การตอบสนองทางชีวภาพของปูนา *Esanthelphusa nani* (Naiyanetr, 1984) ต่อสารฆ่าวัชพืชในนาข้าว จังหวัดน่าน

นายรชตะ มณีอินทร์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2555

BIOLOGIC RESPONSES OF RICE FIELD CRAB *Esanthelphusa nani* (NAIYANETR, 1984) TO HERBICIDES IN PADDY FIELDS, NAN PROVINCE

Mr. Rachata Maneein

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Zoology
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Thesis Title	BIOLOGIC RESPONSES OF RICE FIELD CRAB *Esanthelphusa nani* (NAIYANETR, 1984) TO HERBICIDES IN PADDY FIELDS, NAN PROVINCE
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รชตะ มณีอินทร์ : การตอบสนองทางชีวภาพของปูนา *Esanthelphusa nani* (Naiyanetr, 1984) ต่อสารฆ่าวัชพืชในนาข้าว จังหวัดน่าน (BIOLOGIC RESPONSES OF RICE FIELD CRAB *Esanthelphusa nani* (NAIYANETR, 1984) TO HERBICIDES IN PADDY FIELDS, NAN PROVINCE) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: อ. ดร. นพดล กิตนะ , อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร. วิเชฏฐ์ คนซื่อ , ผศ. ดร. ปกรณ์ วรานุศุภากุล , 114 หน้า.

ปัจจุบันมีการใช้สารฆ่าวัชพืชอย่างแพร่หลายในปริมาณมากในจังหวัดน่าน สารฆ่าวัชพืชอาจส่งผลกระทบต่อสิ่งมีชีวิตที่ไม่ใช่เป้าหมาย จึงจำเป็นต้องตรวจการปนเปื้อนและผลกระทบต่อสัตว์ที่อาศัยในพื้นที่เกษตรกรรม การตรวจสอบการปนเปื้อนเบื้องต้นในสิ่งแวดล้อมด้วยเทคนิคโครมาโตกราฟีตรวจพบสารแอทราซีนในน้ำในพื้นที่ใช้สารฆ่าวัชพืช เมื่อศึกษาเพิ่มเติมโดยใช้ปูนาเป็นสัตว์เฝ้าระวังการปนเปื้อนและตรวจสอบการตอบสนองทางชีวภาพที่เกี่ยวข้อง โดยเก็บปูนาจากพื้นที่ศึกษา 2 แห่ง คือ พื้นที่นาอ้างอิงที่ไม่มีการใช้สารฆ่าวัชพืช และพื้นที่นาที่มีการใช้สารฆ่าวัชพืชเป็นประจำ นำมาตรวจ สอบการปนเปื้อนสารฆ่าวัชพืช เอ็นไซม์ขจัดพิษและลิปิดเปอร์ออกซิเดชัน (LPO) ตลอดจน ขนาดสัณฐานและน้ำหนักของปูนา พบว่ามีสารฆ่าวัชพืช แอทราซีน ไกลโฟเสต และพาราควอตปนเปื้อนในเนื้อเยื่อ โดยมีปริมาณแอทราซีนแตกต่างอย่างมีนัยสำคัญระหว่างพื้นที่ เมื่อตรวจสอบการทำงานของเอ็นไซม์กลูตาไทโอนเอสทรานสเฟอเรส (GST) พบว่าปูนาทั้งเพศผู้และเพศเมียในพื้นที่ปนเปื้อนมีระดับ GST สูงกว่าปูนาในนาอ้างอิงอย่างมีนัยสำคัญ แม้ว่าระดับ LPO ซึ่งใช้แสดงถึงความเสียหายของเซลล์จะไม่แตกต่างกันระหว่างพื้นที่ แต่มีสห สัมพันธ์อย่างมีนัยสำคัญกับระดับ GST และปริมาณแอทราซีนที่ปนเปื้อน จากการตรวจสอบน้ำหนักสัมพัทธ์พบว่าปูนาในพื้นที่ปนเปื้อนมีน้ำหนักน้อยกว่าปูนาในพื้นที่อ้างอิงอย่างมีนัยสำคัญ การตรวจสอบ ลักษณะภาวะรูปร่างสองแบบตามเพศในปูนา ได้แก่ พื้นที่หน้าท้องและขนาดก้ามใหญ่ พบว่า ปูนาเพศผู้ในพื้นที่ปนเปื้อนมีขนาดเล็กกว่าปูนาที่อยู่ในพื้นที่อ้างอิง การวิเคราะห์สหสัมพันธ์แสดงให้เห็นว่าปริมาณแอทราซีนที่ปนเปื้อนในเนื้อเยื่อปูนามีความเชื่อมโยงกับการลดขนาดของก้ามใหญ่ และการเพิ่มระดับ GST และ LPO ของปูนาในพื้นที่ปนเปื้อน ผลการศึกษาในสัตว์เฝ้าระวังนี้สามารถนำมาใช้ประเมินผลกระทบจากสารฆ่าวัชพืชต่อสิ่งมีชีวิตที่ไม่ใช่เป้าหมายที่อาศัยในพื้นที่เกษตรกรรมได้

ภาควิชา ชีววิทยา	ลายมือชื่อนิสิต .....
สาขาวิชา สัตววิทยา	ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....
ปีการศึกษา 2555	ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....
	ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

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KEYWORDS : ATRAZINE / SENTINEL SPECIES / CRAB / MORPHOMETRY / GLUTATHIONE *S*-TRANSFERASE / LIPID PEROXIDATION

RACHATA MANEEIN: BIOLOGIC RESPONSES OF RICE FIELD CRAB *Esanthelphusa nani* (NAIYANETR, 1984) TO HERBICIDES IN PADDY FIELDS, NAN PROVINCE.
ADVISOR: NOPPADON KITANA, Ph.D.,
CO-ADVISOR: ASST. PROF. WICHASE KHONSUE, Dr. Hum. Env.,
ASST. PROF. PAKORN VARANUSUPAKUL, Ph.D., 114 pp.

Nowadays, herbicides have been widely used with increasing intensity in Nan Province. Since herbicides may pose adverse effects on non-target organism, it is crucial to monitor herbicide contamination and potential health hazards to animal living in agricultural area. Environmental screening with chromatographic techniques showed that detectable level of atrazine was found in water of the herbicide utilization area. In addition, a rice field crab *Esanthelphusa nani* was selected as a sentinel species for herbicide contamination and related biologic responses. Crabs were collected from two study sites: a reference site where no herbicides were used and a contaminated site where herbicides were used routinely. Crabs were subjected to analyses for herbicide contamination, changes in detoxifying enzyme activity, lipid peroxidation and morphometric and gravimetric analyses. Herbicide residue analysis showed that detectable levels of atrazine, glyphosate and paraquat was found in the crab tissue, with significant site-related difference in level of atrazine. Activities of hepatopancreas glutathione *S*-transferase (GST), a crucial detoxifying enzyme, of both male and female crabs were significantly elevated in the contaminated site compared to the reference site crabs. Although level of lipid peroxidation, an indicative of cell damage, was not significantly different between sites, it strongly correlated with levels of GST and atrazine in the crab. Relative body weight of male crabs in the contaminated site was significantly lower than those from the reference site. Sexually dimorphic traits of the crab including abdominal area and major claw size of male crabs from the contaminated site were also significantly smaller compared to those of the reference site animals. Correlation analysis indicated that atrazine in crab tissue was associated with decreased major claw size as well as increased hepatopancreas GST activity and LPO levels of crabs in the contaminated area. The results of this sentinel study could be used for assessing a potential impact of herbicide contamination on non-target organisms in agricultural environment.

Department : Biology Student's Signature .....
Field of Study : Zoology Advisor's Signature .....
Academic Year : 2012 Co-advisor's Signature .....
Co-advisor's Signature .....

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CHAPTER I

INTRODUCTION

Agriculture is a common activity in Nan Province, northern part of Thailand. A variety of agrochemicals such as fertilizers, plant hormones and pesticides has been employed to enhance yield of cultivation. A major group of herbicides, especially atrazine, glyphosate and paraquat, has been widely used in this area. Although agrochemicals result in excellent yield, most of them can be contaminated in environment after intensive or frequent uses. In addition, it has been reported that atrazine, glyphosate and paraquat can be contaminated in water and soil (Solomon et al., 1996; Cheah et al., 1998; US EPA, 2006).

Persistency of herbicides in the environment is relatively low. However the herbicides may cause chronic adverse effects to non-target organism, especially animals. It was previously reported that herbicides can directly pose adverse effects on both vertebrates such as inducing hermaphroditism and demasculinization in frog (Hayes et al., 2002) and invertebrates such as increased occurrence of intersex in population of *Daphnia* sp. (Stoeckel et al., 2008). It is thus important to monitor degree of herbicide contamination in environment and its effects in a representative animals living in areas with potential contamination.

Using animal as a sentinel species is one of environmental monitoring approaches that involves an examination of relationship between chemical contamination and biologic response of animal living in contaminated area in order to assess the effect of the contamination on animal health and the environment (National Research Council, 1991). Some crustaceans, particularly crabs, have been successfully used as a sentinel species. Example of this application included using mud crab *Chasmagnathus granulata* to monitor organochorine pesticide

contamination in mangrove ecosystem (Souza et al., 2008), and using a freshwater crab *Eriocheir sinensis* to monitor effect of dioxin and polychlorinated biphenyls contamination in freshwater environment (Clark et al., 2009).

In this study, the rice field crab *Esanthelphusa nani* (Naiyanetr, 1984) was chosen to be a sentinel species for assessing the impact of herbicide utilization in rice fields, Nan Province, since the crab is native to paddy field habitat with direct exposure to herbicide contaminated water and sediment through gill (gas exchange) and mouth (detritivore; Doolgindachbaporn, 2001). Two study sites were designated for this study including a contaminated site which is a paddy field with intensive herbicide utilization, and a reference site which is an organic rice farm with at least 7 years of no herbicide usage.

The first part of this study was aimed to screen for herbicide contamination in soil and water of these two study sites using chromatographic techniques. Crabs were sampled from these two study sites with different degree of herbicide utilization on monthly basis. Subsequently, sensitive enzyme-linked immunosorbent assays were used to determine levels of herbicide contamination in crab body tissue (Chapter III).

In animal sentinel system, it is necessary to measure biological changes in sentinel species at different levels such as morphological, physiological, or biochemical changes in relation to toxicant contamination in the environment and use these changes as biomarkers (National Research Council, 1991). Biotransformation of xenobiotic substance is an important process in animal body and can be used as a biomarker of exposure or biomarker of effect (Kappus, 1986). The biotransformation and related processes were examined in the second part of this study and used as a biomarker of effect for herbicide contamination (Chapter IV). Hepatopancreas of the

rice field crab *E. nani* was examined for biologic responses in form of changes in activity of an antioxidant enzyme, glutathione *S*-transferase and cell damage based on lipid peroxidation.

Since herbicides were reported to pose adverse effects on morphology of both vertebrates (Hayes et al., 2002) and invertebrates (Stoeckel et al., 2008), overall health status and change in some morphological traits of the rice field crab living in agricultural areas with different degree of herbicide utilization were examined in the last part of this study (Chapter V).

Association between tissue residue of herbicides and changes in these biologic responses could be used for assessing potential impact of herbicide contamination on non-target organisms living in agricultural environment, including human.

Objectives

1. To compare levels of tissue residue of herbicides in the rice field crab living in agricultural areas with different degree of herbicide utilization.
2. To compare biologic response in forms of detoxifying enzyme activity, lipid peroxidation and morphological parameters of the rice field crab living in agricultural areas with different degree of herbicide utilization.
3. To determine association between herbicide contamination in the crab and its biologic responses to herbicide exposure in agricultural areas.

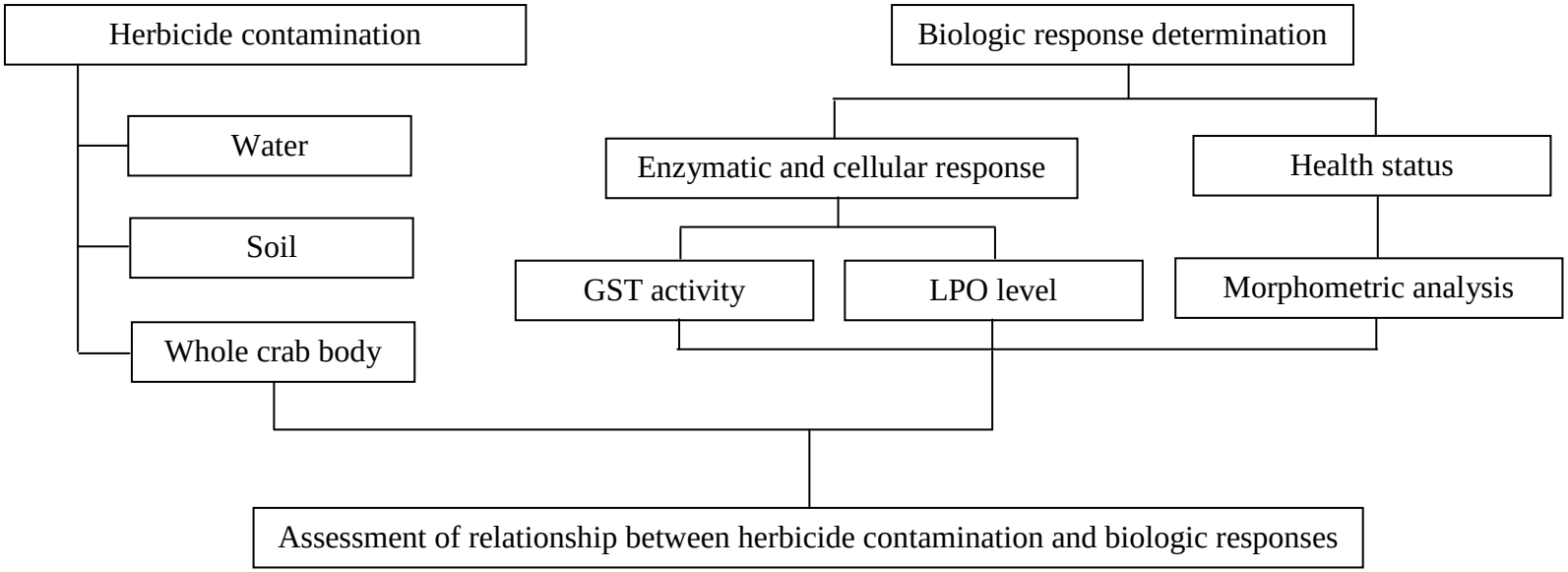


Figure 1.1 Scope of this study: Biologic responses of the rice field crab *Esanthelphusa nani* (Naiyanetr, 1984) to herbicides in paddy fields, Nan Province

CHAPTER II

LITERATURE REVIEW

2.1 Agriculture in Nan Province

Agriculture is one of the major human activity in Nan Province with approximately 1,122 km^2 out of 11,472 km^2 of the total area being used for agricultural purpose. Rice farming is regarded as the largest amount of agricultural land use or 29.58% of all agricultural area in Nan Province (Thadaniti and Prajuabmoh, 2005). In low land area such as Wiang Sa District, Nan Province, more than 95 % of the land is used for wide array of cultivations such as maize, tobacco, rubber tree, soy bean, orange, litchi, and rice (Wiang Sa District Agricultural Extension Office [DAEO], 2012).

These agricultural activities, especially rice cultivation, engage an intensive and varying degree of agrochemicals utilization including pesticides and fertilizers for enhancing crops. It has been reported that 1,274,100 kg of agrochemicals was imported to Nan Province annually. These agrochemicals included herbicides (1,172,700 kg; 92.04%), chemicals for plant diseases (56,600 kg; 4.44%) and insecticides (44,800 kg; 3.52%) (Chanphong, 2008; Figure 2.1)

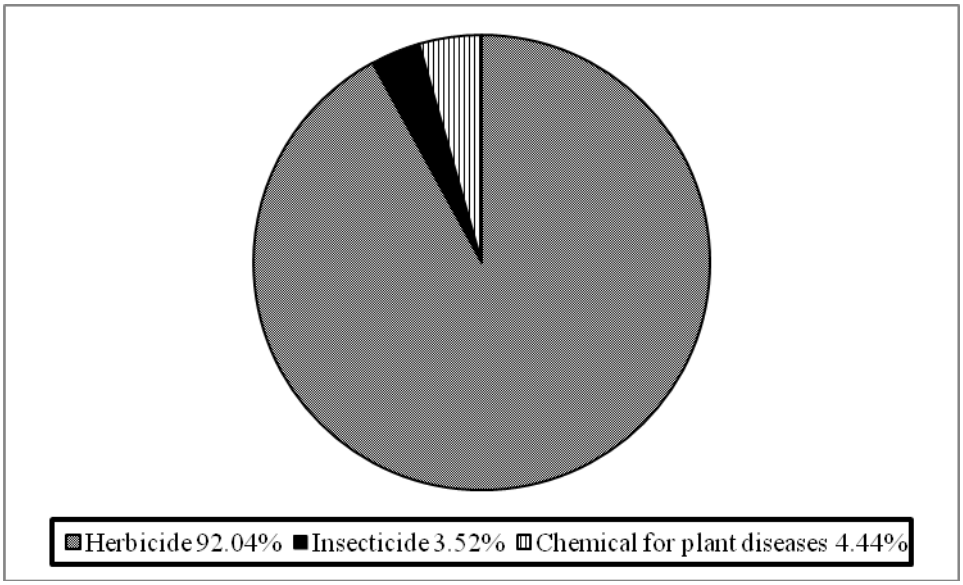


Figure 2.1 Percentage of agrochemicals imported to Nan Province in 2008 (Chanphong, 2008)

The common herbicides used in the rice fields were glyphosate and paraquat (Panuwet et al., 2012 and Wongwichit et al., 2012). In addition, data from field survey at Wiang Sa District also showed that atrazine was also used in the rice fields together with glyphosate and paraquat. Utilization of these herbicides was usually employed intensively as herbicide-mixture depending on crop cycle in each year.

2.2 Herbicides Property

2.2.1 Atrazine

Atrazine is a systemic triazine herbicide with the chemical name of 6-chloro-N2-ethyl-N4-isopropyl-1,3,5-triazine-2,4 diamine (Figure 2.2). Atrazine is a common herbicide used to control broad leaf weed by inhibiting photosynthesis via competition with plastoquinone II at its binding site in the electron transport process in photosystem II (Devine et al., 1993). Atrazine is the most generally used herbicide in the USA (Hayes et al., 2002). However, it has been reported that atrazine is an

endocrine disrupting chemical (EDC) capable of interference with function of sex hormones in all class of vertebrate (Hayes et al., 2011).

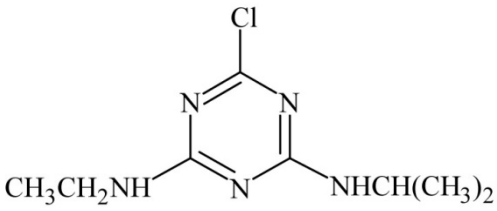


Figure 2.2 Structural formula of atrazine

The most important degradation pathway of atrazine is microbial decomposition in oxygenated conditions. Atrazine is slowly degraded in water due to its ability to defend from microbial degradation with its s-triazine ring (Solomon et al., 1996). It can resist direct aqueous photolysis and abiotic hydrolysis. As the result, atrazine has a relatively long (1-2 years) half-life in water. Atrazine is more stable and less preventive from microorganism in soil than water. As the result, its half-life in soil is only about 3-4 months (US EPA, 2003b). Because of the relatively low adsorption in soil of atrazine, it can be leached from soil into surface and groundwater. In Thailand environment, it has been reported that the half-life of atrazine is more than 60 days and it can contaminate for upto 7-8 months in soil at agricultural area (Suvanakhetnikhom, 2004).

Degradation products of atrazine in water are hydroxyatrazine and desethylatrazine which could be detected frequently in ground water at low levels (Plimmer, 2001). While degradation products of atrazine in soil are 2-chloro-4-amino-6-isopropylamino-1,3,5-triazine, 2-chloro-4-ethylamino-6-amino-1,3,5-triazine, 2-chloro-4-amino-6-amino-1,3,5-triazine, 2-hydroxy-4-ethylamino-6-

isopropylamino-1,3,5-triazine, and the main metabolite, 2-hydroxy-4-amino-6-isopropylamino-1,3,5-triazine (WHO, 1996).

Atrazine not only affects on all class of vertebrate, but it also affects on many invertebrates in varying degree of adverse effect. Low concentration of atrazine (20 µg/L) has been linked with adverse effects on freshwater fauna, including benthic insects (Dewey, 1986). Some effects were considered indirect. For example, richness of benthic insect species was declined significantly with atrazine addition (Dewey, 1986). A marine copepod (*Acartia tonsa*) was the most sensitive aquatic animal tested against atrazine with a 96-hour LC-50 of 94 µg/L. In addition, survival rate of some crustaceans, especially *Daphnia* sp., is sensitive for long-term chronic exposure of atrazine (Macek et al., 1976).

Atrazine is rapidly degraded in box crabs (*Sesarma cinereum*) feeding on smooth cord grass (*Spartina alterniflora*) grown in radiolabeled atrazine solution. After 10 days, only 1.2% of the total radioactivity in the crab was unchanged atrazine, compared to 24% in the food source. The accumulation of water-soluble atrazine metabolites (86% of total radioactivity) in *Sesarma* sp. suggested that glutathione conjugation, or a comparable pathway, was responsible for the complete degradation and detoxification of atrazine in crabs (Davis et al., 1979; Pillai et al., 1979). Atrazine does not appear to be a serious threat to crabs in Chesapeake Bay, where water concentrations of 2.5 µg/L atrazine have been recorded. However, it could have an indirect effect on crabs by decreasing the algal population which composes a portion of their diet (Plumley et al., 1980).

2.2.2 Glyphosate

Glyphosate is an herbicide which is well known as Roundup® in commercial name. Its chemical name is N-phosphonomethyl glycine. Glyphosate herbicide was ranked as the most imported herbicide in Thailand in 2012 (Office of Agriculture Regulation; OAR, 2012). Glyphosate is generally applied to suppress annual and perennial weeds. Glyphosate inhibits the enzyme 5-enolpyruvyl shikimate-3-P synthetase in synthesis of essential aromatic amino acids in plants and some microorganisms (Devine et al., 1993).

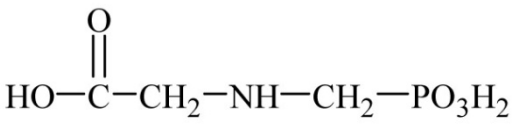


Figure 2.3 Structural formula of glyphosate

Glyphosate is well soluble in water at 15,700 mg/L at 25 ºC. It could be degraded by some microbes. Although glyphosate is highly water solubility, it has a very low rate of movement into groundwater. Most residue of glyphosate usually contaminate in surface waters. Because of glyphosate's hydrolysis and photolysis resistance in aquatic environment, it has longer half-life in water than in soil (Moore et al, 1987). The half-life of glyphosate in water is about 60 days comparing to 7 days in soil (US EPA., 1993). Soil microorganisms can degrade glyphosate to aminomethylphonic acid derivative and use it as a source of carbon, nitrogen and phosphorus (Petit et al., 1995). As a result, glyphosate is generally degraded in soil.

Although glyphosate has been reported to be slightly toxic to aquatic animals, some formula of glyphosate is reported to show very high toxicity to variety of aquatic fauna. Toxicity of the formulated product MONO818 was reported to be

toxic to the invertebrate *Daphnia magna* (US EPA, 1993). Chronic toxicity to aquatic invertebrates (*Daphnia magna*) includes a decrease in the mean number of young/adult/reproductive day in the upper three concentration levels (96, 186, 378 mg/L; Monsanto Co., 1995).

2.2.3 Paraquat

Paraquat dichloride is classified as bipyridylniums. It is one of the most extensively used herbicides in the world (Eisler, 1990). Its chemical formula is N,N′-dimethyl-4,4′-bipyridinium dichloride. Paraquat destroys cell membranes and inhibits photosynthesis by contacting to leaves of weed, without residual soil activity. Un-contacted weed can regrowth with undamaged parts of perennials. Paraquat is used for controlling both terrestrial and aquatic weeds.

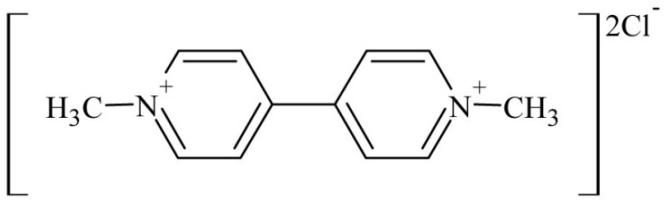


Figure 2.4 Structural formula of paraquat

Paraquat can dissolve well in water with solubility of 620,000 mg/L (US EPA. 1997). It has been usually found in both surface waters and groundwater. Although paraquat is rapidly photodegraded in water by UV light and also biodegraded by aquatic microorganisms with the resulted half-life of several weeks (Eisler, 1990), there was a review that a half-life of paraquat could be between 2 and 820 years depending on sunlight and depth of water (Watts, 2011). Some of paraquat can be degraded by microorganism, but most of paraquat is degraded by UV light. The

derivatives of paraquat degraded by UV light are 1-methy-4-carbox-pyridinium ion, 4-picolinic acid, hydroxyl-4-picolinic acid, N-formylglycone, malic acid and oxalic acid (Eisler, 1990). In contrast, paraquat is very strong and stable to binds with soil particles. It tends to remain stable for a long time in an inactive state (Eisler, 1990). Unlike situation in water, a half-life of paraquat in soil accounts for many years (Watts, 2011). However, there was a report that paraquat on top soil can be rapidly degraded in similar rate to paraquat in water (Eisler, 1990).

Assessment of a paraquat toxicity on several freshwater invertebrates suggested that paraquat is moderately toxic to *Daphnia magna*, amphipods, stoneflies, mayflies, or midges (US EPA, 1997). Most studies have demonstrated that paraqaut could induce oxidative stress by generating reactive oxygen species (ROS; Gómez-Mendikute and Cajaraville, 2003; El-Shenawy, 2009; Lushchak et al., 2009)

Table 2.1 Properties of herbicides, their half-life, maximum residue limit of herbicides in drinking water and meat, and reported value for herbicide contamination in soil and water in USA

	Atrazine	Glyphosate	Paraquat
Properties - Compound - Melting point - Water solubility	 Triazine 172-175 °C 33 mg/L at 25°C	 Organophosphorous 200 °C 12 g/L at 25°C	 Bipyridylium 175-180 °C 561 g/L at 25°C
Half-life in water	578 days	7-14 days	7-30 days
Half-life in soil	13-161 days	60 days	More than 10 years
Reference	US EPA, 2003b	Petit et al., 1995	US EPA, 1993
Maximum contaminant level in drinking water	0.003 mg/L	0.7 mg/L	1.0 mg/L
Reference	US EPA, 2009	US EPA, 2009	Hemilton et al., 2003
Maximum residue limit in food	0.04 mg/kg	0.05 mg/kg	0.005 mg/kg
Reference	HCPMRA, 2010	CODEX, 2006	CODEX, 2006

2.3 Animal Sentinel System

An animal sentinel system is a system that data on animals exposed to contaminations in the environment are regularly and systematically collected and analyzed to identify potential health hazards to other animals or human. Sentinel system may be classified according to what they designed to monitor such as exposure or effect, the types of animals used, the environment in question, or whether the animals are in their natural habitat or are purposely placed in an environment in question (National Research Council/NRC, 1991).

Sentinel system may be designed to reveal environment contamination, to monitor contamination of the food chain, or to investigate the bioavailability of contamination from environmental media. These types of systems can be designed to facilitate assessment of health hazards resulting from such exposure e.g. to provide early warning of human health risks or can involve deliberate placement of sentinel animals at a selected to permit measurement of environmental health hazards. Some sentinel system can be used to indicate both exposure and hazards (NRC, 1991).

Companion animals, domestic livestock, laboratory rodents, and free ranging or captive wild animals and fish are all potentially useful for sentinel systems. Animal can be used to monitor any type of environment, including home, work places, farms, and natural ecosystems. They can be observed in their natural habitats or placed in work places or site of suspected contamination (NRC, 1991).

Various species of animals (domestic, wild, and exotic animals) are potentially useful as animal sentinels. Several attributes of an animal contribute to its suitability as a sentinel species could be listed as follows:

1. A sentinel species should have a territory or home range that overlaps the area to be monitored. If a small and discrete location, such as a hazardous-waste site, is to be monitored, it would not be appropriate to use an animal that range over many square kilometers and visits the site only occasionally or an animal that visits several contaminated site (NRC, 1991).

2. A sentinel species should be easily enumerated and captured. For example, small mammals, such as mice and voles, are easier to capture than large mammals, and their population characteristics and dynamics are easier to assess over a short period. The size of an animal can be important in itself. If an animal is large enough,

various types of monitoring devices can be attached to transmit radio signal to indicate location, allow determination of whether the animal is alive, or permit collect of data on physiologic characteristics and exposure (NRC, 1991).

3. A sentinel species must have sufficient population size and density to permit enumeration. Rare species might not be suitable to employ for sentinel species, because they are difficult to locate for capture. The population size of a sentinel species should be large enough to monitor the adverse effect (NRC, 1991).

4. A sentinel species should have a measurable response (including accumulation of tissue residues) to the agent or class of agents in question. The animal might be exquisitely sensitive to an agent, or undergo physiological or behavioral changes in response to the agent (NRC, 1991).

2.4 Biomarker

In animal sentinel system, it is necessary to measure biological changes in sentinel species at different levels such as morphological, physiological, or biochemical changes in relation to toxicant contamination in the environment and use these changes as biomarkers (National Research Council, 1991).

Biomarker is broadly defined as an indicator signaling events in biologic system. It has been classified as biomarker of exposure, biomarker of effect and biomarker of susceptibility (NAS/NRC, 1989).

A biomarker of exposure is a xenobiotic compound or its metabolite or the byproduct of an interaction between the xenobiotic substances and target molecule or cell which is measure within the exposed organism.

A biomarker of effect is classified as any measurable physiology, biochemistry, pathology or other alternation with the exposed organism that can be recognized as an established or potential health impairment or disease.

A biomarker of susceptibility is an indicator of an innate or acquired limitation of an organism's ability to respond to the specific exposure of xenobiotic substance.

Biotransformation of xenobiotic substance is an important process in animal body and can be used as a biomarker of exposure or biomarker of effect (Kappus, 1986). Phases I of the 2-phase biotransformation process involves oxidation of organic xenobiotics and generation of superoxide radical via a redox process (Boutet et al., 2004). The resulted superoxide radical can be dissolved in water and eliminated out of the body. However, this radical may directly attack and damage cell membrane. Thus, an antioxidant defense system is needed to protect biomolecules from these harmful effects. Phase II biotransformation involves conjugation of the toxic superoxide xenobiotics with antioxidant substrates e.g. glutathione, ascorbic acid, uric acid and vitamin E by an aid of antioxidant enzymes such as catalase and glutathione *S*-transferase (GST) (Stoeckel et al., 2008). This causes the conjugated xenobiotics to become less harmful and can be eliminated out of animal body in water soluble forms. Therefore, changes in factors involving in biotransformation processes are expected in animals living in xenobiotics contaminated environment.

2.4.1 Glutathione *S*-transferase (GST)

GST is a multigene family of isoenzymes that catalyze the conjugation of electrophilic compounds to GSH (glutathione). The diversity of substrates accommodated by GST is a result of both the relatively non-specific nature of the

binding site for the hydrophobic substrate and the existence of numerous isoforms of GST (Salinas and Wong, 1999). GST metabolize carcinogens, environmental pollutants, drugs and a broad spectrum of other xenobiotics. Although microsomal forms of GST have been detected (Morgenstein and DePierre, 1988), GST activity is mainly occurred in the cytosol.

GST plays roles as a major endogenous detoxifying enzyme to defense against oxidative stress. As some reactive oxygen species (ROS) such as hydroxyalkenals and basepropenals, breakdown products of lipid peroxidation or DNA hydroperoxides, are harmful (Hayes and Pulford, 1995), GST can react directly to ROS via a sensitive SH-group. Therefore, this enzyme has a specific role in oxidative stress and has been used as markers of effect (Xia et al., 1996).

2.4.2 Lipid peroxidation (LPO)

Lipid peroxidation can be a major contributor to the loss of cell function under oxidative stress situations. It has been reported that peroxidation leads to calcium release and uncontrolled activation of calcium-dependent proteases and lipases in microsomal membranes. Bindoi (1988) has suggested that peroxidation and permeabilization of mitochondrial membranes can induce disruption of cellular energetic. Moreover, accumulation of lipoperoxidation products in mammalian pathologic conditions can indicate the participation of oxygen radicals in the abnormal molecular mechanisms.

Most toxic xenobiotic compounds, such as metals, pesticide, etc, are well known as the toxic componds with oxidative stress generation and augmenting of LPO levels (Viarengo et al., 1999; Choi et al., 2000). $R^{\bullet}$ is an initiator radical (such

as $^{\bullet}$OH). It is able to attach to lipid structure on cell membrane and change the structure of lipid to be LOO$^{\bullet}$, a lipid peroxide radical (Orrenius et al, 1990). This structure of LOO$^{\bullet}$ is regarded to be an unstable configuration on cell membrane that could result in cellular collapsing. Cellular and ultrastructure study of Orrenius et al. (1990) revealed pathological damage linking to LPO levels. When the enzymatic defensive (vitamin C, superoxide, GST etc) is limited, the free redical generated by toxic xenobiotics could damage to macromolecular level (Monserrat et al, 2006). As a result, lipid peroxidation has been extensively used as a marker of effect.

2.5 Rice Field Crab as a Sentinel Species

Classification of the rice field crab is as follows:

Kingdom Animalia

Phylum Arthropoda

Subphylum Crustacea

Class Crustacean

Superorder Brachyura

Order Decapoda

Family Gecarcinucidae

Genus Esanthelphusa

Species *Esanthelphusa nani (*Naiyanetr, 1984*)*

Figure 2.5 Rice field crab *Esanthelphusa nani* (Naiyanetr, 1984)

Esanthelphusa nani, a rice field crab, was firstly described based on specimens caught at Nan Province (Naiyanetr, 1984). Its carapace is quite smooth and broad with epigastric cristae and separated by Y-shaped groove. There are 4 epibranchial separating from epigastric cristae tooth that is domain characteristics of *Parathelphusa*. Postorbital cristae end beyond outer edge of distinctly sharp postorbital cristae like a H-shaped depression. Third maxilliped with flagellum is longer than width of merus. Strongly asymmetrical characteristics of chelipeds (major claw and minor claw) is occurred in larger males. There is strong sharp carpus inner distal spine of the chelipeds. There is a T-shaped segment 7 with lateral margins slightly concave (neck-like appearance) in male abdomen.

The rice field crab shows several attributes of a sentinel species as suggested by the National Research Council (NRC, 1991). The crab live and forage in the rice field habitat. It could be exposed with a group of herbicides that applied in the rice field. Crab is easily enumerated and captured in dusk time during wet season. In the dry season, it inhabits in the ground with distinct hole location. The crab shows long life span of upto several years with relatively fast reproductive maturity. Therefore,

population size of the crab can be increased every year (Doolgindachbaporn, 2001), and should be sufficient for sentinel study. The crabs can also respond to various classes of xenobiotics (MacFarlane et al., 2000; Monserrat et al., 2006). Moreover, the rice field crab is also used for human consumption by local farmer, making it an important link of contaminant transfer through the food chain.

CHAPTER III

HERBICIDE CONTAMINATION IN ENVIRONMENTAL SAMPLES AND TISSUE OF THE RICE FIELD CRABS LIVING IN AGRICULTURAL AREAS WITH DIFFERENT DEGREE OF HERBICIDE UTILIZATION

3.1 Introduction

Agriculture is a common activity in Nan Province, northern part of Thailand. A variety of agrochemicals such as fertilizers, hormones and pesticides has been employed to enhance yield of cultivation. Herbicides, especially atrazine, glyphosate and paraquat, have been widely used in this area. Although agrochemicals result in excellent yield, most of them can be contaminated in environment after intensive or frequent uses. With its wide array of half-life, intensive utilization of atrazine, glyphosate and paraquat could lead to contamination in both water and soil (US EPA, 1993, 2003b; Petit et al., 1995).

Persistency of herbicides in the environment is relatively low, however the herbicides may cause chronic adverse effects to non-target organism, especially animals. It was previously reported that herbicides can directly pose adverse effects on both vertebrates such as inducing hermaphroditism and demasculinization in frog (Hayes et al., 2002) and invertebrates such as increased occurrence of intersex in population of *Daphnia* sp. (Stoeckel et al., 2008). It is thus important to monitor degree of herbicide contamination in environmental compartment as well as biological component of Nan Province.

Agricultural soil and water were used as the environmental compartment for screening of herbicide contamination. For biological component, a rice field crab *Esanthelphusa nani* (Naiyanetr, 1984), was used as a sentinel species for herbicide contamination in paddy fields of Nan Province since it is native to rice field

habitat with direct exposure to herbicide contaminated water and sediment through gill (gas exchange) and mouth (detritivore).

Objectives of this study were to 1) screen for herbicide contamination in soil and water of rice fields with different degree of herbicide utilization, and 2) determine herbicide contamination levels of the rice field crab inhabiting these two study sites.

3.2 Materials and Methods

3.2.1 Study sites

Two rice fields in Wiang Sa District, Nan Province were chosen as study sites based on their similarities in geographic locations, weather conditions, agricultural activities and presence of *E. nani*. A potential contaminated site (location: 47Q 068772, UTM 2054283) is a rice field in San Sub-district with intensive herbicide utilization, while a reference site (location: 47Q 0686779, UTM 2047187) is a rice field in Lai-nan Sub-district with no history of herbicide usage for 7 years.

3.2.2 Soil and water collection

Collection of soil and water were carried out every 3 month in July 2010, October 2010, January 2011 and April 2011. At both sites, 1 kg of composited soil sample collected from the top 10 cm layer of the rice field was collectedand kept in acetone-rinsed high density polyethylene plastic boxes. Composited water sample (2 L) was collected from surface water of the field in July and October 2010 and collected from surface water of irrigation canal in January and April 2011 when water was not available in the field. The water samples were kept in acetone-rinsed high density polyethylene plastic bottles. After collection, environmental samples were covered with aluminum foil and kept at 4 ^{o}C until delivery to a laboratory for analysis. Herbicide residues in soil, sediment and water were screened by chromatographic

techniques by Central Laboratory (Thailand) Co., Ltd., an ISO/IEC 17025 accredited institutes for food testing by the National Bureau of Laboratory Quality Standards.

3.2.3 Crab collection

Ten crabs (5 male and 5 female crabs with carapace width > 30 mm.) were caught from each study site by visual encounter survey during night time (from dusk until 11 PM). The crab sample collections were carried out on monthly basis from July 2010 to June 2011. Crab samples were transported to a laboratory at the Chulalongkorn University Forest and Research station, Wiang Sa District, Nan Province. Then the crabs were euthanized in ice slurry and cut into 2 parts using the line of symmetry as an incision line. The left part of the body was used for contamination analysis (this chapter) and the right part of the body was used for enzymatic and cellular response analysis (Chapter IV).

3.2.4 Determination of herbicides in crab body

The crab samples were stored at -20 °C until contamination analysis of herbicide. The frozen crab samples were lyophilied to complete dryness in a freeze drier (FreeZone® Model 7753501). Individual crab was grinded into powder with a blender. Overview of chromatographic and enzyme-linked immunosorbent (ELISA) methods for determination of these herbicides is listed as follows.

3.2.4.1 Determination of atrazine

3.2.4.1.1 Determination of atrazine in environmental sample

Screening method for atrazine in environmental sample was carried out according to an in house method of Central Laboratory (Thailand) Co., Ltd. as follows. Ten grams of wet soil sample treated with 10 g of NaCl and 10 mL of deionized water was extracted with 10 mL of acetronitrile before addition with 2 g of

anhydrous magnesium sulfate ($MgSO_4$). The extracted sample was centrifuge at 1,560 xg (Heraeus®, Megafuge® 1.0R) at 5 ºC for 5 min. Only 5 mL of supernatant was transferred to evaporation under stream of nitrogen gas. The content was adjusted to 2 mL with ethyl acetate and subjected to treatment with 0.5 grams of anhydrous $MgSO_4$ and 0.5 g of primary secondary amine (PSA). After precipitation, the upper part of solution was filtered through 0.22 μm syringe filter before further analysis.

Water sample (500 mL) was pre-treated with 20 g of sodium chloride (NaCl) and subjected to extraction with dichloromethane (CH_2Cl_2) for three times (100: 50: 50 mL). After the extracted sample was dried up in an evaporator, the sample was adjusted to 2.5 mL of volume by ethyl acetate before further analysis.

Residue of atrazine in the extracted sample was quantified by gas chromatography-mass spectrometry (GC-MS; Agilent Technologies 6890 N) using Mass Selective Detector (MSD) and a DB-5ms capillary column (0.25 μm internal diameter x 30 m length, 0.25 μm film thickness) with injection volume of 2 μL and flow rate of 1.1 mL/min. The injector was initially set at 210 ºC in 10.69 Psi. The oven temperature was initially set at 80 ºC in 2 min, and then it was programmed to increase up to 280 ºC with rate of 14 ºC/min and held for 10 min. The total run time was 31 min. The limit of detection (LOD) for atrazine residue was 0.01 mg/kg in soil and 0.01 mg/L in water.

3.2.4.1.2 Determination of atrazine in crab tissue

Extraction protocol for crab tissue was modified from the extraction of mussel tissue according to Jacomini et al. (2003). A hundred milligrams of crab powder sample was reconstituted with 1 mL deionized water, extracted with 200 μL of 1.5 M

NaOH and 4 mL of dichloromethane, and shaken vigorously for 20 min. Extracted sample was centrifuged at 1,800 xg for 5 min at room temperature. Three milliliters of organic phase was transferred into a clean tube. After the extracted sample was dried up in an evaporator under stream of N_2, the sample was reconstituted with 100 μL of methanol and shaken vigorously. Finally, the reconstituted sample was added with deionized water to get 1 mL of final volume and mixed thoroughly before stored at 4^{o}C until assay for atrazine. A recovery sample was spiked with 5 ng of atrazine and processed using the above extraction procedure.

Atrazine contamination of the crab tissue was determinated by an enzyme-linked immunosorbent (ELISA) assay developed by Abraxis. A triazine-antibody coated microtiter plate was brought to room temperature before use. A 25 μL aliquot of assay buffer was added into each individual well of the plate using a muti-channel pipette. Twenty five microliters of standard (0, 0.05, 0.10, 0.25, 1.0, 2.5 and 5.0 ng/mL), sample and control were added in duplicate into the wells. A 50 μL aliquot of enzyme conjugate solution (triazine-horseradish peroxidase-conjugate) was added to the individual wells successively using a muti-channel pipette, and the plate was incubated on an orbital shaker (PSU 2-T mini shaker) for 30 min at room temperature. The microtiter plate was washed with 250 μL of washing buffer in each well for 3 times. Remaining buffer in the well was removed by patting the plate dry on a paper towel. The plate was added with 100 μL of substrate solution (hydrogen peroxide and a chromogen: 3,3',5,5'- tetramethylbenzidine; TMB) to each well using a muti-channel pipette. The plate was incubated on horizontal shaker for 15 min at room temperature. After incubation, 50 μL of diluted sulfuric acid was added to stop

reaction in the wells. Finally, the ELISA plate was read for absorbance at 450 nm by the Multiskan EX microplate reader.

Mean absorbance value was calculated for each of the atrazine standards. Then, the mean absorbance was used for calculating percentage bound (%B/B0) of atrazine to antibody on plate by dividing with the mean absorbance value of zero standard. Percentage bound (%B/B0) of standards were used to construct a standard calibration curve by plotting the %B/B0 of each standard on a vertical linear (Y) axis versus the corresponding atrazine concentration on horizontal logarithmic (X) axis using Microsoft Excel. Concentrations of samples were determined from the linear regression equation of the standard curve. Detection limit of the kit for atrazine was 0.04 ng/mL (90% B/B0) or calculated to be 0.53 µg/kg dry weight of crab sample. The recovery of atrazine extraction in the atrazine-spiked crab sample was 95.79%.

3.2.4.2 Determination of glyphosate

3.2.4.2.1 Determination of glyphosate in environmental sample

The extraction method of environmental sample for glyphosate was modified from Börjesson and Torstensson (2000). Ten grams of soil sample was extracted twice with 25 µL of 1M NaOH, centrifugated 4,333 xg (Heraeus®, Megafuge® 1.0R) for 30 min. The supernatant was filtered through a F1 Whatman filter. The pooled extracts was treated with 4.2 µL of concentrated HCl, diluted with water and adjusted to pH 2.0. After 1 hour of incubation at room temperature, a clear upper part of extracts was subjected to similar treatments to water sample as follows.

Water sample (200 mL) was adjusted to pH 2.0 and subjected to ion-exchange and clean-up in Chelex 100 column followed by AG1-X8 column. Then, the sample was evaporated to dryness under vacuum and sequentially treated with water and

water-methanol-HCl (160: 40: 2.7) between each evaporation cycle. After the last evaporation under stream of nitrogen, sample was derivatized for 1 hour at 100 °C after treatment with trifluoacetic anhydride and trifluoroethanol. Finally, the sample was evaporated under nitrogen and redissolved in ethyl acetate prior to analysis.

Residue of glyphosate in extracted sample was determined by high performance liquids chromatography (Agilent HPLC 1100 series) with post-column derivatizer (Pickering PCX 5200) and silica hydrophilic interaction chromatography column (silica HILIC; Atlantis; 2.1 mm internal diameter x 150 mm length and 3 μm film thickness). The injection volume was 20 μL with column flow rate at 0.2 mL/min and post-column deriveatizer. The mobile phase was 100 mM ammonium formate pH 7.3 and acetonitrile (60:40 v/v) with column temperature setting at 40 ºC. The stop time was 12 min and post time was 3 min. The limit of detection (LOD) for glyphosate residue was 0.01 mg/kg in soil and 0.005 mg/L in water.

3.2.4.2.2 Determination of glyphosate in crab tissue

The glyphosate extraction protocol of crab tissue was modified from the procedure of Alferness and Iwata (1994). One hundred milligrams of crab sample was reconstituted with 200 μL of deionized water, and shaken vigorously. The reconstituted sample was extracted with 100 μL chloroform and 400 μL 0.1 N HCl, and shaken vigorously for 5 min. The mixed sample was treated with 0.004 gram sodium sulfate and shaken strongly for a few min before centrifuged at 1,000 xg for 10 min at room temperature. The supernatant (400 μL) was transferred to a new microtube and added with 400 μL chloroform, and shaken vigorously for 5 min. The mixture was centrifuged at 1,000 xg for 10 min at room temperature. A 350 μL of supernatant in aqueous phase was transferred to a new microtube, adjusted to pH 7.0

with 9 μL of 1 N HCl and stored at 4^{o}C until ELISA assay of glyphosate. A recovery sample was prepared by reconstituting sample with 200 μL of 4 ng/mL glyphosate solution (0.8 nanogram glyphosate equivalent) before processed with above extraction method.

Glyphosate levels of standards, control and crab samples were determined by enzyme-linked immunosorbent assay (ELISA) kit of Abraxis. Initially, all standards, control and extracted crab samples had to be derivatized in order to enable an attachment to a specific glyphosate antibody of the kit. Two hundreds and fifty microliters of standards or samples was mixed with 100 μL of diluted derivatization reagent, and incubated at room temperature for 10 min.

Microtiter plate coated with goat-anti rabbit antibody was prepared to room temperature before use. Fifty microliters of anti-glyphosate antibody solution was added to each well of the microtiter plate. Then, 50 μL of the derivatized standard (0, 0.75, 0.2, 0.5, 1.0 and 4.0 ng/mL), control and crab samples were added in duplicate into each well of the plate. The microtiter plate was covered with parafilm and incubated at room temperature for 30 min on an orbital shaker. After incubation, 50 μL of enzyme conjugate solution (horseradish peroxidase; HRP labeled glyphosate analog dilute in a buffered solution) was added to the individual wells, and the plate was incubated at room temperature for 60 min on the orbital shaker. After the incubation, the content in the well was removed, and the well was washed with 250 μL of washing buffer for 3 times. After the last wash, the plate was patting dry on stack of paper towels. Next a 150 μL solution of hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine in organic base was successively added to each well to generate color. After 20 min of incubation, a 100 μL of diluted sulfuric acid was added to each

well to stop the reaction of color development. Finally, the microplate reader was employed to read absorbance of the color in the wells at 450 nm within 15 min after adding the stopping solution.

Mean absorbance value was calculated for each standard glyphosate. The mean absorbance was used for calculating percentage bound (%B/B0) of glyphosate to antibody on plate by dividing with the mean absorbance value of zero standard. Percentage bound (%B/B0) for each standard was used to construct a standard calibration curve by plotting the %B/B0 for each standard on vertical linear (Y) axis versus the corresponding glyphosate concentration on horizontal log (X) axis with Microsoft Excel. Concentrations of samples were determined from the linear regression equation of the standard curve. The limit of detection for glyphosate ELISA is 0.05 ppb or equivalent to 0.45 µg/kg dry weight of crab sample. The recovery of glyphosate extraction in glyphosate-spiked crab sample was 14.71%.

3.2.4.3 Determination of paraquat

3.2.4.3.1 Determination of paraquat in environmental sample

Screening method for paraquat in environmental sample was carried out according to an in house method of Central Laboratory (Thailand) Co., Ltd. as follows. Soil sample (25 g) was treated with deionized water, octane-2, sulfuric acid and glass bead before refluxed in close system for 5 hours. After cool down, the sample was filtered through filter paper and Celite before cleaned up through solid phase extraction (SPE) silica. The sample was evaporated to dryness and adjusted to volume with mixture of 100 mM ammonium formate pH 3.7 and acetronitrile (3:2). The sample was filtered through syringe nylon filter (0.22 µm) before further analysis.

Water sample (100 mL) was adjusted to pH 9.0, cleaned up through solid phase extraction (SPE) silica and then stored in a mixture of hydrochloric acid and methanol (9:1). After that, the sample was evaporated to dryness and adjusted to volume with mixture of 100 mM ammonium formate pH 3.7 and acetronitrile (3:2). The sample was filtered through syringe nylon filter (0.22 μm) prior to analysis. Residue of paraquat was quantified by high performance liquids chromatography with diode array detector (HPLC-DAD; Agilent 1100) and an Atlantis HILIC silica column (3μm film thickness, 2.1 internal diameter x 150 mm length) with injection volume of 10 μL and flow rate of 0.2 mL/min. The limit of detection (LOD) for paraquat was and 0.01 mg/kg for soil and 0.01 mg/L for water.

3.2.4.3.2 Determination of paraquat in crab tissue

Extraction protocol for paraquat in crab sample was modified from the procedure of Brown et al. (1996) and Quick et al. (1990). One hundred milligrams of crab sample was added with 200 μL deionized water and then shaken vigorously for 1 minute by Vortex mixer. The reconstituted sample was added extracted with 200 μL of hexane and 600 μL of 10% trichloroacetic acid (TCA) before shaken vigorously for 5 min. The mixed sample was centrifuged at 2,000 xg for 15 min at room temperature. An aqueous phase (550 μL) of the extracted sample was transferred to a new microtube. The sample pellet was re-extracted with 250 μL of 10% TCA and shaken vigorously for 5 min. The extracted sample was centrifuged at 2,000 xg for 15 min at room temperature, and the aqueous phase (200 μL) was transferred and mixed with the first extraction to get 750 μL of total volume. Four hundreds microliters of hexane was added to the tube in order to remove fat in the sample, and the tube was shaken vigorously for 5 min. The mixture was centrifuged at 2,000 xg for 15 min at

room temperature. Only 700 µL of aqueous phase was collected and added with 300 µL of 2M Tris-hydrochloric acid buffer (pH 7.0) to adjust the pH to 7.0. A recovery sample was spiked with 1.5 ng of standard paraquat and processed with the same procedure above.

Concentration of paraquat in crab tissue was determined by paraquat ELISA kit of Abnova. Initially, a 96-well microtiter plate coated with rabbit anti-paraquat antibodies and all reagents were adjusted to room temperature (25 ^{o}C) before use. Twenty five microliters of standard paraquat (0, 0.375, 0.75, 2.5 and 7.5 ng/mL) and the extracted-samples were added in duplicate into the microtiter plate. Next, 100 µL of paraquat-horseradish peroxidase conjugate was added into individual well. Then, the plate was covered with parafilm, mixed in a rapid circular motion for 30 seconds and incubated at room temperature for 30 min on an orbital shaker. After incubation, the plate was washed with 250 µL washing buffer for 3 times to remove the unbound conjugate. Then, 100 µL of substrate/color solution (hydrogen peroxide and a chromogen: 3,3',5,5'- tetramethylbenzidine; TMB) was added into the individual wells. After that the plate was covered with parafilm, mixed and incubated at room temperature for 15 min on the orbital shaker. After incubation, 100 µL of stop solution (3M of hydrochloric acid) was added into the wells. Finally, the plate was read for absorbance at 450 nm using a microplate ELISA reader.

After ELISA procedures, mean absorbance value was calculated for each standard paraquat. The mean absorbance was used for calculating percentage bound (%B/B0) of paraquat to antibody on plate by dividing with the mean absorbance value of zero standard. Percentage bound (%B/B0) for each standard was used to construct a standard calibration curve by plotting the %B/B0 for each standard on vertical linear

(Y) axis versus the corresponding paraquat concentration on horizontal log (X) axis with Microsoft Excel. Concentrations of paraquat in samples were determined from the linear regression equation of the standard curve. The recovery of extraction in the paraquat-spiked crab sample was 58.47%.

3.2.5 Data analysis of herbicide contamination in crab tissue

All contamination data of each sex and each site were assessed for normality of distribution by Shapiro test. The data passing the normality test was compared for sex-related difference in each site by Student *t*-test, while other data was compared by Mann-Whitney U-test. If the data showed no significant difference between sexes, the data of both sexes were combined into a new data set. Next step, the normal distributed data of each site was assessed for seasonal difference in each site by one-way ANOVA with Tukey's HSD Post Hoc test, while the other was assessed for seasonal difference by Kruskal-Wallis test. In case of no significant difference among month, the data of every season were grouped to new data set. Finally, the *t*-test / U-test was employed to test for site-related difference between the contaminated site and the reference site. Flowchart of analysis is showed in Figure 3.1.

3.2.6 Pearson's correlation analysis between contaminations

Pearson's correlation test was used to correlate between contaminants presented in crab tissue in each individual crab.

All statistical analysis's were performed using R version 2.15.2 (2012-10-26: Copyright© 2012 The R Foundation for Statistical Computing).

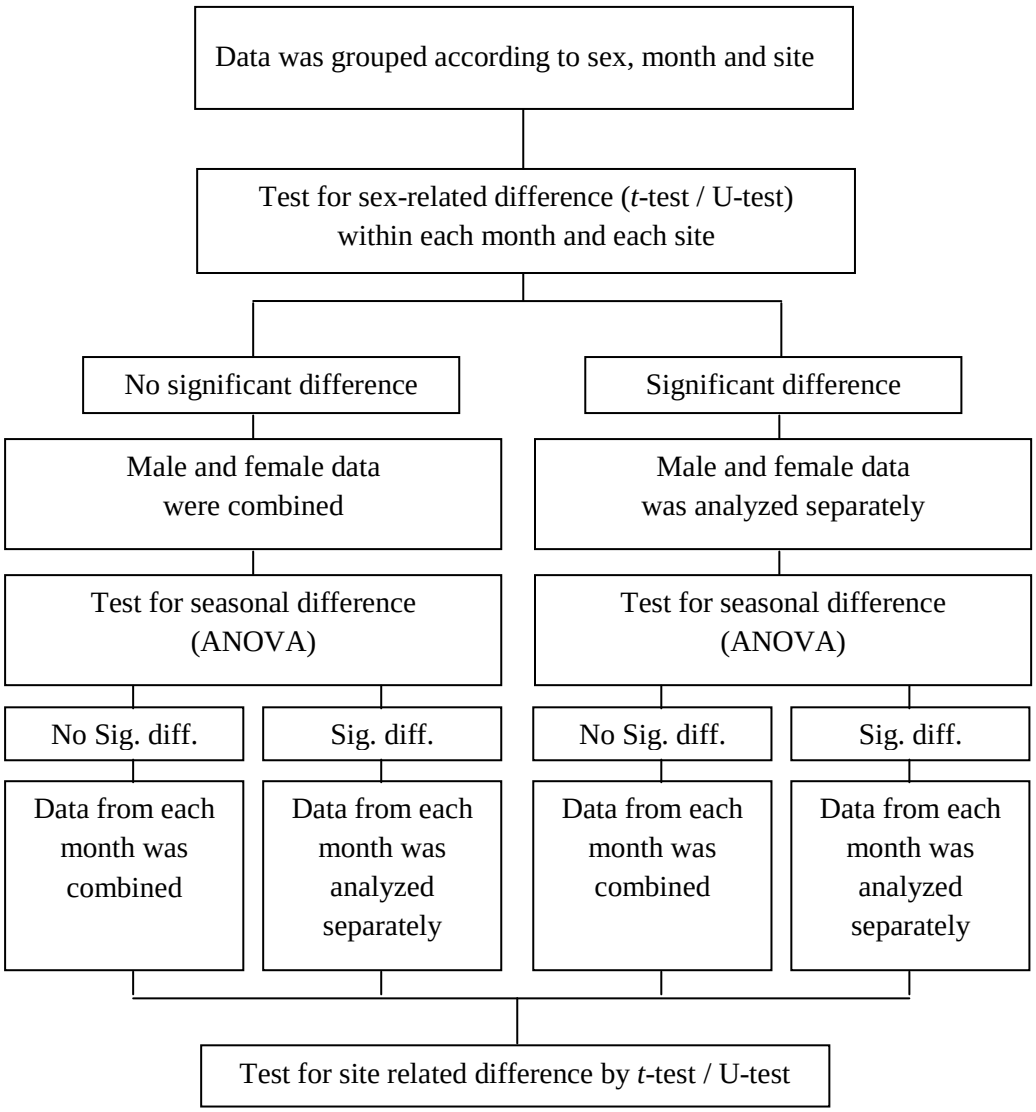


Figure 3.1 Flowchart of statistical analyses for this study

3.3 Result and Discussion

3.3.1 Contamination of atrazine

3.3.1.1 Environmental contamination of atrazine

The result of atrazine screening in water showed that 0.15 mg/L was found in contaminated site only in dry season (January 2011), or the beginning of crop cycle (Table 3.1), while screening in soil sample showed no detectable levels of atrazine. This is probably due to its rapid degradation, rapid transport in environment or limit of detection method. In natural environment, atrazine residues can be degraded by a large group of microorganisms. As the result, half-life of atrazine in soil can be as low as 13 days (US EPA, 2003b) and it can be rapidly degraded in open environment (Jones et al., 1982). In addition, during rice farming activities in wet season, paddy field water tended to constantly flowed from paddy field to nearby river by agricultural water runoff. So, atrazine residue could be rapidly transferred out of the application site (Anderson et al., 2002). Nevertheless, the detection of atrazine in the contaminated site water was an obvious evidence of intensive and constant atrazine utilization. In agricultural areas in USA where atrazine has been used intensively, contamination of atrazine at 1.068 mg/kg in soil (Douglas et al., 1993) and 0.000054-0.018 mg/L in water have been reported (Larson et al., 1995). It is of interest to note the much lower concentration found in water at the US agriculture area possibly due to difference in GC-MS detection limit (0.01 mg/L in this study vs. 0.005µg/L in Larson et al. (1995)).

Table 3.1 Atrazine contamination in environmental samples of the reference and the contaminated agricultural areas in Nan Province, Thailand during July 2010 to April 2011

Atrazine	Reference site (Lai-Nan District)		Contaminated site (San District)	
	Soil (mg/kg)	Water (mg/L)	Soil (mg/kg)	Water (mg/L)
Jul.-2010	<0.01	<0.01	<0.01	<0.01
Oct.-2010	<0.01	<0.01	<0.01	<0.01
Jan.-2011	<0.01	NA	<0.01	0.15
Apr.-2011	<0.01	<0.01	<0.01	<0.01

Note: Limit of detection was 0.01 mg/kg in soil and 0.01 mg/L in water
NA = Not available

3.3.1.2 Residue of atrazine in crab tissue

The result of ELISA for atrazine showed that residue of atrazine was found in tissue of crab from both reference and contaminated sites in the range of 2-20 µg/kg dry weight (Table 3.2 and Figure 3.3). Since the rice field crab was used as food for local farmers, this contamination could pose some risk for human consumption. Given the fact that the weight of freeze dried specimen was approximately 54.36% of the wet weight, the range of atrazine contamination in crab tissue could be estimated at 1.09 to 10.87 µg/kg wet weight. These levels of contamination were still lower than the maximum residue limited of atrazine in food (40 µg/kg wet weight; HCPMRA, 2010).

Table 3.2 Atrazine contamination in male and female crabs from the reference and the contaminated agricultural areas of Nan Province, Thailand during July 2010 to April 2011

Atrazine	Reference site (Lai-Nan District)		Contaminated site (San District)	
	Male (µg/kg)	Female (µg/kg)	Male (µg/kg)	Female (µg/kg)
Jul.-2010	6.52±2.93	7.49±3.17	12.29±0.29	8.24±4.98
Oct.-2010	5.68±1.67	8.52±3.84	9.52±2.99	18.81±2.90
Jan.-2011	3.35±1.13	6.56±0.74	6.18±1.22	7.85±1.75
Apr.-2011	9.20±3.61	8.20±5.06	14.73±4.33	10.44±3.06

Note: Limit of detection of analytical method was 0.53 µg/kg dry weight.
Recovery of extraction was 95.79%.

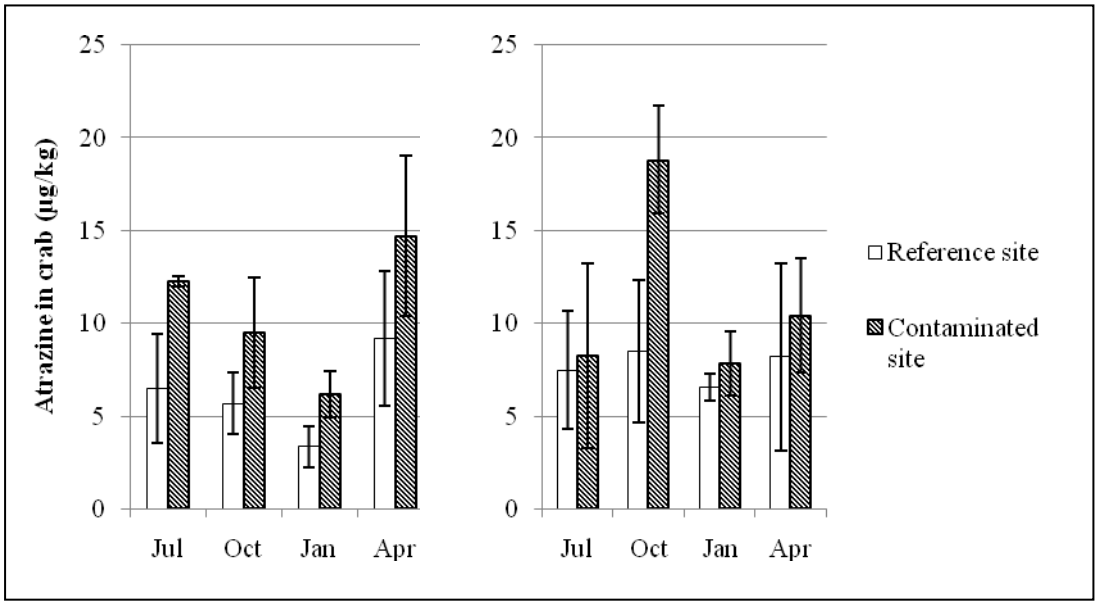


Figure 3.2 Atrazine contamination in male crab (left, n=3 each) and female crab (right, n=3 each) from the reference and contaminated sites during July 2010 to April 2011. There was no significant difference (*t*-test / U-test; $p > 0.05$) between sites.

Since there was no significant sex-related and seasonal difference in the atrazine residue data, comparison for site-related difference was made with the

combined data of two sexes and 4 months in each site. It was found that level of atrazine residue in tissue of crabs from the contaminated site was significantly higher than those of the reference site (Figure 3.3). Similar to mussel and fish (Jacomini et al., 2003; Gunkel and Streite, 1980), the rice field crab, *E. nani*, could be regarded as an important atrazine accumulator in agricultural environment. Moreover, the higher level of atrazine in crabs from the contaminated site could indicate the higher application of atrazine in agricultural activity in this area.

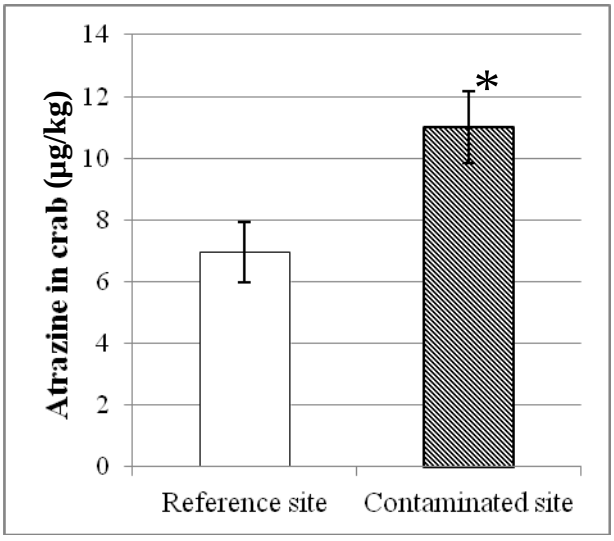


Figure 3.3 Atrazine contamination in tissue of crabs from the reference site (6.94±0.98 µg/kg dry weight, n=24) and the contaminated site (11.00±1.15 µg/kg dry weight, n=24) during July 2010 to April 2011. Significant difference between sites (U-test) was indicated by an asterisk (*).

3.3.2 Contamination of glyphosate

3.3.2.1 Environmental contamination of glyphosate

Screening for glyphosate in environment sample showed that the level in soil and water samples were below the limit of detection (Table 3.3). This could be due to the fast degradation time of glyphosate in nature, yielding half-life in soil of only 60 days (Petit et al., 1995). Although microbial degradation of glyphosate was harder than that of atrazine, glyphosate is more soluble in water than atrazine (WHO, 1994;

US EPA, 2006) causing it to be more transferable to river by agriculture run off during rice farming activities in wet season. As a result, the concentration of glyphosate contamination in study sites could be lower than the limit of detection (lower than 0.005 mg/L), but not indicating that the study sites were clean from glyphosate contamination.

Table 3.3 Glyphosate contamination in environmental samples of the reference and contaminated agricultural areas of Nan Province, Thailand during July 2010 to April 2011

Glyphosate	Reference site (Lai-Nan District)		Contaminated site (San District)	
	Soil (mg/kg)	Water (mg/L)	Soil (mg/kg)	Water (mg/L)
Jul.-2010	<0.01	<0.005	<0.01	<0.005
Oct.-2010	<0.01	<0.005	<0.01	<0.005
Jan.-2011	<0.01	NA	<0.01	<0.005
Apr.-2011	<0.01	<0.005	<0.01	<0.005

Note: Limit of detection was 0.01 mg/kg in soil and 0.005 mg/L in water
NA = Not available

3.3.2.2 Residue of glyphosate in crab tissue

The result of ELISA showed that glyphosate was contaminated in tissue of crabs from both the reference and the contaminated sites in the range of 0.70-2.90 µg/kg dry weight (Table 3.4). Although, the recovery of extraction was only 14.71%, the contamination levels were still more than the limit of detection for ELISA (0.45 µg/kg dry weight). Therefore, it is of importance to note that the actual concentration of glyphosate contamination in crab tissue could be much higher than these values. Given the fact that the weight of freeze dried specimen was approximately 54.36% of

the wet weight and the recovery of extraction was 14.71%, the range of glyphosate contamination in crab tissue could be estimated at 2.58 to 10.72 µg/kg wet weight. These levels of glyphosate contamination were still lower than the maximum residue limited of glyphosate in food (50 µg/kg wet weight, CODEX, 2006).

The data of glyphosate contamination in crab tissue showed both sex-related and seasonal differences. Therefore, these data were analyzed separately for each sex and season (Figure 3.4). There was no significant difference between sites in any period of this study. Since glyphosate has high solubility in water, it could be carried by rain, fog, as well as runoff from one agricultural area to another (US EPA, 1993). Detectable levels of glyphosate in crab tissue at both sites indicate that glyphosate utilization in these areas were widespread than expected.

Table 3.4 Glyphosate contamination in male and female crabs form the reference and the contaminated agricultural areas of Nan Province, Thailand during July 2010 to April 2011

Glyphosate	Reference site (Lai-Nan District)		Contaminated site (San District)	
	Male (µg/kg)	Female (µg/kg)	Male (µg/kg)	Female (µg/kg)
Jul.-2010	1.06±0.07	1.64±0.03	1.59±0.27	0.97±0.05
Oct.-2010	1.20±0.21	1.39±0.31	1.27±0.16	2.46±0.12
Jan.-2011	1.11±0.22	1.44±0.10	1.76±0.26	1.14±0.16
Apr.-2011	1.41±0.15	1.05±0.26	1.75±0.49	1.35±0.11

Note: Limit of detection was 0.45 µg/kg dry weight.
Recovery of extraction was 14.71%.

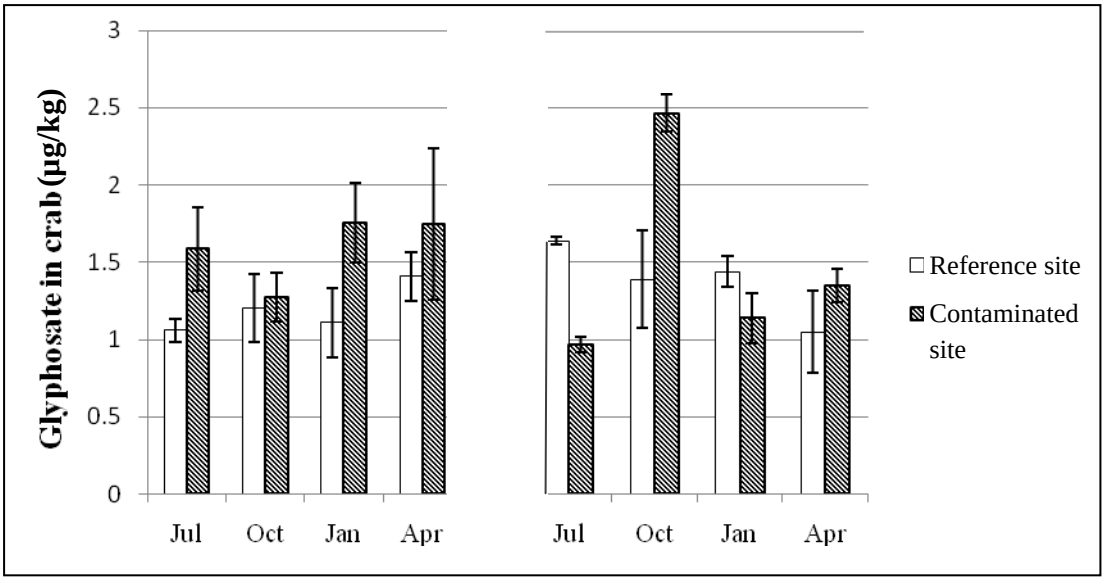


Figure 3.4 Glyphosate contamination in male crab (left, n=3 each) and female crab (right, n=3 each) from the reference and contaminated sites during July 2010 to April 2011. There was no significant difference (*t*-test / U-test; $p > 0.05$) between sites.

3.3.3 Contamination of paraquat

3.3.3.1 Environmental contamination of paraquat

The screening for paraquat contamination in environment showed no detectable levels at any site and any period (Table 3.5). Unlike atrazine and glyphosate, paraquat has a much longer half-life of up to a few years in soil (US EPA, 1993). Its high sorption to soil could defend it against microorganism degradation (Cheah et al., 1998), and potentially against chemical extraction (Knight and Denny, 1970) in this screening method. Furthermore, paraquat is more polar with positive electricity and more soluble in water than other herbicide. This property could rapidly transferred paraquat to agriculture runoff (US EPA, 1997). Although paraquat contamination in water (0.19-3.95 µg/L) has been reported in marsh areas of spain (Fernández et al., 1998), it is of interest to note the difference in method detection limit (0.001 mg/L in this study vs. 0.05-0.08 µg/L in Fernández et al. (1998)).

Therefore, no detectable level of paraquat in this study could not indicate that these study sites were clean from paraquat contamination.

Table 3.5 Paraquat contamination in environmental samples of the reference and contaminated agricultural areas of Nan Province, Thailand during July 2010 to April 2011

Paraquat	Reference site (Lai-Nan District)		Contaminated site (San District)	
	Soil (mg/kg)	Water (mg/L)	Soil (mg/kg)	Water (mg/L)
Jul.-2010	<0.01	<0.05	<0.01	<0.05
Oct.-2010	<0.01	<0.05	<0.01	<0.05
Jan.-2011	<0.01	NA	<0.01	<0.05
Apr.-2011	<0.01	<0.05	<0.01	<0.05

Note: Limit of detection was 0.05 mg/kg in soil and 0.01 mg/L in water.
NA = Not available

3.3.3.2 Residue of paraquat in crab tissue

The result of ELISA for paraquat showed that detactable amount of paraquat was found in tissue of crabs from both the reference and contaminated sites with the range of 26 to 60 µg/kg dry weight (Table 3.6 and Figure 3.5). Given the fact that the weight of freeze dried specimen was approximately 54.36% of the wet weight and the recovery of extraction recovery was 58.47%, the range of paraquat contamination in crab tissue could be estimated at 24.17 to 55.78 µg/kg wet weight. These values of paraquat contamination were much higher than the maximum residue limit in food (5 µg/kg wet weight, CODEX 2006). Since the rice field crab was used as food for local farmers, this level of paraquat contamination could pose some serious risk for human consumption. Therefore, it is of important to note that the rice field crab in these

agricultural areas is not suitable for consumption. Although effects of cooking on the decrease in concentration of chemical contaminants in food have been evidenced in several pesticide (Soliman, 2001; Bayen et al., 2005; Schecter et al., 2006), it still unknown whether cooking the rice field crab could reduce the amount of contaminated paraquat in the crab tissue. This aspect of herbicide degradation through cooking is thus needed to be studied since the crab is regarded as stable food people in northern part of Thailand.

Table 3.6 Paraquat contamination in male and female crabs form the reference and the contaminated agricultural areas of Nan Province, Thailand during July 2010 to April 2011

Paraquat	Reference site (Lai-Nan District)		Contaminated site (San District)	
	Male (µg/kg)	Female (µg/kg)	Male (µg/kg)	Female (µg/kg)
Jul.-2010	42.89±5.40	40.23±4.40	44.13±0.46	34.00±3.77
Oct.-2010	36.30±1.50	38.00±2.62	38.81±2.66	41.23±1.74
Jan.-2011	38.39±6.23	31.53±3.36	44.28±12.12	35.76±2.65
Apr.-2011	39.70±9.86	31.61±2.73	42.22±1.68	41.71±2.04

Note: Limit of detection was 0.61 µg/kg dry weight.
Recovery of extraction was 58.47%.

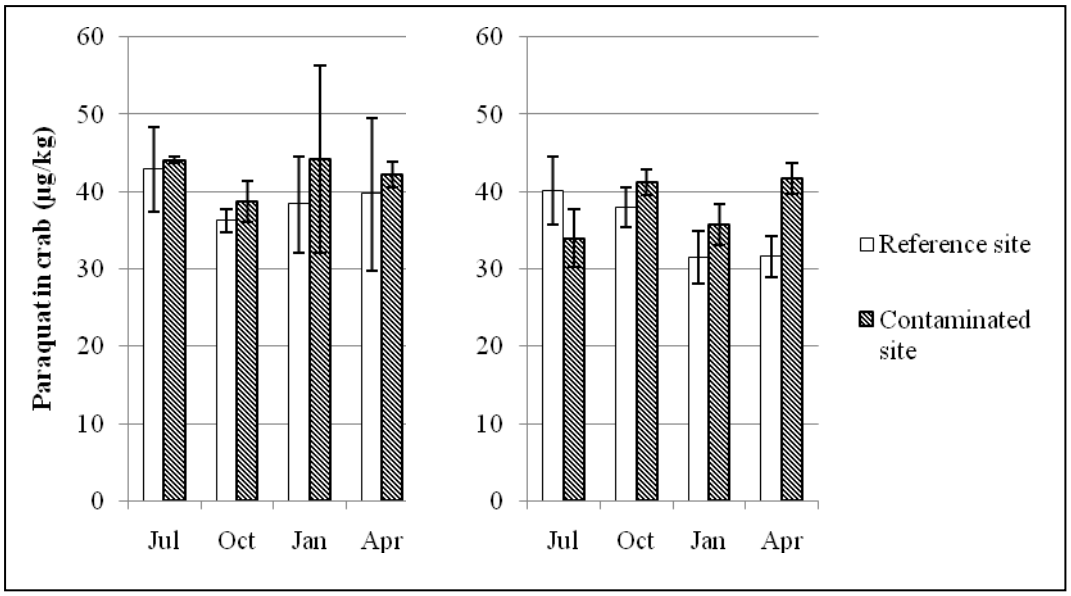


Figure 3.5 Paraquat contamination in male crab (left, n=3 each) and female crab (right, n=3 each) from the reference and contaminated sites during July 2010 to April 2011. There was no significant difference (*t*-test / U-test; $p > 0.05$) between sites.

Since there was no significant sex-related and seasonal difference in the paraquat residue, data, comparison for site-related difference was made with the combined data of two sexes and 4 months in each site. Although the level of paraquat contamination in the contaminated site was slightly higher than those in the reference site, the levels were not significantly different between sites (Figure 3.6).

In vertebrate, especially rat and mice, the accumulation of paraquat in tissue was evidenced. Most rat tissues can accumulate and eliminate paraquat within a few days except lung which used longer time for elimination (Litchfield et al., 1973). Kidney of the rat was found to play major role as organ for excreting of this herbicide (Ghan et al., 1997). In invertebrate, there is a few literature of paraquat accumulation. Earthworm, major soilfauna, did not significantly accumulate paraquat (Eisler, R., 1990). Because of its great solubility in water, paraquat could be excreted by earthworm excretion organ like renal of the rat. In this study, the detectable level of paraquat was found in the rice field crab, possibly indicating the constant paraquat

utilization in these areas. Although no herbicide has been used in the reference site for more than 7 years, paraquat residue in soil is relatively stable with half-life of more than 10 years (US EPA, 1993). As a result, residue of paraquat in soil might be bio-available (EC, 2002) of the rice field crab that feed on organic debris in soil of the rice field.

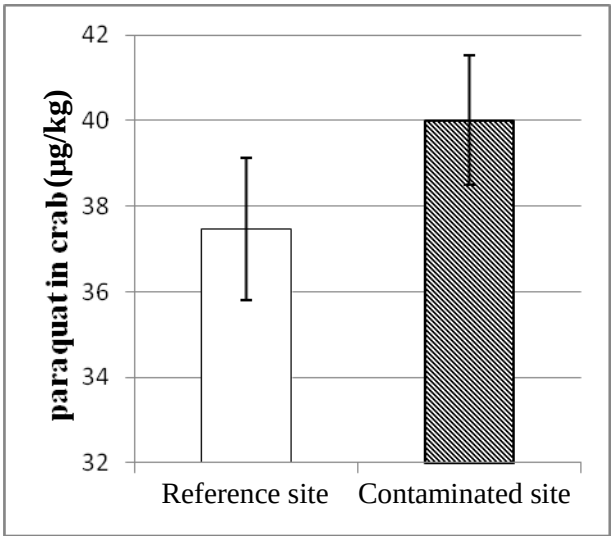


Figure 3.6 Paraquat contamination in tissue of crabs from the reference site (37.46±1.67 µg/kg dry weight, n=24) and the contaminated site (40.00±1.51 µg/kg dry weight, n=24) during July 2010 to April 2011. There was no significant difference (U-test; $p > 0.05$) between sites.

3.3.4 Association between herbicide residues in crab tissue

Pearson's correlation coefficient showed association between residues of these herbicides in crab tissue (Table 3.7). There were significantly positive correlation between residue of atrazine versus glyphosate (Pearson's correlation coefficient = 0.3957, $p< 0.05$) and glyphosate versus paraquat (0.3359, $p<0.05$). The association between these herbicides could reflect complex utilization in the study sites.

Table 3.7 Pearson's correlation coefficients correlating herbicide residues in tissue of crab living in agricultural areas of Nan Province (n=44). Significant correlation between herbicide residues was indicated by an asterisk (*).

Herbicide	Atrazine	Glyphosate	Paraquat
Paraquat	0.0084 p= 0.957	0.3359* p= 0.025	1.0000 p= 0.000
Glyphosate	0.3957* p= 0.008	1.0000 p= 0.000	
Atrazine	1.0000 p= 0.000		

3.4 Conclusion

Environmental screening for herbicide contamination showed that only atrazine was found to be contaminated in water sample of the potentially contaminated site, indicating an evidence of herbicide utilization in the area. The low level, yet detectable, amounts of atrazine, glyphosate and paraquat were found in tissue of crabs from both the reference and contaminated sites, with the significant site-related difference in the level of atrazine. Moreover, the level of paraquat residue in crabs from both sites was much higher than the maximum residue limit in food. These results indicated that a prolonged and intensive herbicide utilization could lead to contamination in environment as well as tissue of the crab or the non-target organism living in the agricultural environment.

CHAPTER IV

CHANGES IN GLUTATHIONE *S*-TRANSFERASE ACTIVITY AND LIPID PEROXIDATION LEVEL OF THE RICE FIELD CRABS LIVING IN AGRICULTURAL AREAS WITH DIFFERENT DEGREE OF HERBICIDE UTILIZATION

4.1 Introduction

Herbicide utilization in agriculture activity has been commonly practiced throughout Nan Province. A major group of herbicide, especially atrazine, glyphosate and paraquat, has been used intensively in agricultural environment. These herbicides have also been found to accumulate in crab tissue (Chapter III). Although, primarily aimed at eliminating weed in crop cultivation, the herbicide was also found to affect a variety of physiological responds in aquatic animals. In frog, it has previously reported that herbicides can directly pose adverse effects on reproductive organ such as inducing hermaphroditism and demasculinization (Hayes et al., 2002). In fish, some herbicides have been found to affect hematology (Hussein et al., 1996) and metabolism (Prasad et al., 1995). In freshwater invertebrate, the herbicides have been shown to affect gill function in crab (Silvestre et al., 2002). It is thus important to monitor its effects in a representative animals living in areas with potential herbicide contamination.

Using animal as a sentinel species is one of environmental monitoring approaches that involve an examination of relationship between chemical contamination and biologic response of animal living in contaminated area in order to assess the effect of the contamination on animal health and the environment (National Research Council, 1991). Some crustaceans, particularly crabs, have been successfully used as a sentinel species. Example of this application included using

mud crab, *Chasmagnathus granulata*, to monitor organochorine pesticide contamination in mangrove ecosystem (Souza et al., 2008), and using a freshwater crab, *Eriocheir sinensis*, to monitor effect of dioxin and polychlorinated biphenyls contamination in freshwater environment (Clark et al., 2009).

In animal sentinel system, it is necessary to measure biological changes in sentinel species at different levels such as morphological, physiological, or biochemical changes in relation to toxicant contamination in the environment and use these changes as biomarkers (National Research Council, 1991). Biotransformation of xenobiotic substance is an important process in animal body and can be used as a biomarker of exposure or biomarker of effect (Kappus, 1986). Phases I of the 2-phase biotransformation process involves oxidation of organic xenobiotics and generation of superoxide radical via a redox process (Boutet et al., 2004). The resulted superoxide radical can be dissolved in water and eliminated out of the body. However, this radical may directly attack and damage cell membrane. Thus, an antioxidant defense system is needed to protect biomolecules from these harmful effects. Phase II biotransformation involves conjugation of the toxic superoxide xenobiotics with antioxidant substrates e.g. glutathione, ascorbic acid, uric acid and vitamin E by an aid of antioxidant enzymes such as catalase and glutathione *S*-transferase (GST) (Stoeckel et al., 2008). This causes the conjugated xenobiotics to become less harmful and can be eliminated out of animal body in water soluble forms. Therefore, changes in factors involving in biotransformation processes are expected in animals living in xenobiotics contaminated environment.

Since the phase II conjugation is enzymatic respond, it has a limit of enzymatic conjugation. When the response is overwhelmed by free radical generation

during the xenobiotic exposure, the free radical without conjugation may directly attack and damage cell membrane (Monserrat et al., 2006). One of the major contributors to loss of cell function on oxidative stress situation is called lipid peroxidation (LPO) (Hermes-Lima et al., 1995).

In this study, a rice field crab *Esanthelphusa nani* (Naiyanetr, 1984), was used as a sentinel species for herbicide contamination in paddy fields of Nan Province since it is native to paddy field habitat with direct exposure to herbicide contaminated water and sediment through gill (gas exchange) and mouth (detritivore). Objective of this study was to examine biologic responses in forms of changes in activity of an antioxidant enzyme, glutathione *S*-transferase, and cell damage in form of lipid peroxidation of the crab living in agricultural areas with different degree of herbicide utilization.

4.2 Materials and Methods

4.2.1 Study sites

Two paddy fields in Wiang Sa District, Nan Province were chosen as study sites based on their similarities in geographic locations, weather conditions, agricultural activities and presence of *E. nani*. A potential contaminated site (location: 47Q 068772, UTM 2054283) is a rice field in San Sub-district with intensive herbicide utilization while a reference site (location: 47Q 0686779, UTM 2047187) is a rice field in Lai-nan Sub-district with no history of herbicide usage for 7 years.

4.2.2 Crab collection

Ten crabs (5 male and 5 female crabs with carapace width > 30 mm.) were caught from each study site by visual encounter survey during night time. The crab collections were carried out on monthly basis from July 2010 to June 2011. After transportation to laboratory at the Chulalongkorn University Forest and Research station, Wiang Sa District, Nan Province, the crabs were euthanized in ice slurry and cut into 2 parts using the line of symmetry as an incision line. The left part of the body was used for contamination analysis (Chapter III) and the right part of the body was used for enzymatic and cellular response analysis (this chapter).

Carapace of the right part of crab body was removed and the crab was dissected for hepatopancreas on ice (4°C). The hepatopancreas was divided into 2 parts and used for determination of GST activity and LPO level. After removal, the hepatopancreas was kept in microtubes and stored immediately at -20°C before further analysis.

4.2.3 Determination of glutathione *S*-transferase activity (GST)

4.2.3.1 Protein extraction and total protein determination

The hepatopancreas tissue was homogenized in extraction buffer (Tris-HCl 0.05 M, KCl 0.15 M, pH 7.4; Appendix D) at 4°C, and the homogenate was centrifuged at 12,000 xg at 4°C for 30 minutes. Cytosolic fraction in the supernatant was collected and subjected to determination of total protein concentration by Bradford's assay in a 96-well plate using stock of 25 µg/mL bovine serum albumin (BSA) as a standard protein. The BSA standard and hepatopancreas extract were diluted with phosphate buffered saline (PBS; Appendix D). For BSA standard, serial dilution was performed to get concentration from 0.78125 to 25 µg/mL. For

hepatopancreas extract, the extract was diluted at 1:100 and 1:1000 dilutions. One hundred microliters of standards, blank and samples were added in duplicate into each well. A 100 µL aliquot of Bradford solution (Sigma) was added into the individual wells successively using a muti-channel pipette. The microplate was shaken with PSU 2-T mini shaker for a few minutes at room temperature before measuring for absorbance by Multiskan EX microplate reader at 595 nm (Bradford, 1976). The average absorbance of each standard was corrected by subtracting with an average absorbance of PBS blank. A standard calibration curve was constructed by plotting the corrected absorbance on a vertical (Y) axis versus the corresponding BSA concentration on horizontal (X) axis followed by linear regression analysis using Microsoft Excel. The protein concentrations of samples were determined using linear regression equation of the standard curve. The protein concentration in every sample was adjusted to 1 mg/mL with PBS prior to GST activity assay.

4.2.3.2 Determination of GST activity

Glutathione *S*-transferase activity in the extracted sample was determined by spectrophotometry according to Habig et al. (1974). First, working solution of glutathione (24.5 mL of 100 mM Tris-HCl mixed with 250 µL of 200 mM L-glutathione in deionized water; Appendix D) was freshly prepared before each use. The working solution was added into 1 mL cuvette (Hellma) and used as a test cuvette. In addition, 990 µL of PBS was added into another 1 mL cuvette and used as a blank cuvette. Each cuvette was added with 20 µL of hepatopancreas extract and 10 µL of 100 mM 1-chloro-2, 4-dinitrobenzene (CNDB; Appendix D). The reaction between glutathione and CDNB facilitated by GST was monitored at 25°C in an UV-visible spectrophotometer (Thermo Electron, GENESYS 6). Change in absorbance at

340 nm was monitored for five minutes and used for calculation of the GST activity as follows.

$$\text{The specific enzyme activity (}\mu\text{mol/mL/min)} = \frac{\Delta A340 \times V}{E\ mM \times V\ ezm}$$

$$\Delta A340 = \frac{A340\ (\text{Final read}) - A340\ (\text{initial read})}{\text{Reaction time (min)}}$$

V = the reaction volume (1mL)

E mM = the extinction coefficient for CDNB conjugation at 340 nm ($9.6nM^{-1}$)

V ezm = volume of enzyme (20 µL)

4.2.4 Determination of lipid peroxidation level (LPO)

4.2.4.1 Lipid extraction

Initially, the hepatopancreas was weighed for 0.5 gram and mixed with 0.5 mL of cold absolute methanol (1:9 w/v) before homogenization for a 2 minutes at 4 ºC (Monserrat et al., 2003). The homogenate sample was centrifuged at 1,000 xg at 4°C for 10 minutes. Lipid in the supernatant was collected and kept at -20 ºC before further analysis.

4.2.4.2 Determination of lipid peroxidation in extracted sample

A FOX assay (ferrous oxidation/xylenol orange) is a fast and accurate method for measuring level of LPO. The method of FOX assay based on the oxidation of Fe (II) by lipid peroxides under acidic conditions has been proposed by Hermes-Lima et al. (1995). Monserrat et al. (2003) has modified the FOX using microplate reader (Multiskan EX microplate reader) instead of cuvette. To apply this method for determination of LPO in hepatopancreas of the rice field crab, preliminary trials were performed to find 1) an optimal sample volume, 2) an optimal incubation time after sample addition and 3) an optimal incubation time after CHP addition (Appendix E).

For each individual sample, it is needed to run 3 sets of reaction simultaneously including: 1) sample reaction, 2) blank reaction, and 3) sample blank reaction. Each well of the duplicated sample reaction consisted of 80 µL of 1 mM $FeSO_4$, 30 µL of 250 mM H_2SO_4, 30 µL of 1 mM xylenol orange and 155 µL of deionized water to get 375 µL of volume. Lipid sample (5 µL) was added into sample reaction well to get 380 µL final volumes (all reagents are listed in Appendix D). The duplicate blank reaction contained similar reagents to the sample reaction except that $FeSO_4$ was replaced by deionized water. The duplicated sample blank reaction was also consisted of similar reagents to the sample reaction except that the lipid sample was replaced by deionized water. The microplate was incubated at room temperature for 240 minutes and the absorbance was determined at 595 nm using a microplate reader. Afterward, 10 µL of 0.5 mM CHP (5 nanogram CHP equivalent) was added into each well and the absorbance was determined at 595 nm at 30 minutes after CHP addition. A modified equation of Hermes-Lima et al. (1995) and Monserrat et al. (2003) were used for lipid peroxides content calculation:

$$\text{CHP equiv./mg wet wt} = \frac{\Delta A595 \text{ sample}}{\Delta A595_{CHP}} \cdot n \cdot \frac{\text{Final volume}}{\text{sample vol} \cdot HF \cdot CF}$$

$\Delta A595$ sample = A595 of sample before CHP addition - A595 of blank before CHP addition

$\Delta A595_{CHP}$ = A595 of sample after CHP addition - A595 of sample before CHP addition

n = amount of CHP (5 nanogram)

HF = homogenization factor relative to the volume of buffer used per gram of tissue (1:9 = 10)

CF = correction factor based on the absorbance increment as a result of tissue sample

$$= \frac{\text{A595 of sample blank after CHP addition - A595 of sample blank before CHP addition}}{\text{A595 of sample after CHP addition - A595 of sample before CHP addition}}$$

4.2.5 Analysis of GST and LPO data

GST or LPO data of each sex and each site were assessed for normality of distribution by Shapiro test. First, the data that passed the normality test was compared for sex-related difference by Student *t*-test, while the unpassed data was compared by Mann-Whitney U-test. In case of no significant difference between sex, the data of both sexes were grouped as the new data set. Next, the normal distributed data of each site was assessed for seasonal difference by one way ANOVA with Tukey's HSD Post Hoc test, while the other was assessed for seasonal difference by Kruskal-Wallis test. In case of no significant difference among months, the data of GST or LPO in every season were grouped as the new data set. Finally, the *t*-test or U-test was employed to test for site-related difference in GST or LPO data. The outline of data analysis was illustrated in Figure 3.1 of Chapter III.

4.2.6 Pearson's correlation analysis

Pearson's correlation test was used to correlate between herbicide residues in crab tissue and biologic response (i.e. levels of GST and LPO in hepatopancreas).

Statistical analyses were performed using program R version 2.15.2 (2012-10-26: Copyright (C) 2012 The R Foundation for Statistical Computing).

4.3 Result and discussion

4.3.1 Change in glutathione *S*-transferase activity

Reactive oxygen species (ROS) are normally generated in cells through several processes such as oxidative mitochondrial electron transport, metabolism of xenobiotics and inflammation. As a result, antioxidant compounds including vitamins and glutathione, as well as anti-oxidant enzyme such as GST are normally provided for body defense (Gate´ et al., 1999). In the reference site animal, GST in

hepatopancreas ranged from 0.7 to 1.3 μmol/min/mg protein in male crab and 0.5 to 1.5 μmol/min/mg protein in female crab. In contaminated site, GST in hepatopancreas ranged from 0.7 to 1.3 μmol/min/mg protein in male crab and 0.5 to 1.5 μmol/min/mg protein in female crab. Since data on GST activity showed the sex-related and seasonal differences, comparisons between sites were performed separately for each sex and season. The result of GST activity comparison by *t*-test showed that the GST activity of male (Figure 4.1) and female (Figure 4.2) crabs in contaminated site were significantly ($p<0.05$) higher than those in the reference site during July and October 2010. Moreover, it was found that the GST level of female crabs in contaminated site in January 2011 was also significantly higher than those in the reference site.

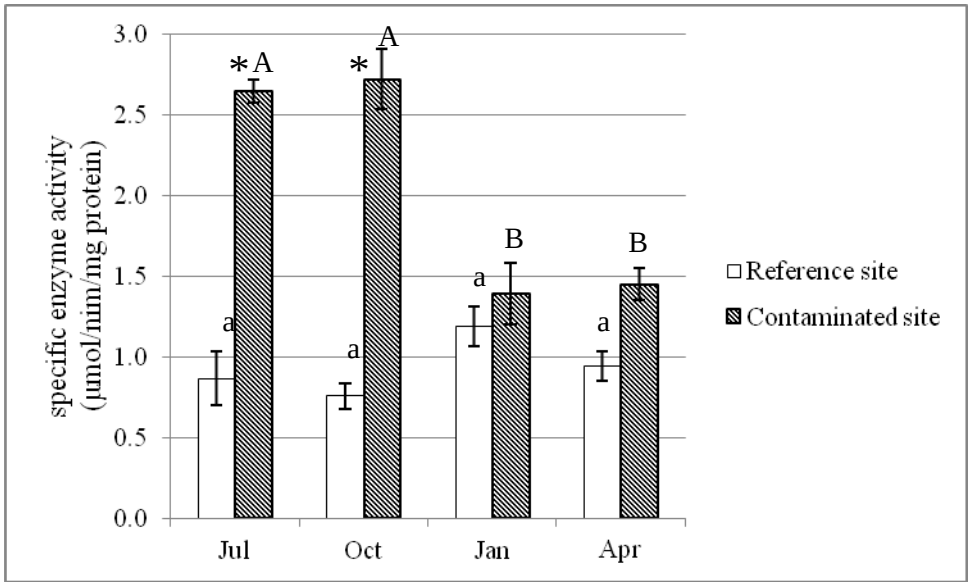


Figure 4.1 Glutathione-*S*-transferase activity (mean ± S.E.M.) in hepatopancreas of **male** *Esanthelphusa nani* caught from reference site (n=5 each) and contaminate site (n=5 each) at Nan Province, Thailand during July 2010 - April 2011. Significant difference (t-test, $p<0.05$) between site is indicated by an asterisk (*). Significant difference (ANOVA with Turkey HSD, $p<0.05$) between months in each site is indicated by different alphabets.

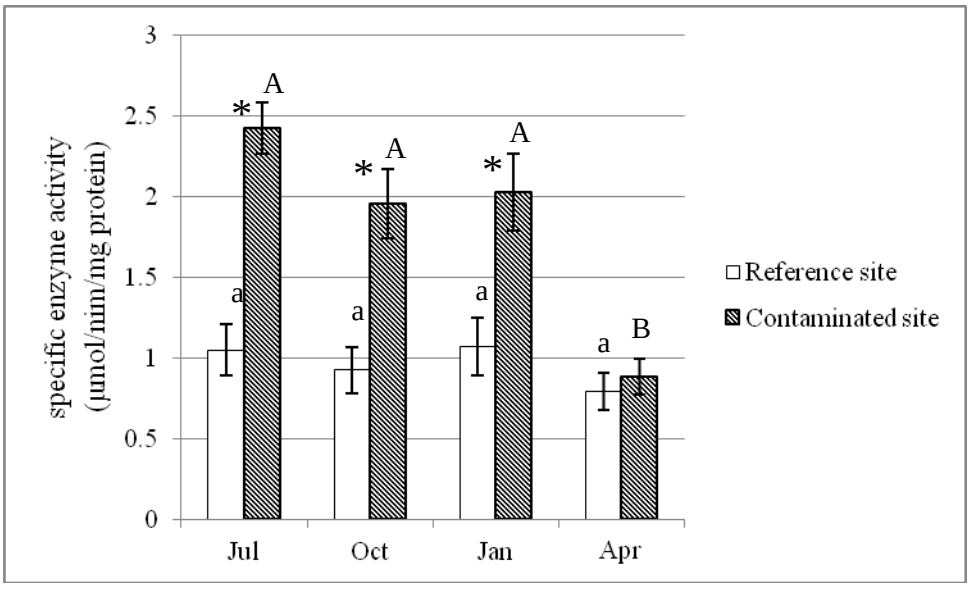


Figure 4.2 Glutathione-*S*-transferase activity (mean ± S.E.M.) in hepatopancreas of **female** *Esanthelphusa nani* caught from reference site (n=5 each) and contaminate site (n=5 each) at Nan Province, Thailand during July 2010 - April 2011. Significant difference (t-test, $p<0.05$) between site is indicated by an asterisk (*). Significant difference (ANOVA with Turkey HSD, $p<0.05$) between month in each site is indicated by different alphabets.

The GST activity in crab of the contaminated site significantly decreased in April 2011 (Figure 4.1 and 4.2, ANOVA with Turkey HSD, $p<0.05$) compared to other months. Although the precipitation data indicated that April 2011 was in the early wet season (Appendix A), there was no agricultural activity in the paddy field at both sites. As the result, the rice field crabs were found to estivate in soil in order to prevent moisture loss from its body during January-April 2011 (field observation). The crab usually digged a 1.0-1.5 meter deep hole in the ground. It is possible that this estivation could prevent it from exposure to herbicide contamination. In addition, the result of GST activity of crab in the reference site showed the consistent level throughout the year with no change during the estivation period. This level could indicate a baseline level of GST activity for defense against naturally occurred ROS.

In vertebrates, particularly in human and rat, there have been many reports about association between herbicide exposure and biotransformation process. Atrazine and its metabolites, desethylatrazine and desisopropylatrazine, were reported to be detoxified by conjugation with glutathione via GST detoxification enzyme in human liver (Joo et al., 2010). *In vitro* paraquat exposure was reported to increase GST activity in mouse keratinocyte via oxidative stress pathway (Black et al., 2008). Although, there are less number of literature on invertebrate biotransformation, variety of biotransformation processes has found in invertebrates, such as phase II conjugation in aquatic Decapoda crustaceans (Ikenaka et al., 2007). Presence of glutathione conjugation and GST species has also found in shore crab *Carcinus maenas* (Gowland et al., 2002). Moreover, accumulation of glyphosate and induction of GST activity were recorded in blackworm *Lumbriculus variegates* (Valeska et al., 2009). Therefore, it is highly possible that GST is responsible for biotransformation of herbicides as well as their metabolites. Since GST activity is closely linked with oxidative stress (Yin et al., 2000), higher levels of GST activity in *E. nani* living in an intensive herbicide utilization area suggested that the crabs living in contaminated area was subjected to exposure to herbicide residue and was under oxidative stress condition. The result of this study indicated that GST activity in hepatopancreas of the rice field crab could be used for assessing extent of herbicide contamination and potential impacts of low level contamination to organisms in agricultural environment.

4.3.2 Changes in lipid peroxidation level (LPO)

Herbicides are well known as the toxic compounds with oxidative stress generation and augmenting of LPO levels (Joo et al., 2010). LPO levels in

hepatopancreas of crab ranged from 15 to 47 nmole CHP/g of wet tissue in the reference site and 22 to 50 nmole CHP/g of wet tissue in the contaminated site. There was no significant difference between sexes of each site, so data of both sexes were pooled for further analysis. Since there were significant seasonal differences, comparison between site was performed separately in each season. It was found that significant difference between study sites was not found in this study (Figure 4.3).

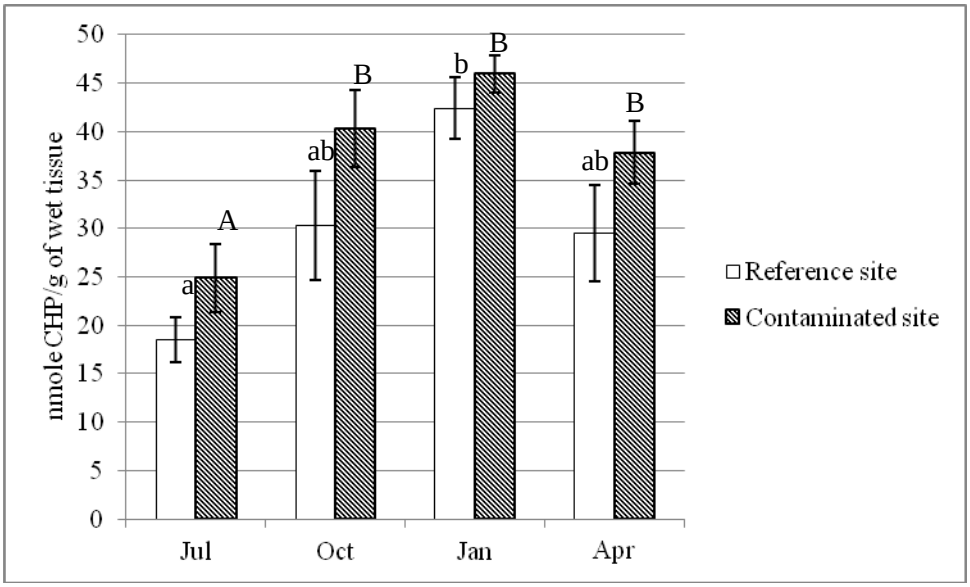


Figure 4.3 Lipid peroxidation level (mean ± S.E.M.) in hepatopancreas of *Esanthelphusa nani* caught from the reference site (n=10 each) and contaminate site (n=10 each) at Nan Province, Thailand during July 2010 - April 2011. Significant difference (ANOVA with Turkey HSD, $p<0.05$) between month in each site is indicated by different alphabets.

One way analysis of variance of LPO levels in hepatopancreas of crabs from both sites showed a similar trend of seasonal variation. LPO level in hepatopancreas was at the lowest level in July 2010 and gradually increased in October 2010, to the relatively higher level in January 2011 and gradually decreased in April 2011. Since there was no previous report on seasonal change in LPO level in any crab, discussion on the seasonal change in LPO level found in *E. nani* was based on field and

laboratory observations. It was observed that hepatopancreas of the crab was small and dark brown in color in July 2010, or the end of estivation. In contrast, the hepatopancreas was large and yellowish in color in January 2011 or the onset of estivation. Since hepatopancreas of crab consists of glycogen, protein and lipid (Vonk, 1960), this color difference could indicate the different amount of lipid content of hepatopancreas between July 2010 and January 2011. According to the lipid extraction protocol, it was unlikely to normalize for the different amount of lipid in the tissue prior to LPO assay. Therefore, different amount of lipid could possibly affect on variation of LPO level in different season. However, this hypothesis cannot be guaranteed without data of relationship between lipid content and LPO level in hepatopancreas of the crab.

Many reports have been suggested that atrazine, glyphosate and paraquat can increase LPO level via generating a group of reactive oxygen species (Nwani et al., 2010; Larsen et al., 2012; Hara et al., 1991). Comparison of LPO levels in hepatopancreas of crab between the reference and the potentially contaminated sites showed no significant difference, while comparison of GST levels showed significantly higher levels of GST activity in hepatopancreas of crabs living in the contaminated site. This indicates that the detoxifying enzyme, GST, alone was sufficient for the crab to respond to herbicide contamination.

4.3.3 Association between herbicide contaminations in crab tissue and biologic responses

Pearson's correlation coefficient has shown association between herbicide contaminations in crab tissue and GST activity, and association between herbicide contaminations in crab tissue and LPO level (Table 4.1). There was a significantly positive correlation between atrazine residue versus GST activity (Pearson's

correlation coefficient = 0.3054, $p<0.05$), and atrazine residue versus LPO level (0.2907, $p<0.05$). Moreover, the Pearson's correlation coefficient has also shown positive correlation between GST activity and LPO level (0.4038, $p<0.05$).

The correlation of GST activity and LPO level indicated the link between these enzymatic and cellular response of the crab body. The associated pattern of atrazine and biologic responses could indicate the effect of atrazine on the rice field crab. With atrazine exposure, it seemed that the crab would expose to reactive oxygen species generated by herbicide contamination in the crab body. As a result, increase in activity of GST was occurred in order to cope against atrazine contaminations in its body. Furthermore, the increased amount of atrazine residue seemed to increase cell damage as indicated by the increase LPO level.

There was an unexpected negative correlation between paraquat residue and GST level (-0.3549, $p<0.05$). This negative correlation could indicate that high level of paraquat in crab tissue could inhibit activity of GST. Inhibition of GST activity in the presence of high concentration of xenobiotics has been reported in heavy mental, especially $CdCl_2$. Exposure to high $CdCl_2$ concentration and reduced activity of the GST, possibly due to high toxicity of $CdCl_2$ to cell (Çoban et al., 1996; Casalino et al., 2006)

Table 4.1 Pearson's correlation coefficients correlating between herbicide residues in crab tissue and biologic responses, levels of GST and LPO in hepatopancreas (n=44). Significant correlation between herbicide was indicated by an asterisk (*).

Data	Atrazine	Glyphosate	Paraquat	GST	LPO
LPO	0.2907* p= 0.045	0.1814 p= 0.181	-0.0556 p= 0.719	0.4039* p= 0.004	1.0000 p= 0.000
GST	0.3054* p= 0.035	0.0674 p= 0.664	-0.3549* p= 0.020	1.0000 p= 0.000	
Paraquat	0.0084 p= 0.957	0.3359* p= 0.025	1.0000 p= 0.000		
Glyphosate	0.3957* p= 0.008	1.0000 p= 0.000			
Atrazine	1.0000 p= 0.000				

4.4 Conclusion

Using the rice field crab as a sentinel species for herbicide contamination, biologic responses in forms of changes in activity of glutathione *S*-transferase, and cell damage in form of lipid peroxidation were examined in crab living in agricultural areas with different degree of herbicide utilization. Since GST activity is closely linked with oxidative stress (Yin et al., 2000), the higher GST activity in hepatopancreas of crabs living in the contaminated site could indicate that the crabs were under more severe oxidative stress condition than crabs living in the reference site. Although the LPO level in the contaminated site was slightly higher than the LPO level in the reference site, there was no significant difference of LPO level between study sites in any period. This indicated that the response of biotransformation enzyme (i.e. GST and other) was sufficient to detoxify herbicide residue in the crab body. It is of importance to note the significant associations between atrazine residue versus GST activity and LPO level. These results suggested that the rice field crabs living in herbicide utilization areas was exposed to reactive oxygen species as a result of herbicide contamination.

CHAPTER V

CHANGES IN HEALTH STATUS AND MORPHOMETRIC PARAMETERS OF THE RICE FIELD CRAB LIVING IN AGRICULTURAL AREAS WITH DIFFERENT DEGREE OF HERBICIDE UTILIZATION

5.1 Introduction

Intensive herbicide utilization especially atrazine, glyphosate and paraquat, in agriculture activity in Nan Province has been documented. Residues of herbicides have been detected in environment. These herbicides have also been found to accumulate in tissue of the rice field crab living in agricultural area (Chapter III). Increases in glutathione *S*-transferase, a phase II biotransformation enzyme, activity in hepatopancreas of paddy crab in herbicide utilization area were observed. Moreover the relationship between herbicide contaminations in crab tissue and detoxifying enzyme activity has been evidenced (Chapter IV). However, an association between herbicide contaminations and change in morphology and health status of the crab has not yet been documented.

Atrazine, glyphostae, and paraquat are a group of herbicide used in agricultural activity of Nan Province (Thammachoti et al., 2012). These herbicides were found to have adverse effects on morphology of the non-target organism. It has been reported that atrazine is an endocrine disrupting chemical (EDC) capable of interference the function of sex hormones and may cause morphological alterations in all class of vertebrate (Hayes et al., 2011). Glyphosate was found to cause shell abnormalities and abnormal mantle morphology in the Pacific oyster *Crassostrea gigas* (Mottier et al., 2013). Paraquat is major cause of lung morphological changes in rat with evidence of hypertrophy/hyperplasia correlated to level of paraquat (Stewart et al., 1979).

Morphologic characters in crustaceans have been employed to assess effect of a variety of xenobiotics (Sangalang and Jones, 1997; Takahashi et al., 2000, Vandenberg et al., 2003). However, there is a lag of research using crustacean morphological characters for determining effect of herbicide contamination on crustacean health status.

In this chapter, overall health status as determined from relative body weight and morphometric analysis of some external morphology of the rice field crab *Esanthelphusa nani* living in agricultural areas with different degree of herbicide utilization were assessed. Association between herbicide contamination and changes in these parameters were further analyzed.

5.2 Materials and Methods

5.2.1 Study sites

Two paddy fields in Wiang Sa District, Nan Province were chosen as study sites based on their similarities in geographic locations, weather conditions, agricultural activities and presence of *E. nani*. A potential contaminated site (location: 47Q 068772, UTM 2054283) is a rice field in San Sub-district with intensive herbicide utilization, while a reference site (location: 47Q 0686779, UTM 2047187) is a rice field in Lai-nan Sub-district with no history of herbicide usage for 7 years.

5.2.2 Crab collection

Ten crabs (5 male and 5 female crabs with carapace width > 30 mm.) were caught from each study site by visual encounter survey during night time (from dusk until 11 PM). The sample collections were carried out on monthly basis from July 2010 to June 2011. Crab samples were transported to a laboratory at the

Chulalongkorn University Forest and Research station, Wiang Sa District, Nan Province. Crabs were subjected to body weight measurement with a digital balance (Ohaus Pioneer Analytical Balances PA214, accuracy at 0.0001 g) and measurement of their carapace width (Figure 5.1) with Mitutoyo digital vernier caliper (accuracy at 0.01 mm). The larger claw in each crab was selected as a major claw to be measured for the claw size with the digital vernier caliper (Figure 5.2). Crabs were eutharized in ice sherry and their abdominal plates (Figure 5.3) were removed and stored in 70 % ethanol for further analysis. Remaining crab body was dissected and shored at -20 ºC for analysis on herbicide contamination (Chapter III) and enzymatic and cellular responses (Chapter IV)

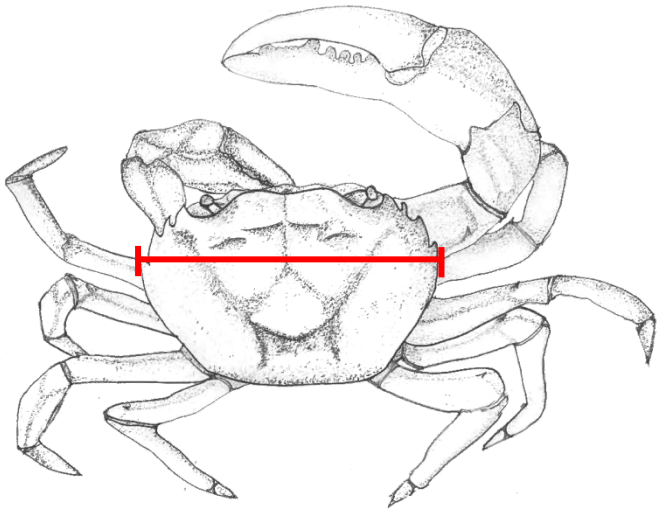


Figure 5.1 Illustration of external morphology of the rice field crab, *E. nani*. The outermost serration on carapace was used as landmarks for carapace width measurement.

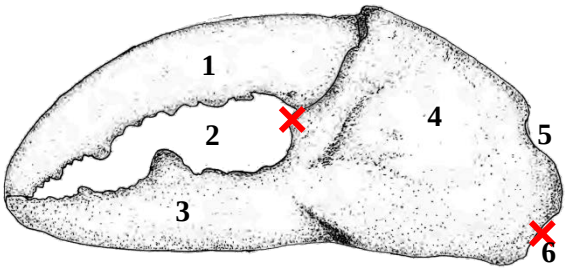


Figure 5.2 Illustration of major claw of the rice field crab, *E. nani*. The two X marks were used as landmarks for measurement of the claw size. Numbers on the claw indicate different morphological characters including 1) dactyl, 2) gape, 3) pollex, 4) manus, 5) upper carpal cavity and 6) lower carpal cavity.

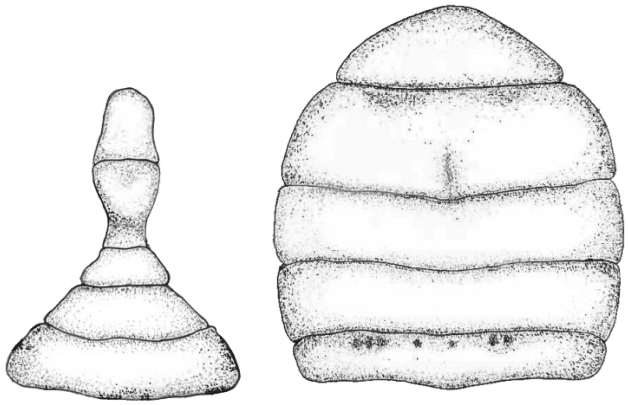


Figure 5.3 Illustration of abdominal morphology of male (right) and female (left) rice field crab, *E. nani*.

5.2.3 Measurement of relative body weight

Relative body weight could be used to assess overall health status of animals (Armstrong and Lund, 1996). In the crab, it was calculated by using a ratio between the body weight and the carapace width as illustrated in the equation.

$$\text{Relative body weight} = \frac{\text{Body weight (g)}}{\text{Carapace width (mm)}}$$

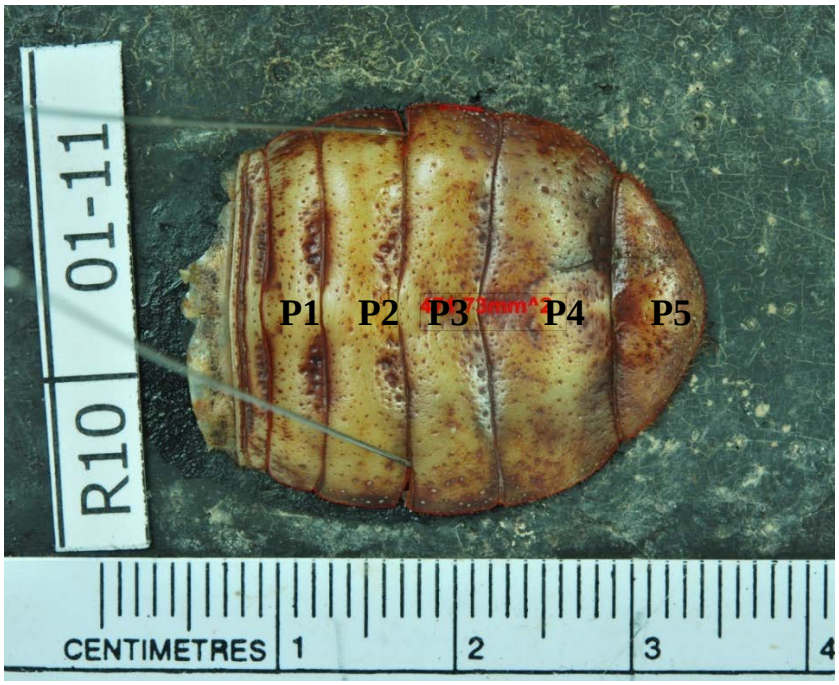
5.2.4 Measurement of a major claw size

The major claw of each crab was selected and claw size was measured with the Mitutoyo digital vernier caliper (Figure 5.2). According to Rosenberg (2001), landmarks for claw size included 1) the articulation of dactyl and manus, and 2) the upper attachment points at the edge of carpal cavity. Distance between these two landmarks was used as the major claw size.

5.2.5 Measurement of abdominal area

The preserved abdominal plate was placed flatly on a stage and photographed with Nikon D300 digital camera with reduced distortion by using Nikon AF 60 mm D macro lens. Based on the digital picture of abdominal plates of each individual crab (Figure 5.4), outline of these plates was initially digitized. Then, total area was calculated using Zeiss AxioVision Rel. 4.8 program.

A.



B.

Figure 5.4 Representative of digital pictures of male (A) and female (B) crab abdominal plates. Five plates (P1-P5) of abdomen were recorded and used for total abdominal area calculation by Zeiss AxioVision Rel. 4.8 program. .

5.2.6 Analysis of morphometric data

Data on relative body weight, abdominal area and major claw size of each sex and each site were assessed for normality of distribution by Shapiro test. First, the data passing the normality test was compared for sex-related difference by Student *t*-test, the unpassed data was compared for sex-related difference by Mann-Whitney U-test. In case of no significant difference, the data were grouped as a new data set. Next, the normally distributed data of each site was assessed for seasonal difference by ANOVA with Tukey's HSD Post Hoc test, while the other was assessed by Kruskal-Wallis test. In case of no significant difference among months, the data in every season were grouped as a new data set before further analysis. Overview of data analysis is shown in Figure 3.1 (Chapter III).

The *t*-test / U-test was employed to test for site-related difference in relative body weight. An analysis of covariance (ANCOVA) using carapace width as a covariate was employed to test site-related difference in abdominal area and major claw size of the crab from the contaminated site and the reference site.

4.2.6 Pearson's correlation analysis

To normalize for difference in body size, major claw size and abdominal area were transformed by dividing with the carapace width before further analysis. Pearson's correlation test was used to correlate between herbicide residues in crab tissue and changes in morphometric parameters including relative body weight, abdominal area and major claw size

All statistic analyses were employed using program R version 2.15.2 (2012-10-26: Copyright (C) 2012 The R Foundation for Statistical Computing), except an analysis of covariance (ANCOVA) that was done by Sigma Plot 11.0.

5.3 Result and discussion

5.3.1 Overall health status

Body weight of the rice field crab caught in this study varied from 11.27 grams up to 45.45 grams. To normalize for body size difference, the ratio of body weight and carapace width was used as the relative body weight and subjected to further comparison. There were sex-related and seasonal differences in relative body weight data. Therefore, site comparison was performed separately for each sex and month.

Relative body weight of male crab ranged from 0.34 g/mm to 0.94 g/mm. The comparison of relative body weight in male crab between sites showed significantly higher relative body weight of male crab in the reference site than those in the contaminated site in August 2010, September 2010 and May 2011 (Figure 5.5). In most, if not all, of the sampling month, the relative body weight of male crab showed trend toward higher relative body weight of the reference site crab compared to those of the contaminated site crab.

Unlike the male crab, the relative body weight of female crab ranged from 0.39 g/mm to 0.82 g/mm. Significant difference between sites of relative body weight in female crab was not found (Figure 5.6).

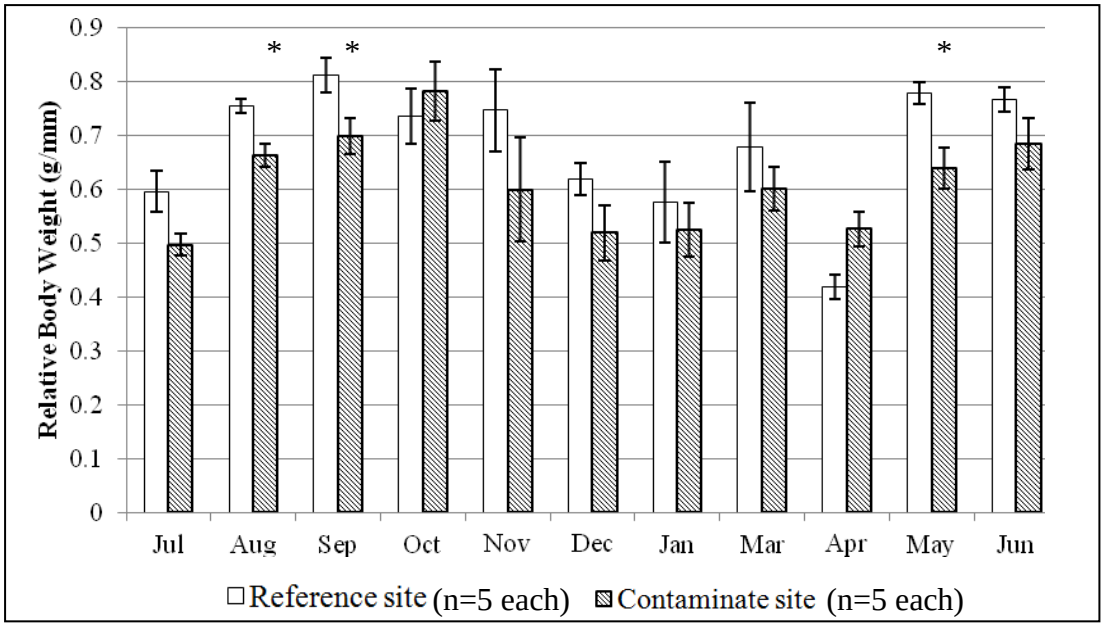


Figure 5.5 Relative body weight of **male** crab, *Esanthelphusa nani*, caught from the reference and the contaminated sites at Nan Province, Thailand during July 2010-June 2011. Significant difference (*t*-test, $p<0.05$) between site is indicated by an asterisk (*).

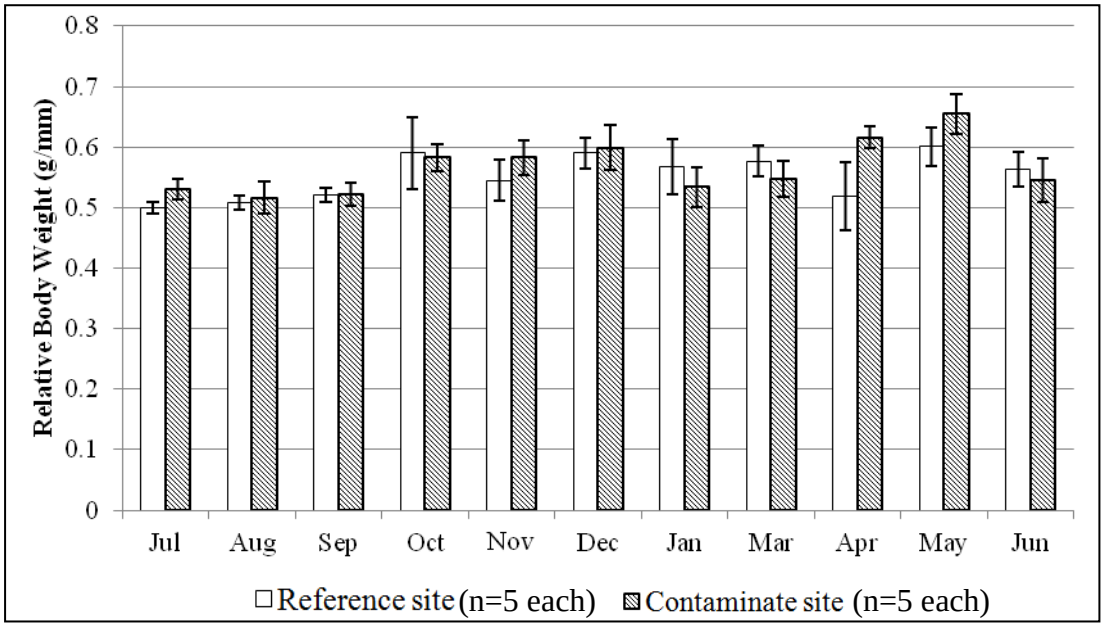


Figure 5.6 Relative body weight of **female** crab, *Esanthelphusa nani*, caught from the reference and the contaminated sites at Nan Province, Thailand during July 2010-June 2011.

The relative body weight is the simplest form to measure the health status in animals. Relative body weight in human, as known as body mass index (BMI), is used to show basic health by comparing standard mean of BMI in population (Kantachuvessiri, 2005). The BMI could be used for early assessment of body conditions and diagnosis of some common disease. The similar technique is applied widely in study of animal especially in veterinary field (Armstrong and Lund, 1996). Database of the relative body weight could be used as a guideline for health status in each species. Furthermore, the relative body weight has been employed in rat to assess its health status as a result of herbicide exposure. The loss of relative body weight was correlated with an increase in the impact of paraquat (Sharp et al., 1972).

In the rice field crab study, male crab living in the herbicide utilization area tended to has lesser relative body weight compared to male crab living in the reference area. These suggest the negative impact of herbicide contamination on health status of paddy crab. However, it is of importance to note the absence of significant difference in female crab relative body weight between sites, indicating lesser interference from herbicides in the female crabs. As suggested by Huxley (1924), the relative body weight has also been implicated with morphological changes. It is possible that this sexual discrepancy could be due to difference in some sexually dimorphic traits related to body weight, especially the claw size.

5.3.2 Morphometric parameters of the rice field crab

5.3.2.1 Abdominal area of the rice field crab

Since abdominal plate of the crab is part of an exoskeleton, size and shape of this hard shell are unlikely to be affected by season. As the result, the abdominal area data of every month was combined and subjected to comparison of the site-related

difference. However, data of each sex was analyzed separately to accommodate the sexual dimorphism in this trait.

In male, an estimated marginal means of abdominal area with carapace width of 42.41mm were 176.14±1.37 mm^2 in the reference site and 171.93±1.44 mm^2 in the contaminated site crabs, respectively. There was a significant site-related difference, with the greater abdominal area of male crab in the reference site compared to those in the contaminated site (Figure 5.7). In female, the estimated marginal means of abdominal area with carapace width of 42.56 mm were 590.97±5.88 mm^2 in the reference site crab and 607.20±5.99 mm^2 in the contaminated site crab. The abdominal area of female crab from the contaminated site was relatively larger than those of the reference site crab. However, there was no significant difference between sites (Figure 5.8).

In male crab, an abdominal plate is a location where male gonopod attached to (Brian, 2005). This reduced abdominal plate size of the crab could affect on size of the gonopod. Change in the gonopod size may further influence on reproductive success during crab copulation. In female crab, an abdominal plate plays an important role as location for hatching eggs and releasing larva (De Vries and Forward, 1991). Moreover, the abdominal area is regarded as an important area where location of spermatophore in male crab and spermathecal opening in female crab is situated (Hill et al., 2008). This role of abdominal area is thus linked to reproductive success in a crab. Change in size of abdominal area of both the male and female rice field crab could affect the reproduction and the crab population in the future.

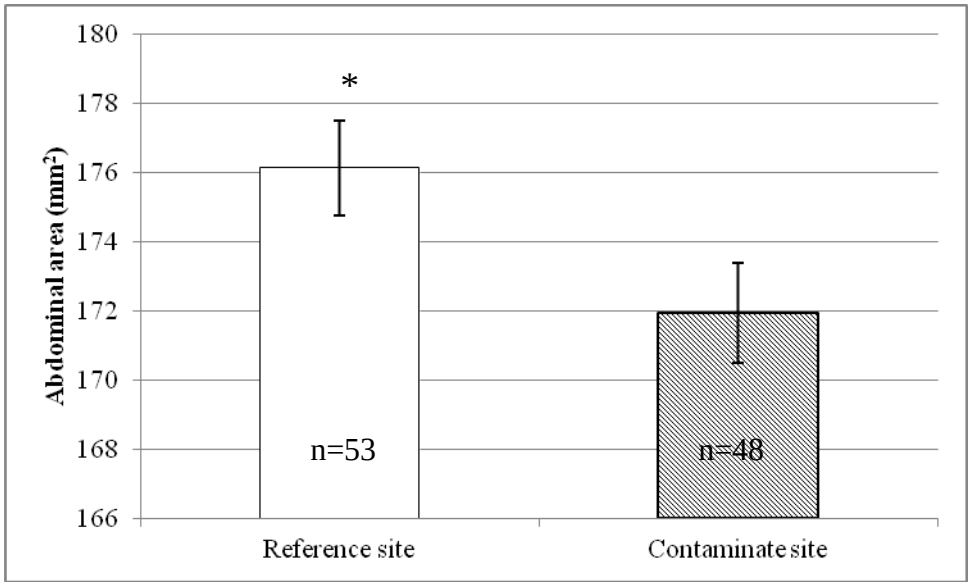


Figure 5.7 Estimated marginal mean of total abdominal area of **male** crab, *Esanthelphusa nani*, caught from reference and contaminated sites at Nan Province, Thailand during July 2010-June 2011. Significant difference (ANCOVA, $p<0.05$) between site is indicated by an asterisk (*). Covariate carapace width was estimated at 42.41 mm.

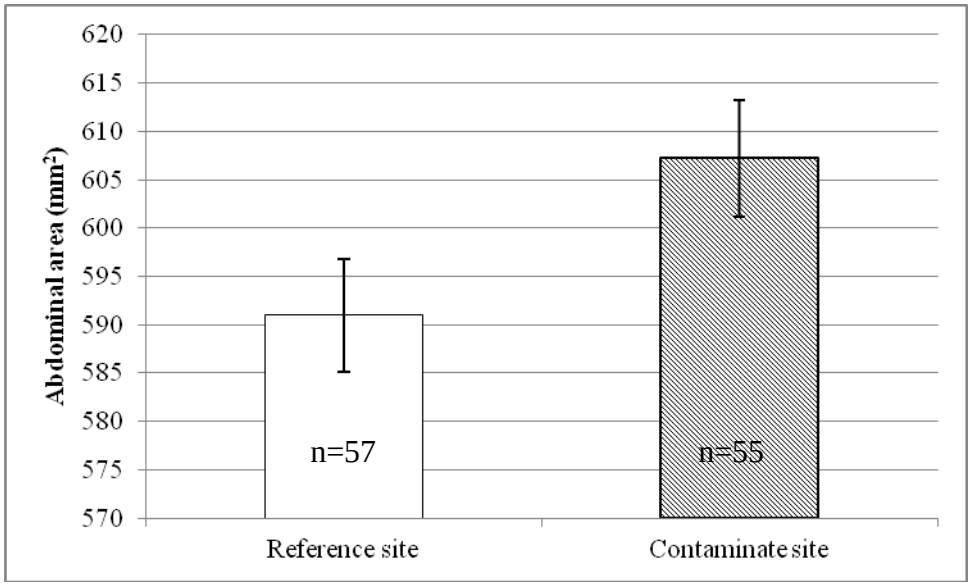


Figure 5.8 Estimated marginal mean of total abdominal area of **female** crab, *Esanthelphusa nani*, caught from reference and contaminated sites at Nan Province, Thailand during July 2010-June 2011. Covariate carapace width was estimated at 42.36 mm.

5.3.2.2 Major claw size of the rice field crab

Since difference in claw size between left and right is regarded as a sexual dimorphic trait, site comparison was performed separately for male and female. In the male crab, an estimated margined means of major claw size were 20.21±0.36 mm in the reference site crab and 19.10±0.37 mm in the contaminated site crab (carapace width of 42.47 mm). There was a significant difference between sites. On the other hand, an estimated margined means of major claw size in female was not significantly different between sites with the value of 13.99±0.25 mm in the reference site crab and 13.95±0.25 mm in the contaminated site crab (carapace width of 42.26 mm).

Chelipeds of a crab play an important role in agonistic encounters between conspecifics (Salmon and Hyatt, 1983). Some species use major claw to show aggressive interactions (Mariappan et al., 2000). Crab claws are important to enhance chance of survival. The reduced in size of the major claw in the rice field crab could decrease the intraspecific competition. In addition, there is a document of body weight associated with an increase in cheliped size (Hartnoll, 1974; Fielding and Haley, 1976). The significant loss in relative weight of the male crab could possibly due to the reduce in size of the major claw in the crab.

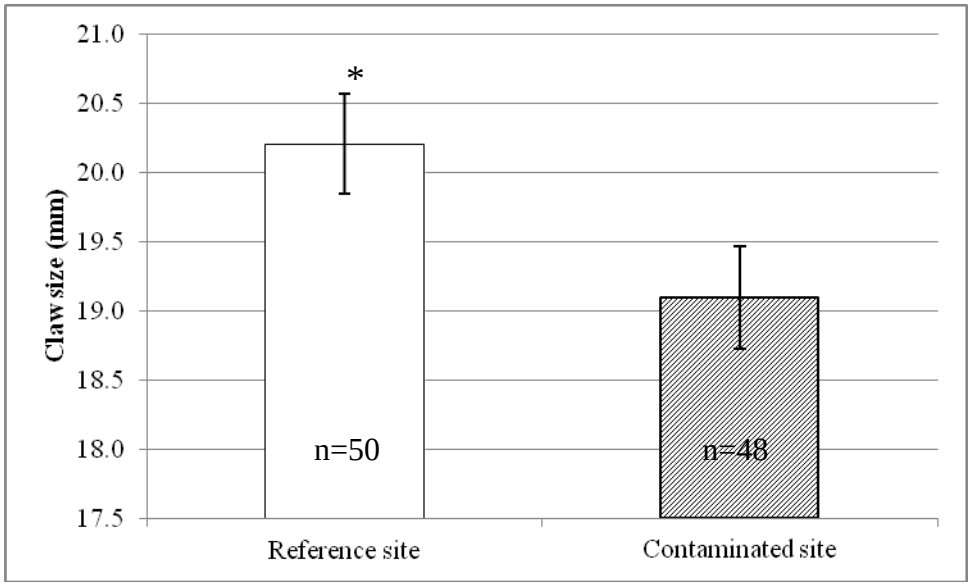


Figure 5.9 Estimated marginal means of major claw size of **male** crab, *Esanthelphusa nani*, caught from reference and contaminated sites at Nan Province, Thailand during July 2010-June 2011. Significant difference (ANCOVA, $p<0.05$) between site is indicated by an asterisk (*). Covariate carapace width was estimated at 42.47 mm.

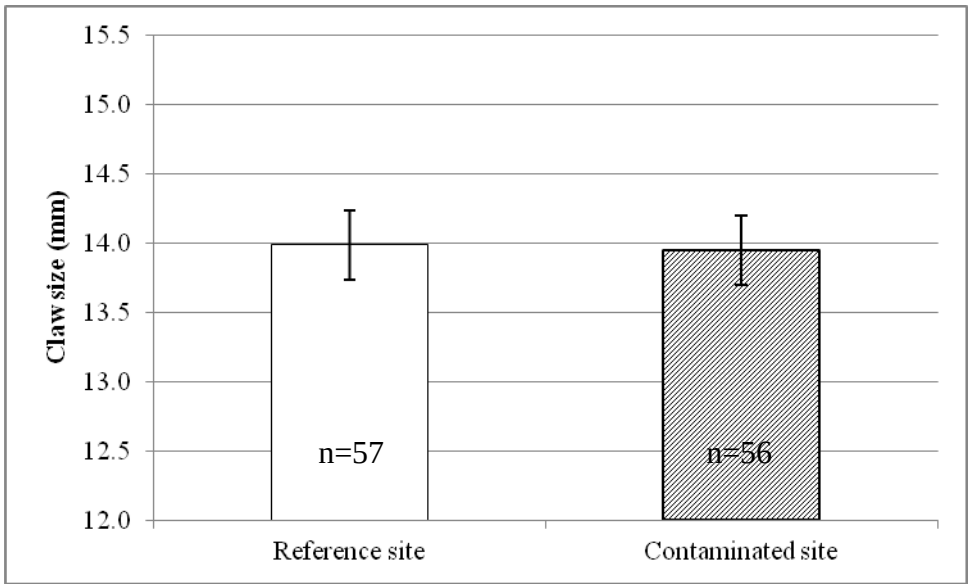


Figure 5.10 Estimated marginal means of major claw size of **female** crab, *Esanthelphusa nani*, caught from reference and contaminated sites at Nan Province, Thailand during July 2010-June 2011. Covariate carapace width was estimated at 42.26 mm.

Sexual dimorphism has been report in crab (Yamaguchi, 1973). In *E. nani*, sexually dimorphic traits include difference in size and shape of abdomen (Figure 5.2) and claw size (Figure 5.1). Male and female crabs similarly have five segments of abdomen, but the abdomen of the female crab is broader than those of the male crab. In the male crab, claw size shows a bilateral asymmetry with one larger claw (major claw) on one side and one smaller claw (minor claw) on another side. On the other hand, the claw size in female shows a bilateral symmetry with relatively similar size of claw.

These sexually dimorphic traits of crab are developed under hormonal control (Charniaux-Cotton, 1955). Major hormone that acts on sex-determination and influences on secondary sexual characteristics in crab is an androgenic gland hormone produced in androgenic gland locating at the distal portion of sperm duct (Charniaux-Cotton, 1955; Takentomi and Nishikawa, 1996). Androgenic gland hormone is necessary for morphotypic progression in male crab such as developing of male secondary characteristics and inhibiting of female secondary characteristics and vitellogenesis (Sagi et al, 1990; Sagi et al., 2002; Manor et al., 2004). In addition, an ovarian hormone is also responsible for the normal development of female secondary sexual characteristics (Nagamine and knight, 1987).

Many reports have been suggested that the vertebrate steroids many affect on reproduction of different crustacean species by stimulating of ovarian maturation (Subramoniam, 2000; Tsukimura et al., 2000; Summavielle et al., 2003; Gunamalai et al., 2006). Insecticides and xenobiotics have also been reported to interfere with hormonal response of crustaceans (Gagne et al., 2005; Tuberty and McKenney, 2005).

Study on effect of tributylin on freshwater crab clearly revealed that TBT can induce imposex in both male and female crabs (Takahashi et al., 2000).

For herbicide, researches on herbicide toxicity in crustaceans showed acute/chronic effects in many species including atrazine, glyphosate and paraquat (Chu and Lau, 1994; Lukančič, et al., 2010; Deepananda et al., 2011). Dodson et al., (1999) reported that atrazine can influence on sex determination of crustacean offspring. However, data on effect of herbicide on hormonal response of crustaceans is still limited.

The results of this study showed potential associations between herbicide contamination and changes in sexually dimorphic traits of the rice field crab. Potential trend of demasculinization was found in the male paddy crab with reduced abdominal area and the major claw size. In contrast, potential trend of hyperfeminization, i.e. larger abdominal area, was occurred in female crab. These evidences indicate the potential interference of herbicide contamination with normal development of sexually dimorphic trails of the crabs.

5.3.3 Association between herbicide contaminations in crab tissue and gravimetric/ morphometric parameters

Correlations between herbicide contaminations in crab tissue and gravimetric and morphometric parameters are shown in Table 5.1. Significant negative correlation was found between atrazine residue in crab tissue versus the major claw size (Pearson's correlation coefficient = -0.3512, $p<0.05$). This negative association suggests that herbicide contamination could play role in change in crab morphology.

Pearson's correlation coefficients showed significant associations among morphological traits. First, the major claw size showed significantly negative correlation with abdominal area. In nature, the narrow abdominal area as well as

large major claw are presented in male crab. While the wide abdominal area as well as small major claw are evidenced in female crab. Therefore, this association confirms the assumption that *E. nani* is sexually dimorphic, and the dimorphic traits include abdominal area and the major claw size. In addition, the major claw size also showed significantly positive correlation with the relative body weight. This indicates significant contribution of claw size to the body weight.

Table 5.1 Pearson's correlation coefficients correlating between herbicide contaminations in crab tissue and gravimetric / morphometric parameters (n=44). Significant correlation between herbicide was indicated by an asterisk (*).

Data	Atrazine	Glyphosate	Paraquat	Body weight	Abdominal area	Claw size
Claw size	-0.3512* p= 0.017	-0.1353 p= 0.393	0.0759 p= 0.633	0.5321* p< 0.001	-0.6867* p< 0.001	1.0000 p=0.000
Abdominal area	0.0945 p= 0.523	0.0937 p= 0.545	-0.2377 p= 0.099	-0.0816 p= 0.581	1.0000 p= 0.000	
Body weight	-0.2354 p= 0.107	-0.1463 p= 0.343	-0.0109 p= 0.944	1.0000 p= 0.000		
Paraquat	0.0084 p= 0.957	0.3359* p= 0.025	1.0000 p= 0.000			
Glyphosate	0.3957* p= 0.008	1.0000 p= 0.000				
Atrazine	1.0000 p= 0.000					

5.4 Conclusion

Overall health status and morphometric analyses of some external morphology of the rice field crab *Esanthelphusa nani* living in agricultural areas with different degree of herbicide utilization were assessed. Over all health as determined by the relative body weight of the crab showed significant site-related difference. Male crab living in the herbicide utilization area tended to lose relative body weight. The significant reduction in size of the major claw in male crab was also observed. Since the major claw size is positively correlated with the relative body weight, this association could indicate that the size of major claw influence on relative body weight. The result of abdominal area comparisons also showed significant reduction in abdominal area of male crab from the contaminated site compared to those in the reference site. Correlation study clearly showed that the rice field crab is sexually dimorphic, with important trails including abdominal area and claw size possibly developed under hormonal control. These results suggested that herbicide, especially atrazine, utilization seemed to play roles in morphological changes including demasculinization of the male crab and feminization of the female crab.

CHAPTER VI

GENERAL CONCLUSION

Utilization of herbicides, especially atrazine, glyphosate and paraquat in agricultural activity throughout Nan Province has been well documented. Since herbicides can directly pose adverse effects on non-target organisms, it is thus important to monitor degree of herbicide contamination in environmental compartments and its effects on a representative animals living in areas with potential contamination. In this study, assessment of herbicide contamination and potential adverse effects was performed in two rice fields with and without herbicide utilization (contaminated site and reference site) in Wiang Sa District, Nan Province. Screening for herbicides in environmental samples (soil and water) of these study sites was conducted. Rice field crab, *Esanthelphusa nani* (Naiyanetr, 1984), was used as a sentinel species for herbicide contamination and potential health effect on non-target animal. The crab caught from these two study sites was subjected to analyses for herbicide contamination in tissue, glutathione *S*-transferase (GST) activity and lipid peroxidation (LPO) level in hepatopancreas, and assessment of health status and change in morphometric parameters.

The screening for herbicide residue in environmental sample showed detectable amount of atrazine in water of the contaminated site, indicating that herbicide contamination was evidenced in agricultural area with intensive herbicide utilization. Using highly sensitive ELISA techniques for contaminant analysis, it was found that atrazine, glyphosate and paraquat were presented in tissue of crab from both contaminated site and reference site. Particularly, the paraquat residue in crab tissue was much higher than the maximum residue limit in food (CODEX 2006), suggesting that the rice field crab in these areas were not suitable for human

consumption. Site comparison revealed that the level of atrazine in crab tissue was significantly higher in the contaminated site compared to those of the reference site, suggesting that the rice field crab could be used as a potential atrazine accumulator, and the higher levels of herbicide residue in crab tissue were corresponded with the herbicide utilization in agriculture activity.

Activity of GST, a detoxifying enzyme in phase II of biotransformation, was used for assessing oxidative stress status in the rice field crab. GST activities in hepatopancreas of the crab were significantly higher in the contaminated site compared to those of the reference site, suggesting that the rice field crab living in the contaminated site was under a more severe oxidative stress than those living in the reference site. FOX assay was employed to determine LPO levels in the rice field crab. LPO is normally used as a marker of cell damage linking with oxidative stress. Comparison of LPO level in the crab hepatopancreas showed no significant difference between sites, indicating that the GST was sufficient to detoxify the reactive oxygen species and protect against oxidative stress. In addition, there were positive correlations between atrazine contamination in crab tissue vs. GST activity and LPO level, demonstrating the link between increased herbicide residue in the crab tissue and increased in GST activity and LPO level in its hepatopancreas.

Relative body weight of the paddy crab was used as a simple marker of overall health status of the rice field crab. The relative body weights were significantly higher in the reference site crab compared to the contaminated site crab. Morphometry of the sexually dimorphic traits including major claw size and abdominal area were performed to examine influence of herbicide on morphology of the rice field crab. The major claw size of male crab living in the reference site was

significantly larger than those in the contaminated site. Since there was a significantly positive correlation between relative body weight and major claw size, losing of the relative body weight could be due to the decrease in major claw size in male crab of the contaminated site. The abdominal area of male crab in the reference site was also significantly larger than those of the contaminated site. Although abdominal area of the female crab was not significantly different between sites, the trend of larger abdominal area or feminized female crab was suggested in crab living in the contaminated site. Moreover, there was a significantly negative association between major claw size and atrazine residue in the crab tissue, suggesting that reduction of major claw size in the male crab living in the contaminated site could be linked to the increase in atrazine contamination.

From this study, the rice field crab has shown a potential to be used as a sentinel species for evaluating effect of herbicide contamination on non-target organism. The rice field crab was a decent herbicide accumulator and could respond to herbicide contamination at different biological levels including enzymatic, cellular and morphological levels. Importantly, associations between herbicide residues in the tissue and biologic responses of the crab were observed (Table 6.1), suggesting that different degree of biologic responses could be expected in the presence of gradient of herbicide utilization in the field. It is also possible that some adverse effects found in the rice field crab may play roles in its survival and reproduction in the future. The results of this sentinel study provided concrete evidences that an intensive herbicide utilization could lead to environmental and biological contamination and adverse effects to the non-target organism living in agricultural environment.

Table 6.1 Pearson's correlation coefficients between herbicide contamination in tissue and biologic responses the rice field crab, *Esanthelphusa nani*, living in agricultural areas of Nan Province, Thailand. Significant correlation between parameters (n=44) is indicated by an asterisk (*).

Data	Atrazine	Glyphosate	Paraquat	GST	LPO	Body weight	Abdominal area	Major claw size
Major claw size	-0.3512* p= 0.017	-0.1353 p= 0.393	0.0758 p= 0.633	0.0005 p= 0.997	0.0173 p= 0.909	0.5321* p< 0.001	-0.6867* p< 0.001	1.0000 p= 0.000
Abdominal area	0.0945 p= 0.523	0.0937 p= 0.545	-0.2377 p= 0.099	-0.1002 p= 0.098	0.1349 p= 0.360	0.0816 p= 0.581	1.0000 p= 0.000	
Body weight	-0.2354 p= 0.107	-0.0109 p= 0.343	-0.0109 p= 0.944	-0.0614 p= 0.679	0.0197 p= 0.895	1.0000 p= 0.000		
LPO	0.2907* p= 0.045	0.1813 p= 0.238	-0.0556 p= 0.719	0.4039* p=0.004	1.0000 p= 0.000			
GST	0.3054* p= 0.035	0.0674 p= 0.664	-0.3548* p= 0.020	1.0000 p= 0.000				
Paraquat	0.0083 p= 0.957	0.3359* p= 0.025	1.0000 p= 0.000					
Glyphosate	0.3958* p= 0.008	1.0000 p= 0.000						
Atrazine	1.0000 p= 0.000							

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Appendices

Appendix A

General climate description in Nan Province during sampling period

(July 2010-June 2011)

General climate description in Nan Province during sampling period

In this study period (July 2010 to June 2011), the average air temperature was 26.06 ºC and average total rainfall in each month was 158.85 mm. Description of wet and dry seasons in this study was determined based on the climate diagram plot between mean temperature and total rainfall of each month (Walter, Harnickell, and Mueller-Dombois, 1975). The climate during this sampling period was described by climograph as listed below:

1. July 2010-September 2010 was determined as late wet period
2. October 2010-December 2010 was determined as early dry period
3. January 2011- March 2011 was determined as late dry period
4. April 2011-June 2011 was determined as early wet period

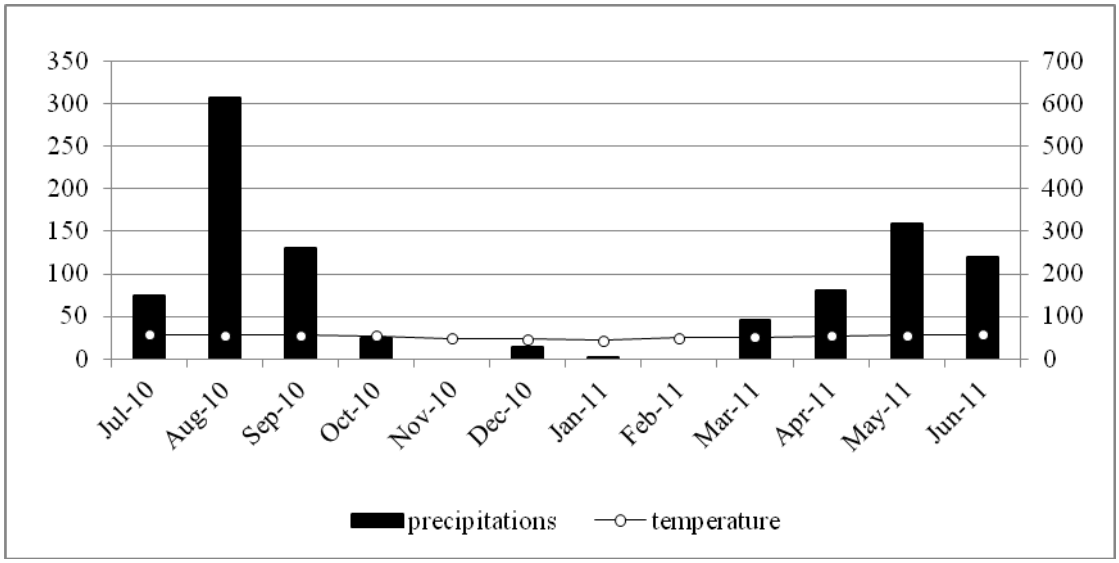

Figure A1 Climate diagram (climograph) of Nan Province during sampling period (July 2010 – June 2011)

Appendix B

Number of the rice field crab, *Esanthephusa nani*, collected

in the sampling period (July 2010-June 2011)

Table B1 Total number of rice field crab, *Esanthephusa nani*, collected from study sites at Nan Province in each month during July 2010-June 2011

Site / Month	Reference site (Lai-Nan District)		Contaminated site (San District)	
	Male	Female	Male	Female
July 2010	5	5	5	5
August 2010	5	5	5	5
September 2010	5	5	5	5
October 2010	5	5	5	5
November 2010	3	5	2	5
December 2010	5	5	3	5
January 2011	5	5	5	5
February 2011	0	5	0	2
March 2011	5	5	3	5
April 2011	3	5	2	5
May 2011	5	5	5	5
June 2011	5	5	5	5

Appendix C

Performance data of enzyme linked immunosorbent assay (ELISA) for determining of herbicide contaminations

I. Performance data of enzyme linked immunosorbent assay (ELISA) kit of Abraxis® for determining of atrazine contamination

Precision

Coefficients of variation (CVs) for standards: <10%, CVs for samples: <15%

Sensitivity

The detection limit for atrazine is 0.04 ng/mL (90% B/B0). The middle of the test (50% B/B0) is at about 0.7 ng/mL. Determinations close to the middle of the tests give the most aaccurate results.

Specificity

The cross-reactivity of the Abraxis Glyphosate Plate Assay for various related analogoues are followed below:

atrazine	100 % (per definition)
ametryn	1.5 %
deethylatrazine	3.08 %
hydroxyatrazine	0.01 %
propazine	96 %
simazine	14.3 %
terbutylazine	0.33 %

The ELISA for atrazine also recognizes, beside atrazine, proazine. Cross-reactivities with pesticide classes other than triazines have not been observed.

Standard curve

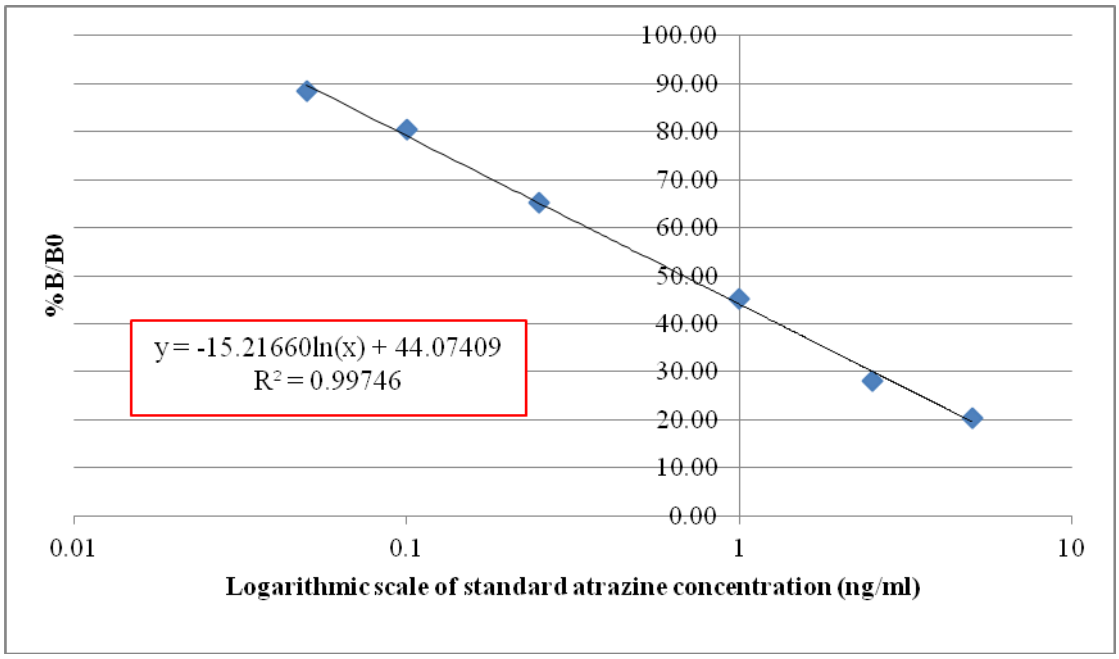

Figure C1 Representative standard calibration curve of Abraxis Atrazine Plate Assay used for the determination of atrazine in crab tissue

II. Performance data of enzyme linked immunosorbent assay (ELISA) kit of Abraxis® for determining of glyphosate contamination

Precision

Coefficients of variation (CVs) within assay: 5.5-12.2%; CVs between assay: 8.0-16.9%

Sensitivity

The Abraxis Glyphosate Plate Assay has an estimated minimum detectable concentration based on a 90% B/B0 of 0.05 parts per billion (ppb).

Specificity

The cross-reactivity of the Abraxis Glyphosate Plate Assay for various related analogoues can be expressed as the least detectable dose (LDD) which is estimated at 90% B/B0 or as the dose required for 50% absorbance inhibition (50% B/B0).

B/B0 of compounds	LDD (ppb)	50% (ppb)
Glyphosate	0.05	0.5
Glyphosine	50	3,000
Glufosinate	2,000	70,000
AMPA	35,000	1,000,000
Glycine	>10,000	1,000,000

The following compounds demonstrated no reactivity in the Abraxis Glyphosate Plate Assay at concentrations up to 1000 ppb: aldicarb, aldicarb sulfoxide, aldicarb sulfone, acetochlor, alachlor, atrazine, ametryn, benomyl, butylate, captan, crabaryl, carbendazim, carbofuran, cyanazine, 2,4-d, 1,3-dichloropropene, dinoseb, MCPA,

metolachlor, metribuzin, pentachlorophenol, picloram, propazine, samazine, terbuzin, terbufos, thiabendazole and thiophanate.

Standard curve

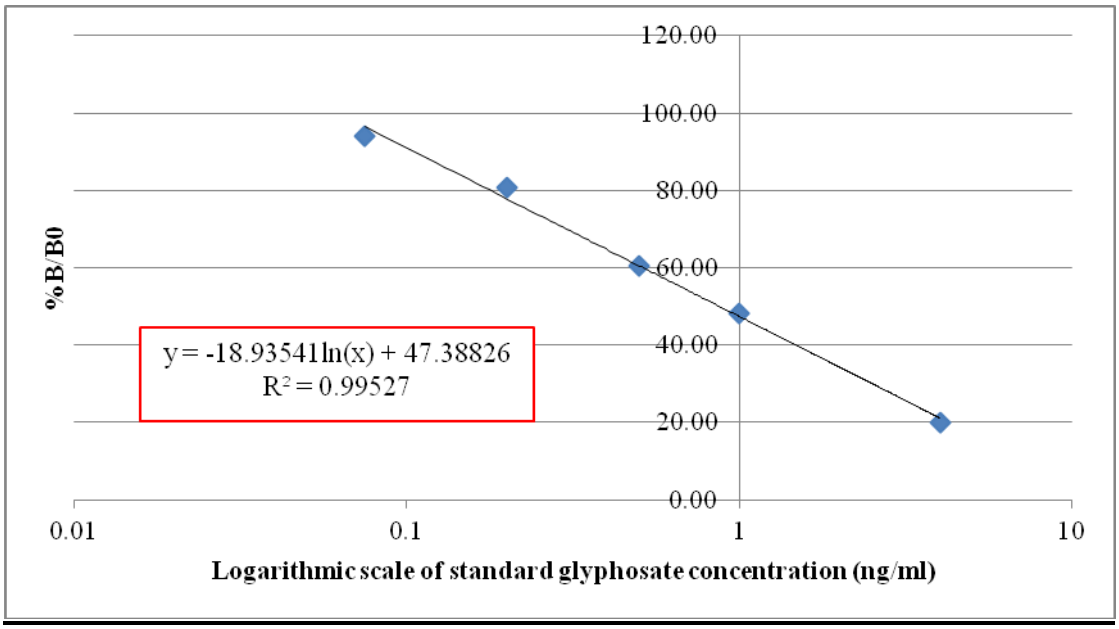

Figure C2 Representative standard calibration curve of Abraxis Glyphosate Plate Assay used for the determination of glyphosate in crab tissue

III. Performance data of enzyme linked immunosorbent assay (ELISA) kit of Abnova® for determining of paraquat contamination

Precision

Not available

Sensitivity

The Abnova® Paraquat Plate Assay has an estimated minimum detectable concentration based on a 90% B/B_O of 0.05 parts per billion (ppb).

Specificity

Not available

Standard curve

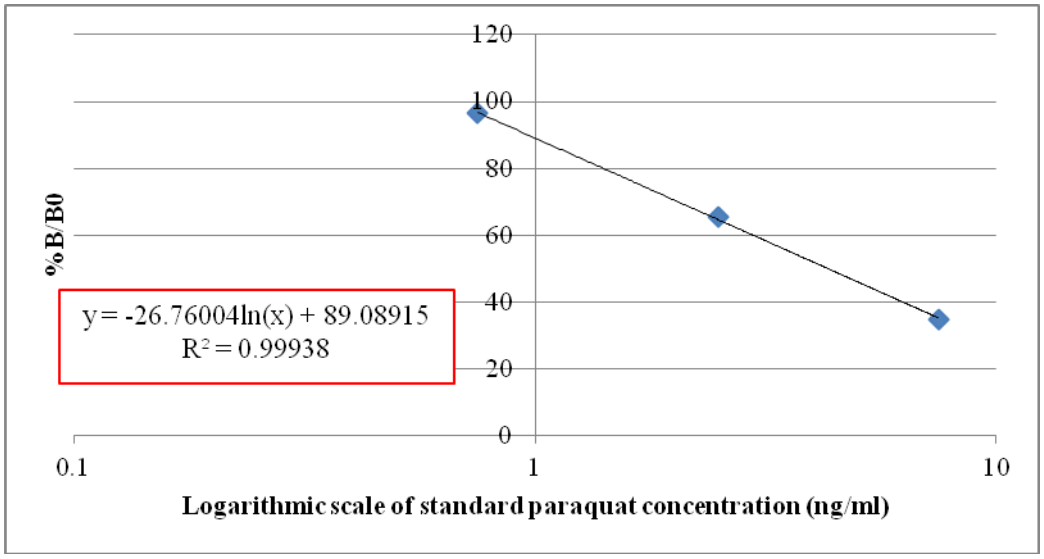

Figure C3 Representative standard calibration curve of Abnova® Paraquat Plate Assay used for the determination of paraquat in crab tissue

Appendix D

Chemical reagents preparation for

glutathione *S*-transferase activity assay

and

FOX assay

I. Solution for glutathione *S*-transferase assay (GST)

1. Extraction buffer (Tris-HCl 0.05 M, KCl 0.15 M, pH 7.4)
   - Triz-HCl 7.88 g
   - KCl 11.184 g
   - dH_2O to make 1,000 mL of final volume
2. Buffer solution for total protein measurement

   (phosphate bufferd saline (PBS), pH 7.2)
   - NaCl 8.0 g
   - KCl 0.2 g
   - Na_2HPO_4 1.44 g
   - KH_2PO_4 0.24 g
   - dH_2O to make 1,000 mL of final volume
3. Stock 1M L-glutathione
   - L-glutathione 0.0033 g
   - Deionized water to make 100 mL of final volume
4. Stock 100 mM Tris-HCl
   - Tris-HCl 15.76 g
   - dH_2O to make 1,000 mL of final volume
5. Working solution of glutathione (freshly preparing and used within 1 hour)
   - 100 mM Tris-HCl 24.5 mL
   - 200 mM L-glutathione 250 µL
6. Stock 100 mM 1-chloro-2, 4-dinitrobenzene
   - 1-chloro-2, 4-dinitrobenzene 0.2056 g
   - 95% ethanol to make 10 mL of final volume

II. Solution for FOX assay (lipid peroxidation determination)

1. Stock 100 mM $FeSO_4$ (prepared immediately before use)
   - $FeSO_4$ 0.2780 g
   - dH_2O to make 10 mL of final volume
2. Working reagent 1 mM $FeSO_4$ (prepared immediately before use)
   - Stock 100 mM $FeSO_4$ 0.1 mL
   - dH_2O to make 10 mL of final volume
3. Stock 250 mM H_2SO_4
   - 95% conc. H_2SO_4 w/v 133.2 µL
   - dH_2O to make 10 mL of final volume
4. Stock 100 M xylenol orange
   - Xylenol orange 0.7606 g
   - dH_2O to make 10 mL of final volume
5. Working reagent 1 mM xylenol orange
   - Stock 100 M xylenol orange 100 µL
   - dH_2O to make 10 mL of final volume
6. Stock 1 M of cumene hydroperoxide
   - 80% cumene hydroperoxide (density=1.03g/mL) 147.76 µL
   - dH_2O to make 1 mL of final volume
7. Working solution 0.5 mM of cumene hydroperoxide

   (5 nmole of cumene hydroperoxide in 10 µL)
   - Stock 1 M of cumene hydroperoxide 10 µL
   - dH_2O to make 20 mL of final volume

Appendix E

Optimal conditions for determining lipid peroxidation by FOX assay

Optimal conditions for determining lipid peroxidation by FOX assay

A FOX assay (ferrous oxidation/xylenol orange) is a fast and accurate method for measuring level of LPO. The method of FOX assay based on the oxidation of Fe (II) by lipid peroxides under acidic conditions has been proposed by Hermes-Lima et al. (1995). Later, Monserrat et al. (2003) has modified the FOX assay using microplate instead of cuvette. Briefly, reaction mixture consists of 1 mM $FeSO_4$, 250 mM H_2SO_4, 1 mM xylenol orange and deionized water. Upon addition of sample tissue extract, the microplate was incubated at room temperature and the absorbance was determined at 595 nm using a microplate reader. Afterward, 5 ng of cumene hydroperoxide (CHP) was added into each well and the absorbance was determined at 595 nm at some point after CHP addition. To apply this method for a certain tissue extract, it is recommended to find 1) an optimal sample volume, 2) an optimal incubation time after sample addition and 3) an optimal incubation time after CHP addition.

I. Optimal sample volume for FOX assay

In the first trial, volumes of the crab tissue extract for FOX assay were initially tested according to Monserrat et al. (2003) at 10 µL, 20 µL, 30 µL, and 40 µL. Absorbance of the reaction mixture was read at 595 nm by a microplate reader every 30 minutes until 180 minutes. These absorbance values were plotted on vertical axis against the corresponding time on horizontal logarithmic axis using Microsoft Excel Program (Figure C1).

Since the microplate reader used in this study (Multiskan EX) can measure absorbance in the range of 0.001-2.000A, with the best accuracy at the middle of the range (1.000). Therefore, the optimal volume should give an absorbance as close to

1.000 as possible. In these cases, the volume of 10 µL seemed to be the best candidate. However, since there will be another spike in absorbance value after the addition of CHP. Moreover, this trial was based on only one sample with no known LPO level. Some other samples may give much higher level of LPO and result in much higher absorbance value. As the result, the volume of 5 µL was chosen as the optimal volume for this assay.

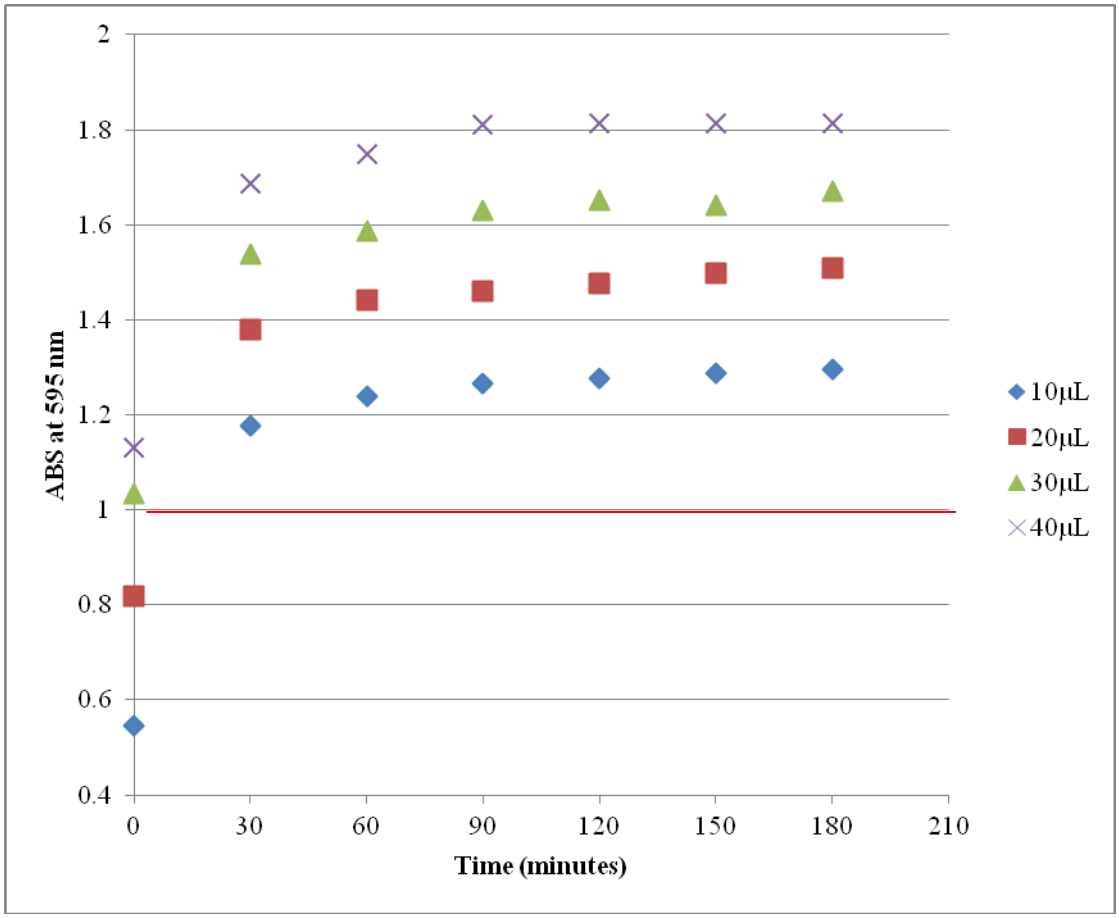

Figure E1 Change in absorbance at 595 nm of the reaction mixture of the FOX assay (before CHP addition) along 180-minute incubation time. Four volumes of tissue extract (10 µL, 20 µL, 30 µL, and 40 µL) were tested in this study.

II. Optimal incubation time after sample addition for the FOX assay

The optimal incubation time (after tissue extract addition) for FOX assay was chosen based on time in which oxidation of Fe(II) is stable. The plots of absorbance increments (Y axis) versus sample volumes (X axis) were created. The linear trend line of absorbance related to sample volumes was chosen as stable time or optimal incubation time (Figure C2). Based on this initial trial, even the longest incubation time (180 minutes), the trend line was still slightly curved. As the result, it was suggested that the optimal incubation time should be higher than 180 minutes.

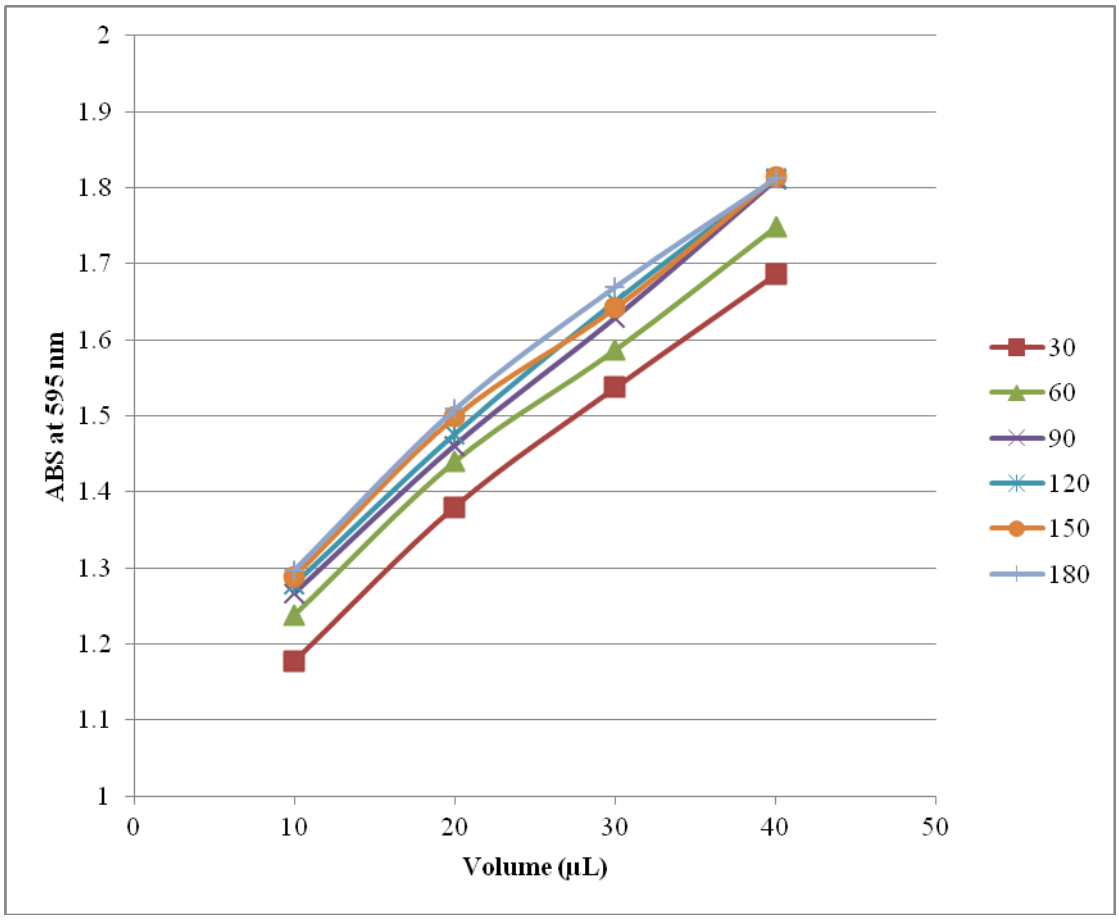

Figure E2 Linearity between absorbance at 595 nm and sample volumes at different incubation times.

III. Optimal incubation time after CHP addition for the FOX assay

This test was aimed to 1) validate that the volume of 5 µL was the optimal sample volume, and 2) find an optimal incubation time after sample addition based on results of larger pool of samples. In addition, this test was also aimed to find an optimal incubation time after CHP addition. Incubation times were read in every 30 minutes until 270 minutes. Afterward, CHP was added in an individual sample and the absorbance was read every 15 minute until 90 minutes. Changes in absorbance were plotted on Y axis versus incubation time on X axis (Figure C3)

It was found that sample volume of 5 µL did yield measurable value within the range of microplate reader (0.001-2.000). After tissue extract addition, stable reaction was observed during 210-270 minutes. After CHP addition, the reaction was stable at 30 minutes after CHP addition.

As the result of these trials, the optimal conditions for the FOX assay in this study included:

- sample volume of 5 µL
- incubation time after tissue extract addition of 240 minutes
- incubation time after CHP addition of 30 minutes.

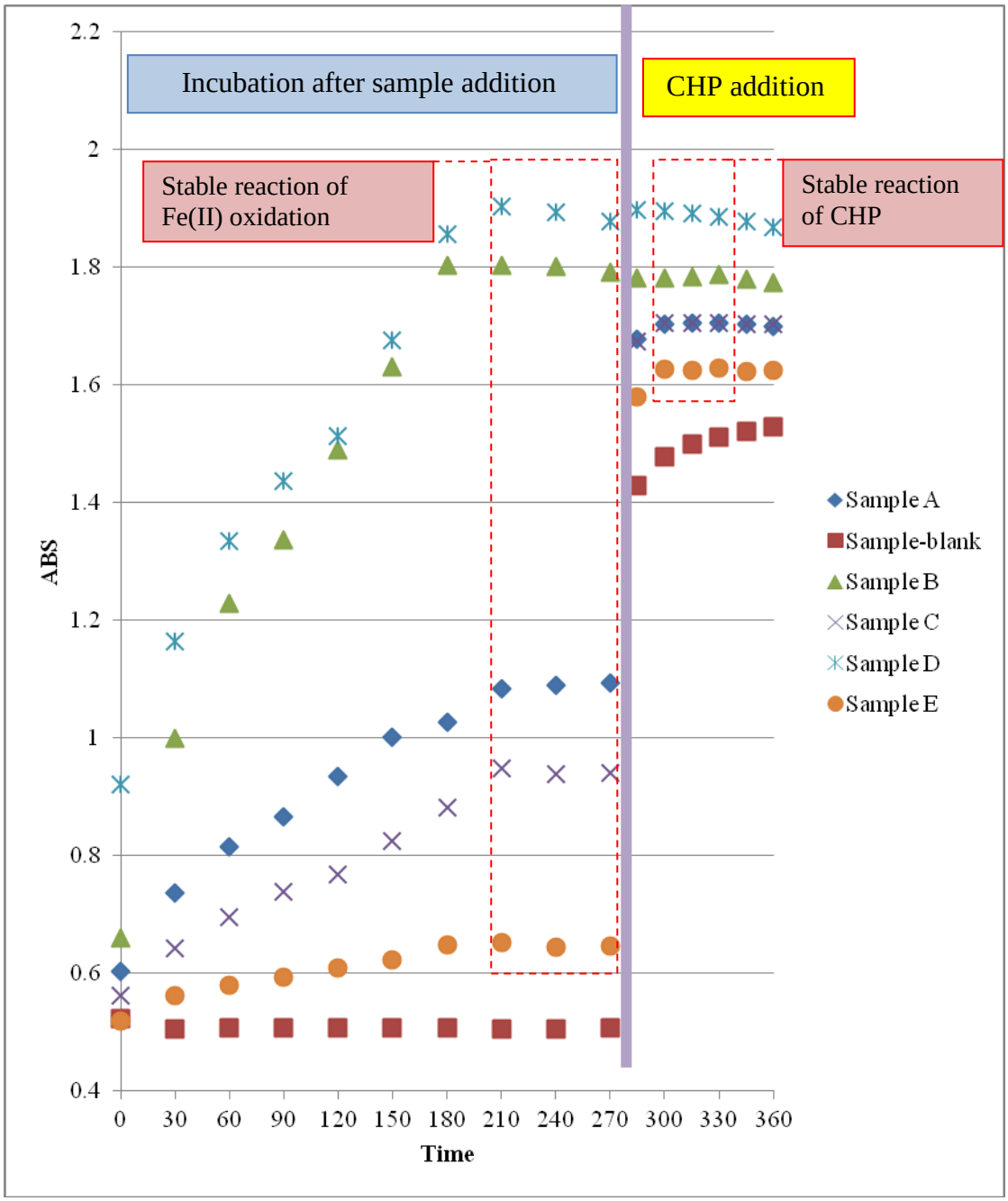

Figure E3 Changes in absorbance at 595 nm of the FOX assay during 270-minute incubation times (after sample addition) and 90-minute incubation times (after CHP addition).

BIOGRAPHY

Mr. Rachata Maneein was born on September 13, 1986 in Rayong province, Thailand. He received a bachelor degree in zoology from the Department of Biology, Faculty of Science, Chulalongkorn University in 2009. After his graduation, he continued his study for master degree in zoology at the same institute. As he interested in using animal as a sentinel species for environmental pollution, he chose to work on using rice field crab as the sentinel species for herbicide contamination in Nan Province for his graduate research. During this work, he carried out his research in the field and at the Chulalongkorn University Forest and Research station, Wiang Sa District, Nan Province for more than a year as part of the Chulalongkorn University Centenary Academic Development Plan 2008-2012 to encourage graduate student to get a first hand experience in field environment as well as perceiving the country's need in real life situations. During his graduate study, he has presented parts of his work in form of oral and poster presentations in the national and international conferences including the 5th International Congress of Chemistry and Environment at Port Dickson, Malaysia in 2010 (oral presentation), the 32nd Annual Meeting of the Society of Environmental Toxicology and Chemistry North America at Boston, M.A., U.S.A. in 2011 (poster presentation), the 1st National Symposium on Biodiversity Management, Nonthaburi, Thailand in 2012 (poster presentation) and the 17th Biological Sciences Graduate Congress, Bangkok, Thailand in 2012 (poster presentation). He has also published a part of his work as a research article in *Research Journal of Chemistry and Environment*, an international research journal listed in the Science Citation Index database.