Phytotoxicity of Atrazine Herbicide to Fresh Water Macrophyte Duckweed (*Lemna perpusilla* Torr.) in Thailand

On-Anong Phewnil[1]*, Nipon Tungkananurak[1], Supamard Panichsakpatana[2], Bongotrat Pitiyont[3]

[1]*College of Environment, Kasetsart University, Bangkok 10900, Thailand.*
[2]*Faculty of Agriculture, Kasetsart University, Bangkok 10900,Thailand*
[3]*Faculty of Science, Kasetsart University, Bangkok 10900, Thailand*

Abstract

Atrazine herbicides are of potential ecotoxicological concern to freshwater in many countries, as a result of cultivating maize, sorghum, pineapple, and sugarcane. Few data are available on the toxicity of atrazine to duckweed (*Lemna perpusilla* Torr.), which is the most common species of macrophyte in Thailand. This study tries to determine the toxicity of atrazine individually to freshwater macrophyte, *Lemna perpusilla* Torr. (duckweed). The growth rate of *Lemna perpusilla* Torr. decreased with increasing atrazine concentration, with the concentration of atrazine inhibiting growth by 50% (EC_{50}) being 13,487 µg/L. The EC_{50} value for the exposure of *Lemna perpusilla* Torr. was significantly affect concentration of 16,000-32,000 µg/L. Therefore the growth rate of *Lemna perpusilla* Torr. reduced when atrazine concentration were present range 250-32,000 µg/L, observed within 24 hours, which were more tolerant than *Lemna minor* (EC_{50} = 250 µg/L). These results have important potential implications for the protection of freshwater ecosystems through the derivation of national water quality guidelines.

Key words: toxicity/ atrazine/ herbicide/ duckweed

1. Introduction

Atrazine has been widely applied in several cultivated areas for the control of broad-leaf annual weeds in maize, sorghum, pine apple, and sugarcane fields. These compounds have been applied directly to the soil and sprayed over cultivated areas, which has led to residues in the environment. In 2007, approximately 3,686,650 kg of atrazine (s-triazine) were imported into Thailand, making it the 7th most imported pesticide (Department of Agriculture, 2007). As a result, there are atrazine residues in the upland soils that may be transported into aquatic ecosystems. Atrazine functions by inhibiting photosynthesis in weeds; however, it may cause similar effects in aquatic ecosystems that receive atrazine via runoff or leaching. Duckweed is a member of the Lamnacae family of floating plants that grow fast and spread quickly. It is easy to access to treatment due to the fact that duckweed are resistant to poisoning. However, it is often found to have a toxic response like any other aquatic plant. Therefore, it is used as a bioassay to test the toxicity of the herbicide in aquatic ecosystem such as surface water and toxic sediments in lakes or rivers. However, it can be found by location and raving in natural surface waters (Freshwater Fishery Division, 1995) and can be used as an agent to test the toxicity of atrazine in aquatic ecosystem. Accordingly, this study was conducted to determine atrazine toxicity in fresh water macrophyte duckweed (*Lemna perpusilla* Torr.), which is the most common duckweed in Thailand. There is currently no set standard in the toxicity of atrazine in surface water. Although Thailand has been using atrazine in large quantities

*Corresponding author
E-mail: fgraonp@ku.ac.th

and causing contamination in surface water. In particular, the toxicity to the aquatic plants which are the primary producers, will cause an imbalance of the aquatic ecosystem. The results obtained in this study may then be used to develop atrazine standards for the surface water of Thailand and to reduce pesticide residues in the environment in the future.

2. Methodology

2.1 Test Applications

Lemna perpusilla Torr. is a small aquatic macrophyte widely distributed in fresh water in Thailand. It is the most common species of the family Lemnaceae. This duckweed is an ideal organism for assessing aquatic phytotoxicity of many herbicides. The duckweed toxicity test is useful, especially for assaying the air-water interface (Lower and Lewis, 1990). It is generally described as a simple, sensitive, and cost-effective test. Duckweed is a floating plant which may underestimate the toxicity of atrazine herbicide.

2.2 Culturing Test Organism

Samples of duckweed were collected from natural environment conditions in a wetland site in Phetchaburi Province. However, laboratory testing was conducted on duckweed. Duckweed was grown by adding Hoagland's nutrient weekly in a 15-L aquarium at least 2 weeks before test. Hoagland's nutrient was prepared to a 10-L culture solution, to which was added 100 ml of the nutrient solutions to tap water and kept at 40-mm water depth or more. A constant cool-white fluorescent light (4,300 lux at the duckweed floating level) was provided. After that, this culturing maintained a temperature at 25$\pm$2°C and stock culture was transferred to a freshly prepared nutrient solution monthly.

2.3 Toxicity Test Procedure

The experiment used static conditions since a solution with low microbial population, high toxic concentration, and low volatility. In range-finding tests, a series of concentrations, usually at ratios of 10 as 0.25, 2.50, 25, 250, and 2,500 µg/L, respectively were examined. A definitive test on the basis of range-finding test results were devised, in which eight concentrations of sample in a ratio of 2 as 250, 500, 1,000, 2,000, 4,000, 8,000, 16,000, and 32,000 µg/L, respectively were used in the experiments and each test was performed in quadruplicate and included a negative control containing only duckweed nutrient solution as well as a positive control containing 35 mg/L potassium chromate in range-finding tests and 250, 500, 1,000, 2,000, 4,000, 8,000, 16,000, and 32,000 µg/L in definitive test (ASTM, 1991; APHA,AWWA, and WEF, 1992; EPA, 1996; Environmental Canada, 1998; OECD, 2006). The same amount of nutrients, which was 1 ml of each nutrient stock solution to 100 ml sample, was added to all control and test samples. The test vessels that contain 15 ml test solution (or control sample) and 12 or more duckweed fronds were covered with glass dishes. Duckweed specimens, with only healthy-looking colonies, containing two fronds of approximately equal size per colony, were taken from stock cultures which were grown under the same conditions, illuminated with continuous cool-white-fluorescent light at 4,300 lux and incubated at 25$\pm$2°C. Duckweed specimens observation period was 7 days.

2.4 Test Results

After 96 hrs., Duckweed plants were observed under a lighted magnifying glass (2X or higher) for symptoms including chlorosis (loss of pigment), necrosis (localized dead tissue), and colony breakup. The most commonly used and seemingly reliable method was frond increase and frond area, a quantity value directly reflecting growth rate with image-analysis green value. Other methods that were used include biomass (fresh weight and dry weight) and plant colony counts. Chlorophyll content was measured with chlorophyll meter, Minolta SPAD-502 model (OEDC, 2006). Duckweed specimens in the control sample for comparison by ANOVA-test and Pair t-test were reported for affecting fronds.

2.5 Calculation

A sample was toxic as compared with the water control. Express toxicity in percent inhibition relative to the control as follow:

$$\%I_r = \frac{(\mu_c) - \mu_t)}{\mu_c} x100$$

When % I_r = % Inhibition of growth rate

μ_c = Average value in control sample
μ_t = Average value in test sample

Results may be graphed using linear plots. The concentration-effect relationship was sigmoid. IC_{50} and IC_{90} values were determined by graphical or statistical methods. The slope of dose-response relationship was toxicant-specific and thus could be valuable information.

3. Results and Discussion

3.1 Range-Finding Test

3.1.1 Frond Area

Toxicity test of atrazine in duckweed (*Lemna perpusilla* Torr.), represented fresh water macrophyte, showed that relative growth rate of duckweed decreased in 7 days as atrazine concentration increased but there was also no significance between each concentration (0.25, 2.5, 25, 250, and 2,500 µg/L, respectively) and the control sample (Figure 1).

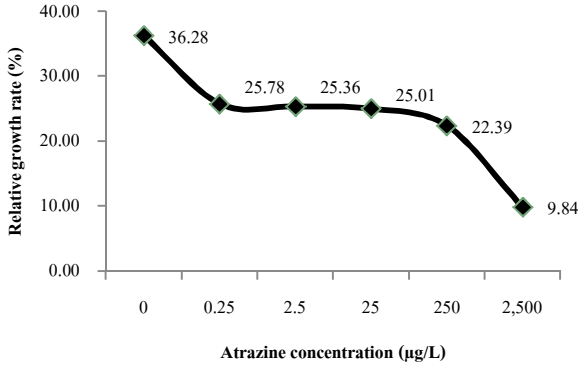

Figure 1 Relation between relative growth rates of frond area of duckweed (*Lemna perpusilla* Torr.) and atrazine concentration.

3.1.2 Frond number

The toxicity of atrazine in test samples in range 0.25-2,500 µg/L had increasing abnormal fronds related to increased atrazine concentration(Figure2). Symptom of atrazine in duckweed (*Lemna perpusilla* Torr.) included chlorosis more than necrosis and dead fronds (Figure 3). After 24 hours could observe symptoms of the duckweed could be observed, especially in the 2,500 µg/L test sample. Atrazine concentration had a positive relation to chlorosis (p=0.017, df=41), necrosis (p=0.006, df=41), and dead fronds (p=0.011, df=41), respectively. The results coincided that atrazine ranged 0.25-2,500 µg/L toxic to aquatic plant by photo-synthesis inhibition that showed symptoms as chlorosis (loss of pigment) and necrosis (localized dead tissue), but it was not an acute effect. Statistics test showed that only the atrazine 2,500 µg/L test sample had highly significant in pair t-test (p=0.001, df=3) between test samples and control sample. It indicated that atrazine 2,500 µg/L inhibited the growth rate of duckweed (*Lemna perpusilla* Torr.) more than atrazine in low concentration test samples.

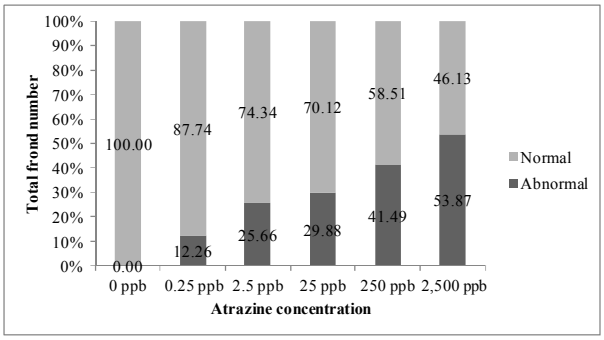

Figure 2 Relation between atrazine concentration and average abnormal frond of duckweed (*Lemna perpusilla* Torr.)

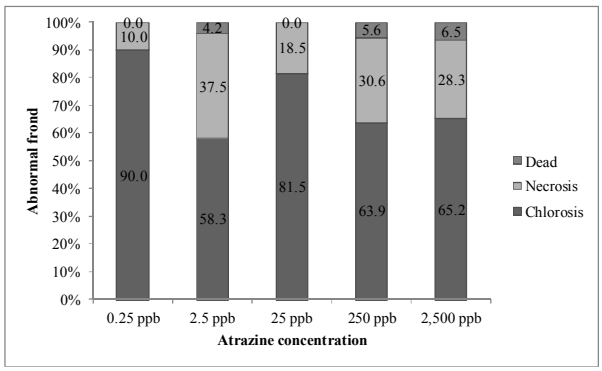

Figure 3 Relation between atrazine concentration and average chlorosis, necrosis, and dead frond of duckweed (*Lemna perpusilla* Torr.)

3.1.3 Chlorophyll Content

Chlorophyll content was measured in fronds of duckweed (*Lemna perpusilla* Torr.) in 7 days after started the test. The trend of chlorophyll content decreased via atrazine concentration increased (Figure 4) indicated atrazine toxic to chlorophyll content of duckweed, which related to symptom of chlorosis after 24 hour after test starting. Atrazine concentration of 2,500 µg/L had highly significance in pair t-test (p = 0.031, df =3). The results related to Wang (1990), reported growth of common duckweed (*Lemna minor)* had negative relation with atrazine concentration, no significance, but chlorophyll a/b decreased when atrazine concentration increased.

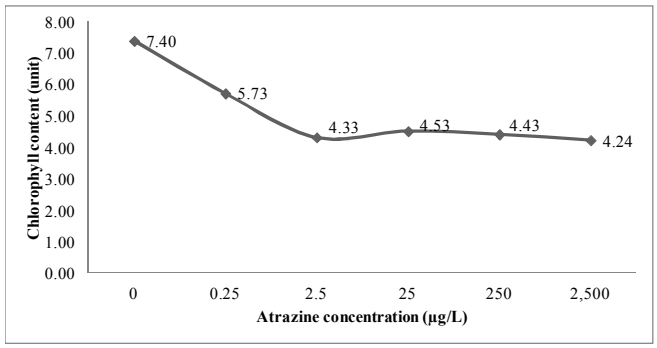

Figure 4 Relation between average chlorophyll content of duckweed (*Lemna perpusilla* Torr.) and atrazine concentration

3.1.4 Biomass

Test samples of atrazine concentration with ranges from 0.25 to -2,500 µg/L showed that fresh weight and dry weight decreased (Figure 5), when compared to the control sample, but it had no significance, exceptfor the 2,500 µg/L sample of atrazine ($p = 0.027$, $df = 3$). It may be concluded that atrazine 2,500 µg/L inhibited the growth of duckweed (*Lemna perpusilla* Torr.) and a low concentration of atrazine may not directly effect duckweed (*Lemna perpusilla* Torr.) withina period of 7 days.

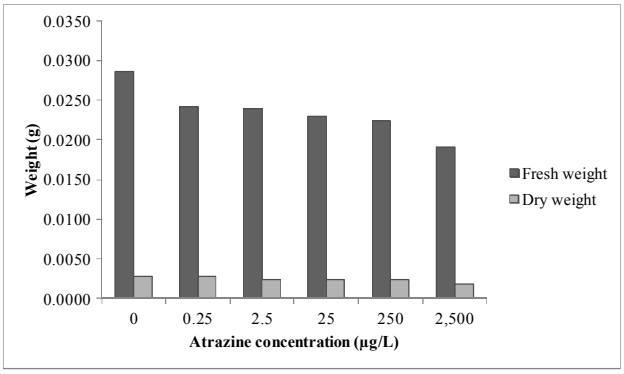

Figure 5 Relation between atrazine concentration and average biomass of duckweed (*Lemna perpusilla* Torr.)

3.1.5 Toxicity of Duckweed (Lemna perpusilla Torr.) in Range-Finding Tests

Percentage of inhibition of growth rate considered to symptoms including chlorosis, necrosis, and chlorophyll content. The results showed each atrazine concentration related to growth rate of duckweed in differentiation. The concentration ranged from 0.25 to 250 µg/L of atrazine and showed a slightly different of percentage of inhibition of growth rate. In other hand, Atrazine 2,500 µg/L had a percentage of inhibition of growth rate of 59.15% (Figure 6). The graph of relation between the percentage of inhibition of growth rate of duckweed (*Lemna perpusilla* Torr.) and atrazine concentration showed a linear equation as $y = 0.011 x + 31.82$ ($R^2 = 0.8952$). Accordingly, the toxicity of atrazine in duckweed occurred in high concentration (2,500 µg/L of atrazine), the definitive test should be done in a concentration above 2,500 µg/L of atrazine.

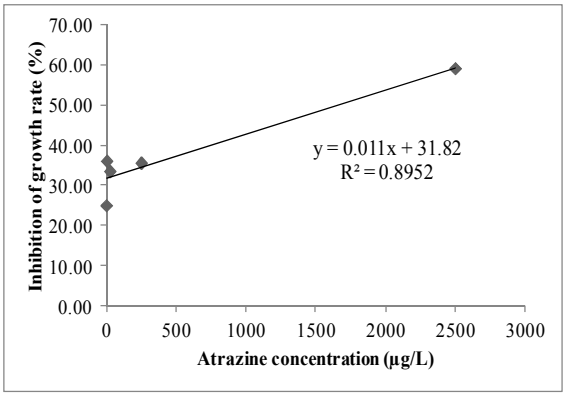

Figure 6 Relation between atrazine concentration and inhibition of growth rate of duckweed (*Lemna perpusilla* Torr.)

3.2 Definitive Test

The results in range-finding tests found that atrazine inhibited growth of duckweed (*Lemna perpusilla* Torr.) Also observed symptoms of duckweed frond included chlorosis, necrosis, dead fronds, chlorophyll content, and biomass, but frond areas were not clarified. The results indicated that atrazine 250 µg/L is toxic to duckweed (*Lemna perpusilla* Torr.). For these reasons, atrazine concentration ranged from 250 to 32,000 µg/L were used in definitive tests which the results showed as follows:

3.2.1 Frond Area

Atrazine concentration levels between 250-32,000 µg/L and potassium chromate 250-32,000 µg/L had a negative relation with the frond area of duckweed (*Lemna perpusilla* Torr.), although the frond area of duckweed had a positive relation with a time period of 7 days (Figure 7 and Figure 8). In the other hand, frond area changing was not significant in ANOVA-test and pair t-test between each concentration of atrazine or potassium chromate test samples and control samples.

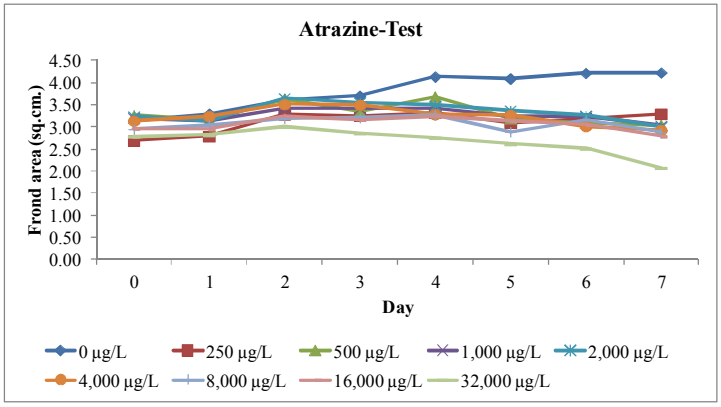

Figure 7 Relation between atrazine concentration and average frond area of duckweed (*Lemna perpusilla* Torr.) in 7 days

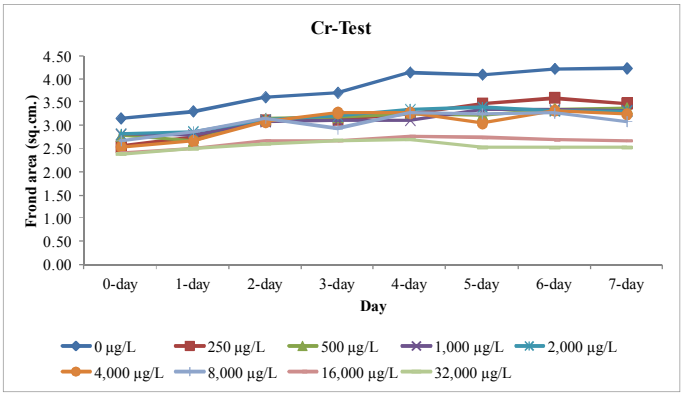

Figure 8 Relation between potassium chromate concentration and average frond area of duckweed *(Lemna perpusilla* Torr.) in 7 days

3.2.2 Frond Number

Duckweed (*Lemna perpusilla* Torr.) showed toxic symptoms to atrazine concentration levels of between 250-32,000 µg/L which was a positive relationship between atrazine concentration and abnormal fronds including; chlorosis, necrosis, and dead fronds (Figure 9). These symptoms occurred within 24 hours after starting tests and chlorosis frond decreased as well as necrosis frond, and dead frond increased within 7 days of testing. The results of the symptoms of fronds were highly significant in chlorosis fronds ($p=1.89x10^{-7}$, df=55), necrosis fronds (p= 0.002, df = 55), and dead fronds ($p= 3.43x10^{-5}$, df =55), respectively (Figure 10). The symptoms indicated that atrazine concentration within the range of 250 to 32,000 µg/L is toxic to duckweed but it did not have an acute effect in the test period. For the potassium chromate solution, positive tests, showed toxicity trends as atrazine and potassium chromate solution ranged from 16,000 to 32,000 µg/L were highly significant, when compared between control samples and test samples (p = 0.0011, p = 0.001; df = 3).

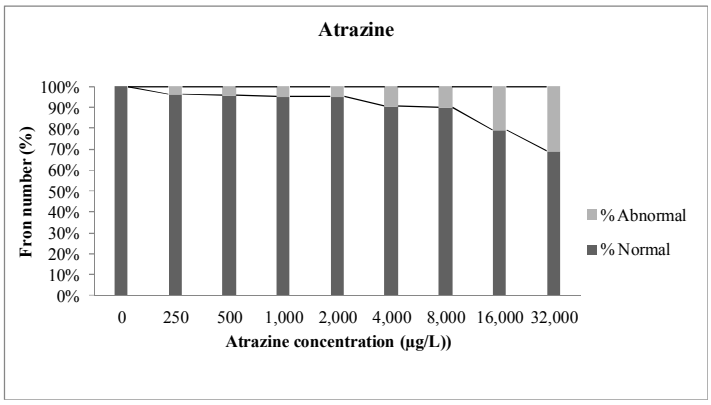

Figure 9 Relation between atrazine concentration and average frond number of duckweed (*Lemna perpusilla* Torr.)

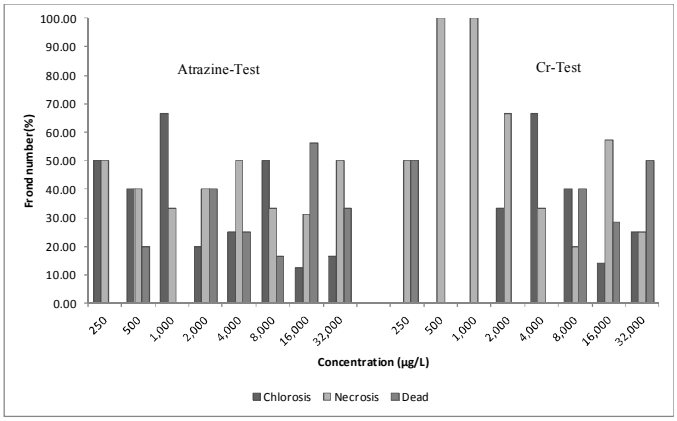

Figure 10 Relation between atrazine concentration and potassium chromate concentration at levels of 250-32,000 µg /L and chlorosis, necrosis, and dead frond of duckweed (*Lemna perpusilla* Torr.)

3.2.3 Chlorophyll Content

Chlorophyll content was measured in duckweed fronds by chlorophyll meter, Minolta model SPAD-502, in 7-days of the test. The detected chlorophyll content in duckweed frond a decreasing trend line via atrazine or potassium chromate concentration increased (Figure 11). The symptoms of chlorosis and necrosis of fronds revealed with these results, where chlorosis fronds were observed within 24 hours after the test started. The results of data statistic analysis showed the chlorophyll content in each level of atrazine and potassium chromate concentration were different by a highly significant amount, when compared with control samples. From these results it can be can be concluded that atrazine and potassium chromate are toxic to chlorophyll content in fronds of duckweed, which revealed that atrazine also effects chlorophyll a/b of common duckweed (*Lemna minor*) while atrazine concentration increased (Wang, 1990).

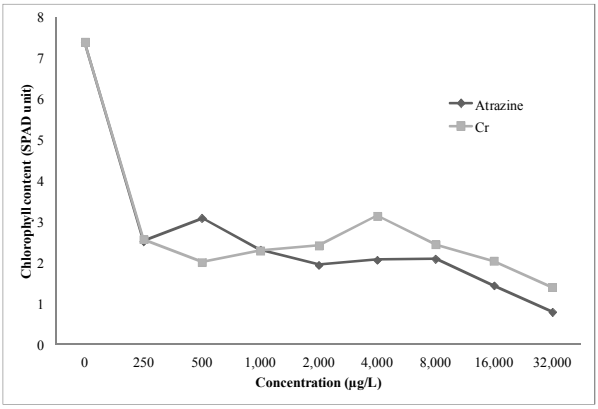

Figure 11 Relation between atrazine and potassium chromate concentration at levels of 250-32,000 µg/L and average chlorophyll content of duckweed (*Lemna perpusilla* Torr.)

3.2.4 Biomass

Figure 12 and Figure 13 showed that fresh weight and dry weight of duckweed (*Lemna perpusilla* Torr.) which was tested with atrazine and potassium chromate in the range of 250-32,000 µg/L were decreased but it was not significant in ANOVA-Test. On the other hand, the pair t-test between control samples and test samples showed that only test samples of atrazine at levels of 16,000-32,000 µg/L were highly significant.

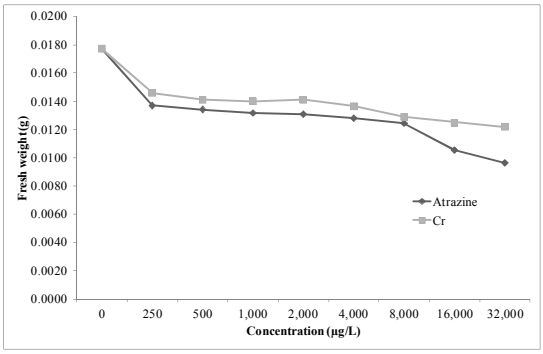

Figure 12 Relation between atrazine and potassium chromate concentration a level 250-32,000 µg/L and average fresh weight of duckweed (*Lemna perpusilla* Torr.)

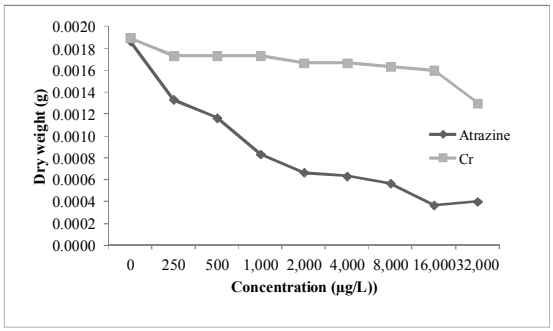

Figure 13 Relation between atrazine and potassium chromate concentration a level 250-32,000 µg/L and average dry weight of duckweed (*Lemna perpusilla* Torr.)

3.2.5 Toxicity in Duckweed (Lemna perpusilla Torr.)

Because frond area were not clarified to indicate toxicity of atrazine in duckweed (*Lemna perpusilla* Torr.), so the calculation for percentage of inhibition of growth rate considered only frond numbers, chlorophyll content, and biomass (fresh weight and dry weight). Atrazine concentration ranges of 250 to 32,000 µg/L inhibited growth of duckweed at different rates and duckweed was growth inhibited by atrazine and potassium chromate at concentration levels of 32,000 µg/L around 68.53% and 51.48%, respectively. The graph in Figure 14 showed a relationship between percentage of inhibition of growth rate of duckweed and atrazine concentration which is a linear equation as follows: $y = 0.011 x + 35.164$ ($R^2 = 0.9294$) and Figure 15 showed relationship between percentage of inhibition of growth rate of duckweed and potassium chromate concentration which is also a linear equation as follows: $y = 0.0006x + 33.699$ ($R^2 = 0.9525$). From this equation, the EC_{50} of atrazine in duckweed (*Lemna perpusilla* Torr.) could be alculated as 13,487 µg/L and EC_{90} as 49,851 µg/L.

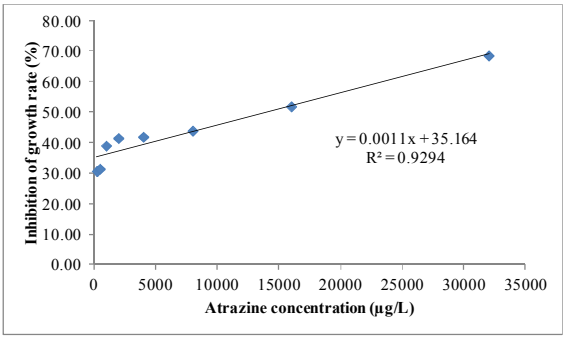

Figure 14 Relation between atrazine concentration at level 250-32,000 µg L^{-1} and percentage of inhibition of growth rate of duckweed (*Lemna perpusilla* Torr.)

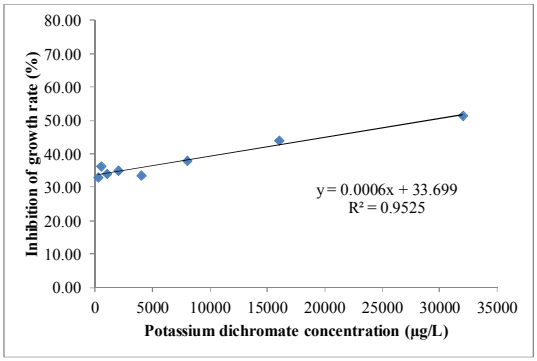

Figure 15 Relation between potassium chromate concentration at levels of 250-32,000 µg L^{-1} and percentage of inhibition of growth rate of duckweed (*Lemna perpusilla* Torr.)

The value of EC_{50} revealed that triazine herbicide inhibited growth of duckweed (*Lemna minor*) more than 50% while atrazine concentration at levels of 0-250 µg/L (Wang, 1990) did not significantly negatively effect growth rate, but observed symptoms could occurr within 96 hours. In addition, the duckweeds (*Lemna perpusilla* Torr.) response to heavy metal such as; as nickel and cadmium where the growth of duckweed decreased when heavy metal concentrations increased (Pukngam et al., 2007). From the results it was revealed that duckweed (*Lemna perpusilla* Torr.) responses to atrazine included frond numbers (symptom of frond), decreasing chlorophyll content, and decreasing biomass more than frond area but it was more tolerant than common duckweed (*Lemna minor*), which EC_{50} = 250 µg/L (Wang, 1990) and submersed aquatic plant *Potamogeton perfoliatus* L., which EC_{50} = 80 µg/L (Jones et al., 1986). However, this test indicated duckweed (*Lemna perpusilla* Torr.) was inhibited by atrazine in photosystem II in chloroplast of photosynthesis process, so the plant had symptoms in its leaf. Atrazine affected the growth of aquatic plants in the electron transfer of the Photosystem II (PSII) of photosynthesis process. PSII was supramolecular pigments in chloroplast, which served to pass electrons from water to plastoquinone (PQ) and release oxygen (Giardi et al., 2001; Bell and Duke, 2005; Graymore et al., 2001). Bioconcentration factor of atrazine in *Lemna minor* was 0.78, but biodegradation of atrazine by the Lemna minor significance at concentrations of 10 µg/L. The rate of decomposition of biological was 16.1 percent in seven days, which explained the process of GSH conjugation in biotransformation of atrazine in the duckweed Accordingly, duckweed was an organism that responded to atrazine

which the symptoms was shown within 96 hours of toxicity bioassay. Duckweed was the most sensitive organism tested, being equally affected by atrazine causing algal phytotoxicity (Peterson et al., 1994). However, the toxicity of pesticides in several groups of aquatic plants had different kinds of plants, which must be taken into consideration. The use of an uncertainty factor is necessary to provide an acceptable margin of safety in evaluating the hazard presented by these chemicals to the aquatic environment. (Peterson et al., 1994; Fairchild et al., 1998; Chi et al., 2007)

4. Conclusion

Phytotoxicity of atrazine in duckweed (*Lemna perpusilla* Torr.) at concentration levels of 250-32,000 µg/L could inhibit growth of duckweed within the range of 30.68 to 68.53% , which EC_{50} equal 13,487 µg/L. Atrazine symptoms that could be observed in fronds, included; chlorosis, necrosis, and dead fronds, chlorophyll content, and biomass at atrazine concentration levels of 16,000-32,000 µg/L but could not observed in frond area. Because of duckweed (*Lemna perpusilla* Torr.) was more tolerant than common duckweed (*Lemna minor*), the study of atrazine acute toxicity in duckweed (*Lemna perpusilla* Torr.) should be tested at concentration levels of 32,000 µg L^{-1} and above. The test at low concentrations should be longer than 7 days to determine toxic period and observed symptoms of duckweed.

5. Acknowledgements

The authors would like to express their sincere gratitude to the King's Royally Initiated Laem Phak Bia Environmental Research and Development Project for their cooperation. This study was funded by the EIA Project, College of Environment, and Kasetsart University.

6. References

American Public Health Association, American Water Works Association, and Water Environment Federation (APHA, AWWA, and WEF). 1992. **Standard Methods for Examination of Water and Wastewater**. USA: American Public Health Association.

ASTM. 1991. **Conducting Static Toxicity Tests with *Lemna gibba*. Guide E 1415-91.** Annual Book of ASTM Standards. Section 11 Water and Environmental Technology. Volume 11.04. Philadelphia, USA.

Bell, A.M. and Duke, N.C. 2005. Effects of Photosystem II inhibiting herbicides on Mangroves preliminary toxicology trials. **Marine Pollution Bulletin** 51(1-4): 297-307.

Chi, Z., Qing, Z.A., Dong, X.W., Min, L. A., and Shao., Y. 2007. Biodegradation of atrazine and toxicological effects on the aquatic plant *Lemna minor*. **Fresenius Environmental Bulletin** 16(4): 379-384.

Department of Agriculture. 2007. **The Use of Agricultural Chemicals in Thailand**. Division of Agricultural Toxic Substance, Bangkok, Thailand.

Environmental Canada. 1998. **Biological Test Method: Test for Measuring the Inhibition of Growth Using the Freshwater Macrophyte *Lemna minor* Report EPS 1/RM/37**. Canada.

EPA. 1996. **Ecological Effects Test Guideline OPPTS 850.4400 Aquatic Plant Toxicity Test**

Using *Lemna* sp., Tier I and II, EPA 712-C-96-156. USA.

Fairchild, J.F., Ruessler, D.S., and Carlson, A.R. 1998. Comparative sensitivity of five species of macrophyte and six species of algae to atrazine, metibuzine, alachlor and metolachlor. **Environmental Toxicology** 17(9): 1830-1834.

Freshwater Fisheries Division. 1995. **Aquatic Plants in Thailand**. Department of Fisheries, Ministry of Agriculture and Cooperatives, Bangkok.

Giardi, M.T., Koblízek, M., and Masojídek, J. 2001. Photosystem II-based biosensors for the detection of pollutants. **Biosensors and Bioelectronics** 16(9-12): 1027-1033.

Graymore, M., Stagnitti, F., and Allinson, G. 2001. Impacts of atrazine in aquatic ecosystem. **Environment International** 26: 483-495.

Jones, T.W., Kemp, W.M., Estes, P.S., Stevenson., J.C. 1986. Atrazine uptake, photosynthetic inhibition, and short-term recovery for the submersed vascular plant, *Potamogeton perfoliatus* L. **Archives of Environmental Contamination and Toxicology** 15: 277-283.

Lower, W.R. and Lewis, M.A. 1990. Common macrophyte species used in pesticide toxicity test reported in open literature. In: Wang W., W. Gorsuch, and W.R. Lower. (eds): **Plants for Toxicity Assessment** (pp.77-97). Japan.

Peterson, H.G., Boutin, C., Martin, P.A., Freemark, K.E., Ruecker, N.J., and Moody, M.J. 1994. Aquatic phytotoxicity of 23 pesticides applied at expected environmental concentration. **Aquatic Toxicology** 28(3-4): 275-292.

Pukngam, S., Kereeratnikom, A., Jamrassang, T., Sukpunnaoun, U., and Khodchapakdee, A. 2007. **Efficiency of cattail (*Typha angustifolia* L.) and duckweed (*Lemna perpusilla* Torr.) in heavy metal treatment in water by biological synthetic-water treatment processing**. The 19th Thaksin University Annual Conference, Pattalung, Thailand.

Wang, W. 1990. Literature review on duckweed toxicity testing. **Environmental Research** 52(1): 7-22.