4-2 [e] White Leghorn-8d, 100ppm DDT (egg yolk)	5.6	pre- feeding (1a-3a)	1	0	0	0	0	0
		during feeding (4b-11b)	1	51.1	79	754	884.1	0.06
		post feeding1 (12c-14c)	2	80.1	83.3	926.0	1089.3	0.07

Appendix 7. Egg yolk DDT and derivatives analysis during DDT feeding of chickens (Continued.)

Diet	Dosage (mg/kg-d) [a]	Date	Chicken #	Egg yolk (μg/kg)			Total DDT [b]	DDE/Total DDT
				DDE	DDD	DDT		
Study 5-1 ISA Brown-8d 10ppm DDT	0.6	1/27/ 2010	1	0.7	0.0	5.8	6.5	0.11
			2	0.4	0.0	4.0	4.4	0.09
			3	0.6	0.0	5.3	5.9	0.1
			5	0.5	0.0	6.8	7.3	0.07
			Mean±SD	0.6±0.1	0.0±0.0	5.5±1.2	6.0±1.2	0.09±0.02
		1/29/ 2010	1	1.9	0.0	19.4	21.3	0.09
			2	1.3	0.0	13.4	14.7	0.09
			3	2.3	0.0	24.9	27.2	0.08
			4	1.1	0.0	9.9	11.0	0.1
			5	1.9	0.0	22.1	24.0	0.08
			Mean±SD	1.7±0.5	0.0±0.0	17.9±6.2	19.6±6.7	0.09±0.01
		Study 5-1 overall Mean±SD		1.2±0.7	0.0±0.0	12.4±7.9	13.6±8.6	0.09±0.01

Appendix 7. Egg yolk DDT and derivatives levels in DDT feeding of chickens (Continued.)

Diet	Dosage (mg/kg-d)	Date	Chicken #	Egg yolk (μg/kg)			Total DDT	DDE/Total DDT
				DDE	DDD	DDT		
Study 5-2 ISA Brown-8d 100ppm DDT	6.2	2/1/ 2010	1	4.0	0.0	37.9	41.9	0.1
			3	2.7	0.0	27.3	30.0	0.09
			4	1.7	0.0	21.9	23.6	0.07
			5	3.1	0.0	35.1	38.2	0.08
			Mean±SD	2.9±1.0	0.0±0.0	30.6±7.3	33.4±8.2	0.09±0.01
		2/3/ 2010	1	11.6	0.0	108.6	120.2	0.1
			2	17.6	0.0	159.8	177.4	0.1
			3	12.8	0.0	154.3	167.1	0.08
			4	7.6	0.0	105.4	113.0	0.07
			5	13.8	0.0	173.4	187.2	0.07
			Mean±SD	12.7±3.6	0.0±0.0	140.3±31.2	153.0±34.1	0.08±0.02
		2/8/ 2010	1	15.0	7.0	129.0	151.0	0.10
			2	21.0	8.0	217.0	246.0	0.09
			3	12.0	10.0	128.0	150.0	0.08
			4	21.0	8.0	244.0	273.0	0.08
			5	48.0	15.0	491.0	554.0	0.09
			Mean±SD	23.4±14.3	9.6±3.2	241.8±148.7	274.8±165.6	0.09±0.01
		Study 5-2 overall Mean±SD		13.7±11.8	3.4±5.1	145.2±121.5	162.3±137.2	0.09±0.01

Appendix 7. Egg yolk DDT and derivatives levels in DDT feeding of chickens (Continued.)

Diet	Dosage (mg/kg-d)	Date	Chicken #	Egg yolk (μg/kg)			Total DDT	DDE/Total DDT
				DDE	DDD	DDT		
Study 5-3 ISA Brown-8d 300ppm DDT	18.6	2/10/ 2010	1	24.0	11.0	149.0	184.0	0.13
			2	30.0	9.0	211.0	250.0	0.12
			3	21.0	17.0	159.0	197.0	0.11
			4	18.0	7.0	143.0	168.0	0.11
			5	28.0	13.0	219.0	260.0	0.11
			Mean±SD	24.2±4.9	11.4±3.8	176.2±36.0	211.8±40.9	0.11±0.01
		2/12/ 2010	1	170.0	74.0	1162.0	1406.0	0.12
			2	108.0	35.0	884.0	1027.0	0.11
			3	59.0	37.0	458.0	554.0	0.11
			4	106.0	28.0	1001.0	1135.0	0.09
			5	53.0	22.0	442.0	517.0	0.1
			Mean±SD	99.2±47.1	39.2±20.3	789.4±325.2	927.8±384.0	0.11±0.01
		2/14/ 2010	1	164.0	95.0	1078.0	1337.0	0.12
			2	123.0	44.0	1063.0	1230.0	0.1
			3	75.0	62.0	578.0	715.0	0.1
			4	45.0	21.0	646.0	712.0	0.06
			5	202.0	88.0	1960.0	2250.0	0.09
			Mean±SD	121.8±63.8	62.0±30.7	1065.0±550.9	1248.8±629.2	0.10±0.02
		2/16/ 2010	1	164.0	93.0	1065.0	1322.0	0.12
			2	138.0	60.0	1109.0	1307.0	0.11
			4	89.0	27.0	828.0	944.0	0.09
			5	130.0	69.0	1124.0	1323.0	0.10
			Mean±SD	130.3±31.1	62.3±27.3	1031.5±138.0	1224.0±186.8	0.11±0.01
		Study 5-3 overall Mean±SD		91.9±58.6	42.7±29.9	751.5±481.1	886.2±561.8	0.11±0.01

Appendix 7. Egg yolk DDT and derivatives levels in DDT feeding of chickens (Continued.)

Diet	Dosage (mg/kg-d)	Date	Chicken #	Egg yolk (μg/kg)			Total DDT	DDE/Total DDT
				DDE	DDD	DDT		
Study 5-4 ISA Brown-8d 1000ppm DDT	62	2/18/ 2010	1	127.0	64.0	772.0	963.0	0.13
			2	138.0	46.0	993.0	1177.0	0.12
			3	71.0	48.0	445.0	564.0	0.13
			4	104.0	27.0	857.0	988.0	0.11
			5	101.0	44.0	791.0	936.0	0.11
			Mean±SD	108.2±26.0	45.8±13.2	771.6±202.1	925.6±223.2	0.12±0.01
		2/20/ 2010	1	116.0	65.0	745.0	926.0	0.13
			2	167.0	61.0	1352.0	1580.0	0.11
			3	57.0	44.0	401.0	502.0	0.11
			4	192.0	46.0	1648.0	1886.0	0.1
			5	156.0	75.0	1429.0	1660.0	0.09
			Mean±SD	137.6±52.7	58.2±13.1	1115.0±520.9	1310.8±576.1	0.10±0.01
		2/23/ 2010	1	575.0	284.0	2873.0	3732.0	0.15
			2	176.0	64.0	1578.0	1818.0	0.10
			3	113.0	90.0	1003.0	1206.0	0.09
			4	221.0	87.0	2197.0	2505.0	0.09
			5	153.0	60.0	1317.0	1530.0	0.10
			Mean±SD	247.6±187.1	117.0±94.3	1793.6±746.1	2158.2±1001.8	0.11±0.03
		Study 5-4 overall Mean±SD		164.5±121.9	73.7±60.6	1226.7±664.4	1464.9±824.5	0.11±0.02

Appendix 7. Egg yolk DDT and derivatives levels in DDT feeding of chickens (Continued.)

Diet	Dosage (mg/kg-d)	Date	Chicken #	Egg yolk (μg/kg)			Total DDT	DDE/Total DDT
				DDE	DDD	DDT		
Study 5-5 ISA Brown-6d 3000ppm DDT	186	2/26/ 2010	1	142.0	72.0	872.0	1086.0	0.13
			2	154.0	56.0	1098.0	1308.0	0.12
			3	213.0	158.0	1850.0	2221.0	0.10
			4	171.0	63.0	1559.0	1793.0	0.10
			5	171.0	70.0	1414.0	1655.0	0.10
			Mean±SD	170.2±26.9	83.8±42.0	1358.6±383.9	1612.6±440.1	0.11±0.01
		2/28/ 2010	1	266.0	111.0	1486.0	1863.0	0.14
			2	295.0	102.0	1922.0	2319.0	0.13
			4	219.0	85.0	1755.0	2059.0	0.11
			5	313.0	107.0	2058.0	2478.0	0.13
			Mean±SD	273.3±41.0	101.3±11.4	1805.3±246.3	2179.8±272.8	0.13±0.01
		Study 5-5 overall Mean±SD		216.0±62.8	91.6±31.8	1557.1±389.7	1864.7±462.7	0.12±0.02

Appendix 7. Egg yolk DDT and derivatives levels in DDT feeding of chickens (Continued.)

Diet	Dosage (mg/kg-d)	Date	Chicken #	Egg yolk (μg/kg)			Total DDT	DDE/Total DDT
				DDE	DDD	DDT		
Study 5-6 ISA Brown	(No DDT)	3/3/2010	2	929.0	348.0	3611.0	4888.0	0.19
			4	1004.0	338.0	3637.0	4979.0	0.20
			5	1032.0	329.0	3689.0	5050.0	0.20
			Mean±SD	988.3±53.3	338.3±9.5	3645.7±39.7	4972.3±81.2	0.20±0.01
		3/5/2010	2	841.0	301.0	3454.0	4596.0	0.18
			4	1154.0	404.0	3735.0	5293.0	0.22
			5	1327.0	473.0	3968.0	5768.0	0.23
			Mean±SD	1107.3±246.3	392.7±86.6	3719.0±257.4	5219.0±589.5	0.21±0.03
		3/6/2010	2	1497.0	389.0	6132.0	8018.0	0.19
			4	1180.0	335.0	4811.0	6326.0	0.19
			5	1340.0	362.0	5794.0	7496.0	0.18
			Mean±SD	1339.0±158.5	362.0±27.0	5579.0±686.2	7280.0±866.4	0.18±0.01
		3/7/2010	2	1372.0	362.0	4912.0	6646.0	0.21
			4	1343.0	344.0	4811.0	6498.0	0.21
			5	891.0	227.0	3286.0	4404.0	0.2
			Mean±SD	1202.0±269.7	311.0±73.3	4336.3±911.0	5849.3±1253.9	0.21±0.01
		3/9/2010	2	539.0	211.0	2402.0	3152.0	0.17
			4	831.0	262.0	3651.0	4744.0	0.18
			5	470.0	177.0	2111.0	2758.0	0.17
			Mean±SD	613.3±191.6	216.7±42.8	2721.3±818.2	3551.3±1051.5	0.17±0.01
		3/12/2010	2	565.0	181.0	2092.0	2838.0	0.20
			4	836.0	241.0	2975.0	4052.0	0.21
			5	487.0	132.0	1755.0	2374.0	0.21
			Mean±SD	629.3±183.2	184.7±54.6	2274.0±630.0	3088.0±866.5	0.20±0.01

Appendix 7. Egg yolk DDT and derivatives levels in DDT feeding of chickens (Continued.)

Diet	Dosage (mg/kg-d)	Date	Chicken #	Egg yolk (μg/kg)			Total DDT	DDE/Total DDT
				DDE	DDD	DDT		
Study 5-6 ISA Brown	(No DDT)	3/17/ 2010	2	834.0	185.0	2487.0	3506.0	0.24
			3	309.0	85.0	658.0	1052.0	0.29
			4	563.0	153.0	1606.0	2322.0	0.24
			5	475.0	112.0	1461.0	2048.0	0.23
			Mean±SD	545.3±219.4	133.8±44.1	1553.0±749.4	2232.0±1009.5	0.24±0.03
		3/21/ 2010	2	2175.0	456.0	6200.0	8831.0	0.25
			3	2941.0	725.0	7211.0	10877.0	0.27
			5	2283.0	409.0	6943.0	9635.0	0.24
			Mean±SD	2466.3±414.6	530.0±170.5	6784.7±523.8	9781.0±1030.8	0.25±0.02
		3/25/ 2010	2	3272.0	515.0	8574.0	12361.0	0.26
			3	4932.0	891.0	10158.0	15981.0	0.31
			4	1957.0	327.0	4015.0	6299.0	0.31
			5	3850.0	522.0	9861.0	14233.0	0.27
			Mean±SD	3502.8±1239.1	563.8±236.1	8152.0±2842.4	12218.5±4214.1	0.29±0.03
		3/28/ 2010	2	4213.0	643.0	9723.0	14579.0	0.29
			4	5476.0	803.0	11557.0	17836.0	0.31
			5	2444.0	353.0	6053.0	8850.0	0.28
			Mean±SD	4044.3±1523.0	599.7±228.1	9111.0±2902.6	13755.0±4549.3	0.29±0.02
		3/31/ 2010	2	853.0	121.0	1824.0	2798.0	0.3
			3	1104.0	211.0	1914.0	3229.0	0.34
			4	1347.0	190.0	2846.0	4383.0	0.31
			5	1075.0	119.0	2616.0	3810.0	0.28
			Mean±SD	1094.8±202.1	160.3±47.3	2300.0±507.8	3555.0±690.4	0.31±0.03

Appendix 7. Egg yolk DDT and derivatives levels in DDT feeding of chickens (Continued.)

Diet	Dosage (mg/kg-d)	Date	Chicken #	Egg yolk (μg/kg)			Total DDT	DDE/Total DDT
				DDE	DDD	DDT		
Study 5-6 ISA Brown	(No DDT)	4/3/ 2010	2	1405.0	179.0	2839.0	4423.0	0.32
			3	2198.0	405.0	3652.0	6255.0	0.35
			4	2414.0	318.0	4696.0	7428.0	0.32
			5	1712.0	247.0	3870.0	5829.0	0.29
			Mean±SD	1932.3±458.0	287.3±96.9	3764.3±723.3	5983.8±1240.9	0.32±0.02
		4/6/ 2010	2	1197.0	146.0	2420.0	3763.0	0.32
			3	1427.0	219.0	2429.0	4075.0	0.35
			5	812.0	89.0	1810.0	2711.0	0.3
			Mean±SD	1145.3±310.7	151.3±65.2	2219.7±354.8	3516.3±714.7	0.33±0.03
		4/8/ 2010	2	953.0	119.0	2016.0	3088.0	0.31
			3	1483.0	243.0	2352.0	4078.0	0.36
			5	839.0	79.0	1724.0	2642.0	0.32
			Mean±SD	1091.7±343.7	147.0±85.5	2030.7±314.3	3269.3±735.0	0.33±0.03
		Study 5-6 overall Mean±SD		1569.2±1169.8	310.4±186.2	4137.8±2577.2	6017.4±3858.7	0.26±0.06

[a] DDT dosage=DDT dose in diet*daily feed consumption/body weight. Daily feed consumption for White Leghorn hens was approximately 80g (out of 100g) and for ISA Brown hens was approximately 120g (out of 150g). Average chicken weight for each study was listed in Appendix 4. [b] Total DDT=sum of (DDT+DDD+DDE); [c] Whole egg DDT derivatives were analyzed in Study 3-1 and 3-2. A conversion factor of 0.75 could be used to convert whole egg DDT level into yolk level: $C_{yolk}=C_{whole\ egg}/0.75$; [d] Each DDT feeding period included three stages: "a" represents pre-DDT control feeding day; "b" represents DDT feeding day; "c" represents post DDT control feeding day; [e] Study 4-1 was not included. No egg was obtained during this study period due to molting of chickens.

Appendix 8. Bacteria aerobic culture results

Study #	Specimen ID	Results[a]
Antibiotic Study 1[b]	Control-1	Mixed flora lg# Enterobacter spp. Mod# Mixed Coliforms Mod#
	Control-2	Mixed flora lg# Mixed Coliforms Mod# Escherichia coli Mod#
	Treatment-1	Mixed flora lg# Escherichia coli Lg# Aerococcus sp. Lg#
	Treatment-2	Mixed flora lg# Mixed Coliforms Mod# Escherichia coli Mod# Enterococcus spp. Mod#
Antibiotic Study 2	Control-1	Mixed flora Mod# Escherichia coli Mod# Proteus swarming
	Control-2	Mixed flora Mod#
	Control-3	Mixed flora Mod#
	Treatment-1	Mixed flora Mod# Escherichia coli Mod#
	Treatment-2	Mixed flora Mod# Escherichia coli Sm#
	Treatment-3	Mixed flora Mod# Escherichia coli Sm#
	Treatment-4	Mixed flora Mod# Escherichia coli Mod#
	Treatment-5	Mixed flora Mod# Escherichia coli Mod#

[a] Size of bacterial colony was determined by vision examination. Sm#, Mod#, and Lg# were used to represent small, moderate, and large quantity of bacterial colonies. [b] Feces were collected from the studied chickens and control chickens kept in a separate chicken house.