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Gene Expressions of the *Dhb*, *Vtg*, *Arnt*, *CYP4*, *CYP314* in *Daphnia magna* Induced by Toxicity of Glyphosate and Methidathion Pesticides

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1. Introduction

Pesticides, the chemicals used commonly in agriculture to control pests, pathogens and weeds, have been contributing to the serious contamination of the aquatic environment through spray drift, volatilization, drainage and leaching (Cerejeira et al., 2003; Pereira et al., 2009). Among various pesticides, methidathion is a highly toxic insecticide used to control a wide spectrum of agricultural insect and mite pests. In the other hand, glyphosate, the active ingredient in many commercial weed-killing formulation (e.g., Roundup), is widely used in agricultural, silvicultural and urban environment (Borggaard, K.O., and Gimsing, L.A., 2008). The results are an increasing detection of these pesticides in the environment, especially in aquatic system may have some ecotoxicological impacts on non-target aquatic organisms (Tsui et al., 2003; Vorkamp et al., 2002). *D. magna*, a freshwater crustacean, has been used extensively to evaluate the toxic effects of chemical on aquatic system (EPA, 2002) because of their high sensitivity to a wide range of chemicals, a short lifecycle, and ease of manipulation in the laboratory. In addition, the daphnia are ubiquitous and play a key role in aquatic food web (Soetaert et al., 2006). Conventionally, the toxicity assays (e.g., acute or chronic toxicity tests) have widely been used to evaluate the aquatic toxicity as well as the adverse impacts of the toxic chemicals on aquatic organisms based on the phynotic endpoints such as the survival, growth, and reproduction (Colleen et al., 2005; Heckmanna et al., 2007). These body responses result from some molecular responses (e.g., gene expression) in organisms that expose to a toxic environment. Therefore, changing in gene expression in the organisms should happen first, and be a more sensitive indicator to the toxic chemicals than the body responses (Jo et al., 2008; Le et al., 2010; Le et al., 2011). Notably, when an organism is exposed to a toxic environment, the metabolic activity in the organism will change to overcome the adverse effects (Ankley and Villeneuve, 2006). Certain genes, possessing the particular functions, express differently in organisms that are exposed to toxicants. For instance, hemoglobins (*Dhb*), the protein are distributed widely in all organisms, are composed of multiple two-domain chains with a relatively normal oxygen binding activity when found in the hemolymph of *D. magna* (Tokishita et al., 1997; Anderson et al., 2008). Vitellogenin (*Vtg*) is a major lipoprotein in many oviparous animals and has

been used as a useful biomarker to examine the hazardous effects of endocrine disrupting compounds (EDCs) (Jones et al., 2000; Kato et al., 2004). Aryl hydrocarbon receptor nuclear translocator (*Arnt*) activates the transcription of the genes that encode the enzymes involved in metabolizing aryl hydrocarbons, such as dioxin and endocrine disruptors in mammalian cells and marine, freshwater and avian species (Tokishita et al, 2006). Hence, the *Arnt* gene probably responds to the altered metabolic effect of exposure to aryl hydrocarbons. Cytochrome P450s (CYPs) are a large and ubiquitous super-family of heme proteins that are encoded by receptor-dependent transcriptional activation genes, and are a class of proteins that respond to the hazardous effect of toxic chemicals (Snyder, 2000). CYPs are categorized into 4 different families, i.e., mitochondrial, CYP2, CYP3-like, and *CYP4* family (Bradfield et al., 1991; Baldwin et al., 2009). In the present study, the hazardous effects of two selected pesticides (i.e., glyphosate, and methidathion) on *D. magna* were examined by studying the changes in the gene expressions of five stress responsive genes, including *Dhb, Arnt*, *Vtg*, *CYP4*, and *CYP314* using the method of reverse transcription polymerase chain reaction (RT-PCR). This technique is a simple and effective tool to study the gene expression (Stephen et al., 2008). The expression level of a gene is measured by determining the mRNA amount generated by that gene via the transcription. Through the gene expression analysis, the molecular responses of *D. magna* exposed to pesticides can be determined to provide an insight into the action mode of chemicals.

The aim of this study was to evaluate toxicity of the two pesticides and to analyze their adverse effects on the expression patterns of different genes in *D. magna*. Thereby, the mechanisms controlling the gene expressions were indirectly studied in response to the addition of pesticides, i.e., glyphosate and methidathion. Five different genes including hemoglobin (*Dhb*) (Ha and Choi, 2009), vitellogenin (*Vtg*) (Jones et al., 2000), aryl hydrocarbon receptor nuclear translocator (*Arnt*) (Tokishita et al., 2006), cytochrome P450 *CYP4* family (*CYP4*) (Scharf et al., 2001), and cytochrome P450 mitochondrial family 314 (*CYP314*) (Shen et al., 2003) were selected for this study to quantify the expression level and analyze the physiological changes and different expression mechanisms for each of the genes.

2. Experimental methods

2.1 Daphnia magna culture

D. magna (the Korea Institute of Toxicology, Daejeon, Korea) were cultured and handled according to the USEPA manual (US Environmental Protection Agency, 2002). The organisms were cultured at 20 ± 1°C in 2 L beakers containing 1.5 L of hard reconstituted water (HRW) prepared by adding 0.12 g/L $MgSO_4$, 0.192 g/L $NaHCO_3$, 0.008 g/L KCl, 0.12 g/L $CaCO_3$ into deionized water distilled using a Minipore Milli-Q apparatus. This HRW was controlled at a pH of 8.2 ± 0.2 and aerated for at least 24 h prior to use. Three times per week, the medium for the *D. magna* culture was renewed with fresh HRW and fed with algae (*Chlorella vulgaris*) and YTC (a mixture of yeast, cerophyll, and Trout chow) purchased from Aquatic Biosystem Inc. (Colorado, US). The number of *D. magna* was adjusted to about 30 to 50 organisms per 2 L culture vessel. A photoperiod of 16 h light : 8 h darkness was applied.

2.2 Acute toxicity test

Acute toxicity test was performed following the standard US EPA manual (2002) to determine the lethal endpoint caused by glyphosate and methidathion (Fluka, US). In all of

the tests, the less-than-24h-old *D. magna* neonates collected from the less-than-30 d-old female organisms were used for the experiment. Ten neonates per test vessel were exposed to several concentrations of glyphosate and methidathion. Each test concentration was prepared using a volume of 50ml with three replicates and maintained at 20 ± 1 °C during a 24 h photoperiod of 16 h light : 8 h darkness without any feeding.

2.3 Chronic toxicity test

The chronic effects on the physiological responses of *D. magna* were studied in this experiment. All of the test conditions were performed according to the US EPA protocol (2002). One daphnia neonate (less than 24-h old) was individually cultured in a 50ml vessel and continuously exposed to three sublethal concentrations (1/100LC50, 1/50LC50, 1/10LC50) of the pharmaceuticals for 21 days. Ten replicates were conducted for each chemical concentration. The experimental conditions and feeding regime were similar to the conditions that were described for the daphnia culture. The test solutions were renewed every two days. The oxygen concentration and the pH of the HRW were checked weekly to ensure that they were not affected by the biological responses. The growth, survival, and reproduction were the three endpoints for evaluating the chronic effects of the pharmaceuticals on the physiological responses of *D. magna*. The growth was determined by measuring the length of the daphnia after the 21 day test using a microscope (Olympus SZX9, Japan). Mortality was observed everyday at the same time. The reproduction was evaluated using two different parameters, the number of offspring per tested organism and the time of first reproduction.

2.4 Exposure experiments to analyze the gene expression

To study the effect of acute toxicity of chemical on the gene expression, the tested concentrations of glyphosate and methidathion were LC5, LC10, LC20, and LC50, which were defined as the concentrations where the percentages of dead testing daphnia were 5, 10, 20, and 50%, respectively. These sublethal concentrations were determined previously through the acute toxicity test. This exposure test was performed according to the USEPA manual 2002. The 20d-old daphnia collected from less than 30-d old mother daphnia were used in this experiment. Before being exposed to the chemicals, the organisms were starved for 24 h under the culturing conditions described above. After that, the exposure tests were conducted using ten organisms per 200ml test solution volume in 500ml vessel with three replicates. Test conditions were controlled at 20 ± 1 °C for 24 h with a photoperiod of 16-h light : 8-h darkness. In the other hand, effect of chronic toxicity of glyphosate and methidathion on the gene expression was studied on all daphnia used for the 21d chronic toxicity assay. Particularly, at the day 21 of the chronic assay, all alive tested organisms were collected into the 1.5ml eppendorf tube and then isolated the total RNA using Trizol method. Next, the amount of messenger RNA for each specific gene, which is the result of the gene expression, was measured using the semi-quantitative reverse transcriptase polymerase chain reaction (RTPCR).

2.5 Isolation of RNA samples and Semi-quantitative reverse transcriptase-polymerase chain reaction

A semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was used to analyze the gene expression in *D. magna* that were exposed to pesticides. The pesticide-

treated daphnia were harvested, and the total RNA was isolated using Trizol method. After isolation, the concentrations of the total RNA in the samples were measured using a Qubit kit (Invitrogen, US). The mRNA levels were determined using the semi-quantitative RT-PCR with a TaKaRa RNA PCR™ Kit (TaKaRa Inc., Japan). In the case of *D. magna*, the PCR primers were designed for six genes, where the actin gene was used as an internal standard (Table 1). On the other hand, five specific genes were used to analyze the expression patterns in *D. magna* after exposure to the pesticides. The PCR experiments were carried out using 30 cycles with a T Gradient Thermocycler (Biometra, Germany) to amplify the specific genes. All of the RNA samples were also used without a RT step for PCR to detect the presence of the genomic DNA contamination. No DNA was detected at any time. The PCR products were visualized on an EtBr-stained agarose gel. The intensities of DNA band observed on the UV light are quantified for the gene expression level using GelScope 1.5 software (Imageline Inc., USA). The PCR was carried out three different times to confirm the reproducibility of the results. All of the results were reported as mean values. The relative expression level of a gene is determined by dividing the expression level of that gene to that of an internal standard (i.e., actin gene).

Genes	Description	Gene bank accession No.	Primer (5′-3′)	Amplicon length (bp)
Dhb	Hemoglobin	AAC47544	Forward: CACCACTGTGACTACCACTG Reverse: CAGCTTTCTTGAGGTTTTTG	506
Vtg	Vitellogenin	AB252738	Forward: ATGCTGAGAACACCGTCTAC Reverse: GTGGGTCTTGTAGTCGTCAT	530
Arnt	Aryl hydrocarbon Receptor Nuclear Translocator	AB242866	Forward: CAGTTTCTGGAGAGGTTACG Reverse: GGTGGAACTACAGGTGATTG	508
CYP4	Cytochrome P450 4 family	AB257772	Forward: ATGGATTTACCAACAAGGTG Reverse: AGGTAATACGAGCAGATGGA	502
CYP314	Cytochrome P450 314 family	AB257771	Forward: ACGCGTAGTGAAAGTGATTT Reverse: TTACAGTATGATCCCCAAGG	203
Act	Actin	AJ292554	Forward: GAGACCGTCTACAACTCGAT Reverse: GTGTCGACAGAGACAATGAG	491

Table 1. Primer sequences of the genes used for the RT-PCR

2.6 Statistical analysis

All of the data were obtained from three independent samples carried out simultaneously for error analysis, and the results were shown along with the standard deviations and

correlations between the cell mortality and the experimental conditions. The data were analyzed using a Sigma Plot (SPS Chicago, IL. USA), and a p value < 0.05 was considered significant. The probit method, which is a parametric statistical method for estimating with 95% confidence limits, was used to calculate the sub-lethal concentrations of pesticides such as LC5, LC10, LC20, and LC50.

3. Results and discussion

3.1 Acute toxicity of glyphosate and methdiathion

The cellular toxicities of glyphosate and methidathion as pesticides were investigated using an acute 24-h toxicity test with 10 neonates of *D. magna* (less than 24-h old). In Figure 1, the mortality percentages of the test organisms increased in a sigmoid-curve relationship with increasing concentrations of glyphosate and methidathion. In particular, all of the neonates died when exposed to 350 mg/L glyphosate, as well as 0.10 mg/L methidathion. Based on the regression curves of the acute toxicity data, LC5, LC10, LC20, and LC50 were determined as the lethal concentrations of a chemical when the percentage of *D. magna* mortality was 5%, 10%, 20% and 50%, respectively. These values related to the lethal concentrations showed a 95% confidence interval. In this study, four lethal concentrations were used to show the specific response patterns of *D. magna* to glyphosate and methidathion through the analysis of the gene expression patterns using a semi-quantitative RT-PCR.

3.2 Effects of acute toxicity of glyphosate and methidathion on gene expression

To examine the effects of glyphosate on the gene expression in *D. magna*, ten 20-d old organisms were exposed to four different LC values (LC5, LC10, LC20 and LC50) 190, 202, 214, and 234 mg/L for 24 hours. In this study, the *Act* gene was selected as an internal standard gene. The expression level of the *Act* was constant in all experimental conditions (data not shown). The relative expression levels of the five selected genes were determined by normalizing to the *Act* expression level (Fig. 2). Three of the five genes selected in this study, excluding *Vtg* and *CYP314*, showed sensitive responses to glyphosate. Particularly, *Arnt* and *CYP4* were down-regulated after glyphosate exposure, and although *Dhb* transiently increased at 190 mg/L of glyphosate, the expression patterns of *Dhb* decreased with increasing concentrations of glyphosate.

On the other hand, four different LC values (LC5, LC10, LC20 and LC50) of methidathion, i.e., 0.024, 0.029, 0.034 and 0.044 mg/L were also used to study the expression patterns of the five selected genes in this study (Fig. 3). The effect of methidathion on the expression of *Dhb* and *Arnt* was the same as glyphosate, indicating the reduced gene expression after methidathion exposure. Interestingly, *CYP4* and *CYP314*, which are categorized into the CYP family, had significantly different expression patterns for glyphosate and methidathion. These results showed that exposure of *D. magna* to glyphosate led to a down regulated expression of the *CYP4* gene but no significant responses in the expression of the *CYP314* gene. However, in exposure to the increasing concentrations of methidathion, the *CYP4* gene expression did not differ, but the expression of *CYP314* gradually decreased compared to control.

Cytochrome P450s (*CYPs*), a large and ubiquitous super-family of heme proteins encoded by receptor-dependent transcriptional activation genes, are a class of proteins that respond to the hazardous effects of toxic chemicals (Snyder, 2000). Although both *CYP4* and *CYP314* belong to the same group of functional proteins, their different families elicited different susceptibilities to glyphosate. *CYP4*, which metabolizes endogenous compounds such as

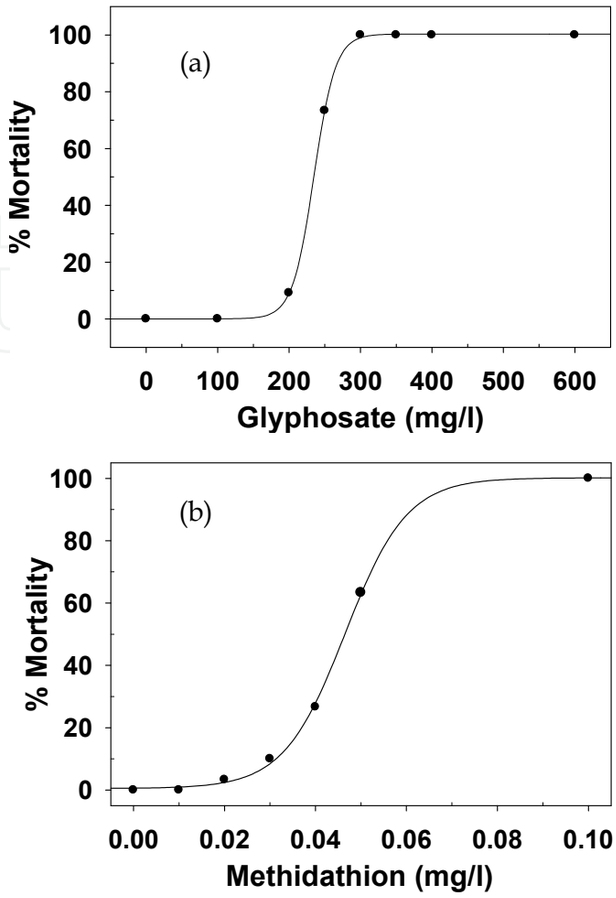

Fig. 1. Acute toxicity assay of *D. magna* for glyphosate (a) and methidathion (b).

fatty acids and steroids (Brandfield et al., 1991), is used for insecticide resistance in insects (Pittendrigh et al., 1997; Scharf et al., 2001). On the other hand, *CYP314* was subcategorized into mitochondrial CYPs and is required for the conversion of ecdysone to its active form (Shen et al., 2003). Therefore, based on these results, glyphosate probably creates more serious effects in fatty acids and steroids metabolisms and the ecdysis of *D. magna* would be influenced by methidathion.

Hemoglobin (*Dhb*), an important protein in many advanced organisms, is required for the formation of the constitutive part of their oxygen transport system (Aderson et al., 2008). Its expression is usually increased by most of the tested chemicals that reduce the oxygen level in aquatic systems (Ha et al., 2009). However, the increasing oxygen consumption is not always associated with an up-regulated expression of the hemoglobin gene (Anderson et al., 2008). For instance, the exposure of Chironomus midge to atrazine herbicide resulted in the elevated oxygen consumption in atrazine-treated midge that associated with a decreased expression level of the *Hb* genes (Anderson et al., 2008). In this study, the expression level of *Hb* in *D. magna* that was exposed to glyphosate slightly increased at a low concentration (LC5) and decreased gradually in response to the higher concentrations (LC20, LC50) in Figure 2a. Similarly, the expression of the *Hb* gene was significantly down regulated in *D. magna* after methidathion exposure (Fig. 3a).

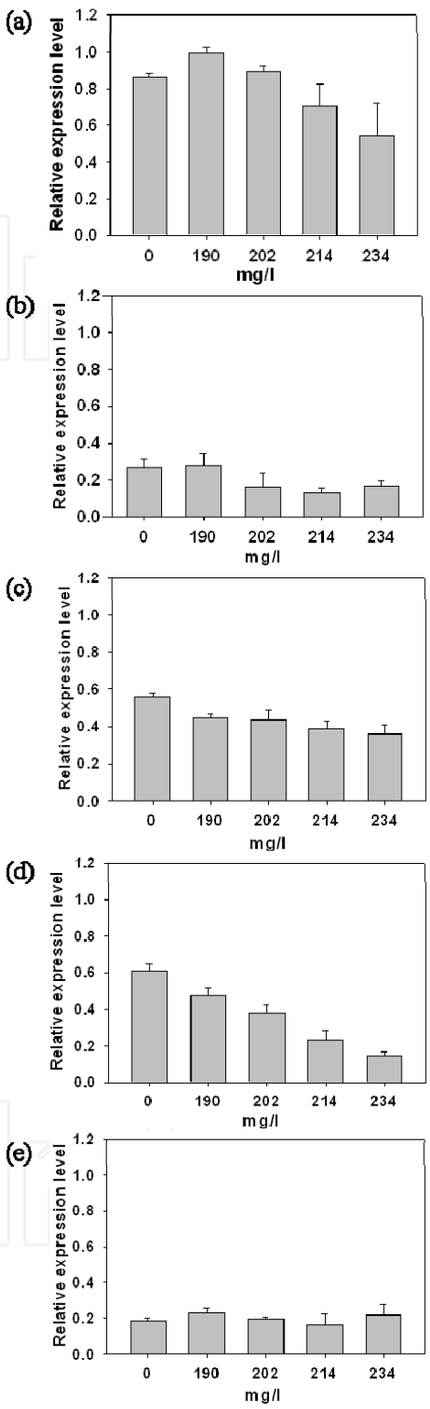

Fig. 2. Relative expression levels of 5 selected genes after 24h exposures of 0, 190, 202, 214, and 234 mg/L glyphosate. (a) *Dhb*, (b) *Vtg*, (c) *Arnt*, (d) *CYP4* and (e) *CYP314*. All of the data correspond to the expression level relative to the *Act* gene.

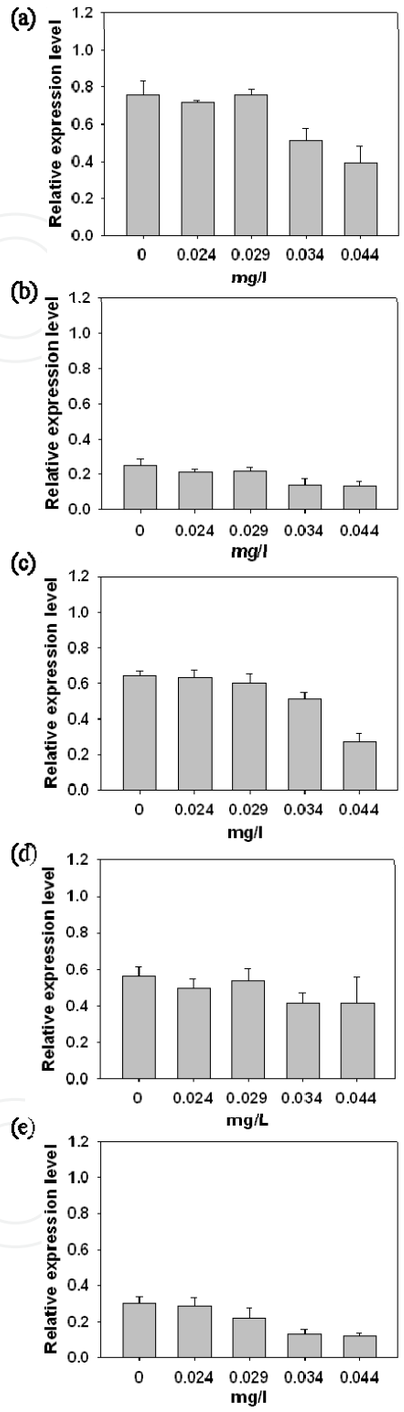

Fig. 3. Relative expression levels of 5 selected genes after 24h exposures of 0, 0.024, 0.029, 0.034, and 0.044 mg/L methidathion. (a) *Dhb*, (b) *Vtg*, (c) *Arnt*, (d) *CYP4* and (e) *CYP314*. All of the data correspond to the expression level relative to the *Act* gene.

The aryl hydrocarbon receptor nuclear translocator (*Arnt*) activates the transcription of the genes that encode the enzymes involved in metabolizing aryl hydrocarbons, such as dioxin and endocrine disruptors in mammalian cells and marine, freshwater and avian species (Tokishita et al., 2006). In this study, the expression of *Arnt* in *D. magna* gradually decreased in the dose-dependent response to both glyphosate (Fig. 2c) and methidathion, leading to the metabolism of the toxic compounds tested in this study (Fig. 3c).
Finally, vitellogenin is the precursor of the egg-yolk protein, vitellin, which provides an energy supply for embryo development in oviparous animals. Vitellogenin is considered as a biomarker for estrogenic compound because the production of vitellogenin is controlled by some estrogen hormones (Jones et al., 2000; Matozzo et al, 2008). In this study, no significant differences were discovered for the vitellogenin expression in *D. magna* in response to the sublethal concentrations of glyphosate (Fig. 2b) and methidathion (Fig. 3b) for the 24h duration. These results indicated that neither the acute toxicity of glyphosate nor methidathion induced estrogenically.

3.3 Chronic toxicity of glyphosate and methdiathion

The chronic toxicity of pesticides (i.e., glyphosate and methidathion) was examined based on their adverse impacts on the survival, growth and reproduction of *D. magna* after 21d exposing to sublethal concentrations. Three sublethal concentrations were selected for the chronic test included 1/100, 1/50, and 1/10 of LC50 glyphosate and methidathion in Table 2, and 3, respectively.

Glyphosate (ppm)	Suvival (%)	Body length (mm)	Time of first reproduction (day)	Number of offsprings per female (organism)
Control	100	3.87±0.18	13.2±1.03	28.8±8.26
2.34	100	3.86±0.16	13.0±1.05	28.2±11.3
4.68	100	3.90±0.24	13.6±1.33	25.3±7.65
23.4	100	3.45±0.15	15.4±0.97	11.3±2.54

Table 2. Survival and reproduction of *D. magna* after a 21d exposure to glyphosate

Methidathion (ppb)	Suvival (%)	Body length (mm)	Time of first reproduction (day)	Number of offsprings per female (organism)
Control	100	3.89±0.08	12.6±1.2	49.5±14.1
0.02% ethanol	100	3.77±0.16	12.8±0.8	48.8±9.70
0.44	100	3.88±0.06	13.9±1.9	46.6±16.6
0.88	90	3.74±0.11	14.3±2.8	38.0±25.1
4.4	0	na	na	0

Table 3. Survival and reproduction of *D. magna* after a 21d exposure to methidathion

The addition of 0.02% ethanol into culture media, the highest content of ethanol among all experiments, was also tested in comparison with control because ethanol was used to

prepare stock solution of methidathion. There were no significant differences of *D. magna* behaviors between addition of 0.02% ethanol and control (Table 3).

Except the highest of these tested concentrations (1/10 LC50), which gave an obvious effect on the survival and reproduction of organisms, the other concentrations including 1/100 and 1/50 LC50 showed a little difference compared to control. After 21d chronic toxicity experiment, the survival of daphnia exposed to 1/100, 1/50, 1/10 LC50 glyphosate were all equal to control (100%) while those exposed to methidathion were 100%, 90%, and 0%, respectively. The body length of daphnia organisms exposed to the low concentrations (1/100 and 1/50 LC50) of both pesticides were not significantly different from those of control. However, the size of daphnia exposed to 1/10 LC50 glyphosate was only 3.45±0.15mm, and about 10.85% smaller than that of control daphnia, which was 3.87±0.18mm. In case of exposing to 1/10LC50 methidathion, the size of daphnia could not be measured because all of the tested organisms died before the day 21.

Additionally, the reproduction ability of daphnia, which was represented by two tesed parameters including time-of-first-reproduction and number-of-offsprings, was also examined by the 21d chronic toxicity experiment. As shown in the table 2, no significant differences in these parameters were observed for the daphnia exposed to both 1/100 and 1/50 LC50 glyphosate compared to the control organisms. But, the time-of-first-reproduction was delayed from 13.2±1.03d to 15.4±0.97d and the number-of-offspring was dramatically reduced from 28.8±8.26 offsprings to 11.3±2.54 offsprings when daphnia was exposed to the 1/10LC50 glyphosate during 21d experiment. In case of methidathion, while the 1/10LC50 was high enough to kill all organisms before their reproduction, the 1/100LC50 was too weak to make any obvious effects on the reproduction ability (Table 3). In case of the exposure to 1/50LC50 methidathion, there was slightly adverse influence on the reproduction of organisms. Particularly, the time-of-first-reproduction was delayed from 12.6±1.2d to 14.3±2.8d and the number-of-offspring was reduced from 49.5±14.1 offsprings to 38.0±25.1 offsprings. The results indicated that only the 1/10LC50 concentration, which is highest among the tested concentrations, significantly affected on the physiological behaviors of organisms while the 1/100 and 1/50LC50 gave almost no obvious influences on the survival, and reproduction of *D. magna*.

3.4 Effects of chronic toxicity of glyphosate and methidathion on gene expression

The gene expressions of the five genes in *D. magna* were investigated by RTPCR technique using the total RNA isolated from organisms exposed to glyphosate and methidathion for 21 days. Among these examined genes, *Dhb* was the only gene of which expression level was not changed in daphnia following the 21d exposures to all tested doses including 2.34, 4.68, and 23.4 mg/L glyphosate compared to control (Fig. 5a) while the expression level of *CYP4* gene was slightly increased in response to those glyphosate concentrations (Fig. 5d). However, the other genes such as the *Vtg*, *Arnt*, and *CYP314* genes were significantly up-regulated after 21d exposing to glyphosate (Fig. 5b, 5c, 5e). The expression level of these genes substantially rose when the exposing concentration increased from 0 to 4.68 mg/L, and kept maintaining when it increases from 4.68 mg/L to 23.4 mg/L. Ethanol is the solvent used to dissolve methidathion and prepare some stock solutions for the chronic toxicity experiment. The 0.02% ethanol, a maximum percentage of ethanol exposed to tested *D. magna* in all experiments, was examined to confirm its effects on the gene expression. As shown in the Figure 4, there were no significant differences of the expression level of all five selected genes in *D. magna* after a 21d exposure to 0.02% ethanol compared to the control.

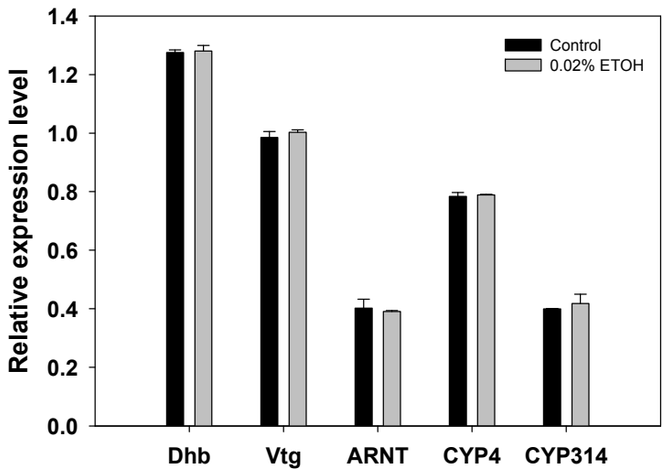

Fig. 4. Effects of 20% ethanol on the relative expression levels of 5 selected genes.

In case of methidathion, the gene expression of the five genes can be examined for only daphnia exposed to 0.44 and 0.88 μg/L because all daphnia exposed to 4.4 μg/L methidathion were died before the day 21 (Fig. 6). Unlike glyphosate, which was tolerantly influenced on the gene expression, methidathion significantly induced the expression level of the five genes when daphnia exposed to low concentration (0.44 μg/L), but reduces the expression level of those genes when daphnia exposed to the higher concentration (0.88 μg/L). It can be explained that methidathion may damage the normal functions of DNA in *D. magna* when the exposure concentration reaches to the 0.88 μg/L. In the other hand, the exposure to the 0.44 μg/L methidathion up-regulated the expression of the *Dhb*, *Vtg*, and *CYP314* genes stronger than that of the *Arnt* and *CYP4* genes. Notably, while the chronic toxicity assay based on the conventional endpoints such as growth, survival, and reproduction gave out some general information about the impacts of glyphosate and methidathion on the physiological behaviors of *D. magna*, the study on expression pattern of the five selected genes including *Dhb*, *Vtg*, *Arnt*, *CYP4*, and *CYP314* may provide a better insight into the action mode of these pesticides.

Hemoglobin is an important protein for the oxygen transport system of animals. Its expression is usually upregulated by the chemicals that reduce the oxygen level in aquatic environment (Ha et al. 2009). In this study, the expression level of *Dhb* was not changed after 21d exposure to the three concentrations of glyphosate (Fig. 5a), but slightly upregulated in response to 0.44 μg/L methidathion (Fig. 6a). This indicates that methidathion may have more possibility than glyphosate to inhibit the regular interaction between hemoglobin and free oxygen molecule in the aquatic environment. Vitellogenin, which its production is controlled by some estrogen hormones (Jones et al., 2000; Matozzo et al, 2008), is considered as a biomarker for estrogenic compound. In this study, while expression level of *Vtg* gene reduced in response to acute toxicity of glyphosate (Fig. 2b) and methidathion (Fig. 3b), that increased when exposing organism to the low concentration of these pesticides for 21days (Fig. 5b and 6b). This indicates that chronic toxicity of both glyphosate and methidathion may stimulate the synthesis of some estrogenic compounds in *D. magna.* The aryl hydrocarbon receptor nuclear translocator (*Arnt*), which plays a role in the synthesis of the enzymes involved in metabolizing aryl hydrocarbons (Tokishita et al.,

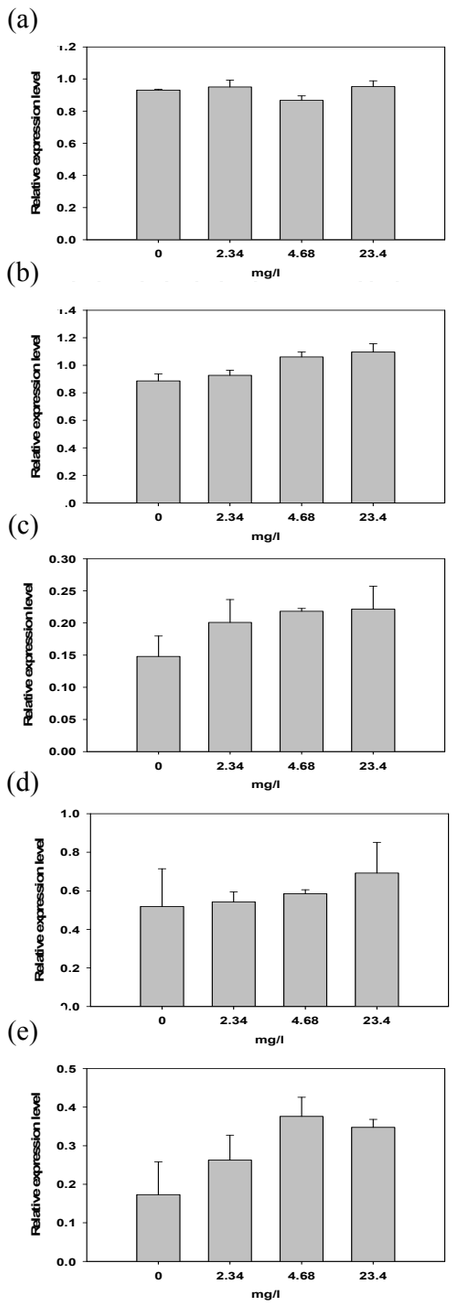

Fig. 5. Relative expression levels of 5 selected genes after 21d exposures of 0, 2.34, 4.68 and 23.4 mg/L glyphosate. (a) Dhb, (b) Vtg, (c) Arnt, (d) CYP4 and (e) CYP314. All of the data correspond to the expression level relative to the Act gene.

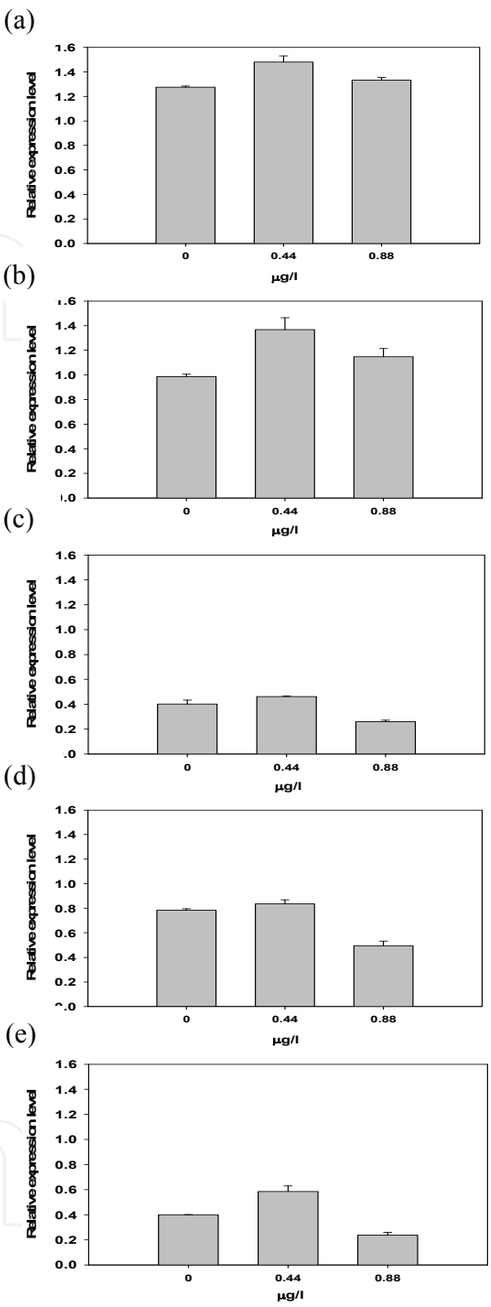

Fig. 6. Relative expression levels of 5 selected genes after 21d exposures of 0, 0.44, and 0.88 µg/L methidathion. (a) Dhb, (b) Vtg, (c) Arnt, (d) CYP4 and (e) CYP314. All of the data correspond to the expression level relative to the Act gene

2006), was differently regulated by the short-time and long-time exposure to both glyphosate and methidathion (Fig. 2c, 3c, 5c, and 6c). Particularly, the expression level of *Arnt* gradually decreased in *D. magna* when these organisms were exposed to the high doses of pesticides for 24h (Fig. 2c, and 3c), but it slightly increased when exposing to the low concentration of glyphosate (2.34μg/L) and methidathion (0.44μg/L) for 21d (Fig. 5c, and 6c). These results suggested that the low concentrations of glyphosate and methidathion could activate the expression of the *Arnt* gene in *D. magna* while the exposure to high dose of these pesticides even just for the short time can post some adverse effects on the molecular responses in organism. Although both proteins encoded by *CYP4* and *CYP314* genes belong to a same class of proteins that respond to the hazardous effects of toxic chemicals (Snyder, 2000), their different families caused different susceptibilities to pesticides. Particularly, in the 21d exposure to the low concentrations of glyphosate (Fig. 5d) and methidation (Fig. 6d), expression level of *CYP4* gene was slightly raised while that of *CYP314* was substantially increased compared to control. Therefore, *CYP314*, which is required for the conversion of ecdysone to active form (Shen et al., 2003), could be a more sensitive indicator to glyphosate and methidathion than *CYP4*, which metabolizes endogenous compounds such as fatty acids and steroids (Brandfield et al., 1991).

4. Conclusions

Glyphosate and methidathion, which have widely been used in agriculture to protect the crop productivity, are contaminating the aquatic environment through some main routes such as spray drift, volatilization, drainage, and leaching (Cerejeira et al., 2003; Pereira et al., 2009). Commonly, the toxicity tests based on the physiological responses (e.g., survival, growth rate, and reproduction) have been used to examine the biological effects of chemicals (Sakai, 2002; De Schamphelaere, 2004). In this study, the results indicated that both glyphosate and methidathion posted some adverse effects on the non-target aquatic organisms (e.g., *D. magna*) by both the acute and chronic toxicity. However, these physiological responses primarily result from molecular responses such as changes in gene expressions that happen in organisms in exposure to toxicants. Therefore, the changes in gene expressions should be more sensitive indicators than conventional endpoints to assess toxicity of chemicals. While *D. magna* showed specific response patterns according to the toxic effects caused by glyphosate and methidathion, the manners of expression for *Dhb*, *Vtg*, *Arnt*, *CYP4* and *CYP314* were quite different in *D. magna*. In the 24h exposure, the high concentrations of glyphosate and methidation may damage the regular functions of DNA in *D. magna*, and that result in the significant down-regulation of expression of the selected genes. *CYP4* and *CYP314* most sensitively responded as biomarkers to identify glyphosate and methidathion among the various pesticides. However, the results of the 21d exposure indicated that the low concentration of these pesticides induced the gene expression in daphnia. Among the five selected genes, *Vtg* and *CYP314* genes are substantially upregulated by both glyphosate and methidathion in long time exposure. Probably, the daphnia has a specific mechanism to adapt to the toxic influences of both glyphosate and methidathion. Therefore, the results of this study suggested that the differences between the gene expression patterns caused by glyphosate and methidathion must be considered to further understand the action mode of these chemicals and the specific molecular responses in *D. magna* as well. In further study, the effects of these toxic chemicals on the expression level of some other genes (not only the five genes used in this study) as well as their protein

expression level should be investigated to understand molecular mechanisms underlying the ability of *D. magna* to adapt to the toxic environments.

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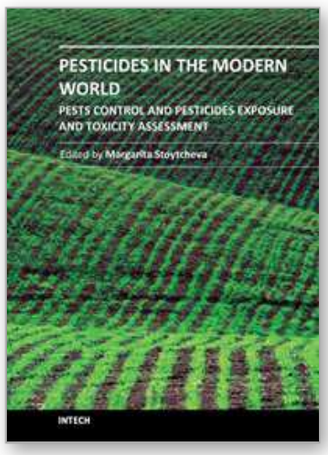

Pesticides in the Modern World - Pests Control and Pesticides Exposure and Toxicity Assessment

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The present book is a collection of selected original research articles and reviews providing adequate and up-to-date information related to pesticides control, assessment, and toxicity. The first section covers a large spectrum of issues associated with the ecological, molecular, and biotechnological approaches to the understanding of the biological control, the mechanism of the biocontrol agents action, and the related effects. Second section provides recent information on biomarkers currently used to evaluate pesticide exposure, effects, and genetic susceptibility of a number of organisms. Some antioxidant enzymes and vitamins as biochemical markers for pesticide toxicity are examined. The inhibition of the cholinesterases as a specific biomarker for organophosphate and carbamate pesticides is commented, too. The third book section addresses to a variety of pesticides toxic effects and related issues including: the molecular mechanisms involved in pesticides-induced toxicity, fish histopathological, physiological, and DNA changes provoked by pesticides exposure, anticoagulant rodenticides mode of action, the potential of the cholinesterase inhibiting organophosphorus and carbamate pesticides, the effects of pesticides on bumblebee, spiders and scorpions, the metabolic fate of the pesticide-derived aromatic amines, etc.

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