การประเมินความเชื่อมโยงระหว่างการปนเปื้อนของสารกำจัดศัตรูพืชกลุ่มออร์แกโนคลอรีน และผลต่อการสืบพันธุ์ของเต่านา (*Malayemys macrocephala*) ในบริเวณลุ่มแม่น้ำเจ้าพระยาตอนล่าง ประเทศไทย

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AN ASSESSMENT OF THE ASSOCIATION OF ORGANOCHLORINE PESTICIDE CONTAMINATION AND REPRODUCTIVE EFFECTS ON THE SNAIL-EATING TURTLE (*Malayemys macrocephala*) IN THE LOWER CHAO PHRAYA RIVER BASIN, THAILAND

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SARUN KEITHMALEESATTI: AN ASSESSMENT OF THE ASSOCIATION OF ORGANOCHLORINE PESTICIDE CONTAMINATION AND REPRODUCTIVE EFFECTS ON THE SNAIL-EATING TURTLE (*Malayemys macrocephala*) IN THE LOWER CHAO PHRAYA RIVER BASIN, THAILAND ADVISOR : NOPPADON KITANA, PH.D., CO-ADVISOR : ASST. PROF. PAKORN VARANUSUPAKUL, PH.D., 143 pp.

Organochlorine pesticides (OCPs) are persistent and bioaccumulative toxicants that may cause reproductive impairments in wildlife as well as human. It is thus important to examine the extent of contamination and the potential reproductive effects on the animals living in area with history of OCP uses. In this study, the snail-eating turtle (*Malayemys macrocephala*) is used as a sentinel for OCP contamination in the Chao Phraya River Basin because it is a long-lived vertebrate with a home range overlapping a potentially affected area. Study on nesting and reproductive biology showed that *M. macrocephala* has clutch size of 3-10 eggs, egg size of 22 x 38 mm and egg weight of 11.6 g. Egg incubation in a temperature-controlled incubator resulted in incubation period of 82-186 days with hatching success of 36-54%. A novel non-invasive sexing technique has been developed based on a morphometric study and can be used to predict sex of juvenile turtle with an accuracy of 85.3%. Incubation at different temperatures resulted in different sex ratio suggesting that *M. macrocephala* has temperature-dependent sex determination. Analyses for OCP residues in nest soil, female blood and complete clutch of eggs revealed detectable levels in nest soil and turtle eggs. OCP residue analysis in eggs showed that 5 groups of OCPs (HCHs, chlordanes, DDTs, endosulfans, and aldrin and dieldrin) were detected in turtle eggs, and HCHs was the most predominant residues. Egg incubation at 29 °C revealed the hatching success of 66.95% and the neonate survival rate of 92.40%. Although no correlation between OCP levels and survival rate was found, the levels of Σchlordane in eggs were positively correlated with percentage of hatchling that requires assistance after egg pipping in order to emerge from eggshell. Levels of ∑HCH in eggs were found to associate with an increased incidence of deformities in *M. macrocephala*. Levels of OCP residue in eggs were also found to skew the sex ratio toward male-biased ratio. Overall, the results indicated that, although OCPs had been banned, their low level contamination is still present in central agricultural plain of Thailand. Associations between OCP residues and hatching success, deformities and sex ratio of the turtles suggest the potential ecological risk of OCP contamination on reproductive health and population status of the long-lived vertebrate living in area with history of OCP utilization.

Field of Study : Environmental Science Student's Signature Sarun Keithmaleesatti
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AN ASSESSMENT OF THE ASSOCIATION OF ORGANOCHLORINE PESTICIDE CONTAMINATION AND REPRODUCTIVE EFFECTS ON THE SNAIL-EATING TURTLE (*Malayemys macrocephala*) IN THE LOWER CHAO PHRAYA RIVER BASIN, THAILAND
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สารกำจัดศัตรูพืชกลุ่มออร์แกโนคลอรีน มีฤทธิ์ตกค้างยาวนานในสิ่งมีชีวิต และสามารถก่อให้เกิดผลเสียต่อระบบสืบพันธุ์ของสัตว์ป่าและมนุษย์ได้ การตรวจสอบการปนเปื้อนและผลต่อการสืบพันธุ์ของสัตว์ที่อาศัยในพื้นที่ที่มีประวัติการใช้สารกำจัดศัตรูพืชจึงมีประโยชน์ต่อการบ่งบอกการตกค้างในธรรมชาติและผลกระทบที่อาจเกิดต่อสัตว์ป่า ในการศึกษาครั้งนี้ได้เลือกใช้เต่านา (*Malayemys macrocephala*) ซึ่งเป็นสัตว์มีกระดูกสันหลังที่มีอายุยืนและอาศัยในพื้นที่ที่มีประวัติการใช้สารกำจัดศัตรูพืชกลุ่มออร์แกโนคลอรีน เป็นตัวเฝ้าระวังการปนเปื้อนในพื้นที่ลุ่มแม่น้ำเจ้าพระยาตอนล่าง การศึกษาชีววิทยาการสืบพันธุ์และวางไข่ของเต่านา แสดงให้เห็นว่าเต่านาสามารถวางไข่ได้ครั้งละ 3-10 ฟอง โดยไข่มีขนาดเฉลี่ยกว้าง 22 มิลลิเมตร ยาว 38 มิลลิเมตร และมีน้ำหนักเฉลี่ย 11.6 กรัม การฟักไข่ในตู้ควบคุมอุณหภูมิแสดงให้เห็นว่าไข่เต่านาใช้เวลาในการฟัก 82-186 วัน และมีอัตราการฟักร้อยละ 36-54 ข้อมูลจากการศึกษามอร์ไฟเมตรีของลูกเต่าสามารถนำมาพัฒนาเป็นวิธีการตรวจสอบเพศที่มีความแม่นยำสูงถึงร้อยละ 85.3 ข้อมูลจากการฟักไข่ที่อุณหภูมิต่างกันทำให้ได้สัดส่วนเพศของเต่าที่ต่างกัน แสดงให้เป็นว่าเต่านามีรูปแบบการกำหนดเพศโดยอาศัยอุณหภูมิ เมื่อนำดินจากบริเวณที่เต่าวางไข่ และ เก็บตัวอย่างเลือดของเต่าตัวเมีย และ ตัวอย่างไข่เต่ามาวิเคราะห์การปนเปื้อนของสารกำจัดศัตรูพืชกลุ่มออร์แกโนคลอรีน พบว่ายังมีการปนเปื้อนของสารกลุ่มดังกล่าวในดินและไข่เต่าอยู่ โดยพบการปนเปื้อนของสารกำจัดศัตรูพืช 5 กลุ่ม ในไข่เต่า คือ เอชซีเอช คลอเดน ดีดีที เอนโดซัลแฟน และ อัลดรินและดีลดริน โดยเอชซีเอชเป็นกลุ่มที่พบมากที่สุดเมื่อนำไข่เต่ามาฟักที่อุณหภูมิคงที่ 29 องศาเซลเซียส พบว่ามีอัตราการฟักร้อยละ 66.95 และลูกเต่าที่ได้มีอัตราการอยู่รอดร้อยละ 92.40 เมื่อนำข้อมูลการปนเปื้อนมาวิเคราะห์สหสัมพันธ์กับผลต่อการสืบพันธุ์ พบว่าปริมาณออร์แกโนคลอรีนที่ปนเปื้อนไม่มีความสัมพันธ์กับอัตราการอยู่รอด อย่างไรก็ดีปริมาณของคลอเดนในไข่เต่ามีความสัมพันธ์กับสัดส่วนลูกเต่าที่ไม่สามารถออกจากไข่ได้เองหลังการฟัก และปริมาณเอชซีเอชในไข่เต่ามีความสัมพันธ์กับสัดส่วนลูกเต่าที่มีลักษณะผิดปกติ นอกจากนี้ยังพบว่าปริมาณสารกำจัดศัตรูพืชที่เพิ่มขึ้นมีผลทำให้สัดส่วนเพศของลูกเต่ามีเพศผู้มากขึ้น ผลการศึกษาโดยรวมแสดงให้เห็นว่า สารกำจัดศัตรูพืชกลุ่มออร์แกโนคลอรีนยังคงตกค้างอยู่ในพื้นที่เกษตรกรรมในภาคกลางของประเทศไทย แม้ว่าจะถูกห้ามใช้มาเป็นเวลานานแล้วก็ตาม โดยปริมาณที่ตกค้างมีความเชื่อมโยงกับการออกจากไข่ที่ผิดปกติ สัดส่วนของลูกเต่าที่มีลักษณะผิดปกติ และสัดส่วนเพศของลูกเต่า แสดงให้เห็นถึงความเสี่ยงทางนิเวศวิทยาของการปนเปื้อนสารกำจัดศัตรูพืชกลุ่มออร์แกโนคลอรีนกับการสืบพันธุ์และประชากรของสัตว์มีกระดูกสันหลังที่มีอายุยืนยาวที่อาศัยในพื้นที่ที่มีประวัติการใช้สารกำจัดศัตรูพืชกลุ่มนี้

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CONTENTS

Pages

ABSTRACT IN THAI …… iv

ABSTRACT IN ENGLISH ……..... v

ACKNOWLEDEMENTS …….. vi

CONTENTS …… vii

LIST OF TABLES …….. x

LIST OF FIGURES …… xiii

CHAPTER I INTRODUCTION ……. 1

1.1 Rationale 1

1.2 Objectives 4

1.3 Hypothesis 4

CHAPTER II LITERATURE REVIEWS ……. 5

2.1 Organochlorine Pesticide.. 5

2.2 Snail-eating Turtle *Malayemys macrocephala* 20

2.3 Ecotoxicology of organic contaminants in reptile..... 24

2.4 Endocrine disruptor 26

Pages

CHAPTER III NESTING BIOLOGY, SEXUAL SIZE DIMORPHISM AND EFFECTS OF INCUBATING TEMPERATURE ON DEVELOPMENT OF THE SNAIL-EATING TURTLE *Malayemys macrocephala* FROM THE LOWER CHAO PHRAYA RIVER BASIN, THAILAND..... 31

3.1 Introduction ……..……..…… 31

3.2 Materials and Methods ……..…….. 33

3.3 Results …….……. 40

3.4 Discussions ……..……. 51

3.5 Conclusions …….……. 55

CHAPTER IV CONCENTRATION OF ORGANOCHLORINE PESTICIDES IN NEST SOIL, EGG AND BLOOD OF SNAIL-EATING TURTLE *Malayemys macrocephala* FROM THE CHAO PHRAYA RIVER BASIN THAILAND..... 56

4.1 Introduction ……..…… 56

4.2 Materials and Methods ……..…….. 58

4.3 Results …….……. 65

4.4 Discussions ……..……. 71

4.5 Conclusions 73

Pages

CHAPTER V ASSOCIATION OF ORGANOCHLORINE PESTICIDE CONTAMINATION IN EGG AND REPRODUCTIVE EFFECTS ON THE SNAIL-EATING TURTLE *Malayemys macrocephala* IN THE LOWER CHAO PHRAYA RIVER BASIN, THAILAND..... 74

5.1 Introduction ……..…… 74

5.2 Materials and Methods ……..…….. 76

5.3 Results …….……. 84

5.4 Discussions ……..……. 95

5.5 Conclusions …….……. 98

CHAPTER VI CONCLUSIONS 100

REFERENCES 104

APPENDICES 122

APPENDIX A 123

APPENDIX B 137

APPENDIX C 140

BIOGRAPHY 143

LIST OF TABLES

Table Pages

2.1: Hormone disrupting effects of organochlorine pesticides 27

3.1: Soil type in the snail – eating turtle nest at Bang Ban distrinct, Phra Nakhon Si Ayutthaya province, Thailand. 40

3.2: Nest characteristics of in *M. macrocephala* at Bang Ban distrinct, Phra Nakhon Si Ayutthaya province, Thailand. 42

3.3: Clutch size and egg morphology of snail-eating turtle egg at Bang Ban distrinct, Phra Nakhon Si Ayutthaya province, Thailand..... 43

3.4: Morphological characteristics of juvenile turtle that are significantly different between sex..... 44

3.5: Effects of incubation temperature on incubation duration and hatching success..... 46

3.6: Hatching success and survival rate of juvenile turtles after egg incubation at three different temperatures..... 47

3.7: *G* statistic for the log-likelihood ratio goodness of fit test for the sex ratio of the snail-eating turtle (*M. macrocephala*) hatched from eggs incubated at three different temperatures..... 51

4.1: Organochlorine pesticide residues (ng/mL wet weight) in nest soil of *M. macrocephala* eggs at Phra Nakhon Si Ayutthaya province, Thailand 66

Table **Pages**

4.2: Organochlorine pesticide residues (ng/g wet weight) in three complete clutches of *M. macrocephala* eggs at Phra Nakhon Si Ayutthaya province, Thailand..... 68

4.3: Residues of DDT (ng/g wet weight) in three complete clutches of *M. macrocephala* eggs at Phra Nakhon Si Ayutthaya province, Thailand..... 69

4.4: Organochlorine pesticide residues (ng/mL wet weight) in adult blood of *M. macrocephala* at Phra Nakhon Si Ayutthaya province, Thailand..... 70

5.1: Data (mean ± S.E.) of *M. macrocephala* eggs collected from Bang Ban district, Phra Nakhon Si Ayutthaya province and Rangsit agricultural area (Khlong 7), Khlong Luang district, Pathum Thani province, Thailand..... 84

5.2: The concentration (mean ± S.E.) of organochlorine pesticides (OCPs) in egg of *M. macrocephala* collected from Bang Ban district, Phra Nakhon Si Ayutthaya province and Rangsit agricultural area (Khlong 7), Khlong Luang district, Pathum Thani province, Thailand..... 85

5.3: The concentration (mean ± S.E.) of organochlorine pesticides (OCPs) in egg of *M. macrocephala* collected from the lower Chao Phraya river basin, Thailand..... 87

Table **Pages**

5.4: Incubation time and hatching success of *M. macrocephala* eggs collected from agricultural areas in the lower Chao Phraya river basin, Thailand..... 88

5.5: The number of *M. macrocephala* survived between one day to 12 months. Turtles were hatched from eggs collected from agricultural areas in the lower Chao Phraya river basin, Thailand..... 89

5.6: Incidence of deformed morphology of *M. macrocephala* hatched from eggs collected from agricultural areas in the lower Chao Phraya river basin, Thailand..... 93

5.7: *G* statistic for the log-likelihood ratio goodness of fit test for the sex ratio of the snail-eating turtle hatched from eggs collected from agricultural areas in the lower Chao Phraya river basin, Thailand..... 95

LIST OF FIGURES

FIGURES **Pages**

3.1: Study area at Bang Ban district, Phra Nakhon Si Ayutthaya province, Thailand 34
3.2: Nest of snail – eating turtle at Bang Ban district, Phra Nakhon Si Ayutthaya province, Thailand..... 35
3.3: Positions (1-5) on eggshell that were cut and measured for eggshell thickness..... 36
3.4: Egg incubation in a microprocessor-controlled incubator..... 37
3.5: Diagram and names of turtle shells referred in the morphometric study..... 38
3.6: Turtle with assisted hatching 39
3.7: Cover and hole of the snail-eating turtle nest..... 41
3.8: The turtle nest before open the cover and after open the cover..... 41
3.9: Morphological characteristics used in sex determining equation..... 45
3.10: Normal hatching..... 48
3.11: Assisted hatching..... 49
3.12: Dead turtle due to desiccation..... 49
3.13: Sex ratio of *M. macrocephala* hatched from eggs incubated at three different temperatures..... 50
4.1: Study area in Bang Ban district, Phra Nakhon Si Ayutthaya province, Thailand 59

FIGURES **Pages**

5.1: Study areas at Bang Ban district, Phra Nakhon Si Ayutthaya province and Khlong 7, the Rangsit agricultural area, Khlong Luang district, Pathum Thani province, Thailand..... 77

5.2: In assisted hatching, the hatchling cannot move out of the egg by itself because egg membrane was dried or sticky..... 81

5.3: Dendogram of *M. macocephala* eggs collected from the lower Chao Phraya River Basin. Eggs can be divided into 2 groups according to pattern of OCP residues..... 87

5.4: Severe deformity that turtle cannot hatch and died in egg..... 91

5.5: Carapace asymmetry..... 91

5.6: Plastron asymmetry..... 91

5.7: Carapace scute in excess of 38 pieces 91

5.8: Carapace scute in shortage of 38 pieces..... 92

5.9: Deformed carapace..... 92

5.10:Tail disappear..... 92

5.11: Dwarf..... 92

5.12: Lower jaw disappear..... 92

5.13:Sex ratio of *M. macrocephala* hatched from eggs collected from agricultural areas in the lower Chao Phraya river basin, Thailand..... 94

CHAPTER I

INTRODUCTION

1.1 Rationale

After the end of the World War II, a number of synthetic pesticides such as organochlorine, organophosphate and carbamate have been heavily used to protect crops in Thailand. Organochlorine pesticides (OCPs) such as dichlorodiphenyl trichloroethane (DDT) was the most popular pesticide used for agricultural and public health purposes due to its broad spectrum, highly persistent and low price (Perry *et al*., 1998). However, there have been several reports on adverse effects of the OCPs on reproductive system of non-target organisms since 1950s. The reports indicated that the OCPs can be transferred through food chain with an increasing concentration in the higher consumer. The OCPs can be accumulated in fat tissue of animals and the biological degradation of these chemicals is very slow. The body burden of the OCPs and their effects on reproductive system have been found in organisms at higher trophic levels such as bird of prey, mammal and human since 1960s (Walker *et al*., 2001; and Cunningham, Cunningham and Woodworth, 2007). As a result, OCPs were banned in many countries including Thailand since 1980s (Thirakhupt *et al.*, 2006). In addition, many countries have agreed on the restriction of manufacturing, selling and using of OCPs following the Stockholm Convention on Persistent Organic Pollutants (POPs) in 2001 (NIP/POPs, 2005).

Due to the persistence of the organochlorine pesticides, there have been many reports on the low level contamination of OCPs in most, if not all, environmental matrices even though the use of those OCPs had been banned in the area. The recent studies have suggested that even the low level of OCP residues (part per billion, ppb) may interfere with structure or function of endocrine system and cause adverse effects to animal reproduction and development at the level of progeny and populations (Damstra *et al*., 2002; Guillette *et al*., 1994 and Guillette *et al*.,1996). The endocrine disruption has been reported in both wildlife observation and laboratory experiments. The increased incidence of endocrine-related health problem in human also suggests that human population may be at risk from these chemicals as well.

Although the OCPs had been banned in Thailand since 1980s, residues of these chemicals are still detectable in Thai aquatic and terrestrial ecosystems (Siriwong, 2006 and Keithmaleesatti, 2007a). It is thus important to examine the extent of contamination and the potential reproductive effects on the long-lived animals living in the area with prior history of the OCP uses.

In this study, the snail-eating turtle *Malayemys macrocephala* is used as a sentinel for OCP contamination in ecosystem because it is a long-lived vertebrate with a homerange that overlap the agricultural area with history of OCP uses (i.e. rice-field habitat). *M. macrocephala* is a common freshwater turtle that widely distributes in Thailand, especially in the central agricultural plain of Thailand (Srinarumol, 1995). The long life span and

high position in trophic level of the turtles make them susceptible to long term bioaccumulation of OCPs in the body parts including their eggs.

Since the information on nesting, reproductive and developmental biology of the snail-eating turtle in Thailand is still limited, a study on these aspects was initially carried out to provide standard basis on normal values of this species. The parameters of interest include nest characteristics, clutch size and egg characteristics. A morphometric study was carried out to develop a novel non-invasive sexing technique to be used for juvenile turtle. Effects of incubating temperature on sex ratio of turtles were also studied at three different temperatures in a controlled environment in laboratory.

Next, analyses for organochlorine pesticide residues were performed on nest soil, blood and eggs of *M. macrocephala* in order to examine the extent of OCPs contamination in rice-field habitats of the Chao Phraya river basin. At this stage, OCP residue analysis in turtle eggs was performed as complete clutch to provide information on variation in maternal transfer of OCP residues into eggs in the clutch.

Finally, analyses of OCP residues were performed in representative of turtle eggs collected from two areas with intensive agricultural activities in the lower Chao Phraya river basin. Further information on hatching success, survival rate, deformities and sex ratio were gathered from the remaining eggs subjected to temperature-controlled incubation. Then, the association between levels of OCPs and the reproductive and developmental success of the turtles were assessed.

1.2 Objectives

1. To study some reproductive biology and nesting ecology of the snail-eating turtle *M. macrocephala* in the lower Chao Phraya river basin, Thailand.

2. To examine the organochlorine pesticide contamination in some tissues of the snail-eating turtle in agricultural areas in the lower Chao Phraya river basin, Thailand.

3. To assess the association of organochlorine pesticide contamination and some reproductive effects on the snail-eating turtle.

4. To evaluate the ecological risk of organochlorine pesticide contamination on reproductive health and developmental success of the snail-eating turtle.

1.3 Hypothesis

Some organochlorine pesticides that had been used in the lower Chao Phraya river basin leave their residues as low level contaminants in eggs and blood of the snail eating turtle *M. macrocephala*. This low level contamination affects the reproductive health of the turtle including hatching success, developmental abnormalities, sex ratio and survival of the neonate turtle.

CHAPTER II

LITERATURE REVIEWS

2.1 Organochlorine pesticide

Organochlorine pesticide (OCP) is a large group of synthetic chemicals with considerable diversity of structure, property and usage. They are stable organic compounds of very low water solubility and high lipophilicity. Some of them are highly persistent in their original forms or as stable metabolites and are considered acting as environmental hormones, which disrupt the reproductive cycle of humans and wildlife (Colborn and Smolen, 1996).

Most OCPs including dichloro-diphenyltrichloroethane (DDT), aldrin, dieldrin, endrin, chlordane, heptachlor, hexachlorobenzene, mirex and toxaphene are classified as persistent organic pollutants (POPs) according to Stockholm Convention. The use of these compounds for agriculture, public health and other purposes had been banned because of the hazards to human health and to the environment. However, some of these chemicals are still frequently used or used on a limited scale in some developing countries (Thirakhupt *et al*., 2006; NIP/POPs Coordination, 2005).

2.1.1 Organochlorine pesticide groups

Organochlorine pesticides have been identified by carbon ordering of their molecular structures and have been divided into three groups (Thirakhupt *et al*., 2006):

(1) Diphenyl aliphatic group

Diphenyl aliphatics include compounds such as dichloro-diphenyltrichloroethane (DDT) and its related compounds, methoxychlor and dicofol. The insecticidal properties of DDT were discovered by Paul Muller of Ciba-Geigy in 1939. DDT is one of the more well known insecticides and was mainly used for vector control during World War II. It came to be widely used thereafter for the control of agricultural pests, vectors of disease (e.g. malarial mosquitoes) and ectoparasites of farm animals including industrial and household insect pests. Because of its low water solubility, DDT has been formulated as an emulsifiable concentrate for application as a spray. DDT has an acute oral LD_{50} of 250 mg/kg for rats and is considered to be moderately toxic to vertebrates. It has been shown to cause eggshell thinning in some sensitive species of birds at very low doses and has estrogenic effects that can cause endocrine disruption in animals (Colborn, vom Saal and Soto, 1993). Kelthane (dicofol) is an example of a pesticide related in structure to DDT which has been marketed as an acaricide. Kelthane has weak insecticidal activity with limited persistence, but there is evidence that it may also act as an endocrine disruptor in vertebrates.

(2) Chlorinated cyclodienes group

Chlorinated cyclodiene insecticides were introduced during the 1950s. Some of them have both high toxicity to vertebrates and marked biological persistence thus, giving rise to some serious environmental problems. Chlorinated cyclodienes are synthesized by the Diels-Alder reaction. Aldrin, dieldrin, and heptachlor are examples of cyclodiene insecticides with acute oral LD_{50} for rats of about 40-60

mg/kg. Chlordane is a similar chemical, but is of lower vertebrate toxicity. Endrin and, to a lesser extent, endosulfan are of very high vertebrate toxicity, but limited biological persistence. In general, the cyclodienes resemble DDT in being stable lipophilic solids of very low water solubility, but differ from it in their mode of action. Endosulfan is an exception to this rule having appreciable water solubility. Cyclodienes were introduced into the western countries during the 1950s and were used in diverse formulations for many different purposes. Because of their water insolubility, emulsifiable concentrates and wettable powders were the formulations normally used for spraying. Sprays were used to control certain crop pests and to control vectors to prevent spread of diseases. Cyclodienes were also used in dips and sprays to control ectoparasites of livestock and were widely used as seed dressings for cereals and other crops. The use of aldrin, dieldrin and heptachlor for the latter purpose has caused very serious ecological consequences through food chains and food webs including contamination in soil, water and groundwater.

(3) Hexachlorocyclohexanes (HCH) group

HCH has similar properties to other organochlorine insecticides, but it is 100 times more polar and water soluble than DDT. HCH is classified into alpha, beta, gamma and delta isomers. Emulsifiable concentrates of HCH have been used for controlling agricultural pests and parasites on farm animals. It has also been used as an insecticidal seed dressing. HCH is moderately toxic to rats (LD_{50} 60-250 mg/kg).

2.1.2 Organochlorine pesticides properties (Thirakhupt *et al*., 2006)

Aldrin and dieldrin are the common names of two structurally similar compounds that were once used as insecticides. They are chemicals made in the laboratory and do not occur naturally in the environment. The scientific name for aldrin is 1,2,3,4,10,10-hexachloro-1,4,4α,5,8,8α-hexahydro-1,4-endo,exo-5,8-dimethanonaphthalene. Technical-grade aldrin is composed of not less than 85.5% aldrin. The trade names used for aldrin include Aldrec, Aldrex, Drinox, Octalene, Seedrin and Compound 118. The scientific name for dieldrin is 1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4α,5,6,7,8,8α-octahydro-1,4-endo,exo-5,8-dimethanonaphthalene. Technical-grade dieldrin is composed of not less than 85% dieldrin. The trade names used for dieldrin include Alvit, Dieldrix, Octalox, Quintox and Red Shield.

Pure aldrin and dieldrin are white powders while technical-grade aldrin and dieldrin are tan powders. Aldrin and dieldrin slowly evaporate in the air. Aldrin evaporates more readily than dieldrin. Both aldrin and dieldrin have mild chemical odors. Residues have been found in soil, water and buildings where these compounds were used to kill termites. They also have been found in plants and animals near the hazardous waste sites.

Aldrin and dieldrin can enter the environment from accidental spills or leaks from storage containers at waste sites. In the past, aldrin and dieldrin were disseminated into the environment when farmers used these compounds to kill crop pests and when exterminators used them to kill termites. Aldrin and dieldrin are still present in the

environment from these past uses. Sunlight, other physical factors, and microorganisms in the environment can convert aldrin to be dieldrin. Therefore, dieldrin can be found in places where aldrin was originally released. Dieldrin in soil or water is degraded very slowly. It sticks to the soil and may exist there unchanged for many years. Water does not easily wash dieldrin off soil. Dieldrin does not dissolve in water very well and is, therefore, not found in water at high concentrations. Most dieldrin in the environment has attached to soil and to sediments at the bottom of lakes, ponds and streams. Dieldrin can migrate long distances by attaching to dust particles which can then be transported by the wind. In the air, dieldrin is converted to photodieldrin within a few days. Plants can take up dieldrin from the soil and store it in their leaves and roots. Fish and animals that consume dieldrin-contaminated materials store a large amount of the dieldrin in their fat tissue. Many carnivorous animals have level of dieldrin in their fat tissues many times higher than herbivorous animals.

Hexachlorocyclohexane (HCH) also known as benzene hexachloride (BHC), is a synthetic chemical that exists in four chemical forms called isomers. The different isomers are named according to the position of the hydrogen atoms in the structures. One of these forms, gamma-HCH (or γ-HCH, commonly called Lindane), is produced and used as an insecticide on fruit, vegetables, and forest crops. It is also used as a topical treatment for head and body lice and also for scabies mites which cause contagious skin diseases. It is a grayish or brown amorphous solid which vaporizes approximately 100 times faster than DDT. The vapor is colorless and has a slight musty odor. The substance has not been produced in the United States since 1976. However, imported γ-HCH is available in the United States for insecticide use as

dust, powder, liquid and concentrate. It is also available as a lotion, cream and shampoo to control scabies mites and head lice.

Technical-grade HCH, a mixture of several chemical forms of HCH, consists of about 10-15% γ-HCH as well as the alpha (α), beta (β), delta (δ), and epsilon (ε) forms of HCH. α-, β-, γ- and δ- HCH have been found in the soil and surface water at hazardous waste sites. In the air, the different forms of HCH can be present as a vapor or attached to small particles such as soil and dust; the particles may be removed from the air by rain. γ-HCH can remain in the air for almost 17 weeks depending on moisture in the air and temperature. In soil, sediment and water, it is broken down to less toxic substances by algae, fungi and bacteria. Generally, HCH isomers are broken down quickly in water; in natural water samples, γ-HCH does not remain for much longer than 30 days. γ-HCH is not usually found in drinking water. The persistence time of the HCH isomer in soil is not known.

Chlordane is a man-made chemical that was used as a pesticide in the United States from 1948 to 1988. It is sometimes referred to by the trade names Octachlor® and Velsicol 1068®. Chlordane is a thick liquid with color ranging from clear to amber depending on its purity. It may have no smell or a mild irritating smell. Chlordane is not a single chemical, but a mixture of many related chemicals of which about 10 are major components. Some of the major components are trans-chlordane, cis-chlordane, beta-chlordene, heptachlor and trans-nonachlor. Chlordane does not dissolve in water. Therefore, before it can be used as a spray, it must be placed in water with emulsifiers (soap-like substances) which results in a milky-looking mixture. From 1983 until 1988, chlordane's approved use was to control household pests such as

termites in buildings. The pesticide was applied underground around the foundation of the buildings. When chlordane was used in the soil around a house, it killed termites that came in contact with it. When used as a pesticide on crops, lawns and gardens, and also to control termites in houses, chlordane enters the environment. In the soil, it attaches strongly to particles in the upper layers of soil and is unlikely to enter into groundwater. If degradation occurs, the process is very slow. Chlordane is known to remain in some soils for over 20 years. Persistence is greater in heavy, clayey or organic soil than in sandy soil. Most chlordane is lost from soil by evaporation. Evaporation is more rapid from light sandy soils than from heavy soils. Half of the chlordane applied to the soil surface may evaporate in a few days. Evaporation is much slower after chlordane penetrates into the soil. In the water, some chlordane attaches strongly to sediment and particles in the water column and some is lost by evaporation. It is not known whether much degradation of chlordane occurs in water or in sediment. Chlordane is degraded in the atmosphere by reacting with sunlight and with some chemicals. However, it is sufficiently long lived that it may travel long distances and be deposited on land or in water far from its source. Chlordane or the chemicals that chlordane converted to have accumulated in fish, birds and mammals. It has persisted in the environment for many years and is still found in food, air, water and soil. Chlordane is still commonly found in some form in the fat tissues of fish, birds mammals and almost all humans.

DDT (1,1,1-trichloro-2,2-bis (*p*-chlorophenyl) ethane) is a pesticide that was once widely used to control insects on agricultural crops and insects that carry diseases like malaria and typhus, but is now used in only a few countries to control malaria. Technical-grade DDT is a mixture of three forms *p,p'*-DDT (85%), *o,p'*-DDT (15%) and *o,o'*-DDT

(trace amounts). All of these are white, crystalline, tasteless and almost odorless solids. Technical-grade DDT may also consist of DDE (1,1-dichloro-2,2-bis (*p*-chlorophenyl) ethylene) and DDD (1,1-dichloro-2,2-bis (*p*-chlorophenyl) ethane) as contaminants. DDD was also used to kill pests, but to a far lesser extent than DDT.

One form of DDD (*o,p'*-DDD) has been used medically to treat cancer of the adrenal gland. Both DDE and DDD are degraded products of DDT. Most DDT in the environment is a result of past use. DDD was also used as a pesticide to a limited extent in the past. DDT still enters the environment because of its current use in other areas of the world. DDE is only found in the environment as a result of contamination by and degradation of DDT. DDD also enters the environment during the breakdown of DDT. Large amounts of DDT were released into the air and on soil or water when it was sprayed on crops and forests to control insects. DDT was also sprayed in the environment to control mosquitoes. DDT, DDE and DDD may also enter the air when they evaporate from contaminated water and soil. DDT, DDE and DDD in the air will then be deposited on land or surface water. This cycle of evaporation and deposition may be repeated many times. As a result, DDT, DDE and DDD can be carried long distances in the atmosphere. These chemicals have been found in bogs, snow and animals in the Arctic and Antarctic regions, far away from where they were used. Some DDT can enter the soil from waste sites. DDT, DDE and DDD may occur in the atmosphere as a vapor or attach to solid particles in the air. Vapor phases of DDT, DDE and DDD may be broken down in the atmosphere due to reactions caused by sunlight. The half-life of these chemicals in the atmosphere as vapors (the time it takes for one-half of the chemical to turn into something else) has been calculated to be approximately 1.5-3 days.

However, in reality, this half-life estimate is too short to account for the ability of DDT, DDE and DDD to be carried for the long distances reported. DDT, DDE and DDD have lasted in the soil for a very long time. Potentially, they may last for hundreds of years. DDT is broken down slowly to DDE and DDD by the microbial degradation. These chemicals may also evaporate into the air and then be deposited in other places. They stick strongly to soil, and therefore, generally, remain in the surface layers of soil. Some soil particles with attached DDT, DDE or DDD may get into rivers and lakes through runoff. Only a very small amount, if any, will seep into the ground and migrate into groundwater.

The length of time that DDT will last in soil depends on many factors including temperature, type of soil, and whether the soil is wet. DDT lasts for a much shorter time in the tropics where the chemical evaporates faster and where microorganisms degrade it faster. DDT disappears faster when the soil is flooded or wet than when it is dry. DDT also disappears faster when it initially enters the soil. Later on, the evaporation slows down and some DDT moves into small spaces in the soil particles where are very difficult for microorganisms to reach and break it down efficiently. In tropical areas, DDT, DDE and DDD may disappear in much less than a year. In temperate areas, half of a deposit initially present usually disappears in about 5 years. However, in some cases, the half will remain for 20, 30 or more years. In surface water, DDT binds to particles in the water, then settles and is deposited in the sediment. DDT is taken up by small organisms and fish in the water. It accumulates to high levels in fish and marine mammals (such as seals and whales), reaching levels many thousand times higher than existing in the water. In these animals, the highest levels of DDT are found in their adipose tissues. DDT in the bottom sediment can also be absorbed by

some water plants and by the aquatic animals which consume those plants. DDT metabolites can be transported through food webs to top consumers such as humans.

Endosulfan is a man-made insecticide used for control of a number of insects on food crops such as grains, tea, fruits and vegetable; and also on nonfood crops such as tobacco and cotton. It is also used as a wood preservative. Endosulfan is sold as a mixture of two different forms of the same chemical (referred to as alpha- and beta-endosulfan). It is a cream to brown colored solid that may appear crystalline or as flakes. It has a distinct odor similar to turpentine. Endosulfan does not burn. Endosulfan enters the air, water and soil during the manufacturing process or when used as a pesticide. Endosulfan is often applied to crops using sprayers. Endosulfan in the air may travel long distances before it lands on crops, soil or water. Endosulfan on crops generally degrades down within a few weeks. Endosulfan released into the soil usually attaches to soil particles. Endosulfan found near hazardous waste sites is usually found in the soil. Endosulfan in soil evaporates into the air where it is broken down. However, it may persist in soil for several years before being broken down. Rainwater can wash endosulfan that is attached to soil particles into surface water. Endosulfan does not dissolve easily in water. Most endosulfan in surface water is attached to soil particles floating in the water or attached to soil at the bottom. The small amount of endosulfan that dissolves in water degrades over time. Depending on the conditions in the water, endosulfan may be broken down within 1 day or it may take several months. Some endosulfan in surface water evaporates into the air and is degraded. Because it is water insoluble, only very small amounts of endosulfan are found in groundwater (water below the soil surface; for example, well water). Animals inhabiting

endosulfan-contaminated water areas can build up endosulfan in their bodies. The amount of endosulfan in their bodies may be several times greater than that in the surrounding water.

Endrin is a white, solid, almost odorless substance that was used as a pesticide to control insects, rodents and birds. Endrin has not been produced or sold for general use in the United States since 1986. Little is known about the properties of endrin aldehyde, an impurity and the breakdown product of endrin, or endrin ketone, which is a product of endrin when it is exposed to sunlight. Endrin does not dissolve very well in water. It has been found in groundwater and surface water but only at very low levels. It is more likely to cling to the bottom sediment of rivers, lakes and other bodies of water. Endrin is generally not found in the air except when it was applied to fields during agricultural applications. The persistence of endrin in the environment depends highly on local conditions. Some estimates indicate that endrin persist in soil for over 10 years. Endrin may also be broken down by exposure to high temperatures (230 °C) or sunlight to form primarily endrin ketone and endrin aldehyde. However, the amount of endrin is broken down to endrin aldehyde or endrin ketone is very small (less than 5%). It is not known what happens to endrin aldehyde or endrin ketone once they are released to the environment.

Heptachlor is a synthetic chemical that was used in the past for killing insects in homes, buildings and on food crops. It has not been used for these purposes since 1988 in the United States. There are no natural sources of heptachlor or heptachlor epoxide. Trade names for heptachlor include Heptagran®, Heptamul®, Heptagranox®, Heptamak®, Basaklor®, Drinox®, Soleptax®, Gold Crest H-60®, Termide® and

Velsicol 104®. Heptachlor is both a breakdown product and a component of the pesticide chlordane (approximately 10% by weight). Pure heptachlor is a white powder. Technical-grade heptachlor is a tan powder and has a lower level of purity than pure heptachlor. Technical-grade heptachlor is the form of heptachlor used most often as a pesticide. Heptachlor smells somewhat like camphor. It does not burn easily and does not explode. It also does not dissolve easily in water.

Heptachlor epoxide is a breakdown product of heptachlor. It was not manufactured and was not used as an insecticide like heptachlor. Like pure heptachlor, heptachlor epoxide is a white powder that does not explode easily. Heptachlor epoxide is a degradation product converted by microorganisms in the environment. When heptachlor enters animal and human bodies, it will be metabolized to heptachlor epoxide. This profile describes these two chemicals together because about 20% of heptachlor is changed within hours into heptachlor epoxide in the environment and in the human body.

Heptachlor or heptachlor epoxide can be found in the soil and the air around buildings treated for termites. It has dissolved in surface water or groundwater, or evaporated into the air near hazardous waste sites. Heptachlor or its by-product, heptachlor epoxide, could be found in plants and animals near hazardous waste sites. Although heptachlor is no longer used to kill insects on crops or in homes and buildings, it is still approved by US EPA for killing fire ants inside power transformers.

Heptachlor entered the soil and surface water when farmers used it to kill insects in seed grains and on crops. It also entered the air

and soil when professional insect exterminators and homeowner used it to kill termites. Heptachlor sticks to soil very strongly and evaporates slowly into the air. It does not dissolve easily in water. Heptachlor epoxide dissolves more easily in water than heptachlor does and evaporates slowly from water. Like heptachlor, heptachlor epoxide also sticks to soil. Both heptachlor and heptachlor epoxide can travel long distances in the wind from places where they are released such as treated fields or manufacturing sites. In soil and water, heptachlor is degraded by microorganisms to be more harmful substance such as heptachlor epoxide. Heptachlor in the soil can be taken up by plant roots. Heptachlor in the air can be deposited on plant leaves. Heptachlor epoxide is broken down very slowly in the environment. It can exist in the soil and the water for many years. Animals that consume plants containing heptachlor absorb and convert heptachlor to heptachlor epoxide in their bodies. Both heptachlor and heptachlor epoxide are accumulated in fish and in cattle and humans. Heptachlor epoxide accumulates in their fat tissues. Some studies show that heptachlor epoxide can exist in fat tissues for 3 years after exposed. Most of the degradation products of heptachlor are thought to be less harmful than heptachlor itself. However, in laboratory studies in animals, heptachlor epoxide is more harmful than heptachlor.

Methoxychlor also known as DMDT, Marlate® or Metox®, is a manufactured chemical now used in the United States for controlling insects. Methoxychlor is effective against flies, mosquitos, cockroaches and a wide variety of other insects. This insecticide is used on agricultural crops and livestock, including animal feed, barns and grain storage bins. Some pesticide products that consist of methoxychlor are used for controlling insect pests in gardens or on pets.

Pure methoxychlor is a pale-yellow powder that has a slightly fruity or musty odor. It does not readily evaporate into air or dissolve in water. Pest control operators usually dissolve methoxychlor in a petroleum-based liquid and apply it as a spray, or they mix it with other chemicals and apply it as a dust. Application of methoxychlor as an insect killer accounts for most of the methoxychlor that enters the environment. Since the use of methoxychlor depends on cultivating season, the amount that is released to the environment tends to be greater during pest control period. Some methoxychlor is released to the environment from chemical plants where methoxychlor is made or from manufacturing sites that formulate products consisting of methoxychlor. A small amount may also be released from hazardous waste sites of where it has been disposed.

Methoxychlor does not occur naturally in the environment. Most methoxychlor enters the environment when it is applied to forests, agricultural crops, and farm animals. It can be applied to forests and crops by aerial spraying. This process causes methoxychlor contamination of nearby land and water. Methoxychlor released into the air will eventually settle to the ground although some may travel long distances before settling. Rain causes methoxychlor to settle to the ground more quickly.

Once methoxychlor is deposited on the ground, it binds to the soil. This makes methoxychlor move slowly from one place to another place. However, soil particles that combined with methoxychlor can be blown by the wind or be carried by rainwater or melting snow into rivers or lakes. Most methoxychlor exists in the outermost top layer of soil, but some of the breakdown products may move deeper into the ground. Smaller amounts of methoxychlor in the air may settle directly

into rivers, lakes, and other surface water. Once methoxychlor is in the water, it usually binds to sediment or organic matter and settles to the bottom.

Methoxychlor is broken down in the environment by several processes. However, these processes are slow and may take months. In the soil, some methoxychlor is broken down by bacteria and other microorganisms, and some is broken down by reactions to water or materials in soil. In air and water, some methoxychlor is usually broken down by sunlight. Methoxychlor is also broken down by reactive chemicals normally present in the air. Some of the breakdown products are capable of producing harmful effects similar to those effects caused by exposure to methoxychlor, for example, exhibit estrogenic activity in animals.

Methoxychlor can accumulate in some living organisms including algae, bacteria, snails, clams and some fish. However, most fish and animals convert methoxychlor into other substances that are rapidly released from their bodies, thus methoxychlor does not usually build up in the food chain.

Toxaphene known as camphechlor, chlorocamphene, polychlorocamphene or chlorinated camphene is usually found as a solid or gas. In its original form, toxaphene is a yellow to amber waxy solid that smells like turpentine. It does not burn and evaporates when in solid form or when mixed with liquids. Toxaphene was used to control insect pests on cotton and other crops. It was also used to control parasites on livestock and to kill unwanted fish in lakes.

Toxaphene enters the environment after it is applied to a crop or poured into a lake. Toxaphene can enter the air (by evaporation), the soil (by sticking to soil particles) and the water (from runoff after rains). It may also enter the environment from hazardous waste sites or when it accidentally spills or leaks during storage or transportation. It does not dissolve well in water, so it is more likely to be found in air, soil or the sediment at the bottom of lakes and streams. If toxaphene is found in surface water or groundwater, it is usually at very low levels. Once toxaphene is in the environment, it can last for years because of slow degradation. This indicates that there is still a chance of being exposed to toxaphene in the United States and many countries including Thailand even though it has not been widely used for over 10 years. Because toxaphene is broken down slowly, exposure could probably be to the original material.

Toxaphene levels may be high in some predatory fish and mammals because of accumulation in the fat tissues. For example, when a raccoon eats a contaminated fish, some of the toxaphene in the fish is transferred to the raccoon. The more contaminated fish the raccoon eats the more toxaphene it acquires. This implies that even when toxaphene levels are low or confined to a certain area, they could be high in individual animals.

2.2 Snail-eating Turtle (*Malayemys macrocephala*)

2.2.1 Taxonomic and conservation status

In Thailand, there are 6 families with 28 species of turtles. Thirteen species of freshwater turtle is classified in Family Bataguridae

or Geoemydidae (Thirakhupt, 2000 and Bonin, Devaux and Dupré, 2006). The snail-eating turtle can be classified as follows:

Kingdom Animalia
Phylum Chordata
Class Reptilia
Order Chelonia (Testudines)
Family Geoemydidae (Bataguridae)
Genus *Malayemys*
Species *Malayemys macrocephala*

The turtle in genus *Malayemys* (Lindholm, 1931) is endemic, native and common in Thailand. It has also been reported in mainland of Southeast Asia such as in Cambodia, Vietnam and Malaysia (Srinarumol, 1995). According to a report by Brophy (2004) on geographic variation and systematics of the turtle in genus *Malayemys*, the genus can be classified into 2 groups including *M. subtrijuga* (Schlegal and Müller, 1844) and *M. macrocephala* (Gray, 1859). Although both species are native to Southeast Asia, *M. subtrijuga* restricts its distribution in the Mekong River Basin, while *M. macrocephala* distributes in the Chao Phraya and Mae Klong River Basins, coastal area of south-eastern Thailand and the Malay Peninsula.

Since the revised systematics of *Malayemys* is relatively recent (Brophy, 2004), most of the information on *Malayemys* turtle is usually referred to the species *subtrijuga* (Wermuth and Mertens, 1961, Taylor, 1970 and Bonin *et al*., 2006). The common name and local name of *Malayemys* turtle included Tao Na (Thai), Rua Ba Go (Vietnamese), Tao Saam San (Lao), Andoeuk Sakal (Khmer), Jelebu

Siput (Bahasa Malaysia) and Kura-Kura Pemakan Siput (Bahasa Indonesia) (Stuart, Van Dijk and Hendrie, 2001).

M. subtrijuga is protected by CITES Appendix II and the Wild Animals Reservation and Protection Act B.E. 2535 of Thailand. International Union for Conservation of Nature and Natural Resources (IUCN) classified it as a threatened vulnerable species (IUCN, 2007). However, *M. macrocephala* has not yet been protected by any national and international law.

2.2.2 Description

Bonin *et al.* (2006) described the characteristics of the turtle in genus *Malayemys* as follows. The carapace is oval and modestly domed, and three discontinuous carapacial keels form a small knob on each of the larger scutes. The median keel extends the length of the five vertebral scutes, whereas the lateral keels rarely reach as far as the fourth costals. The carapace length is less than 200 mm. The posterior marginals are not serrated. The dark to light brown color of carapace tends toward chestnut, and a fine yellow line borders the carapace. The small knobs on the keels are darker than the rest of the carapace. The plastron is unhinged and narrower than the carapace with a strong anal notch. The posterior lobe is shorter than bridge. A large dark brown blotch is present on each of the plastral scutes, and two more dark blotches lie over the bridges. The head is rather large and is black, with several light or even whitish stripes. The first stripe extends from the nostrils and passes over the eyes on its way to the neck. Another extends from each corner of the snout and curves downward to pass below the eye and rejoin the neck. Two more light bands cross the tympana behind

the eyes. A large scale covers the crown of the head, and the area posterior to this is covered with numerous small scales. The limbs and the tail are grey to black, with a yellow edge on the outer borders.

2.2.3 Habits and habitats

The turtle in genus *Malayemys* was found in wetland habitat including estuarines, swamps, river and rice-fields (Srinarumol, 1995 and Thirakhupt, 2000). Additionally, Bonin *et al.* (2006) reported that this turtle may be found in a muddy bottom and abundant aquatic vegetation zone and it is also often found on land. It is carnivorous and eats large numbers of small snails (hence the vernacular name), earthworms, aquatic insects, crustaceans and small fish. In Thailand, the snail-eating turtle is a common freshwater turtle that widely distribute in Thailand, especially in the central agricultural plain (i.e. rice-field) of Thailand (Srinarumol, 1995).

2.2.4 Reproductive and nesting biology

M. macrocephala (previously reported as *M. subtrijuga*) could lay 3-6 eggs per clutch (Srinarumol, 1995) to 5-10 eggs per clutch (Nutphand, 1979). The incubation period at 26-32 ºC was 97-292 days; the hatching success was 38.89% and the 5-month survival rate was 65% (Srinarumol, 1995). The dimension of *M. subtrijuga* (potentially *M. macrocephala*) eggs was 40-45 x 20-25 mm. (Smith, 1931) or 44 x 22 mm. (Ewert, 1979).

2.3 Ecotoxicology of organic contaminants in reptiles

2.3.1 Contaminant residues of organochlorine pesticides in reptilian population.

Reports on body burden of the organochlorine pesticides (OCPs) and their reproductive effects in non-target organisms, especially birds, during 1960s had been reviewed in a book entitled "Silent Spring" by Carson (1962). In 1980, Hall published a review of the levels and effects of OCPs in reptiles, whereas an overview of contaminant included OCPs, Polychlorinated Biphenyls (PCBs), Polychlorinated dibenzodioxins (PCDDs) and Polychlorinated dibenzofurans (PCDFs) and effects in turtles was published by Meyers-schöne and Walton (1994). Most studies are isolated reports of residue levels in reptiles and have been conducted primarily in North America. There were few long-term studies of contaminant levels in reptiles (Portelli and Bishop, 2000).

(1) Freshwater turtles and Marine turtles

Turtles are known as potential bioindicators of chemical contamination because of their wide geographic distribution, relatively long life span, and tenancy of a variety of habitats (Meyers-schöne and Walton, 1994 and Meyers-schöne *et al*., 1993). OCPs, PCBs, PCDDs and PCDFs have been determined in turtle tissues that are highly lipid including fat, eggs, liver, testes, brain, heart, kidney, pancreas and lung (Portelli and Bishop, 2000). The turtles in North America that were subjected to analysis for organic chemicals in tissues include snapping turtle *Chelydra serpentina* (Stone *et al*., 1980; Hebert *et al*., 1993 and

Bishop *et al*., 1995), midland painted turtle *Chrysemys picta*, Blanding's turtle *Emydoidea blandingii*, map turtle *Gratemys geographica* and spiny softshell *Apalone spiniferus* (Bishop and Gendron, 1998).

In addition, the pattern of OCPs distribution in sea turtle tissues is similar to that of other turtles, the highest concentrations occurring in fat followed by liver, kidney and muscle (Rybitski *et al*., 1995). The species of sea turtles that have been used for organic contaminant analysis in tissues and eggs include loggerhead sea turtle *Caretta caretta* and green sea turtle *Chelonia mydas* (McKim and Johnson, 1983), Kemp's ridley turtle *Lepidochelys kempi* (Rybitski *et al*., 1995).

(2) Snake

Snake tissues have high lipid content, similar to turtle tissues (Portelli and Bishop, 2000). Two species of water snakes *Nerodia rhombifera* and *N. cyclopion* were studied for OCPs in fat and muscle (Sabourin *et al*., 1984). In addition, blotched water snakes *Natrix erythrogaster*, common water snakes *Natrix sipedon*, ribbon snakes *Thamnophis proximus* and cottonmouths *Agkistrodon piscivorus* from the contaminated Brazos area possessed similarly high levels of OCPs in their fat (Fleet, Clark and Plapp, 1972). In the Great Lakes, Meeks (1968) reported that a DDT residue is found in the fat of water snake *Natrix sipedon*.

(3) Lizards

Studies on organic contaminant in lizards were mainly focused on OCP residues. Culley and Applegate (1967) detected DDE residue in tail muscle and egg of whiptail lizards *Gnemidophorus* spp. White and Krynitsky (1968) reported high levels of DDE in whiptail lizards at Rio Grande and Pecos River, New Mexico and Texas. Lambert (1997) used lizards and frogs as bioindicators of contamination in the Hargesia, Somaliland.

(4) Alligator and Crocodile

Eggs of alligator and crocodile have been used in several studies on organic residues. The famous case is concentration of chlorinated hydrocarbon in American alligator *Alligator mississippiensis* eggs from various lakes in Florida in 1984 and 1985 (Heinz, Percival and Jennings, 1991). In addition, eggs of Nile crocodile *Crocodylus niloticus* from Zimbabwe was also used to detect chlorinated hydrocarbon (Phelps *et al*., 1986).

2.4 Endocrine disruptor

Endocrine disruptor is a synthetic or natural chemical that can mimic or block the sex hormone or otherwise interfere with normal hormone activity, often at extremely small dose. The chemicals that are known as endocrine disrupting chemicals (EDCs) include OCPs such as aldrin, chlordane, DDT and derivative and polychlorobiphenyls (PCB). Moreover; some heavy metal (e.g. cadmium, lead) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD) are also classified as

endocrine disrupting chemicals. Example of OCPs that can exert endocrine disrupting properties and their reported effects are shown in Table 2.1 (California PSR and CALPIRG, 1998).

Table 2.1: Hormone disrupting effects of organochlorine pesticides

Chemicals	Effects
DDT and metabolite	Androgen antagonist
Methoxychlor	Estrogenic; metabolite interferes with sexual development, reproduction, and behavior of bird and mammals Increases aggressive behavior (mouse) (DDT, methoxychlor on day 11-17 of pregnancy)
Endosulfan	Binds to the estrogen receptor and, in cell cultures, stimulates the growth of estrogen-sensitive breast cancer cells
Lindane	Accumulates in the ovarian follicles, fallopian tubes, and uterus of test animal; Most investigators conclude that lindane has anti-estrogenic properties.
Dicofol	Causes feminization of male embryos, abnormal submissive behavior in male offspring, and impaired reproductive success (birds); contaminated with DDT; strongly competes for binding site of the thyroid hormone.

There are at least five mechanisms by which environmental contaminants are able to disrupt vital functions of the endocrine system (Keith, 1997).

1. Some contaminates are similar enough in structure to hormones that they are able to bind to cellular receptors designed to be targets for natural hormones. This causes unpredictable and abnormal cell activity.

2. Other contaminants appear to block these binding sites so that hormones are unable to bind them, thus impairing normal cell activity.

3. Some contaminants induce the creation of extra receptor sites in the cell. The consequence can be an amplification of the impact of hormones on cell activity.

4. Contaminants can interact directly and indirectly with natural hormones, changing the hormones message and thus altering cell activity.

5. The natural pattern of hormones synthesis can be disrupted by contaminants, resulting in an improper balance or quantity of circulating hormones.

Apparently some contaminants can trigger the mixed function oxidase system (MFO), which is responsible for the production of enzymes involved in the biosynthesis of sex steroids. In addition, the MFO produces enzymes that influence the synthesis of new hormones and the breakdown of hormones. These enzyme activities control the level of sex hormones circulation in the body. By inducing the MFO to produce the controlling enzymes, contaminants are able to drastically alter the level of sex hormones in the body (Keith, 1997).

2.4.1 Endocrine disruption in reptiles

Many chlorinated hydrocarbons interfere with hormonal and endocrine function in wildlife. The findings in reptiles involved the case of declining population of the *Alligator mississippiensis* at Lake Apopka, Florida, USA (Guillette *et al.*, 1994 and Cunningham *et al.*, 2007). The

surveys showed that 90 percent of the alligator eggs laid in each year was infertile, and only about half of the few that hatched survived for more than two weeks. Male hatchlings had shrunken penises and unusually low level of testosterone. Female alligator had highly elevated estrogen levels and abnormal ovaries. The potential cause of these abnormalities seems to be the DDT spill in the lake during 1980s, along with pesticide-laden runoff from the adjacent farms. These led to a high level of DDE, a persistent metabolite of DDT, in the alligator tissue and eggs. The similar findings were also reported in the Florida red-belly turtle *Chrysemys nelsoni* (Guillette *et al.*, 1994).

Data on endocrine disruption in reptiles in the field and laboratory, although limited to only alligators and turtles, have provided important supports for the endocrine-disruptor hypothesis (Guillette, 2000). The modern-use pesticides including dicofol and atrazine are presents some affinity for the alligator estrogen receptors (aER) (Vonier *et al.*, 1996). The molecular data suggest that potential environmental estrogens are present in reptilian eggs. Furthermore, the data indicate that the chemicals are mimicry or inhibitor of hormone in reptiles (Guillette, 2000).

Many reptiles including turtles and crocodiles are reported to have temperature-dependent sex determination (Valenzuela and Lance, 2004). This environmental influence can be overcome, through male to female sex reversal, by treating eggs with estrogen. Genetic factors associated with sex determination and thus gonadal development, in reptiles are present and are still under intense study. But the role of temperature and sex steroid has been established in a number of species. Alligators and several turtle species exhibit male-to-

female sex reversal if developing embryos are exposed to an estrogenic compound during a specific period of development, usually the second third of the embryonic period. A single pulse of estradiol-17β (E_2) given to alligator or turtle eggs incubated at male-producing temperatures can induce the development of apparently normal females. Intersex individuals are produced when the concentration of an estrogenic compound is below a specific threshold. That is, with intermediate treatment levels, an embryo could exhibit female Müllerian ducts and a testis or even an ovotestis (Guillette, 2000).

Bergeron, Crews and McLachlan (1994) showed that treatment on eggs of the red-eared slider turtle *Trachemys scripta* with less than 9 ppm (100 µg) of 2',4',5'-trichloro-4-biphenylol resulted in 100% sex reversal whereas treatment with 2',3',4',5'-tetrachloro-4-biphenylol stimulated total sex reversal in 50% of the embryos and partial sex reversal (intersex) in 21% of the embryos. Importantly, they also showed that mixtures of these PCBs can actually produce a synergistic response. In other words, greater sex reversal was achieved at lower exposure concentration when two PCBs were mixed together. However, treatment of the sea turtle *Chelonia mydas* with p,p'-DDE less than 543 ng/g did not induce sex reversal at the dose examined (Podreka *et al.*, 1998).

CHAPTER III

NESTING BIOLOGY, SEXUAL SIZE DIMORPHISM AND EFFECTS OF INCUBATING TEMPERATURE ON DEVELOPMENT OF THE SNAIL-EATING TURTLE *Malayemys macrocephala* FROM THE LOWER CHAO PHRAYA RIVER BASIN, THAILAND

3.1 Introduction

In Thailand, there are 6 families with 28 species of turtles. The turtle in genus *Malayemys* (Lindhom, 1931) is endemic, native and common in Thailand. Its distribution has also been reported in mainland of Southeast Asia such as Cambodia, Laos, Vietnam and Malaysia (Srinarumol, 1995). It was found in wetland habitat including swamps, estuarine, river and rice-fields (van Dijk and Thirakhupt, 1994 and Srinarumol, 1995). Bonin, Devaux and Dupré. (2006) reported that this turtle may be found in a muddy bottom and aquatic vegetation zone and also often found on land. It is carnivorous and eats large numbers of small snails (hence the vernacular name), aquatic insects, earthworms, crustaceans and small fish.

Although *M. macrocephala* is a native and common turtle in Thailand, the information on reproductive biology such as nesting ecology, clutch size, egg size and hatching success is still lacking. A few previous reports showed that *M. macrocephala* (previously reported as *M. subtrijuga*) could lay 3-6 eggs per clutch (Srinarumol, 1995) to 5-10 eggs per clutch (Nutphand, 1979). The dimension of *M. subtrijuga*

(potentially *M. macrocephala*) eggs was 40-45 x 20-25 mm (Smith, 1931) or 44 x 22 mm (Ewert, 1979). The incubation period at 26-32 ºC was 97-292 days; the hatching success was 57.14% and the 5-month survival rate was 65% (Srinarumol, 1995).

Sexual dimorphism is a condition in which the males and females in a species are different in morphological traits such as coloration, size or other features (Bury, 1979). Patterns of sexual size dimorphism are correlated with habitat type and male mating strategy (Berry and Shine, 1980). In aquatic species, males are usually smaller than females, possibly due to the small size in males evolves to increase mobility, or because selection for increased fecundity may result in increased female size. In adult *M. subtrijuga* (potentially *M. macrocephala*), Srinarumol (1995) reported that adult male turtle is significantly smaller than the female. Comparison on width of tail base in relation to carapace length showed that males have significantly larger tail than the female. In juvenile turtle, however, the sex is not obviously externally dimorphic rendering problem in sex identifying of hatching or juvenile turtles (Valenzuela *et al.*, 2004).

In reptiles, incubation temperature is an important ecological factor influencing sex determination and embryonic development (Zhu, *et al*. 2006). Majority of reptiles, especially turtle, exhibited a temperature-dependent sex determinations (TSD) in which sex of hatchlings depend on temperature of the nest during incubation (Bull and Vogt, 1979; Georges, 1988; Booth *et al*., 2004 and Valenzuela and Lance, 2004). TSD patterns in turtle are different among species. From 79 species of turtle examined through incubation at controlled temperatures, 64 turtles have TSD and 15 have genotypic sex

determination (GSD). However, sex determination pattern of the majority (more than 70%) of 257-280 turtle species, including *M. macrocephala*, remains untested (Ewert, Etchberger and Nelson, 2004).

In this study, study on nesting biology of the snail-eating turtles *M. macrocephala* from the lower Chao Phraya River Basin was carried out. The parameters of interest include nest characteristics, clutch size and egg characteristics. A morphometric study was carried out to develop a non-invasive technique to identify sex of the juvenile turtle. Next, effects of incubating temperature on sex ratio as well as hatching success and growth and survival of juvenile turtles were studied at three different temperatures in a controlled environment in laboratory.

3.2 Materials and Methods

3.2.1 Study area and study period

Field surveys were conducted in three nesting seasons of *M. macrocephala* including Season 1 (November 2005 to March 2006), Season 2 (December 2006 to March 2007) and Season 3 (December 2007) at Bang Ban district, Phra Nakhon Si Ayutthaya province, central part of Thailand (100 km north of Bangkok; Figures 3.1).

3.2.2 Characteristics of the snail-eating turtle nest

Nests of turtles at the study site were located by visual encountered surveys. Upon locating the freshly laid egg nest (Figure 3.2), nest dimension (length, width and depth) was measured with metric ruler. Ambient and nest temperature and relative humidity were measured

with digital thermo-hygrometer. In addition, temperature on the eggshell surface was recorded with infrared digital thermometer.

Number of eggs in the nest was recorded as clutch size. Every eggs was labeled by pencil and removed from the nest. The eggs were transported in a styrofoam box with cushion to the laboratory at the Department of Biology, Faculty of Science, Chulalongkorn University, Bangkok, Thailand until further use. Nest media, i.e. soil from the bottom of the nest, were collected and analyzed for characteristics of soil particles (performed by the Land Development Department, Ministry of Agriculture and Cooperatives).

Figure 3.1: Study area (★) at Bang Ban district, Phra Nakhon Si Ayutthaya province, Thailand.


Figure 3.2: Nest of the snail-eating turtle at Bang Ban district, Phra Nakhon Si Ayutthaya province, Thailand.

3.2.3 Characteristics of the snail-eating turtle eggs

Eggs were measured for length and width by vernier caliper (Mitutoyo, Japan) and weight by an electronic balance. Eggs collected in Season 3 (December 2007; n=22) were used as representative for eggshell thickness measurement. Eggs were opened and cut into small piece at five positions (Figure 3.3). Eggshell pieces were fixed on glass slide and examine under a light microscope. Thickness of eggshell piece was measured from the calcareous and membranous layers with an aid of ocular and stage micrometers. Thickness of each egg was report as average thickness of eggshell at 5 positions.

3.2.4 Egg incubation

Upon arrival in the laboratory, eggs were randomly placed in trays containing 1:1 vermiculite: water (weight: volume) and kept in a microprocessor-controlled incubator (Siam Incubator, Thailand Figure 3.4). Tray of water was placed inside the incubator to maintain relative

humidity at above 80%. Temperature and humidity inside the box were monitored using a digital thermo-hygrometer. Every three days, water was sprayed on vermiculite to increase humidity.

Eggs collected in Season 1 were incubated at 27-29 ˚C until hatch. The neonate turtles from this season were used for morphometric study on sexual size dimorphism. Eggs collected from Season 2 were used to study effects of incubating temperature on sex ratio of the neonate turtles. In this experiment, eggs from each clutch were randomly divided into 3 groups (n=39 eggs per group). Eggs in each group were kept in a microprocessor-controlled incubator at 26˚C, 29˚C or 32˚C until hatch. According to Bull, Vogt and McCov (1982) and Wibbels, Bull and Crews (1991) these are male-producing (26˚C), pivotal (29˚C) and female-producing (32˚C) temperatures for most of the freshwater turtle.

Figure 3.3: Positions (1-5) on eggshell that were cut and measured for eggshell thickness.

3.2.5 Sexual size dimorphism and determining of juvenile turtle sex

A morphometric study was carried to examine sexual size dimorphism of the juvenile snail-eating turtle. Thirty four juvenile turtles (age 8-10 months) derived from Season 1 eggs were measured for morphological characteristics by vernier caliper (Mitutoyo). The 26 morphological characteristics (Figure 3.5) included head width, head length, carapace width, straight carapace length, maximum carapace length, nuchal width, nuchal length, plastron width, straight plastron length, right plastron length, left plastron length, right bridge length, left bridge length, humeral width, upper femoral width, lower femoral width, lower anal width, straight upper femoral to lower anal length, straight right upper femoral to lower anal length, straight left upper femoral to lower anal length, upper femoral to right lower anal length, upper femoral to left lower anal length, tail width, precloacal length, postcloacal length and tail length. After measurement, these juvenile turtles were subjected to cold anaesthetized and sacrificed by decapitation. Sex of these juvenile turtles was identified based on the presence of ovary or testis.

Figure 3.4: Egg incubation in a microprocessor-controlled incubator

3.2.6 Effect of temperature on hatching success and survival rate

Incubation period and hatching success were monitored. In this study, the hatching was classified into two groups. The first group is normal hatching when turtle can hatch by itself. The second group is assisted hatching when turtle required assistance after egg piping in order to emerge from eggshell (Figures 3.6).

Hatchlings were kept individually in a plastic box containing dechlorinated tap water and stored in a room with ambient temperature of 25-28 ˚C. After seven days, hatchling turtle were fed with turtle pellets (Tetra Reptomin®) 3 times per week. Water in the plastic box was changed and the box was cleaned on weekly basis. Survival rates were monitored as number of turtle that hatched and survived for one day, seven days, one month to one year. The turtles were measured for width and length of carapace and body weight on monthly basis.

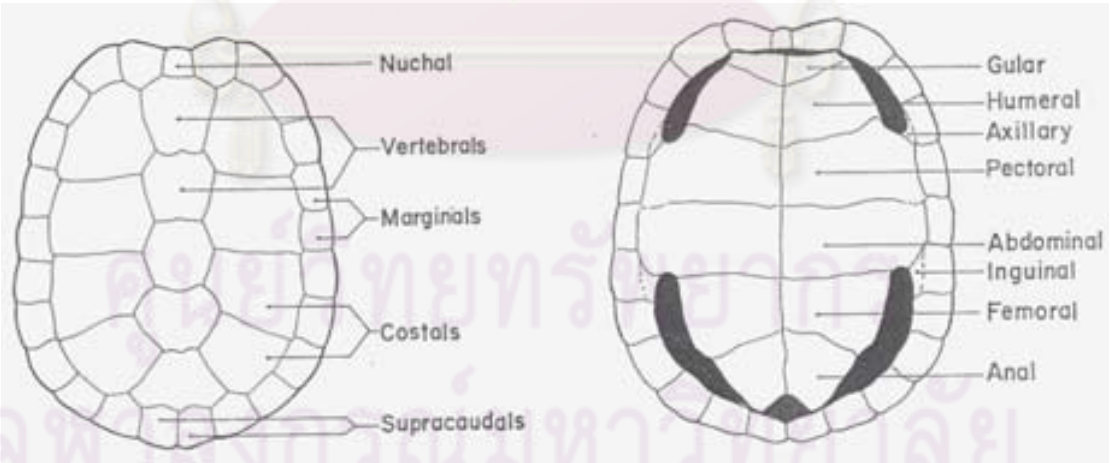

Figure 3.5: Diagram and names of turtle shells referred in the morphometric study (Srinarumol, 1995).

3.2.7 Effect of temperature on sex ratio

The effect of temperature on sex determination of the snail-eating turtle was examined in juvenile turtles hatched from Season 2 eggs. Juvenile turtles were measured for 5 morphological characteristics (see results 3.3.3) by vernier caliper (Mitutoyo). Sex was determined from anatomical examination in dead specimens or predicted from sex determining equation (see result 3.3.3).

Figure 3.6: Turtle with assisted hatching

3.2.8 Statistical analyses

All data were tested for normality using the Kolmogorov-Smirnov test and presented as descriptive statistics (mean and standard error of the mean). In the morphometric study, means of 26 morphological characteristics were determined by independent sample t-test. The morphological traits that showed significant sex-related difference were used in logistic regression analysis. For sex ratio study, *G* statistic for the log-likelihood ratio goodness of fit test (*G* test) was used to determine whether the sex ratio of each temperature group is deviated from the expected sex ratio. A significant difference is

reported at $p \leq 0.05$. Statistical analyses were done using SigmaStat and SPSS for Windows.

3.3 Results

3.3.1 Nest characteristics

The characteristics of snail-eating turtle nests were u-shape hole with cover made from soil and plant materials (Figures 3.7-3.8). Analysis of physical characteristics of the nest soild revealed that soil type of the nest was silt clay (SiC) or clay (C) (Table 3.1).

Table 3.1: Soil type in the snail-eating turtle nest at Bang Ban district, Phra Nakhon Si Ayutthaya province, Thailand.

	Turtle nest		
Soil physical characteristics	1	2	3
Particle size, mum (USDA)			
Sand: 2.0 - 0.05 (%)	2.4	2.2	14.0
Silt: 0.5 - 0.005 (%)	43.2	32.7	34.3
Clay: < 0.005 (%)	54.5	65.0	51.7
Texture class	SiC	C	C

The averages of width, length and depth of 19 turtle nest (Season 2) were 8 cm, 8 cm and 10.9 cm, respectively. Mean of 19 nest temperature and humidity were 30.4 ^{o}C and 67.1 %, respectively. The eggshell temperatures of the snail-eating turtle clutches were randomly measured at three points in field. The average eggshell temperatures (mean ± S.E.) were 24.6 ± 0.6 ^{o}C (Table 3.2). Additionally, the averages ambient temperature and humidity at study area were 32.6 ^{o}C and 59.8

%, respectively (Table 3.2). The results showed the temperature in turtle nests was lower than the ambient temperature while the humidity in turtle nests was higher than the ambient humidity. The result indicates that the adult female turtle tended to select dry area to lay the eggs.

Figure 3.7: Cover and hole of the snail-eating turtle nest

Figure 3.8: The turtle nest before open the cover and after open the cover

Table 3.2: Nest characteristics of *M. macrocephala* at Bang Ban district, Phra Nakhon Si Ayutthaya province, Thailand.

Clutch	Egg per clutch	Nest size (cm.)			Temperature (°C)			Humanity (%)	
		Width	Length	Depth	Ambient	Nest	Eggshell	Ambient	Nest
1	5	10	8	14	26.5	23.3	21.3	37.0	27.2
2	10	11	6	15	26.4	23.2	21.5	38.0	27.6
3	7	6	7	8	31.0	29.3	22.8	55.0	35.7
4	7	11	11	13	28.0	29.1	23.4	59.0	37.1
5	6	8	10	9	29.8	30.1	23.0	61.0	38.0
6	6	6	7	10	28.5	28.6	22.4	76.0	42.3
7	8	7	7	10	29.3	29.6	23.0	69.0	40.5
8	5	9	11	14	28.6	28.7	23.8	69.0	40.5
9	6	8	7	10	30.7	27.8	22.8	72.5	41.0
10	5	10	8.5	11	30.7	30.9	24.2	72.5	42.5
11	5	8	9	11	29.0	29.1	23.0	66.0	39.4
12	5	7	7	11	30.0	30.7	23.9	68.0	40.9
13	6	9	9.5	9	35.7	32.9	25.9	60.0	39.6
14	7	8	7	12	34.6	32.7	26.4	59.8	39.6
15	4	6	6	10	38.0	33.9	27.3	58.0	39.7
16	9	7.5	8	11	38.5	32.1	27.2	58.0	39.1
17	6	8	9.5	12.5	39.2	36.6	29.4	55.1	40.4
18	4	7	8	10	42.3	36.0	29.4	50.7	38.7
19	6	5.5	5.5	6.5	42.0	33.6	27.4	50.7	37.2
Mean	6.2	8.0	8.0	10.9	32.6	30.4	24.6	59.8	38.3

3.3.2 Clutch size and egg morphology

Clutch sizes of snail-eating turtle in these three seasons (n=36 clutches) were 3-10 egg per clutch (Table 3.3). The egg is elongate and white in color. Egg size (mean ± S.E.) including width, length and weight (n=214 eggs) were 22.19 ± 0.11 mm, 38.28 ± 0.19 mm and 11.61 ± 0.15 g, respectively. Eggshell thickness was measured in representative eggs collected in Season 3 (n=22 eggs). Average eggshell thickness of *M. macrocephala* was 0.315 ± 0.006 mm.

Table 3.3: Clutch size and egg morphology of snail-eating turtle egg at Bang Ban district, Phra Nakhon Si Ayutthaya province, Thailand.

Turtle Egg	Season		
	1 (2005)	2 (2006)	3 (2007)
Number of clutch	14	19	3
Number of eggs	75	117	22
Clutch size (egg/nest)	3-8	4-10	6-8
Weight (g) (Mean ± S.E.)	12.03 ± 0.25	11.30 ± 0.02	11.88 ± 0.56
Width (mm.) (Mean ± S.E.)	22.56 ± 0.18	21.88 ± 0.15	22.57 ± 0.37
Length (mm.) (Mean ± S.E.)	38.66 ± 0.34	38.14 ± 0.24	37.67 ± 0.73
Eggshell thickness (mm; Mean ± S.E.)	N/A	N/A	0.315 ± 0.006

3.3.3 Sexual size dimorphism and determining of juvenile turtle sex

Seventeen juvenile male (carapace range 43.43-64.94 mm) and seventeen juvenile female turtles (carapace range 42.58-65.18 mm) were measured for 26 morphological characteristics. Comparison of

these traits by Independent sample t-test showed that 3 characters including tail width, postcloacal length and tail length were significantly different between male and female turtles. Juvenile male turtle exhibited greater tail width, tail length and postcloacal length than those of juvenile female (Table 3.4).

Table 3.4: Morphological characteristics of juvenile turtle that are significantly different between sex.

Morphological Characteristics	Mean ± S.E. (mm.)		Independent sample t-test *p-value*
	Male	Female	
Tail width	5.16 ± 0.26	4.21 ± 0.22	0.009
Postcloacal length	7.27 ± 0.36	6.08 ± 0.37	0.027
Tail length	13.87 ± 0.82	11.32 ± 0.41	0.011

In order to develop a novel non-invasive sexing technique, the logistic regression equation was created using 5 morphological characteristics (Figure 3.9) that were normally distributed according to Kolmogorov-Smirnov test including carapace width, mid-line carapace length, plastron width, postcloacal length and tail length (p = 0.051, 0.200, 0.200, 0.200 and 0.096, respectively). As a result, the logistic regression (n=34) can be showed in the following logit P equation:

logit P = -6.408 - (0.908 carapace width) - (0.355 mid-line carapace length) + (1.364 plastron width) + (0.478 postcloacal length) + (0.958 tail length)

Based on the above equation, sex of the juvenile snail-eating turtle can be predicted by the following sex determining equation:

$$\text{Juvenile snail-eating turtle sex (p)} = 1 / (1 + e^{-\text{logit P}})$$

Sex can be predicted as male turtle when p is equaled to or greater than (≥) 0.5, and can be predicted as female turtle when p is less than 0.5. The correlation coefficient for this relationship (r^2) is equaled to 0.383 and the percent accuracy is 85.3%.

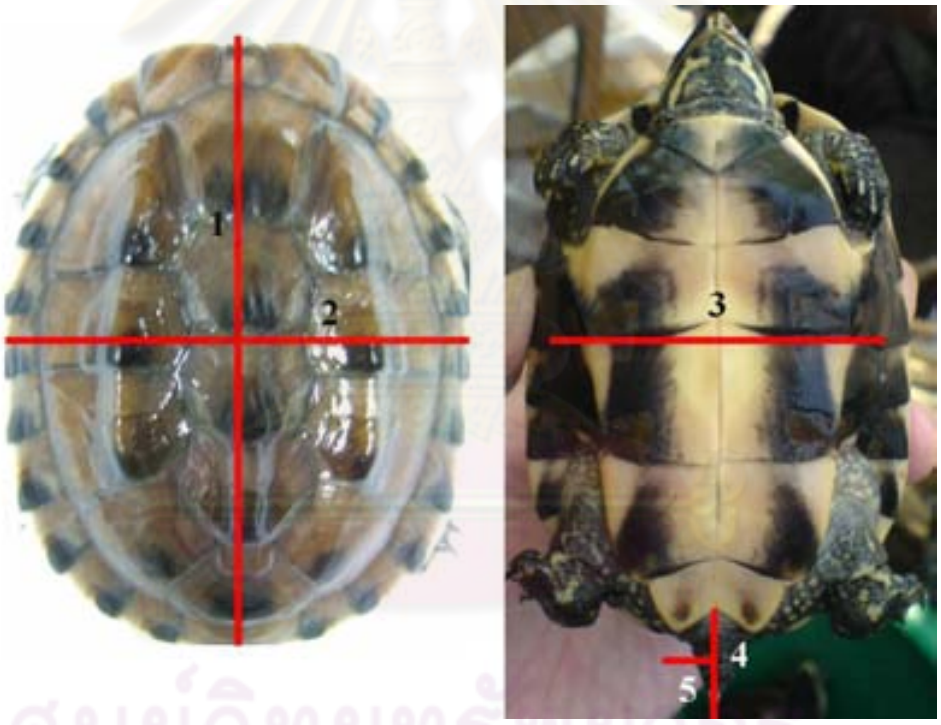

Figure 3.9: Morphological characteristics used in sex determining equation (1 - mid-line carapace length, 2 - carapace width, 3 - plastron width, 4 - tail length, 5 - postcloacal length).

3.3.4 Effect of temperature on hatching success and survival rate

Overall, the incubation period ranged from 82 to 186 days. The results indicated that the incubation temperatures seemed not to affect the incubation duration. However, at high temperature, the incubation time was relatively shorter than low temperature (Table 3.5).

Table 3.5: Effects of incubation temperature on incubation duration and hatching success.

Temperature	Incubation period (days)	Hatching success (%)
Low temperature (26 ^{o}C)	105 – 165 (n=39)	56.4%
Pivotal temperature (29 ^{o}C)	98 – 176 (n=39)	56.4%
High temperature (32 ^{o}C)	82 – 186 (n=39)	35.9%

The percentage of hatching success at 26 ^{o}C, 29 ^{o}C and 32 ^{o}C were 56.41, 56.41 and 35.90, respectively. The results indicated that high temperature dramatically reduced the hatching success of the turtle. To further examine the effect of temperature on the hatching success, the hatching success was categorized into two patterns including 1) normal hatching when turtle can hatch by itself (Figure 3.10) and 2) assisted hatching when turtle required assistance after egg piping in order to emerge from eggshell (Figure 3.11). In the assisted hatching, if no help was given, the hatchling body would fuse to in egg membrane and probably lead to death (Figures 3.12).

Table 3.6 shows that incubation at 29 ^{o}C resulted in higher proportion of normal hatching (63.36%), while incubation at either low

(26 ^{o}C) or high temperature (32 ^{o}C) resulted in more turtle with assisted hatching (59.09% and 71.42 %, respectively).

Table 3.6: Hatching success and survival rate of juvenile turtles after egg incubation at three different temperatures.

Stage	Incubation temperatures		
	26 ^{o}C	29 ^{o}C	32 ^{o}C
Number of eggs	39	39	39
Number of hatchings	22	22	14
- Normal hatching	9	14	4
(incubation period, days)	105-165	98-176	91-131
- Assisted hatching	13	8	10
(incubation period, days)	121-165	99-150	82-186
Number of hatchling (one day)	22	22	14
Survival			
seven days	21	20	11
one month	21	20	10
two months	21	20	10
three months	21	20	10
four months	21	20	10
five months	21	20	10
six months	21	20	10
seven months	21	20	10
eight months	21	20	10
nine months	21	19	10
ten months	21	19	10
eleven months	21	19	10
twelve months	21	19	10

Survival rates of hatchling from different incubating temperature were monitored for 1 year. The mini life table of the snail-eating turtle incubated at different temperature is shown in Table 3.6. The overall percentages of one year survival were 95.45 % at 26 ^{o}C,

86.36 % at 29 ^{o}C and 71.42 % at 32 ^{o}C (Table 3.6). The hatchling stage in this study referred to turtle that hatched and survived for at least one day. The results indicated that high mortality occurred during the first week. The hatchling at high temperature died 21.42% within seven days, while after seven days all hatchlings survived. Further examination indicated that neonate turtles that died during the first week were all from assisted hatching group (data not shown).

Figure 3.10: Normal hatching.

Figure 3.11: Assisted hatching.

Figure 3.12: Dead turtle due to desiccation.

3.3.5 Effect of temperature on sex ratio

Juvenile snail-eating turtles hatched from eggs incubated at three difference temperatures were measured for 5 morphological characteristics and applied to logit P regression, followed by sex determining equation. Sex ratio of turtles from each incubating temperature is shown in Figure 3.13.

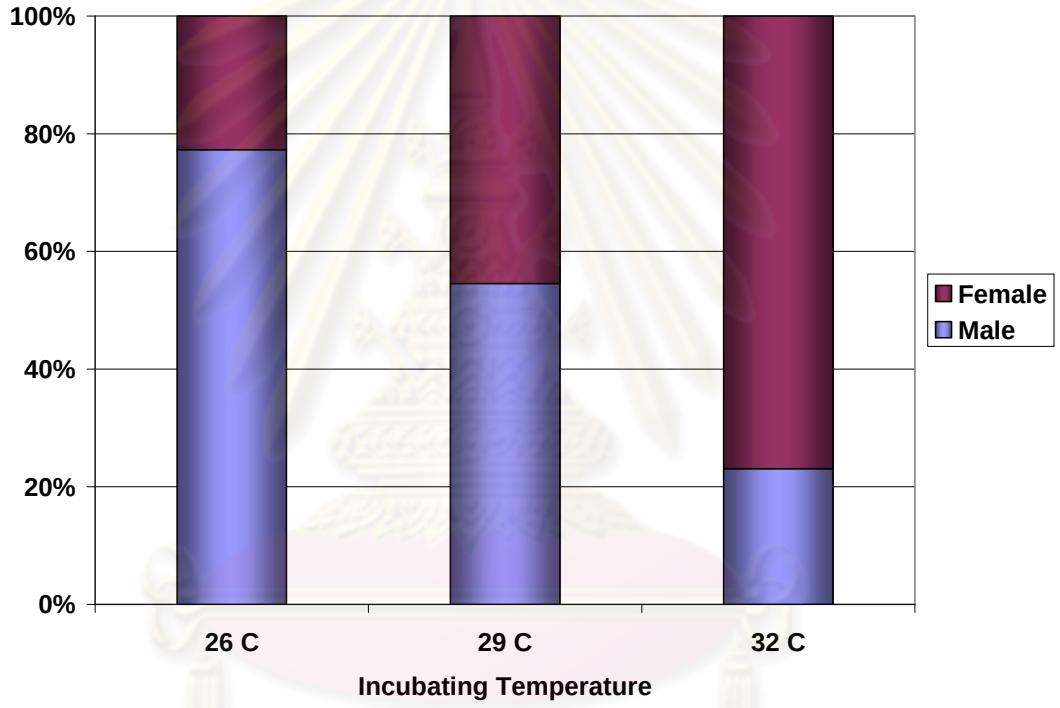

Figure 3.13: Sex ratio of *M. macrocephala* hatched from eggs incubated at three different temperatures.

Table 3.7 shows *G* tests for goodness of fit at three expected sex ratio of (2 males: 1 female, 1 male: 1 female, 1 male: 2 females). It was found that sex ratio of turtles incubated at low temperature (26 ^{o}C) fits with 2 males: 1 female ratio. Incubation at pivotal temperature (29 ^{o}C) resulted in 1 male: 1 female sex ratio, while incubation at high temperature (32 ^{o}C) gave rise to 1 male: 2 females sex ratio.

Table 3.7: *G* statistic for the log-likelihood ratio goodness of fit test for the sex ratio of the snail-eating turtle *M. macrocephala* hatched from eggs incubated at three different temperatures.

Parameters	Sex ratio		
	2 males: 1 female	1 male: 1 female	1 male: 2 females
G of 26 ^{o}C	0.720 ($p > 0.1$)	5.756 ($p < 0.1$)	15.984 ($p < 0.1$)
G of 29 ^{o}C	0.925 ($p > 0.1$)	0.045 ($p > 0.1$)	3.335 ($p < 0.1$)
G of 32 ^{o}C	8.579 ($p < 0.1$)	2.877 ($p < 0.1$)	0.247 ($p > 0.1$)

3.4 Discussion

This study reports important data on reproductive and nesting biology of the snail-eating turtle, a native and common freshwater turtle species in Thailand and Southeast Asia. The results on nest physical characteristics reflected elaborate nest site selection of the gravid females so that the temperature and humidity would be favorable for egg incubation. The results on soil particle analysis indicated that nesting sites were mainly silt clay or clay. Since central part of Thailand is known as an important flood plain with high deposit rate, this information provides further proof that the lower Chao Phraya river basin is an important breeding area of the snail-eating turtle.

Clutch size of *M. macrocephala* in this study (3-10 eggs) was similar to previous reports in *Malayemys* turtles (Nutphand, 1979; Srinarumol, 1995; Thirakhupt, 2000 and Bonin *et al*. 2006), and similar to other small and medium size freshwater turtle in Thailand such as

Cuora amboinensis (3-5 eggs per clutch; Nutphand, 1979), *Hieremys annandalii* (5-8 eggs per clutch; Thirakhupt, 2000).

Compared to previous reports, egg width of *M. macrocephala* in this study was similar to other reports but the egg length was less than those reported by Smith (1931) and Ewert (1979). For eggshell thickness, no previous data of this species is available for comparison. However, the eggshell thickness is not different from other freshwater turtle such as *Kinosternon flavescens* (Packard, Iverson and Packard 1984), *Chinemys reevesii* (You and Wang, 1993) and *Mauremys mutica* (Zhu *et al*., 2006). The statistical analysis indicates that the eggshell thickness is not correlated with the egg size.

In *M. subtrijuga* (potentially *M.* macrocephala), Srinarumol (1995) reported that the incubation period is 97-292 days at ambient temperature (25-32 ^{o}C). The incubation period in this study ranged from 82 to 186 days. Results from these two studies indicate that incubation time of *M. macrocephala* eggs is relatively longer than those of other freshwater turtles. Godley *et al.* (2001) reported that the incubation duration was an integration of the speed of development throughout incubation. Although the effect of temperatures on incubation period was not conclusive in this study, at high temperature, the incubation time was relatively shorter than low temperature. This is similar to other studies in freshwater turtle such as *Mauremys mutica* (Zhu *et al*., 2006) and *Emydura signata* (Booth *et al*., 2004) that showed the effect of temperature on embryonic development.

Many studies have reported that incubation temperature is a significant factor that affected embryo development, hatching rate of

turtle, some hatchling morphological characteristics and post-hatching growth (Brooks *et al*., 1991; Rhen and Lang, 1999; Booth *et al*., 2004 and Fordham, Georges and Corey, 2007). In addition, high temperature is harmful to embryonic development and may decrease survival rate of the turtle (Zhu *et al*., 2006). Moreover, the high and low incubating temperatures produce slow growing turtles compared to turtles from pivotal temperature (Rhen and Lang, 1999). In this study, the result on hatching success at the high temperature showed similarly low percentage as other studies. However, the hatching successes at low and intermediate temperatures were not markedly different. In addition, the high temperature also gave rise to the high mortality of hatchling, especially during the first week after hatch. The data on survival rate showed that one week after hatching is critical period for turtle mortality. The survival rate is relatively stable afterward. Therefore, if care is given properly, it is possible to raised *M. macrocephala* in captivity.

Similar to other freshwater turtle, sex of juvenile *M. macrocephala* is difficult to determine because of the lack of distinct sexually dimorphic traits and heteromorphic sex chromosomes (Ceriani and Wyneken, 2008). However, the current morphometric study revealed that male and female juvenile *M. macrocephala* shows significant difference in morphological characteristics at the tail. The result indicates that tail of juvenile is an important trait that can be used to identify sex of juveniles. This study further attempted to develop a novel non-invasive sexing technique based on the morphometric data. The analysis by logistic regression resulted in a sex determining equation that could predict sex of the juvenile at the accuracy of more than 85%. This approach is an important alternative to predict sex of juveniles with just a simple measurement of some morphological characteristics.

The current findings on effect of incubation temperature on sex ratio of freshwater turtle is the first record of *M. macrocephala* (Ewert *et al*., 2004) and the first record of such study for freshwater turtle in Thailand. The sex ratio of juvenile *M. macrocephala* showed temperature-dependent pattern. Incubation at low temperature (26 ^{o}C) produced higher proportion of male, while incubation at high temperature (32 ^{o}C) resulted in higher proportion of female. The result indicates that *M. macrocephala* has TSD (temperature-dependent sex determination).

Since sex of freshwater turtle is influenced by the incubating temperature, it is thus important to find the pivotal temperature for species of interest. The intermediate temperature or pivotal temperature is defined as the constant temperature of sex ratio parity (1 male to 1 female; Ewert *et al*., 2004). The pivotal temperature of freshwater turtle such as *Pseudemys* sp. (Ewert *et al*., 2004), *Mauremys mutica* (Zhu *et al*., 2006) and *Chinemys reevesii* (Du *et al*., 2007) is 29 ^{o}C. While, the pivotal temperature of some freshwater turtles including *Chrysemys picta* and *Trachemys decorata* is 26-28 ^{o}C (Ewert *et al*., 2004). The results in this study indicated that 29 ^{o}C should be the pivotal temperature of *M. macrocephala* since the resulting sex ratio fits with 1 male: 1 female sex ratio. It is of interest to note that 29 ^{o}C is also the most suitable for egg incubation due to its high proportion of normal hatching. This finding is similar to a study in *Chinemys reevesii* which have high hatching success at the pivotal temperature of 28-30 ^{o}C (Du *et al*., 2007).

3.5 Conclusion

M. macrocephala is a native and common freshwater turtle in Thailand. Agricultural areas at Bang Ban district, Phra Nakhon Si Ayutthaya province, central part of Thailand is an important breeding area of *M. macrocephala*. The reproductive and nesting biology data in this study shows that *M. macrocephala* has clutch size of 3-10 eggs, egg size of 22 x 38 mm and egg weight of 11.6 g. A morphometric study and logistic regression analysis revealed that a sex determining equation can be developed and used to predict sex of juvenile *M. macrocephala* at the accuracy of 85.3%. Egg incubation in a temperature-controlled incubator resulted in incubation period of 82-186 days with hatching success of 36-54%. Egg incubation at different temperature resulted in different sex ratio suggesting that *M. macrocephala* has temperature-dependent sex determination. Incubating temperature also affected hatchling success, mortality rate and survival rate but showed no distinct effect on incubation period.

CHAPTER IV

CONCENTRATION OF ORGANOCHLORINE PESTICIDES IN NEST SOIL, EGG AND BLOOD OF SNAIL-EATING TURTLE *Malayemys macrocephala* FROM THE CHAO PHRAYA RIVER BASIN THAILAND

4.1 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 very low water solublility and high lipophilicity (Perry *et al*., 1998). Due to their persistence and inexpensive price, the OCPs such as DDT (dichloro-diphenyltrichloroethane), 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 (Walker *et al*., 2001 and Cunningham, Cunningham and Woodworth, 2007). 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 (Thirakhupt *et al.*, 2006).

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 (ppb, part per billion) may interfere with structure or function of endocrine system and cause adverse effects to animal reproduction and development (Damstra *et al.*, 2002). The 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* (Guillette, *et al.*, 1994 and Guillette, *et al.*,1996). 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 (Hopkins, 2006). Eggs and blood of several turtle species have been used for monitoring the persistent organic pollutants such as loggerhead sea turtle *Caretta caretta* (Keller, Kucklick and McClellan-Green, 2004a and Alava *et al.*, 2006) and the common snapping turtle *Chelydra serpentina serpentina* (Bishop *et al.*, 1991; Bishop *et al.*, 1998 and de Solla, Fernie and Ashpole, 2008). Since contamination in egg yolk may result in OCPs absorption into embryos during development (Bishop *et al.*, 1995), 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 (Siriwong, 2006) and little egret's egg (Keithmaleesatti *et al*., 2007a). 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 (Srinarumol, 1995 and Brophy, 2004). The turtle is carnivorous and eats large number of small snails, earthworms, aquatic insects, crustaceans, and small fish (Bonin, Devaux and Dupré, 2006). 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.

4.2 Materials and methods

4.2.1 Study area

The study area is a floodplain nearby the Chao Phraya river at Bangban district, Phra Nakhon Si Ayutthaya province (~100 kilometers north of Bangkok, Figure 4.1), Thailand. 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.

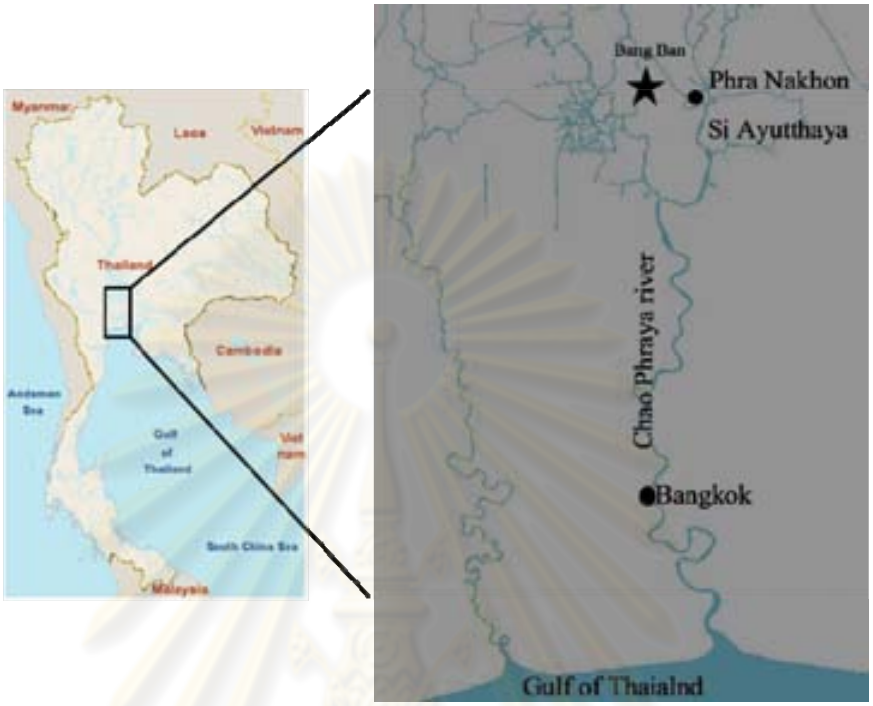

Figure 4.1: Study area (★) in Bang Ban district, Phra Nakhon Si Ayutthaya province, Thailand.

4.2.2 Sample collection

(1) Egg 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.

(2) Blood collection

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.

4.2.3 Pesticide standards and reagents

A standard solution containing nineteen organochlorine pesticides (α- hexachlorocyclohexane (HCH), β-HCH, γ-HCH, δ-HCH, heptachlor, aldrin, heptachlor epoxide, γ-chlordane, endosulfan I, α-chlordane, dieldrin, 4,4' DDE, endrin, endosulfan II, 4,4' DDD, 4,4' DDT, endrin aldehyde, endosulfan sulfate, and endrin ketone) and 2 surrogates (2,4,5,6-tetrachloro-m-xylene: TCMX) and decachlorobiphenyl: DCBP) were obtained from Restek, U.S.A. Pesticide grade solvents such as dichlormethane, diethyl ether, petroleum eather, and 95 % n-hexane was purchased from Lab Scan Asia. SPE-florisil cartridges of 1000 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. Lastly, washed glassware was baked in an oven at 250 ◦C overnight and rinsed with acetone before each use.

4.2.4. Sample Preparation for OCP Determination

(1) OCP in Turtle Nest Soil

Methods for an analysis of OCP in nest soil was modified from (Siriwong, 2006). 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 (Pridmore *et al*., 1992). 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 (Siriwong *et al*., 2007). To remove sulfur contamination as previously described in (Pan *et al*., 2004), 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 elutes were combined and collected in a concentrator tube for evaporation to 1.5 mL under gentle stream of nitrogen prior to quantification with a gas chromatography equipped with micro electron capture detector (GC-μECD).

(2) OCP in Turtle Egg

Methods for determination of OCP in the snail-eating turtle eggs was modified from (Siriwong, 2006). 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 (Siriwong *et al*., 2007). 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.

(3) OCP in Turtle Blood

Methods for OCP determination in turtle blood was adapted from (Lino *et al*., 1998). 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 (Siriwong *et al*., 2007). 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.

4.2.5 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 (Siriwong *et al*., 2007). 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.

4.2.6 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, 50 and 100 ng/mL. Calibration standards were reestablished and checked 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 and DCBP) were determined for every sample. Method detection limits (MDLs) in this study was determined from spiked OCP standard at 50 ng/g wet weight (n=7 for blood and eggs samples and n = 6 for soil samples).

4.2.7 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.

4.3 Results

4.3.1 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. 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 51.2-102.8 % with RSD of 3.2-9.7 %. 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 82.0-118.0 % with relative standard deviation (RSD) of 2.5-4.9 %. 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 42.1-116.7 % with RSD of 2.5-12.2 % (Appendix B).

4.3.2 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 4.1). The average recoveries of pesticide surrogate (TCMX and DCBP) were 85.6% and 96.6% respectively.

Table 4.1: Organochlorine pesticides residues (ng/g dry weight) in nest soil of *M. macrocephala* at Phra Nakhon Si Ayutthaya province, Thailand.

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

Note: ND = not detected

4.3.3 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 mm. width, 37.68 ± 0.73 mm. 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 4.2). The concentrations of ∑HCHs and ∑Chlordanes were not significantly different among eggs as well as among nests (F-test, $p > 0.05$ and ANOVA, $p > 0.05$).

However, the concentration of DDTs was varied among eggs and among nests (Table 4.3). 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, $p > 0.05$).

4.3.4 OCP Contamination in Turtle Blood

The average recovery of pesticide surrogate (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 (Table 4.4).

Table 4.2: Organochlorine pesticide residues (ng/g wet weight) in three complete clutches of *M. macrocephala* eggs at Phra Nakhon Si Ayutthaya province, Thailand.

Nest	Chemicals	Min - Max (ng/g) wet wt.	Mean ± S.E. (ng/g) wet wt.
1 n = 8	TCMX (%)	87.3 - 95.1	92.6
	∑ HCHs	7.3 - 21.0	12.9 ± 1.7
	∑Chlordanes	ND - 13.5	9.9 ± 1.0
	∑DDTs	21.2 - 500.7	98.5 ± 57.8
	∑Endrins	ND	ND
	∑Endosufans	ND	ND
	aldrin and dieldrin	ND	ND
	DCBP (%)	33.9 - 84.5	48.8
2 n = 8	TCMX (%)	84.5 - 119.1	101.9
	∑ HCHs	9.4 - 25.3	16.3 ± 2.0
	∑Chlordanes	ND - 16.5	13.1 ± 1.3
	∑DDTs	26.6 - 75.7	38.0 ± 5.8
	∑Endrins	ND	ND
	∑Endosufans	ND	ND
	aldrin and dieldrin	ND	ND
	DCBP (%)	21.9 - 41.7	30.0
3 n = 6	TCMX (%)	109.1 - 115.0	111.8
	∑ HCHs	12.3 - 20.7	16.5 ± 1.4
	∑Chlordanes	ND - 17.9	12.1 ± 2.0
	∑DDTs	34.1 - 600.2	293.6 ± 99.0
	∑Endrins	ND	ND
	∑Endosufans	ND	ND
	aldrin and dieldrin	ND	ND
	DCBP (%)	23.2 - 36.1	28.9

Note: ND = not detected

Table 4.3: Residues of DDT (ng/g wet weight) in three complete clutches of *M. macrocephala* eggs at Phra Nakhon Si Ayutthaya province, Thailand.

Egg number	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.	98.5 ± 57.8	38.0 ± 5.8	293.6 ± 99.0

Note: Egg number is arbitrary and does not refer to the order of oviposition.

N/A = not applicable.

Table 4.4: Organochlorine pesticide residues (ng/mL wet weight) in adult blood of *M. macrocephala* at Phra Nakhon Si Ayutthaya province, Thailand.

Chemical	Female								Male		
	1	2	3	4	5	6	7	8	1	2	3
TCMX (%)	80.0	80.9	80.5	87.3	101.1	83.9	83.4	89.6	98.8	107.7	83.5
∑HCH (ng/mL)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
∑Chlordane (ng/mL)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
∑DDT (ng/mL)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
∑Endrin (ng/mL)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
∑Endosulfan (ng/mL)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Aldrin and dieldrin (ng/mL)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
DCBP (%)	95.6	93.5	101.3	94.5	117.8	118.3	117.9	114.3	121.2	116.2	117.3

Note: ND = not detected

4.4 Discussions

Although the OCPs had been banned in Thailand (Thirakupt *et al*., 2006), the OCP residues were still detectable in Thai ecosystem (Siriwong, 2006, Keithmaleesatti *et al*., 2007a and Siriwong *et al*., 2007). Our findings on OCP contamination in nesting soil of the freshwater turtle were similar to prior studies in the nearby areas (Chulintorn, 2006), and at a watershed nearby the Mae Klong river (Poolpak *et al*., 2008). 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 (Bishop *et al*., 1995, Alava *et al*., 2006 and Keithmaleesatti *et al*., 2007b). 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 (Wu *et al*., 2006). The current study was 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 4.2). 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 (Bishop *et al*., 1995), crocodile (Wu *et al*.,

2006) and bird (Ormerod and Tylor, 1990). The higher level of OCP residue was associated with higher lipid content of the egg (Wu *et al*., 2006). 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 (Wu *et al*., 2006).

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 (Rauschenberger *et al*., 2004a and Rauschenberger *et al*., 2004b). 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 (Caňas and Anderson, 2002). 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* (de Solla *et al*., 1998), *Phoca hispida* and *Erignathus barbatus* (Bang *et al*., 2001), *Larus hyperboreus* (Bustnes *et al*., 2001), *L. argentatus* (Norstrom *et al*., 1986) and *Caretta caretta* (Keller *et al*., 2004a). Additonally, Keller *et al*., (2004b) reported that blood was a suitable alternative to fatty tissues for monitoring OCPs since it was 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 The Association of Official Agricultural Chemists standard ([AOAC], 1993), 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 (Bishop *et al*., 1994) which stated that the majority of contaminants deposited in snapping turtle eggs were from the recent diet instead of body adipose stores.

4.5 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.

CHAPTER V

ASSOCIATION OF ORGANOCHLORINE PESTICIDE CONTAMINATION IN EGG AND REPRODUCTIVE EFFECTS ON THE SNAIL-EATING TURTLE *Malayemys macrocephala* IN THE LOWER CHAO PHRAYA RIVER BASIN, THAILAND

5.1 Introduction

The use of organochlorine pesticides (OCPs) had started from 1949 to 1990s (Perry *et al.*, 1998). In Thailand, OCPs had been heavily used for agricultural and public health. Particularly, DDT was the first pesticide that had been used for malaria control since 1949 (Thirakhupt *et al.*, 2006). Despite the fact that organochlorine pesticides (OCPs) had been banned in Thailand for several decades, their residues are still detectable in Thailand ecosystem including water, soil, sediment, shellfish, shrimp, fish and little egret's eggs from 1987 to 2006 (Siriwong, 2006; Keithmaleesatti *et al*., 2007a).

Because OCPs are long lasting and bioaccumulative in ecosystem, the OCP residues can pose a risk of causing adverse effects to human health and environment. Recently, 8 OCPs including aldrin, heptachlor, hexachlorocyclohexane, chlordane, dieldrin, endrin, DDT and toxaphene have been included in the list of 12 Persistent Organic Pollutant (POPs) under the Stockholm's convention, suggesting their significance as prime contaminants in global scale (Untied Nations Environmental Programme (UNEP), 2006). In addition, several OCPs are identified as

endocrine disrupting chemicals (EDCs) that relate to sex reversal and abnormalities in reproductive functions of many vertebrate (Damstra *et al.*, 2002).

Many long-lived reptiles, such as crocodile and turtle, have been used as bioindicators of environmental contamination (Hopkins, 2006). OCPs contaminated in reptile tissue have been linked with several adverse effects on the reproductive functions. American alligator *Alligator mississippiensis* in the Lake Apoka, an area with history of dicofol spill, showed reduced penis size. Likewise, male Florida red-belly turtles *Chrysemys nelsoni* lived in this area also showed several sex abnormalities (Guillette, *et al*., 1994 and Guillette, *et al*., 1996). Since OCPs can be transferred from adult female to egg yolk (Alava *et al*., 2006), many studies showed that reptile's eggs can be used as biomonitoring of OCP residues in the environment and their potential effects on the animals. Previous reports on OCP residues and/or reproductive impairment included studies in loggerhead sea turtle *Caretta caretta* (Alava *et al*., 2006) and the common snapping turtle *Chelydra serpentina serpentina* (Bishop *et al*., 1998 and de Solla, Fernie and Ashpole, 2008).

The snail-eating turtle *Malayemys macrocephala* is the native and common freshwater turtle found in many parts of Southeast Asia and Thailand. It can be found in wetland habitat such as canals, ponds and rice-field (Srinarumol, 1995 and Brophy, 2004). This species is carnivorous turtle feed on a large number of small snails, earthworm, aquatic insects, crustaceans, and small fish (Bonin, Devaux and Dupré, 2006).

Although the OCPs have been banned in Thailand, Siriwong (2007) reported that their residues is still present in water and sediment at Rangsit agricultural area, Pathum Thani province. At the same time, Chulintorn (2006) detected these pesticides in the canal at Bang Ban district, Phra Nakhon Si Ayutthaya province. These two areas are located in the lower Chao Phraya river basin which is also an important nesting site of *M. macrocephala*. It is thus important to examine the extent of OCP contamination and the potential reproductive effects on this long-lived animals.

The objectives of this study were to 1) investigate the level of OCP contamination in *M. macrocephala* eggs collected from areas with prior history of OCP uses, and 2) assess the association between OCP contamination in eggs and potential effects on hatching success, survival, hatchling deformities, and sex ratio of the turtles.

5.2. Materials and methods

5.2.1 Study areas

The study areas are floodplain nearby the Chao Phraya River at Bang Ban district, Phra Nakhon Si Ayutthaya province (~100 kilometers north of Bangkok, Thailand) and Rangsit agricultural area (Khlong 7), Khlong Luang district, Pathum Thani province (~70 kilometers northeast of Bangkok, Thailand) (Figure 5.1). At Bang Ban district, rice is the predominant crop in this area with its field covering more than 90% of the land use. Rangsit agricultural area is an important agricultural fields (predominantly rice field) near Bangkok with a man-made irrigation-network-system of 14 sub-canals (Khlong). These areas

are important breeding ground of the snail-eating turtle in central part of Thailand.

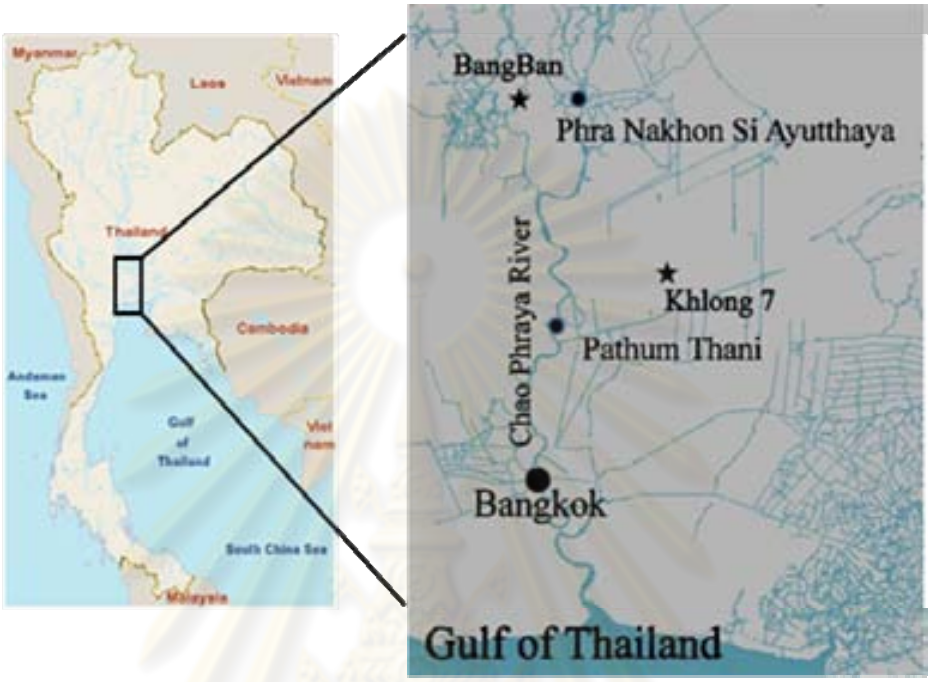

Figure 5.1: Study areas (★) at Bang Ban district, Phra Nakhon Si Ayutthaya province and Khlong 7, the Rangsit agricultural area, Khlong Luang district, Pathum Thani province, Thailand.

5.2.2 Turtle egg collection

In nesting season of the snail-eating turtle (December 2006 to March 2007), 15 clutches of eggs from Bang Ban district and 9 clutches of eggs from Khlong 7, Rangsit agricultural area were collected from the field. One egg per clutch was randomly selected for OCP residue analysis. Egg content (yolk and egg white) was kept at -36 °C until analysis for OCPs. The remaining eggs in each clutch were subjected to incubation and further studies on hatching success, survival, deformities and sex ratio (see 5.2.7 and 5.2.8).

5.2.3 Pesticide standards and reagents

A standard solution containing nineteen organochlorine pesticides (α- hexachlorocyclohexane (HCH), β-HCH, γ-HCH, δ-HCH, heptachlor, aldrin, heptachlor epoxide, γ-chlordane, endosulfan I, α-chlordane, dieldrin, 4,4' DDE, endrin, endosulfan II, 4,4' DDD, 4,4' DDT, endrin aldehyde, endosulfan sulfate, and endrin ketone) and 2 surrogates (2,4,5,6-tetrachloro-m-xylene: TCMX) and decachlorobiphenyl: DCBP) were obtained from Restek, U.S.A. Pesticide grade solvents such as dichlormethane, petroleum eather, diethyl ether, and 95 % n-hexane was purchased from Lab Scan 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. Lastly, washed glassware was baked in an oven at 250 °C overnight and rinsed with acetone before each use.

5.2.4 Sample preparation for OCP determination

Methods for determination of OCP in the snail-eating turtle eggs was modified from Siriwong (2006). The whole egg content (egg yolk and albumin) was homogenized in a 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 Siriwong *et al*. (2007). 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 a gas chromatograpy equipped with micro-electron capature detector (GC-μECD).

5.2.5 Chromatographic conditions

A gas chromatography 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 Siriwong *et al*. (2007). 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.

5.2.6 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, 50 and 100 ng/mL. Calibration standards were reestablished and checked 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 and DCBP) were determined for every sample. Method detection limits (MDLs) in this study spiked the OCP standard at 50 ng/g wet weight at 7 time. The limit of detections (LODs) and the limit of quantitations (LOQs) were in the range of 0.01 - 0.04 ng/mL and 0.04 - 0.13 ng/mL, respectively. The method detection limits (MDLs) (n=7) at 50 ng/g wet wt. of this study were 4.77 - 9.99 ng/g (wet wt.). Recovery of this study was 82 - 118 % and relative standard deviation (RSD) was 2.5 - 4.9 %. The methods were considered to be adequate for the analytical protocols here in the determination of OCPs following the Peer Verified Methods Program of AOAC (1993).

5.2.7 Hatching success, deformities and survival rate

Upon arrival in the laboratory, eggs were randomly placed in trays containing 1:1 vermiculite: water (weight: volume) and kept at 29 °C in a microprocessor-controlled incubator (Siam Incubator, Thailand). Tray of water was placed inside the incubator to maintain relative humidity at above 80%. Temperature and humidity inside the box were monitored using a digital thermo-hygrometer. Every three days, water was sprayed on vermiculite to increase humidity. Incubation period, hatching success, were monitored.

In this study, the hatching turtle was classified to two groups. The first group is normal hatching when turtle can hatch by itself. The second group is assisted hatching when turtle required assistance after egg piping in order to emerge from eggshell (Figure 5.2). In addition, neonate turtle was examined for pattern of deformity after one day of age.

Figure 5.2: In assisted hatching, the hatchling cannot move out of the egg by itself because egg membrane was dried or sticky.

Hatchlings were kept individually in a plastic box containing dechlorinated tap water and stored in a room with ambient temperature of 25-28 ˚C. After seven days, hatchling turtle were fed with turtle

pellets (Tetra Reptomin®) 3 times per week. Water in the plastic box was changed and the box was cleaned on weekly basis. Survival rates were monitored as number of turtle that hatched and survived for one day, seven days, one month to one year. The turtles were measured for width and length of carapace and body weight on monthly basis.

5.2.8 Sex ratio

Juvenile turtles were measured for 5 morphological characteristics (carapace width, mid-line carapace length, plastron width, postcloacal length and tail length) by vernier caliper (Mitutoyo, Japan). Sex was determined from anatomical examination in dead specimens or predicted from sex determining equation. Briefly, the measured parameters were used in the following logit P equation:

logit P = -6.408 - (0.908 carapace width) - (0.355 mid-line carapace length) + (1.364 plastron width) + (0.478 postcloacal length) + (0.958 tail length)

Then, sex of the juvenile snail-eating turtle can be predicted by the following sex determining equation:

Juvenile snail-eating turtle sex (p) = $1 / (1 + e^{-\text{logit P}})$

Sex can be predicted as male turtle when p is equaled to or greater than (≥) 0.5, and can be predicted as female turtle when p is less than 0.5.

5.2.9 Statistical analyses

Nineteen organochlorine pesticides were classified to six groups. ∑HCHs were defined as the sum of α-HCH, β-HCH, γ-HCH and δ-HCH, and ∑Chlordanes as the sum of heptachlor, heptachlor epoxide, γ-chlordane, and α-chlordane. ∑DDTs were classified as the sum of 4,4' DDE, 4,4' DDD and 4,4' DDT and ∑endrins as the sum of endrin, endrin aldehyde, and endrin ketone. ∑endosulfans were defined as the sum of endosulfan I, endosulfan II and endosulfan sulfate. The last groups were the sum of aldrin and dieldrin. The mean concentrations of OCPs residue were used to determine the differences between two sites using independent sample t-test.

Since the pattern of OCP contamination in eggs showed no site-related difference, data on OCP residues of 24 clutches of eggs was subjected to cluster analysis (PC-ORD) in order to classify these eggs into groups according to pattern of OCP contamination.

The associations between OCPs residues and the hatching success or hatching deformities were analysed by indepentdent sample t-test and Pearson's correlation. λ^2-test were used to compare number of survival in both areas. For sex ratio study, *G* statistic for the log-likelihood ratio goodness of fit test (*G* test) was used to determine whether the sex ratio of each group is deviated from the expected sex ratio. A significant difference is reported at $p \leq 0.05$.

5.3 Results

5.3.1 Turtle egg collection

The total number of *M. macrocephala* eggs at both sites was 144 eggs from 24 clutches. There were 15 clutches of eggs from Bang Ban district with clutch size of 4-8 eggs, and 9 clutches of eggs from Rangsit agricultural area with clutch size of 4-7 eggs. The egg width, egg length and egg weight at both area were not significantly different (Indepdent sample t-test, $p > 0.05$; Table 5.1).

Table 5.1: Data (mean ± S.E.) of *M. macrocephala* eggs collected from Bang Ban district, Phra Nakhon Si Ayutthaya province and Rangsit agricultural area (Khlong 7), Khlong Luang district, Pathum Thani province, Thailand.

Parameters	Bang Ban district (n=97 eggs)	Rangsit area (n=45 eggs)	t-test *p-value*
Clutch size (eggs)	4-8	4-7	N/A
Width (mm.)	22.16 ± 0.10	21.76 ± 0.18	0.598
Length (mm.)	38.42 ± 0.41	38.94 ± 0.41	0.062
Weight (g)	11.61 ± 0.19	11.43 ± 0.29	0.431

5.3.2 Organochlorine pesticide contamination in turtle egg

The mean concentration of OCPs in *M. macrocephala* eggs from Bang Ban district, Phra Nakhon Si Ayutthaya province (n=15) and Khlong 7, Rangsit agricultural area, Pathum Thani province (n=9) are summarized in Table 5.2. The mean concentration of ∑Chlordanes, ∑DDTs, ∑endosulfans, and the sum of aldrin and dieldrin in eggs from

Bang Ban district were greater than those found in eggs from Khlong 7, Rangsit agricultural area. ∑HCHs in eggs from Bang Ban district were less than those found in eggs from Khlong 7, Rangsit agricultural area. Nevertheless, mean of all OCPs was not significantly different between two sites (independent sample t-test, $p > 0.05$; Table 5.2).

Table 5.2: The concentration (mean ± S.E.) of organochlorine pesticides (OCPs) in egg of *M. macrocephala* collected from Bang Ban district, Phra Nakhon Si Ayutthaya province and Rangsit agricultural area (Khlong 7), Khlong Luang district, Pathum Thani province, Thailand.

Pesticides	Average concentration ng/g (wet wt.)		t-test
	Bang Ban district	Rangsit area	*p-value*
∑HCHs	62.3 ± 8.2	82.3 ± 7.4	0.112
∑Chlordanes	17.0 ± 2.1	15.2 ± 1.4	0.531
∑DDTs	27.9 ± 6.8	< MDL	- [a]
∑Endrins	< MDL	8.1	- [a]
∑Endosulfans	26.1 ± 6.2	20.4 ± 1.2	0.455
Aldrin and dieldrin	20.5 ± 4.1	15.4 ± 1.3	0.315

Note: [a] Independent sample t-test cannot be computed because at least one of the groups is empty.

At both sites, ∑HCHs, the sum of α-, β-, γ-, and δ-HCH, was the most predominant among six groups of OCPs. The range of ∑HCHs detected in all eggs was 39.5 - 106.5 ng/g (wet wt.) in eggs from Rangsit agricultural area, and 32.9 - 142.7 ng/g (wet wt.) in eggs from Bang Ban district. ∑Chlordanes, the sum of heptachlor, heptachlor

epoxide, γ-chlordane, and α-chlordane, was in the range of 10.7 - 21.5 ng/g (wet wt.) in eggs from Rangsit agricultural area, and up to 33.2 ng/g (wet wt.) in eggs from Bang Ban district. ∑endosulfans, the sum of endosulfan I, endosulfan II and endosulfan sulfate, ranged from less than MDL to 38.4 ng/g (wet wt.) in eggs from Bang Ban district, and less than MDL to 22.1 ng/g (wet wt.) in eggs from Rangsit agricultural area. The sum of aldrin and dieldrin was found to range from below MDL to 64.5 ng/g (wet wt.) in eggs from Bang Ban district, and below MDL to 19.8 ng/g (wet wt.) in eggs from Rangsit agricultural area. It is of important to note that ∑DDTs, the sum of 4,4' DDE, 4,4' DDD and 4,4' DDT, was found only in eggs from Bang Ban district. At both sites, the concentration of ∑endrins, the sum of endrin, endrin aldehyde, and endrin ketone, was less than MDL.

Since the pattern of OCP contamination in eggs (Table 5.2) showed no site-related difference, data on OCP residues of 24 clutches of eggs was subjected to cluster analysis in order to classify these eggs into groups according to pattern of OCP contamination. It was found that cluster analysis could classified these 24 eggs into 2 groups (Figure 5.3). Comparison of OCP residues in eggs showed that group I tended to have higher concentration of OCPs than group II. Means concentration of ∑HCHs in group I were significantly higher ($p < 0.05$) than those of group II (Table 5.3). Therefore, to examine the association between OCP contamination and reproductive effects in turtle, this grouping criteria was used in all subsequent analysis.

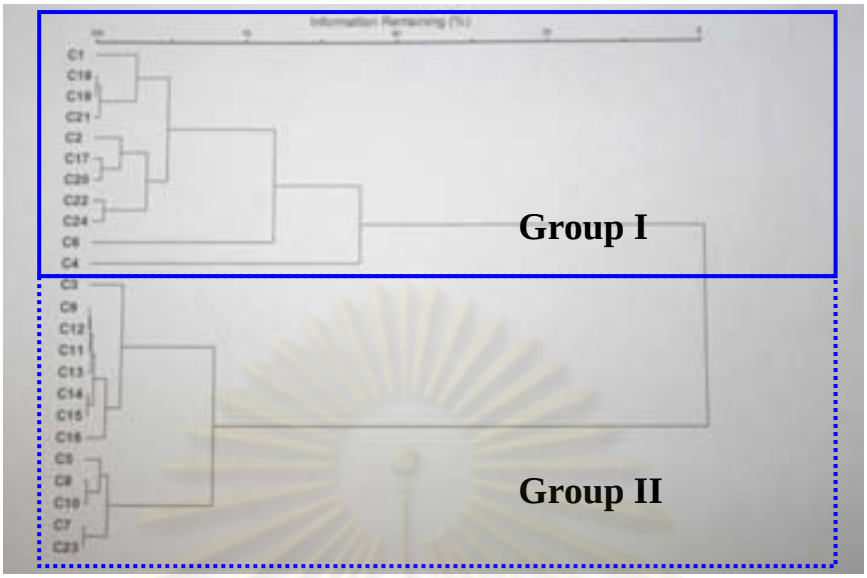

Figure 5.3: Dendogram of *M. macocephala* eggs collected from the lower Chao Phraya river basin. Eggs can be divided into 2 groups according to pattern of OCP residues.

Table 5.3: The concentration (mean ± S.E.) of organochlorine pesticides (OCPs) in egg of *M. macrocephala* collected from the lower Chao Phraya river basin, Thailand.

Pesticides	Group I (n=11) ng/g (wet wt.)	Group II (n=13) ng/g (wet wt.)	*p-value*
∑HCHs	96.7 ± 5.5	47.1 ± 3.9	***p* < *0.05*** [a]
∑Chlordanes	18.8 ± 2.6	14.4 ± 1.3	*p > 0.05* [b]
∑DDTs	34.7	21.1	- [c]
∑Endrins	8.1	-	- [c]
∑Endosulfans	24.3 ± 3.6	18.1	- [c]
Aldrin and dieldrin	21.2 ± 5.0	15.8 ± 0.9	*p > 0.05* [b]

Note: [a] Independent sample t-test; [b] Mann-Whitney U-test.

[c] Independent sample t-test cannot be computed because at least one of the groups is empty.

5.3.3 Association between OCP contamination and hatching success and survival rate

For *M. macrocephala* eggs collected from the lower Chao Phraya river basin, incubation periods at 29 ^{o}C were 69-199 days, and the overall hatching success were 66.95 %. It was found that hatching success was not significantly different between groups (Table 5.4).

Table 5.4: Incubation time and hatching success of *M. macrocephla* eggs collected from agricultural areas in the lower Chao Phraya river basin, Thailand.

Parameters	Group I	Group II	Total	*p –value*
Number of clutch	11	13	24	N/A
Total eggs	67	77	144	N/A
Incubated eggs	54	64	118	N/A
Incubation time (days)	69-189	83-199	69-199	N/A
Hatching success - number hatched - percent hatched	 40 (74.07%)	 39 (60.94%)	 79 (66.95%)	$p > 0.05^{b}$
Normal hatching	22 (55.00%)	33 (84.61%)	55	$p > 0.05^{a}$
Assisted hatching	18 (45.00%)	6 (15.39%)	26	$\boldsymbol{p < 0.05}^{b}$

Note: [a] Independent sample t-test; [b] Mann-Whitney U-test

In general, it was found that the proportion of normal hatching was higher than the assisted hatching in both groups. Comparison between groups showed that there was no significant difference in normal hatching incidence. However, Man-Whitney U-test indicated that eggs in group I (potentially higher OCP contamination), had higher number of assisted hatching than group II (lower OCP contamination).

Furthermore, Pearson's correlation analysis showed that there was a significant relationships between amount ∑chlordanes in eggs and the percentage of assisted hatching (p = 0.001).

Table 5.5: The number of *M. macrocephala* survived between one day to 12 months. Turtles were hatched from eggs collected from agricultural areas in the lower Chao Phraya river basin, Thailand.

	Number of turtles	
Stage	Group I (11Clutches)	Group II (13 Clutches)
one day	40	39
seven days	38	38
1 month	38	38
2 months	38	38
3 months	37	37
4 months	37	37
5 months	37	37
6 months	37	37
7 months	37	37
8 months	37	37
9 months	37	36
10 months	37	36
11 months	37	36
12 months	37	36

The results in Table 5.5 showed that survival rate of *M. macrocephala* in both groups were 92.40 % in 1 year. The critical time for hatchling is during one to seven days after hatching when mortality was usually high. It was found that 3 hatchlings (50% of the total death) died during this period. But, there was no different pattern of survival between groups.

5.3.4 Association between OCP contamination and deformities

Examination of *M. macrocephala* morphology revealed several deformities in turtles from both groups. Some deformities in this study were so severed that the hatchling can not hatch and died in egg (Figure 5.4). Deformities were found in hatchlings from both normal hatching and assisted hatching. There were 8 types of deformities found with hatchlings in this study. The deformities were classified and rated according to Bell, Spatila and Congdon (2006) into 3 classes. The *minor* deformities (Figure 5.5-5.8) that are not likely to affect survival included 1) carapace asymmetry, 2) plastron asymmetry, 3) carapace scute in excess of 38 pieces and 4) carapace scute in shortage of 38 pieces. The *moderate* deformities (Figure 5.9-5.10) that could lower the chances of survival included 1) deformed carapace and 2) tail dissappear. Although this type of deformities could affect the survival, there are still some adut turtle with some of these conditions. Finally, the *lethal* deformities (Figure 5.11-5.12) that greatly reduced the chance of survival included 1) dwarf and 2) lower jaw disappear. Normally, no adults has been seen with these lethal conditions.

Figure 5.4: Severe deformity that turtle can not hatch and died in egg.

Figure 5.5: Carapace asymmetry.

Figure 5.6: Plastron asymmetry.

Figure 5.7: Carapace scute in excess of 38 pieces.

Figure 5.8: Carapace scute in shortage of 38 pieces.

Figure 5.9: Deformed carapace.

Figure 5.10: Tail disappear.

Figure 5.11: Dwarf.

Figure 5.12: Lower jaw dissappear

Table 5.6: Incidence of deformed morphology of *M. macrocephala* hatched from eggs collected from agricultural areas in the lower Chao Phraya river basin, Thailand.

Morphological Characteristics	Group I	Group II	Total (n)
Normal	1	5	6
Deformities[a]	39	34	73
Minor deformities			
Carapace asymmetry	*15*	*10*	
Plastron asymmetry	*9*	*3*	
Carapace scute more than 38 pieces	*12*	*14*	
Carapace scute less than 38 pieces	*0*	*1*	
Moderate deformities			
Carapace deformed	*2*	*4*	
Tail disappearance	*0*	*1*	
Lethal deformities			
Dwarf	*1*	*0*	
Lower jaw disappearance	*0*	*1*	

Note: [a] In case of more than one deformities was observed, only the dominant charactertistics was recored.

The results in Table 5.6 showed that deformities were found in 79 hatchlings (92.40%) from both groups. Most of the hatching turtles were found with minor deformities including 35.61% of carapace scute in excess of 38 pieces and 34.25% of carapace asymmetey. It is of interest to note the report on rare deformities of the hatchlings including tail and lower jaw disappearance. Pearson's correlation analysis showed a significant correlation between ∑HCHs and percentage of abnormal morphology (p = 0.01). However, there was no correlation between other OCPs and the incidence of deformities.

5.3.5 Association between OCP contamination and sex ratio

Juvenile *M. macrocephala* hatched from eggs incubated at 29 °C were measured for 5 morphological characteristics and applied to logit P regression, followed by sex determining equation. Sex ratio of turtles from each groups is shown in Figure 5.13.

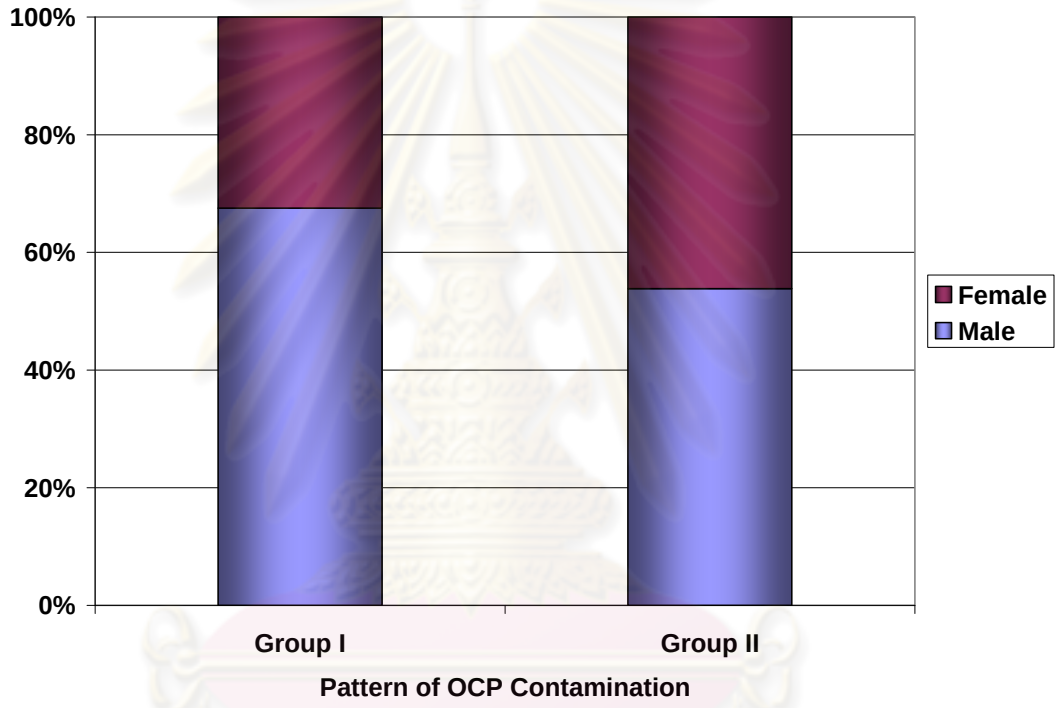

Figure 5.13: Sex ratio of *M. macrocephala* hatched from eggs collected from agricultural areas in the lower Chao Phraya river basin, Thailand.

Table 5.7 shows *G* tests for goodness of fit at three expected sex ratio (2 males: 1 female, 1 male: 1 female, 1 male: 2 females). It was found that sex ratio of turtles in group I followed the 2 males: 1 female sex ratio, while sex ratio of turtles in group II follows the to 1 male: 1 female sex ratio. Based on our previous observation (Chapter III), incubation at 29 °C is usually resulted in 1 male: 1 female sex ratio.

However, sex ratio of turtle in group I (potentially with higher OCP residue) seemed to skew toward 2 males: 1 female ratio.

Table 5.7: *G* statistic for the log-likelihood ratio goodness of fit test for the sex ratio of the snail-eating turtle hatched from eggs collected from agricultural areas in the lower Chao Phraya river basin, Thailand.

Parameters	Sex ratio		
	2 males: 1 female	1 male: 1 female	1 male: 2 females
G of Group I	0.003 ($p > 0.1$)	4.303 ($p < 0.1$)	18.035 ($p < 0.1$)
G of Group II	2.232 ($p > 0.1$)	0.103 ($p > 0.1$)	6.082 ($p < 0.1$)

5.4 Discussions

This current study reports on the use of freshwater turtle eggs as sentinel of organochlorine pesticide (OCP) contamination in ecosystem in the lower Chao Phraya river basin, Thailand. Snail-eating turtle *M. macrocephala* eggs were subjected to analysis for OCP contamination and investigation for hatching success, hatchling deformities, survival rate and sex ratio. The results showed that, although all of these OCPs had been banned in Thailand, detectable levels of these OCPs are still present in turtle eggs indicated that they have been persisted in agricultural fields for long periods of time. This is similar to previous study by Siriwong *et al*. (2007) showing that residue of OCPs was present in aquatic ecosystem at Rangsit Khlong 7, Khlong Luang district, Pathum Thani province, Thailand.

Since *M. macrocephala* is an important secondary consumer in wetland ecosystems, OCPs probably accumulated in *M. macrocephala*

because of trophic transfer. In eggs of amphibian, reptile and bird, route of chemical contamination such as OCPs, PCBs and trace metal could be from maternal transfer or accumulation directy from the environment (Mitchelomore, Rowe and Place, 2006). In many studies, maternal transfer is the most important route of exposure (Bishop *et al.*, 1998). Verreault *et al*. (2006) reported that ∑PCB, ∑OC, ∑PDBE in *Larus hyperboreus* eggs were positively associated with concentrations in female plasma. The importance of contaminant transfer from nesting substrate to egg has not been adequately investigated (Hopkins, 2006). However, based on the current study (see Chapter IV) and study by de Solla and Fernie (2004), the contamination in nesting soil is relatively low and not associated with OCPs found in turtle eggs. These suggest the possibilty that maternal transfer is the major cause of OCPs contamination in turtle eggs.

The hatching success of the snail-eating turtle was previously reported by Srinarumol (1995). The hatching success of 57.14% was obtained from incubation at ambient temperature (26-32 ^{o}C). In this study, the higher hatching success (66.95%) is probably due to better control of incubating temperature and humidity. In *Chinemys reevesii*, incubation at high tempuature (32 ^{o}C and 34 ^{o}C) also resulted in lower hatching success compared to incubation at lower tempuature of 24 ^{o}C to 28 ^{o}C (Du *et al.*, 2007). Alternatively, this could be due to different in locality of egg collection.

Significant correlations in the presences of certain the OCPs groups in turtle eggs may indicate the common pattern of pesticide use by farmers in these region or may indicate the common route of ecological transfer into turtle. This suggests a potential association

between the extent of the OCPs contamination and the reproductive effects on the long-lived animals living in the area with prior history of the OCPs utilization. This studies further revealed the association of OCP contamination with hatching success, hatchling deformities and sex ratio.

Data on concentration of OCP residue and deformities in *M. macrocephala* is not previously available. de Solla *et al*. (2008) reported that *Chelydra serpentina* showed no correlation between high contamination of PCBs and OCPs and deformities or hatching success. In addition, no siginifcant positive correlation between deformities and exposure of PCBs and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalents (TCDD-EQ) was found within a sample size of *Phalacrocorax auritus* clutches (Larson *et al*., 1996). However, in this study, the results showed that there were correlation between the OCPs residues (especially ∑HCH) in egg and increased incidence of deformities in *M. macrocephala*. Furthermore, It is of importance to note that the levels of Σchlordanes residue in eggs were positively correlated with percentage of hatchling that require assistance after egg pipping in order to emerge from eggshell (Pearson correlation coefficient = 0.650, $p < 0.05$).

Levels of OCP contamination in turtle eggs showed no specific site-related difference, i.e. different levels of contamination can be found within one specific sites. This indicates that problems of OCP contamination in the lower Chao Phraya river basin are more widespread than previously thought. This study successfully used a cluster analysis approach to categorize eggs into groups according to pattern of contamination.

Assessment of the association between OCP contamination and sex ratio of the hatchlings revealed that different pattern of OCP contamination leads to different sex ratio of the hatchlings. It was found that sex ratio of turtles in group I (potentially with high OCP contamination) followed the 2 males: 1 female sex ratio, while sex ratio of turtles in group II follows the 1 male: 1 female sex ratio. Based on our previous observation, incubation at 29 °C is usually resulted in 1 male: 1 female sex ratio. However, sex ratio of turtle in group I (potentially with higher OCP residue) seemed to skew toward 2 males: 1 female ratio. This indicats that OCP contamination in turtle eggs could interfere with normal gonadal developmental process of the developing turtle embryos.

5.5 Conclusion

Eggs of long-lived animals such as turtles is usually used as bioindicators of environmental contamination. In this study, eggs of the snail-eating turtle *Malayemys macrocephala*, the most common species in rice field habitat in Thailand, were studied concentration of organochlorine pesticides (OCPs), a group of agricultural persistent organic pollutants (POPs), were measured in eggs collected from two agricultural areas at Phra Nakhorn Si Ayutthaya (n=15) and Pathum Thani province (n=9), central part of Thailand in breeding season of 2006 to 2007. One egg per clutch was randomly selected and analyzed for 19 OCP residues by gas chromatography with micro-electron captured detector. The remaining eggs were incubated in a microprocessor-controlled incubator at 29˚C with humidity in excess of 80%. It was found that 5 groups of OCPs were detected in turtle eggs, and hexachlorocyclohexane (HCH) was the most predominant

residues. Hatching success of these eggs was 66.95% and survival rate of the neonate turtle was 92.40%. Although no correlation between OCPs levels and hatching success or survival rate was found, it is of importance to note that the levels of Σchlordane residue in eggs were positively correlated with percentage of hatchling that require assistance after egg pipping in order to emerge from eggshell. Levels of ∑HCH in eggs were found to associate with an increased incidence of deformities in *M. macrocephala*. High levels of OCPs residue in eggs could skew the sex ratio of the eggs incubated at pivotal temperature toward male producing temperature. These suggests a potential association between the extent of OCP contamination and the reproductive effects on the long-lived animals living in the area with prior history of OCP utilization.

CHAPTER VI

CONCLUSION

Organochlorine pesticides (OCPs) are known to be persistent and bioaccumulative toxicants (Perry *et al.*, 1998). The recent studies have suggested that even the low level of OCP residues may interfere with endocrine system and cause adverse effects to animal reproduction and development (Damstra *et al*., 2002). The endocrine disruption has been reported in both wildlife observation and laboratory experiments. The increased incidence of human endocrine-related health problems also suggests that human population may be at risk as well.

Although the OCPs had been banned in Thailand since 1980s, their residues are still detectable in several ecosystems (Siriwong, 2006 and Keithmaleesatti, 2007a). It is thus important to examine the extent of contamination and the potential reproductive effects on the animals living in the area with prior history of the OCP uses. In this study, the snail-eating turtle *Malayemys macrocephala* is used as a sentinel for OCP contamination in ecosystem because it is a long-lived vertebrate with a home range overlapping a potentially affected area (i.e. rice-field habitat). *M. macrocephala* is a common freshwater turtle that widely distribute in Thailand, especially in the central agricultural plain (Srinarumol, 1995). The long life span and high position in trophic level make it susceptible to long term bioaccumulation of OCPs in body parts including their eggs.

Initially, investigation on nesting, reproductive and developmental biology of the snail-eating turtle in Thailand was carried out with a population in agricultural areas at Bang Ban district, Phra Nakhon Si Ayutthaya province, central part of Thailand, an important breeding area of *M. macrocephala*. The reproductive and nesting biology data in this study shows that *M. macrocephala* has clutch size of 3-10 eggs, egg size of 22 x 38 mm. and egg weight of 11.6 g. A morphometric study and logistic regression analysis revealed that a sex determining equation can be developed and used to predict sex of juvenile turtle with an accuracy of 85.3%. Egg incubation in a temperature-controlled incubator resulted in incubation period of 82-186 days with hatching success of 36-54%. Egg incubation at different temperature resulted in different sex ratio suggesting that *M. macrocephala* has temperature-dependent sex determination (Ewert *et al.*, 2004; Valenzuela and Lance, 2004). Incubating temperature also affected hatchling success, mortality rate and survival rate but showed no distinct effect on incubation period.

Next, analyses for OCP residues were performed on nest soil, blood and complete clutch of eggs of *M. macrocephala* from Phra Nakhon Si Ayutthaya province to examine the extent of OCPs contamination in rice-field habitats of the Chao Phraya river basin. Although OCPs were not detected in blood of turtles, the analyses showed that HCHs, chlordanes and DDTs were found in every egg. Moreover, endosulfans were also found in nest soil in addition to those OCPs found in eggs. Although blood has been used to monitor the contamination in wildlife due to its properties as a good representative of exposure levels and a suitable alternative to fatty tissues (Keller *et al.*, 2004b), the levels of OCPs in turtle blood in this study was not detectable. It is possible that the contamination is below the detection limits or the level of OCPs in

M. macrocephala blood is not a good indicative of contamination in other tissues. This suggests that the contaminants deposited in eggs could be from the diet instead of body adipose stores (Bishop *et al*., 1994). The results indicated that although these OCPs 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.

Finally, concentrations of OCPs were measured in turtle eggs collected from two agricultural areas at Phra Nakhorn Si Ayutthaya province and Pathum Thani province, central part of Thailand. It was found that 5 groups of OCPs (HCHs, chlordanes, DDTs, endosulfans, and aldrin and dieldrin) were detected in turtle eggs, and HCHs was the most predominant residues. Incubation of eggs in a temperature-controlled incubator at 29 °C revealed that hatching success of these eggs was 66.95% and survival rate of the neonate turtle was 92.40%. Although no correlation between OCPs levels and hatching success or survival rate was found, it is of importance to note that the levels of Σchlordane residue in eggs were positively correlated with percentage of hatchling that require assistance after egg pipping in order to emerge from eggshell. Levels of ∑HCH in eggs were found to associate with an increased incidence of deformities in *M. macrocephala*. High levels of OCPs residue in eggs was also found to skew the sex ratio of the eggs incubated at pivotal temperature toward male producing temperature.

Overall, the results indicated that low level contamination (part per billion, ppb) of OCPs is still present in central part of Thailand. The results showed a potential association between the OCP contamination and hatching success, deformities and sex ratio of the

snail-eating turtle. These suggest the ecological risk of OCP contamination on reproductive health, developmental success, and potentially the population of the long-lived vertebrate living in the area with prior history of OCP utilization.

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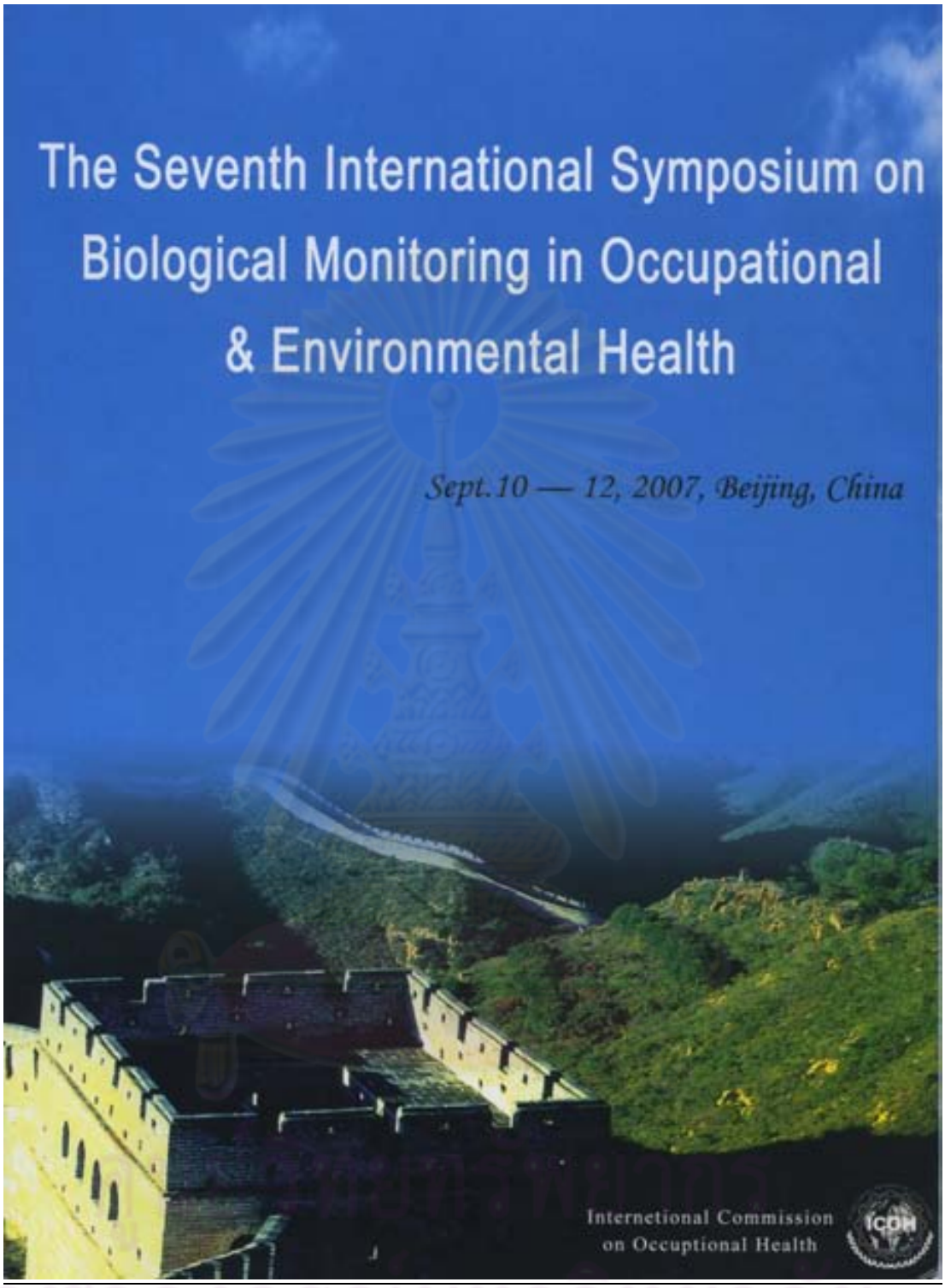
APPENDICES

APPENDIX A

Presentation and Publication



Organochlorine Pesticide Residues in Egg of the Snail-eating Turtle *Malayemys macrocephala* from the Lower Chao Phraya River Basin, Thailand

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Abstract

Organochlorine pesticides (OCPs) were used over the past few decades in Thailand's agricultural and public health activities. The OCPs are known to be the persistent and bioaccumulative toxicants that may cause reproductive impairments in wildlife as well as human. The current study uses eggs of the snail-eating turtle (*Malayemys macrocephala*) as a sentinel, to monitor environmental contamination of OCPs. *M. macrocephala* is a long-lived animal that commonly distributes in rice field habitat in central part of Thailand. Representative eggs of each turtle nest were collected from two agricultural areas in the Chao Phraya River Basin, Thailand (Phra Nakhorn Si Ayutthaya Province; n = 15 and Pathum Thani Province; n = 9) in the nesting season of 2006-2007. The eggs were extracted by an accelerated solvent extractor (ASE), and nineteen OCP residues were analyzed by gas chromatography with micro-electron captured detector (GC-μECD). Method detection limits (MDL) were in the range of the 4.77 – 9.99 ng/g wet weight. The recovery and relative standard deviation (RSD) were 82 – 118 % and 2.48 – 4.90 %, respectively. It was found that, among 19 OCPs examined, residues of 12 OCPs and 6 OCPs were detected in turtle eggs from Phra Nakhorn Si Ayutthaya Province and Pathum Thani Province, respectively. At both areas, total hexachlorocyclohexane (HCH) were most predominant among the organochlorine pesticides with a mean concentration of upto 98.68 ng/g wet weight. Pairwise comparison of OCP residues in turtle eggs from these two areas showed no significant difference between areas ($p > 0.05$). Although all of these OCPs had been banned in Thailand, their detectable levels in turtle eggs indicate that they can persist in agricultural fields for long periods of time. Overall, the results of using turtle eggs as biomonitoring systems for persistent organic pollutants (POPs) 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.

Key words: *Malayemys macrocephala*; eggs; organochlorine pesticides; accelerated solvent extractor; gas chromatography

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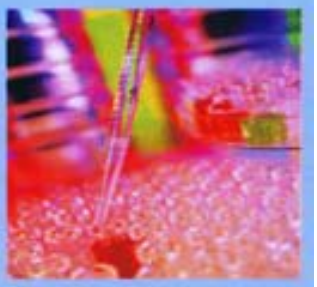

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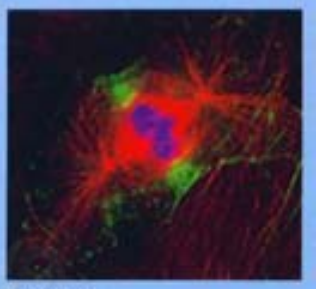

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Association of Organochlorine Pesticide Residues in Egg and Developmental Effects on the Snail-eating Turtle *Malayemys macrocephala* from the Lower Chao Phraya River Basin, Thailand

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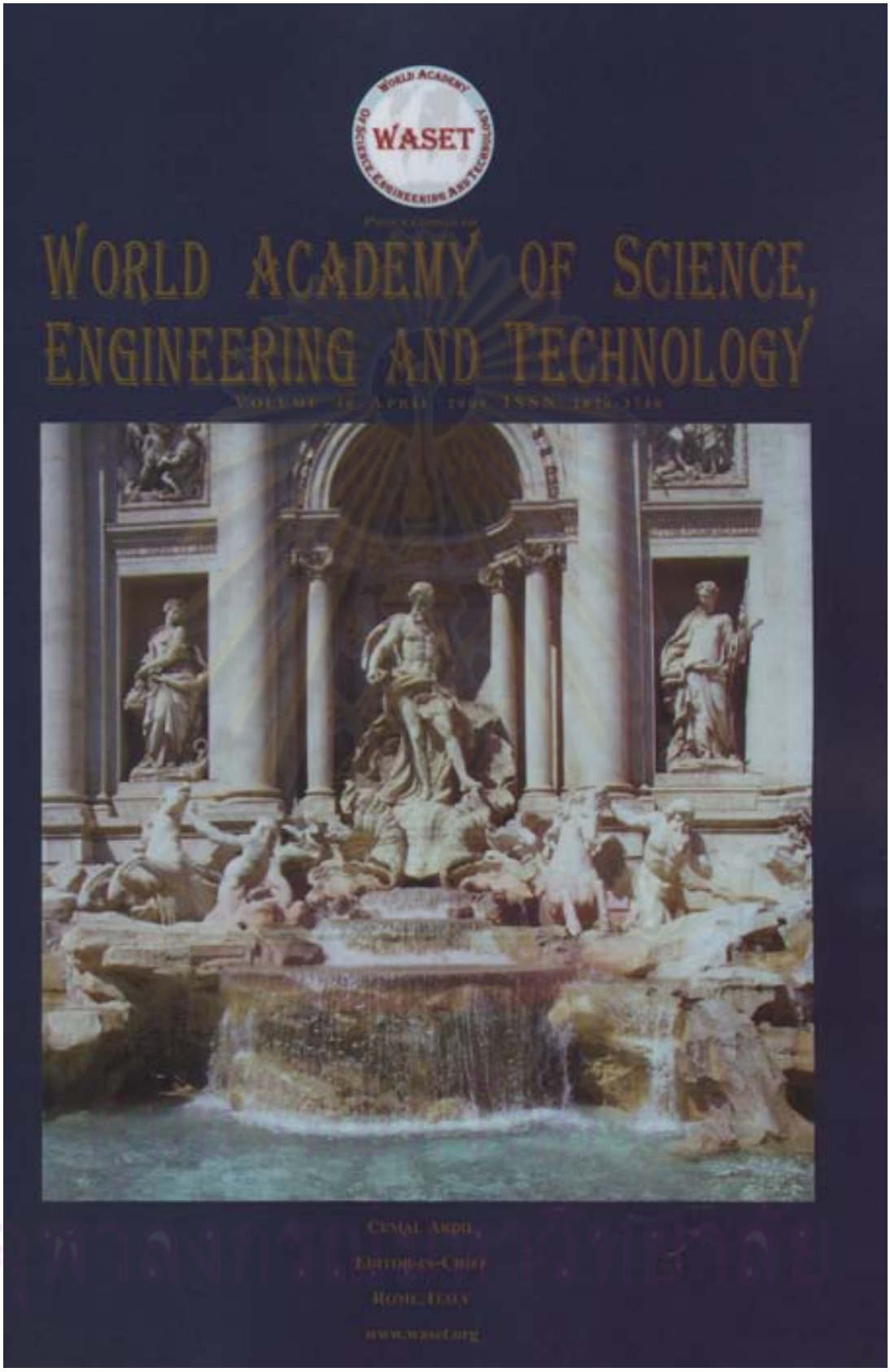
The organochlorine pesticides (OCPs) are known to be the persistent and bioaccumulative toxicants that may cause reproductive impairments in wildlife as well as human. The current study uses eggs of the snail-eating turtle (*Malayemys macrocephala*) as a sentinel, to monitor environmental contamination of OCPs and assess associations of OCP residues and developmental effects on the turtle. *M. macrocephala* is a long-lived animal that commonly distributes in rice field habitat in central part of Thailand. Turtle eggs were collected from 2 agricultural areas in the Lower Chao Phraya River Basin, Thailand during nesting season of 2006-2007. One egg per clutch (n=24) was randomly selected and subjected to extraction and analysis for 19 OCP residues by gas chromatography with micro-electron captured detector (GC-μECD). The remaining eggs were placed in trays containing 1:1 vermiculite:water (weight:volume) and kept in a microprocessor-controlled incubator at 29°C with humidity in excess of 80%.

It was found that 13 OCPs were detected in turtle eggs, and gamma-hexachlorocyclohexane (γ-HCH) was the most predominant residues with the concentration of upto 95.88 ng/g wet weight. The analysis also showed significant positive correlations in the presences of certain OCP groups in turtle eggs (Σendosulfan v.s. ΣDDT, Σendosulfan v.s. aldrin & dieldrin, and ΣDDT v.s. aldrin & dieldrin). Hatching success of these eggs was 66.10% and 7-day survival rate of the neonate turtle was 62.71 %. Although no correlation between

OCP levels and hatching success or survival rate was found, it is of importance to note that the levels of Σchlordane residue in eggs were positively correlated with percentage of hatchling that require assistance after egg pipping in order to emerge from eggshell. This suggests a potential association between the extent of OCP contamination and the reproductive effects on the long-lived animals living in the area with prior history of OCP utilization.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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 dichloromethane, diethyl ether, petroleum esther, 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 °C for 2 min, and programmed to increase to 280 °C at 12 °C /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 °C. 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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APPENDIX B

Limit Of Detections (LOD), Limit Of Quantitations (LOQ) and Method Detection Limits (MDLs) of Soil Nest, Egg and Blood of *M. macrocephala*

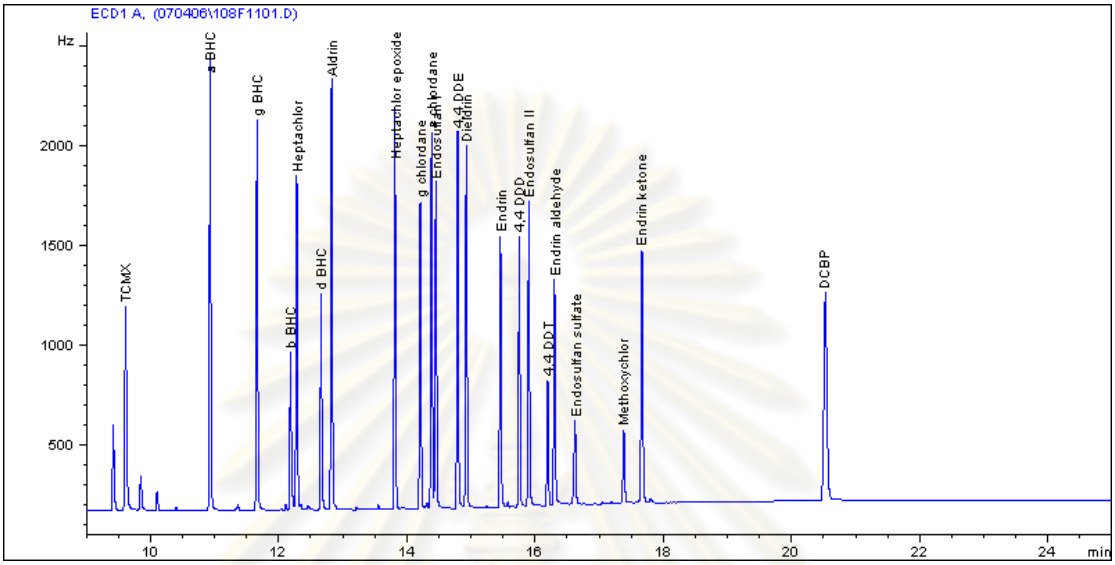
			Soil Nest			Turtle Egg			Turtle Blood		
Organochlorine Pesticide	LOD ng/mL	LOQ ng/mL	MDL ng/g dry wt.	Recovery %	RSD %	MDL ng/g wet wt.	Recovery %	RSD %	MDL ug/mL	Recovery %	RSD %
Tetrachlor-m-xylene	0.04	0.13	3.94	72.9	5.35	4.77	96.8	2.48	4.84	75.7	6.58
α-HCH	0.01	0.05	4.94	79.4	6.15	7.32	104.4	3.61	4.91	77.7	6.51
γ-HCH	0.03	0.11	2.64	82.2	3.18	7.82	109.5	3.38	5.35	84.6	6.50
β-HCH	0.02	0.07	6.25	84.8	7.30	5.83	117.5	2.55	5.30	73.7	7.40
Heptachlor	0.02	0.06	4.73	83.2	5.64	7.00	118.5	3.04	6.34	81.6	8.00
δ-HCH	0.04	0.12	6.58	88.9	3.66	8.45	113.8	3.82	6.27	103.3	3.13
Aldrin	0.03	0.09	2.56	51.2	4.95	6.35	96.2	3.40	4.96	42.1	12.15
Heptachlor epoxide	0.02	0.08	5.13	70.0	7.26	9.80	113.1	4.46	4.54	65.6	7.12
γ-Chlordane	0.02	0.08	6.52	66.5	9.71	5.82	105.6	2.60	4.34	61.1	7.31
α-Chlordane	0.02	0.07	4.97	74.5	6.61	6.93	105.7	3.38	4.91	64.6	7.82

			Soil nest			Turtle Egg			Turtle Blood		
Organochlorine Pesticide	LOD ng/mL	LOQ ng/mL	MDL ng/g dry wt.	Recovery %	RSD %	MDL ng/g wet wt.	Recovery %	RSD %	MDL ug/mL	Recovery %	RSD %
4,4 DDE	0.02	0.06	5.51	73.4	7.44	9.35	112.5	4.28	6.33	69.4	9.39
Dieldrin	0.02	0.06	5.03	78.2	6.37	7.43	105.4	3.63	4.99	71.0	7.23
Endrin	0.02	0.05	5.16	76.3	6.69	7.01	114.2	3.16	3.89	71.6	5.59
4,4 DDD	0.02	0.05	3.54	102.8	3.41	7.27	101.1	3.70	6.51	106.4	6.30
Endosulfan II	0.01	0.05	5.68	60.2	9.34	4.78	81.8	2.57	1.52	62.2	2.52
4,4 DDT	0.01	0.04	7.87	86.9	4.48	9.99	96.7	4.90	5.75	68.5	4.31
Endrin aldehyde	0.01	0.04	4.41	65.3	6.68	8.45	94.1	4.62	3.27	42.4	7.92
Endosulfan sulfate	0.01	0.04	6.84	94.8	3.57	7.58	106.8	2.50	9.91	116.7	4.48
Methoxychlor	0.01	0.04	6.59	66.0	4.94	9.36	108.2	4.45	8.60	69.8	6.37
Endrin ketone	0.01	0.04	7.15	83.7	4.23	5.96	114.9	2.67	10.50	82.2	6.58
Decachlorobiphenyl	0.02	0.06	5.99	86.1	6.88	5.65	100.0	2.90	5.19	79.4	6.73

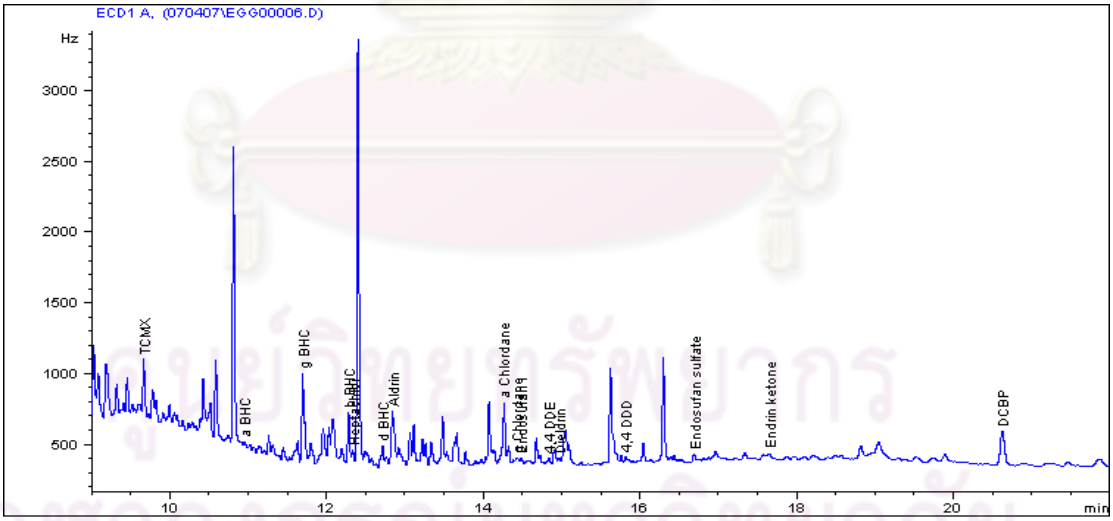
APPENDIX C

Chromatogram of Organochlorine Pesticides

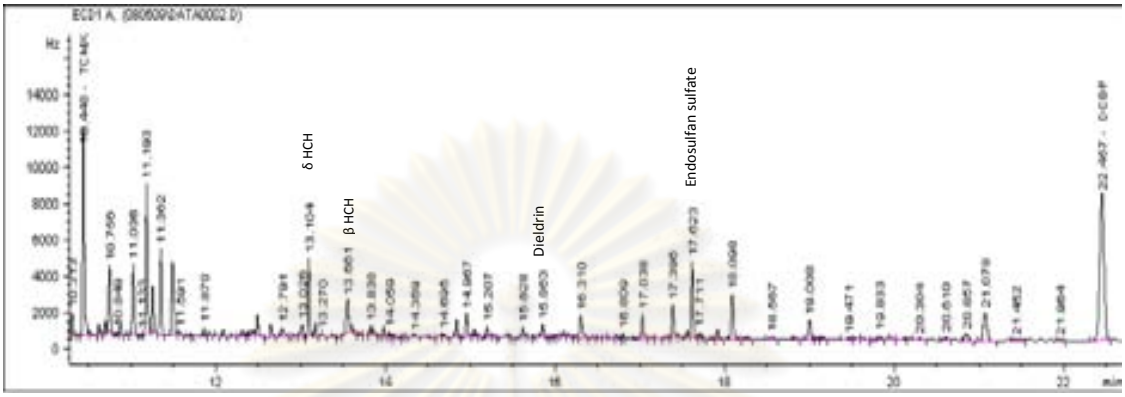
1. Organochlorine pesticides standard and surrogate standard at 50 µg/mL (Column DB 35 Ms).



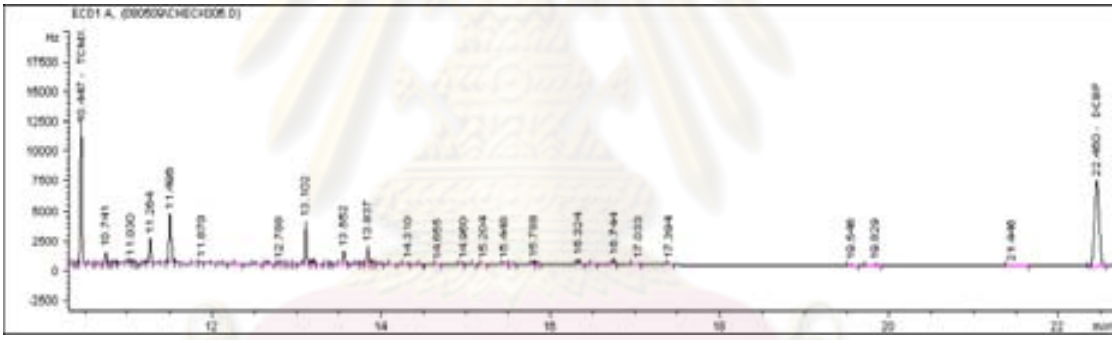
2. Organochlorine pesticides contamination in Snail-eating turtle egg’s.



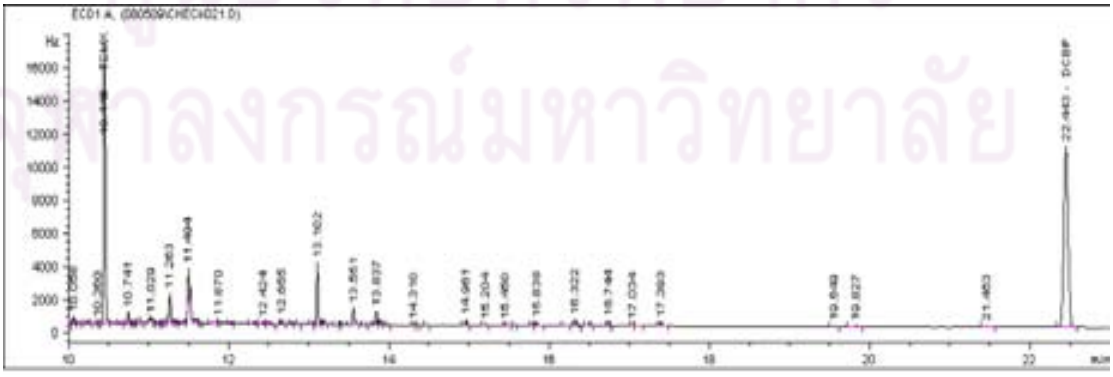
3. Organochlorine pesticides contamination in nest soil from Bang Ban district, Phra Nakhon Si Ayutthaya province, Thailand.



4. Organochlorine pesticides contamination in adult female snail-eating turtle blood .



5. Organochlorine pesticides contamination in adult male snail-eating turtle blood.



BIOGRAPHY

Mr. Sarun Keithmaleesatti was born on the 22nd of May 1978, in Khon Kaen province. He graduated a Bachelor of Science degree in Environmental Science from the Faculty of Science, Khon Kaen University in 1999. He received a scholarship from the University Development Commission (UDC) in 2001 to continue his graduate study, and finished a Master of Science degree in Environmental Science from the Graduate School, Chulalongkorn University in 2003. Later on, he has been appointed as a lecturer in the Department of Environmental Science, Faculty of Science, Khon Kaen University since 2003. He has started his Ph.D. study at the Inter-department of Environmental Science, Graduate School, Chulalongkorn University under support from the UDC scholarship since 2005.