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Contamination of Organochlorine Pesticides in Nest Soil, Egg, and Blood of the Snail-eating Turtle (*Malayemys macrocephala*) from the Chao Phraya River Basin, Thailand

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***Abstract*—**Organochlorine pesticides (OCPs) are known to be persistent and bioaccumulative toxicants that may cause reproductive impairments in wildlife as well as human. The current study uses the snail-eating turtle *Malayemys macrocephala,* a long-lived animal commonly distribute in rice field habitat in central part of Thailand, as a sentinel to monitor OCP contamination in environment. The nest soil, complete clutch of eggs, and blood of the turtle were collected from agricultural areas in the Chao Phraya River Basin, Thailand during the nesting season of 2007-2008. The novel methods for tissue extraction by an accelerated solvent extractor (ASE, for egg) and liquid-liquid extraction (for blood) have been developed. The nineteen OCP residues were analyzed by gas chromatography with micro-electron captured detector (GC-μECD). The validated methods have met requirements of the AOAC standard. The results indicated that significant amounts of OCPs are still contaminated in nest soil and eggs of the turtle even though the OCPs had been banned in this area for many years. This suggested the potential risk to health of wildlife as well as human in the area.

***Keywords*—**Gas chromatography, persistent organic pollutants, rice field, sentinel species.

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I. Introduction

ORGANOCHLORINE pesticides (OCPs) are the synthetic organic insecticides that contain carbon, chlorine and hydrogen. They are highly persistent in the organism and environment because of their low water solubility and high lipophilicity [1]. Due to their persistence and inexpensive price, the OCPs such as dichloro-diphenyltrichloroethane (DDT), aldrin, endosulfan had been widely used for pest control. In addition, DDT was regarded as significant measure to control malaria during World War II. However, it was later found that OCP residues were slowly released into aquatic and terrestrial ecosystems. These residues can be transferred and biomagnified through food chain. Thus significant levels of OCPs could be accumulated and caused adverse health effects in animals at higher trophic levels, including human [1]-[3]. As a result, the OCPs had been banned in many countries (1970 in Sweden; 1971 in Japan; 1972 in USA). In Thailand, use of OCPs started from 1949 to 1990s. In particular, DDT was the first pesticide that had been used for malaria control in the country since 1949. Later on, OCPs had been banned in Thailand during 1980s to 2004 [4].

Due to the persistence of the OCPs, low level contamination of OCPs in environment was reported, even though the use of those OCPs had been banned in the area. Recent studies have suggested that even the low level of OCP residues (part per billion) may interfere with structure or function of endocrine system and cause adverse effects to animal reproduction and development [5]. OCP contamination in animal tissues was linked to adverse effects on reproductive system such as reduced penis size of American alligator *Alligator mississippiensis* in Lake Apopka, Florida, USA, and abnormality in reproductive functions of Florida red-belly turtles *Chrysemys nelsoni* [6], [7]. Since human population may be similarly at risk from these chemicals, it is crucial to monitor the degree of OCP contamination in ecosystem.

The tissue residues of many long-lived reptiles such as crocodile and turtle have been widely used as bioindicators for environmental contamination. The information could be of

importance to identify potential health hazards to other animals as well as human [8]. Eggs and blood of several turtle species have been used for monitoring the persistent organic pollutants such as loggerhead sea turtle *Caretta caretta* [9], [10] and the common snapping turtle *Chelydra serpentina serpentina* [11], [12]. Since contamination in egg yolk may result in OCPs absorption into embryos during development [13], turtle eggs could be further used to determine the link between levels of contamination and the potential reproductive and developmental effects.

Although OCPs had been banned in Thailand for many years, their residues were still detectable in components of aquatic and terrestrial ecosystems including water, soil, sediment, shellfish, shrimp, fish [14] and little egret's egg [15]. It is thus important to examine the extent of contamination in organisms living in the area with history of OCP use.

Snail-eating turtle, *Malayemys macrocephala*, is the native and the most common freshwater turtle in Thailand and Southeast Asia. It can be found in wetland habitats such as canals, ponds and rice fields [16], [17]. The turtle is carnivorous and eats large number of small snails, earthworms, aquatic insects, crustaceans, and small fish [18]. In this study, nest soil, egg and blood of *M. macrocephala* are used as sentinels for OCP contamination in ecosystem of the lower Chao Phraya River Basin, central part of Thailand where agricultural activities is extensive.

II. Materials and Methods

A. Study Area

The study area is a floodplain nearby the Chao Phraya River at Bangban district, Phra Nakhon Si Ayuthaya province (~100 kilometers north of Bangkok, Fig. 1). Rice is the predominant crop in this area with its field covering more than 90% of the land use. The area is an important breeding ground of the snail-eating turtle in central part of Thailand.

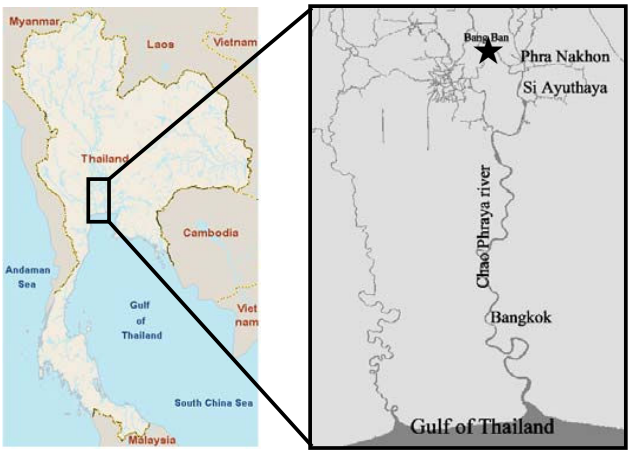

Fig. 1 Study area (★) in Bang Ban district, Phra Nakhon Si Ayuthaya province, central part of Thailand

B. Sample Collection

During December 2007 to January 2008, three nests of the snail-eating turtle in the study area were located by visual encounter surveys. Turtle nests were measured for dimension (width, length and depth). Ten grams of nest soil were sampled from each nest. Freshly laid eggs were collected as a complete clutch and measured for width, length and weight. Egg content was kept at -36 °C until analysis for OCPs.

Nine adult female and three adult male turtles were captured from rice fields in the study area and transported to the laboratory animal facility at the Department of Biology, Faculty of Science, Chulalongkorn University. After 1-week acclimatization, turtles were subjected to cold anesthetization in ice slurry and blood withdrawal. Three milliliters of blood was taken from dorsocervical sinus of each turtle using a 20-ga needle and a heparinized syringe. Blood samples were centrifuged at 150 xg for 10 min, and the plasma was collected and kept frozen at -36 °C until analysis for OCP. Animal handling procedures in this study have been approved by Chulalongkorn University Animal Care and Use Committee.

C. Pesticide Standards and Reagents

A standard solution containing nineteen organochlorine pesticides including α-hexachlorocyclohexane (HCH), β-HCH, γ-HCH, δ-HCH, heptachlor, heptachlor epoxide, γ-chlordane, α-chlordane, 4,4' DDE, 4,4' DDD, 4,4' DDT, endrin, endrin aldehyde, endrin ketone, endosulfan I, endosulfan II, endosulfan sulfate, aldrin, dieldrin, and 2 surrogates (2,4,5,6-tetrachloro-m-xylene: TCMX and decachlorobiphenyl: DCBP) were obtained from Restek, USA. Pesticide grade solvents such as dichlormethane, diethyl ether, petroleum eather, and 95% n-hexane were purchased from LabScan Asia. SPE-florisil cartridges of 1,000 mg were purchased from Alltech. All Pyrex® glassware and Teflon® centrifuge vial were well-cleaned with laboratory detergent purchased from EMC-IMEX, then sequentially rinsed with distilled water. Washed glassware was baked in an oven at 250 °C overnight and rinsed with acetone before each use.

D. Sample Preparation for OCP Determination

OCP in Turtle Nest Soil – Methods for an analysis of OCP in nest soil was modified from [19]. Soil sample was mixed and dried in a circulating air at the room temperature without exposure to sunlight for 7 days. Dried sample was ground and sieved (500 μm) to remove stones and shells [20]. An approximately 5 g of the sample was mixed with 5 g of anhydrous sodium sulfate and held at the room temperature for ~20 min prior to extraction. The sample was placed into a 34-mL vessel of Accelerated Solvent Extractor (ASE, Dionex Oakville, ON, Canada) in which the cellulose paper was added with 1 g of activated copper powder. A 50 μL aliquot of 1 μg/mL TCMX and DCBP was spiked into every sample as surrogates. The samples spiked with 50 μL of 1 μg/mL OCP standard solution were used for recovery studies. A mixture of 95% n-hexane: dichloromethane (1:1 v/v) was used as extracting solvent. The ASE was operated in static mode

for 2 cycles by first preheated the sample for 5 min and extracted at 100°C with pressure of 1,500 psi for 10 min, after which, the sample was purged with nitrogen for 60 s. The extract was collected in a collecting vessel and evaporated to 1 mL by Turbo Vap II (Zymark) before cleaned up with SPE-florisil cartridge as described in [19]. To remove sulfur contamination as previously described in [21], the cleaned up technique included packing 1 g of anhydrous sodium sulfate layer and 1 g of activated copper powder on top of 1,000 mg of SPE-florisil cartridge. A 10 mL of each 6%, 15%, and 50% of diethyl ether in petroleum ether was used for elution, successively. The eluates were combined and collected in a concentrator tube for evaporation to 1.5 mL under gentle stream of nitrogen prior to quantification with gas chromatography-electron capture detector (GC-μECD).

OCP in Turtle Egg – Methods for determination of OCP in the snail-eating turtle eggs was modified from [19]. The whole egg content (egg yolk and albumin) was homogenized in metal cup. Approximately 1 g of egg content was mixed thoroughly with 5 g of anhydrous sodium sulfate and held for dryness at room temperature for ~20 min prior to extraction with ASE. A 50 μL aliquot of 1 μg/mL TCMX and DCBP was spiked into every egg sample as surrogates. Egg samples spiked with 50 μL of 1 μg/mL OCP standards were used for recovery studies. A mixture of 95% n-hexane: dichloromethane (1:1 v/v) was used as extracting solvent. The ASE was operated in static mode for 2 cycles by first preheated the sample for 5 min and extracted at 100°C with pressure of 1,500 psi for 10 min, after which, the sample was purged with nitrogen for 60 sec. The extract was collected in a collecting vessel and evaporated to 1 mL by Turbo Vap II before cleaned up with SPE-florisil cartridge as described in [19]. A 10 mL of each 6%, 15%, and 50% of diethyl ether in petroleum ether was used for elution, successively. The eluates were combined and collected in a concentrator tube for evaporation to 1.5 mL under a gentle stream of nitrogen prior to quantification with GC-μECD.

OCP in Turtle Blood – Methods for OCP determination in turtle blood was adapted from [22]. One mL of turtle plasma was transferred to 10–mL Teflon® centrifuge vial. A 50 μL aliquot of 1 μg/mL TCMX and DCBP was spiked into every plasma sample as surrogates. Plasma samples spiked with 50 μL of 1 μg/mL OCP standard solution were used for recovery studies. Five milliliters of the mixture of 95% n-hexane: acetone (9:1) was added to sample and mixed thoroughly for 1 min. The mixture was extracted for 5 min by centrifuge at 1,520 xg. The organic phase (upper level) was transferred to evaporation tube, and the lower phase was re-extracted using the same condition. The extract was collected in a collecting vessel and evaporated to 1 mL by Turbo Vap II before cleaned up with SPE-florisil cartridge as described in [19]. A 10 mL of each 6%, 15%, and 50% of diethyl ether in petroleum ether was used for elution, successively. The eluates were combined and collected in a concentrator tube for evaporation to 1.5 mL under stream of nitrogen prior to analysis with GC-μECD.

E. Chromatographic Conditions

A gas chromatograph equipped with micro electron capture detector (Agilent 6890N GC-μECD) and a DB-35MS fused silica capillary column (30 m length, 0.25 mm i.d., 0.25 μm film thickness, 35% diphenyl polysiloxane (J&W Scientific)) were used for quantification. Sample quantification was using multiple external standards following [19]. One microliter of sample was injected into the GC-μECD on a splitless mode with 0.75 min vent delay. The injector and detector temperature were maintained at 260 °C and 300 °C, respectively. The oven temperature was initially set at 100 ºC for 2 min, and programmed to increase to 280 ºC at 12 ºC /min and held for 10 min. The total run time was calculated to be 27 min. Helium (UHP grade, 99.999%) was used as a carrier gas with flow rate of 2 mL/min. Nitrogen (UHP grade) was set at 60 mL/min as a make-up gas. The pesticide data were processed by Hewlett Packard Chemstation software.

F. Quality Control

A stock of 10 μg/mL standard mixture containing 19 pesticides was prepared in 99% n-hexane and stored at -4 ºC. Working mixtures of standard solutions between 1 to 100 ng/mL were prepared in 99% n-hexane. Calibration curves of OCPs were prepared at concentrations of 1, 2, 5, 10, and 50 ng/mL. Calibration standards were reestablished every 10 samples. All measurements were performed within the range of linearity found for each compound. Organochlorine pesticides were identified by comparison of retention times with standards and confirmed on a DB-1701 fused silica capillary column (14% cyanopropylphenyl and 86% diphenyl polysiloxane; 30 m length, 0.32 mm internal diameter and 0.25 μm film thickness (J&W Scientific)). Blank and duplicate samples (matrix spiked samples) were included in every batch. Recoveries of 2 surrogates (TCMX, DCBP) were determined for every sample.

G. Statistical Analysis

The concentrations of OCPs were classified and reported as ∑HCH (sum of α-HCH, β-HCH, γ-HCH and δ-HCH), ∑chlordane (sum of heptachlor, heptachlor epoxide, γ-chlordane, and α-chlordane), ∑DDT (sum of 4,4' DDE, 4,4' DDD and 4,4' DDT), ∑endrin (sum of endrin, endrin aldehyde, and endrin ketone), ∑endosulfan (sum of endosulfan I, endosulfan II and endosulfan sulfate), and sum of aldrin and dieldrin. Descriptive statistical analysis (mean and standard error of the mean) and one-way analysis of variance (ANOVA) were used in this study.

III. Results

A. Quality Control

Limit of detections (LODs) and limit of quantitations (LOQs) of organochlorine pesticides determined by GC-μECD were in the range of 0.01-0.04 ng/mL and 0.04-0.13 ng/mL, respectively. The method detection limits (MDLs) of OCPs in soil were 2.64-7.87 ng/g dry weight (n=6) at 50 ng/g

dry weight. The recoveries were 51.21-102.82 % with RSD of 3.18-9.71 %. The MDLS of OCPs in turle egg were 4.77-9.99 ng/g wet weight (n=7) at 50 ng/g wet weight. The recoveries of turtle eggs spiked OCPs were 82-118 % with relative standard deviation (RSD) of 2.48-4.90 %. The MDLs of OCPS in the turtle blood were 1.52-10.50 µg/L (n=7) at 50 µg/L. The recoveries of turtle blood spiked OCPs were 42.07-116.66 % with RSD of 2.52-12.15 %.

B. OCP Contamination in Turtle Nest Soil

The snail-eating turtle nest in the study area was in a cup shape with dimension of 5-10 cm width, 7-11 cm length and 7-15 cm depth. The concentrations of organochlorine pesticide residues in nest soil were in the range of 7.8 to 31.5 ng/g dry weight (Table I). The average recovery of pesticide surrogates (TCMX and DCBP) were 85.6% and 96.6% respectively.

TABLE I
ORGANOCHLORINE PESTICIDE RESIDUES (NG/G DRY WEIGHT) IN NEST SOIL OF *M. MACROCEPHALA* AT PHRA NAKHON SI AYUTHAYA PROVINCE, CENTRAL PART OF THAILAND

Chemicals	Nest #1	Nest #2	Nest #3	Mean ± S.E.M.
TCMX (%)	81.1	83.9	91.9	85.6
Σ HCH (ng/g)	31.5	27.2	30.9	29.9 ± 1.3
Σ chlordane (ng/g)	8.7	7.3	14.1	10.0 ± 2.1
Σ DDT (ng/g)	ND	ND	ND	ND
Σ endrin (ng/g)	ND	ND	ND	ND
endosulfan (ng/g)	7.8	12.2	14.0	11.3 ± 1.8
Aldrin & dieldrin (ng/g)	11.4	7.2	3.3	7.3 ± 2.3
DCBP (%)	96.2	95.8	97.7	96.6

ND = not detected

TABLE II
ORGANOCHLORINE PESTICIDE RESIDUES (NG/G WET WEIGHT) IN THREE COMPLETE CLUTCHES OF *M. MACROCEPHALA* EGGS AT PHRA NAKHON SI AYUTHAYA PROVINCE, CENTRAL PART OF THAILAND

Nest	Chemicals	Min - Max	Mean ± S.E.M.
#1 (n=8)	TCMX (%)	87.3 - 95.1	92.6
	Σ HCH (ng/g)	7.3 - 21.0	12.9 ± 1.7
	Σ chlordane (ng/g)	ND - 13.5	9.9 ± 1.0
	Σ DDT (ng/g)	21.2 - 500.7	98.5 ± 57.8
	Σ endrin (ng/g)	ND	ND
	endosulfan (ng/g)	ND	ND
	Aldrin & dieldrin (ng/g)	ND	ND
	DCBP (%)	33.9 - 84.5	48.8
#2 (n=8)	TCMX (%)	84.5 - 119.1	101.9
	Σ HCH (ng/g)	9.4 - 25.3	16.3 ± 2.0
	Σ chlordane (ng/g)	ND - 16.5	13.1 ± 1.3
	Σ DDT (ng/g)	26.6 -75.7	38.0 ± 5.8
	Σ endrin (ng/g)	ND	ND
	endosulfan (ng/g)	ND	ND
	Aldrin & dieldrin (ng/g)	ND	ND
	DCBP (%)	21.9 - 41.7	30.0
#3 (n=6)	TCMX (%)	109.1 -115.0	111.8
	Σ HCH (ng/g)	12.3 - 20.7	16.5 ± 1.4
	Σ chlordane (ng/g)	ND - 17.9	12.1 ± 2.0
	Σ DDT (ng/g)	34.1 - 600.2	293.6 ± 99.0
	Σ endrin (ng/g)	ND	ND
	endosulfan (ng/g)	ND	ND
	Aldrin & dieldrin (ng/g)	ND	ND
	DCBP (%)	23.2 - 36.1	28.9

ND = not detected

C. OCP Contamination in Turtle Egg

Clutch size of the turtle in this area ranged from 6-8 eggs with the average size of 22.57 ± 0.36 cm width, 37.68 ± 0.73 cm length and 11.87 ± 0.56 g weight. The analysis for OCP residues in three complete clutches showed that HCHs, chlordanes and DDTs were found in all clutches (Table II). The concentrations of ∑HCHs and ∑Chlordanes were not significantly different among eggs as well as among nests (F-test, $p > 0.05$ and ANOVA, $p > 0.05$).

TABLE III
RESIDUES OF DDT (NG/G WET WEIGHT) IN THREE COMPLETE CLUTCHES OF *M. MACROCEPHALA* EGGS AT PHRA NAKHON SI AYUTHAYA PROVINCE, CENTRAL PART OF THAILAND

Egg Number[1]	Nest 1 (n=8)	Nest 2 (n=8)	Nest 3 (n=6)
1	41.5	75.7	553.4
2	29.9	26.9	600.7
3	26.3	26.6	40.4
4	500.7	30.4	264.1
5	77.1	30.7	34.1
6	21.2	39.6	269.2
7	35.1	44.4	N/A
8	55.8	29.9	N/A
Mean + S.E.M.	98.5 ± 57.8	38.0 ± 5.8	293.6 ± 99.0

[1]Egg number is arbitrary and does not refer to the order of oviposition.
N/A = not applicable

However, the concentration of DDTs was varied among eggs and among nests (Table III). In nest number 3, the high concentration of DDT was found in 4 out of 6 eggs. As a result, there were significantly difference in DDT concentration among eggs in this clutch (F – test, $p < 0.05$ and ANOVA, $p > 0.05$).

D. OCP Contamination in Turtle Blood

The average recovery of pesticide surrogates (TCMX and DCBP) in turtle blood were 88.8% and 109.8% respectively. However, OCPs were not detected in blood of any of the turtles caught from the field in this study area.

IV. DISCUSSION

Although the OCPs had been banned in Thailand [4], the OCP residues were still detectable in Thai ecosystem [14], [19]. Our findings on OCP contamination in nesting soil of the freshwater turtle were similar to prior studies in the nearby areas [23], and at a watershed nearby the Mae Klong River [24]. Since the lower Chao Phraya River basin is regarded as the major area for rice plantation in Thailand, the OCP residues are potentially results of the past and ongoing agricultural activities in the area.

Using turtle eggs as biomonitoring systems for environmental contamination of persistent organic pollutants have been established in many areas of the world [10], [13], [25]. However, there are very few studies on OCP contamination performed in complete clutch of eggs. The first report on reptile eggs was studied in complete clutch of Morelet's crocodile (*Crocodylus moreletii*) eggs from Belize [26]. The current study is the first report on OCP contamination in complete clutches of the snail-eating turtle eggs. The complete clutch analysis showed interesting pattern

of OCP contamination in turtle eggs. It was found that OCP residues distributed relatively even to every egg with some variation in the level of contamination. The significant levels of ∑HCH, ∑chlordane, and ∑DDT were found in every clutch (Table II). Concentrations of ∑HCHs and ∑chlordane were similar among eggs, while the concentration of ∑DDT in some clutch showed intra-clutch variation. The intra-clutch variations in OCP residues have been previously reported in turtle [13], crocodile [26] and bird [27]. The higher level of OCP residue was associated with higher lipid content of the egg [26]. The inter-clutch variation was also found in *M. macrocephala* eggs, potentially dued to the different levels of OCP in female turtle as previously suggested by [26].

The OCP contamination in turtle eggs may be from maternal transfer to the yolk or from the environment i.e. the nesting soil. It has been previously reported that OCPs could be transferred from fat to developing follicles in mothers during vitellogenesis and yolk production [28], [29]. In regard to the nesting soil, it was reported that the OCPs can be uptake from the nest material to snake eggs and the concentration of most OCPs increased from week 4 to week 6 [30]. Although the OCP residues could be detected in the nest soil at the areas with the activities of using pesticides to control pest in the rice field, their up taken into eggs required decent amount of time. Since the current study used freshly laid eggs for OCP analysis, the probability of OCP contamination from nest soil being uptake into egg is thus unlikely.

Animal blood has been used to monitor the xenobiotics contamination in wildlife such as *Chelydra serpentina serpentina* [31], *Phoca hispida* and *Erignathus barbatus* [32], *Larus hyperboreus* [33] and *Caretta caretta* [9]. Reference [34] reported that blood is a suitable alternative to fatty tissues for monitoring OCPs since it is a good representative of the exposure levels of target tissues. In the current study, although the significant amounts of OCPs were found in turtle eggs, the levels of OCPs in blood of adult female and male turtles caught from the rice field was not detectable. It is possible that the contamination in blood is below the detection limits (1.52-10.50 µg/L). Alternatively, since the accuracy and precision of the current analytical method are acceptable by AOAC standard [35], it is possible that the level of OCPs in blood of *M. macrocephala* is not a good representative of contamination in other tissue. This is similar to previous report [36] which stated that the majority of contaminants deposited in snapping turtle eggs were from the recent diet instead of body adipose stores.

V. Conclusion

Concentrations of organochlorine pesticides have been successfully measured in nest soil, complete clutch of eggs, and blood of the common freshwater turtle lived in rice field habitat in the Chao Phraya River Basin, Thailand. The results indicated that although all of these pesticides had been banned in Thailand for many years, their detectable levels in nest soil and turtle eggs indicate that they can persist in agricultural fields for long period of time. Overall, the results of using turtle tissues as biomonitoring systems for persistent organic pollutants in the environment may provide a significant linkage to our understanding of the potential environmental risk to health and reproductive success of wildlife as well as human.

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