ความเชื่อมโยงระหว่างการปนเปื้อนสารฆ่าวัชพืช และผลทางระบบสืบพันธุ์ใน หอยกาบน้ำจืด *Uniandra contradens* ในพื้นที่เกษตรกรรม จังหวัดน่าน

นายธงชัย ฐิติภูรี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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ปีการศึกษา 2555

ASSOCIATION BETWEEN HERBICIDE CONTAMINATION AND REPRODUCTIVE EFFECTS IN FRESHWATER MUSSEL *UNIANDRA CONTRADENS* IN AGRICULTURAL AREAS, NAN PROVINCE

Mr. Tongchai Thitiphuree

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Zoology
Department of Biology
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Thesis Title	ASSOCIATION BETWEEN HERBICIDE CONTAMINATION AND REPRODUCTIVE EFFECTS IN FRESHWATER MUSSEL *UNIANDRA CONTRADENS* IN AGRICULTURAL AREAS, NAN PROVINCE
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ธงชัย ฐิติภูรี: ความเชื่อมโยงระหว่างการปนเปื้อนสารฆ่าวัชพืช และผลทางระบบสืบพันธุ์ในหอยกาบน้ำจืด *Uniandra contradens* ในพื้นที่เกษตรกรรม จังหวัดน่าน. (ASSOCIATION BETWEEN HERBICIDE CONTAMINATION AND REPRODUCTIVE EFFECTS IN FRESHWATER MUSSEL *UNIANDRA CONTRADENS* IN AGRICULTURAL AREAS, NAN PROVINCE).

อ.ที่ปรึกษาวิทยานิพนธ์หลัก: อ.ดร. นพดล กิตนะ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: อ.ดร. จิรารัช กิตนะ, ผศ.ดร. ปกรณ์ วรานุศุภากุล, 157 หน้า.

จังหวัดน่านในภาคเหนือของประเทศไทยเป็นพื้นที่เกษตรที่อุดมสมบูรณ์ การเพาะปลูกพืชหมุนเวียนในพื้นที่ทำให้มีการใช้สารเคมีทางการเกษตรอย่างแพร่หลาย โดยเฉพาะสารฆ่าวัชพืช ในการตรวจสอบผลของการใช้สารฆ่าวัชพืชต่อการปนเปื้อนสู่สิ่งแวดล้อมทางน้ำ ได้นำตัวอย่างดินตะกอนและน้ำจากอ่างเก็บน้ำในพื้นที่เกษตรกรรมของจังหวัดมาตรวจสอบการปนเปื้อนแอทราซีนด้วยเครื่อง GC-MS และไกลโฟเสตกับพาราควอตด้วยเครื่อง HPLC พบว่ามีการปนเปื้อนแอทราซีนในน้ำ (<0.01-0.16 ไมโครกรัมต่อมิลลิลิตร) และดินตะกอน (<0.01-0.23 ไมโครกรัมต่อกรัม) ในการศึกษาการปนเปื้อนในสิ่งมีชีวิต ได้นำหอยกาบน้ำจืด *Uniandra contradens* ที่เก็บตัวอย่างทุกสามเดือนในช่วง กรกฎาคม พ.ศ. 2553 ถึงมิถุนายน พ.ศ. 2554 มาตรวจการปนเปื้อนภายในตัวด้วยเทคนิค ELISA พบว่ามีการปนเปื้อนของแอทราซีน (1.26-88.86 นาโนกรัมต่อกรัมน้ำหนักแห้ง) ไกลโฟเสต (5.34-11.90 นาโนกรัมต่อกรัมน้ำหนักแห้ง) และพาราควอต (23.30-55.97 นาโนกรัมต่อกรัมน้ำหนักแห้ง) ในทุกตัวอย่างที่ศึกษา และเมื่อนำตัวอย่างหอยกาบที่เก็บในแต่ละเดือนมาตรวจสอบสุขภาวะโดยรวมโดยใช้ค่าน้ำหนักสัมพัทธ์พบว่ามีค่าแตกต่างกันในแต่ละช่วงของปี โดยแสดงสหสัมพันธ์เชิงลบกับปริมาณของแอทราซีนและไกลโฟเสตที่พบในหอยกาบ เมื่อตรวจสอบระบบสืบพันธุ์ของหอยกาบโดยใช้ลักษณะกายวิภาคและชีวเคมี พบว่าหอยกาบเพศผู้และเพศเมียมีการสร้างเซลล์สืบพันธุ์ตลอดทั้งปี และพบช่วงที่ระบบสืบพันธุ์เพศเมียเจริญสูงสุด กล่าวคือ มีเซลล์ไข่เจริญ มีระดับไวเทลโลเจนินสูงสุด และมีการสะสมไข่ในเหงือก ลักษณะที่แสดงถึงฤดูวางไข่เหล่านี้พบในช่วงฤดูแล้งซึ่งเป็นช่วงเวลาเดียวกับที่พบการปนเปื้อนแอทราซีนในน้ำ จึงแสดงโอกาสเสี่ยงและผลกระทบต่อระบบสืบพันธุ์และการเจริญของหอยกาบ เมื่อทดลองใช้หอยกาบน้ำจืดเป็นสัตว์เฝ้าระวังในพื้นที่แหล่งน้ำที่มีกิจกรรมการเกษตรแตกต่างกัน พบว่าหอยกาบน้ำจืดเป็นตัวเฝ้าระวังที่สามารถบ่งบอกถึงระดับการปนเปื้อนและแสดงการตอบสนองที่มีสหสัมพันธ์กับการปนเปื้อนสารฆ่าวัชพืช ซึ่งข้อมูลจากการศึกษานี้อาจนำมาใช้เป็นสัญญาณเตือนถึงอันตรายของสารฆ่าวัชพืชต่อสัตว์น้ำ และอาจใช้แสดงความเชื่อมโยงกับความเสี่ยงต่อระบบสืบพันธุ์ในสัตว์ชนิดอื่นที่อาศัยในพื้นที่รวมทั้งมนุษย์

ภาควิชา ….ชีววิทยา…….. ลายมือชื่อนิสิต.....

สาขาวิชา ….สัตววิทยา……. ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก……

ปีการศึกษา ……2555……... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม……

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม……

5272324823: MAJOR ZOOLOGY
KEYWORDS: SENTINEL SPECIES / BIVALVE / TISSUE RESIDUE / CONDITION FACTOR / GEMETOGENESIS / SPAWNING

TONGCHAI THITIPHUREE: ASSOCIATION BETWEEN HERBICIDE CONTAMINATION AND REPRODUCTIVE EFFECTS IN FRESHWATER MUSSEL *UNIANDRA CONTRADENS* IN AGRICULTURAL AREAS, NAN PROVINCE. ADVISOR: NOPPADON KITANA, Ph.D. CO-ADVISOR: JIRARACH KITANA, Ph.D., ASST. PROF. PAKORN VARANUSUPAKUL, Ph.D., 157 pp.

Nan Province, in the northern part of Thailand, is a fertile area for agricultural activities. Crop rotations in this area lead to widely uses of agrochemicals especially herbicides. To examine whether an intensive use of herbicide could lead to contamination in aquatic environment, sediment and water samples collected from an agricultural catchment in Nan Province were screened for atrazine (GC-MS), paraquat and glyphosate (HPLC). The results showed that detectable levels of atrazine residue were found in water (<0.01-0.16 µg/mL) and sediment (<0.01-0.23 µg/g) of the reservoir. To examine an extent of contamination in biological samples, a freshwater mussel *Uniandra contradens* collected quarterly from the reservoir during July 2010 to June 2011 were examined for herbicide contamination by enzyme-linked immunosorbent assay. The results showed that detectable levels of herbicide including atrazine (1.26-88.86 ng/g dry weight), paraquat (23.30-55.97 ng/g dry weight) and glyphosate (5.34-11.90 ng/g dry weight) were found in every mussel examined. Monthly sampling and health monitoring of the mussels showed a seasonal change in condition factor, an indicative of overall health, with a significant negative correlation with atrazine and glyphosate residue in the tissue. Reproductive activities as determined by gross- and microanatomical as well as biochemical markers showed that mature sperms can be found throughout the year, indicating prolonged male gametogenic activities. A similar pattern was observed in female mussels with an exception in dry season when oocytes became larger, the vitellogenin level was at peak and swelling gills with mature egg deposition was evident. These indicatives of a spawning period occurred in dry season when atrazine contamination was evident, indicating a potential window of susceptibility of the mussel reproduction and development. Using *U. contradens* as a sentinel of contamination in two reservoirs with different agricultural activities, it was further proven that the mussel could be used as an effective biomonitor with a measurable and correlative response to herbicide contamination. The data from this study could be used as an early warning of the effects of herbicide contamination on freshwater animals and maybe used as a potential link to predict the reproductive health risk of other organisms including human living in this area.

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

Nan, a province in the northern part of Thailand, is known as an origin of several rivers and tributaries such as Nan River as well as a fertile area for agricultural activities. Seasonal cultivation in this area involves an intensive utilization of agrochemicals, especially herbicides (atrazine, glyphosate and paraquat). The continuous application of these herbicides in large amount could lead to environmental contamination and accumulation in aquatic organisms (Uno et al., 2001). In some situations, range of contamination could extend beyond aquatic habitats to bay tributaries (Lehotay et al., 1998) or into marine environment (Haynes et al., 2000). It is thus important to monitor an extent of contamination and potential health effects to animals living in the aquatic environment.

Mussels are invertebrate animals of phylum Mollusca. They are greatly depending on quality of aquatic environment. The mussel has complete life cycle in water as bottom dweller and filter feeder on plankton and organic matters through water column (Dillon, 2000). This life history makes the mussel susceptible to xenobiotic exposure and accumulation chemical residues into their body. This residue can be transfer to human by food chain because many kinds of mussels are used for human consumption. Thus, the mussel is regarded as a suitable monitoring species for xenobiotic contamination in environment.

In the past, many marine mussels were used as sentinel species for xenobiotic contamination. The International Mussel Watch project is one of the successful case studies. The project has systematically used the mussel *Mytilus edulis* as surrogate animal for the contamination along the coastal area of the Europe, Taiwan, Canada and the United States since 1978 (Jernelov, 1996). In addition, there was also a report on the use of two bivalves *Donax trunculus* and *Brachidontes pharaonis* for monitor pollutants in the

Mediterranean Coast of Israel and use *Pteria aegyptia* in the northern tip of the Gulf of Aqaba, Red sea (Bresler et al., 2003). Furthermore, *Mytilus edulis* and *Macoma balthical* have been used for monitoring organochlorine and heavy metal contamination in the southern coast of Finland (Lehtonen et al., 2006) and *Mytilus galloprovincialis* was used for monitoring the environmental contamination in the Northwest Portuguese Coast (Moreira and Guilhermino, 2005).

Freshwater mussels were also used for monitoring xenobiotic contaminations in environment. Several bivalves such as *Corbicula fluminea* and *Diplodon fontaineanus* have been selected for monitoring ametryn contamination in the river of São Paulo State of Brazil (Jacomini et al., 2010). In addition, several studies reported on link between xenobiotic accumulation in mussels and adverse health effects in their organ systems, suggesting the potential use of freshwater mussels as a sentinel species of environmental health hazards from xenobiotic contamination (Ji et al., 2006; Sheehan and power, 1999; Won et al., 2005).

According to the U.S. National Research Council, an animal selected to be used as a sentinel species should 1) show a measurable response to contaminant, 2) have home range overlap with contaminated site, 3) have large population size, and 4) be easily enumerated and captured (NRC, 1991). In a sentinel system, therefore, appropriate biomarkers should be examined in order to provide important biological information on potential impacts of xenobiotic contamination on the health of organisms and ecosystems.

The biological markers are classified into 3 types including 1) biomarker of exposure 2) biomarker of effect and 3) biomarker of susceptibility. Reproductive health is regarded as one of an important biomarker of effect for the environmental contamination (ATSDR, 2007). The use of reproductive biomarkers in sentinel system for xenobiotic contamination has been reported in several mollusk species. Effect on reproductive system such as imposex was reported in marine gastropod living in tributyltin contaminated area (Bettin et

al., 1996). Intersex was found in male clam *Scrobicularia plana* exposed to xenobiotic contaminant (Chesman and Langsto, 2006; Gomes et al, 2009). Hatching rate of snail *Marisa cornuarietis* was found to be affected by herbicide contamination (Sawasdee and Köhler, 2009). Decrease in growth rate of juvenile bivalves (Bringolf et al., 2007a; 2007b) and degeneration of oocytes in female gastropod (Bacchetta et al., 2002) were also reported as potential effects of pesticide contamination.

Recently, the specific biomarkers are combined with sentinel species system in several reports for providing the important information and evaluation on the potential effect of contamination and health effect of organisms in the ecosystem (Van der Oost et al., 1997). The biochemical responses in several molecules have been measures in the mussels such as vitellogenin-like proteins and glutathione-s-transferase (Gagné et al., 2001; 2004; Won et al., 2005).

Approach of this study

Nan Province is a fertile area for agricultural activities with intensive utilization of agrochemical especially herbicide. The continuous application of these herbicides in large amount could lead to environmental contamination and accumulation in aquatic organisms. In this study, the major study site was an agricultural catchment or Nong Bua reservoir located in San Subdistrict, Wiang Sa District, Nan Province. This reservoir was constructed for many agricultural activities and drained by several run-offs from surrounding areas. The minor study site was a public catchment or Nong Luang reservoir located in Lai Nan Subdistrict, Wiang Sa District, Nan Province. This reservoir is located in a native village with sporadic agricultural activities but could be affected by Nan River during flooding season. The herbicide residues were determined in environmental samples (water and sediment) and mussels for providing information of the exposure with herbicide contamination. The reproductive activities of the mussel are examined in order to provide baseline

information and insight into a potential window of susceptibility to herbicide contamination. The association between herbicide contamination and reproductive activities were correlated for potential utilization of *Uniandra contradens*, a common species that widely distribute in aquatic habitat close to agricultural area of Nan Province, as a sentinel species of herbicide contamination.

General objectives of the study

1. To monitor contamination of herbicide in freshwater mussel *Uniandra contradens* living in agricultural catchments, Nan Province
2. To examine reproductive activities of the freshwater mussel *Uniandra contradens* living in agricultural catchments, Nan Province
3. To evaluate association between herbicide contamination and reproductive effects of the mussel living in agricultural catchments, Nan Province

Chapter II
Literature Review

1. Herbicide Utilization in Thailand

An intensive agricultural activity of Thailand has been transformed into industry since 1970s. The mass production of agricultural product in similar fashion to the industry lead to utilization of agrochemicals for increasing the yield of crop production, reducing pest and time (Panuwet et al., 2012). In five year period (during 2007-2011), Thailand had imported agrochemicals for more than 645.91 tons. Herbicides have been well documented as a dominant chemicals in many reports (Figure 2.1). The amount of imported herbicides has increased dramatically from 68.82 to 97.96 tons during 2007-2011. Glyphosate, paraquat and atrazine are a major class of imported chemicals during these years (OAR, 2011).

In northern part of Thailand, it is well known as an origin of several tributaries such as Ping River, Wang River, Yom River and Nan River. Agricultural areas have been widely expanded toward riverine areas of these main rivers. Farmers in Thailand are cultivating rice as a major crop, and using insecticides to control pest in this major plant. On the contrary, herbicides are widely used in other crop to control weed (Sapbamrer et al., 2011). In Nan Province, a number of agrochemicals were applied to several agricultural activities. Herbicide (1,172.7 tons) was predominantly used for improving yield of crop production (Chanphong, 2008). The amount of herbicide utilization in rice crop and corn of Nan Province ranged from 90.5 to 854.7 tons (Chanphong, 2008). The major type of herbicide utilization of maize farmers in Na Noi, Wiang Sa, Muang and Santisuk Districts was paraquat (67.7%; Nan Provincial Agricultural Office, 2005). Further, interview of maize farmers also revealed the major class of herbicide as paraquat (60.9%) and glyphosate (39.1%) (Wongwichit et al., 2012).

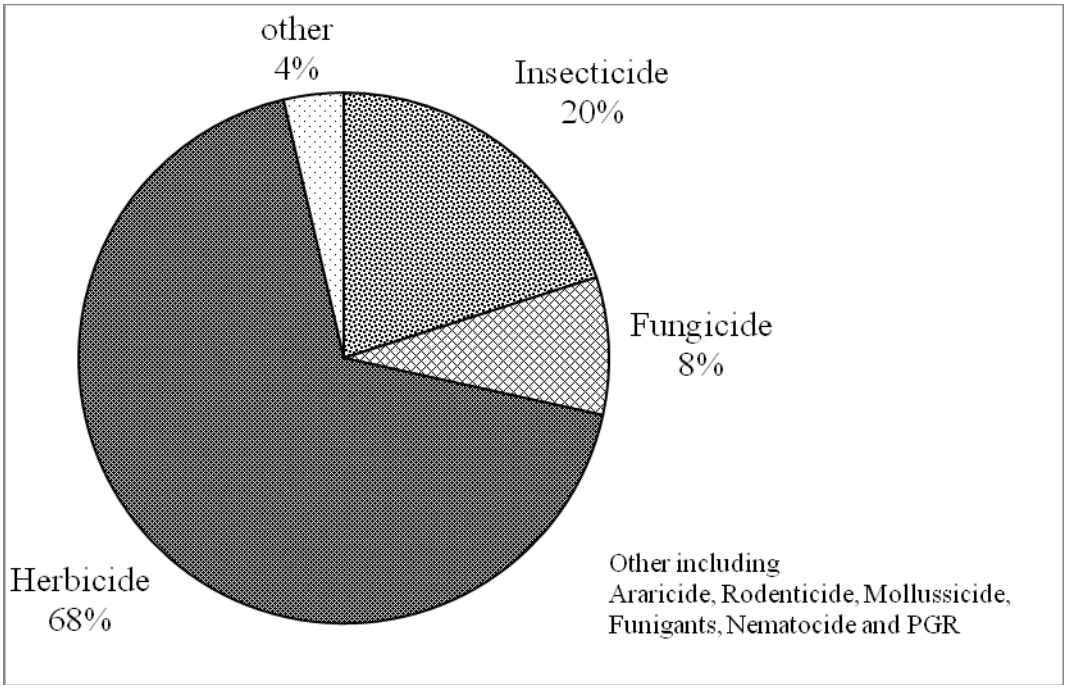
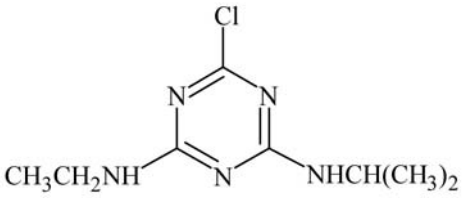


Figure 2.1: Proportion of the imported agrochemicals in Thailand during 2007 to 2011 (OAR, 2011)

2. Herbicide Property and Fate

2.1 Atrazine

Atrazine is a systemic triazine herbicide. This compound was used during pre-emergence to control broadleaf weeds. Atrazine has a chemical name as 2-chloro-4-ethylamino-6-isopropyl-amino-1-*s*-triazine. Atrazine is a white crystalline solid and slightly soluble in water (33 mg/L at 25°C).



Empirical Formula:	$C_8H_{14}ClN_5$
Molecular Weight:	215.7
CAS No.:	1912-24-9

Figure 2.2: Structure of atrazine with some properties (USEPA, 2003)

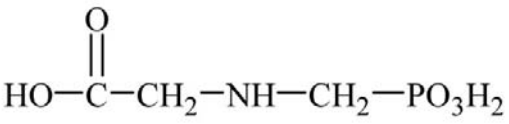
Properties of atrazine prevent it from strongly adsorb to sediments and may be isolated from the water current. Atrazine can be degraded in the water with slow rate of hydrolysis and aqueous photolysis (Solomon et al., 1996). The persistent of atrazine in an aqueous system is the result of *s*-triazine ring which defense its degradation from microorganisms (Solomon et al., 1996). The environmental half-life of atrazine has been documented to be more stable at normal pH. In the pH range of 2.9 to 7.0, half-life of atrazine was 34.8 to 742 days (Khan, 1978). Atrazine can be degraded into hydroxyatrazine compounds and chlorinated atrazine compounds such as desethylated atrazine, desisopropyl atrazine and diaminodhlorotriazine (USEPA, 2003). But the major product of atrazine hydrolysis was hydroxyatrazine (Khan, 1978)

There have been several organisms documented to be affected by atrazine in many way, especially endocrine disruption (Allran and Karasov, 2001; Hayes et al., 2003). Endocrine system of several mollusk species were reported to show an adverse effect upon exposure to agrochemicals

contamination (Chesman and Langsto, 2006; Gomes et al., 2009). Atrazine was reported to show a chronic effect in a low level (0.1 µg/mL) on ramshorn snail *Marisa cornuarietis* to cause a reduction in hatching rate (Sawasdee and Köhler, 2009). At the higher concentration of atrazine (>3.8 µg/mL), glochidia and juvenile of mussel *Lampsilis siliquoidea* was found to reduce in growth rate (Bringolf et al., 2007a; 2007b). Furthermore, atrazine also showed its potential to be accumulated in bivalves *Anodontites trapesialis* and *Corbicula fluminea* (Jacomini et al., 2003; 2006).

2.2 Glyphosate

Glyphosate is classified into a derivative glycine group. Glyphosate is a non selective herbicide that was used post-emergence for vegetation control, even in forestry. Glyphosate has a chemical name as N-phosphonomethyl glycine. Several trade names were report such as Roundup, Rodeo, Shackle etc. This compound can be well soluble in water with solubility of 15,700 mg/L at 25°C at pH 7.0 (USEPA, 1993).



Empirical Formula:	$C_3H_8NO_5P$
Molecular Weight:	169.07
CAS No.:	38641-94-0

Figure 2.3: Structure of glyphosate with some properties (USEPA, 1993)

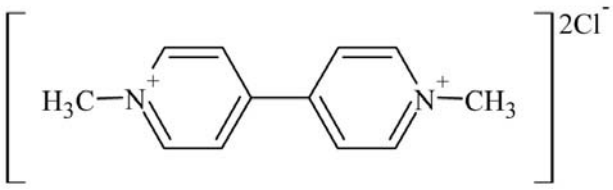
Glyphosate is a resistant compound to hydrolysis, thermal decomposition and photolysis (Moore et al., 1983). Glyphosate can be biodegraded by bacteria and inactivated by adsorption to clay (Petit et al., 1995). Glyphosate is completely degraded in soil by micro organisms and produced major metabolized of aminomethylphosphonic acid (AMPA).

Balthazor and Hallas (1986) studied an industrial activated sludge and showed that glyphosate can be transformed to AMPA as a first product of the reaction. Muller et al. (1981) reported that glyphosate can be metabolized in both aerobic and anaerobic conditions in soil that are closely associated with microbial activity. Glyphosate has a half-life of 8.1 days in anaerobic conditions. Afterward, the AMPA was a major metabolized product (USEPA, 1993). Giesy et al. (2000) showed that half-life of glyphosate exposed with microorganisms could range from 7 to 14 days in soil.

Glyphosate is slightly toxic to aquatic fauna, but several of its formula showed relatively higher toxicity in aquatic organism (Folmar et al., 1979). Bringolf et al. (2007) showed the high sensitivity of early stage of *Lampsilis siliquoidea* at both glochidia (48 hr, EC50: 2.9 mg/L) and juvenile (48 hr, EC50: 5.9 mg/L). Conners and Black. (2004) showed acute toxicity of Roundup in freshwater mussel *Utterbackia imbecillis*, and reported the EC50 at 24 hours of 13.5 mg/L in this mussel.

2.3 Paraquat

This compound is member of bipyridylniums. The structure of paraquat is formed in a complex salt known as a paraquat dichloride. Paraquat dichloride has chemical name as 1,1’-dimethy1-4,4’-bibipyridinium dichloride. Several trade names have been established such as Gramoxone, Destrone, Herboxone etc. Paraquat can be well soluble in water with solubility of 620,000 mg/L at 25°C (USEPA, 1997).



Empirical Formula: $C_{12}H_{14}Cl_2N_2$

Molecular Weight: 257.2

CAS No.: 1919-42-5

Figure 2.4: Structure of paraquat dichloride with some properties (USEPA,1997)

Paraquat is strongly adsorbed to soils and sediments resulting in an unavailable biological form. It is not degraded significantly for many years, except in surface soils (Eisler, 1990). In top soils, approximately 50% of paraquat is loosed by photodecomposition within 3 weeks (Eisler, 1990). In freshwater habitat, it could be decreased rapidly from the water current at about 50% in 36 hour and 100% in 4 weeks (Eisler, 1990). The strong binding of paraquat to soil interaction is the major factors that decrease the motilities of the herbicide leaching to the water (Smith and Mayfield, 1978). Paraquat adsorption is not significantly affected by soil pH, but could be modified by soil porosity, moisture content, residence time, and adsorption capacity (Smith and Mayfield, 1978).

Paraquat can be metabolized by bacteria and produced end product of carboxylated 1-methylpyridinium ion (Funderburk and Bozarth, 1967). Photodecomposition can be activated on top soil (Smith and Mayfield, 1978). Christian et al. (1985) also suggested that 20-50% of paraquat on top soil can be degraded in about 3 weeks. A major product from photodegradation in soils by UV light was 1-methyl-4-carboxypyridinium ion, 4-picolinic acid, hydroxyl-4-picolinic acid, N-formylglycine, malic acid and oxalic acid, respectively (Eisler, 1990).

Several reports showed toxicity of paraquat on mollusk species. Haley (1979) showed that paraquat was toxic to eggs and newly hatched snails *Bulinus truncates*, *Biomphalaria alexandrina* and *Lymnaea calliaudi.* In ribbed mussels *Geukensia demissa*, paraquat showed an ability of inducing an anti-oxidant enzymes that demonstrated the response in oxidative stress in other species (Wenning et al., 1988). Bacchetta et al. (2002) reported that paraquat showed an adverse effect on fertility of *Physa fontinalis* by decreasing an amount of egg laying and increasing degeneration of oocyte in female snail.

Table 2.1: Summary of herbicide properties and some effects on non-target organism

Characteristics	Atrazine	Glyphosate	Paraquat
Half-life			
• **Soil**	o 3-4 month (laboratory) (US EPA, 2003) o Photolysis in soil = 330 days (sediment) (US EPA, 2003)	o Biodegradation in soil = 60 days (Petit and Cabridenc, 1995)	o Relative stable (EC, 2003)
• **Water**	o >100 days at 20°C (WHO, 1996), 41-237 days (Solomon et al., 2008), 578 days (US EPA, 2003) o Stable to sunlight at pH 7.0 (US EPA, 2003) o Overall = 608 days (US EPA, 2003)	o 7-14 days (Giesy, 2000) o Resistant to hydrolysis (Petit and Cabridenc, 1995)	o Relative stable at pH 5, 7 and 9, 30 days at 25 °C and 40 °C. (EC, 2003) o Relative stable at visible wavelengths in environment (EC, 2003)

Table 2.1: Summary of herbicide properties and some effects on non-target organism (continued)

Characteristics	Atrazine	Glyphosate	Paraquat
Toxicity			
• **Mammal**			
o Rat	o LD_{50} = 1,869-3,080 mg/kg (US EPA, 2003)	o LD_{50} = >5,000 mg/kg (Williams et al., 2000)	o LD_{50} = 93.4 mg/kg (EC, 2003)
• **Fish**			
o Rainbow trout (*Oncorhynchus mykiss*)	o LC_{50} = 5,300 µg/L (96 hr) (US EPA, 2003)	o LC_{50} = 8.3 mg/L at 12 °C (24, 96 hr) of Roundup formula (Folmar et al., 1979)	o LD_{50} = 19 mg as/l bw (96 hr) (EC, 2003)
• **Invertebrate**			
o Daphnids (*Daphnia magna*)	o LC_{50} = 720 µg/L (48 hr) (US EPA, 2003)	o LC_{50} = 3.0 mg/L at 22 °C (48 hr) of Roundup formula (Folmar et al., 1979)	o EC_{50} = 4.4 mg as/l (48 hr) (EC, 2003)
o Midge (*Chironomus tentans*)	o LC_{50} = 1000 µg/L (96 hr) (US EPA, 2003)	o LC_{50} = 18 mg/L at 22 °C (48 hr) of Roundup formula (Folmar et al., 1979)	o LC_{50} = 1325 mg/L (24 hr) (Ha and Choi, 2008)

Table 2.1: Summary of herbicide properties and some effects on non-target organism (continued)

Characteristics	Atrazine	Glyphosate	Paraquat
Maximum residue limit in food o **Poultry meat** o **Meat (from mammals other than marine mammals)**	o 0.04 mg/kg (HCPMRA, 2010) o 0.04 mg/kg (HCPMRA, 2010)	o 0.05 mg/kg (CODEX, 2006) o 0.05 mg/kg (CODEX, 2006)	o 0.005 mg/kg (CODEX, 2006) o 0.005 mg/kg (CODEX, 2006)
Maximum contaminant level in drinking water	o 0.003 µg/L (US EPA, 2009)	o 0.7 mg/L (US EPA, 2009)	o 1.0 µg/L (Hemilton et al., 2003)

3. Sentinel Species

The animals systems can be used to predict the potential health effects of the contamination in an environment to other animals and human. This idea has been used for environmental risk assessment because of the fact that the animal is exposed to chemical contaminants in habitats shared or comparable with humans at the same concentrations. Furthermore, animal could respond to chemical insulting in the forms of pathologic conditions such as behavioral changes, reproductive disruption, immunologic disruption, biochemical as well as anatomic changes. Animals outside the laboratory can yield a more accuracy information at each step in risk assessment. Under appropriate conditions, the use of domestic and wild animals can help to reveal the presence of unknown chemical contaminants in the environment before they cause harmful effects to human. This is due to the fact that domestic and wild animals share the environment and food chain with the human (NRC, 1991) (Figure 2.5).

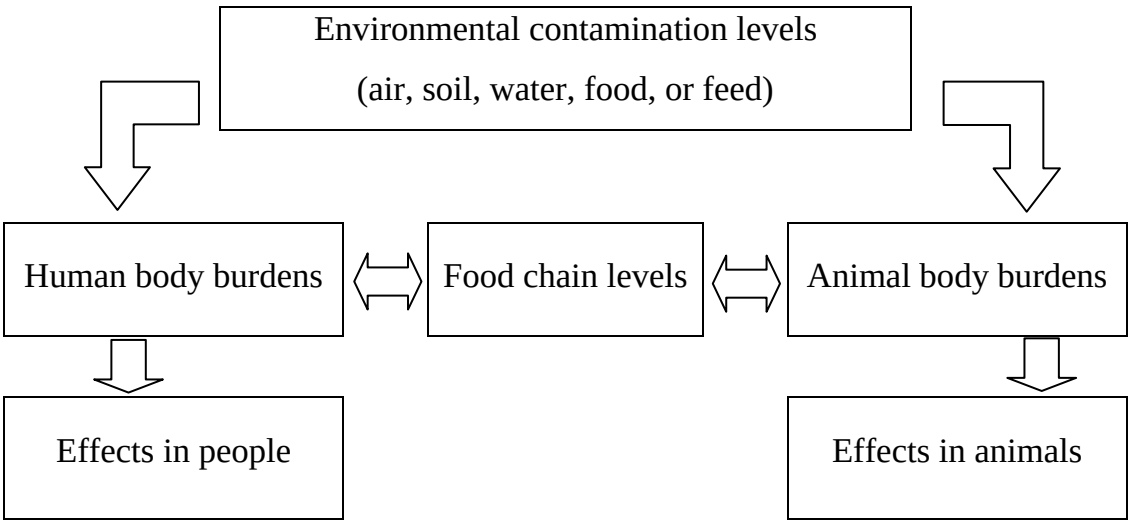


Figure 2.5: The relationship of the environment and the food chain to human and animal health effects (NRC, 1991)

Before an animal sentinels systems is chosen, several characteristics must be selected as followed:

- A sentinel should have a measurable response to the agent in the question (NRC, 1991). The animal might be exquisitely sensitive to an agent, or it might be resistant, and accumulate the agent to a high concentration in its tissues, or undergo physiologic or behavioral change in response to the agent.
- A sentinel should have a territory or home range that overlaps the area to be monitored. If a small and discrete location, it would not be appropriate to use an animal that ranges over many square kilometers and visits the site only occasionally or an animal that visits several contaminated sites. Thus, animals with small home ranges would be appropriately chosen (NRC, 1991).
- A sentinel species should be easily enumerated and captured. This example can be illustrated by the small mammals that are easier to capture than the large mammal. Their population characteristics and dynamics are easier to assess over a short period. Therefore, size of an animal can be important in itself (NRC, 1991)
- A sentinel species must have sufficient population size and density to permit enumeration. In general, the population of a sentinel species should be large enough to sustain the harvesting required by a monitoring study without major adverse impact (NRC, 1991).

4. Biological Marker

Biological marker or biomarkers are broadly described as an indicator of changes in biologic systems. They have been classified into 3 types as biomarkers of exposure, effect and susceptibility (ATSDR, 2007)

A biomarker of exposure is a foreign molecule or productions of an interaction between a foreign molecule and some materials in the cell of an organism including metabolize. The accumulation of certain chemicals in animal body is regarded as one case of the studies.

A biomarker of effect is described as a responsible within an organism such as biochemical, physiologic, or other alterations. The presence of any measurable responses can be recognized as a potential health impairment or disease. The reproductive effect is also regarded as an important biomarkers.

A biomarker of susceptibility is an indicator of an inherent of an organism's ability to respond to the exposure of a specific xenobiotic substance. The organism may show an impairment on intrinsic genetic or other characteristics. The expression of detoxification enzyme gene is a good example for this kind of biomarker.

5. Freshwater Mussel

Freshwater mussels have a laterally compressed body enclosed by a shell in two hinged parts and have no head or rasping tongue. The gills have evolved to a specialized organ for feeding and breathing. Most freshwater mussels bury themselves in sediment where they are safe from predation. The shell of a mussel compose of calcium carbonate as a major chemicals. The two shells of a mussel are joined together at one edge by a ligament. Adult shell sizes vary from a millimeter to over a meter in length, but the majority of species do not exceed ten centimeters. Freshwater mussels live in many types of habitat, ranging from small ditches, ponds, lakes, canals, and rivers. Most mussels are filter feeders, using their gills to capture particulate food such as phytoplankton from the water column (Dillon, 2000; Brandt, 1974).

5.1 Freshwater Mussel Reproduction

Sexes are usually separated in many bivalves, but some hermaphrodites are often found. The gonads are located close to the intestines of the visceral mass. Spawning was triggered by environmental factors such as day length, water temperature or the presence of sperm in the water column. Freshwater mussels in Unionid group have a different life cycle from the other group. Sperm is released into the water and enter into a female's gills with the inhalant siphon. The internal fertilization was found in female gill. Eggs hatch into glochidia larvae that develop within the female's shell. Then glochidia are released and attached themselves parasitically to the gills or fins of a fish host. After several weeks they drop off their host, metamorphose and develop into juveniles on sediment. An advantage of this processes make them able to disperse upstream with their temporary hosts rather than being constantly swept downstream by the water flow (Dillon, 2000).

5.2 Vitellogenin

Vitellogenesis is the central event of egg maturation in all oviparous animals. Yolk protein precursors are produced in large amounts by female tissue, accumulated by developing oocytes through receptor-mediated endocytosis, and eventually used as a source of nutrients for developing embryos. During vitellogenesis, females devote most of their resources to the synthesis to yolk protein precursors, which are usually produced extraovarially in tissue such as the nematode intestine, the insect fat body, and the vertebrate liver (Raikhel and Sappington, 2002).

Vitellogenin and yolk proteins of the mollusk have been isolated and characterized only in a few species. Vitellogenin and ferritin are the principal precursor proteins of molluscan yolk, and ferritin is the major yolk protein of certain freshwater snails (Bottke, 1982). Apparently yolk ferritin, an iron transporter of molluscan blood, evolved from the same ancestor as the soma isoform. This differs from other invertebrates in which plasma iron is transported by transferrin. Immunological and molecular methods (Barre et al., 1991; Bride et al., 1992) have indicated that the heterosynthetic mechanism of yolk production also characterizes the classical lipovitellin polypeptides found in the eggs of *Helix aspersa*, a ground dwelling pulmonate snail. These proteins probably originate by proteolytic cleavage of a vitellogenin synthesized in the digestive gland of the adult (Bride et la., 1992)

3.3 Freshwater Mussel as Sentinel Species

Since the mid 1970, several researchers have been used bivalves for monitoring chemical contamination along coastal marine waters. Because of their ability of sedentary habitat and complete life cycle in the water resulting in bioaccumulation of pollutants, mussels appear to be appropriate as a sentinel species for monitoring contaminant in aquatic organisms (Farris and Van Hassel, 2005). The Mussel Watch program was established in the mid 1970s by USEPA. This program used mussels and oysters as sentinel species for

monitoring the local levels of several classes of pollutants such as synthetic organics, fossil fuel compounds and several trace elements (Jernelov, 1996). As a results of this project initiatives, several report have been continuously produced to monitor the environmental contamination in estuary environment (Gomes et al., 2009) to aquatic environment (Sheehan and Power, 1999; Won et al., 2005; Ji et al., 2006). The contaminant successfully monitored by the mussels ranged from organochlorine pesticide contamination (Lopes et al., 1992: Avelar et al., 1991) and triazine herbicide contamination (Lahatay et al., 1998; Jacomini et al., 2006).

5.4 Description and Distribution of *Uniandra contradens*

Kingdom Animalia
Phylum Mollusca
Class Bivalvia
Order Unionoidea
Family Unionidae
Genus *Uniandra*
Species *Uniandra contradesn* (Haas,1913)

General characters of the species include shell to be more of less elongately ovate, with rounded or moderately tunicate anterior end and tapering posterior end. Beaks is prominent, with angular umbonal sculpture. Shell is smooth or partly, often completely, sculptured with irregularly concentric wrinkles. Left valve is present with 1 small, compressed pseudocardinal and 2 long, thin lamelliform laterals, while right valve is present with 2 short pseudocardinals and long lateral. Supraanal opening is separated from the anal opening. Marsupial is present in the outer demibranchs, and in parts of the inner demibranchs. Glochidia are usually ovate (Brandt, 1974).

Figure 2.6: Morphology of *Unandra contradens* collected from Nan Province

This common and widely distributed genus (*Uniandra*) contains 20 species, attributed to it by several authors in the last 100 years. A critical revision may find two or three of them to be valid species, some of them were acceptable as races, but most of them was regarded as synonym. Distributions of these mussels are common from central of China southwards to the Malayan Peninsular, Sumatra, Vietnam, and Cambodia including Thailand (Brandt, 1974).

Chapter III

Herbicide Contamination in Environment and Freshwater Mussel *Uniandra contradens* Living in an Agricultural Catchment, Nan Province

1. Introduction

Nan Province is a fertile area for several agricultural activities. Seasonal cultivation in this area involves an intensive utilization of agrochemicals, especially herbicides (atrazine, glyphosate and paraquat). The continuous application of these herbicides in large amount could lead to environmental contamination and accumulation in aquatic organisms (Uno et al., 2001). It is thus important to monitor an extent of contamination in environment and in animals living in the aquatic environment.

Freshwater mussel is an invertebrate that greatly depend on quality of freshwater environment as an animal that has complete life cycle in water, as a filter feeder on plankton and organic matters in water and as a bottom dweller in sediment (Dillon, 2000). This life history makes the mussel susceptible to xenobiotic exposure and accumulation of chemical residues into their body (Uno et al., 2001; Jacomini et al., 2003). The freshwater mussel is thus regarded as a suitable monitoring species for xenobiotic contamination in agricultural area. Examples of this include the use of several freshwater bivalves as biomonitoring species for herbicide contamination (Uno et al., 2001; Jacomini et al., 2003; 2006). In addition, several studies reported on link between xenobiotic accumulation in mussels and adverse health effects in their organ systems, suggesting the potential use of freshwater mussels as a sentinel species of environmental health hazards from xenobiotic contamination (Sheehan and Power, 1999; Won et al., 2005; Ji et al., 2006).

Although herbicide utilization is widely dispersed in several areas of Nan Province, but the information of herbicide contaminations in environmental and biological samples are still scare**.** In this study, herbicide residue analyses were performed to monitor the contamination of herbicide in

environmental samples. Next, a freshwater mussel *Uniandra contradens*, a common freshwater mussel species that widely distribute in aquatic habitats close to agricultural area of Nan Province, was selected as a sentinel species for determination of herbicide residue.

Objective

- To screen herbicide contamination in environmental samples and examine the extent of herbicide contamination in freshwater mussels living in an agricultural catchment at Nan Province

2. Materials and methods

2.1 Study site

The study site was located in San Subdistrict, Wiang Sa District of Nan Province, Thailand. Seasonal cultivations of corn, cucumber gourd, rice, sesame and soybean can be found throughout the area. Among patches of agricultural area, Nong Bua reservoir (18˚30'35.39" N, 100˚46'4.48" E) was constructed to be used as a catchment for run-off water from surrounding agricultural patches before flowing to the adjacent Nan River during wet season, and a reservoir for agricultural activities during dry season. Several aquatic animals are inhabited in the reservoir including a freshwater mussel *Uniandra contradens*, a sentinel species in this study.

2.2 Environmental sample collection

Environmental samples (sediment and water) from Nong Bua reservoir was collected every three months in July-2010, October-2010, January-2011 and April-2011. Composited samples of sediment (1 kg) were collected from surroundings of the mussel habitat. A 1 L of water sample was collected under water at 15 cm depth. Both sediment and water samples were collected and stored in a plastic box and a high density polyethylene bottle, respectively. Every container was washed with laboratory detergent, rinsed with acetone and air dried at room temperature before each use. These containers were wrapped with aluminum foil to avoid sunlight and stored in a refrigerator at 4°C until further analysis.

2.3 Mussel collection

Mussels were collected by hand on monthly basis from Nong Bua reservoir during July 2010 to June 2011. Mussel samples were transported to a laboratory at the Chulalongkorn University Forest and Research Station at Nan Province. Morphological data including wet body weight, shell length and shell width were measured and recorded. The mussels were euthanized in ice

slurry and dissected to separate the soft part from the shell. The soft part of mussel collected in July 2010, October 2010, January 2011 and April 2011 was kept frozen at -20 °C and used for herbicide residue analysis.

The frozen mussel tissue was freeze-dried (FreeZone 7753501) until complete dryness. Three mussels were combined as a composited sample, and three composited samples per sex were analyzed in each month. The composited mussel samples were grinded with mortar and pestle before storage in desiccators with silica gel for humidity control until further analysis.

2.4 Extractions and determinations of herbicide contamination in the environmental samples

Herbicide residues in sediment and water were analyzed 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. Sediment and water sample were subjected to extraction process according to an in-house method of the company. Overview of extraction methods for these herbicide residue analyses are listed as follows.

Atrazine

A 10 g of wet sediment sample was treated with 10 g of sodium chloride (NaCl) and 10 mL of deionized water. The mixed sample was extracted with 10 mL of acetronitrile (CH_3CN; HPLC grade) before addition with 2 g of anhydrous magnesium sulfate ($MgSO_4$). The extracted sample was centrifuged at 3,000 rpm (Heraeus®, Megafuge® 1.0 R), 5 °C for 5 minutes, and 5 mL of supernatant was transferred to evaporate under stream of nitrogen gas. The content was adjusted to the volume of 2 mL ethyl acetate ($CH_3COOCH_2CH_3$) and subjected to treatment with 0.5 g 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 nylon filter before further analysis with gas chromatography with mass spectrometry.

A 500 mL of water sample was pre-treated in reparatory funnel with 20 g of sodium chloride (NaCl) and subjected to extraction with dichloromethane (CH_2Cl_2) for three times (100: 50: 50 mL) in round-bottle flask. The extracted sample was dried up in an evaporator. The sample volume was adjusted to 2.5 mL 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; selected ion monitoring mode) and a DB-5ms capillary column (0.25 mm internal diameter x 30 m length and 0.25 μm film thickness). Two microliters of samples was injected into the GC-MS with 2.5 minutes solvent delay. The injector was initially set at 210 °C and 10.69 Psi. The oven temperature was initially set at 80 °C for 2 minutes, and programmed to increase to 280 °C at the rate of 14 °C/minute and held for 10 minutes. The total run time was calculated to be 31 minutes. Helium was used as a carrier gas with a flow rate of 1.1 mL/min. The limit of detection (LOD) for atrazine residue was 0.01 μg/mL in water and 0.01 μg/g in sediment.

Glyphosate

Sample preparation for glyphosate analysis was based on methods by Börjesson and Torstensson (2000). In brief, a 10 g of sediment sample was extracted twice with 25 μL of 1 M NaOH and centrifuged at 5,000 rpm (Heraeus®, Megafuge® 1.0 R) for 30 minutes. The supernatant was filtered through a F1 Whatman filter paper. The pooled extract was treated with 4.2 mL of concentrated HCl, diluted with 200 mL of water and adjusted to pH 2.0. After 1 hour of incubation at room temperature, a clear upper part of the extract was subjected to similar treatments to water sample as followed.

A 200 mL of water sample 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 1 mL of water and water-methanol-HCl (160: 40: 2.7) between each evaporation cycle. After the last evaporation under stream of nitrogen gas, sample was derivatized for 1 hour at 100 °C after treatment with trifluoacetic anhydride and trifluoroethanol. Finally, the sample was evaporated under nitrogen and re-dissolved in ethyl acetate prior to analysis with HPLC.

Determination of glyphosate residue in the extracted sample was quantified by high performance liquid chromatography (HPLC; Agilent 1100, Germany) with post-column derivatization (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). A 20 mL of sample was injected into the HPLC with post-column derivatization with 0.2 mL/min. of column flow rate control. The stop time was 12.0 minutes and post time was 3.0 minutes. The column temperature was held at 40 °C. The mobile phase was 100 mM ammonium formate, pH 7.3 and acetonitrile (NH_4HCO_2:CH_3CN; 60:40 v/v). The limit of detection (LOD) for glyphosate residue was 0.005 μg/mL in water and 0.01 μg/g in sediment samples.

Paraquat

A 25 g of sediment sample was treated with 10 mL of deionized water, 1 mL of octane-2, 35 mL of sulfuric acid and glass bead before refluxed in a close system for 5 hours. After cooled down to room temperature, the sample was filtered through filter paper (No. 4, Bluchner) and Celite before cleaned up through solid phase extraction (SPE) silica. The sample was evaporated to dryness and adjusted the volume to 2 mL with mixture of 100 mM ammonium formate, pH 3.7 and acetronitrile (ratio 60:40). The sample was filtered through syringe nylon filter (0.22 μm) before further analysis with HPLC.

A 100 mL of water sample was adjusted to pH 9.0 with 10 N of NaOH, cleaned up through solid phase extraction (SPE) silica and then stored in 10 mL of a mixture of 8 N hydrochloric acid and methanol (ratio 9:1). After that, the

sample was evaporated to dryness and adjusted the volume to 2 mL with mixture of 100 mM ammonium formate, pH 3.7 and acetronitrile (ratio 60:40). The sample was filtered through syringe nylon filter (0.22 μm) prior to analysis with HPLC.

Quantification of paraquat residue in the extracted sample was performed by high performance liquid chromatography (HPLC; Agilent Technologies 1100, Germany) using diode-array detector with a broad spectrum 190-400 nm and silica hydrophilic interaction chromatography column (silica HILIC; Atlantis; 2.1 mm internal diameter x 150 mm length and 3 μm film thickness). A 10 μL of sample was injected into the HPLC-DAD with 0.2 mL/min. of column flow rate control. The stop time was 12.0 min. and post time was 3.0 min. The column temperature was held at 40 °C. The mobile phase was 100 mM ammonium formate, pH 7.3 and acetonitrile (NH_4HCO_2:CH_3CN; 60:40 v/v). The limit of detection (LOD) for paraquat residue was 0.01 μg/mL in water and 0.01 μg/g in sediment.

2.5 Extractions and determinations for herbicide contamination in the mussel sample

Quantification of herbicide residue in the mussel was examined with enzyme-linked immunosorbent assay (ELISA). This test is based on an immunological recognition of herbicide residue by a specific antibody. Therefore, specificity and sensitivity of the assay are suitable for the quantification even in a low concentration range. The atrazine and glyphosate kits were purchased from Abraxis LCC and paraquat kit was purchased from Abnova. These kits are originally designed to check the contaminations of herbicides residue in the water sample. In order to use these ELISA kits, the mussel sample was thus subjected to extraction and reconstitution in water before analysis. The overview of extraction and assay methods are listed as followed.

Atrazine

The tissue was extracted according to a modified method of Jacomini et al. (2003). Briefly, a 100 mg of lyophilized tissue was mixed with 1 mL of ultrapure water (Merck) before extraction with 4 mL of dichloromethane (HPLC grade, Fisher®) adjusted to slightly base with 1.5 M NaOH. After centrifugation at 1,800 xg for 5 minutes, 3 mL of organic phase was transferred to a clean tube and dried with an evaporator (TurboVap® II) under stream of nitrogen gas (Ultra high purity nitrogen grade, 99.999%). The residues were reconstituted with 100 µL of methanol (HPLC grade, Merck), followed by 900 µL of ultrapure water to make final volume of 1 mL. The samples were stored at -20°C until analysis with ELISA kit. To check for recovery of extraction, 50 µL of standard atrazine solution (100 ng/mL) was added to a representative sample before proceed with the subsequent steps.

ELISA kit for determination of atrazine residue was obtained from Abraxis LLC. Assay was performed according the company's protocol. Briefly, 25 µL of assay buffer was added to an individual well of a microtiter plate coated with rabbit anti-triazine antibody. Then, 25 µL of the samples and standard atrazine solutions (0, 0.05, 0.1, 0.25, 1.0, 2.5 and 5.0 ng/mL) was loaded in duplication into the designated well. Fifty microliters of the triazine-horseradish peroxidase conjugate was added into each well, and the plate was incubated on an orbital shaker for 30 minutes at room temperature. After incubation, the plate was washed three times with washing buffer solution, and loaded with 100 µL of substrate/color solution (hydrogen peroxide and 3,3',5,5'–tetramethylbenzidine; TMB) into each well. The plate was incubated on the orbital shaker for another 15 minutes at room temperature before 50 µL of a stop solution (sulfuric acid) was added into each well. Absorbance at 450 nm was measured by a microplate reader (Multiskan EX).

To calculate atrazine concentration, mean absorbance of each duplicated standards and samples was calculated first. Then, percent bound ($\%B/B_0$) was calculated for each standard and sample by dividing the mean absorbance value

of the standard and sample with the mean absorbance of the zero standard (standard 0; 0 ng/mL). A standard curve was constructed by plotting the $\%B/B_0$ on y-axis and logarithmic atrazine concentrations on x-axis. Standard calibration curves of atrazine were linear from 0-5.0 ng/mL with r^2 of 0.97035 to 0.99660. The atrazine concentration of each sample was determined by interpolation from this standard curve and shown as ng/mL (ppb) of atrazine residue. Based on this assay, the detection limit for atrazine residue in mussel tissue was 0.53 ng/g dry weights, and the recovery of extraction was 87.41 %.

Glyphosate

Mussel tissue was extracted according to the modified method of Alferness and Iwata (1994). A 100 mg of dried tissue was mixed with 200 µL of ultrapure water (Merck) before extraction with 100 µL of chloroform (Merck) and 400 µL of 0.1 N hydrochloric acid (HCl) for 5 min. After that 0.004 g of sodium sulfate (Merck) was added to the sample, and an additional 2 minutes of shaking was required for maceration. The homogenates were centrifuged at 1,000 xg for 10 minutes at room temperature, and 400 µL of aqueous extract was collected and transferred to a clean tube. The aqueous phase was re-extracted with 400 µL of chloroform for 2 minutes, and centrifuged at 1,000 xg for 10 minutes at room temperature. Finally, a 350 µL aliquot of the aqueous extract was adjusted to pH 7.0 with 1 N sodium hydroxide (NaOH). The samples were stored at -20°C until analysis with ELISA kit. To check for recovery of extraction, a 200 µL of standard glyphosate solution (4.0 ng/mL) was added to the lyophilized sample instead of the ultrapure water before proceeding with the subsequent steps.

Prior to ELISA protocol, every sample and glyphosate standard solution was subjected to pretreatment by derivatization. Briefly, a 250 µL aliquot of the extracted sample and glyphosate standard solution (S_0-S_5; 0, 0.075, 0.2, 0.5, 1.0 and 4.0 ng/mL) were mixed with 1 mL of assay buffer. Then, a 100 µL of derivatization reagent was added to each tube and the tube was shaken

immediately for 15 seconds. The derivatized samples and standards were incubated at room temperature for 10 minutes before assay with ELISA.

ELISA kit for determination of glyphosate was obtained commercially from Abraxis LLC. Assay was performed according to the company's protocol. Briefly, 50 µL of the rabbit anti-glyphosate antibody in buffered saline solution was added to individual well of a microtiter plate coated with goat anti-rabbit antibody. After that, 50 µL of derivatized samples and standards (0, 0.075, 0.2, 0.5, 1.0 and 4.0 ng/mL) were loaded in duplication into each individual wells. A 50 µL of glyphosate enzyme conjugate (horseradish peroxidase (HRP) labeled glyphosate analog diluted in a buffered solution) was loaded into each wells and incubated at room temperature on an orbital shaker for 97 minutes. After incubation, the plate was washed three times with washing buffer solution, and loaded with 150 µL of substrate/color solution (hydrogen peroxide and 3,3',5,'-tetramethylbenzidine) into each well. The plate was incubated for another 20 minutes at room temperature before 100 µL of a stop solution (sulfuric acid) was added into each well. Absorbance at 450 nm was measured by a microplate reader (Multiskan EX).

To calculate glyphosate concentration, mean absorbance of each duplicated standards and samples was calculated. Then, percent bound ($\%B/B_0$) was calculated for each standard and sample by dividing the mean absorbance value of the standard and sample with the mean absorbance of the zero standard (standard 0; 0 ng/mL). A standard curve was constructed by plotting the $\%B/B_0$ on y-axis and logarithms of glyphosate concentrations on x-axis. Standard calibration curves of glyphosate were linear from 0-4.0 ng/mL with r^2 of 0.99064 to 0.99935. The glyphosate concentration of each sample was determined by interpolation from this standard curve and shown as ng/mL (ppb) of glyphosate residue. Based on this assay, the detection limit for glyphosate residue in mussel tissue was 0.21 ng/g dry weights, and the recovery of extraction was 37.78 %.

Paraquat

The extraction protocol was modified from Brown et al. (1996) and Quick et al. (1990). A 100 mg of the lyophilized tissue mixed with 200 µL of ultrapure water (Merck) was extracted with 200 µL of hexane and 600 µL of 10% trichloroacetic acid (Merck) in a vortex mixer for 5 minutes. The homogenate was centrifuged at 2,000 xg for 15 minutes at room temperature. A 550 µL of the aqueous extract was transferred into a clean tube. The pellets were re-extracted with 300 µL of 10% trichloroacetic acid in a vortex mixer for 5 minutes. After centrifugation at 2,000 xg for 15 minutes at room temperature, 200 µL of aqueous extract was collected and combined to make the final volume of 750 µL. The combined aqueous phase was extracted with 400 µL hexane and centrifuged at 2,000 xg for 15 minutes at room temperature. A 700 µL of aqueous extract was collected and mixed with 300 µL of 2M tris-hydrochloric acid buffer (pH 8.5). Finally, pH of the extract was adjusted to 7.0 with 6N hydrochloric acid. All samples were stored at -20°C until analysis with ELISA kit. To check for recovery of extraction, 200 µL of standard paraquat solution (7.5 ng/mL) was added to the lyophilized sample instead of ultrapure water before proceeding with the subsequent steps.

ELISA kit for determination of paraquat was obtained commercially from Abnova. Assay was performed according the company's protocol. Initially, 25 µL of samples and paraquat standard solutions (S_0-S_4; 0, 0.375, 0.75, 2.5 and 7.5 ng/mL) were loaded in duplication into each well of a microtiter plate coated with rabbit anti-paraquat antibody. One hundred microliters of paraquat-horseradish peroxidase conjugate (PRQ-HRP) was loaded into each well, and the plate was incubated at room temperature on an orbital shaker for 30 minutes. After incubation, the plate was washed three times with washing buffer solution, and loaded with 100 µL of substrate/color solution (hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine) into each well. The plate was incubated on the orbital shaker at room temperature for 15 minutes. Finally, a 100 µL of the stop solution (3M hydrochloric acid) was

added into each well, and the plate content was measured for the absorbance at 450 nm using a microplate reader (Multiskan EX).

To calculate paraquat concentration, mean absorbance of each duplicated paraquat standards and samples was determined. Then % inhibition was calculated as shown in this equation.

$$\% \text{ Inhibition} = 100 - \left(\frac{\text{Mean absorbance of standard}}{\text{Mean absorbance of zero standard}} \right) \text{X } 100$$

A standard curve was plotted using the % inhibition placed on y-axis and logarithms of paraquat concentration placed on x-axis. The standard calibration curves of paraquat were linear from 0-0.75 ng/mL with r^2 of 0.97754 to 0.99938. Concentration of paraquat in each sample was determined by interpolating from this standard curve and shown as ng/mL (ppb) of paraquat residue. The detection limit of the ELISA kit for paraquat residue analysis is 9.89 ng/g dry weights, and the recovery of extraction was 51.17%.

2.6 Statistical analysis

All parameters were tested for normal distribution and homogeneity of variance. Comparison between sexes was performed by Student's *t*-test, while seasonal variation were compared by one way analysis of variance (ANOVA) followed by Student-Newman-Keuls multiple comparison methods.

3. Results and discussion

The result of chromatographic analysis showed that residues of atrazine can be found in sediment (0.23 µg/g) and water (0.16 µg/mL) of Nong Bua reservoir in late dry season (January 2011; Table 3.1). Detectable amount of atrazine in this season is not unexpected since it was a beginning of new crop cycle when agrochemicals utilization was at peak. Presence of atrazine at levels lower than the limit of detection in other periods could be due to the relatively low sensitivity of GC-MS (LOD: 0.01 µg/mL in water and 0.01 µg/g in sediment). However, given the fact that atrazine is relatively stable with half-life in surface water of more than 200 days (ATSDR, 2003), the levels found in this study pose potential concerns over its effect to aquatic life since it is quite close to the lowest observed effect concentration for early life stage of fish (0.46 µg/mL; Giddings et al., 2005).

Since bioconcentration of atrazine is unlikely (Giddings et al., 2005), atrazine residue in mussel tissue was thus determined by ELISA in order to yields a more sensitive assay (LOD: 0.53 ng/g dry weight). Detectable levels (1.26-14.59 ng/g dry weight) of atrazine were found in every mussel examined (Figure 3.1 and Table 3.2). Similar to previous studies in *Anodontites trapesialis* and *Corbicula fluminea* bivalves (Jacomini et al., 2003; 2006), these data suggest that *U. contradens* could temporally store atrazine residue presented in the aquatic environment. However, concern on the safety of mussel consumption should be low since levels of atrazine found in the mussel are still much lower than the minimal risk level for oral exposure to atrazine in intermediate duration (0.003 mg/kg/day; ATSDR, 2003). The maximum residue limits for atrazine in meats were reported at 0.04 µg/g (40 ng/g wet weight or equivalent to 292.18 ng/g dry weight of this freshwater mussel species; Health Canada, 2010).

Since there was no sex-related difference in atrazine concentration, male and female data were combined for further statistical analysis. One way ANOVA showed a significant seasonal difference in level of atrazine residue in

the mussel with the highest level found in late wet season (July 2010: overall mean 7.35 ± 1.80 ng/g dry weight). It is interesting to note that the peak of atrazine residue in the freshwater mussel (wet season) did not coincide with the peak of atrazine residue in environmental sample (dry season). The results confirm and suggest that, unlike physical environment, rate of pollutant uptake and loss in sentinel species may vary with physiological stage of the animal (Beeby, 2011). Therefore, monitoring program for environmental contamination should focus on both physical and biological samples in order to predict the potential health impact on organism with more accuracy.

Table 3.1: Levels of atrazine residue screening in environmental samples collected from an agricultural area (Nong Bua reservoir) in Nan Province, Thailand

Samples	July 2010	October 2010	January 2011	April 2011
Water	<0.01 µg/mL N=1	<0.01 µg/mL N=1	0.16 µg/mL N=1	<0.01 µg/mL N=1
Sediment	<0.01 µg/g N=1	<0.01 µg/g N=1	0.23 µg/g N=1	<0.01 µg/g N=1

Remark:

- Limit of detection (LOD; 0.01 µg/g for sediment and 0.01 µg/mL for water)
- N = number of composited sediment sample (1 kg) and water sample (1 L)

Table 3.2: Levels of atrazine residue in the freshwater mussel *U. contradens* collected from an agricultural area (Nong Bua reservoir) in Nan Province, Thailand (Mean ± S.E.M.)

Sex	July 2010 (ng/g)	October 2010 (ng/g)	January 2011 (ng/g)	April 2011 (ng/g)
Male	6.83 (± 2.16) N=3	2.71 (± 0.30) N=3	2.45 (± 0.61) N=3	3.24 (± 0.17) N=3
Female	7.86 (± 3.37) N=3	3.79 (± 0.45) N=3	1.66 (± 0.20) N=3	3.71 (± 0.85) N=3
Combined data	7.35^{A} (± 1.80) N=6	3.25^{B} (± 0.35) N=6	2.06^{B} (± 0.34) N=6	3.48^{B} (± 0.36) N=6

Remark:

- Limit of detection (LOD) is 0.53 ng/g.
- No significant difference between sex ($p>0.05$, *t*-test).
- Significant difference between month ($p<0.05$, one way ANOVA & Student-Newman-Keuls Method) is indicated by difference in superscript letter.
- N = number of composited mussel sample (three mussel were combined as one composited sample)

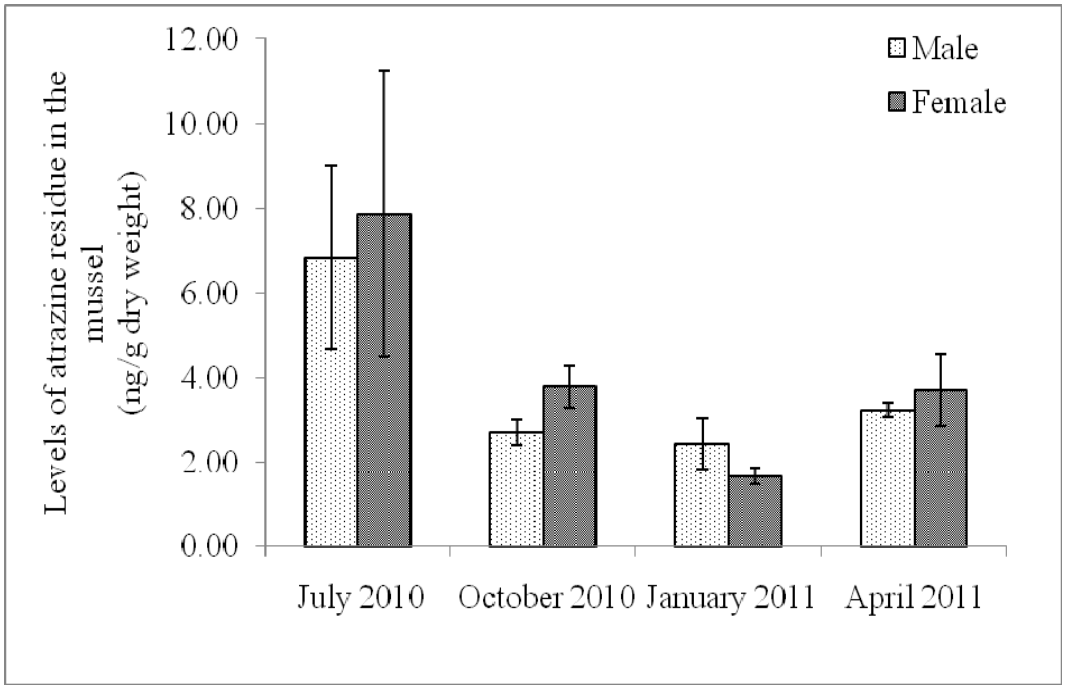


Figure 3.1: Mean ± S.E.M. of atrazine residue in *U. contradens* collected from an agricultural area (Nong Bua reservoir) in Nan Province, Thailand. There was no significant difference between sex ($p>0.05$, *t*-test) and month ($p>0.05$, one way ANOVA & Student-Newman-Keuls Method). N = 3 composited samples per sex in each month.

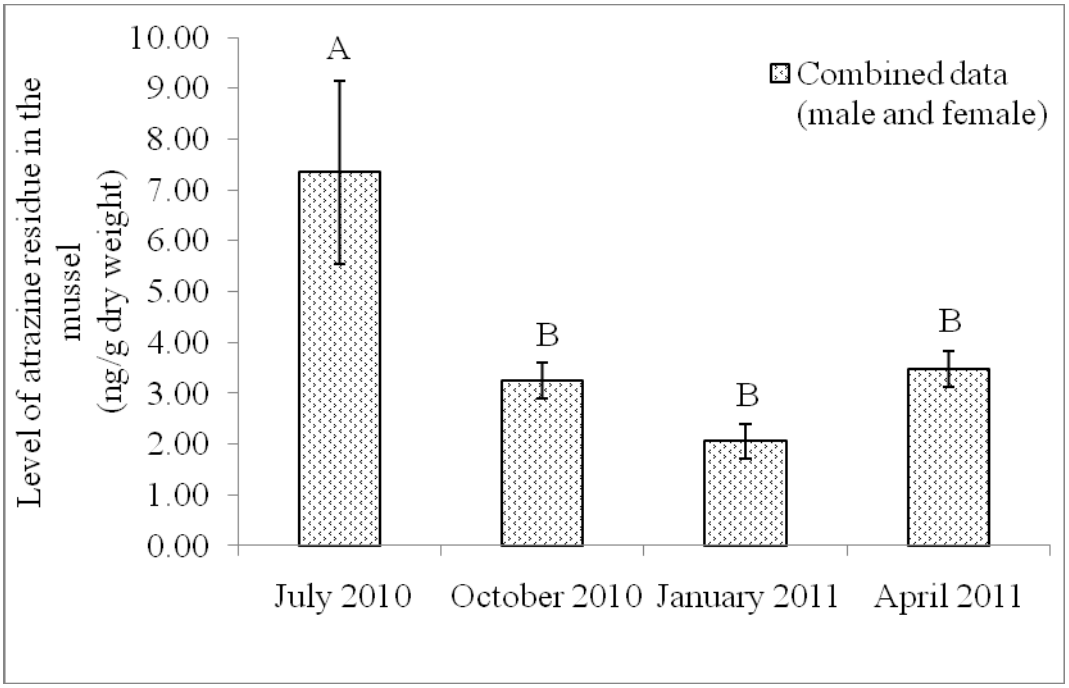


Figure 3.2: Mean ± S.E.M. of atrazine residue in *U. contradens* collected from an agricultural area (Nong Bua reservoir) in Nan Province, Thailand. Significant difference between month ($p<0.05$, one way ANOVA & Student-Newman-Keuls Method) is indicated by difference in superscript later. N = 6 composited samples in each month.

Chromatographic analysis did not show the residues of glyphosate in sediment and water of Nong Bua reservoir in any season (Table 3.3). Presence of glyphosate at the levels lower than the limit of detection in all season may be due to the relatively low sensitivity of HPLC equipment (LOD: 0.005 μg/mL in water and 0.01 μg/g in sediment). Alternatively, this could be due to the short half-life and high degradation rate of glyphosate in the environment. Glyphosate has a half-life in field-water of 7-14 days (Giesy et al., 2000) and field-soil of 32 days (Giesy et al., 2000). It can be rapidly degraded by microorganism in environment (Rueppel et al., 1977). Lund-Høie and Friestad (1986) also suggested that glyphosate could be subjected to photodegradation by UV-light as well.

Since glyphosate was reported to have a low potential for bioaccumulation in several aquatic organism (Giesy et al., 2000), in this study glyphosate residue in mussel tissue was thus determined by ELISA in order to yield a more sensitive assay (LOD: 0.21 ng/g dry weight). Detectable levels (5.34-11.13 ng/g dry weight) of glyphosate were found in every mussel examined (Figure 3.3 and Table 3.4), although residues of glyphosate were not found in sediment and water of Nong Bua reservoir. These data implied that although glyphosate residues can be readily degraded, *U. contradens* mussels can be temporally stored its residue in the tissue. However, concern on the safety of mussel consumption should be low since levels of glyphosate found in the mussel are still much lower than the minimal risk level for reference dose of daily glyphosate exposure (1.75 mg/kg/day; USEPA, 2009), and the maximum residue limits for glyphosate in meats were reported as 0.05 μg/g (50 ng/g wet weight of equivalent to 365.23 ng/g dry weight of this freshwater mussel speceis) (CODEX, 2006).

Since there was a significant sex-related difference in glyphosate concentration ($p<0.05$; *t*-test), male and female data were separated for further statistical analysis. One way ANOVA showed a significant seasonal difference in level of glyphosate residue only in the female mussel with ranges from 6.14-

11.13 ng/g dry weight. The highest level was found in late wet season (July 2010: overall mean 10.44 ± 0.34 ng/g dry weight). It is interesting to note that the peak of glyphosate residue in the mussel albeits its absence in the environmental samples. The results confirm and suggest that, unlike physical environment, rate of pollutant uptake and loss in sentinel species may vary with physiological stage of the animal (Beeby, 2011). Therefore, monitoring program for environmental contamination should focus on both physical and biological samples in order to predict the potential health impact on organism with more accuracy.

Table 3.3: Levels of glyphosate residue screening in environmental samples collected from an agricultural area (Nong Bua reservoir) in Nan Province, Thailand

Samples	July 2010	October 2010	January 2011	April 2011
Water	<0.01 µg/mL N=1	<0.01 µg/mL N=1	<0.01 µg/mL N=1	<0.01 µg/mL N=1
Sediment	<0.01 µg/g N=1	<0.01 µg/g N=1	<0.01 µg/g N=1	<0.01 µg/g N=1

Remark:

- Limit of detection (LOD; 0.01 µg/g for sediment and 0.005 µg/mL for water)
- N = number of composited sediment sample (1 kg) and water sample (1 L)

Table 3.4: Levels of glyphosate residue in the freshwater mussel *U. contradens* collected from an agricultural area (Nong Bua reservoir) in Nan Province, Thailand (Mean ± S.E.M.)

Sex	July 2010 (ng/g)	October 2010 (ng/g)	January 2011 (ng/g)	April 2011 (ng/g)
Male	8.72 (± 0.46) N=3	10.56 (± 1.32) N=3	7.17 (± 0.98) N=3	6.86 (± 0.79) N=3
Female	$10.44^{A,*}$ (± 0.34) N=3	9.00^{AB} (± 1.00) N=3	7.83^{AB} (± 0.68) N=3	6.71^{B} (± 0.57) N=3

Remark:

- Limit of detection (LOD) is 0.21 ng/g.
- Significant difference between sex ($p<0.05$, *t*-test) is indicated by an asterisk.
- Significant difference between month ($p<0.05$, one way ANOVA & Student-Newman-Keuls Method) is indicated by different superscript letter in the same row.
- N = number of composited mussel sample (three mussel were combined as one composited sample)

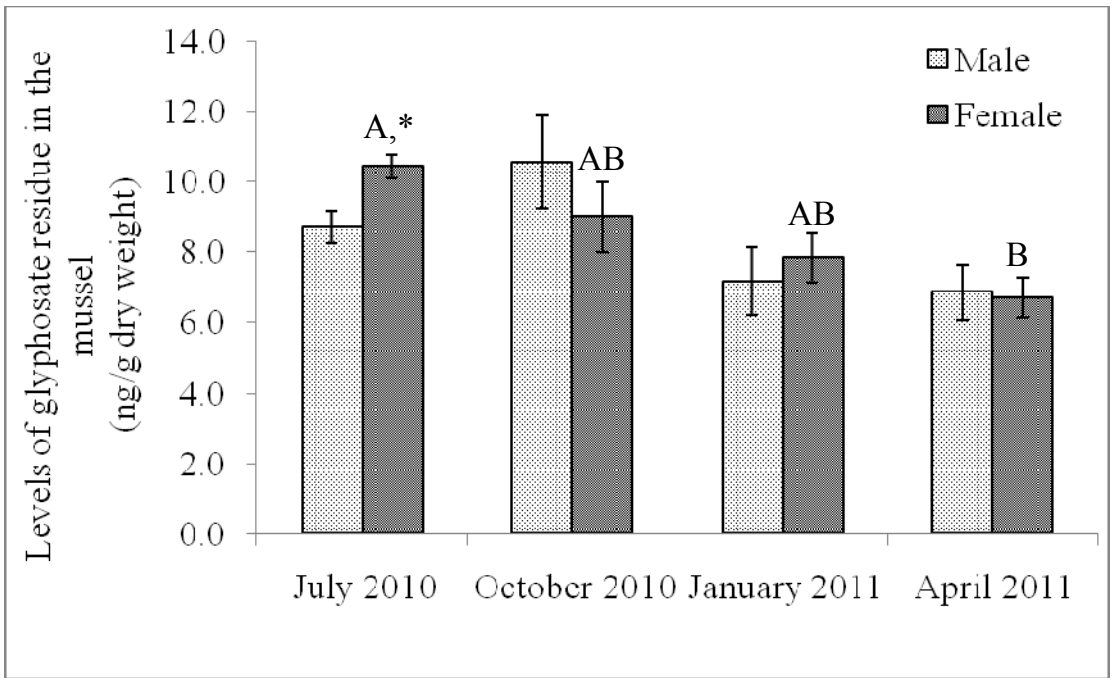


Figure 3.3: Mean ± S.E.M. of glyphosate residue in *U. contradens* collected from agricultural area (Nong Bua reservoir) in Nan Province, Thailand. Significant difference between sex ($p<0.05$, *t*-test) is indicated by an asterisk and month ($p<0.05$, one way ANOVA & Student-Newman-Keuls Method) is indicated by different superscript letter. N = 3 composited samples per sex in each month.

Chromatographic analysis did not show the residues of paraquat in sediment and water of Nong Bua reservoir in any season (Table 3.5). Presence of paraquat at the levels lower than the limit of detection in all season may be due to the relatively low sensitivity of HPLC equipment (LOD: 0.01 µg/mL in water and 0.01 µg/g in sediment). In addition, this could be due to the short half-life and low availability of paraquat in the environment. Paraquat can be degraded rapidly by photolysis within 4 weeks (Petit et al., 1995). Tu and Bollen (1968) also suggested that paraquat could be degraded by microorganism in environment as well. On top on this, Paraquat is well-known to strongly adsorb and form complex with sediment as well as suspension material in the water column making it very difficult to detect (Corwin and Farmer, 1984; Khan, 1978).

Since paraquat residue in the mussel tissue was not detected with sediment and water, it was thus determined by ELISA in order to yields a more sensitive assay (LOD: 9.89 ng/g dry weight). Detectable levels (25.44-55.97 ng/g dry weight) of paraquat residue were found in every mussel examined (Figure 3.4, 3.5 and Table 3.6). These data implied that although paraquat residues can be readily degraded or strongly adsorbed with sediment, *U. contradens* mussels can stored its residue in the tissue. According to the standard guideline of CODEX, the maximum residue limits of paraquat in meats are reported at 0.005 µg/g (5 ng/g wet weight or equivalent to 36.52 ng/g dry weight of this freshwater mussel species; CODEX, 2006). These data showed that paraquat residue in the mussel (25.44 to 55.97 ng/g dry weight) was higher than this standard guideline suggesting that the amount of paraquat residue in *U. contradens* could be harmful to consumer in food chain including human.

Since there was no significant sex-related difference in paraquat concentration, male and female data were combined for further statistical analysis. One way ANOVA also showed no significant seasonal difference in level of paraquat in the mussel ranging from 25.44 to 55.97 ng/g dry weight. It

is of interest to note the different levels of paraquat residue in mussels and the sediment and water. The results confirm and suggest that, unlike physical environment, rate of pollutant uptake and loss in sentinel species may vary with physiological stage of the animal (Beeby, 2011). Therefore, monitoring program for environmental contamination should focus on both physical and biological samples in order to predict the potential health impact on organism with more accuracy.

Table 3.5: Levels of paraquat residue screening in environmental samples collected from agricultural areas (Nong Bua reservoir) in Nan Province, Thailand

Samples	July 2010	October 2010	January 2011	April 2011
Water	<0.01 µg/mL N=1	<0.01 µg/mL N=1	<0.01 µg/mL N=1	<0.01 µg/mL N=1
Sediment	<0.01 µg/g N=1	<0.01 µg/g N=1	<0.01 µg/g N=1	<0.01 µg/g N=1

Remark:

- Limit of detection (LOD; 0.01 µg/g for sediment and 0.01 µg/mL for water)
- N = number of composited sediment sample (1 kg) and water sample (1 L)

Table 3.6 Levels of paraquat residue in the freshwater mussel *U. contradens* collected from agricultural areas (Nong Bua reservoir) in Nan Province, Thailand (Mean ± S.E.M.)

Sex	July 2010 (ng/g)	October 2010 (ng/g)	January 2011 (ng/g)	April 2011 (ng/g)
Male	47.04 (± 4.68) N=3	32.05 (± 5.68) N=3	40.89 (± 3.73) N=3	37.63 (± 3.59) N=3
Female	39.36 (± 2.73) N=3	35.94 (± 1.58) N=3	41.67 (± 2.97) N=3	36.64 (± 0.75) N=3
Combined date	43.20 (± 2.97) N=6	33.99 (± 2.78) N=6	41.18 (± 2.13) N=6	37.13 (± 1.66) N=6

Remark:

- Limit of detection (LOD) is 9.89 ng/g.
- No significant difference between sex ($p>0.05$, *t*-test) and month ($p>0.05$, one way ANOVA & Student-Newman-Keuls Method).
- N = number of composited mussel sample (three mussel were combined as one composited sample).

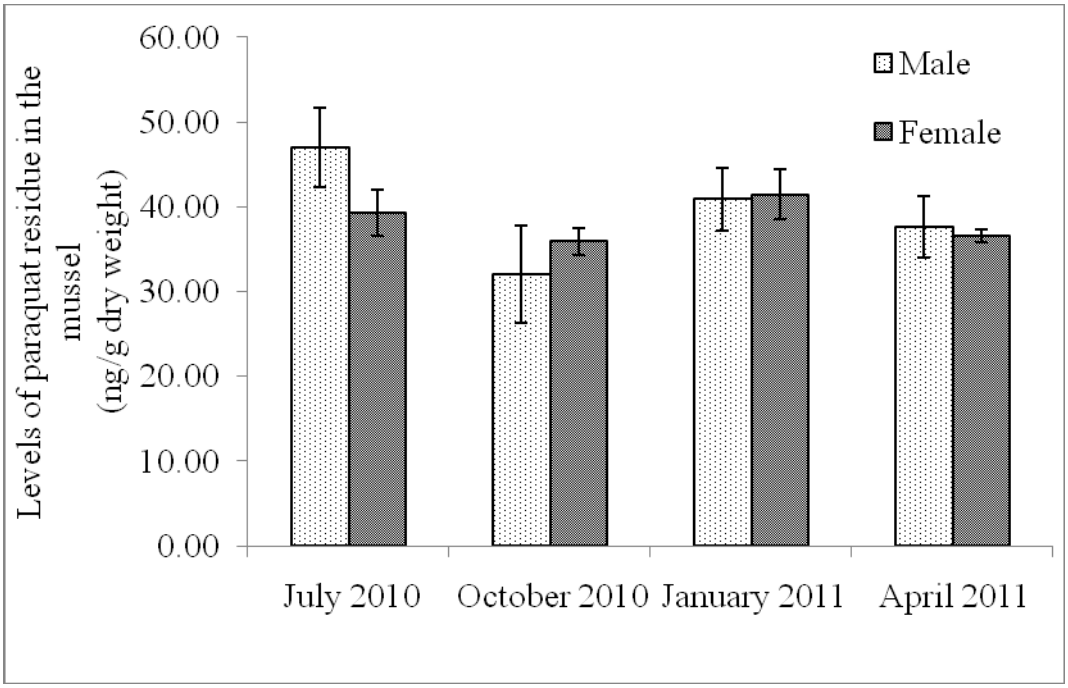


Figure 3.4: Mean ± S.E.M. of paraquat residue in *U. contradens* collected from agricultural area (Nong-Bua reservoir) in Nan Province, Thailand. There was no significant difference between sex ($p>0.05$, *t*-test) and month ($p>0.05$, one way ANOVA & Student-Newman-Keuls Method). N = 3 composited samples per sex in each month.

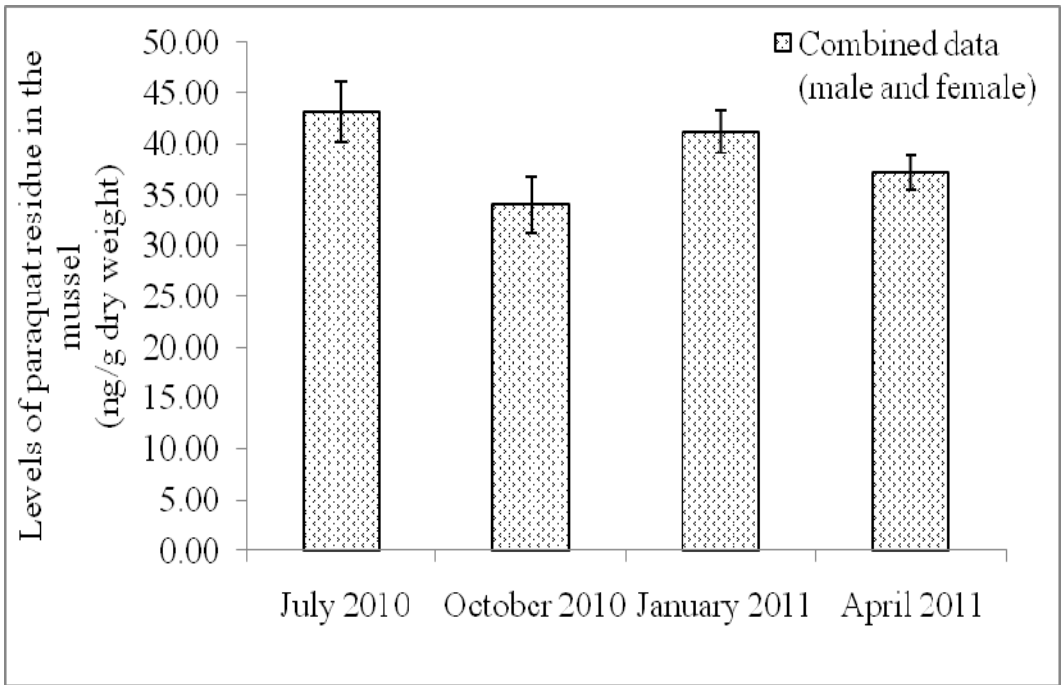


Figure 3.5: Mean ± S.E.M. of paraquat residue in *U. contradens* collected from agricultural area (Nong-Bua reservoir) in Nan Province, Thailand. There was no significant difference between month ($p>0.05$, one way ANOVA & Student-Newman-Keuls Method). N = 6 composited samples in each month.

4. Conclusion

Analysis of environmental samples (sediment and water) was a general method to monitor an environmental contamination. In some situation, this method cannot show detectable level of residue in environmental samples. The application of animals as a sentinel species is good alternative that can be used to indicate the extent of environmental contamination. In this study, the environmental samples (sediment and water) were screened for contamination and mussels were subjected to contaminant analysis. These finding showed that detectable levels of atrazine, glyphosate and paraquat were found in every mussel examined with ELISA, although residues of herbicide in sediment and water were not detectable with GC-MS and HPLC in some period. It is of importance to note that limits of detection were different between ELISA and chromatographic methods, and interpretation on herbicide contamination in environment vs. mussels should be done with cautions. These data implied that albeit its readily degradable, herbicide residues could be temporally stored in *U. contradens* tissue. Thus, the monitoring program for environmental contamination should focus on both physical and biological samples in order to predict the potential health impact on organism with more accuracy

Chapter IV

Reproductive Activities of the Freshwater Mussel *Uniandra contradens* and its Potential Use as a Biomarker for Herbicide Contamination in Nan Province

1. Introduction

Environmental contaminations have been found in several areas especially aquatic environment (Lopes et al., 1992). In order to evaluate and monitor the potential effect of contamination on any organism, several programs have been established including using animal as sentinels of environmental health hazards (Goldberg et al., 1983; Washington, 1984; Philips and Segar, 1986). The sentinel species was selected on the basis of their characteristics as a common species, clearly identification and large population (Phillips and Segar, 1986).

The mussel have been used extensively as sentinels in part because they has complete life cycle in water as bottom dweller and filter feeder on plankton and organic matters through water current (Dillon, 2000). They are shown as a general monitors for particular contaminants in aquatic habits (Phillips and Rainbow, 1993). Especially, in 1978, the International Mussel Watch projects was established to use the mussel *Mytilus edulis* as a sentinel species for the contamination along the coastal area of the world (Jernelov, 1996)

Reproductive health is regarded as one of an important biomarker of effect for the environmental contamination (ATSDR, 2007). Examples of this include the imposex on marine gastropod exposed to tributyltin (Bettin et al., 1996), intersex in male clam *Scrobicularia plana* exposed to xenobiotic contaminant (Chesman and Langsto, 2006; Gomes et al., 2009), decrease hatching rate of snail *Marisa cornuarietis* exposed to herbicide (Sawasdee and Köhler, 2009), and decrease growth rate of juvenile bivalves (Bringolf, 2007a; 2007b) including degeneration of oocytes in female gastropod exposed to herbicide (Bacchetta, 2002).

In Nan Province, northern part of Thailand, herbicides used in agricultural areas may accumulate in environment and cause harmful effects to animals living in the area. In this study, a freshwater mussel *Uniandra contradens*, a common species that widely distribute in aquatic habitats close to agricultural area of Nan Province, was selected as a sentinel species. Condition factor, vitellogenin-like protein level, Gross- and microanatomy of reproductive system were studied in order to provide baseline information on reproductive activities of the mussel and insight into a potential window of susceptibility to xenobiotic contamination.

Objective

- To examine the reproductive activities of *U. contradens* living in an agricultural catchment at Nan Province

2. Materials and methods

2.1 Study site

The study site was an agricultural catchment (Nong Bua reservoir) located in San Subdistrict, Wiang Sa District of Nan Province, Thailand. Seasonal cultivations of corn, cucumber gourd, rice, sesame and soybean can be found throughout near Nong Bua reservoir (18˚30'35.39" N, 100˚46'4.48" E). This reservoir was constructed to be used as a catchment for run-off water from surrounding agricultural patches before flowing to the adjacent Nan River during wet season, and a reservoir for agricultural activities during dry season. Several aquatic animals are inhabited in the reservoir including a freshwater mussel *Uniandra contradens*, a sentinel species in this study.

2.2 Mussel collection, maintenance and processing

The freshwater mussel was obtained from Nong Bua reservoir. There were collected by hand on monthly basis throughout the year from July 2010 to June 2011. After the mussels were collected, they were transported to a laboratory at the Chulalongkorn University Forest and Research Station at Nan Province. The mussels were maintained and acclimatized in plastic tank with aerated natural water overnight (at least 12 hours). Before the mussels were euthanized in ice slurry and dissected to separate the soft part from the shell, there were measured for morphometric (shell length and shell width; Figure 4.1) and gravimetric data.

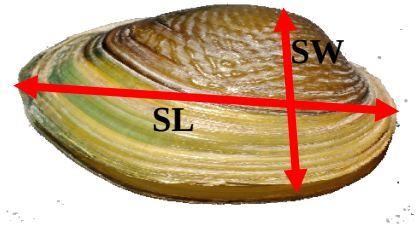


Figure 4.1: Morphometry of *U. contradens* from an agricultural area, Nan Province. SL = shell length, SW = shell width.

2.3 Condition factor of freshwater mussel

After morphometric and gravimetric data of the mussel were obtained, their whole bodies were removed from the shell. The data of shell length and whole body weight without shell of each mussel was used to calculate a condition factor of each mussel as follows (Gagné et al., 2006).

$$\text{Condition factor (C.F.)} = \frac{\text{Whole body weight without shell (g)}}{\text{Shell length (cm)}}$$

2.4 Determination of sex and reproductive maturity of freshwater mussel

Sexes of each mussel were identified based on wet mount slide preparation technique. Briefly, after the soft tissue part was separated from the shell, gonad was punctured with a dissecting needle. Approximately 20 µL of gonadal tissue fluid was transferred to a microscope glass slide and mounted with a cover glass. The wet mount slides of gonadal tissue fluid were examined under light microscope for a presence of motile sperm in male or mature oocyte in female.

2.5 Spawning activity of female freshwater mussel

Since gill of female mussel is a major deposition site of eggs and developing glochidia, spawning activity of each female mussel was identified based on a wet mount slide preparation of gill tissue fluid. After the soft tissue part was separated from the shell, gill morphology was observed before puncture with dissecting needle. Approximately 20 µL of gill tissue fluid was transferred to a microscope glass slide and mounted with a cover glass. The wet mount slides of gill tissue fluid were examined under light microscope for a presence of egg. The score was based on visual grading of gill morphology in relation to presence of egg deposition in the gill as followed:

Score 0 = normal gill with no sign of egg deposition

Score 1 = normal gill with egg deposition

Score 2 = partly enlarged/swelled gill with egg deposition

Score 3 = completely enlarged/swelled gill with egg deposition

2.6 Histology

After the mussels were euthanized in ice slurry, the soft part was dissected from the shell. Whole body samples of the mussel were fixed in Davidson's solution (Dietrich and Krieger, 2009) immediately for 48 hours and then preserved in 70% ethanol until further analysis. The samples were cut (x-section) into small pieces with 2-3 mm thickness and processed through standard paraffin method (Presnell and Schreibman, 1997). The paraffinized tissue were serially sectioned at 5 µm thickness with a rotary microtome (Leica RM2125RT) and attached onto a microscope glass slide. Tissue slides were stained with Delafied's haematoxylin and eosin, dehydrated in graded ethanol, clearing in xylene and mounted in distyrene and tricresyl phosphate in xylene (DPX) prior to examination under a light microscope.

2.7 Vitellogenin analysis

2.7.1 Tissue extraction for vitellogenin analysis

The soft part of the mussel was kept at -20 °C until analysis. Prior to tissue extraction, the tissue was thawn in a refrigerator, and the gonad was dissected on ice. The gonadal tissue was homogenized with 1 mL of extraction buffer (0.05 M of Tris-HCl, pH 7.5 containing 0.2 M NaCl and protease inhibitors cocktail (ethylenediaminetetraacetic acid [EDTA], 4-[2-aminoethyl] benzenesulfonyl fluoride [AEBSF], bestatin, E-46, leupeptin and aprotinin)) for 1 minute. The gonad extract was centrifuged at 12,000 xg for 30 minutes at 4 °C. The supernatant was collected and transferred to clean tube. Before further analysis the sample was stored at -20 °C.

2.7.2 Normalization of protein concentration of the samples

Determination of Total protein concentration in the extracted sample was examined by Bradford assay (Bradford, 1976) modified for 96-well microtiter plate (Redinbaugh and Campbell, 1985). The standard protein was a bovine serum albumin (BSA; Sigma, St. Louis, Mo). The standard protein and gonad extract were diluted with extraction buffer, pH 7.5. Standard BSA concentrations were ranges from 0.78125 to 25.000 µg/mL. The sample of gonad extract was diluted to 1:100, 1:1000 and 1: 10000 with the extraction buffer, pH 7.5 prior to analysis. One hundred microliters of samples (triplicate) and protein standard (duplicate) were loaded into each well. Then, 100 µL of Bradford reagent was added and incubated at room temperature for 10 minutes. Absorbance at 595 nm was read with a microplate reader (Multiskan EX).

Mean absorbance of the duplicate standards and triplicate samples were calculated first. Then, average absorbance of each standards and samples were subtracted with average absorbance of the blank. A standard curve was constructed by plotting the absorbance at 595 nm on y-axis and the protein concentration on x-axis. Standard calibration curves of protein concentration were linear from 0-25 µg/mL with r^2 of 0.97253 to 0.99886. The protein concentration of each sample was determined by interpolation from this standard curve as µg/mL. When the total protein concentration of the gonad was measured, protein concentrations in the samples were adjusted to 1 mg/mL in each gonad extract with the extraction buffer.

2.7.3 Determination of vitellogenin by alkali-labile phosphate

Vitellogenin are complex lipophosphoproteins which rich of phosphate. The vitellogenin associated phosphates are alkali-labile. The fact that the alkali-labile phosphate is strongly correlated with the amount of vitellogenin can be used as an indirect measurement of vitellogenin level. The assay is based on the principle of an ether-extraction, and then lipophosphoproteins are subjected to an alkali treatment with NaOH in order to liberate the labile

phosphates into aqueous phase. The higher levels of alkali-labile phosphate in the gonad extract of the female mussel is presumably corresponded to an increase of free alkali-labile phosphate. The examination of alkali-labile phosphate was modified from Gagné and Blaise (2000) to be used in a 96-well microtiter plate. Detection of phosphate level was performed with detection kit (malachite green kit, R&D Systems) as described belows.

Ether extraction

This step is working on principles of extraction of non-polar molecules (vitellogenin) from the sample into ether. Two hundred and fifty microliters of the gonad extract was added into clean microcentrifuge tubes. The sample was extracted with 500 µL of *t*-butyl methyl ether (Merck) for 30 minutes at room temperature. The homogenate was centrifuge at 10,000 xg for 5 minutes at 4°C. After centrifugation, the ether phase was separated and transfer into another clean microcentrifuge tube.

Phosphate isolation and pH condition

The free phosphate was isolated from phospholipids in the ether phase. Briefly, 400 µL of ether phase was mixed with 200 µL of 2M NaOH and vigorously shaken at 37 °C for 60 minutes to liberate phosphate into the aqueous phase. One hundred and fifty microliters of aqueous phase was collected and adjusted pH to 7.0 by adding 150 µL of 2M Tris-HCl buffer, pH 9.0 and 150 µL of 4M hydrochloric acid (Merck). After pH of each sample was adjusted, the free phosphate was determined with malachite green kit.

Phosphate determination

After the sample was extracted and adjusted pH to 7.0, the sample was analyzed for phosphate level with malachite green kit. The standard phosphate and blank were prepared in 2 M Tris-HCl, pH 9.0. Determination of phosphate

level was applied in a 96-well microtiter plate. Briefly, the standard phosphate solution (1.56, 3.12, 6.25, 12.5, 25 and 50 µmol/L) and blank were loaded into a microtiter plate. Twenty microliters of malachite green reagent A was added into each well , and the plate was incubated at room temperature on an orbital shaker for 10 minutes. Then, 20 µL of malachite green reagent B was added into each well, and the plate was incubated at room temperature on an orbital shaker for 20 minutes. Afterword, the absorbance was measured at 620 nm by a microplate reader (Multiskan EX).

Calculation of phosphate level

Mean absorbance of the duplicated standards and samples was calculated first. Then, average absorbance of each standards and samples were subtracted with average absorbance of blank. A standard curve of phosphate level was constructed by plotting the absorbance (620 nm) on y-axis and phosphate concentration on x-axis. Standard calibration curves of phosphate level were linear from 0-50 µmol/L with r^2 of 0.98799 to 0.99645. The phosphate concentration of each sample was determined by interpolation from this standard curve and shown as µg phosphate / mg protein.

2.8 Statistical analysis

All parameters were tested for normal distribution and homogeneity of variance. Condition factor and alkali labile phosphate of the freshwater mussel was compared between months by Kruskal-Wallis one way ANOVA on ranks followed by Dunn’s multiple comparison methods. Correlation between herbicide contamination and condition factor were tesed with Pearson product moment correlation and Spearman rank correlation was tested the spawning activities and herbicide contamination.

3. Results and discussion

The condition factor was calculated from morphological and gravimetric data. This relative weight is an indicative of growth and can be use to predict the overall health status of the mussel living in the agricultural area. It was found that the mussels collected from Nong Bua reservoir showed a wide variety of size (shell length ranging from 10.85 to 52.07 mm) and weight (whole body without shell ranging from 0.65 to 9.44 g). The mean condition factors in male were ranges from 0.45±0.03 to 1.19±0.02 and female were ranges from 0.46±0.03 to 1.32±0.03. The results of relative weight were found to increase with time indicating that either the mussels were growing during the sampling period or there were seasonal difference in body weight of mussels. Mean comparison of condition factor in each sex showed a significant difference between seasons ($p<0.05$; one way ANOVA on rank & Dunn's method; Table 4.1). The results of this gravimetric-morphometric analysis provided a baseline data for overall health and growth of *U. contradens* and could be used for future evaluation of this sentinel species in this area.

Table 4.1: Mean ± S.E.M. of condition factor of freshwater mussel *U. contradens* collected from an agricultural catchment (Nong Bua Reservoir) in Nan Province, Thailand during July 2010 to June 2011

Sampling Months	Male mussel	Female mussel
July 2010	0.45 ± 0.03 ^{C} (N = 32)	0.46 ± 0.03 ^{D} (N = 10)
August 2010	0.71 ± 0.05 ^{C} (N = 34)	0.80 ± 0.10 ^{CD} (N = 18)
September 2010	0.81 ± 0.05 ^{CF} (N = 24)	0.77 ± 0.02 ^{CD} (N = 31)
October 2010	0.99 ± 0.08 ^{BEF} (N = 38)	1.0 ± 0.03 ^{CE} (N = 26)
November 2010	1.0± 0.03 ^{BD} (N = 51)	1.09 ± 0.03 ^{BE} (N = 44)
December 2010	1.19 ± 0.02 ^{A} (N = 57)	1.32 ± 0.03 ^{A} (N = 33)
January 2011	1.09 ± 0.03 ^{AD} (N = 48)	1.20 ± 0.03 ^{AB} (N = 42)
February 2011	1.08 ± 0.02 ^{AD} (N = 48)	1.18 ± 0.05 ^{ABE} (N = 42)
March 2011	1.03 ± 0.02 ^{BD} (N = 54)	1.06 ± 0.03 ^{BE} (N = 36)
April 2011	1.05 ± 0.03 ^{BD} (N = 53)	1.09 ± 0.03 ^{BE} (N = 37)
May 2011	1.09 ± 0.03 ^{AD} (N = 51)	1.14 ± 0.04 ^{ABE} (N = 39)
June 2011	1.06 ± 0.02 ^{ADE} (N = 48)	1.05 ± 0.03 ^{BE} (N = 42)

Remark:

Significant difference between months ($p<0.05$, one way ANOVA on ranks & Dunn's method) is indicated by difference in superscript letter.

Reproductive activities of *U. contradens* were studied by gross- and microanatomy of gonad in order to obtain a baseline information and insight into a potential window of susceptibility to xenobiotic contamination. Wet mount slide preparation of gonadal tissue fluid showed that most of male mussel had motile sperms in the testis (Figure 4.2A) during July 2010 to January 2011 and eggs of female mussels were present in wet mount slide preparation of gonadal tissue fluid during the entire sampling period (Figure 4.2B).

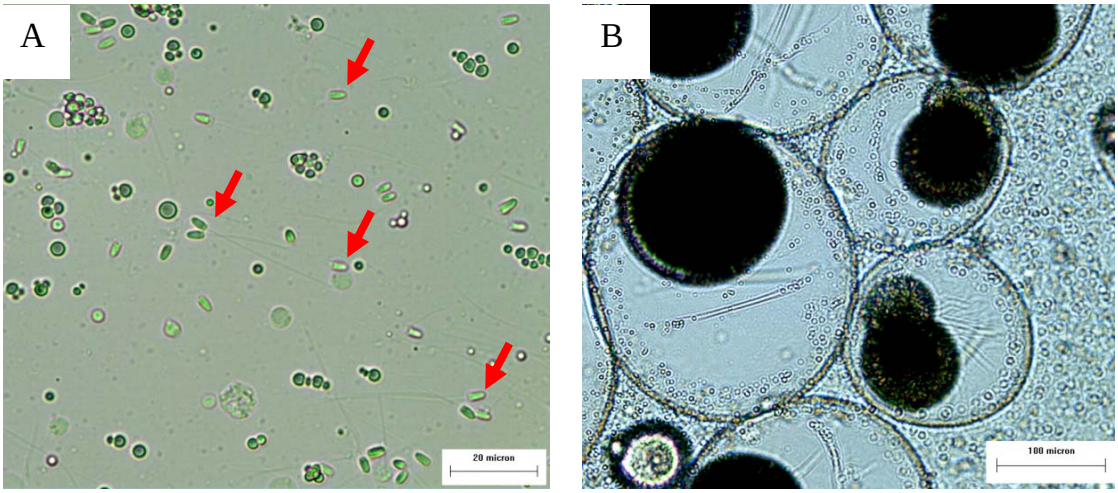


Figure 4.2: Micrographs of gonadal fluid of *U. contradens* collected from Nong Bua reservoir, Nan Province, Thailand. (A) Wet mount slide of testicular tissue fluid showed numerous motile sperms (arrows). (B) Wet mount slide of ovarian tissue showed numerous mature eggs.

In this study the sexual maturation of the mussels was determined in both male and female using gross morphology of the gonad (Figure 4.3). The external form of the mussel gonad was similar in both sexes. The gonad was originated from primordial germ cells located near the posterior end of the body and just ventral to the pericardium (Coe and Tuner, 1938). Expansion of the primordial gonad began in the connective tissue layer adjacent to the digestive tract (Coe and Tuner, 1938). When the mussel was matured, the gonad covered a large area in visceral mass of the body (Figure 4.4). At maturation, the gonad was soft white in color and swelling. It consists of gonadal fluid and germ cells including accessory cells. Both male and female mussels have the gonads with various stages of germ cells distributed from the basal to the lumen.

Histological analysis of the testicular tissue sections showed germ cells at different stages of development including spermatogonia, spermatocyte, spermatid and spermatozoa (Figure 4.4). Spermatogonia are located along the basal part of acini. Germ cell proliferation was observed indicating sexual maturation. These germ cells migrated toward the center of the gonad acini resulting in several layers of germ cells. Spermatogonia are the largest germ cells in the gonadal acini. They are oval in shape with a chromatin clumped nucleus and a small amount of cytoplasm. Spermatocytes are proliferating cells moving toward the lumen from a closely packed cell at the acinar wall. They have round nuclei with dispersed chromatin condensation. Each spermatocyte is surrounded with a relatively small amount of cytoplasm and it is smaller in size than spermatogonia. A number of spermatids are produced and discharged toward the center of acinar lumen. They are small round cells with strongly basophilic chromatin dispersed in the nuclei. Spermatozoa are transformed cells of spermatid. They are frequently found close to the lumen. A spermatozoon has a cylindrical head shape. The middle piece can be found with densely stained of eosin. The acidophilic tail stained pink color and radially projected toward the center of the lumen.

Furthermore, a special gametogenesis of *U. contradens* was described in this study by the observation of morulae germ cell (Figure 4.5). The morulae germ cell was found along the acini of the *U. contradens* testes in every sampling mussel. There on also several report in family Amblemidae, Hyriidae, Margaritiferidae, and Uniodae (Coe and Turner, 1938; Ropes and Stickney, 1965; Matos et al., 1988), but the functions are still not clear. Coe and Turner (1938) described the sperm morulae in *Mya arenaria* and suggested that it may function in the supplying of nutrient to spermatozoa when they become cytolysis. This structure is also studied in *Anodota cygnea* and other bivalves. It was found that they do not produce the normal spermatozoa and there was an evidence of abnormality of spermatogenesis (Mackie, 1978; Rocha and Azevedo, 1990). In contrast, there is a study about morulae germ cell showing that it can be differentiated into the mature spermatozoa. Motos et al. (1998) investigated *Prisodon alatus* and suggested that spermatid morulae can be metamorphosed into mature spermatozoa in lumen of the acini. In *Anodonta grandis*, the sperm morulae was present only in some season and disappeared when the sperm became mature and fully occupied in the acini of the gonad (Van der Schalie and Locle, 1941; Heard, 1975). In bivalve, the mature sperm from atypical spermatogenesis with morulae like cell do not confirm the viability and successful fertilization (Heard, 1975). In addition, it was also reported in *Hyriopsis myersian* and *Hyriopsis bialatus* that the morulae germ cells are classified into three stages consisting of 1) early stage containing spermatogonia with a few cellular content of morulae. 2) middle stage containing several spermatocytes and 3) late stage containing spermatid and spermatozoa (Srakaew et al., 2010; Chatchavalvanich et al., 2006). In this study, the morulae germ cells are found in every *U. contradens* collected year round. This finding may suggested the continuous production of typical and atypical spermatogenesis of tropical zone freshwater mussel. Both type of spermatogenesis are presented and occurred in several acini of the mussel, this

finding are supported by the study in *Hyriopsis myersian* and *Hyriopsis bialatus* (Srakaew et al., 2010; Chatchavalvanich et al., 2006).

In females, different stages of germ cell were found in the acinar wall of the ovary (Figure 4.6-4.7). The stages of maturation of female germ cell were classified according to yolk accumulation into three stages (Coe and Tuner., 1938). First, pre-vitellogenic stage was a small cell located at the basal part of acinus. It contains a basophilic cytoplasm. Second, vitellogenic stage is a larger cell that accumulates nutrients in the lumen of yolk vesicles inside the cytoplasm. The cytoplasm is eosinophic. Third, post-vitellogenic stage was found as an empty acinus or an acinus containing a degenerating oocye.

The results on gonadal examination during July 2010 to June 2010 reveal that gametogenesis occurred in both male and female mussels. The sign of gonadal maturation was evidenced in every sampling periods indicating by the observation at various stages. In males, motile sperms were found whereas in female, oocytes at different stages were found throughout the year. In previous studies, two modes of gametogenesis were reported in bivalve: seasonal gametogenic cycle as in pearl oyster *Pinctada fucata* (Choi and Chang, 2003) and *Scrobicularia plana* (Rodríguez-Rúa et al., 2003) and continuous gametogenesis as in *Hyriopsis bialatus* (Chatchavalvanich et al., 2006). The results from this study suggested that *U. contradens* possesses continuous gametogenesis since all mussels showed a sign of matured germ cells in the gonads in every month examined.

Figure 4.3: Gross morphology of the gonad of freshwater mussel *U. contradens* in Nan province.

(A) General view of the shell

(B) The removed soft body part consisting of gill, gonad (G), foot (Ft), intestine (I) and hepatopancreas (Hp)

(C) Histological structure of the female cross section from the area indicated with black square in Figure 4.3B.

(D) Cross section of mussel after fix with Davidson's solution.

(E) Histological structure of the male cross section containing basophilic stained testis.

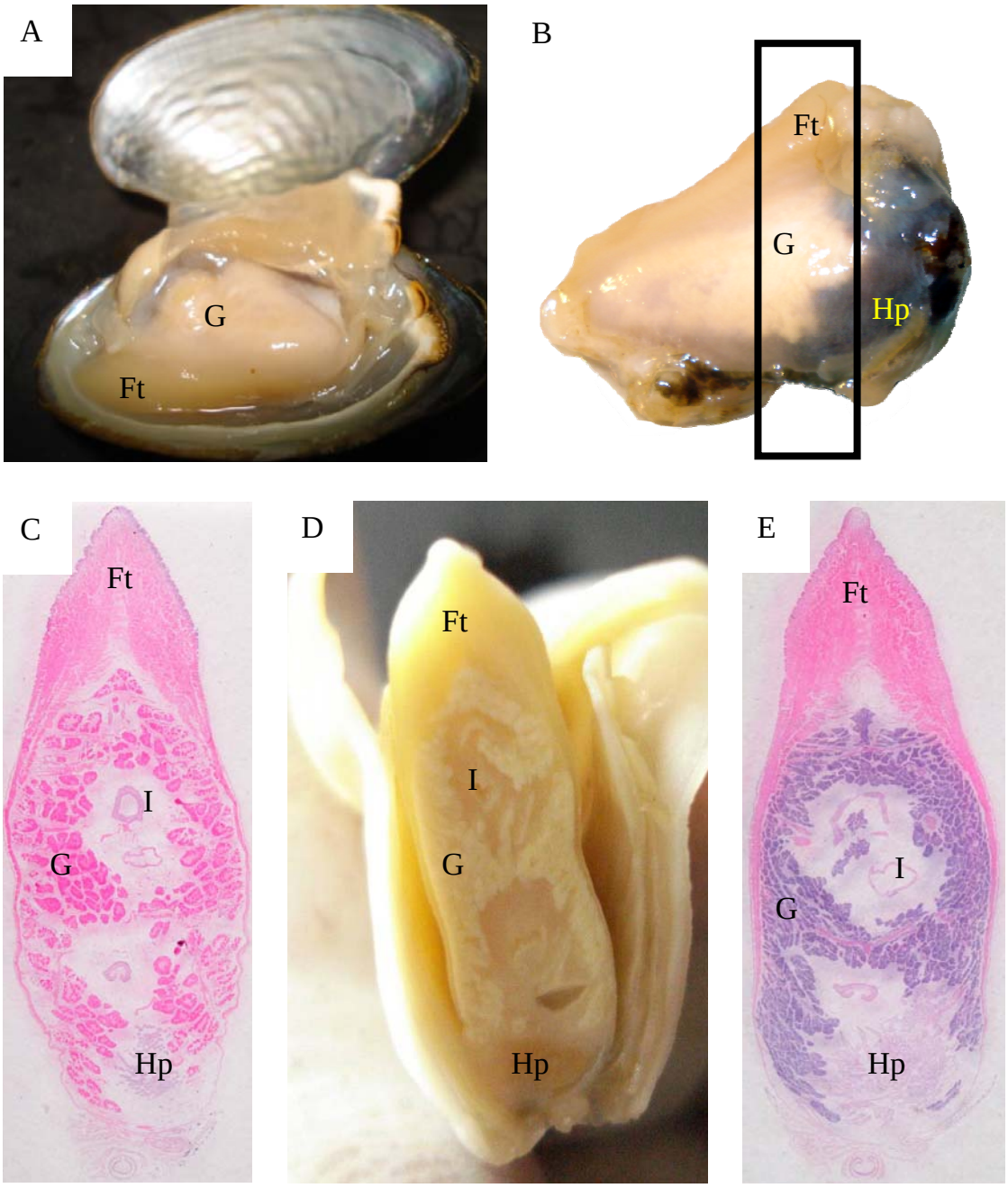

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Figure 4.4: Histology of *U. contradens* testis.

(A) Testicular tissue at low magnification showed overall general structure.

(B) Gametogenesis in acinar wall of the testis. Dark arrows indicated acinar wall. Several types of germ cells lined from the acinar wall toward the center of the lumen consisting of spermatogonia (Sg), spermatocyte (Sc), spermatid (St) and spermatozoa (Sp), H&E stain.

(C) The smear of male gonad fluid stained with H&E. The asterisk indicates head of sperm and the arrows indicate middle piece which densely stained with eosin.

(D) The histology of testicular tissue at high magnification showed detail of germ cell stained with H&E illustrated the spermatogonia located at periphery wall (arrows)

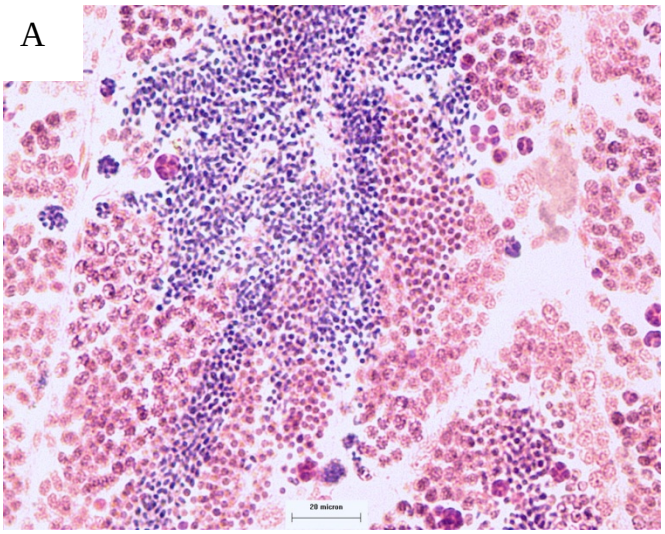
(E) The histology of testicular tissue at high magnification showed detail of germ cell stained with H&E illustrated the spermatocytes (arrow)

(F) The histology of testicular tissue at high magnification showed detail of germ cell stained with H&E illustrated the spermatid (arrow)

(G) The histology of testicular tissue at high magnification showed detail of germ cell stained with H&E illustrated the spermatozoa in lumen of the acinus (asterisks)

(H) Wet mount slide preparation showed motile sperms of male mussel. Dark arrow indicates head and red arrow indicates slender tail.

A



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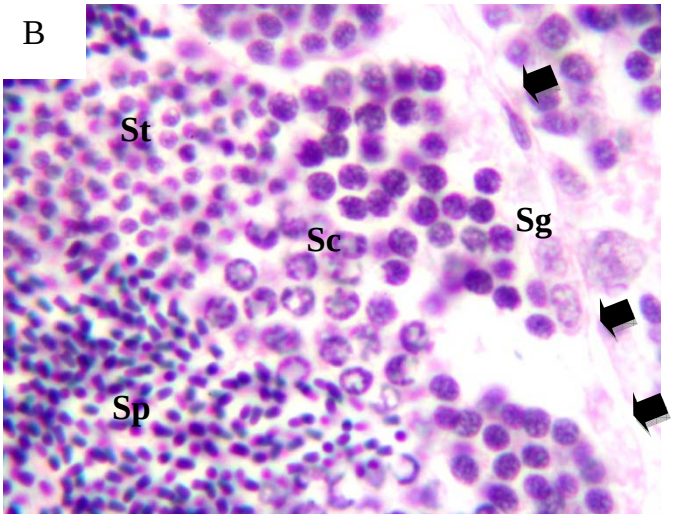

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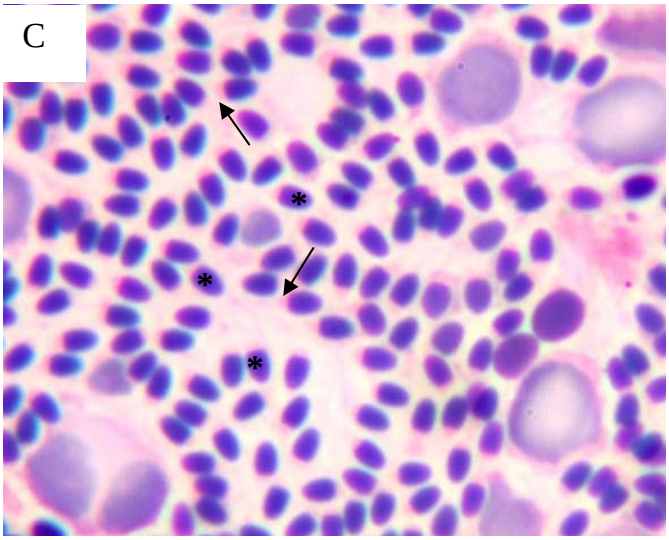

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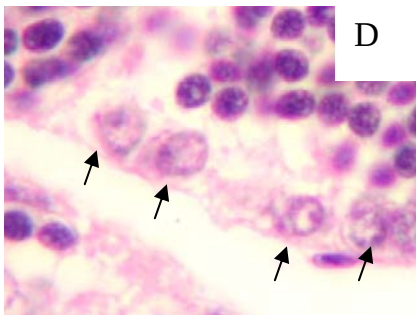

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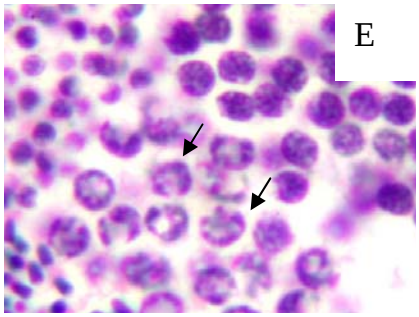

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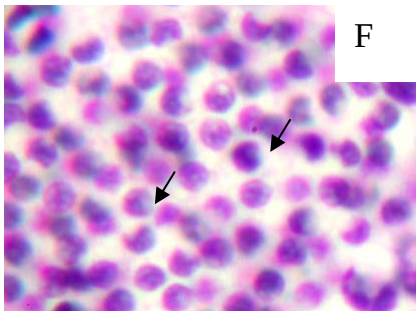

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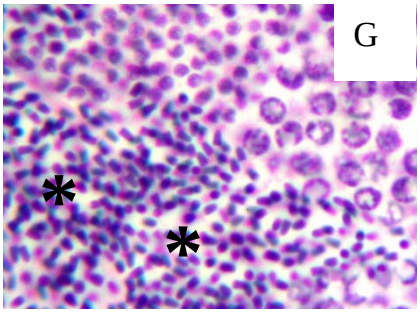

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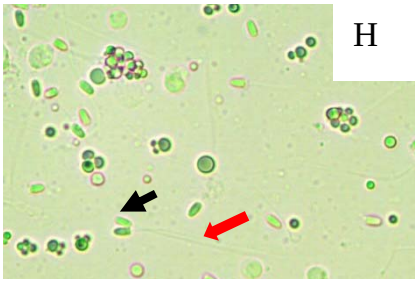

Figure 4.5: Histological structure of *Uniandra contradens* testes.
(A) Several morular germ cells (white arrows), PAS stain
(B) Several morular germ cells (white arrows), H&E stain
(C-F) Different stages of morular germ cells, H&E stain.

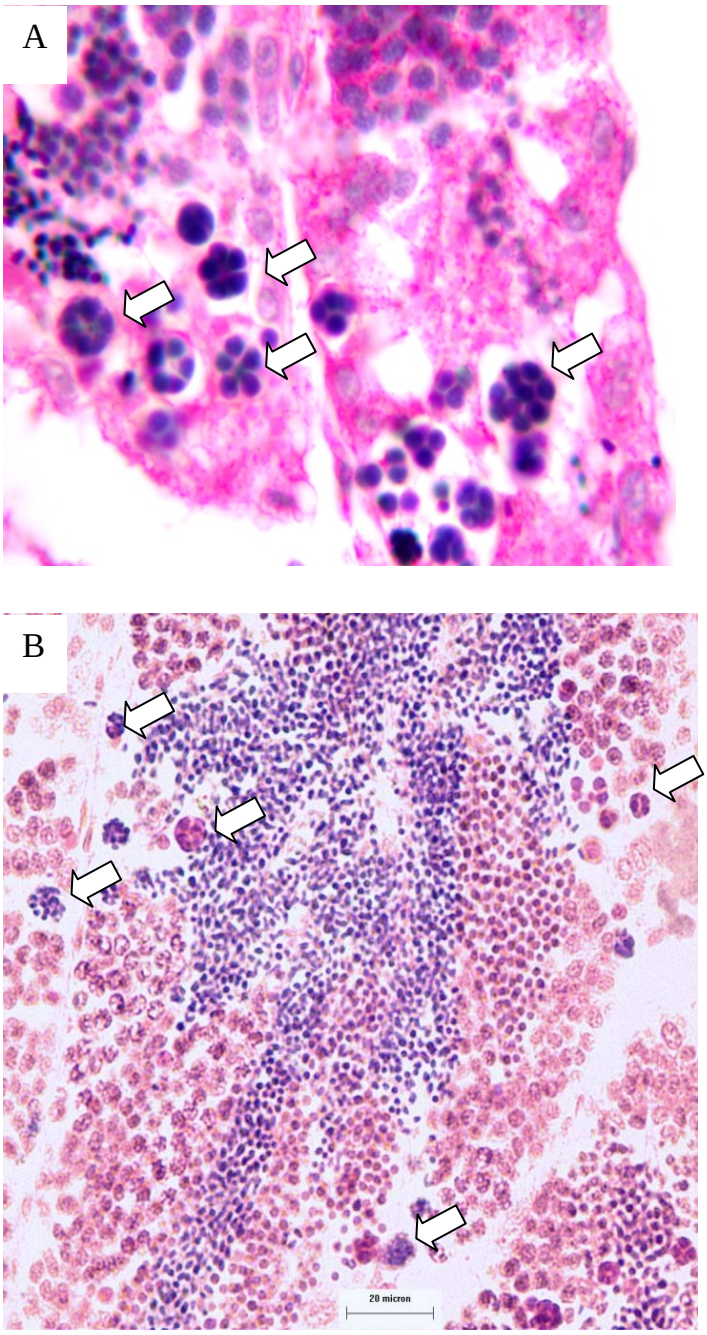
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Figure 4.6: Histological structure of *U. contradens* ovaries.

(A) The basic structure of ovarian tissue containing gonoduct (asterisk)

(B) Structure of gonoduct with spawning egg (asterisk). Dark arrows indicated ciliated columnar cells of the duct epithelium, H&E stain.

(C) The ovarian tissue at low magnification showed overall structure of degenerated acinas after ovulation. H&E stain.

(D) The ovarian tissue at low magnification showed overall structure of acinar wall (dark arrow) with several oocytes (asterisk), H&E stain.

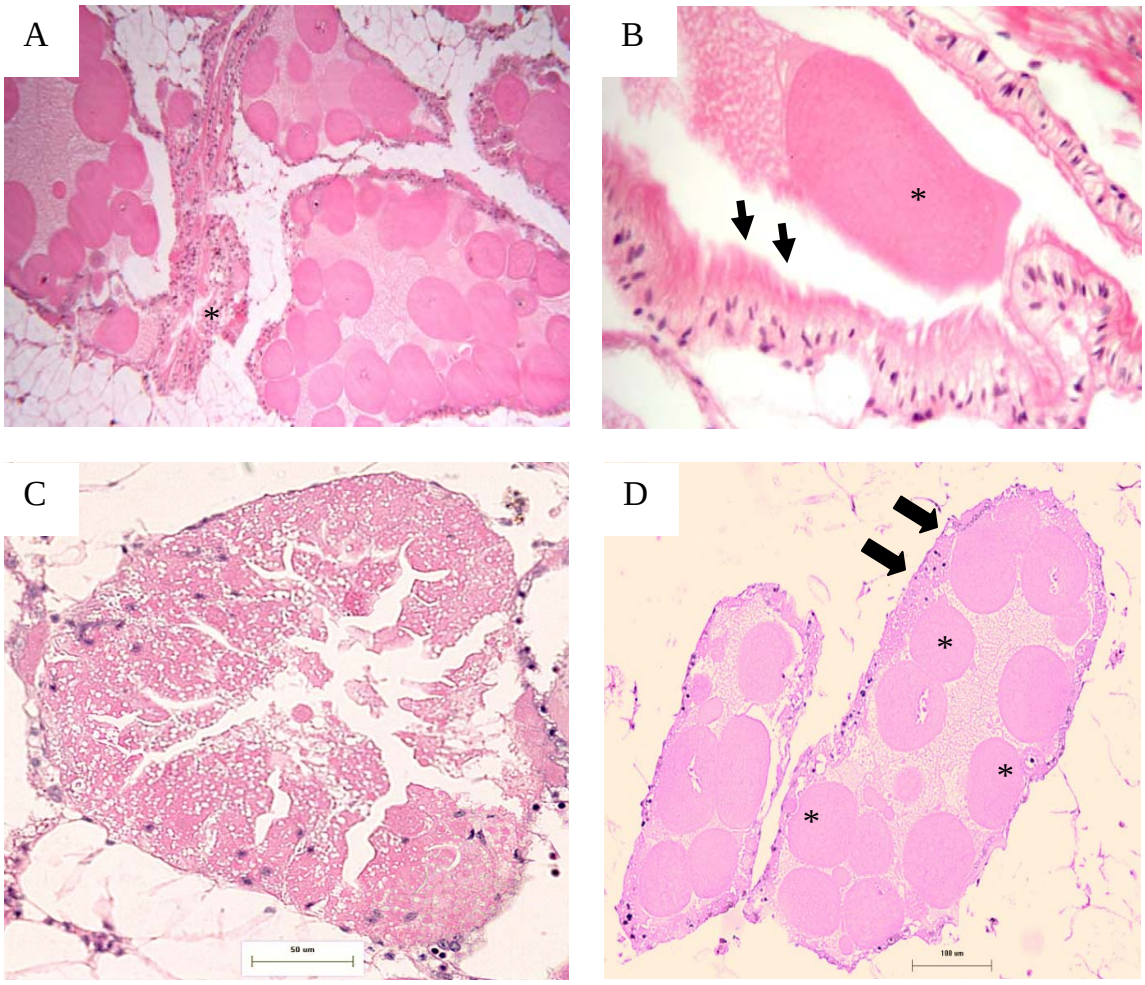
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Figure 4.7: Histological structure of *U. contradens* ovaries.

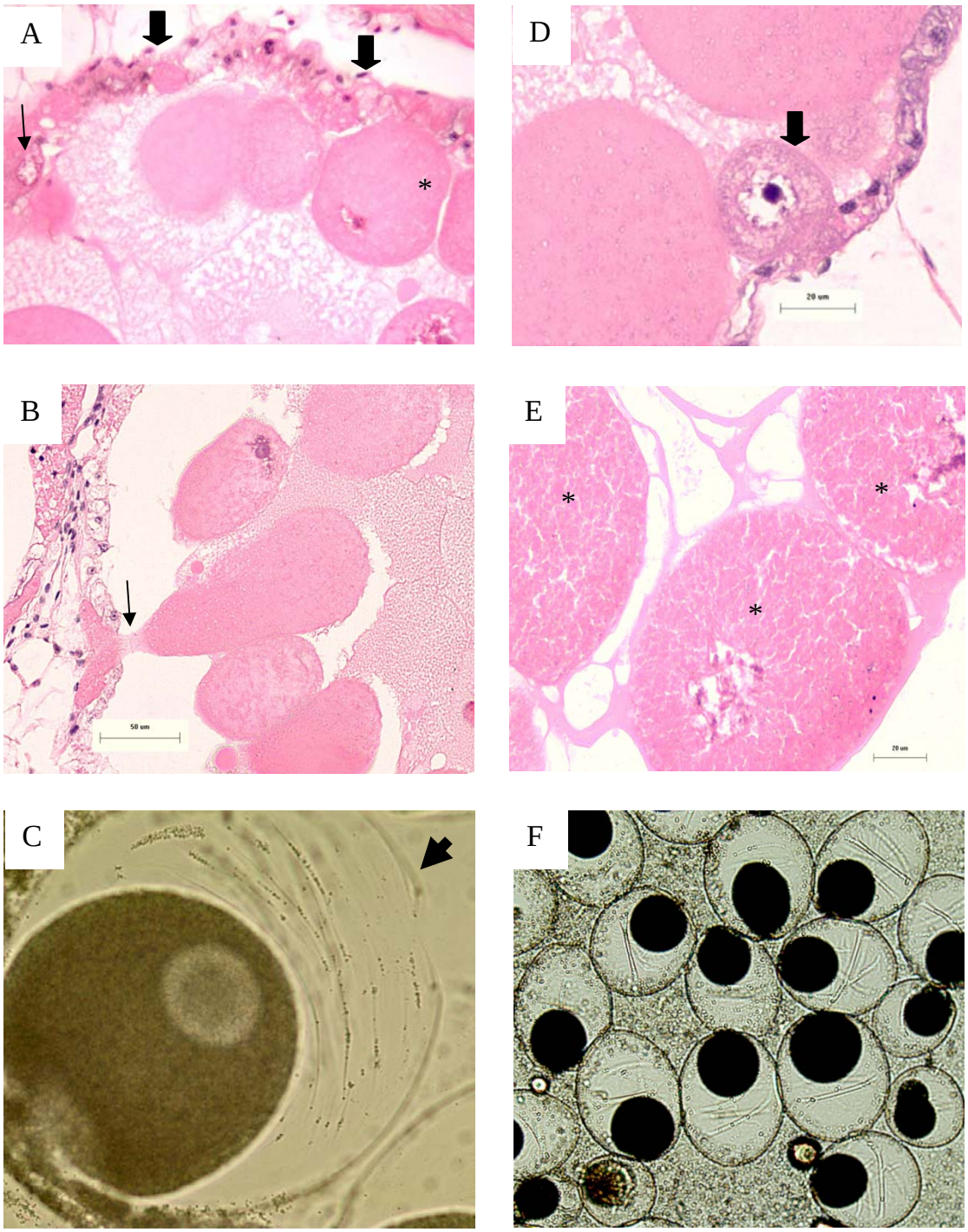
(A) Structure of acinar wall with oogonia (narrow arrow) and follicular cell (dense arrow), asterisk indicated the oocyte.

(B) The ovarian tissue showed growing oocyte attached to the acinar wall with stalk (arrow).

(C, F) Wet mount slide preparation showed oocyte from gonad fluid of female mussel. Dark arrow indicated vitelline membrane.

(D) The ovarian tissue at high magnification showed the pre-vitellogenic oocyte (arrow), H&E stain.

(E) The ovarian tissue at high magnification showed the vitellogenic oocyte (arrow), H&E stain.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Since gill of female mussel is a major deposition site of eggs and developing glochidia (Dillon, 2000), spawning activity of female could be determined by observing deposition of eggs at the gill of female mussels (Chatchavalvanich et al., 2006). Spawning activity of individual female *U. contradens* was scored based on visual grading of gill morphology in relation to presence of egg deposition in the gill. The result in Figure 4.8 showed that, although gametogenesis is continuous in this species, there were temporal differences in spawning activities. Lowest spawning activity was found in female mussels in early wet season (July 2010). Then spawning activity seemed to increase gradually until its peak in early dry season (December 2010 to January 2011).

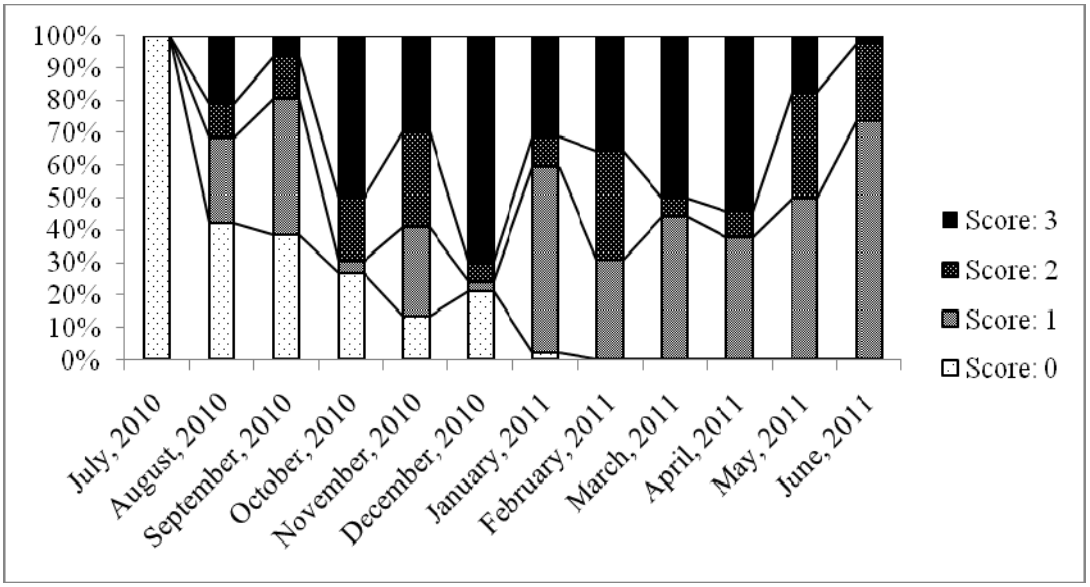

Figure 4.8: Degree of spawning activity of female *U. contradens* collected from Nong Bua reservoir, Nan Province, Thailand during July 2010 to January 2011. The score was based on visual grading of gill morphology in relation to presence of egg deposition in the gill as followed:

Score 0 = normal gill with no sign of egg deposition

Score 1 = normal gill with egg deposition

Score 2 = partly enlarged/swelled gill with egg deposition

Score 3 = completely enlarged/swelled gill with egg deposition

To obtain information on reproductive system of the female mussel, the gonadal vitellogenin level was determined. Vitellogenin is well known as a major precursor for yolk which provides energy reserves for embryo in oviparous organism (Matozzo et al., 2008). In addition, invertebrates also synthesize yolk or vitellin protein from vitellogenin which located in oocytes (Blaise et al., 1999). Vitellogenin can be detected indirectly by the alkali-labile phosphate (ALP) assay. From the result in this study, it was found that the mussels collected from Nong Bua reservoir showed seasonal variation on ALP level throughout the year (July 2010-June 2011) (Figure 4.9). In female, the mean phosphate concentration which an indicative vitellogenin level are significantly highest in December 2010 (0.30±0.14) and lowest in September 2010 (0.03±0.01) during sampling period (July 2010 to June 2011) ($p<0.05$, one way analysis of variance on ranks). These result indicated, although *U. contradens* gametogenesis is continuous, production of yolk protein may be seasonal with the peak of ALP level in only a certain period of the breeding cycle. There are no correlations of ALP level with spawning activities by gill morphology ($p>0.05$; Spearman rank correlation). The results of this alkali-labile phosphate analysis provided a baseline data for reproductive status of *U. contradens* and could be used for future evaluation of this sentinel species in this area.

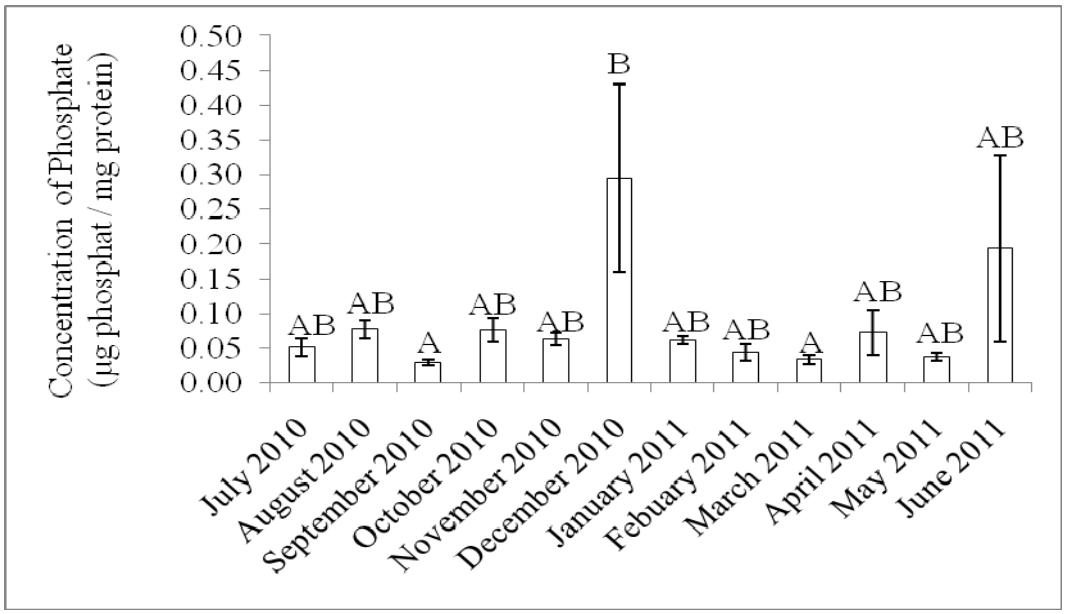

Figure 4.9: Mean ± S.E.M. of phosphate concentration in female mussel collected from agricultural area (Nong Bua reservoir) in Nan province, Thailand. Significant difference between month ($p<0.05$, one way ANOVA on Ranks & Dunn's Method) is indicated by difference in superscript letter.

To monitor change in health status and growth of mussels in this area, gravimetric and morphometric techniques were performed. Mean comparison of condition factor in each sex showed significant differences between months (ANOVA on ranks & Dunn's Method, $p<0.05$; Table 4.1). Further analysis showed a significant negative correlation between atrazine residue in the mussel tissue and the condition factor of both sexes of the mussels (Pearson product moment correlation, $p<0.05$, $r=-0.662$ in male; $r=-0.627$ in female: Table 4.2). These finding indicated potential impact of atrazine on overall health of the mussel.

Since atrazine is known to cause disruption of endocrine and reproductive systems of animal (Allran and Karasov, 2001; Hayes *et al*., 2003), effects on aquatic animals living in the area are thus expected. Previously, contaminations of endocrine disrupting chemicals as a result of agricultural activities were reported to adversely affect several mollusk species (Chesman and Langsto, 2006; Gomes *et al*., 2009). Although atrazine has no acute toxic effect in bivalve (Bringolf *et al*., 2007a; 2007b), chronic effect of low level atrazine exposure have been found in several mollusk species. A low level of atrazine (0.1 µg/mL) was reported to cause a reduction in hatching rate in the ramshorn snail *Marisa cornuarietis* (Sawasdee and Köhler, 2009). While a higher concentration of atrazine (>3.8 µg/mL) was reported to cause a reduction in growth rate of glochidia and juvenile mussels (Bringolf *et al*., 2007a; 2007b).

In addition, analysis of glyphosate residue in their mussel was showed a significant negative correlation between residue in the mussel tissue and the condition factor of female mussels (Pearson product moment correlation, $p<0.05$, $r=-0.775$: Table 4.2). The glyphosate residue in the mussel also shows significant negative correlation with spawning activities as determined by gill morphology (Spearman rank's correlation, $p<0.05$, $r=-0.840$: Table 4.3). These finding indicated potential impact of glyphosate on reproductive health and activities of the mussel.

Glyphosate was reported to has a low potential bioaccumulation in several aquatic organism (Giesy, 2000). In this findings, detectable levels of glyphosate residue (6.71 -10.44 ng/g: Chapter 3) can be found in every sample and could indicate potential health effect to the mussels. In previous study, acute toxicity of Roundup, a formula of glyphosate herbicide, was found in several aquatic organisms (Folmar et al., 1979; Tsui and Chu, 2003). Early life stage of several mussels were also sensitive to glyphosate and its formula. Biringolf et al. (2007) reported that both glochidai and juvenile of *Lampsilis siliquoidea* showed an acute toxicity in the range of 2.9-5.9 mg/L (EC50, 48 hour). In *Utterbackia imbecillis*, the EC50 of glyphosate during 24 hour exposure was reported at 13.5 mg/L (Conners and Black, 2004).

In addition to these herbicides, the observed health effect on mussel from Nong Bua reservoir may be due to direct or synergistic effect of xenobiotic residues in this agricultural area. It is well known that organochlorine pesticides (OCPs) were widely used in most, if not all, of the agricultural area in Thailand. Although OCPs had been banned in Thailand for many years, their residues are still persisted in the environment (Thirakhupt et al., 2006). Using *Uniandra contradens* as a sentinel to OCP contamination, Chayathorn et al. (2011) reported that OCP residues were found in sediment and tissue as well as in accordance with increased level of detoxifying enzyme of mussels living in agricultural area of Central Thailand. .

Table 4.2: Pearson's correlation coefficients correlating herbicide residue and condition factor in the freshwater mussel *Uniandra contradens* in agricultural area of Nan province during July 2010 to June 2011.

	Atrazine	Glyphosate	Paraquat	Condition factor		Alkali labile phosphate
				Male	Female	
Atrazine		0.243 0.26 (N=23)	0.360 0.08 (N=24)	-0.662 0.02 (N=12)	-0.627 0.03 (N=12)	-0.546 0.45 (N=4)
Glyphosate			0.150 0.49 (N=23)	-0.451 0.14 (N=12)	-0.775 0.01 (N=11)	-0.606 0.39 (N=4)
Paraquat				-0.320 0.31 (N=12)	-0.009 0.98 (N=12)	-0.765 0.24 (N=4)

Remark:

- Shaded cells indicate significant correlation ($p<0.05$).
- Values are show in column order by correlation coefficient (*r*), p value (*p*) and number of samples (N) respectively.

Table 4.3: Spearman rank's correlation coefficients correlating herbicide residue and female reproductive activities in the freshwater mussel *Uniandra contradens* in agricultural area of Nan province during July 2010 to June 2011.

Herbicide type	Spawning activities by gill morphology
Atrazine	-0.411 0.17 (N=12)
Glyphosate	-0.840 0.00 (N=11)
Paraquat	-0.209 0.50 (N=12)

Remark:

- Shaded cells indicate significant correlation ($p<0.05$).
- Values are show in column order by correlation coefficient (r), p value (p) and number of samples (N) respectively.

4. Conclusion

In order to provide baseline information and insight into a potential window of susceptibility to xenobiotic contamination, reproductive activities of *U. contradens* living an agricultural catchment were examined. This study revealed the seasonal reproductive activities of *U. contradens*. The presence of atrazine and glyphosate showed a strong negative correlation with a biomarker of health of the mussel. Furthermore, it clearly indicated that glyphosate has a negative correlation with spawning activities of the mussel. It is thus possible that this low level of contamination could pose serious threat to reproductive and developmental activities of the freshwater mussels. The data from this study could be used as an early warning of the effects of herbicide contamination on freshwater animals and maybe used as a potential link to predict the risk on reproductive health of other organisms living in this area.

Chapter V

Site-related Difference in Herbicide Contamination and Associated Biomarkers in the Freshwater Mussel *Uniandra contradens* in Agricultural Catchments, Nan Province

1. Introduction

Since the mid 1970s, several report have been evaluated the contamination along coastal area using bivalves as a sentinel species. The Mussel Watch program was organized by US EPA and utilized mussels and oysters for monitoring of organic chemical, oil and trace elements (Jernelov, 1996). Because of their availability in sedentary habitat, filter feeder and complete life cycle in the water (Dillon, 2000), the bivalves could be regarded as a good receptor for pollutant in the aquatic ecosystem (Uno et al., 2001; Jacomini et al., 2003; Farris and Van Hassel, 2005). Thus, mussels appear to be appropriate as a sentinel species for monitoring xenobiotic contamination in environment, including an agricultural area.

Examples of research using mussels as sentinel of environmental health hazards include the use of *Anodondites trapesialis* to monitor organochlorine pesticide contamination (Lopes et al., 1992), the use of *Crassostrea virginica* for monitoring pesticide cocktail contaminated in bay tributaries (Lehotay et al., 1998), and the use of freshwater bivalves as biomonitoring species for herbicide contamination (Uno et al., 2001; Jacomini et al., 2003; 2006). In addition, several studies reported on a link between xenobiotic accumulation in mussels and adverse health effects in their organ systems, suggesting the potential use of freshwater mussels as a sentinel species of environmental health hazards from xenobiotic contamination (Sheehan and Power, 1999; Won et al., 2005; Ji et al., 2006).

Nan Province is a fertile area for agricultural activities. Seasonal cultivation in this area involves an intensive utilization of agrochemicals, especially herbicides (atrazine, glyphosate and paraquat). The continuous

application of these herbicides in large amount could lead to environmental contamination and accumulation in aquatic organisms (Uno et al., 2001). In this study, two agricultural areas with different agricultural activities were selected as candidate study sites. At the first site, Nong Bua reservoir at San Subdistrict, Wiang Sa District, Nan Province is an area with high activities of seasonal agriculture and intensive use of herbicide. The reservoir was surrounded by many crop rotations such as corn, cucumber, gourd, rice, sesame and soybean. The reservoir was constructed to be used as a catchment for run-off water from surrounding agricultural patches before flowing to the adjacent Nan River during wet season, and a reservoir for agricultural activities during dry season. At the second site, Nong Luang reservoir at Lai Nan Subdistrict Wiang Sa District, Nan Province is a public pond located near a mango plantation with occasional uses of herbicide. The reservoir was a public catchment used by native villager for many activities. This reservoir is seasonally flooded and affected by Nan River during wet season.

To validate the potential use of freshwater mussel as a sentinel species for herbicide contamination, it is thus important to monitor an extent of contamination between sites and potential health effects to animals living in the aquatic environment. In this study, environmental samples (sediment and water) were determined with chromatographic method. Freshwater mussel *Uniandra contradens*, a common species that widely distribute in aquatic habitats close to agricultural area of Nan Province, was selected as a sentinel species. Bioaccumulation, Condition factor and reproductive activities (vitellogenin, gross morphology of gill and gonadal histology) were studied in order to determine a suitable biomarker of effect and susceptibility to this xenobiotic contamination.

Objective

- To evaluate the potential use of *Uniandra contradens* as sentinel species for herbicide contamination in agricultural catchments at Nan Province

2. Materials and methods

2.1 Study sites

Two study sites were located in Wiang Sa District of Nan Province, Thailand as follows.

1) Nong Bua reservoir (18˚30’35.39” N, 100˚46’4.48” E) at San Subdistrict: In this area, corn and rice are the predominant crop with the other crop such as gourd, cucumber, sesame and soybean can be found throughout the year. Therefore, an intensive use of herbicide was evident. This reservoir was constructed to be used as a catchment for run-off water from surrounding agricultural patches before flowing to the adjacent Nan River during wet season, and a reservoir for agricultural activities during dry season.

2) Nong Luang reservoir (18°34’14.81”N, 100°46’34.82”E) at Lai Nan Subdistrict: This is a public reservoir near a mango farm and native village. Use of herbicide was occasionally found in this site since it is mainly conserved for common activities for neighborhoods. However, during the wet season, this reservoir could be affected by runoff from Nan River.

Several aquatic animals are inhabited in these reservoirs including a freshwater mussel *Uniandra contradens*, a sentinel species in this study.

2.2Environmental sample collection

Environmental samples from Nong Bua and Nong Luang reservoir were collect in July-2010. The composited sediment sample (1 kg) and water sample (1 L) were collected and stored in a plastic box and a high density polyethylene bottle, respectively. Every container was washed with laboratory detergent, rinsed with acetone and air dried at room temperature before each use. These containers were wrapped with aluminum foil to avoid sunlight and stored in refrigerator at 4°C until further analysis.

2.3 Mussel collection

The mussels were collected from Nong Bua and Nong Luang reservoir in July-2010. Mussel samples were transported to a laboratory at Chulalongkorn University Forest and Research Station at Nan Province. Morphological data including wet body weight, shell length and shell width were measured and recorded. The mussels were euthanized in ice slurry and dissected to separate the soft part from the shell. The soft part of mussel collected in July-2010 was kept frozen at -20 °C and used for herbicide analysis.

The frozen mussel tissue was freeze-dried (FreeZone 7753501) until complete dryness. Three mussels were combined as a composite sample, and three composite samples per sex were analyzed. The composited mussels were grinded with mortar and pestle before stored in desiccators with silica gel for humidity control until further analysis.

2.4 Extractions and determinations for herbicide contamination in the environmental sample

Herbicide residues in sediment and water were analyzed 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. Sediment and water sample were subjected to extraction process according to an in-house method of the company. Overview of extraction and analytical methods for these herbicide residue analyses are listed in Chapter III and briefly described below.

Atrazine

Both sediment and water samples were processed by liquid-liquid extraction. Sediment was extracted with acetronitrile after pretreated with sodium chloride. Water samples were prepared by extraction with dichloromethane. The residue of atrazine separation was determined by gas

chromatography-mass spectrometry (GC-MS; Agilent Technologies 6890 N) using Mass Selective Detector (MSD; Selected ion monitoring mode) and a DB-5ms capillary column (0.25 mm internal diameter x 30 m length and 0.25 µm film thickness). The limit of detection (LOD) for atrazine residue was 0.01 µg/mL in water and 0.01 µg/g in sediment.

Glyphosate

Sediment and water samples were treated with water in acid condition and cleaned up by solid phase extraction (SPE) using Chelex 100 column and AG1-X8 column. The residue of glyphosate separation was performed in high performance liquids chromatography (HPLC; Agilent 1100, Germany) 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 3µm film thickness). The limit of detection (LOD) for glyphosate residue was 0.005 µg/mL in water and 0.01 µg/g in sediment samples.

Paraquat

Sediment samples were extracted with water in acid condition and cleanup by SPE silica. Water samples were treated with hydrochloric acid and methanol after cleaned up through solid phase extraction. The residue of paraquat was quantified by high performance liquid chromatography (HPLC; Agilent Technologies 1100, Germany) using diode-array detector with a broad spectrum 190-400 nm and silica hydrophilic interaction chromatography column (silica HILIC; Atlantis, 2.1 mm internal diameter x 150 mm length and 3µm film thickness). The limit of detection (LOD) for paraquat residue was 0.01 µg/mL in water and 0.01 µg/g in sediment.

2.5 Extractions and determinations for herbicide contamination in the mussel sample

Quantification of herbicide residue in the mussel was examined with enzyme-linked immunosorbent assay (ELISA). This test is based on an immunological recognition of herbicide residue by a specific antibody. Therefore, specificity and sensitivity of the assay are suitable for the quantification even in a low concentration range. The atrazine and glyphosate kits were purchased from Abraxis LCC and paraquat kit was purchased from Abnova. These kits are originally designed to check the contaminations of herbicides residue in the water sample. In order to use these ELISA kit, the mussel sample was thus subjected to extraction and reconstitution in water before analysis. The overview of extraction methods and assay are listed in Chapter IV and briefly described below.

Atrazine

Mussel samples were extracted with dichloromethane by liquid-liquid extraction. The ELISA was performed to determine atrazine residue in 96-well microtiter plate. The competitive ELISA system was selected using rabbit anti-triazine antibody as a primary antibody. The second antibody was triazine-horseradish peroxidase conjugate. The substrate was hydrogen peroxide and 3,3',5,5'–tetramethylbenzidine. The detection limit for atrazine residue in mussel tissue was 0.53 ng/g dry weights and the recovery of atrazine extraction was 87.41 %.

Glyphosate

Mussel tissue processing was a liquid-liquid extraction with chloroform and water in acid condition. Derivertization of glyphosate was performed before determination with ELISA kit. Double binding and competition mode was selected to determine in a 96-well microtiter plate coated goat anti-rabbit antibody in each well. The primary antibody was rabbit anti-glyphosate

antibody and glyphosate-horseradish peroxidase conjugate was a secondary antibody. The substrate was hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine. The detection limit of the ELISA kit for glyphosate residue analysis was 0.21 ng/g dry weights. The percent recovery of glyphosate extraction was 37.78 %.

Paraquat

Mussel tissue was first extracted with water in acid condition and cleaned up by liquid-liquid extraction using hexane. ELISA was performed in a 96-well microtiter plate coated the primary antibody (rabbit anti-paraquat antibody). The paraquat-horseradish peroxidase conjugate was used as a secondary antibody. The substrate was hydrogen peroxide and 3 ,3' ,5 , 5'-tetramethylbenzidine. The detection limit of the ELISA for paraquat residue is 9.89 ng/g. The percent recovery of paraquat extraction was 51.17 %.

2.6 Condition factor of freshwater mussel

After morphological and gravimetric measures of the mussel, their whole bodies were removed from the shell. The data of shell length and whole body weight without shell of each mussel was used to calculate a condition factor of each mussel as follows (Gagné et al., 2006)

$$\text{Condition factor (C.F.)} = \frac{\text{Whole body weight without shell (g)}}{\text{Shell length (cm)}}$$

2.7 Spawning activity of female freshwater mussel

Since gill of female mussel is a major deposition site of eggs and developing glochidia, spawning activity of each female mussel was identified based on wet mount slide preparation of gill tissue fluid. After the soft tissue part was separated from the shell, gill morphology was observed before

puncture with dissecting needle. The score was based on visual grading of gill morphology in relation to presence of egg deposition in the gill as followed.

Score 0 = normal gill with no sign of egg deposition

Score 1 = normal gill with egg deposition

Score 2 = partly enlarged/swelled gill with egg deposition

Score 3 = completely enlarged/swelled gill with egg deposition

2.8 Histology

Whole bodies of the mussel samples were fixed in Davidson's solution (Dietrich and Krieger, 2009) immediately for 48 hours and then preserved in 70% ethanol until further analysis. The samples were cut (x-section) into smaller pieces with 2-3 mm thickness and processed through standard paraffin method (Presnell and Schreibman, 1997). Tissue slides were stained with Delafied's haematoxylin and eosin prior to examination under a light microscope.

2.9 Vitellogenin analysis

Vitellogenin in the mussel were analyzed by alkali-labile phosphate (Gagné and Blaise, 2000). Gonad tissue was subjected to extraction process, normalization of the total protein concentration and determination of vitellogenin level. Overview of vitellogenin determination in the female mussels are listed in Chapter IV and briefly described below.

Tissue extraction for vitellogenin analysis

Gonad tissue was extracted with extraction buffer (0.05 M of Tris-HCl, pH 7.5 containing 0.2 M NaCl and protease inhibitors cocktail (ethylenediaminetetraacetic acid [EDTA], 4-[2-aminoethyl] benzenesulfonyl fluoride [AEBSF], bestatin, E-46, leupeptin and aprotinin)). The homogenated was centrifuged at 12,000 xg with 4°C for 30 minute before stored at -20 °C until further analysis.

Normalization protein concentration of the samples

Total protein concentration in the gonad extract was determined by Bradford assay (Bradford 1976) modified for a 96-well microtiter plate (Redinbaugh and Campbell, 1985). The standard protein was a bovine serum albumin (BSA) (Sigma, St. Louis, Mo). After total protein determination, the protein concentration in each sample was adjusted to 1 mg/mL with the extraction buffer.

Determination of vitellogenin by alkali-labile phosphate

The examination of alkali-labile phosphate was modified from Gagné and Blaise (2000) in order to use with a 96-well microtiter plate. Free phosphate level was performing with detection kit (malachite green kit, R&D System). The determination was described in Chapter IV and briefly illustrated below.

The sample was extracted with *t*-butyl methyl ether and centrifuged at 10,000 xg for 5 minutes, 4°C. The ether phase was separated and transfer into a clean tube. The free phosphate was isolated from phospholipids in ether phase to aqueous phase by liquid-liquid extraction. The samples were adjusted pH to 7.0 before examined with malachite green kit. The absorbance was measured at 620 nm by using a microplate reader (Multiskan EX).

2.8 Statistical analysis

All parameters were tested for normal distribution and homogeneity of variance. Comparison between sexes and sites were performed by Student's *t*-test. Correlation between herbicide contamination and condition factor were tested with Pearson product moment correlation.

3. Results and discussion

The findings of herbicide contamination in the environmental samples (sediment and water) are presented in Table 5.1-5.3. No residue of herbicides was found in either Nong Bua or Nong Luang reservoirs in July-2010 although the large amount of runoff was expected during this late wet season. An absence amount of herbicide in this month could be due to the relatively low sensitivity of instruments including GC-MS (atrazine LOD: 0.01 µg/mL in water and 0.01 µg/g in sediment), HPLC with post derivertizer (glyphosate LOD: 0.005 µg/mL in water and 0.01 µg/g in sediment) and HPLC-DAD (paraquat LOD: 0.01 µg/mL in water and 0.01 µg/g in sediment). In addition, these herbicides could be degraded by photolysis, hydrolysis or biodegradation with microorganism (ATSDR, 2003; Giesy et al., 2000; WHO, 1994).

Table 5.1 Levels of atrazine residue screening in environmental samples on July 2010 collected from an agricultural areas in Nan Province, Thailand

Sites	Composited samples	
	Sediment	Water
Nong Bua reservoir	< 0.01 µg/g N=1	< 0.01 µg/mL N=1
Nong Luang reservoir	< 0.01 µg/g N=1	< 0.01 µg/mL N=1

Remark:

- Limit of detection (LOD; 0.01 µg/g for sediment and 0.01 µg/mL for water)
- N = number of composited sediment sample (1 kg) and water sample (1 L)

Table 5.2 Levels of glyphosate residue screening in environmental samples on July 2010 collected from an agricultural areas in Nan Province , Thailand

Sites	Composited samples	
	Sediment	Water
Nong Bua reservoir	< 0.01 µg/g N=1	< 0.005 µg/mL N=1
Nong Luang reservoir	< 0.01 µg/g N=1	< 0.005 µg/mL N=1

Remark:

- Limit of detection (LOD; 0.01 µg/g for sediment and 0.005 µg/mL for water)
- N = number of composited sediment sample (1 kg) and water sample (1 L)

Table 5.3 Levels of paraquat residue screening in environmental samples on July 2010 collected from an agricultural areas in Nan Province, Thailand

Sites	Composited samples	
	Sediment	Water
Nong Bua reservoir	< 0.01 µg/g N=1	< 0.01 µg/mL N=1
Nong Luang reservoir	< 0.01 µg/g N=1	< 0.01 µg/mL N=1

Remark:

- Limit of detection (LOD; 0.01 µg/g for sediment and 0.01 µg/mL for water)
- N = number of composited sediment sample (1 kg) and water sample (1 L)

The results of atrazine concentration in *Uniandra contradens* are shown in Figure 5.4. Atrazine residue was determined by ELISA in order to yield a more sensitive assay (LOD: 0.53 ng/g dry weight). It was found that atrazine residue can be found in every mussel samples from both study sites in this period (July 2010). The amount of atrazine residue in the mussel from Nong Luang reservoir ranged from 6.54-11.76 ng/g dry weight in male and 12.18-88.86 ng/g dry weight in female, while atrazine residue of mussel from Nong Bua reservoir ranged from 4.30-11.13 ng/g dry weight in male and 4.16-14.59 ng/g dry weight in female. There was no sex-related difference in atrazine concentration at both study site ($p>0.05$; *t*-test). Although no significant site-difference ($p>0.05$; *t*-test) in atrazine residue was found, the atrazine residue in mussel from Nong Luang seemed to be higher than those of Nong Bua reservoir in both male and female.

The findings of glyphosate concentrations in *U. contradens* are presented in Figure 5.5. Glyphosate residue was determined by ELISA in order to yield a more sensitive assay (LOD: 0.21 ng/g dry weight). The glyphosate residue can be found in every sample examined. The bioaccumulation of glyphosate in the mussels were in the ranges of 6.56-7.45 ng/g dry weight in male and 6.22-7.15 ng/g dry weight in female mussels from Nong Luang reservoir, while the range of 7.80-9.28 ng/g dry weight in male and 10.06-11.13 ng/g dry weight in female mussels were found in the mussel from Nong Bua reservoir. There was a significant sex-related difference ($p<0.05$; *t*-test) in glyphosate concentration in Nong Bua mussels. In addition, both male and female mussel from Nong Bua reservoir showed a significantly higher level of glyphosate ($p<0.05$; *t*-test) than mussel from Nong Luang reservoir.

The results of paraquat concentration in *U. contradens* are shown in Figure 5.6. Determination of paraquat concentration was performed by ELISA in order to yield a sensitive detection assay (LOD: 9.89 ng/g dry weight). It was found that paraquat residue can be found in every sample examined. The paraquat residue in the mussels of Nong Luang reservoir were in the ranges of

28.41-73.01 ng/g dry weight in male and 27.56-31.87 ng/g dry weight in female, whereas the mussels from Nong Bue reservoir showed the ranges of 40.14-55.97 ng/g dry weight in male and 34.76-44.21 ng/g dry weight in female mussels. There was no significant sex-related difference in paraquat concentration in their tissue at both sites ($p>0.05$; *t*-test). The findings also revealed no significant site-related difference between Nong Bua and Nong Luang reservoirs ($p>0.05$; *t*-test), but the levels in Nong Bua mussels seemed to be a bit higher than those of Nong Luang mussels in both sexes.

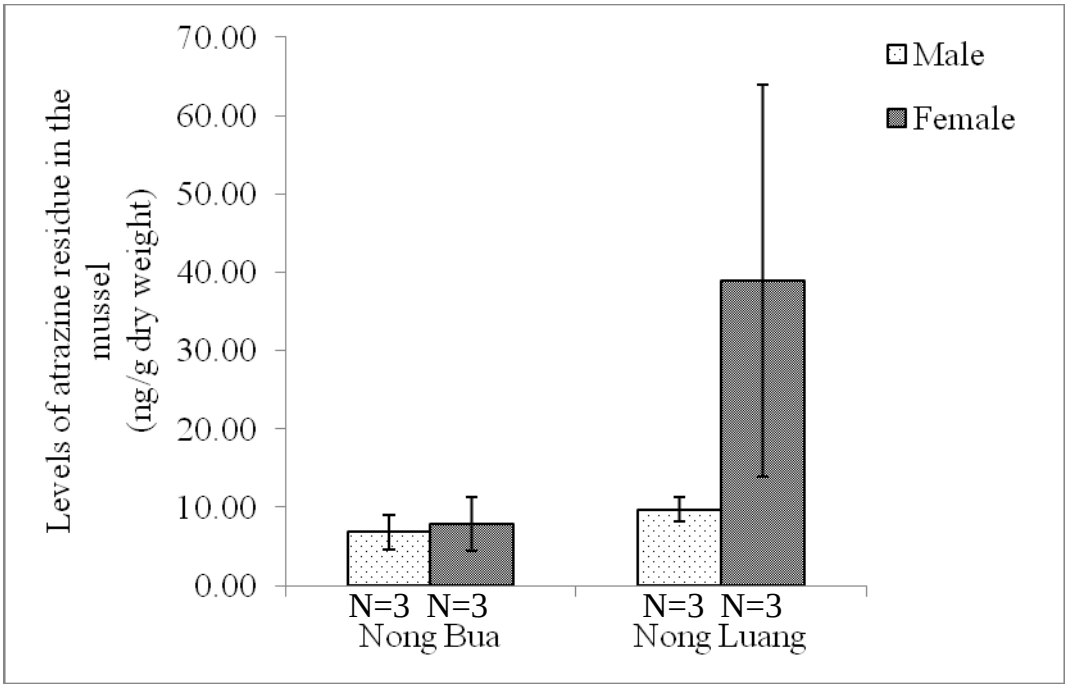

Figure 5.1: Mean ± S.E.M. of atrazine residue in *U. contradens* collected from Nong Bua reservoir and Nong Luang reservoir, Nan Province, Thailand. There was no significant difference between sexes and sites ($p>0.05$, *t*-test). N = 3 composited samples per sex in each site.

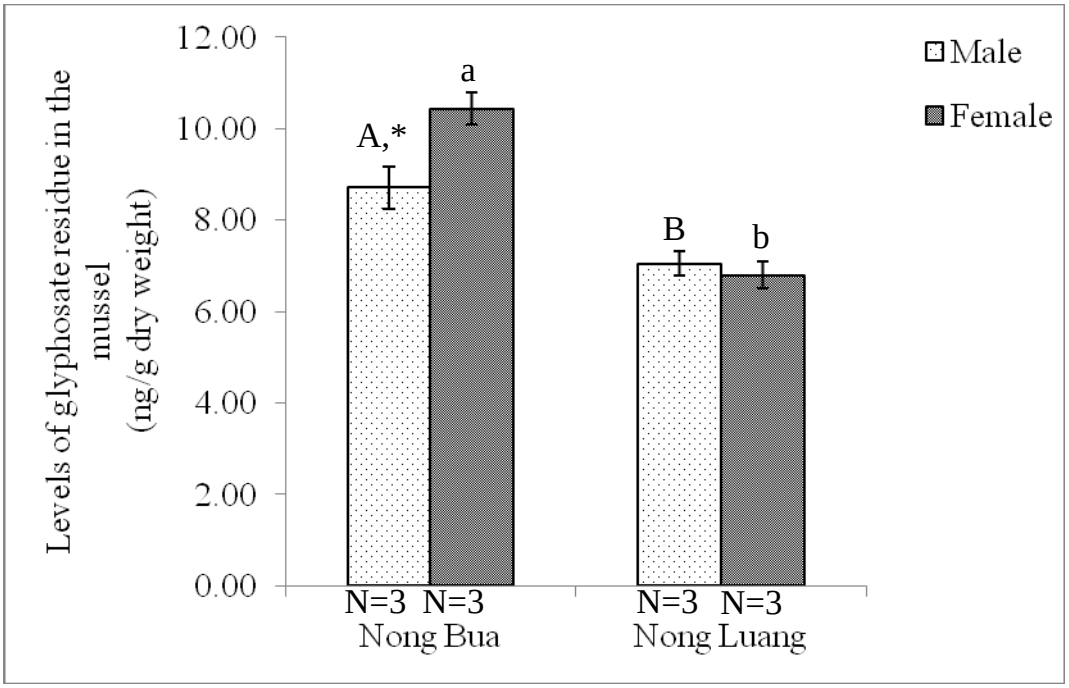

Figure 5.2: Mean ± S.E.M. of glyphosate residue in *U. contradens* collected from Nong Bua reservoir and Nong Luang reservoir, Nan Province, Thailand. Significant difference ($p<0.05$; *t*-test) between sex is indicated by an asterisk and significant differences between sites are indicated by differences in superscript capital letter in male and superscript normal letter in female. N = 3 composited samples per sex in each site.

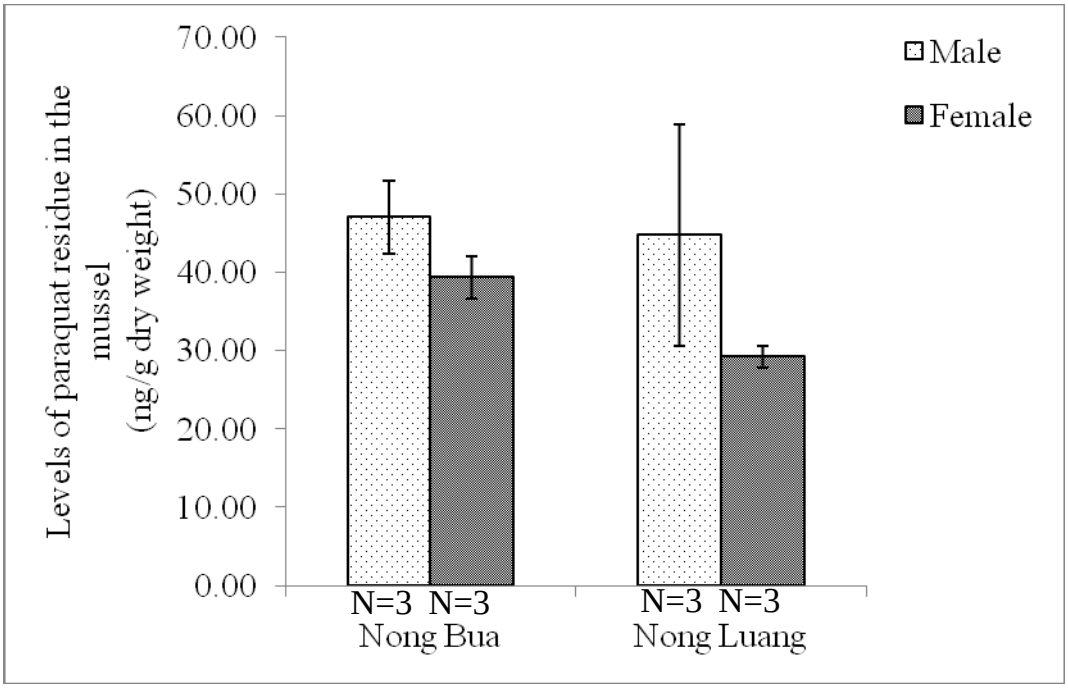

Figure 5.3: Mean ± S.E.M. of paraquat residue in *U. contradens* collected from Nong Bua reservoir and Nong Luang reservoir, Nan Province, Thailand. There was no significant difference between sexes and sites ($p>0.05$, *t*-test). N = 3 composited samples per sex in each site.

To monitor change in health status and growth of mussels in this area, gravimetric and morphometric techniques were performed. The average condition factors of *U. contradens* from Nong Luang and Nong Bua reservoir were compared in July 2010 (Figure 5.7). The mussels from Nong Luang reservoir showed a condition factor in the ranges of 0.32-0.94 in male and 0.33-0.86 in female, while the mussels from Nong Bua reservoir showed the ranges of condition factor from 0.24-1.02 in male and 0.33-0.62 in female. There was no sex-related difference in the condition factor at both sites ($p>0.05$; *t*-test). Mean comparison of condition factor showed that the mussels from Nong Bua reservoir has a significantly lower condition factor ($p<0.05$; *t*-test) than those of Nong Luang mussels in both male (0.449±0.028 vs. 0.649±0.038) and female (0.462±0.030 vs. 0.633±0.0315). Examinations of condition factor in *U. contradens* revealed that the relative weight as an indicative of overall health of the mussel was different between site. These findings could be due to effects of herbicide contamination or just an unequally growth between sites during this study period. Therefore, it is still too early to conclude on this different until further analysis has been done in round year basis.

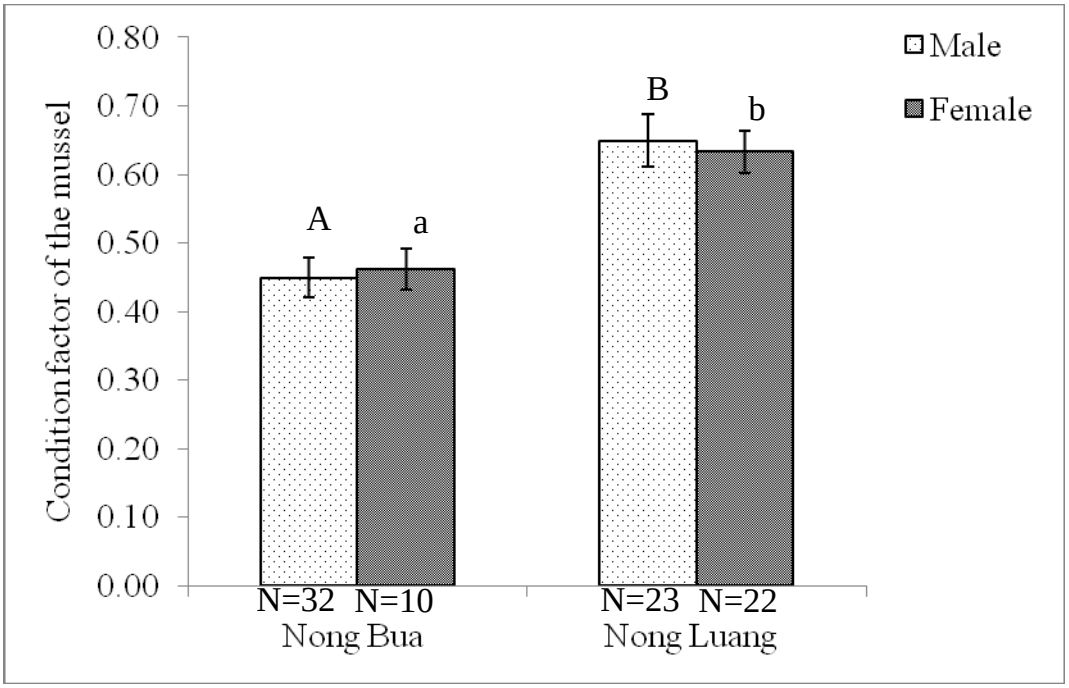

Figure 5.4: Mean ± S.E.M. of condition factor of *Uniandra contradens* collected from Nong Bua reservoir and Nong Luang reservoir, Nan Province in July 2010.

Significant differences between sites ($p<0.05$; *t*-test) are indicated by differences in superscript capital letter in male and superscript normal letter in female.

To obtain the information of reproductive activities, vitellogenin levels were determined by analysis for alkali labile phosphate assay. The average of alkali-labile phosphate of female *U. contradens* from Nong Luang and Nong Bua reservoir were compared in July 2010 (Figure 5.8). The female mussels from Nong Luang reservoir showed ranges of phosphate concentration from 0.04-0.05 µg phosphate/mg protein, whereas the levels in female mussels from Nong Bua reservois ranged from 0.02-0.08 µg phosphate/mg protein. There was no significant site-related difference ($p>0.05$; *t*-test) in this marker. However, of the level of alkali-labile phosphate of mussel from Nong Bua reservoir seemed to relatively higher than those of Nong Luang mussels.

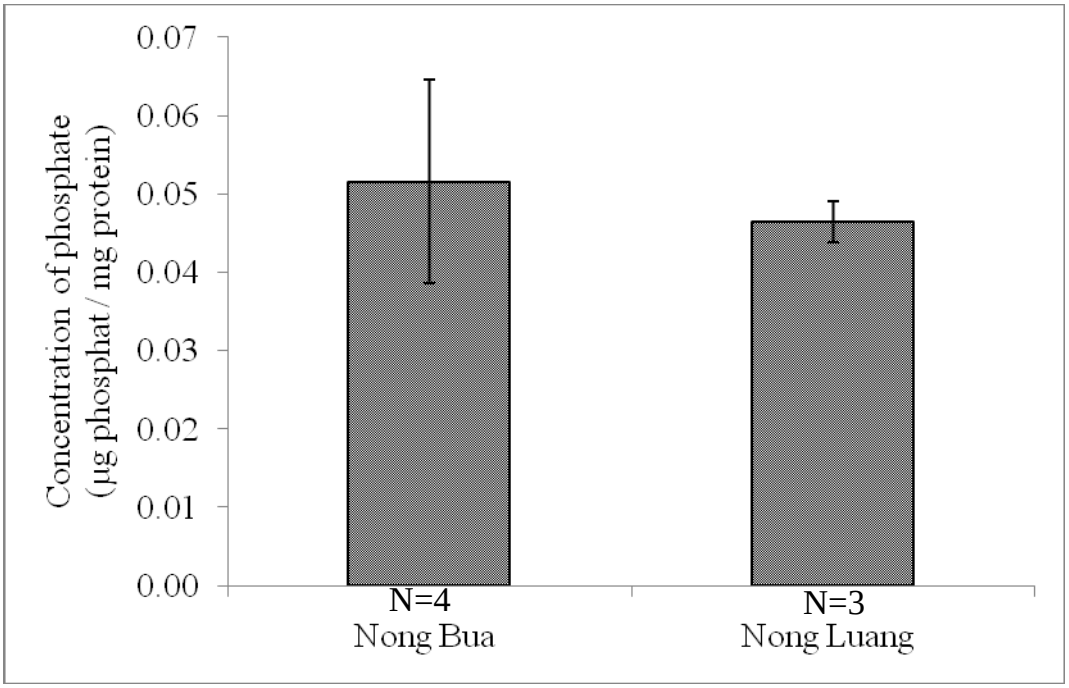

Figure 5.5: Mean ± S.E.M. of phosphate concentration in female mussel *U. contradens* collected from Nong Bua reservoir and Nong Luang reservoir, Nan Province, Thailand.

Reproductive activities of *U. contradens* were determined by gross- and microanatomy of gonad in order to provide baseline information and insight into a potential window of susceptibility to xenobiotic contamination. Wet mounted preparation of gonad tissue fluid showed that most of the male mussel had motile sperms in the testis (Table 5.4) in July-2010 at both Nong Luang and Nong Bua reservoirs. Histological analysis of testicular sections showed germ cells in different stages of development and confirmed the maturities of male mussel (Chapter IV). In females, different stages of germ cells were found in the acinus wall of the ovary (Chapter IV) and eggs were present in wet mounted preparation of gonad tissue fluid (Table 5.4) of both study sites. Since gill of the female mussel is a major deposition site of eggs and developing glochidia, spawning activity of female could be determined by observing deposition of eggs at the gill of female mussels. Spawning activity of individual female *U. contradens* was scored based on visual grading of gill morphology in relation to presence of egg deposition in the gill (chapter IV). The result in Table 5.4 showed that the female mussel of Nong Luang and Nong Bua reservoir had no spawning activity at this period.

Table 5.4: The reproductive activities of *U. contradens* collected in July 2010 from Nong Bua reservoir and Nong Luang reservoir, Nan Province, Thailand.

	Male	Female	Gill morphology			
	Mussel with motile sperm in the gonad	Mussel with oocyte in the gonad	Score			
			0	1	2	3
Nong Bua	23 (N=23)	22 (N=22)	22 (N=22)	-	-	-
Nong Luang	31 (N=31)	11 (N=11)	11 (N=11)	-	-	-

Remark:

The score of gill morphology as major site of egg deposition.

Score 0 = normal gill with no sign of egg deposition

Score 1 = normal gill with egg deposition

Score 2 = partly enlarged/swelled gill with egg deposition

Score 3 = completely enlarged/swelled gill with egg deposition

To obtain a candidate biomarker for herbicide contamination, analysis for association of herbicide residue and the potential biomarkers was performed. In this study, a condition factor was selected as a candidate biomarker since it seemed to be less sensitive to seasonal fluctuation. However, Table 5.5 showed that no significant association between herbicide residue and the condition factor was found ($p>0.05$; Pearson's product moment correlation). It is of importance to note that glyphosate (r=-0.036) and paraquat (r=-0.049) did show trend toward negative association with condition factor of the mussels. These results may indicate the potential use of condition factor as a biomarker for herbicide contamination in the future study. The condition factor is an indicative of overall health of the mussel that could be impacted from herbicide contamination even in a short period. It is also of interest to note the negative correlation of atrazine residue with glyphosate residue (r=-0.443) and paraquat residue (r=-0.178) in the mussel tissues. These finding indicated that these two study sites might have a difference agricultural activity resulted in different patterns of herbicide utilization.

Table 5.5: Pearson's correlation coefficients correlating herbicide residue and condition factor in the freshwater mussel *Uniandra contradens* in agricultural area of Nan Province in July 2010

	Glyphosate	Paraquat	Condition factor
Atrazine	-0.443 0.15 (N=12)	-0.178 0.58 (N=12)	0.287 0.37 (N=12)
Glyphosate		0.163 0.61 (N=12)	-0.036 0.91 (N=12)
Paraquat			-0.049 0.88 (N=12)

Remark:

Values in each cell from top to bottom are 1) correlation coefficient (*r*), 2) p value (*p*) and 3) number of samples (N) respectively.

4. Conclusion

In this part of the study, sentinel systems for herbicide were applied at two reservoirs with different agricultural activities. Different patterns of herbicide utilization between Nong Bua and Nong Luang reservoirs were evident and confirmed by the analysis for herbicide tissue residue in the sentinel species, a freshwater mussel. Although detectable levels of herbicide residue was not presented in environmental samples (sediment and water), but the mussel had shown the ability to accumulate herbicide in their tissue. On top of this, the mussel sentinel system could provide both biomarker of exposure (i.e. herbicide tissue residue) as well as biomarker of effect (condition factor) at the same time. For the effects on reproduction (histology of gonad and gill morphology), the results is not yet conclusive due to seasonal variation (Chapter IV) and should be determined in a long period. Therefore, based on this current study, *U. contradens* has shown certain characteristics of a good candidate as a sentinel species for herbicide contamination, and the mussel condition factor should be used as a suitable biomarker for evaluation the effect in a short term.

Chapter VI
General Conclusion and Recommendation

Agricultural areas in Thailand have been widely expanded toward riverine areas of the main river such as Nan River in Nan Province, northern part of Thailand. Seasonal cultivations in these areas lead to widely use of agrochemicals especially herbicides. Field observations showed that a major group of herbicide used in this area was atrazine, glyphosate and paraquat.

Detectable levels of herbicide contaminations can be found in environment matrix, especially atrazine. An amount of atrazine contaminations was relatively high in late dry season (January 2011) when level of atrazine residue was 160 ng/mL (0.16 µg/mL) in water and 230 ng/g (0.23 µg/mL) in sediment. Further analysis of bioaccumulation in the mussel showed that the freshwater mussels *U. contradens* could store these herbicide residues in their tissue. The amount of herbicide residue in the mussel ranged from 1.26 to 88.86 ng/g dry weight of atrazine, 5.34 to 11.90 ng/g dry weight of glyphosate, and 23.30 to 55.97 ng/g dry weight of paraquat. These quantities of herbicide residue can be found in every mussel samples in every study periods.

Levels of atrazine and glyphosate in the mussel tissue were within the standard guideline for human consumption. However, levels of paraquat residue in the mussel were more than the maximum residue limit (5 ng/g wet weight or equivalent to 36.52 ng/g dry weight in this mussel species; CODEX, 2006). Since *U. contradens* is used as food for local people, it is of importance to provide an early warning of the potential safety concern of mussel consumption to the local community in vicinity of this study site.

Health status and growth of mussels in agricultural area, Nan Province, was determined and shown that the condition factors, an indicative of overall health, of both sex were significantly different between months. Further analysis of the condition factor also showed a negative correlation with atrazine

and glyphosate residue in the mussel tissue, indicating potential impact of these herbicides on overall health of the mussel.

Reproductive activities of *U. contradens* possess continuous gametogenesis since all mussels showed sign of matured germ cells in gonads in every month. However, the spawning activity of female mussels was seasonal and showed a peak in early dry season (December 2010). These results could be used as baseline information and provided insight into a potential window of susceptibility to xenobiotic contamination. Further analysis of gill morphology also showed a negative correlation with glyphosate residue in their mussel, indicating potential impact of glyphosate on the offspring of the mussel.

In this study, detectable levels of atrazine residue were found in Nong Bua reservoir in late dry season (0.16 µg/mL in water and 0.23 µg/g in sediment). This period was also found to be a peak spawning activity of the freshwater mussel species inhabited in this reservoir. It is thus possible that this low level of contamination could pose serious threat to reproductive and developmental activities of the freshwater mussels.

Vitellogenin, a precursor of yolk protein synthesis, was examined in mussel gonad extract. Levels of vitellogenin in *U. contradens* showed significant seasonal variation and peak in early dry season (December 2010), indicating a seasonal reproductive activity of *U. contradens* at molecular levels.

Comparison between sites was study at two reservoirs with different agricultural activities: Nong Bua reservoir as a representative of intensive agricultural area and Nong Luang reservoir as a representative of sporadic agricultural area. Although detectable levels of herbicide residue was not found in environmental samples (sediment and water) of both sites, detectable levels of these three herbicides were found in the mussels from both sites. Detectable levels of atrazine were relatively higher in mussel from Nong Luang reservoir, whereas levels of glyphosate and paraquat were relatively higher in the mussel from Nong Bua reservoir. Further analysis of herbicide residue in

the mussel showed trends of negative correlation between atrazine residue and glyphosate residue (r=-0.443) and paraquat residue (r=-0.178) in the mussel tissues, indicating different pattern of herbicide utilization between these two areas. Condition factor also showed trend of the negative association with glyphosate (r=-0.036) and paraquat (r=-0.049). These results may be implied the potential use of condition factor as a biomarker for herbicide contamination in the future.

The data from this study described the evidence of herbicide contamination in edible freshwater mussel as well as the potential health effects of herbicides on mussel health and reproductive activities. This could be used as an early warning of the effects of herbicide contamination on freshwater animals and maybe used as a potential link to predict the risk on reproductive health of other organisms living in this area including human.

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APPENDIX

Research Dissemination

Research Article

Thitiphuree, T., Kitana, J., Varanusupakul, P. and Kitana, N. 2013. Atrazine contamination and potential health effects on freshwater mussel *Uniandra contradens* living in agricultural catchment at Nan Province. *EnvironmentAsia* 6(1): xx-xx.

Oral Presentation

Thitiphuree, T., Kitana, J., Varanusupakul, P. and Kitana, N. 2011. Atrazine residue and potential effects on reproductive activities of freshwater mussels *Uniandra contradens* in agricultural area of Nan Province, Thailand. *Abstract: the 5th International Congress of Chemistry and Environment*, May 27-29, 2011, Port Dickson, Malaysia. p. 49.

Poster Presentation

Thitiphuree, T., Kitana, J., Varanusupakul, P. and Kitana, N. 2011. Atrazine residue and glutathione s-transferase response of freshwater mussel *Uniandra contradens* living in agricultural catchments, Northern Thailand. Abstract*: the Society of Environmental Toxicology and Chemistry North America 32nd Annual Meeting*, November 13-17, 2011, Boston, MA, USA. p. 366.

Thitiphuree, T., Kitana, J., Varanusupakul, P. and Kitana, N. 2012. Association between herbicide contamination and reproductive effects in freshwater mussl *Uniandra contradens* in agricultural areas, Nan Province. *Abstract, the 1st National Symposium on Biodiversity Management*, February 2-3, 2012, Nonthaburi, Thailand. p. 91.

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Date : September 12, 2012

Dr. Noppadon Kitana, Ph.D.
Department of Biology,
Faculty of Science,
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Dear Dr. Noppadon Kitana,

Thank you very much for submitting the manuscript entitled "Atrazine Contamination and Potential Health Effects on Freshwater Mussel *Uniandra contradens* Living in Agricultural Catchment at Nan Province, Thailand" by Tongchai Thitiphuree, Jirarach Kitana, Pakorn Varanusupakul and Noppadon Kitana for consideration for publication in ***EnvironmentAsia***.

I am pleased to inform you that the manuscript has been accepted to be published in ***EnvironmentAsia*** Vol. 6 No.1 (January 2013) by two independent referees.

Thank you again for your interest in contributing to our journal.

Your sincerely,

Associate Professor Dr. Voravit Cheevaporn
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Atrazine Contamination and Potential Health Effects on Freshwater Mussel *Uniandra contradens* Living in Agricultural Catchment at Nan Province, Thailand

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Abstract

Seasonal cultivation in northern part of Thailand leads to widely uses of agrochemicals especially atrazine herbicide. To examine whether an intensive use of atrazine could lead to contamination in aquatic environment, sediment and water were collected from an agricultural catchment in Nan Province during 2010-2011 and subjected to analysis for atrazine by GC-MS. The results showed that detectable levels of atrazine were found in water (0.16 μg/mL) and sediment (0.23 μg/g) of the catchment. To monitor potential effects of atrazine on aquatic animals, a freshwater mussel *Uniandra contradens* was used as a sentinel species for bioaccumulation and potential health effects. Mussels collected from the catchment during 2010-2011 were subjected to analysis for atrazine residue in tissue and condition factor based on body weight and shell length. The results showed that detectable levels of atrazine were found in mussel tissue with the highest level (8.40 ± 2.06 ng/g) in late wet season when runoff from heavy rain was evidenced. Condition factor, an indicative of overall health, showed a significant negative correlation with atrazine residue in the tissue. This information could be used as part of

the monitoring program for herbicide contamination and potential health effects in agricultural environment.

Keywords: condition factor; freshwater bivalve; GC-MS; herbicide; sentinel species

1. Introduction

Nan, a province in northern part of Thailand, is known as an origin of several rivers and tributaries such as Nan River as well as a fertile area for agricultural activities. Seasonal cultivation in this area involves an intensive utilization of agrochemicals, especially herbicide. The continuous application of herbicide in large amount could lead to environmental contamination and accumulation in aquatic organisms (Uno *et al*., 2001). In some situations, range of contamination could extend beyond aquatic habitats to bay tributaries (Lehotay *et al*., 1998) and marine environment (Haynes *et al*., 2003). It is thus important to monitor an extent of contamination and potential health effects to animals living in the aquatic environment.

Mussel has been regarded as one of the suitable sentinel species since it is an invertebrate that greatly depend on quality of aquatic environment as an animal that has complete life cycle in water, as a filter feeder on plankton and organic matters in water and as a bottom dweller in sediment (Dillon, 2000). This life history makes the mussel susceptible to xenobiotic exposure and accumulation of chemical residues into their body (Uno *et al*., 2001; Jacomini *et al*., 2003). In addition, several studies reported on link between xenobiotic accumulation in mussels and adverse health effects in their organ systems, suggesting the potential use of freshwater mussels as a sentinel species of environmental health hazards from xenobiotic contamination (Sheehan and Power, 1999; Won *et al*., 2005; Ji *et al*., 2006; Boonlue *et al*., 2011).

Since atrazine is regarded as one of the most imported herbicide of Thailand (Panuwet et al., 2012) and its use was evidenced in the field, analysis for atrazine residue in water and sediment of agricultural catchment was performed to monitor the extent of its environmental

contamination in this study. In addition, *Uniandra contradens*, a common freshwater mussel widely distributed in rivers and reservoirs of Thailand as well as Southeast Asia (Brandt, 1974), was selected as a sentinel species to monitor atrazine contamination and potential health effects to aquatic animals.

2. Materials and Methods

2.1 Study site: Study site was located in Wiang Sa District of Nan Province, Thailand. Seasonal cultivations of corn, cucumber gourd, rice, sesame and soybean can be found throughout the area. Among patches of agricultural area, Nong Bua reservoir (18°30'35.39" N, 100°46'4.48" E) was constructed to be used as a catchment for run-off water from surrounding agricultural patches before flowing to the adjacent Nan River during wet season, and a reservoir for agricultural activities during dry season. Several aquatic animals are inhabited in this reservoir including a freshwater mussel *Uniandra contradens*, a sentinel species in this study.

2.2 Mussel collection: The mussels were collected monthly from Nong Bua reservoir during July 2010 to June 2011. Mussel samples were transported to a laboratory at Chulalongkorn University Forest and Research Station at Nan Province. Morphological data including wet body weight, shell length and shell width were measured and recorded. Mussels were euthanized in ice slurry and subjected to sex identification. Briefly, mussel gonadal fluid was drawn onto a glass slide and examined under light microscope for a presence of motile sperm in male or mature oocyte in female. Mussels were dissected to separate soft part from the shell, and the soft part of mussel collected in July-2010, October-2010, January-2011 and April-2011 was kept frozen at -20°C and used for atrazine residue analysis.

2.3 Atrazine residue analysis in environmental sample: Environmental samples (sediment and water) from Nong Bua reservoir was collected every three months in July-2010, October-

2010, January-2011 and April-2011. Composited samples of sediment (1 kg) and water (1 L) samples were stored in plastic box and high density polyethylene bottle, respectively. These containers were wrapped with aluminum foil to avoid sunlight and stored at 4°C until further analysis. Herbicide residues in sediment and water were analyzed 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. Composited samples of sediment and water were subjected to extraction process according to an in-house method of the company. Briefly, wet sediment sample treated with sodium chloride (NaCl) was extracted with acetronitrile (CH_3CN) before addition of anhydrous magnesium sulfate ($MgSO_4$). The extracted sample was centrifuge at 3,000 rpm (Heraeus®, Megafuge® 1.0 R) at 5 °C for 5 minutes, and supernatant was transferred to evaporation under stream of nitrogen gas. The content was adjusted to volume by ethyl acetate ($CH_3COOCH_2CH_3$) and subjected to treatment with anhydrous $MgSO_4$ and primary secondary amine (PSA). After precipitation, the upper part of solution was filtered through 0.22 µm syringe filter before further analysis. Water sample was pre-treated with NaCl and subjected to extraction with dichloromethane (CH_2Cl_2). After the extracted sample was dried up in an evaporator, the sample was adjusted to volume by ethyl acetate before further analysis.

Residue of atrazine in extracted sample was quantified by gas chromatography-mass spectrometry (GC-MS; Agilent Technologies 6890 N) using Mass Selective Detector (selected ion monitoring mode) and a DB-5ms capillary column (0.25 mm internal diameter, 30 m length and 0.25 µm film thickness). Two microliters of sample was injected into the GC-MS with 2.5 min solvent delay. The injector was initially set at 210 °C and 10.69 Psi. The oven temperature was initially set at 80 °C for 2 min, and programmed to increase to 280 °C at the rate of 14 °C/min and held for 10 min. The total run time was calculated to be 31 min. Helium was used as a carrier gas with flow rate of 1.1 mL/min. The limit of detection (LOD) for atrazine residue was 0.01 µg/mL in water and 0.01 µg/g in sediment.

2.4 Atrazine residue analysis in the mussel: The frozen mussel tissue was freeze-dried (FreeZone 7753501) until complete dryness. Three mussels were combined as a composite sample, and three composite samples per sex were analyzed in each month. The tissue was extracted according to a modified method of Jacomini *et al.* (2003) and analyzed by enzyme-linked immunosorbent assay (ELISA). Briefly, 100 mg of lyophilized tissue were mixed with 1 mL of ultrapure water before extraction with 4 mL of dichloromethane adjusted to slightly base with 1.5 M NaOH. After centrifugation at 1,800 xg for 5 minutes, 3 mL of organic phase was transferred to a clean tube and dried with evaporator (TurboVap® II) under stream of nitrogen gas. The residues were reconstituted with 100 µL of methanol and 900 µL of ultrapure water. The samples were stored at -20°C until analysis with ELISA.

ELISA kit for determination of atrazine was obtained commercially from Abraxis LLC. Assay was performed according the company's protocol. Briefly, 25 µL of assay buffer was added to individual well of a microtiter plate coated with rabbit anti-triazine antibody. Then, 25 µL of samples and standard atrazine solutions (0, 0.05, 0.1, 0.25, 1.0, 2.5 and 5.0 ng/mL) was loaded in duplication into the designate well. Fifty microliters of triazine-horseradish peroxidase conjugate was added into each well, and the plate was incubated for 30 minutes at room temperature. After incubation, the plate was washed three times with washing buffer solution, and loaded with 100 µL of substrate/color solution (hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine) into each well. The plate was incubated for another 15 minutes at room temperature before 50 µL of a stop solution (sulfuric acid) was added into each well. Absorbance at 450 nm was measured by a microplate reader (Multiskan EX). Standard calibration curves of atrazine were linear from 0-5.0 ng/mL with r^2 of 0.970 to 0.997. Based on this assay, the limit of detection for atrazine residue in mussel tissue was 0.53 ng/g dry weight, and the recovery of atrazine extraction was 87.41%.

2.5 Condition factor of freshwater mussel: Shell length and whole body weight without shell of each mussel was used to calculate a condition factor of each mussel as follows (Gagné *et al*., 2006): Condition factor = Whole body weight without shell (g) / Shell length (cm)

2.6 Statistical analysis: All parameters were tested for normal distribution and homogeneity of variance. Comparison between sexes was performed by Student's *t*-test, while seasonal variation were compared by one way analysis of variance (ANOVA) followed by Student-Newman-Keuls multiple comparison methods. Condition factor of the freshwater mussel was compared between months by Kruskal-Wallis one way ANOVA on ranks followed by Dunn's multiple comparison methods. Correlation between atrazine residue and condition factor was determined by Pearson product moment correlation.

3. Results and Discussion

The result of chromatographic analysis showed that residues of atrazine can be found in sediment (0.23 µg/g) and water (0.16 µg/mL) of Nong Bua reservoir in late dry season (January 2011; Table 1). Detectable amount of atrazine in this season is not unexpected since it was a beginning of new crop cycle when agrochemicals utilization was at peak. Presence of atrazine at the levels lower than the limit of detection in other periods could be due to the relatively low sensitivity of GC-MS (LOD: 0.01 µg/mL in water and 0.01 µg/g in sediment). However, given the fact that atrazine is relatively stable with half-life in surface water of more than 200 days (ATSDR, 2003), the levels found in this study pose potential concerns over its effect to aquatic life since it is quite close to the lowest observed effect concentration for early life stage of fish (0.46 µg/mL; Giddings *et al*., 2005).

Since bioconcentration of atrazine is unlikely (Giddings *et al*., 2005), atrazine residue in mussel tissue was thus determined by ELISA in order to yields a more sensitive assay (LOD: 0.53 ng/g dry weight). Detectable levels (1.44-16.69 ng/g) of atrazine were found in every

mussel examined (Figure 1). Similar to previous studies in *Anodontites trapesialis* and *Corbicula fluminea* bivalves (Jacomini *et al*., 2003; 2006), these data suggest that *U. contradens* could temporally store atrazine residue presented in the aquatic environment. However, concern on the safety of mussel consumption should be low since levels of atrazine found in the mussel are still much lower than the minimal risk level for oral exposure to atrazine in intermediate duration (0.003 mg/kg/day; ATSDR, 2003).

Since there was no sex-related difference in atrazine concentration, male and female data were combined for further statistical analysis. One way ANOVA showed a significant seasonal difference in level of atrazine residue in the mussel with the highest level found in late wet season (July 2010: overall mean 8.40 ± 2.06 ng/g). It is interesting to note that the peak of atrazine residue in the freshwater mussel (wet season) did not coincide with the peak of atrazine residue in environmental sample (dry season). The results confirm and suggest that, unlike physical environment, rate of pollutant uptake and loss in sentinel species may vary with physiological stage of the animal (Beeby, 2011). Therefore, monitoring program for environmental contamination should focus on both physical and biological samples in order to predict the potential health impact on organism with more accuracy.

To monitor change in health status and growth of mussels in this area, gravimetric and morphometric techniques were performed. Mean comparison of condition factor in each sex showed significant differences between months (ANOVA on ranks & Dunn's Method, $p<0.05$; Table 2). Further analysis showed a significant negative correlation between atrazine residue in the mussel tissue and the condition factor of both sexes of mussels (Pearson product moment correlation, $p<0.05$, r = -0.662 in male; r = -0.627 in female), indicating potential impact of atrazine on overall health of the mussel.

Since atrazine is known to cause disruption of endocrine and reproductive systems of animal (Allran and Karasov, 2001; Hayes *et al*., 2003), effects on aquatic animals living in the area are thus expected. Previously, contaminations of endocrine disrupting chemicals as a result

of agricultural activities were reported to adversely affect several mollusk species (Chesman and Langsto, 2006; Gomes *et al*., 2009). Although atrazine has no acute toxic effect in bivalve (Bringolf *et al*., 2007a; 2007b), chronic effect of low level atrazine exposure have been found in several mollusk species. A low level of atrazine (0.1 µg/mL) was reported to cause a reduction in hatching rate in the ramshorn snail *Marisa cornuarietis* (Sawasdee and Köhler, 2009). While a higher concentration of atrazine (>3.8 µg/mL) was reported to cause a reduction in growth rate of glochidia and juvenile mussels (Bringolf *et al*., 2007a; 2007b).

In addition to atrazine herbicide, the observed health effect on mussel from Nong Bua reservoir may be due to direct or synergistic effect of xenobiotic residues in this agricultural area. It is well known that organochlorine pesticides (OCPs) were widely used in most, if not all, of the agricultural area in Thailand. Although OCPs had been banned in Thailand for many years, their residues are still persisted in the environment (Thirakhupt et al., 2006). Using *Uniandra contradens* as a sentinel to OCP contamination, Boonlue et al. (2011) reported that OCP residues were found in sediment and tissue as well as in accordance with increased level of detoxifying enzyme of mussels living in agricultural area of Central Thailand. .

Overall, this study revealed that atrazine was contaminated in water and sediment of agricultural catchment as well as the freshwater mussel of Nan Province. The presence of atrazine showed a strong negative correlation with biomarker of health of the mussel. These data could be used as part of the monitoring program for herbicide contamination as well as an early warning of the effects of herbicide contamination on freshwater animals.

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We would like to thank member of BioSentinel Laboratory as well as farmers of Nan Province for their assistances in field samplings and related laboratory works. Financial supports have been obtained from the Science for Locale Project under the Chulalongkorn

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Table 1 Levels of atrazine residue in composited environmental samples collected from an agricultural catchment (Nong Bua reservoir) in Nan Province, Thailand during July 2010 to June 2011

Samples	July 2010 (late wet season)	October 2010 (early dry season)	January 2011 (late dry season)	April 2011 (early wet season)
Water	< 0.01 µg/mL* (n = 1)	< 0.01 µg/mL* (n = 1)	0.16 µg/mL (n = 1)	< 0.01 µg/mL* (n = 1)
Sediment	< 0.01 µg/mg* (n = 1)	< 0.01 µg/mg* (n = 1)	0.23 µg/g (n = 1)	< 0.01 µg/mg* (n = 1)

Remark:

* = Limit of detection (LOD; 0.01 µg/g for sediment and 0.01 µg/mL for water)

Table 2: Mean ± S.E.M. of condition factor of freshwater mussel *Uniandra contradens* collected from an agricultural catchment (Nong Bua Reservoir) in Nan Province, Thailand during July 2010 to June 2011

Sampling Months	Male mussel	Female mussel
July 2010	0.45 ± 0.03^{C} (n = 32)	0.46 ± 0.03^{D} (n = 10)
August 2010	0.71 ± 0.05^{C} (n = 34)	0.80 ± 0.10^{CD} (n = 18)
September 2010	0.81 ± 0.05^{CF} (n = 24)	0.77 ± 0.02^{CD} (n = 31)
October 2010	0.99 ± 0.08^{BEF} (n = 38)	1.00 ± 0.03^{CE} (n = 26)
November 2010	1.00 ± 0.03^{BD} (n = 51)	1.09 ± 0.03^{BE} (n = 44)
December 2010	1.19 ± 0.02^{A} (n = 57)	1.32 ± 0.03^{A} (n = 33)
January 2011	1.09 ± 0.03^{AD} (n = 48)	1.20 ± 0.03^{AB} (n = 42)
February 2011	1.08 ± 0.02^{AD} (n = 48)	1.18 ± 0.05^{ABE} (n = 42)
March 2011	1.03 ± 0.02^{BD} (n = 54)	1.06 ± 0.03^{BE} (n = 36)
April 2011	1.05 ± 0.03^{BD} (n = 53)	1.09 ± 0.03^{BE} (n = 37)
May 2011	1.09 ± 0.03^{AD} (n = 51)	1.14 ± 0.04^{ABE} (n = 39)
June 2011	1.06 ± 0.02^{ADE} (n = 48)	1.05 ± 0.03^{BE} (n = 42)

Remark:

Significant difference between month ($p<0.05$, one way ANOVA on ranks & Dunn's method) is indicated by difference in superscript letter.

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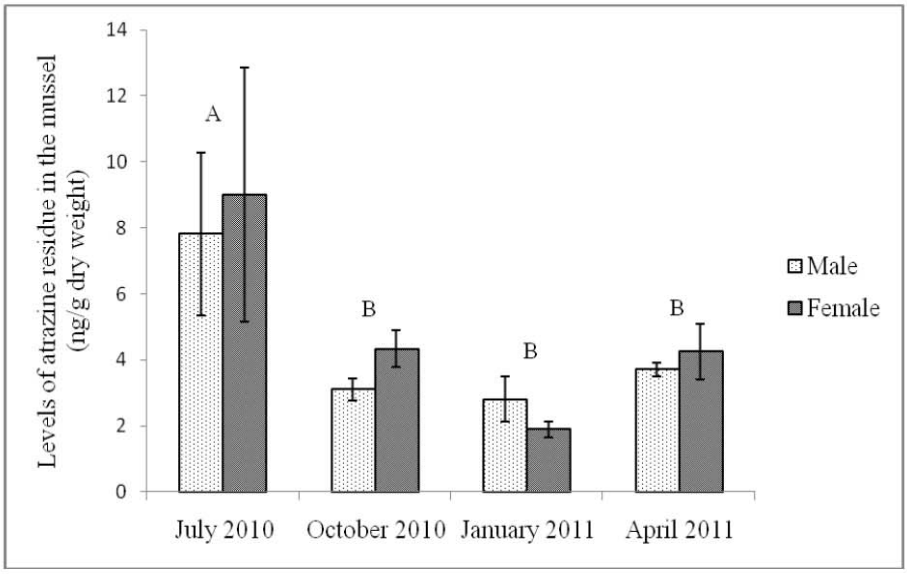

Figure 1: Mean ± S.E.M. of atrazine residue in *Uniandra contradens* collected from agricultural area in Nan Province, Thailand. Significant difference between month ($p<0.05$, one way ANOVA & SNK post hoc) is indicated by difference in superscript letter.

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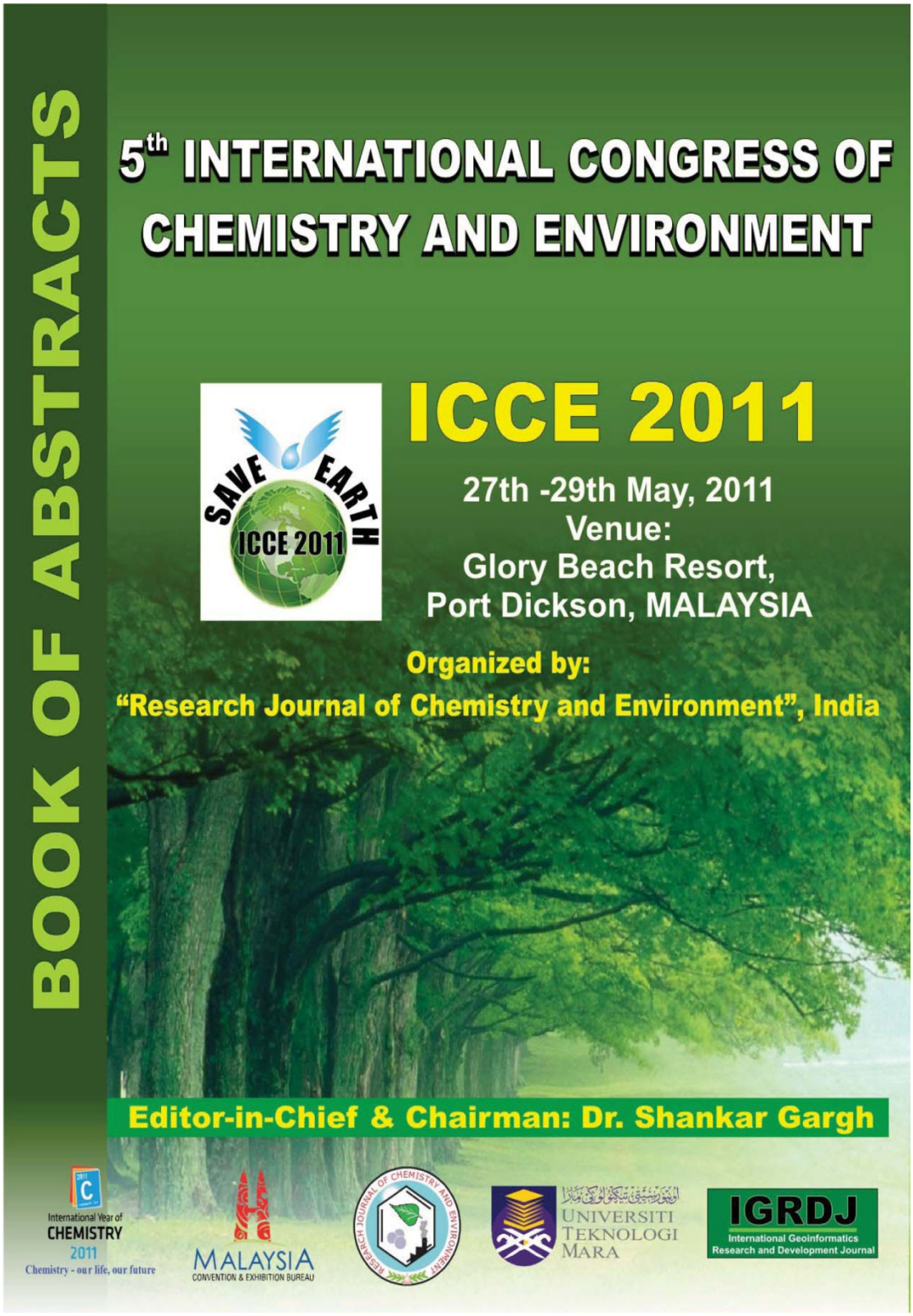
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field crab *Esanthelphusa nani* has been selected as a sentinel species because it lives in paddy fields with direct exposure to herbicide contaminated soil and water. In this study, crabs were collected from two study sites: reference site where no herbicides were used and the contaminated site where herbicides were used routinely. Crabs were subjected to morphometric and gravimetric analyses and the result showed that body weight of male crabs in the reference site is significantly higher than those from the contaminated site (ANCOVA, $p<0.01$). This could indicate association between atrazine contamination and the weight loss of male crab population in the contaminated area. Hepatopancreas of the crab were further determined for activity of glutathione *S*-transferase (GST), a crucial enzyme in biotransformation processes of xenobiotic contaminants. Comparison of the biologic response of crab, in term of hepatopancreas GST activity, to different levels of herbicide contamination will be presented. The results from this sentinel study could be used for assessing a potential impact of herbicide contamination on non-target organisms in agricultural environment.

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Atrazine Residue and Potential Effects on Reproductive Activities of Freshwater Mussels Uniandra contradens in Agricultural Area of Nan Province, Thailand

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Abstract

Nan, a province in the northern part of Thailand, is known as an origin of several rivers and tributaries such as Nan River as well as a fertile area for agricultural activities. Seasonal cultivation in this area leads to widely uses of agrochemicals especially herbicides. These herbicides may accumulate in environment and cause adverse effects to organ systems of animals living in the area. Environmental sample (soil, sediment and water) were collected from agricultural area in July, October 2010 and January 2011 for analysis of atrazine by GC-MS and glyphosate and paraquat by HPLC. The result show that residue of atrazine (0.01 mg/kg) can be found in sediment of Nan River in early wet season (July). Higher levels of atrazine residue were found in water of agricultural area (0.15 mg/L), reservoir (0.16 mg/L) and Nan River (0.15 mg/L) as well as in sediment of reservoir (0.23 mg/kg) and Nan River (0.24 mg/kg) in early dry season (January). Since atrazine is also known to cause disruption of endocrine and reproductive systems of animal, it is thus important to monitor its effects on representative species of animal. In this study, a freshwater mussel Uniandra contradens is used as a sentinel species for potential health effects of herbicide contamination. Freshwater mussels were collected from agricultural area in Nan Province during July 2010 to January 2011. Mussels were examined for condition factor and reproductive activities in term of gonadal development and germ cell maturation as well as spawning activities of female mussels. The results showed that the condition factor of both sexes tended to change according to season. Presence of motile sperm in testis and mature oocytes in ovary at every sampling time suggested a continuous gametogenic mode in this species. However, the female mussels showed temporal differences in spawning activities with the peak in December to January (early dry season). Since detectable levels of atrazine were also found in the environment in this period, it is thus possible that this low level of contamination could pose serious threat to reproductive and developmental activities of the freshwater mussels. The information from this study could be used as an early warning of effects of herbicide contamination on freshwater animals and the potential link to reproductive health of other organism in the area including human.

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components, water soluble fraction of oil, and dispersed oil) on copepod survival and reproduction are used as input for development of numerical models for environmental risk and damage assessment. The accessibility of a continuous copepod culture provides homogeneous specimen in terms of developmental stage and lipid content. This, along with sophisticated experimental systems, is a major foundation for investigating effects of stressors on molecular systems. A 60K oligonucleotide microarray has been developed based on a combination of available NCBI expressed sequence tags (ESTs) and 260,000 ESTs sequenced by FLX454 technologies. Several methods, like 1H-nuclear magnetic resonance and mass spectrometry, have also been developed and applied in order to investigate metabolic profiles and alterations. Together these complementary methods give valuable supporting information to well known fitness-related endpoints, as they contribute with modes of toxic action of stressors, and output data may also be used to determine effect limits. The presentation will include results from experiments aimed at investigating the effects of chemically and mechanically dispersed oil, their modes of toxic action, and proposed effect limits of toxicity based on fitness-related endpoints as well as molecular profiling.

RP095 Atrazine Residue and Glutathione S-Transferase Response of Freshwater Mussel UNIANDRA CONTRADENS Living in Agricultural Catchments, Northern Thailand T. Thitiphuree, Chulalongkorn Univ, Chulalongkorn Univ, Dept of Biology, Faculty of Science; J. Kitana, Chulalongkorn Univ, Dept of Biology, Faculty of Science; P. Varanusupakul, Chulalongkorn Univ, Dept of Chemistry; N. Kitana, Chulalongkorn Univ, Dept of Biology. Northern part of Thailand is known as an origin of several rivers and tributaries as well as a fertile area for agricultural activities. Seasonal cultivation in this area leads to widely uses of agrochemicals especially atrazine herbicide. To examine whether an intensive use of atrazine could lead to contamination in aquatic environment, the sediment and water were collected from agricultural catchment basin in Nan province, northern part of Thailand during 2010-2011 and subjected to analysis for atrazine by gas chromatography-mass spectrometry. The results showed that detectable levels of atrazine residue were found in water (maximum value 0.16 mg/L) and sediment (maximum value 0.23 mg/kg) of the catchment area. Since atrazine is also known to cause disruption of organ systems of animal, it is thus important to monitor its effects on representative species of aquatic animal. In this study, a freshwater mussel Uniandra contradens was used as a sentinel species for potential health effects of atrazine contamination in aquatic environment. Freshwater mussels were collected from the agricultural catchment area during July 2010 to June 2011. Mussels were subjected to tissue residue analysis for atrazine by high performance liquid chromatography and examine for specific activity of glutathione s-transferase (GST), a biotransformation enzyme, in their hepatopancreas by spectrophotometric assay. The results on seasonal profiles of atrazine residue and specific activity of GST will be presented. Association between environmental and tissue residues of atrazine and potential oxidative stress condition of the freshwater mussel will be discussed. The information from this study could be used as an early warning of health effects of low level atrazine contamination on freshwater animals and the potential link to other organism in the area including human.

RP096 Evaluation of Algal Estrogen Exposure Scenario Focusing on Gengorobuna as a High-Exposure Group Y. Hida, The Univ of Shiga Prefecture, Ecosystem Studies, The Univ of Shiga Prefecture; Y. Fujimoto, K. Kitao, M. Nakamura, T. Shibata, H. OOkura, Y. Yamada, S. Kuribayashi, T. Kurata, The Univ of Shiga Prefecture. A class of chemicals called endocrine disruptors, which were pointed out in 1990s, still has been an "unknown" type of risk in the society. While clear cause-effect relationships about endocrine disruptors have not yet been established, in some countries, the concern that the uncertainty in their risk estimation may be excessive large results in the society's decision-making to avoid them completely. Thus, if the current risk assessment methodology of endocrine disruptors is that its do not affect the society's decision-making, efforts should be improved to reduce its uncertainty. In this sense, it is critical to fundamentally characterize endocrine-disrupting action itself using field data. Since 2004, we have shown that algae (phytoplankton), which are ubiquitous in the aquatic environment, have weak estrogenic activities, are taken up passively, and act on gonadal development by temporarily suppressing maturation and the seasonal maturation phase in round Crucian carp (Nigorobuna). In this study, we focused on Gengorobuna as a group that is highly exposed to algae among Crucian carp species, which are supposed to eat zooplankton or aquatic animals generally. Gengorobuna is the only carp which are supposed to eat phytoplankton, and since it has almost twice the number of gill rakers as Nigorobuna, it is probably more affected by algae exposure. Therefore, by comparing gonadal development in these two carp, we can show the one example about the effects of algae on fish based on the actual amount of algae they are exposed to in nature. When 5-month-old Gengorobuna and Nigorobuna maintained under the same cultivation environment with excessive algae flourished were compared, serum vitellogenin (VTG) concentrations in Gengorobuna were lower in both sexes, and the gonadosomatic index (GSI) was skewed to low values, __particularly in males. Seasonal variations in GSI and VTG in mature Gengorobuna in Lake Biwa are nearly always lower than those in Nigorobuna throughout the year in both sexes. Furthermore, in breeding experiments with Gengorobuna exposed to *Chlorella*, which have estrogenic activity, gonadal maturation phase was preceded in a control group of males compared with that in an exposed group and lagged in females, and the difference was clearer in both sexes of Gengorobuna when compared with Nigorobuna. These results are consistent with the hypothetical scenario that fish are exposed to estrogenic substances through algae consumption in nature.

RP097 Integrating Toxicity Test and Experimental Ecosystem Data to Better Define Aquatic Risks D.R. Mount, US Environmental Protection Agency, ORD; R.J. Erickson, USEPA, Mid-Continent Ecology Division. Aquatic risk assessments based on species sensitivity distributions can be uncertain regarding the nature and magnitude of the effects being addressed. The use of inconsistent endpoints and the failure to consider complete effects/exposure relationships contribute to this uncertainty. What percentile is selected as a level of concern in the distribution also represents a major uncertainty. Better definition of risks can be achieved by more fully exploiting information from toxicity tests, using more consistent endpoints, and relating results from toxicity tests to effects in experimental or natural ecosystems. Using atrazine effects on aquatic plants and plant communities as an example, this talk will discuss an index for better describing the level of toxicity to an assemblage of species as a function of concentration and time, and how experimental ecosystem data can be used to support risk characterizations based on this index.

RP098 Putting "Health" Back into Ecosystem Health Assessment K. Munkittrick, Univ of New Brunswick, Canadian Rivers Institute, Univ of New Brunswick, Dept of Biology. There are many different purposes to collecting information, and trying to merge predictive and retrospective frameworks is very difficult. A major challenge is that situations where toxicity is strong show positive results across a spectrum of responses, including laboratory and field approaches. Regardless of the assessment purpose, objectives, approach, design and analysis, indicators have to be practical, simple, and consistent, with a low type II error rate. Laboratory approaches sacrifice ecological relevance, while field approaches have to make compromises in terms of conflicting requirements for protection, detection, reversibility, causality and ecological relevance. Ecosystem health assessment has largely followed a human health medical paradigm, where reference collections seek to define the level of natural variability in terms of spatial and temporal fluctuations that can mask impacts. Historically, definitions of "normal" levels of responses are used to detect deviations from normal and the absence of "health". There is perhaps a more useful paradigm, where the ability of organisms to integrate responses can be used holistically to evaluate performance. Under this effects-based paradigm, study designs seek out natural variability, and a system self-defines its level of health, thresholds of responses, and triggers to inform management decisions. Relationships also translate ecological information into currencies relevant for land use planning, natural resource management and impact mitigation. It represent a simpler site-specific approach that requires a commitment to baseline monitoring, consistency, and commitment to long term planning that is usually absent in current situations.

RP099 Relationships Between Atrazine Contamination and Biologic Response of Paddy Crab, Esanthelphusa nani, in Agricultural Area of Nan Province, Thailand R. Maneein, Chulalongkorn Univ; W. Khonsue, Chulalongkorn Univ, Dept of Biology; P. Varanusupakul, Chulalongkorn Univ, Dept of Chemistry; N. Kitana, Chulalongkorn Univ, Dept of Biology. A variety of herbicides has been used intensively in agricultural activities at Nan province, northern part of Thailand. Although adverse effects on non-target organisms of these herbicides, especially atrazine, have been reported,

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ความเชื่อมโยงระหว่างการปนเปื้อนสารฆ่าวัชพืชและผลทางระบบสืบพันธุ์ในหอยกาบน้ำจืด *Uniandra contradens* ในพื้นที่เกษตร จังหวัดน่าน

ธงชัย ฐิติภูรี[1], จิรารัช กิตนะ[1], ปกรณ์ วรานุศุภากุล[2] และ นพดล กิตนะ[1]
[1]ภาควิชาชีววิทยา และ [2]ภาควิชาเคมี คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย กรุงเทพฯ

การใช้สารฆ่าวัชพืชในการเกษตรอาจนำไปสู่การปนเปื้อนในสิ่งแวดล้อมและส่งผลกระทบต่อสัตว์ ในการศึกษานี้ได้เก็บตัวอย่างดินและน้ำจากพื้นที่เกษตรในจังหวัดน่านในปี พ.ศ.2553-2554 มาตรวจสอบหาสารฆ่าวัชพืชแอทราซีนด้วยเครื่อง GC-MS และ ไกลโฟเสตและพาราควอตด้วยเครื่อง HPLC พบว่าไม่มีการตกค้างของไกลโฟเสตและพาราควอต แต่มีแอทราซีนในน้ำจากนาข้าว (0.15 mg/L) และอ่างเก็บน้ำ (0.16 mg/L) และในดินตะกอนของอ่างเก็บน้ำ (0.23 mg/kg) เมื่อพิจาณาจากการศึกษาที่ผ่านมา พบว่าแอทราซีนสามารถรบกวนการทำงานของต่อมไร้ท่อและระบบสืบพันธุ์สัตว์ จึงมีความจำเป็นต้องเฝ้าระวังผลกระทบที่อาจจะเกิดขึ้น โดยในการศึกษานี้ได้ใช้หอยกาบน้ำจืด *Uniandra contradens* เป็นสิ่งมีชีวิตเฝ้าระวังผลกระทบต่อระบบสืบพันธุ์ เมื่อเก็บตัวอย่างหอยจากอ่างเก็บน้ำหนองบัว ในพื้นที่เกษตรของจังหวัดน่าน ในช่วงกรกฎาคม 2553 – มิถุนายน 2554 มาศึกษาสุขภาวะโดยรวมโดยใช้ค่าน้ำหนักสัมพัทธ์ และศึกษาการเปลี่ยนแปลงในระบบสืบพันธุ์เพื่อที่จะหาช่วงที่มีความไวต่อการได้รับสารปนเปื้อน พบว่าน้ำหนักสัมพัทธ์ของหอยมีการเปลี่ยนแปลงตามฤดูกาล โดยมีค่าต่ำสุดในฤดูฝน และพบว่าในช่วงที่ศึกษา หอยทั้งสองเพศมีภาวะเจริญพันธุ์สูง โดยเพศผู้มีเซลล์อสุจิที่เคลื่อนที่ได้ และเพศเมียมีเซลล์โอโอไซต์ระยะเต็มวัย อย่างไรก็ดีหอยเพศเมียมีรูปแบบการปล่อยไข่ที่แตกต่างกันในรอบปี โดยพบสูงที่สุดในช่วงปลายฤดูฝนถึงต้นฤดูแล้ง ซึ่งเป็นช่วงเดียวกับที่พบแอทราซีนในสิ่งแวดล้อม จึงน่าเกรงว่าสารแอทราซีนที่ปนเปื้อนอาจส่งผลต่อการสืบพันธุ์ และการเจริญของหอยกาบน้ำจืดได้

Association between herbicide contamination and reproductive effects in freshwater mussel *Uniandra contradens* in agricultural area waters, Nan Province

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Widely use of herbicides in cultivation may lead to environment contamination and harmful effects to animals. In this study, environmental samples collected from agricultural areas in Nan Province during 2010-2011 were subjected to analysis for atrazine with GC-MS, and glyphosate and paraquat with HPLC. Although level of glyphosate and paraquat was not detectable, residue of atrazine could be found in water of paddy field (0.15 mg/L), reservoir (0.16 mg/L) and sediment of reservoir (0.23 mg/kg). Since atrazine is known to cause disruption of endocrine and reproductive systems, it is thus important to monitor its effects on animal. Therefore, a freshwater mussel *Uniandra contradens* is used as a sentinel species for potential reproductive effects. Mussels were collected from Nong-Bua reservoir near agricultural areas in Nan Province during July 2010-June 2011. Mussels were examined for condition factor and reproductive activities in order to locate a susceptible period in their life history. The results showed that condition factor tended to change according to season with the lowest level in early wet season. Examination of reproductive activities showed that motile sperm in testis and mature oocyte in ovary were presented at every sampling period. However, the female mussels showed temporal differences in spawning activities with the peak in late wet season to early dry season when detectable levels of atrazine were found. It is thus possible that this contamination could pose serious threat to reproductive and developmental activities of the mussels.

Biography

Mr. Tongchai Thitiphuree was born on the 3rd of May, 1987 at Phuket Province. He graduated a Bachelor of Science in Biology from Department of Biology, Faculty of Science, Chulalongkorn University since 2009. After graduation, he continued his graduate study for Master of Science in Zoology Program at the same department. During his study, he has spent more than a year for field research at Nan Province as part of the CU Centenary Academic Development Plan to encourage graduate students to acquire first hand experience in field environment as well as gain perspectives on the country's need in real life situations. He has given one oral and one poster presentations in the international conferences including the 5th International Congress of Chemistry and Environment, Port Dickson, Malaysia (2011) and the 32nd Annual Meeting of the Society of Environmental Toxicology and Chemistry North America, Boston, Massachusetts, USA (2011).