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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 $\pm$ 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^{o}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^{o}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 (Fig. 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

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)

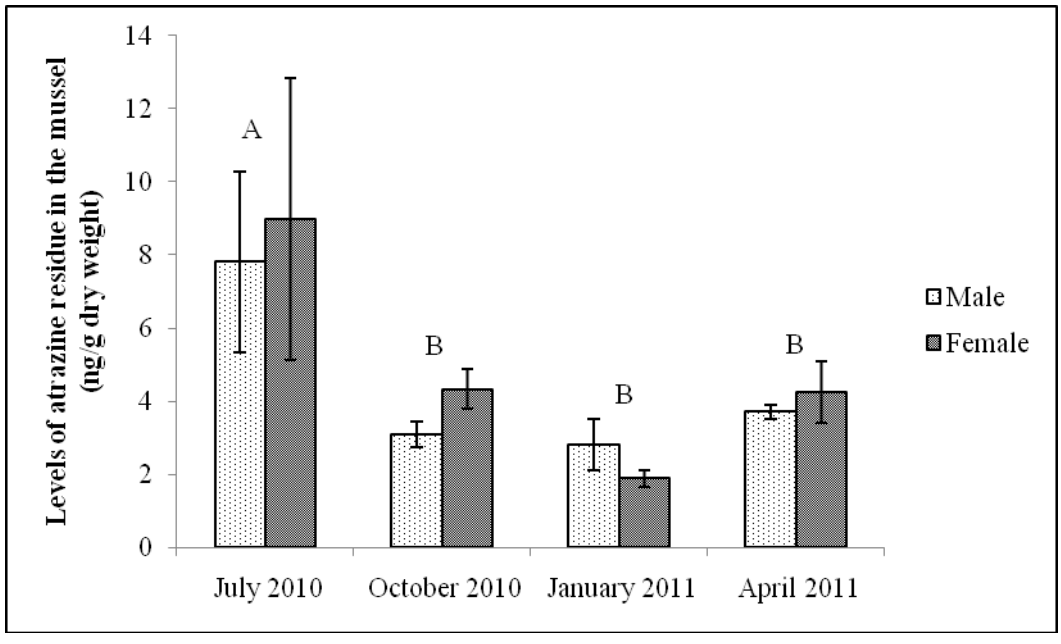

Figure 1. Mean ± S.E.M. of atrazine residue in *Uniandra contradens* collected from agricultural area in Nan Province, Thailand. Significant difference between month ($p<0.05$, one way ANOVA & SNK post hoc) is indicated by difference in superscript letter.

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

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.

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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