Doctoral Dissertation

Ecotoxicogenomics study combined with Cytochrome P450 mutagenesis on sublethal effects of the model herbicide atrazine in *Daphnia magna*

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Chapter 1 General Introduction

1.1. Environmental risk assessment in aquatic system

Over the last few decades, risk assessment has been a widely-practiced field in evaluating the hazard of chemicals either to human health or to ecological system. To examine harmful effects of these chemicals to ecological receptors, laboratory toxicity test is a common practice to perform. This kind of toxicity test can be done easily in a laboratory as long as the compound of interest and model organisms are present. Toxicity test is usually performed by directly exposing the organism to the chemical inside their living medium. In the case of aquatic trophic level as shown in 0, laboratory toxicity tests are usually performed by single-species tests. In these tests, a fish, an invertebrate species or algae is picked as a model organism and chemicals are exposed inside the water. The toxicity of a chemical is then measured

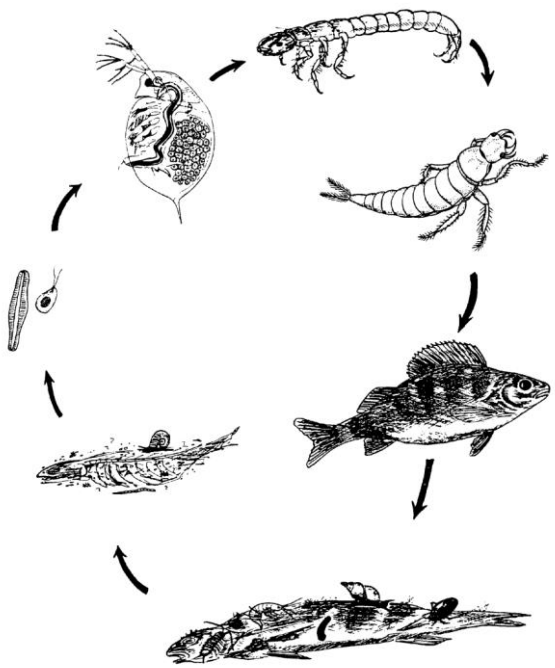

Figure 1. Simplified aquatic food chain
consisting of primary producers (various species of algae), herbivores (daphniids), primary carnivores (caddisfly larvae), secondary carnivores (beetle larvae), tertiary carnivores (fish) and decomposers [1].

through mortality, decreased growth rate and lowered reproductive capacity [1]. The guidelines of these toxicity tests are standardized based on Organisation for Economic Co-operation and Development (OECD) guidelines.

Generally, there are two kinds of toxicity tests, those are acute toxicity test and chronic toxicity test. Acute toxicity is performed by doing exposure in a short period of time, usually less than 24 h, and observing the effect in less than 14 days [2]. By performing acute toxicity test, chemicals concentration that causes lethality of a certain population percentage could be measured. In this case, the endpoint of the test is the amount of population. On the other hand, chronic toxicity test is done in longer period compared to acute toxicity test. This test is performed to examine the sub-lethal effect of chemicals, for example towards organism's reproduction, life cycle or behaviors. In aquatic toxicology, this sub-lethal effect assessment is crucial because it will set the chemical lower concentration limit that can be present in aquatic system. With these two kinds of toxicity tests, minimum and maximum concentration that may present in aquatic system can be decided easily. But, because of its simplicity where only single species is present in the test, it lacks approach toward how the chemicals affect in an ecosystem.

If we look back at aquatic food chain as shown in 0 where different kinds of organisms exist, interaction between organisms is one of the main factors for population change in ecosystem. Some studies reveal that the presence of these interactions together with chemical exposure can result in more severe effect to a certain population [3]–[5]. The concept of direct and indirect effect can also be related to risk assessment. Direct effect refers to the effect of certain chemical to a more sensitive target. For example, herbicide was applied to crop field to kill wild grass or weed. In this case, herbicide target organism is plant organism or microalgae presents in the water. Its non-target organism is organism that may present on the time of herbicide application but actually is not its target. For example, fishes or insects are present in the crop fields. Although these organisms are not the main targets, but these chemicals can also

affect them, which is called indirect effect of chemicals [6]. Another concept of indirect effect is described as when chemical give effects on more tolerant species by a number of ecological mechanisms (for example producer and consumer relationship) [6]. This kind of indirect effect though cannot be detected by simple single species toxicity tests.

Both direct and indirect effect of many chemical compounds are widely studied in aquatic system organism. For herbicide, both its direct effect on phytoplankton and indirect effect on zooplankton are well studied [6]. But how herbicide will affect zooplankton through phytoplankton pathway, especially in sublethal level, is yet to be clarified (Figure 2).

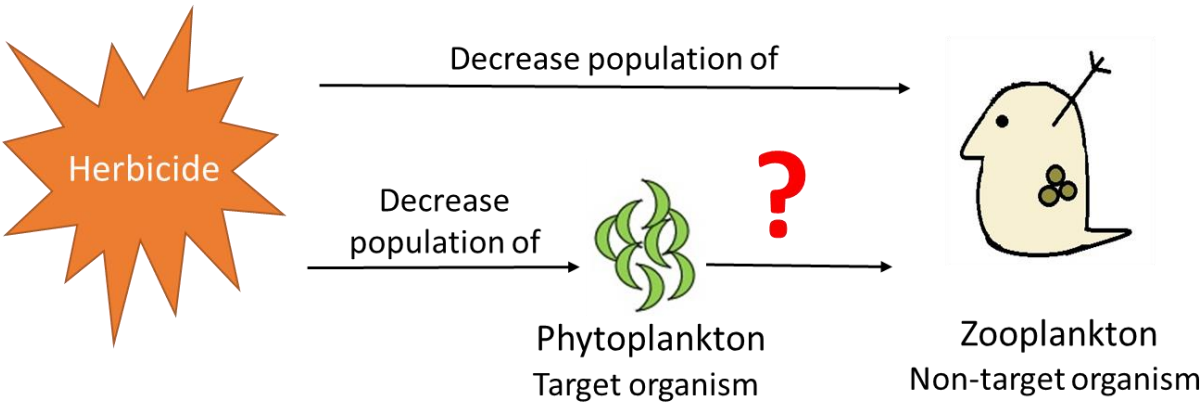

Figure 2. Direct and indirect effect of herbicide

1.2. Herbicide usage in environment

Herbicide is a kind of chemical which is commonly applied for agricultural use. The objective of herbicide application is to eliminate weeds or other unwanted plants which can affect the growth or crops production. These weeds can absorb essential nutrient in the soil or water, therefore resulting in inability of crop plants to get sufficient nutrient for growth. With increasing population of the world estimated to reach 9.3 billion people by 2050 [7], food necessities are increasing as well, undoubtedly increasing the agricultural application of herbicide. In 2017 world-wide, total pesticide usage reached 4 million tons and 400,000 tons

belonged to herbicide [8]. This enormous amount of usage can give side effect to the ecosystem. As the usage of herbicides is increasing, it is necessary to estimate the effect to environment.

Take an example of atrazine, second mostly used herbicide in the USA [9]. Atrazine is a triazine class of herbicide with usage reaching at least 13,000 tons in all over the world [8] that inhibit photosystem II in plant cells, affecting the transport of electron and causing inhibition of photosynthesis [10]. Application of atrazine affects cell membrane, causing death of the plant cells. Atrazine was found to be effective in eliminating weeds in crop fields and its cheap price as well as easy application are the main reasons of why it became one of the most favorite herbicides, although recently its heavy usage was reported to cause effect other than eliminating wild plants. At 2001, atrazine was found to pollute most of drinking water in USA with concentration reaching 3 ppb [11]. Atrazine was also banned in Europe Union [12] implying its usage and effect to living organisms remains controversial.

Although its effects to human and other living organism remains unclear, many researchers suspect it as one of possible endocrine disruptor chemicals (EDC) in animals. A famous yet controversial study about atrazine revealed that exposure of atrazine on frog can cause demasculinization with concentration of 1 ppb [13]. Some other related studies also reveal the sub-lethal effect of atrazine. In the level of primary consumer in aquatic system for example, atrazine was found to increase male production in *Daphnia pulicaria* [14].

1.3. *Daphnia magna* as ecotoxicological model organism

The zooplankton crustacean *D. magna* has long been used as a model for ecotoxicology studies. In freshwater trophic level, it serves as primary consumer, feeding on the primary producer (phytoplankton) by grazing. It has transparent body, making it possible to notice some phenotypic change such as hemoglobin accumulation caused by hypoxic condition [15]. In addition, they are easy to be cultured in laboratory, and has high sensitivity to a wide range of

chemicals, making it a suitable model for risk assessment. Changes of phenotypes such as swimming capability and fecundity are standardized endpoints for the assessment of chemicals using *D. magna* [16], [17]. Its ability to switch its reproduction style from parthenogenesis to sexual reproduction is also a unique key which can be related to some undesirable environmental stressors (Figure 3). Since OECD's acute immobilization test protocol was publicized on 1984, in many earlier studies, researchers focused on *Daphnia*'s response towards chemicals, making this field advances rapidly. Moreover, since its draft genome sequence has been publicly available since 2016 [18] and genetic manipulation methods such as RNA interference [19] and genome editing including the CRISPR/Cas and TALEN systems have been established [20]–[22], revealing their molecular response to chemicals has been an engaging interest [23], [24]. With this, the term "ecotoxicogenomics" was introduced, trying to relate the toxicogenomic approach of aquatic invertebrate model *Daphnia* with ecological relevance (Figure 4) [25]. This term is also representing both mechanistic insight (molecular parameter) and ecological insight (ecosystem) linking between phenotypic and genotypic parameter of the animal model [23].

1.4. Xenobiotic biotransformation in *Daphnia*

In general, biotransformation is the process where both endogenous and exogenous substances that enter the body are changed from hydrophobic to hydrophilic molecules to facilitate elimination from the body [26]. This biotransformation is performed generally by two phases (Figure 5). Phase I reactions, mainly performed by Cytochrome P450 (CYP), involving hydrolysis, reduction and oxidation where these reactions expose or introduce a functional group (such as –OH, $-NH_2$, -SH, -COOH), while in phase II reactions include glucuronidation, sulfonation (sulfation), acetylation, methylation and conjugation with glutathione [27]. By

performing these reactions, the toxic effect of xenobiotic compounds will be less dangerous as it will be more hydrophilic and easier to be excreted from the cells.

Both vertebrates and invertebrates possess this modification system as a defense mechanism toward xenobiotic in their living environment. In vertebrate, especially in human, having knowledge of biotransformation (also commonly known as drug metabolism) is important to know which drug will inhibit or induce the system. By this, it can help minimizing the possible effect of various drug reactions and interactions [28]. While in invertebrate, especially for organisms used in risk assessment, learning more about xenobiotic metabolism will be necessary for designing toxicity test, development of biomarkers, and modelling of chemical fate in ecosystems [29].

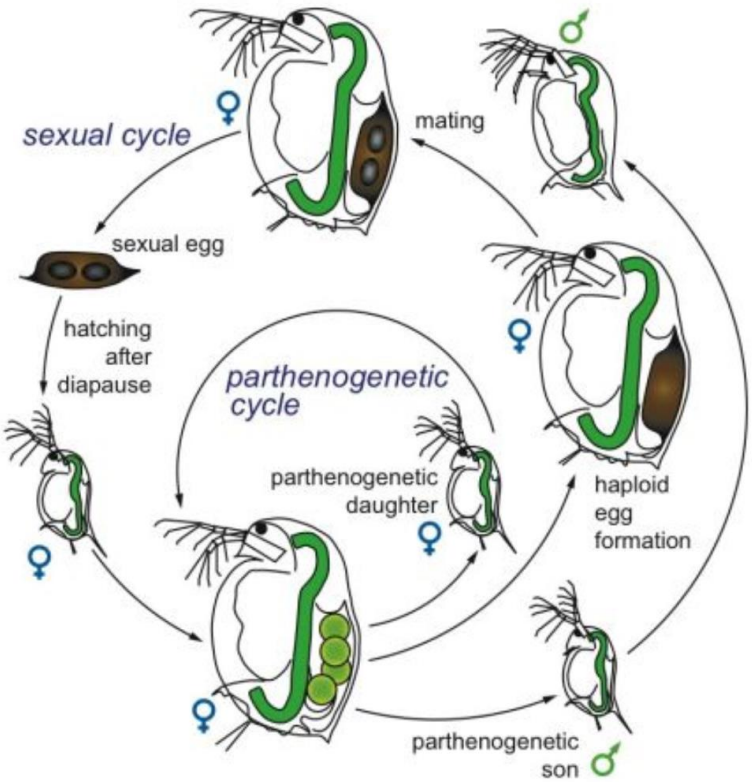

Figure 3. Life cycle of a cyclic parthenogenetic Daphnia [30]
In normal condition, Daphnia goes through asexual cycle (parthenogenetic) producing female clones. In undesirable condition, it will change to sexual cycle by producing male offspring and end up producing resting egg.

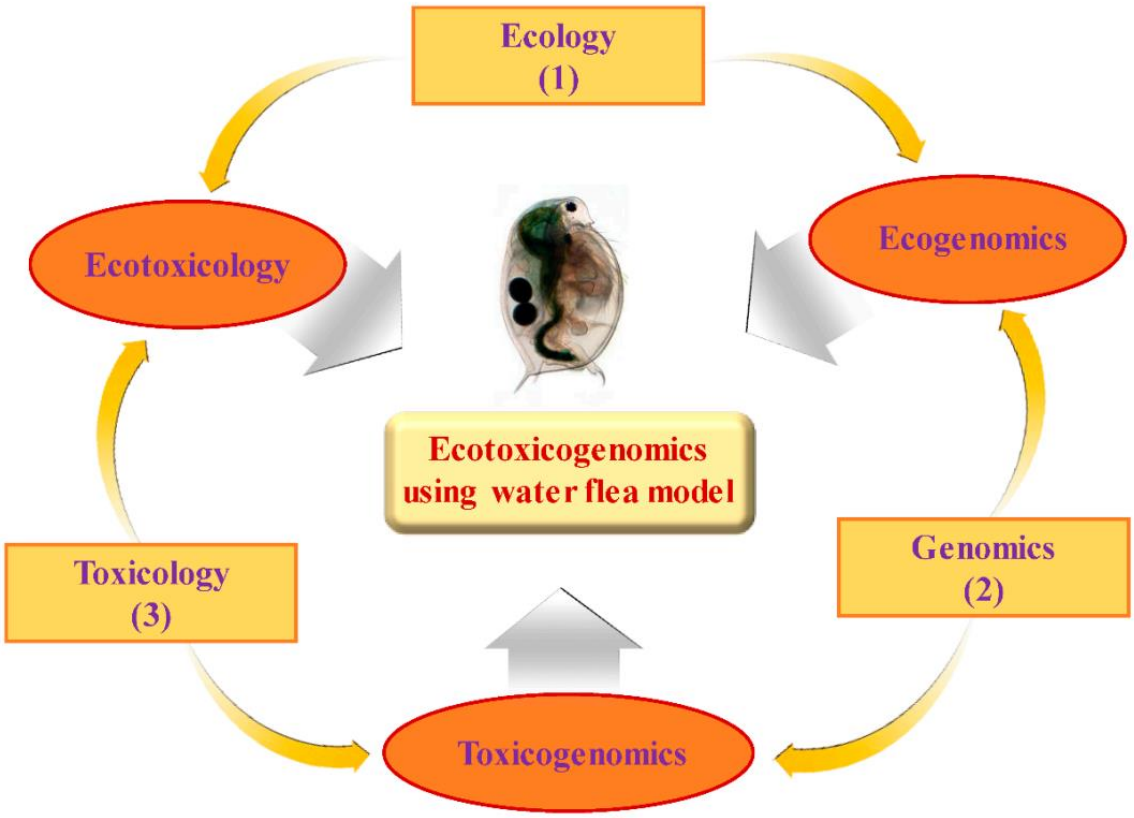

Figure 4. Illustration of conceptual ecotoxicogenomics using Daphnia model [23]
Primary study fields (rectangle) forms interdisciplinary fields (circle). Thick arrows indicate the tools that can be applied in the studies (1) ecological surveys, (2) genomic tools, (3) toxicity test

In *Daphnia*, several studies on xenobiotic biotransformation were performed. *D. magna* is able to metabolize pyrene into 1-hydroxypyrene-sulfate (most probably from phase II) and oxidized pyrene conjugate (from phase I) [31]. Metabolism of pyrene was found to be inhibited by CYP inhibitor, SKF-525A [31], showing CYP involvement in the process. Another study also showed possible CYP-dependent sulfate conjugation, and glucose conjugation (later confirmed to be suppressed under 20-hydroxyecdysone exposure) for testosterone metabolism in *D. magna* [32]. Although CYP involvement was predicted in these studies, the information on which CYP and phase II metabolism gene performing this biotransformation is lacking.

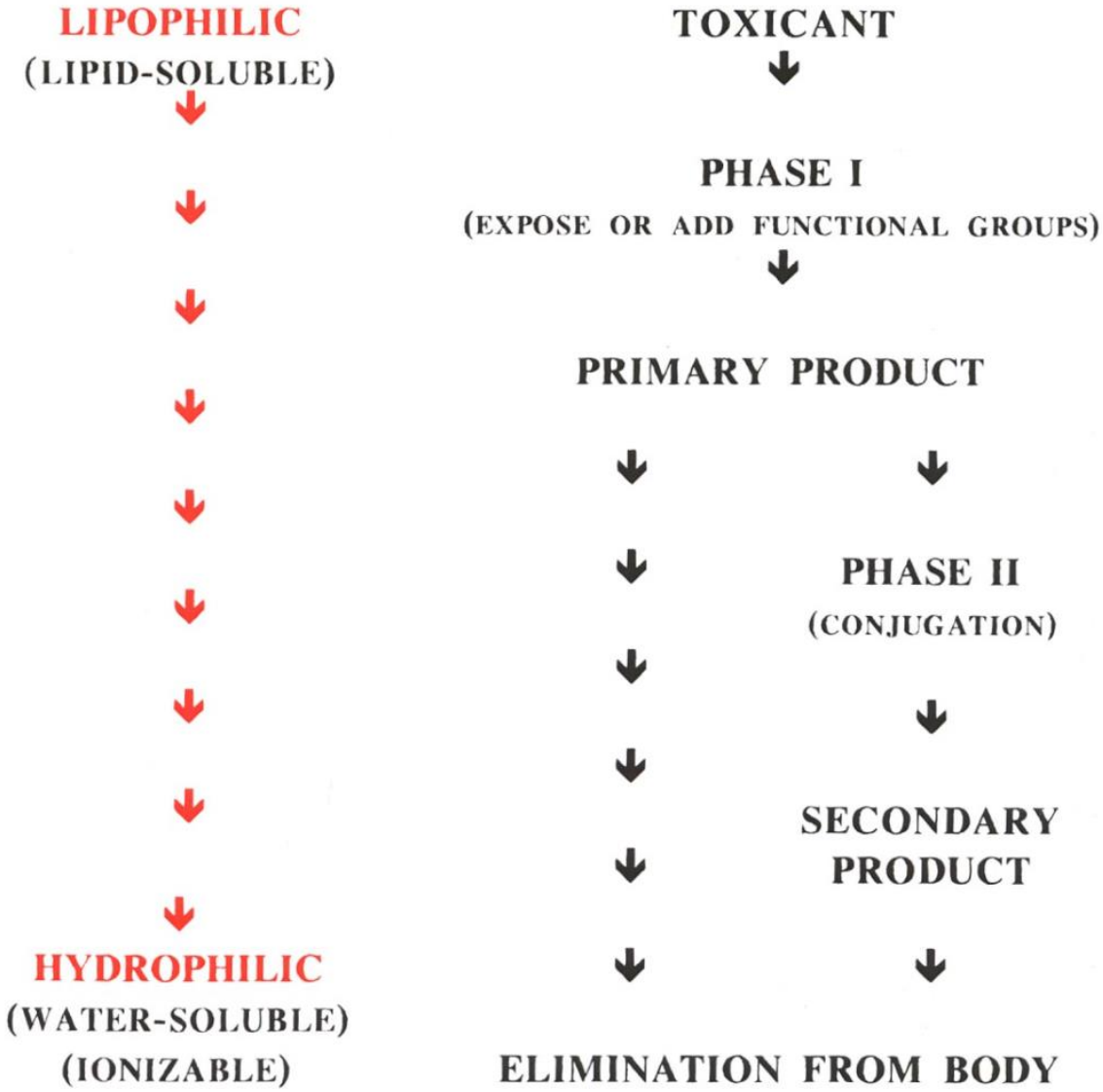

Figure 5. Simple schematic relationship among toxicants, phase I and phase II biotransformation, products, and elimination from the body in relation to lipophilic and hydrophilic characteristics [26]

1.5. Objective of this study

Atrazine as a commonly used herbicide need to be assessed as it has potential to pollute the environment, for example freshwater ecosystem. Moreover, *D. magna* as a primary consumer and microalgae as primary producer may present together in the ecosystem in this possible atrazine polluted scenario. This study aims to identify atrazine sub-lethal effect to *D. magna*,

especially through its food microalgae (Chapter 2). Furthermore, as xenobiotic detoxification is a potential defense mechanism in *D. magna*, exploring more into how xenobiotic will be metabolized especially by CYP is a part which is still unclear. I tried to identify a CYP gene that may have important role in xenobiotic metabolism (using atrazine as a model chemical) and introduce mutation on the candidate gene. Finally, the sensitivity of the mutant to chemicals was examined (Chapter 3).

Chapter 2 Atrazine causes production of non-viable juveniles in *D. magna*

2.1. Introduction

Atrazine is a commonly used herbicide that is often considered to be a potential endocrine disrupting chemicals (EDC), with several initial studies focused on reptiles such as alligators and frogs [13], [33], [34]. In reptiles, the studies focused on the effect of atrazine particularly on demasculinization, or association between presence of intersex individuals and atrazine detection in natural sites (e.g. lake or rivers). Later, in mammals atrazine was identified to affect testis weight increase, testosterone reduction, and significant decrease in number of sperm in mouse [35], [36], though another sub-lethal effect of dimorphic neurodegenerative profile in certain brain region was also found [37]. In invertebrates, atrazine was found to cause azoospermia and oocytes deformation in snail (*Biomphalaria alexandrina*) [38].

Studies of atrazine sub-lethal effect were also done in branchiopod *Daphnia* as ecotoxicological model in freshwater ecosystem. In *D. pulicaria*, atrazine increased the male production shown by higher sex ratio $\left(\frac{\text{number of male offspring}}{\text{number of total offspring}}\right)$ when exposed to minimum of 0.5 ppb atrazine [14]. In *D. magna*, exposure of 500 µg/L atrazine to embryo caused high abnormality percentage [39]. Altogether, these studies showed direct effect of atrazine to *Daphnia*, especially when it is present in the water. In fact, if we look into the ecosystem, the potential exposure will happen not only to the *Daphnia*, but also to its prey, phytoplankton. As some studies on sub-lethal effect by direct exposure of *D. magna* were already done previously, in this study I will focus more into how xenobiotic, especially herbicide atrazine as a model chemical, will affect *D. magna* through food in sub-lethal level.

A previous study from Lampert et al. (1989) [40] is probably the earliest to investigate indirect effect of herbicide on zooplankton by using a complex system featuring both phytoplankton and zooplankton and model herbicide (Figure 6). In treatment A, the

zooplankton medium containing atrazine and unexposed phytoplankton was continuously supplied to zooplankton population. In treatment B, phytoplankton was directly exposed to atrazine. The atrazine-free zooplankton medium and the exposed phytoplankton were used for zooplankton culture. This resulted in 1.5-5 times population declining on B treatment compared to A treatment [40].

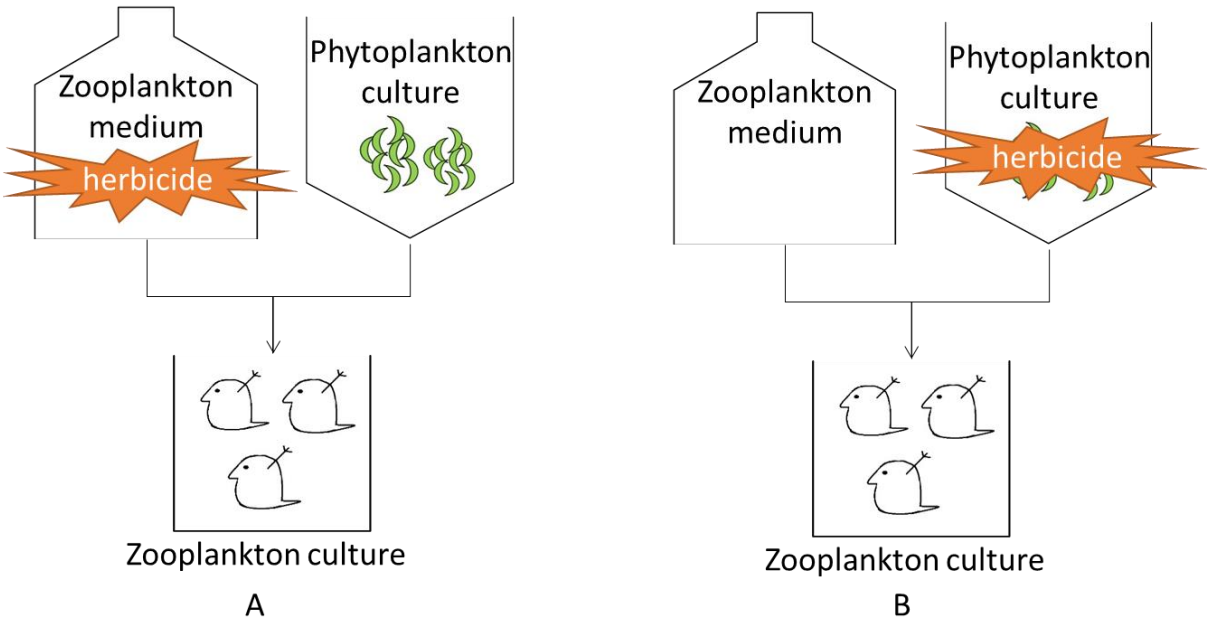

Figure 6. Continuous flow system with herbicide, phytoplankton and zooplankton that present at the same time [40] **(with modification)**

From this study, we can know that when herbicide is present together with its target and non-target organism, it resulted not only in decreasing population of phytoplankton as target organism but also to the non-target organism. Herbicide atrazine in fact has a very high EC_{50} to *D. magna* when it is exposed directly to the atrazine which is 10 mg/L [40]. Meanwhile, with this complex system, effective concentration decreased to 50 µg/L [40]. This endpoint of population size is a very common measure of chemical risk assessment. Though 50 µg/L atrazine can be considered sublethal in a normal toxicity test set up, this complex system gave

a pretty high lethality level. This study though doesn't clarify what other sublethal effect resulted from this system aside of decreasing population.

Sublethal effect which are defined as effects (physiological or behavioral) on individuals that survive exposure, should be considered for a complete analysis of a chemical [41], especially for a commonly used chemicals such as herbicides. In investigating more into the sublethal effect in the previous study's complex system (Figure 6), we should consider what actually happens in the system. I raise three scenarios which may have happened inside this system. First, there is the decreasing of phytoplankton amount (limited food condition to the daphniid). Second, there is the herbicide contaminating the medium directly. Third, there is atrazine-exposed microalgae which enter the zooplankton body and may cause both lethal and sublethal effects.

In this study, I focused on investigating the sublethal effect of model herbicide atrazine in zooplankton *D. magna* through its food. Learning more about this will help us elucidating how atrazine affect *D. magna* sublethally from various route, especially via food route. I used a simpler system where atrazine was exposed to microalgae, harvested and fed to *D. magna* in reproduction test. Survival rate of the exposed daphniids and their fecundity were evaluated based on OECD guideline on *D. magna* reproduction test 2012.

2.2. Materials and methods

2.2.1. Phytoplankton and zooplankton strains

Phytoplankton *Raphidocelis subcapitata* was used in this research. *R. subcapitata* (Korshikov) Hindák NIES-35 was obtained from National Institute for Environmental Studies (NIES), Japan. Zooplankton *D. magna* was also obtained from NIES, Japan. *D. magna* was cultured using Artificial Daphnia Medium (ADaM) [42] with density of 80 daphniids in 5 L ADaM. Culturing tanks were kept in a room maintained at 22°C with 16 h light and 8 h dark

cycle. *D. magna* was fed everyday with $7x10^6$ cells per daphniid and ADaM was changed weekly.

2.2.2. *R. subcapitata* culturing

All *R. subcapitata* culturing was done in C medium (Table 1) [43]. Pre-culture was prepared in 200 mL C medium by picking up colonies from C agar medium. After green color could be seen clearly from culture, concentration of pre-culture was measured by measuring optical density at wavelength of 680 nm (OD_{680}) using Biochrom WPA CO7500 Colorimeter (Tokyo, Japan). OD_{680} then used to measure concentration with equation 2.335×10^7 (OD_{680}) – 1.42 x105 [44]. Batch culture was then prepared by transferring a certain volume from pre-culture stock to a new 500 mL C medium so in this new medium initial concentration reached 5×10^4 cells/mL [17]. Batch culture condition is 22 – 23ºC, light intensity of 96 µEm-2s-1, 14 h light and 10 h dark cycle [45] and continuous supply of 4% CO_2.

To start continuous culture, 6 L C medium was prepared in 6 L media storage bottle. The medium was flowed through silicon tube by peristaltic pump until the culturing flask with flowrate around 500 mL/day. 2 L storage bottle was prepared for harvesting. With this system, 500 mL microalgae culture could be obtained every day. 4% CO_2 gas was supplied to the culturing flask. Air flowrate was maintained at 750 mL/day and CO_2 at 30 mL/day. Millex vent filter unit (Millipore, Billerica, USA) was connected to the air flow to filter air supply. The whole system could be seen in Figure 7.

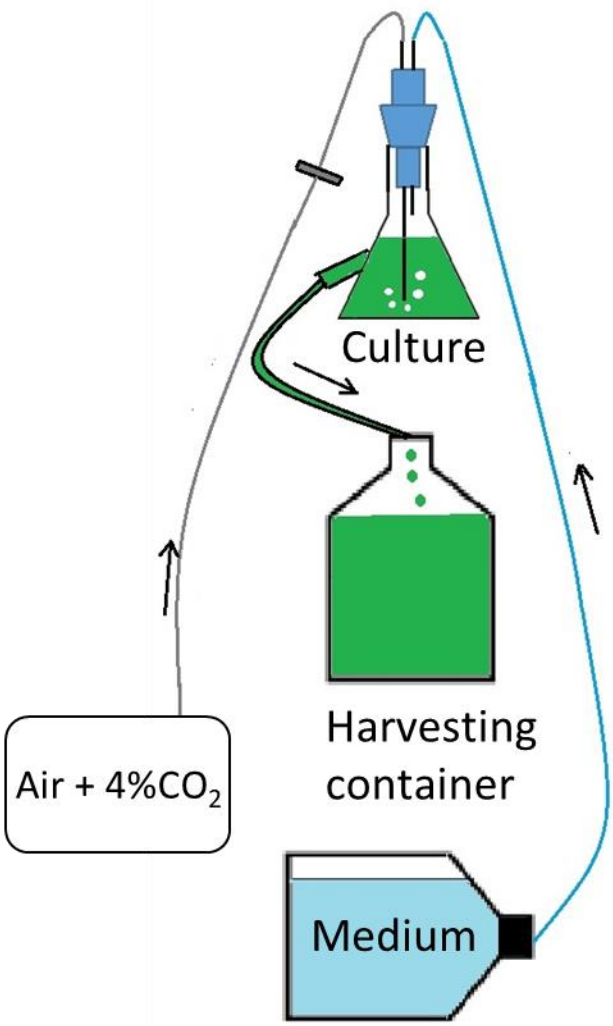

Figure 7. Microalgae continuous culturing system
Top : 500 mL culturing flask, center : Harvesting vessel, bottom : 6 L medium vessel. Medium, air, and harvested biomass flow direction are shown by arrow.

Table 1. C medium recipe

Compound	Weight	Diluted using milliQ to	Volume used for 1 L medium	Provider
KNO_3	5.0 g	50 mL	1 mL	Wako Pure Chemical, Osaka, Japan
β-Na_2glycerophosphate. $5H_2O$	2.5 g	50 mL	1 mL	Apollo Scientific, Manchester, UK
$Ca(NO_3)_2.4H_2O$	7.5 g	50 mL	1 mL	Nacalai Tesque, Kyoto, Japan
$MgSO_4.7H_2O$	2.0 g	50 mL	1 mL	
Tris (hydroxymetyl) aminomethane	2.5 g	50 mL	10 mL	
Agar	1.5% weight of total volume			

After mixing all solutions, pH was measured. 1 N HCl was added so that medium pH reached 7.5. Medium was then autoclaved at 121°C for 20 min. After cooling down, PIV metals and vitamin mix solution was added. 3 mL PIV metals and 1 mL vitamin mix solution was added for 1 L medium. PIV metals and vitamin solution recipe can be seen in Table 2 and Table 3.

Table 2. PIV metals recipe

Compound	Weight	Provider
$Na_2EDTA.2H_2O$	100 mg	Nacalai Tesque, Kyoto, Japan
$MnCl_2.4H_2O$	3.6 mg	
$ZnCl_2$	1.04 mg	
$FeCl_3.6H_2O$	19.6 mg	Wako Pure Chemical, Osaka, Japan
$CoCl_2.6H_2O$	0.4 mg	
$NaMoO_4.2H_2O$	0.25 mg	
Add MilliQ until	100 mL	

Table 3. Vitamin mix solution recipe

Compound	Weight	Diluted using milliQ to	Take from stock to make mix vitamin solution	Provider
Biotin	5 mg	50 mL	5 µL	Nacalai Tesque, Kyoto, Japan
B12	5 mg	50 mL	5 µL	
Thiamine	5 mg	50 mL	500 µL	Wako Pure Chemical, Osaka, Japan
Add MilliQ until		5000 µL		

2.2.3. Microalgae growth inhibition test

Microalgae growth inhibition test was performed to know what concentration of atrazine will fit the condition of getting enough biomass to be harvested for *D. magna* feeding in reproduction test, but also still causing growth inhibition for microalgae. Six different concentration of atrazine were exposed to microalgae culture (50, 100, 150, 300, 450, and 600 µg/L). Microalgae was prepared as batch cultures with 0 µg/L treatment as a control. Atrazine (Wako Pure Chemical, Osaka, Japan) was diluted in dimethylformamide (DMF) (Nacalai Tesque, Kyoto, Japan) because of atrazine's low solubility in water. Atrazine was exposed 48 h after inoculation and all cultures start with initial concentration of 5 x 10^4 cells/mL. DMF was exposed in control treatment with same volume as in exposed cultures. After 72 h of exposure, final concentration was measured by OD_{680}. Growth inhibition was calculated using this formula [46]

$$\mu_{i-j} = \frac{\ln X_j - \ln X_i}{t_j - t_i} (day^{-1}),$$

where:

μ_{i-j} is the average specific growth rate from time i to j;

X_i is the biomass at time i;

X_j is the biomass at time j.

2.2.4. Microalgae continuous culture with atrazine exposure

Two batch cultures of microalgae were prepared in 500 mL flask. After 48 h culturing, one of the cultures was exposed to atrazine with concentration that were decided based on experiment 2.2.3. Control culture was added with DMF with same volume in exposed culture. After 78 h, culturing flasks were connected to continuous supply of microalgae medium. For preparation

of atrazine-exposed microalgae, atrazine was added to the medium. Biomass was obtained every day and centrifuged with 9000 rpm 4°C for 40 min. Microalgae pellet was collected and washed 3 times using MilliQ by centrifugation 9000 rpm 4°C for 10 min each time. Washed pellet was diluted with MilliQ and stored in 4°C before feeding. Cell concentration was measured before proceeding to *C* and *N* content measurement.

2.2.5. Microalgae carbon (*C*) and nitrogen (*N*) content measurement

To check the quality of microalgae that were fed to *D. magna*, *C* and *N* content was measured weekly. This step is also important to decide on how many cells should be fed to *D. magna* because 0.2 mg *C* is needed for feeding to each daphnia every day. Microalgae with amount of 5 x 10^{7} cells was filtered using glass microfiber filters (Whatman, Maidstone, UK) using a small system shown at Figure 8 and heated overnight at 60°C. Samples were then covered with tin foil discs ultralight weight (Elemental Microanalysis, Devon, UK). Samples were analyzed using FLASH 2000 Organic Elemental Analyzer (Thermofisher Scientific, Cambridge, UK). *C* and *N* content were measured by using Eager Xperience Software.

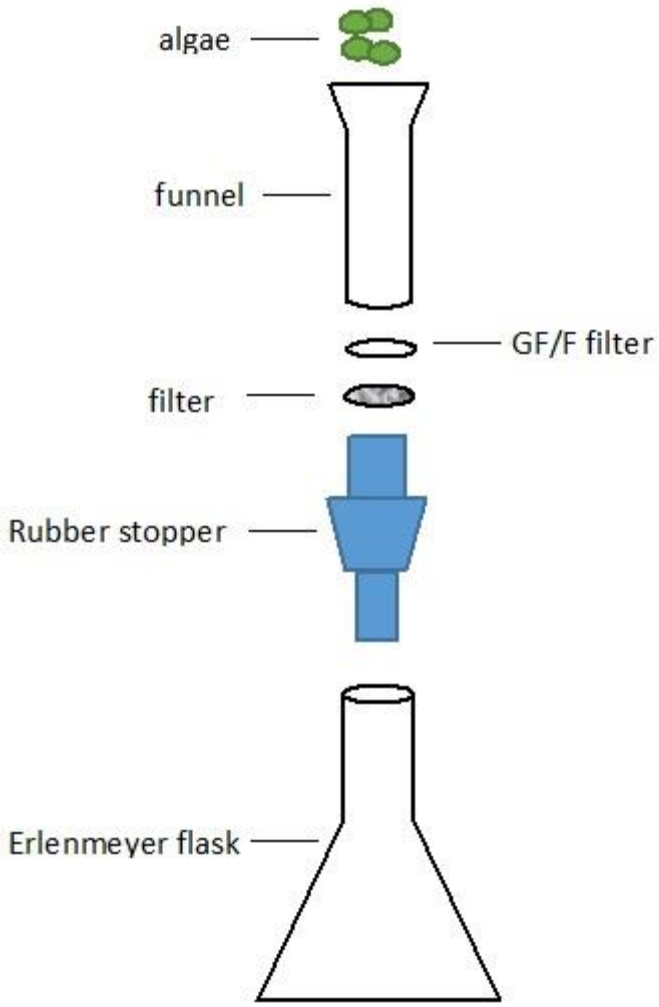

Figure 8. GF/F system to collect microalgae biomass
Filtering system is set up as in the picture. Algae is flowed through funnel, passed by GF/F filter and metal filter. Filtered water will be flowed to the erlenmeyer flask. Biomass was collected on GF/F filter surface will be later dried and used for *C* and *N* analysis.

2.2.6. *D. magna* reproduction test with atrazine exposed microalgae

This experiment was performed to examine the sub-lethal effect of atrazine exposed microalgae on *D. magna*. Reproduction test was performed on 10 daphniids individually for 21 days. Each daphniid was placed inside 50 mL falcon tube (Fisher Scientific, New Hampshire, US). Along the experiment, all daphniids were kept in 22°C incubator with 14 h light and 10 h dark cycle. 0.2 mg *C* of microalgae was fed for each daphnia each day. Artificial Daphnia Medium (ADaM) was used as a culturing medium [42]. For direct exposure on *D. magna*, atrazine was

dissolved in ADaM to reach the concentration equal with decided concentration on experiment 2.2.3. Four different treatments were done for each 10 daphnids: normal microalgae feeding and normal ADaM; atrazine exposed microalgae and normal ADaM; normal microalgae feeding and atrazine supplemented ADaM; atrazine exposed microalgae feeding and atrazine supplemented ADaM. Medium was changed daily, resulting in continuous exposure of atrazine to treated daphnids. During experiment, *D. magna* condition was observed and number of offspring was recorded for 21 days.

2.2.7. Atrazine measurement by Gas Chromatography and Mass Spectroscopy (GCMS)

Analysis was performed in Muranaka Laboratory, Osaka University, to examine atrazine presence inside microalgae cells. Microalgae biomass was harvested and washed three times with distilled water. OD_{680} was measured and 4 x 10^9 cells pellet was prepared. 2 mL ethyl acetate (Nacalai Tesque, Kyoto, Japan) and 1 mL methanol (Nacalai Tesque, Kyoto, Japan) was added. All solvents are 1st Grade Purity (Analytical method):≧99.0%(GC). Samples were vortexed for 5 min and centrifuged with 2400 G for 5 min. Organic solvent phase was recovered. Extraction steps were repeated for three times and all organic solvent phase were collected. One gram Na_2SO_4 (Nacalai Tesque, Kyoto, Japan) was added to crude extract to decrease water content. Na_2SO_4 was washed with 3 mL ethyl acetate. This washing ethyl acetate was added to crude extract. Crude extract was then evaporated using rotary evaporator to dryness and rehydrated with 3 mL of hexane continued with 3 mL of ethyl acetate, each for three times. Hexane phase was eluted in Sep-Pak C18 cartridge (Water, Massachusetts, USA). Ethyl acetate phase also eluted in the same cartridge. Ethyl acetate phase was then evaporated and rehydrated with 1 mL ethyl acetate for GCMS injection sample.

Extracted samples and standard samples were injected to HP-5MS column with splitless injection (5977A Series GC/MSD System, Agilent Technologies, USA). The GC temperature

program was: 90°C held for 2.0 min, then at 10°C/min to 250°C, 250°C held for 5.0 min, and at 40°C/min to 300°C, 300°C held for 5.0 min [47]. Full-scan MS was conducted to confirm atrazine detection. Selected ion monitoring (SIM) mode was used for more sensitive analysis. Two ions at *m/z* 200.1 and *m/z* 215.1 were selected. Analyses were performed using Agilent MassHunter Qualitative Analysis Software.

For atrazine measurement from *D. magna* embryos, I collected 1st until 3rd clutches (300-600 embryos for each clutches) from 80 *D. magna* mothers that were fed with atrazine exposed microalgae. I sent these samples to our collaborator (Nisso Chemical Analysis Service). Our collaborator performed extraction and LC-MS/MS analysis to examine the presence of atrazine inside the embryo samples. Ultrasonic treatment was used for extraction using 1.5 mL acetonitrile in 2 mL sample tube. Crude extract was filtered through 0.45 µm diameter hydrophilic PTFE filter. Filtered extract was applied to LC-MS/MS. Mass spectrometer used is API4000 (AB-SYX) with LC-20AD Pump (Shimadzu Corporation), constant temperature oven CTO-20A (Shimadzu Corporation), and autosampler SIL-20AC (Shimadzu Corporation). LC condition is as follows: Column: Inertsil ODS-SP 2.1 mm I.D. x 150 mm, particle size 3 µm (GL science); Column oven temperature: 40°C, mobile phase Milli-Q and acetonitrile (1:9 ratio); flow rate 0.2 mL/min; injection volume: 20 µL. MS/MS condition is as follows: Ionization method: ESI, Positive; measurement method: MRM; Monitor ion: NA-89: 216.1 (m/z) > 174.2 (m/z). Data processing software used is Analyst 1.6.2.

2.3. Results

2.3.1. Microalgae growth inhibition test

Microalgae growth inhibition test was performed to check which atrazine concentration can be used for exposure experiment. Optimal concentration should be high enough to give inhibition to *R. subcapitata* culture but also low enough to let sufficient biomass production

every day for daphnia feeding. Six different concentrations were picked (50, 100, 150, 300, 450, 600 µg/L) and exposed at 48 h after inoculation. This experiment was divided into two sets. First and second sets included higher (300, 450, 600 µg/L) and lower concentrations (50, 100, 150 µg/L) respectively. Final cell concentration and total inhibition was measured 72 h after exposure. With concentration of 300 until 600 µg/L, as shown in Figure 9, there was a high inhibition where cell concentration almost didn't increase for 72 h after exposure started. When maximum flowrate (F_{max}) that can be used in the system was calculated (Table 4), all the F_{max} value were under 500 mL, which is the limit to maintain stable flowrate of continuous system in Watanabe Laboratory. If these concentrations were picked, there is high possibility that culture washing out will occur, causing not enough biomass production every day.

On the other hand, with lower atrazine concentration of 50, 100 and 150 µg/L, inhibition reaching almost 27% still can be detected. As shown in Figure 9, exposed cultures also still showed exponential phase even after exposure started. All of F_{max} value reached more than 500 mL/day, which means with these atrazine concentration both stable flowrate and specific growth rate (µ) can be maintained in the continuous system. With this condition, similar biomass volume and cell concentrations could be obtained every day. There is also lower probability of culture flushing out to happen. From this experiment, highest concentration where the continuous culture still could be maintained with highest inhibition percentage was picked, which was 150 µg/L.

Table 4. Microalgae inhibition on different atrazine concentration

Atrazine concentration (µg/L)	X after 72 h (cells/mL)	Specific growth rate µ (/hour)	Doubling time (hour)	F_{max} (mL/day)	% inhibition
0	7.12 x10^7	0.0595	11.6	714.2	0%
50	2.10 x10^7	0.0495	14.0	594.2	17%
100	1.46 x10^7	0.0465	14.9	558.3	22%
150	1.00 x10^7	0.0435	15.9	521.5	27%
300	6.54 x10^7	0.0396	17.5	475.4	33%
450	4.20 x10^7	0.0360	19.2	432.3	39%
600	3.97 x10^7	0.0356	19.5	426.8	40%

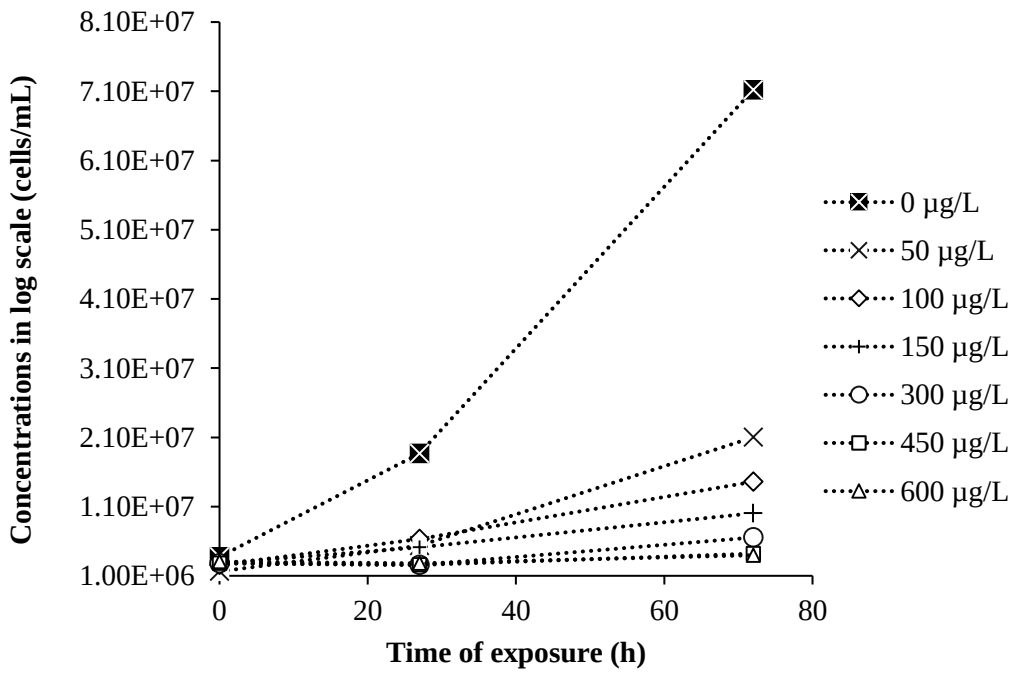

Figure 9. ***R. subcapitata*** **growth when exposed to various concentrations of atrazine**
Graph shown in cell concentrations (log scale) after atrazine exposure for 72 hours. Cell measurement was conducted at 24 and 72 h.

Continuous system for both control microalgae and atrazine exposed microalgae showed stable growth in the means of biomass concentration for 21 days experiment (Figure 10). Stable condition of atrazine exposed microalgae also showed by carbon and nitrogen

content measured weekly over the 21 days experiment. Atrazine exposed microalgae showed no significant changes of *C*:*N* ratio among weekly samples (Figure 11), although control microalgae showed noticeable change of these contents. Possible explanation of this result is because growing inhibition caused by atrazine resulted in slower growth rate, therefore prolonging the age of the exposed culture. On the other hand, normal microalgae cells grew and divided normally, causing the culture to become old faster. Although the change of this quality was quite unexpected, same amount of *C* could be fed to daphniids. Therefore, it's decided to keep continuing the experiment with this continuous system.

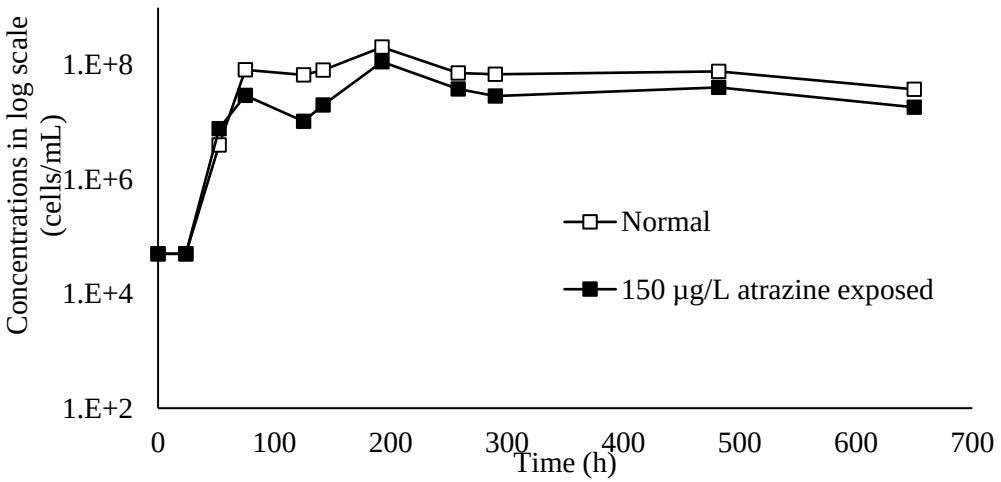

Figure 10. *R. subcapitata* growth in continuous system when exposed to atrazine of 150 µg/L

Graph shown in cell concentration in log scale for 650 h cultivation. Atrazine was exposed at 52 h and continuous culture was started at 75 h.

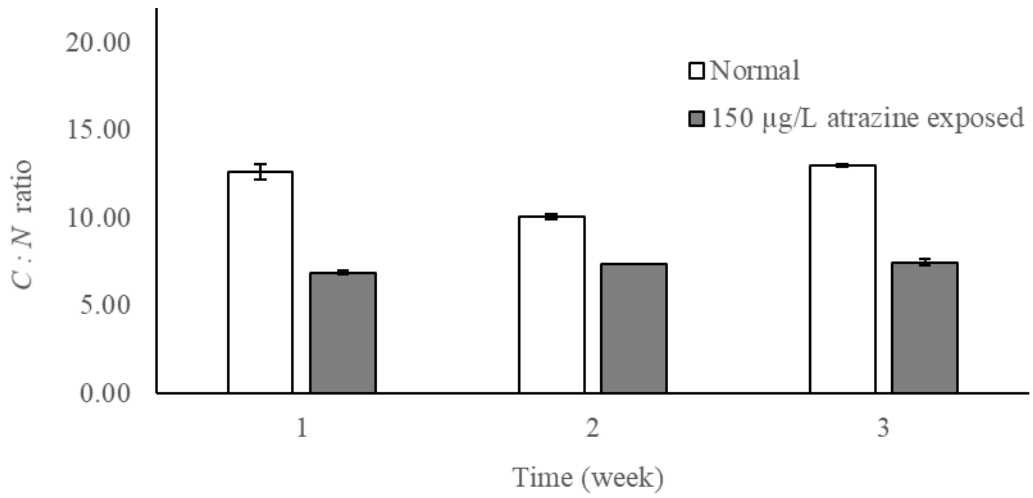

Figure 11. ***R. subcapitata C* : *N* ratio over 3 weeks' exposure of 150 µg/L atrazine**
Graph is shown as *C : N* ratio, calculated from mol *C* divided by mol *N* for each week microalgae data. Week 1 data is for first week feeding of daphniid, and so on until Week 3.

2.3.2. Atrazine measurement in microalgae cells

To examine whether atrazine was retained inside algae cells, GCMS analysis was performed. Algae cells were collected and extracted. Treated extract were then injected to GCMS. SCAN method in MS was used first to check all peaks present in the sample. After getting a peak known as atrazine, SIM method was used to examine peak in much smaller quantity. Atrazine chromatogram obtained by SIM method could be seen on Figure 12. Ion spectra result could be seen from Figure 13, where ion spectra from atrazine exposed microalgae was identified as atrazine based on National Institute of Standards and Technology (NIST) MS Library. From GCMS result it was found that atrazine was retained inside microalgae cells, possibly becoming the reason of offspring non-viable phenotype. This result was in accordance with previous study where it was found that green microalgae has the ability to accumulate atrazine inside its cells [48].

Possible amount of atrazine accumulation was also measured by preparing several concentrations of atrazine standard for making the standard curve that was prepared by using

SIM method. From this calculation, amount of atrazine accumulated was calculated to be 4 x 10^5 pg/cell.

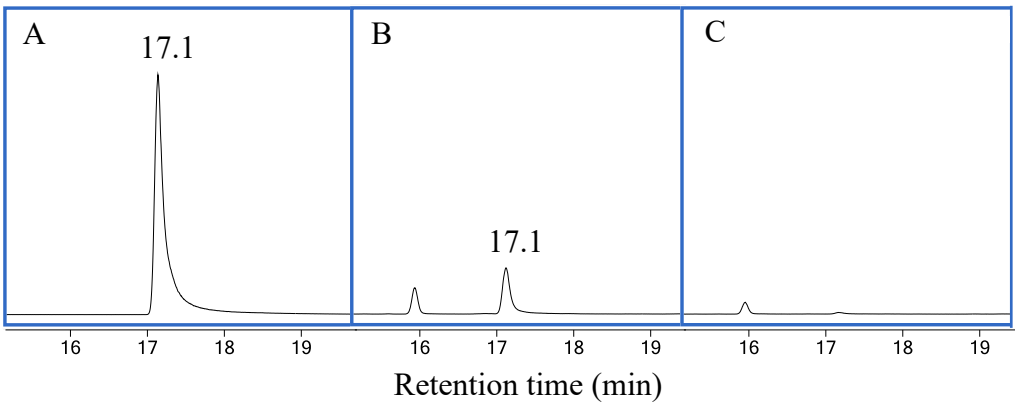

Figure 12. GCMS chromatogram of atrazine detection using SIM method
(A) Atrazine standard 1 ppm (B) atrazine exposed microalgae (C) non-exposed microalgae

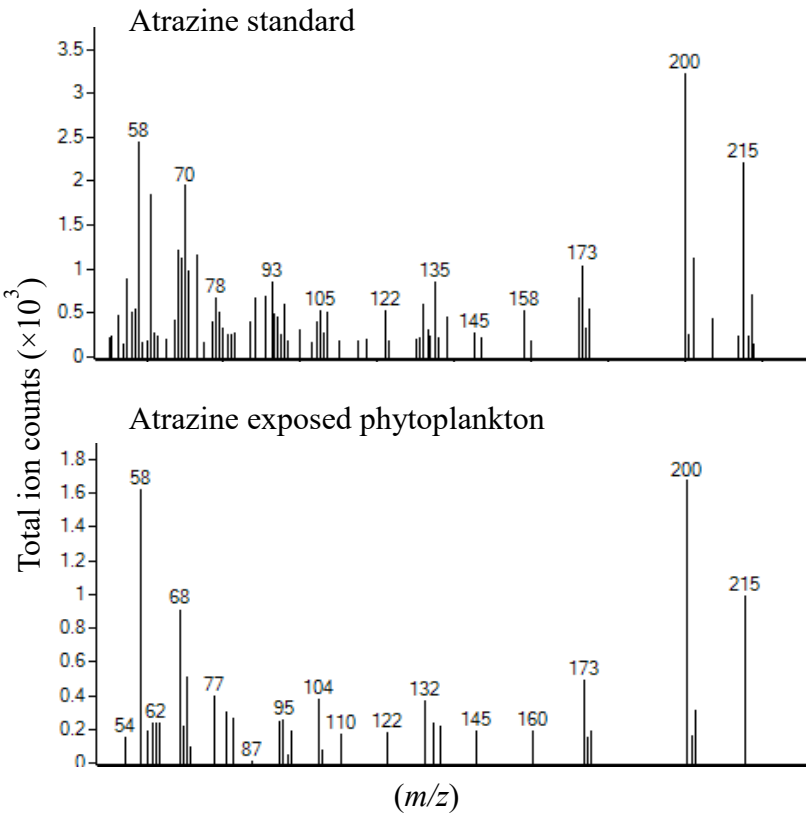

Figure 13. Product ion spectra of (A) atrazine standard and (B) atrazine extracted from atrazine exposed *R. subcapitata*
Full SCAN method and selected ion monitoring (SIM) mode was used. Two ions at *m/z* 200.1 and *m/z* 215.1 were selected for atrazine specific detection

2.3.3. Atrazine exposed *R. subcapitata* increased *D. magna* offspring number

Reproduction test was performed to check the effect of atrazine exposed microalgae to daphniid population and also to individual daphniid. Test was performed for 21 days and four different treatments as mentioned in method section were given to daphniids. From this experiment, two endpoints were examined: parents' survival rate and offspring production. Results showed that either direct exposure of atrazine or the indirect exposure mediated by *R. subcapitata* didn't affect significantly on parent's survival rate as shown in Figure 14. Most of parents were surviving through the test and normal condition. Parents didn't show any abnormality or unhealthy condition and could produce offspring.

Unexpected condition were found on the offspring, where non-viable offspring were found in all of the treatments. Non-viable offspring was found to have abnormality on body shape, second antennae and also spine shell, as shown on Figure 15. Although amount of total offspring is increased in all atrazine treated samples (Figure 16), the production of non-viable offspring also increased significantly (ANOVA, Tukey HSD post hoc, $p<0.05$) from mother that fed with atrazine exposed food as shown by Figure 17. Based on this result, I conclude that atrazine exposed microalgae could give more severe effect on *D. magna* compared to direct exposure of atrazine.

I found that atrazine-exposed algae generally have higher *N* content and lower *C* content, resulted in lower *C : N* ratio (Figure 11). The decrease in *C* content was probably caused by the inability to fix carbon due to the inhibition of photosynthesis. Decrease in *C* content was also reported in other microalgae cells with concentration of atrazine reaching 200 µg/L [49]. Another report also showed decrease in *C : N* ratio caused by atrazine in *Chlamydomonas reinhardtii* [50]; although in this study, the lower *C : N* ratio was solely affected by the decrease in *C* content and a slight decrease in *N* content.

I noticed that, after certain days, non-viable juveniles were not produced, which means that most offspring were alive and could swim. Therefore, the number of the non-viable offspring was counted per each clutch until 5th clutch which is the last clutch for 21 days. As shown in Figure 18, it was found that there was a larger number of nonviable juveniles on 1st clutch. This amount was then decreased on 2nd clutch and 3rd clutch and mostly no more non-viable juvenile were found on 4th and 5th clutch.

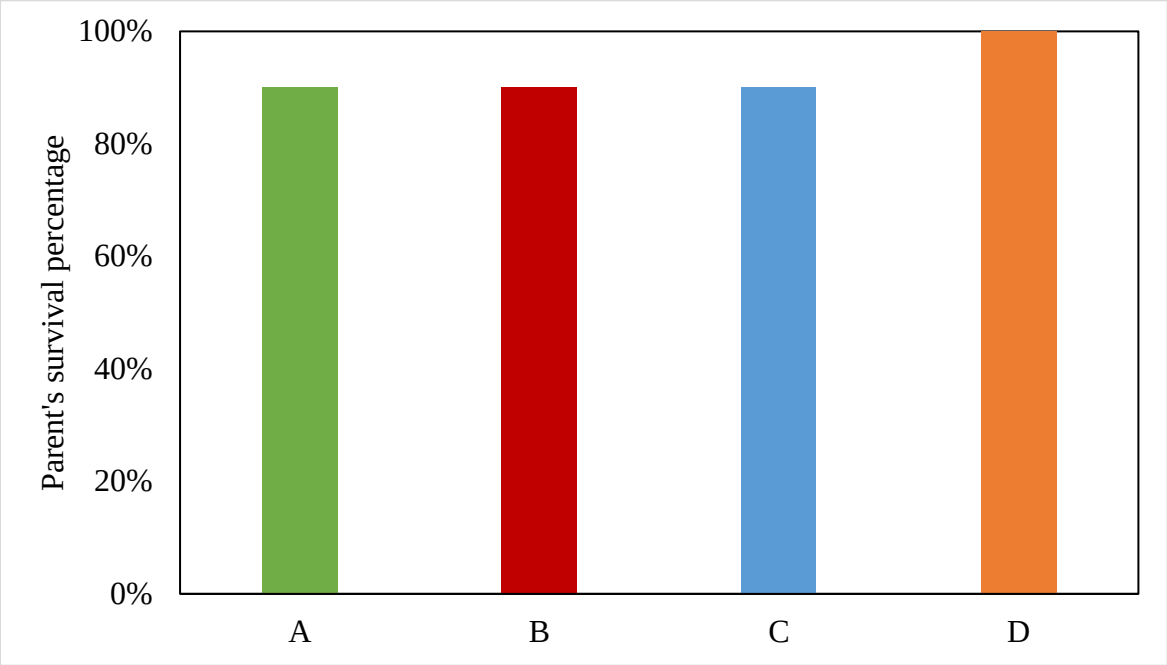

Figure 14. Parents' survival percentage on four different treatments
(A) Normal ADaM and normal microalgae (control), (B) atrazine exposed ADaM and normal microalgae (direct exposure), (C) normal ADaM and atrazine exposed microalgae (indirect exposure), (D) Atrazine exposed ADaM and atrazine exposed microalgae (combined exposure).

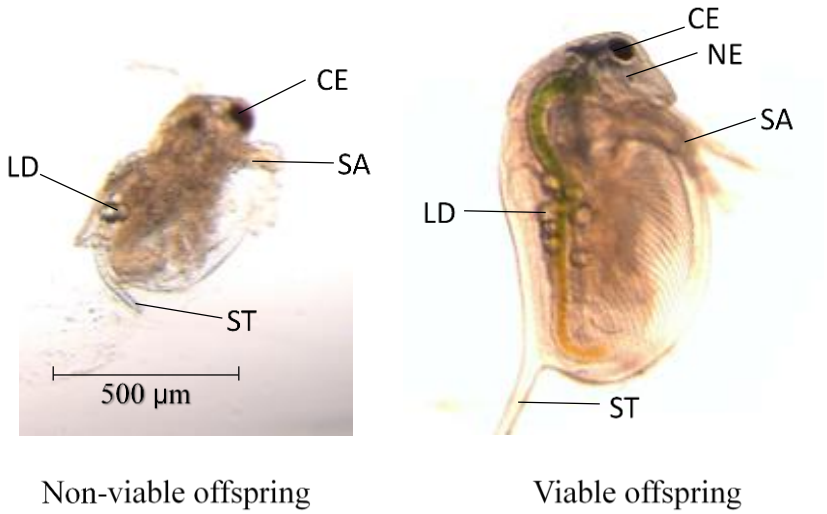

Figure 15. Phenotype of non-viable offspring produced by atrazine treated mother compared with viable one (<24 h since released from brood chamber).
CE: Compound eye; NE: Naupliar eye; SA: Second antennae; ST: Spine tail; LD: Lipid droplets.

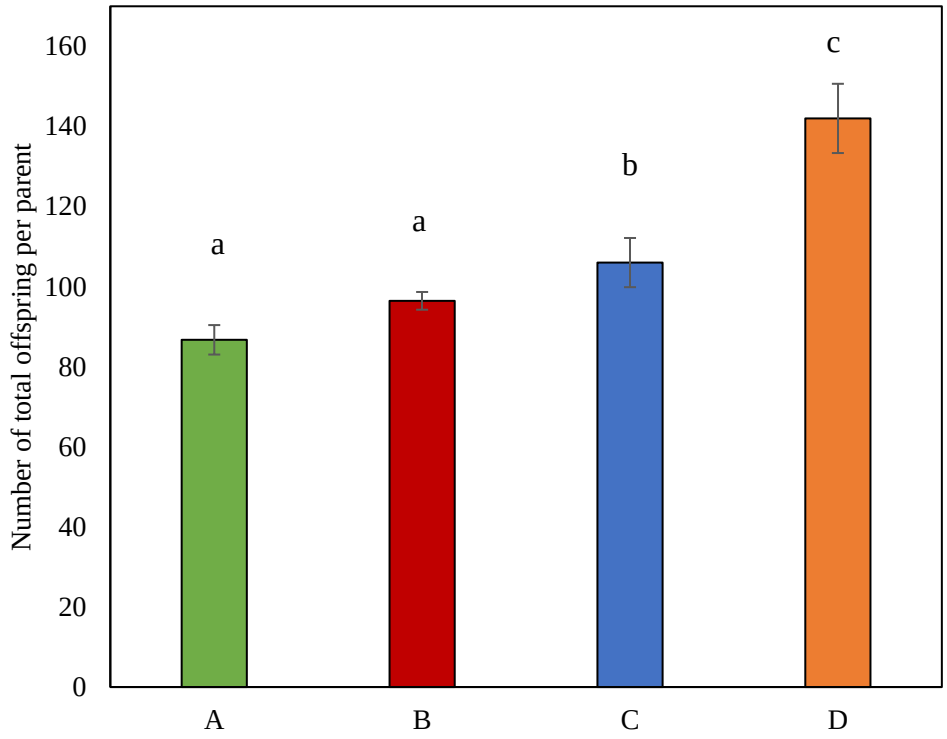

Figure 16. Number of total offspring in each treatment after 21 days.
(A) Normal ADaM and normal microalgae (control), (B) atrazine exposed ADaM and normal microalgae (direct exposure), (C) normal ADaM and atrazine exposed microalgae (indirect exposure), (D) Atrazine exposed ADaM and atrazine exposed microalgae (combined exposure). Different alphabet show significant difference (ANOVA, Tukey HSD, $p<0.05$)

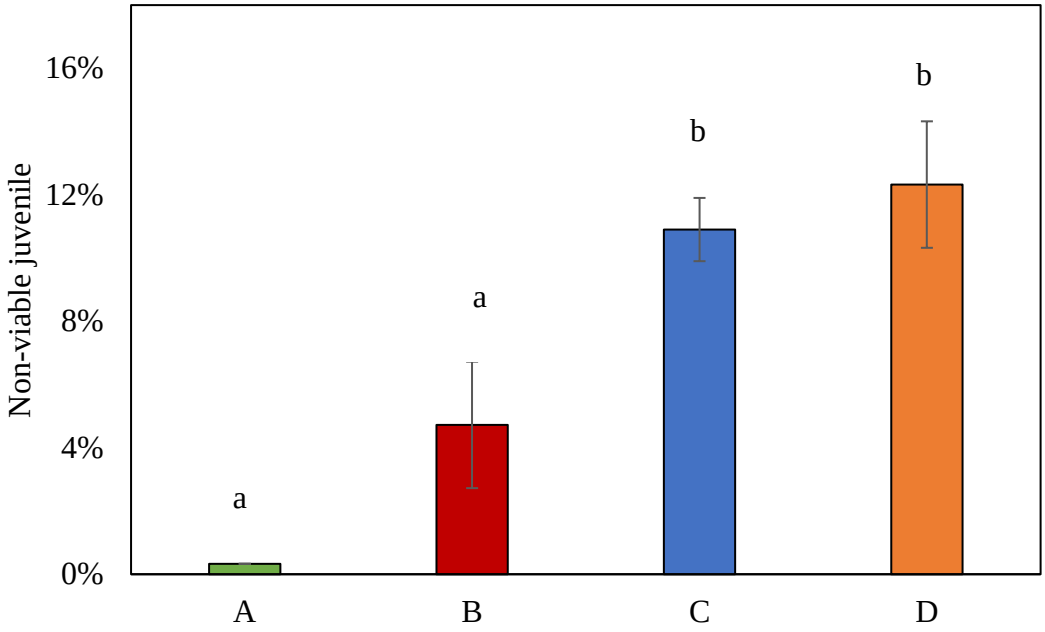

Figure 17. Percentage of non-viable offspring in each treatment.
Different alphabet show significant difference (ANOVA, Tukey HSD, $p<0.05$)
(A) Normal ADaM and normal microalgae (control), (B) atrazine exposed ADaM and normal microalgae (direct exposure), (C) normal ADaM and atrazine exposed microalgae (indirect exposure), (D) Atrazine exposed ADaM and atrazine exposed microalgae (combined exposure).

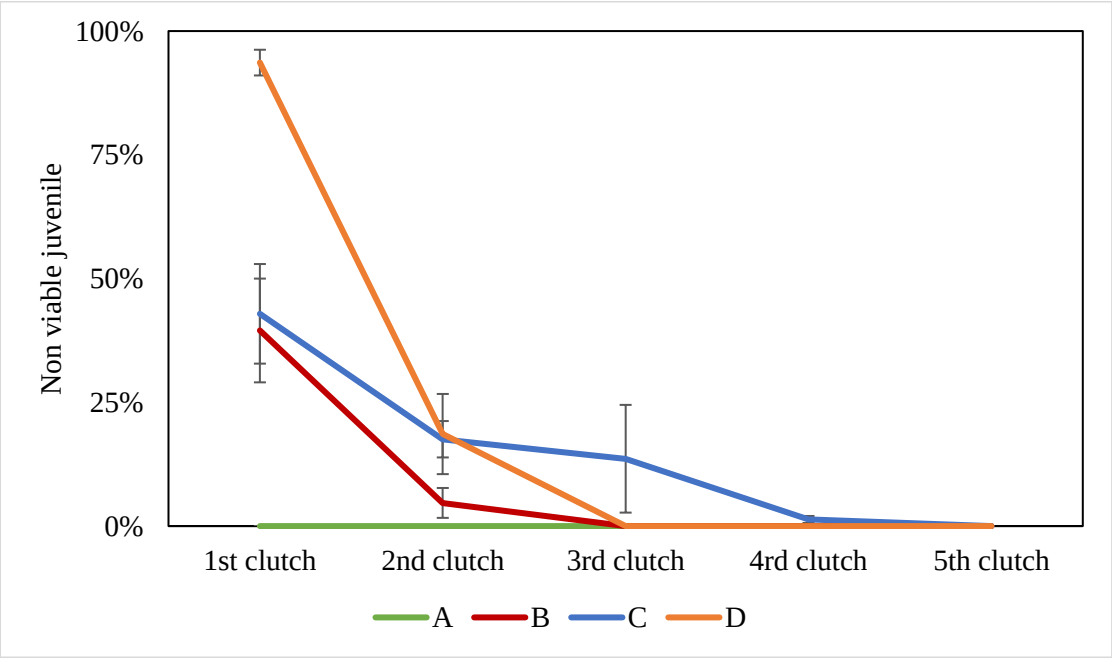

Figure 18. Number of non-viable offspring based on clutch production
(A) Normal ADaM and normal microalgae (control), (B) atrazine exposed ADaM and normal microalgae (direct exposure), (C) normal ADaM and atrazine exposed microalgae (indirect exposure), (D) Atrazine exposed ADaM and atrazine exposed microalgae (combined exposure).

2.3.4. Atrazine content remains the same among *D. magna* clutches

As earlier clutch has more severe phenotype, I hypothesized that the first clutch may have more atrazine content coming from initial accumulation of atrazine 6-7 days prior to ovulation. To confirm whether atrazine was transferred from the parents and present in embryos, I collected embryos sample from parents that were fed with atrazine exposed microalgae. ADaM medium was left untreated to leave detection bias coming from atrazine in the daphniid medium. Based on LC-MS/MS analysis result performed by Nisso Chemical Analysis Service, I found that among 1st until 3rd clutch of treated parents, all clutches had similar atrazine with no significant difference (Figure 19). This result did not represent the phenotype difference between 1st and 3rd clutch, as opposed to my hypothesis. This result suggests that atrazine content in embryos does not contribute to the severity level of embryo phenotype.

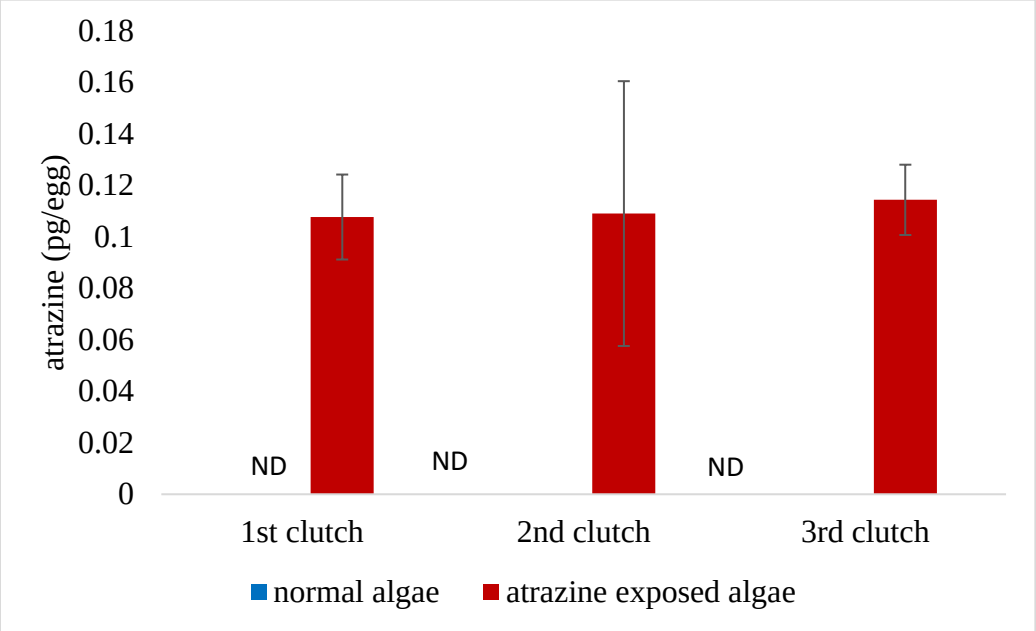

Figure 19. Atrazine content from 1st until 3rd clutch embryos
Atrazine content is shown in pg/egg by dividing total atrazine content in sample with amount of egg in each samples. (student T test, $p>0.05$) (ND = Not detected)

2.4. Discussion

To investigate on how atrazine affects *D. magna* through microalgae, I prepared atrazine exposed phytoplankton, and fed it to daphniid as a part of 21 days reproduction test. An environmentally relevant concentration of 150 µg/L, either exposed to *D. magna* medium or to phytoplankton, resulted in production of non-viable offspring. Based on observation of the non-viable offspring morphology, the inability of the embryo to develop until the final stage of embryogenesis is indicated. The previous study also reported that direct exposure of 0.5 mg/L atrazine led to toxicity on 80% of embryos [39]. Another study showed that direct exposure of 500 µg/L atrazine in 21-days reproduction test resulted in the decreased offspring numbers the in *D. magna* [51] though they didn't mention any non-viable offspring appearance. The production of non-viable offspring in this study has higher percentage in the 1st clutch, a sub-lethal effect that has never been identified before.

This result directed me to hypothesize that there was accumulation of atrazine inside mother's body when growing up and reaching maturity. This accumulation was then mostly released to the first production of offspring, but decreased in later clutches, leading to recovery of the offspring. Releasing of heavy metals to offspring was also found on previous study using *D. magna*, indicating *D. magna* ability of detoxification using reproduction as a pathway [52]. To confirm this hypothesis, I sent embryo samples from mothers that I fed with atrazine exposed phytoplankton to examine atrazine presence in the embryos.

LC-MS/MS result shows that all 3 clutches has similar tiny amount of atrazine remains, which rebut my hypothesis. This indicated that the presence of atrazine in all clutches is probably not the reason of different phenotype of the offspring. Some other reason can be considered, for example the maternal effects, which is a genetic or environmental differences in maternal generation that later expressed to the offspring with different phenotype [53]. Increased tolerance among F1, F2, and F3 generation to toxic *Microcystis* in *D. magna* was

also found when mothers were exposed with the same toxin [54]. Although this previous study did not show the difference of offspring's fitness over the clutches from same generation, it may be possible that transgenerational effects are applied at later clutches.

It may also be possible that detoxification system in mother maturates at later stages as *Daphnia* excretory organ complexity increase with aging [55]. In my result, there was no significant effect of atrazine exposed microalgae to daphniid parents, implying detoxification system may occur. Basically, organism can metabolize and detoxify xenobiotic by performing phase 1 reaction (oxidation, reduction, hydrolytic), and phase 2 reaction (conjugation) [56]. The products from these reactions will be later excreted from cells. Existing xenobiotic metabolism is a common sense which results in vitality of daphniid towards chemicals.

In conclusion, this chapter describes the effect of atrazine in sub-lethal level to *D. magna* through its food. The data suggests mother with different age releases offspring with different initial phenotype, though further investigation need to be performed to find the reason.

Chapter 3. Mutation of the highest expressed CYP gene, *CYP360A8*, increases sensitivity to atrazine in *D. magna*

3.1. Introduction

In previous chapter, one of the atrazine effect in sub-lethal level was described to be the production of non-viable offspring in *D. magna*. The exposed mother first release higher percentage of non-viable juvenile, then in older age release healthier offspring. We can see that even when exposed from juvenile age, atrazine hardly has effect to the exposed daphniid. Instead, it gives development effect to the earlier offspring produced. I hypothesize that recovery of the offspring phenotype by older daphniid rely on the detoxification system of the parent. Certain gene(s), may have role in the better response of older daphniid to atrazine. Studies on detoxification system in *D. magna*, especially in response to herbicide atrazine, though are still limited. This leads me to focus more on the phase 1 of detoxification mechanism in *D. magna* as it is the first reaction of xenobiotic metabolism.

Molecular mechanisms underlying xenobiotic metabolism have been studied for understanding and evaluating impact of chemicals on organisms. This metabolic reaction is divided into two phases, phase I (oxidation, hydrolysis, reduction) and phase II (conjugation). Some of cytochrome P450 (CYP) enzymes play an important role in the phase I reaction, where they perform biotransformation from more hydrophobic to hydrophilic compounds for avoiding accumulation of lipophilic chemicals [57]. CYP genes constitute one of the largest family of genes and, in animals, they are classified into 11 CYP clans each of which is composed of families and subfamilies [58]. These classifications are based on phylogenetics as well as sequence identity. Vertebrates have ten clans (2, 3, 4, 7, 19, 20, 26, 46, 51, and the mitochondrial) [57]. In human, some of the clan 2, the clan 3, and the clan 4 CYPs are used for metabolism of xenobiotics [59], [60].

In the branchiopod crustacean *Daphnia* that is closely related insects [61], the importance of CYP genes for xenobiotics detoxification has also been suggested. CYP activity was detected by the fluorometric assay using 7-ethoxycoumarin as a substrate [62], [63]. A CYP inhibitor, piperonyl butoxide, showed a potential role of CYP for toxaphene detoxification [64]. At a genetic level, several clan 4 CYP genes were cloned and their induction by polyphenols was observed [65]. Following genome sequence determination, all of CYP genes were annotated in *Daphnia pulex* [66]. As well as insects, *D. pulex* CYP genes belong to any of the 4 clans (2, 3, 4, and mitochondrial).

To find out how CYP genes respond to certain xenobiotics, microarray and RNA-seq analyses have been performed in *D. magna*. In juveniles, beta-naphthoflavone up-regulated several CYP genes including a gene that shows similarity to *D. melanogaster* clan 4 *CYP* named *CYP4C3* [67]. Another study showed that juvenile hormone agonist epofenonane exposure increased the expression level of the clan 3 *CYP3A89* ortholog [68]. These studies indicated the importance of CYP genes for chemical response in *D. magna*.

In case of atrazine, a genome-wide analysis by RNA-seq was previously done. In adults, the highest differentially expressed from CYP gene is clan 2 *CYP370A13* (3.4 fold upregulation), while the highest out of detoxification genes is phase II Aldo-keto reductase family 1 member B10/ADH A (14 fold upregulation) in response to atrazine [69]. This previous study didn't give the data on basal mRNA levels (both exposed and unexposed), making it hard to understand the real behavior of detoxification genes in *D. magna*. CYP genes in *D. magna* also had not been fully annotated yet, making it hard to know which CYP gene should be chosen as the gene of interest.

Investigating CYP function by reverse genetic tools can demostrate specific CYP gene function *in vivo* and further help us in understanding the molecular mechanism of xenobiotic detoxification. Similar strategy though has been done to examine the function of certain CYP

gene towards certain xenobiotic. In mouse for example, to understand xenobiotic metabolism, the CYP mutant models have been generated. The *CYP1A1* (clan 2) mutation resulted in more sensitivity to benzo[a]pyrene (BaP), a polycyclic aromatic hydrocarbon [70]. Disruption of the *CYP3A* (clan 3) cluster led to more sensitivity to chemotherapeutic agent docetaxel [71]. Mutants of the CYP6 family belonging to the clan 3 have been established in insects. In the cotton bollworm *Helicoverpa armigera*, the *CYP6AE* gene cluster knockout resulted in higher susceptibility to a group of host plant chemicals and insecticides[72]. In *Drosophila melanogaster*, the knockout mutant of the *CYP6A17* showed higher sensitivity to insecticide deltamethrin [73]. However, any function of CYP genes for detoxification of xenobiotics has not been studied using reverse genetics tools in *Daphnia*. As several genetic manipulation methods such as CRISPR/Cas mediated mutagenesis has been established in *D. magna* [20], [74], it is now possible to further investigate xenobiotics detoxification mechanism by establishing a CYP mutant in *D. magna*.

In this chapter, I tried to identify CYP gene which may have role in general detoxification mechanism in *D. magna*, especially towards herbicide atrazine. Finding an abundant expressed CYP gene is the first criteria, as abundance of CYP is one of the important factors for xenobiotic metabolism [75]. Referring to the sublethal effect of atrazine that I found in Chapter 2, the second criteria is to find a CYP gene which is upregulated in older *Daphnia* as it probably resulted in the production of healthier offspring. For this purpose, I tried to find out the desired CYP gene based on existing RNA-seq data in Watanabe laboratory followed by a temporal RT-qPCR analysis. In this chapter also, I described the CRISPR/Cas-mediated mutagenesis of the CYP gene of interest. The candidate CYP gene function was investigated in CYP mutant by exposing them to some model herbicides and comparing its response to wildtype *D. magna*.

3.2. Materials and Methods

3.2.1. Culture and maintenance of *D. magna*

D. magna was obtained from National Institute for Environmental Studies (NIES), Japan. *D. magna* was cultured using Artificial Daphnia Medium (ADaM) [42] with a density of 80 daphniids in 5 L ADaM. Culturing tanks were kept in a room maintained at 22-23°C with 16 h light and 8 h dark cycle. Each individual of *D. magna* was fed every day with 8 x 10^6 cells of commercially purchased *Chlorella vulgaris* (Oita-medakabiyori; Oita, Japan) per daphniid and the ADaM was changed weekly. The young offspring from culture were removed every day to make sure the same amount of food was supplied to the animal parents.

3.2.2. RNA-sequencing data analysis

RNA-seq data were analyzed by CLC Genomics Workbench software (CLC Bio; Aarhus, Denmark). *D. magna* genome database was used as a reference genome for mapping (http://arthropods.eugenes.org/EvidentialGene/daphnia/daphnia_magna/Genome/dmagna-v2.4-20100422-assembly.fna.gz). Annotation file was also used as the reference of gene locations in the genome sequences (http://arthropods.eugenes.org/EvidentialGene/daphnia/daphnia_magna/Genes/earlyaccess/dmagset7finloc9c.puban.gff.gz). Default mapping criteria were used (Mismatch cost of 2, insertion cost of 3, deletion cost of 3, length fraction of 0.8, similarity fraction of 0.8, maximum number of hits for a read of 10). Data were normalized by calculating Transcripts per Million (TPM), where,

$$TPM = \frac{RPKM \times 10^6}{\sum RPKM}$$

and where RPKM [76] is defined as,

$$RPKM = \frac{total\ exon\ reads}{mapped\ reads\ (million) \times exon\ length\ (KB)}.$$

TPM was calculated for each gene and annotated with *D. magna* gene annotation file. From the annotation file, a gene set was built by a student in our lab as described previously [77]. Some genes consist of two fragments in a distant position in the genome, and these split fragments are treated as two different genes. This resulted in an edited gene set from initial 17,228 to 47,109 unique genes.

Expression browser showing gene lists was then created by selecting gene expression level tracks and associated it with annotation source (created by BLAST2GO based on *D. magna* genome database). RNA-Seq of adult *D. magna* resulted in an average of 26 million reads for each replicate data. About 80% of the total reads could be mapped in pairs to the *D. magna* reference genome. 12% were mapped in broken pairs and 6% were unmapped. Only the reads which mapped in pairs were used further in the analysis.

3.2.3. *D. magna* CYP gene annotation using RNA-seq result

For CYP gene annotation, I used existing RNA-seq data from 12 days old wild-type *D. magna* which RNA was isolated by a senior student in Watanabe Laboratory. Data can be accessed through NCBI Gene Expression Omnibus (GEO) database through accession number of GSE150821 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE150821). Expression browser was created from gene lists (47,109 genes) by using mapped gene expression data (3 data replicates) and associated it with annotation resource from BLAST2GO. This expression browser showed gene expression level with the annotated name based on *D. magna* gene annotation file. To get a list of CYP genes, simple name filter was used in the annotated name column (such as CYP or Cytochrome P450). A list of 62 CYP genes was

obtained by this filter. Among this gene list, 8 genes showed no expression (0 TPM), leaving the remaining 54 CYP genes expressed in adult *D. magna*. A second filter was then used based on expression level (sorting from highest to lowest relative transcript abundance value in TPM). With this filter, I extracted the 5 highest CYP genes expressed in adult *D. magna*. The existence of the 5 highest expressed CYP genes was then confirmed bioinformatically by performing BLAST against the *D. magna* genome database (http://arthropods.eugenes.org/EvidentialGene/daphnia/daphnia_magna/BLAST/) based on scaffold location information in the expression browser. I named these five genes based on *D. pulex* CYP genes with the highest similarity of amino acid sequence [66].

3.2.4. RT-qPCR

Total RNA was isolated using the Sepasol-RNA I Super G (Nacalai Tesque; Kyoto, Japan) and purified with phenol-chloroform extraction and ethanol precipitation. First-strand cDNA was synthesized using the PrimeScript II 1st strand cDNA synthesis kit (Takara, Shiga, Japan) using random primers from the kit. Quantitative PCR was performed by the MX3005P real-time (RT)-PCR System (Agilent Technologies; CA, USA) with the Power SYBR Green PCR Master Mix (Applied Biosystem) with respective primers as well as housekeeping gene primers (Ribosomal protein L32) Table 5. PCR amplification was performed with the condition of 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C and 1 min at 60°C. Respective target mRNA transcript level was normalized to the transcript level of L32. The specificity of primers was confirmed by performing BLAST to the sequences in *D. magna* genome database. Gel electrophoresis and melting curve analyses were performed to confirm correct amplicon size and the absence of nonspecific bands.

Table 5. Primers used for RT-qPCR of 5 selected CYP genes

Gene	Primer sequences (5′ – 3′)	Product size (bp)
CYP360A8	F : GCCCGAAGCCTATACCGTAC R : TCGTTAAAAGGTTCGGCAAC	138
CYP370A10	F : TACGCAATGTAGACGACCGAC R : GGTGTGGCATGATGTTTCTT	96
CYP360A1	F : GCCTTAAACGTTCATTCGTAGG R : AGACGTGACATACCTGTAGGCA	129
CYP4AP4	F : GATCCTCCTTCTGGCCTTAC R : CTAGGACCAGGAATCCGACC	87
CYP360A10	F : GGGACTTTGGAAGAAGAATACTGC R : CTTTCACGAAAATGGCCTTG	140
60S ribosomal protein L32	F : GACCAAAGGGTATTGACAACAGA R : CCAACTTTTGGCATAAGGTACTG	67

bp = base pair

3.2.5. Chemicals

Chemical exposures were performed with atrazine and paraquat dichloride standard 98.0+% (HPLC) (Wako Pure Chemical; Osaka, Japan). For stock solution, paraquat was diluted in water and kept at 4°C. For atrazine, stock solution was prepared using Dimethylformamide (DMF) analytical grade (≥99.0% (GC), Nacalai Tesque, Japan) and kept in -30°C.

3.2.6. In vitro gRNA synthesis.

Template was prepared by the PCR using PrimeStar GXL polymerase (Takara) to attach two oligo DNAs (0) (Table 6). The first oligo contains T7 promoter, 20 bp target from the genome sequence (5´ – cctGGCCGTAACCGCCAACGTTAC – 3´, protospacer adjacent motif shown in lowercase) and common sequence of gRNA scaffold. The second oligo contains gRNA scaffold sequence. The resulted template was purified by the MinElute Column PCR purification kit (QIAGEN). This purified template DNA was used in the *in vitro* gRNA

synthesis using the T7 MEGAscript kit, followed by purification by the Roche Mini Quick Spin RNA Column. Lastly, ethanol precipitation was performed and the resulting gRNA pellet was dissolved in the DNase/RNase-free water.

Table 6. Oligo DNAs used for *CYP360A8* gRNA synthesis

Oligo	Primer sequences (5' – 3')	Length (bp)
First	GAAATTAATACGACTCACTATAGGCCGTAACC GCCAACGTTACGTTTTAGAGCTAGAAATAGC	62
Second	AAAAGCACCGACTCGGTGCCACTTTTTCAAGT TGATAACGGACTAGCCTTATTTTAACTTGCTA TTTCTAGCTCTAAAAC	80

3.2.7. Microinjection.

Before starting microinjection, 0.5 µM Cas9 and 1 µM gRNA were mixed and incubated at 37ºC to form Cas9-gRNA complex. Alexa dye (0.01 mM) was later added to the complex solution as a volume marker. Cas9 protein was prepared as described previously [78]. The gRNA-Cas9 complexes were incubated for 5 min at 37°C and injected into wild type *D. magna* eggs, as described previously [19]. Freshly ovulated eggs used for injection were collected from 2-3 weeks animal mother in 80 mM sucrose containing M4 medium. The eggs were injected by the glass needle with gas pressure from N_2 with an approximate volume of 0.2 nL. After microinjection, all injected eggs were transferred to a 96-well plate and cultured until the juvenile stage.

3.2.8. Genomic DNA extraction and genotyping.

The mutant candidate was collected into a tube (2-3 daphniids of <24 h juvenile) and added with 90 µL of 50 mM NaOH and 1 µL of 10 mg/mL Salmon Sperm DNA as a carrier.

Daphniids were then homogenized 3 times by Φ1.0 zirconium beads using Micro Smash machine at 3000 rpm for 30 s. This lysate was incubated at 95°C for 10 min. After 2-3 min of cooling down, 10 µL of 1 M Tris-HCl (pH 7.5) and 2 µL of 50 mM EDTA were added. This lysate containing genomic DNA was centrifuged at 12000 rpm, 4°C for 5 min before used for further PCR. The targeted region was amplified by PCR with the Ex Taq® Hot Start (TAKARA) with primers: *CYP360A8*-fwd 5´– CGCTACGGTACGTGTAAACATT –3´ and *CYP360A8*-rev 5´– AGCGCCATCTTTTACCAGTA –3´. Amplified PCR products were analyzed by native PAGE electrophoresis and DNA sequencing.

3.2.9. Phenotyping by acute immobilization test.

Acute immobilization test was performed by exposing *D. magna* to atrazine and paraquat dichloride standard 98.0+% (HPLC) (Wako Pure Chemical; Osaka, Japan). For stock solution, paraquat was diluted in water and kept at 4°C. For atrazine, stock solution was prepared using Dimethylformamide (DMF) analytical grade (≥99.0% (GC), Nacalai Tesque, Japan) and kept in -30°C. All of the solution for exposure was made from one stock, later diluted by serial dilution to make a range of medium concentration. For exposure, juveniles were picked up from cultured tanks within 24 h after they were released from the mothers. For the toxicity assay using matured daphniids, 6 days old daphnia were used. All daphniids were put in 6 well plates with medium volume of 10 mL for 5 daphniids. Daphniid was kept at 23°C incubator during exposure. The culturing medium was changed every 24 hours. For juvenile, immobilized animals are those that are not able to swim within 15 seconds, after gentle agitation of the test vessel [16]. For adult daphniid, as there is no standard in OECD, I decided the immobilization as the inability to swim and no movement of thoracic appendages for 10 seconds. For RT-qPCR experiments, 3 daphniids were picked up for one sample and stored at -80°C for further mRNA isolation. For storing of adult daphnia, eggs were flushed before

freezing. I measured EC50 for both *CYP360A8* mutant and wild type using an existing online calculator [79].

3.3. Results

3.3.1. *CYP360A8* is the most abundant CYP transcript in the adult *D. magna*

As there are many CYP genes present in *D. magna*, it is a great matter to decide which gene is important in detoxification mechanism generally and in metabolizing atrazine specifically. Choosing the most abundantly expressed CYP gene is the first criteria and as described in Materials and Methods section, I chose the five most abundantly expressed CYP genes. As shown in Figure 20 with details of gene location and expression level in Table 7, these 5 genes are *CYP360A8* homolog (clan 3), *CYP370A10* homolog (clan 2), *CYP360A1* homolog (clan 3), *CYP4A4* homolog (clan 4), and *CYP360A10* homolog (clan 3). Results are based on RNA-seq data analysis from 3 replicates of 12 days old WT *D. magna* and shown in TPM. The highest expressed CYP is *CYP360A8* which based on *D. pulex* annotation, belongs to CYP3 clan, the clan which often associated to xenobiotic metabolism. To further confirmed that the annotated *CYP360A8* is the *CYP360A8* ortholog in *D. pulex*, I first annotated all of the clan 3 genes transcribed in adults by amino acid similarity and conservation of the syntenic position between each CYP and its surrounding genes among *D. pulex* and *D. magna*. Four orthologs, *CYP360A1*, *CYP360A3*, *CYP360A4*, and *CYP360A6* are present in tandem in one scaffold as reported in *D. pulex* (Figure 22). The other three orthologs, *CYP360A8, CYP360A10*, and *CYP360A11* are located in different scaffolds (Figure 23, Figure 24, Figure 25). *CYP360A8* in *D. magna* shows conserved syntenic position with surrounding genes in *D. pulex*. Based on this, I can confirm that *D. magna CYP360A8* is *D. pulex CYP360A8* ortholog which is priorly annotated [66] and other CYP clan 3 genes in *D. magna* also shows conservation in both amino acid sequence and syntenic position based on *D. pulex* CYP clan 3 annotation.

Table 7. Five highest expressed CYP genes in *D. magna*.

Genomic position	Region	Database identifier[a]	Annotation based on *D. pulex* CYP	WT1 TPM	WT2 TPM	WT3 TPM	WT Mean
scaffold01581	Complement (2052090..2054913)	Cytochrome P450	CYP360A8	223.38	229.04	243.87	232.10
scaffold02452	Complement (682285..685137)	putative Cytochrome P450 305A1	CYP370A10	129.82	92.86	110.61	111.10
scaffold01361	Complement (1358356..1361068)	putative Cytochrome P450 6A18	*CYP360A1*	45.91	39.55	50.65	45.37
scaffold01361	Complement (467631..470909)	putative Cytochrome P450 4AA1	*CYP4AP4*	33.56	37.35	45.07	38.66
scaffold02227	49439..51881	Cytochrome P450 3A8-like	*CYP360A10*	32.39	21.60	30.28	28.09

[a]Database identifier is based on genomic information in the *D. magna* genome database

TPM = Transcripts per million

WT = Wild type

3.3.2. *CYP360A8* is highly expressed by maturation time

I examined developmental expressions of those five genes from Day 0 to Day 12 after released from mothers. Expression of each CYP gene was normalized with that of the ribosomal protein L32 gene (Figure 21). As for the profile in the transcriptome analysis, *CYP360A8* showed the highest expression among the tested five genes. Its expression increased 6 fold in day 4 when ovarian maturation occurred. Later, this gene was constitutively active until Day 12. *CYP4AP4* and *CYP360A10* expressions were more modestly increased in adult stages. The other *CYP370A10* and *CYP360A1* expression levels were lower than the others during life stages. The expression level of *CYP360A8* was estimated as one-third of the ribosomal protein L32 gene expression after Day 4, suggesting abundant and constitutive expression of *CYP360A8*.

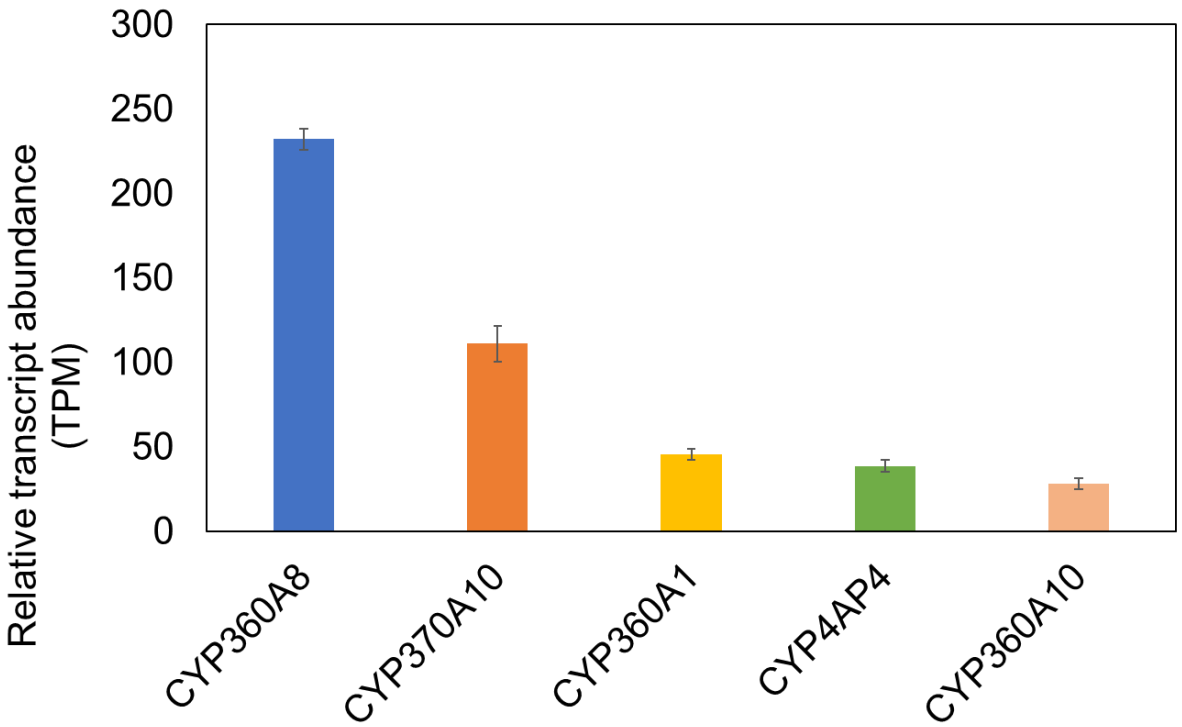

Figure 20. Five highest expressed CYP genes in D. magna based on RNA-Seq analysis. Values are mean relative abundance in TPM ± SEM in three replicates.

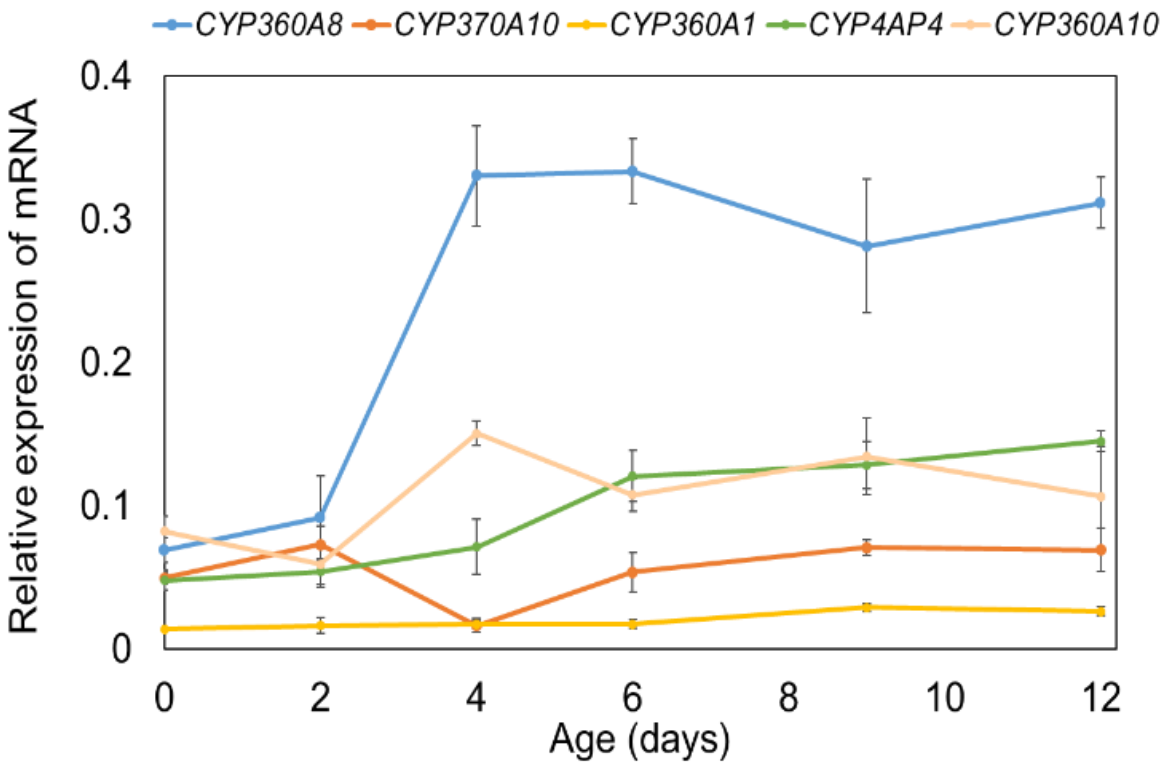

Figure 21. The temporal expression level of 5 highest expressed CYP genes in D. magna confirmed by RT-qPCR.
Data are presented as means ±SEM, *n* = 3/group (9 daphniids). Data are expressed at relative expression to Ribosomal protein L32 gene. Age day 0 is <24h since the daphniid is released from brood chamber; day 4 is starting of ovary formation; by day 6 all daphniid ovulated and has first clutch in the brood chamber

3.3.3. Generation of CYP360A8 mutant by CRISPR/Cas system

The higher abundance of *CYP360A8* transcripts suggested its role in xenobiotic detoxification. Introducing mutation into this gene using CRISPR/Cas system can be a good strategy to find out the function and importance of this gene. To avoid gRNA cross-reactivity among the clan 3 CYP genes that *CYP360A8* belongs, I compared sequence of *CYP360A8* with the other clan 3 CYP genes. Except for *CYP360A11* and *CYP360A3*, I confirmed five conserved motifs of CYP proteins, the WxxxR motif, the GxE/DTT/S motif, the ExLR motif, the PxxPxPE/DRF motif and the PFxxGxRxCxG/A motif in their deduced amino acid sequences [80] (Figure 26).

gRNA was designed at a distance of 27 bp downstream from the start codon (Figure 27). Mismatches of this gRNA sequence to the other CYP clan 3 genes were more than 5 bp with PAM (NGG at 3´ end) (Table 8), indicating that off-target to the other CYP clan 3 genes was prevented as reported previously [81], [82]. I injected 0.5 μM Cas9 protein and 1 μM gRNA into wild type eggs. Of 17 injected eggs, 15 survived to adulthood. I extracted genome DNA from offspring of the survived adults and amplified a region around the gRNA-targeted site by PCR (Figure 27). I established one mutant line that has a monoallelic mutation of 5 bp deletion, which leads to a frameshift mutation and a premature stop codon before the first conserved motif WxxxR (Figure 27).

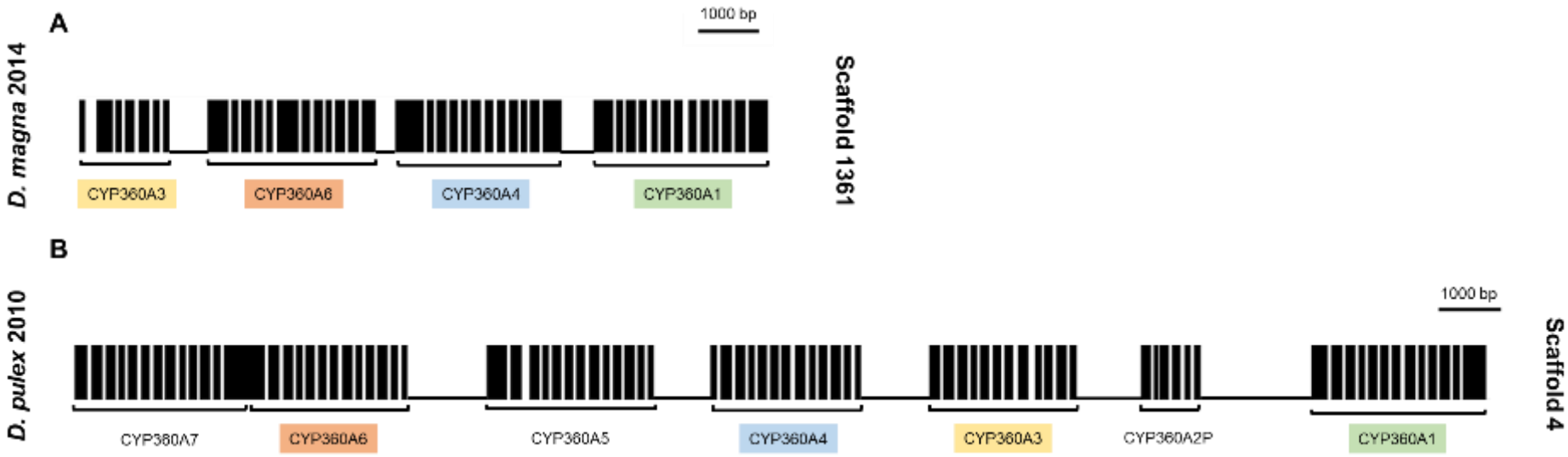

Figure 22. Organization of *CYP360A1* until *CYP360A7* cluster

(A) in *D. magna* (2014 gene database) located in Scaffold 1361, (B) in *D. pulex* (2010 gene database) located in Scaffold 4. Black boxes are exon. The intergenic regions are shown by separating line between genes. Genes encoding ortholog proteins are indicated by colored boxes with respective names. Scale bar is shown in 1000 bp length.

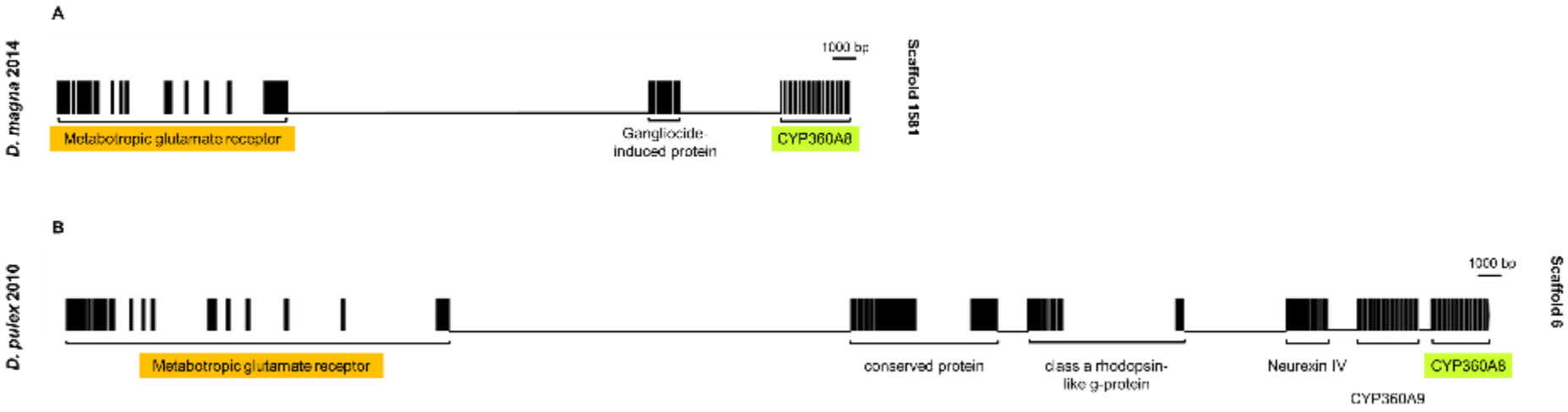

Figure 23. Organization of neighboring genes near *CYP360A8*

(A) in *D. magna* in Scaffold 1581 (2014 gene database), (B) in *D. pulex* in Scaffold 6 (2010 gene database). Black boxes are exon. The intergenic regions are shown by separating line between genes. Genes encoding ortholog proteins are indicated by colored boxes with respective names. Scale bar is shown in 1000 bp length.

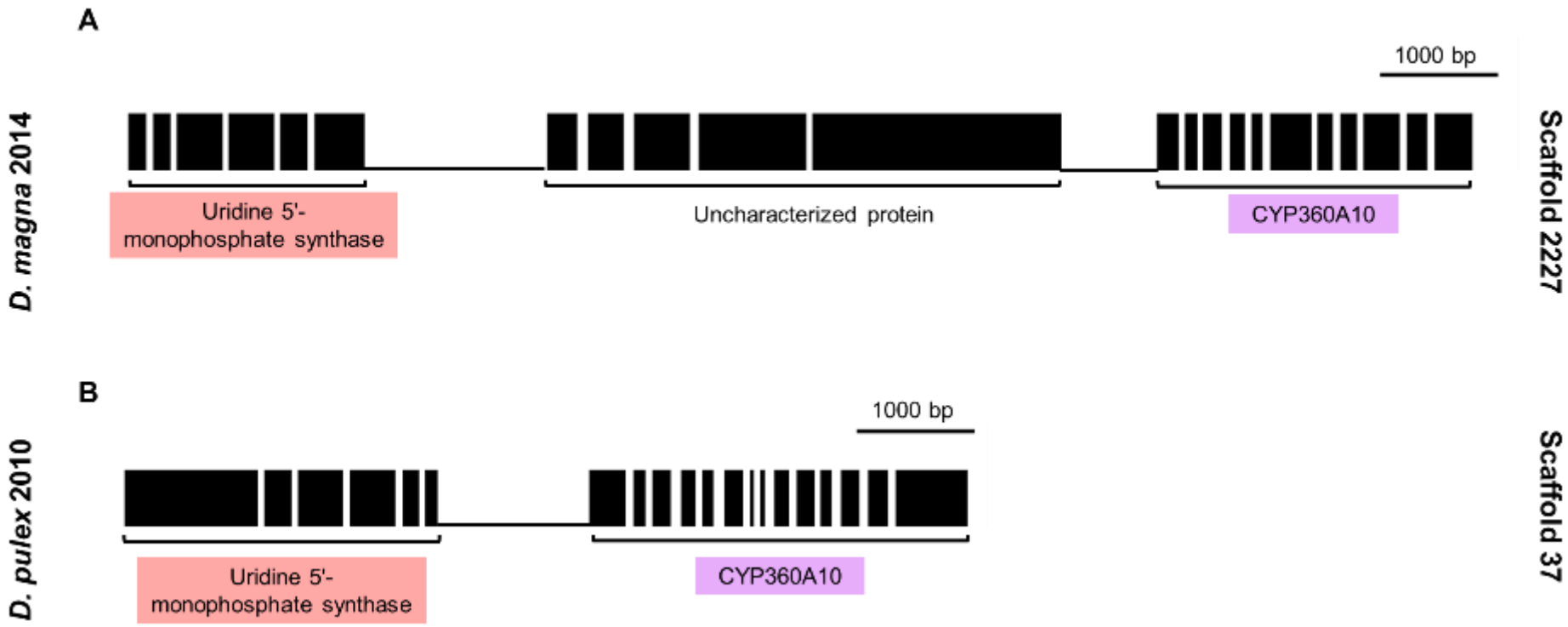

Figure 24. Organization of neighboring genes near *CYP360A10*

(A) in *D. magna* (2014 gene database) located in Scaffold 2227, (B) in *D. pulex* (2010 gene database) located in Scaffold 37. Black boxes are exon. The intergenic regions are shown by separating line between genes. Genes encoding ortholog proteins are indicated by colored boxes with respective names. Scale bar is shown in 1000 bp length.

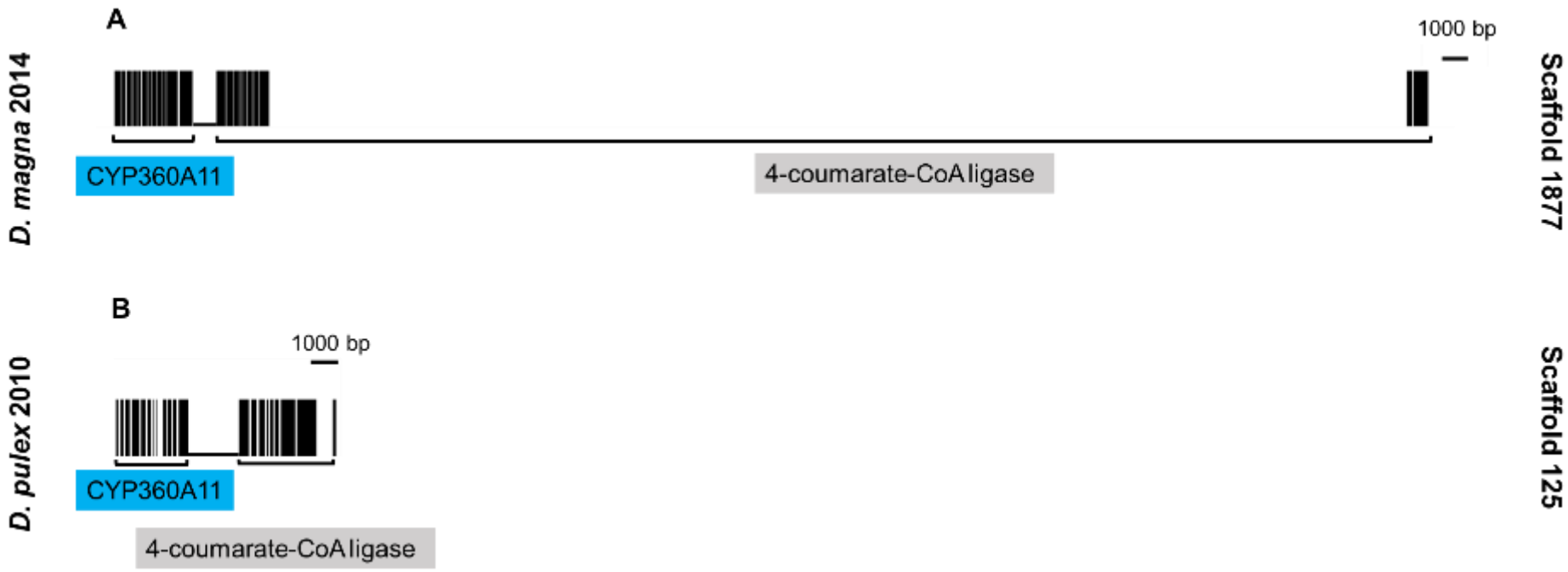

Figure 25. Organization of neighboring genes near *CYP360A11*

(A) in *D. magna* (2014 gene database) located in Scaffold 1877, (B) in *D. pulex* (2010 gene database) located in Scaffold 125. Black boxes are exon. The intergenic regions are shown by separating line between genes. Genes encoding ortholog proteins are indicated by colored boxes with respective names. Scale bar is shown in 1000 bp length.

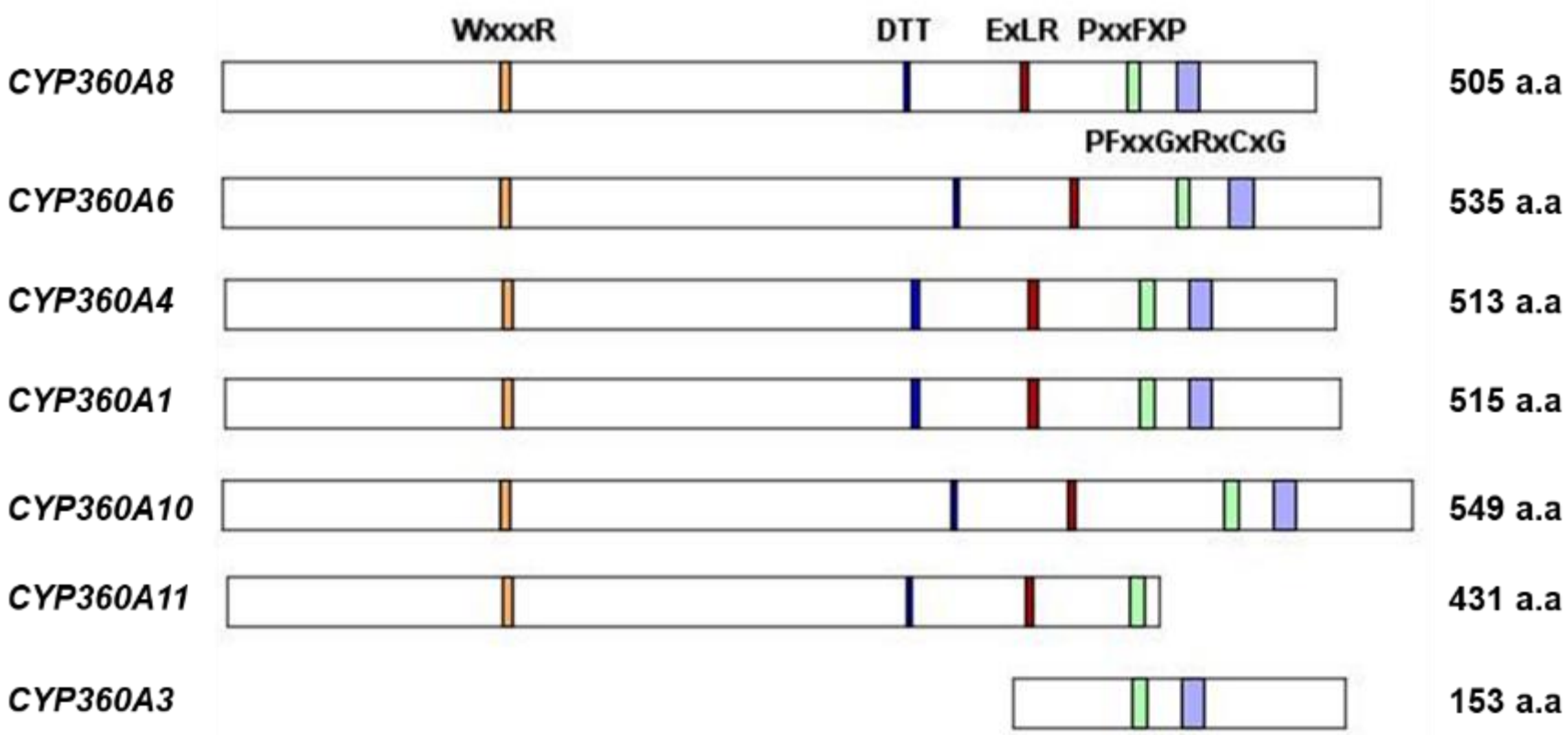

Figure 26. Conserved amino acid motifs in CYP3 clan genes in *D. magna*

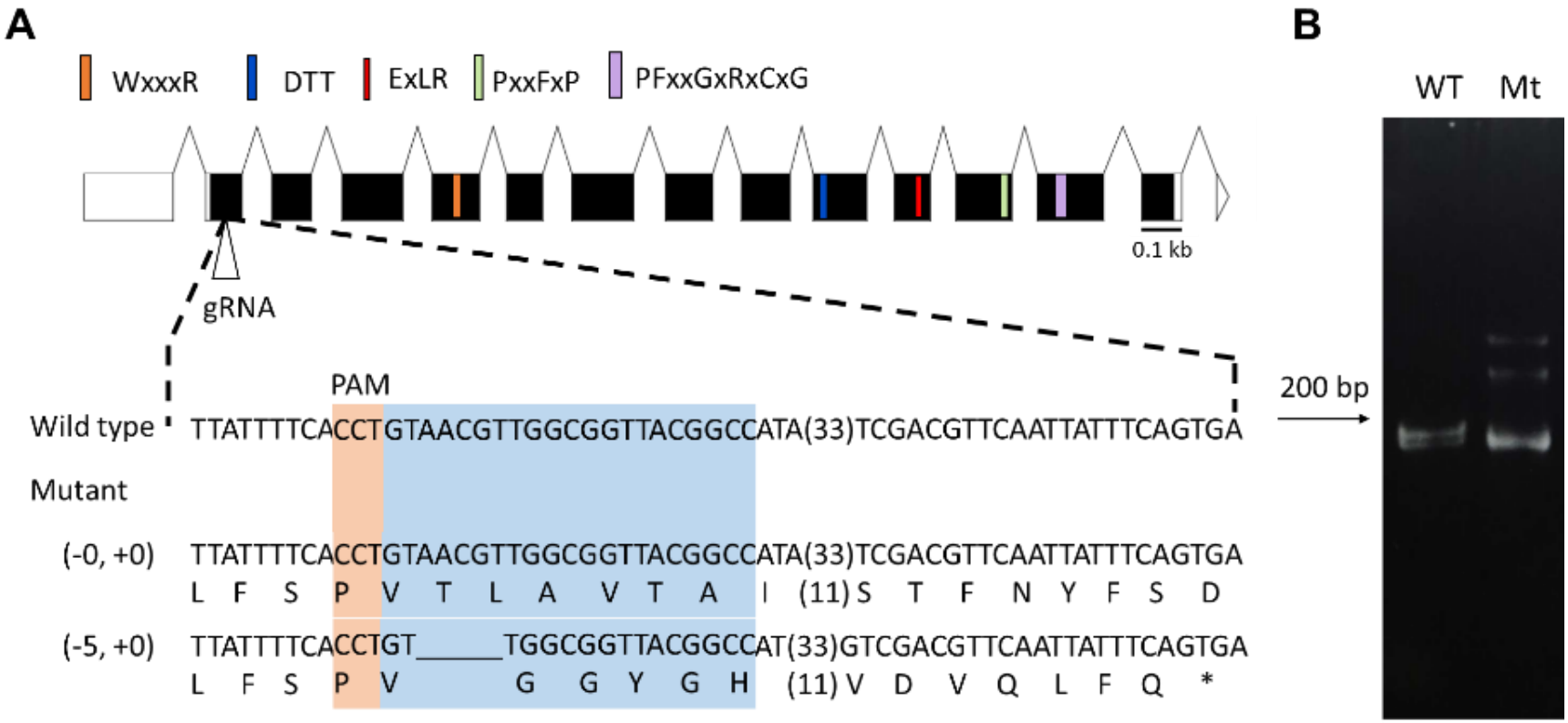

Figure 27. Mutation of *CYP360A8* in *D. magna* by CRISPR/Cas system

(A) Schematic gene structure of *CYP360A8* in wild type and mutant *D. magna* (*CYP360A8*$^{-}$). The target site for gRNA is indicated by the blue box. Deletion is shown by underline without letters. The protospacer adjacent motif (PAM) sequence is colored in pink. (B) PCR for genotyping of wild type (WT) and *CYP360A8*$^{-}$ (Mt). The amplified genomic DNA fragments are shown by native polyacrylamide gel electrophoresis (Native PAGE). Two bands are shown in WT showing variants in an intron in the amplified region. Upper two bands in Mt showing the formation of heteroduplexes, suggesting mutation.

Table 8. Potential off target sites of gRNA in other CYP3 clan genes

	Annotations	On/off target sites	Mismatches (bp)	Location
On target	*CYP360A8*	GGCCGTAACCGCCAACGTTACagg	-	exon
Off target	*CYP360A1*	**TAT**CGT**C**ACC**A**CC**C**A**GAAA**ACagg	10	exon
	CYP360A4	G**A**C**A**GT**T**AC**A**GCC**C**A**A**GT**GG**C**g**gg	9	exon
	CYP360A6	G**ATT**GTAACC**A**CC**C**AC**AGC**ACagg	8	exon
	CYP360A10	G**A**C**G**GT**T**A**TA**GCC**C**A**A**GT**C**AC**g**gg	9	exon
	CYP360A11	GGC**GACG**ACC**AA**C**G**A**AAACGTga**g	15	exon

Genomic sequences of on/off target sites (uppercase) with PAM (NGG, lowercase), the annotations, the number of base pair differences and the locations are shown. Bold letters show mismatched nucleotides.

3.3.4. *CYP360A8* mutant shows higher sensitivity to atrazine and paraquat

To examine the sensitivity of *CYP360A8* mutant to chemicals, I performed acute immobilization test on both wild type and this mutant. Because *CYP360A8* gene activity was increased after Day 4 and its expression level was maintained thereafter, I used two different stages of daphniids, young juvenile (day 0, immature stage when it is released from brood chamber), and adult (day 6, matured stage). Atrazine was first used for exposure to confirm if *CYP360A8* has a role in detoxifying it as I hypothesized in Chapter 2. For atrazine, EC50s could not be examined as it's solubility is low in water (33 mg/L at 25°C) [83] so I only examined the response of daphniid to concentration of 10 until 30 mg/L. For juvenile, significant decrease of survival rate can be seen started from 10 mg/L, as for adult highly significant decrease of survival could be seen on 30 mg/L (Figure 28).

To examine if *CYP360A8* also has a role in general detoxification system in *D. magna*, as it is constitutively expressed in high level, I decided to examine another herbicide with high solubility in water so I won't have limitation on preparing the solution concentration. For this experiment, I chose paraquat. Result showed paraquat EC50s of WT juveniles and adults were 5.5 mg/L and 26.6 mg/L respectively (Figure 29). In both juveniles and adults, the EC50 of the mutants was 1.3 times lower than that of wild type daphniids. The sensitivity to paraquat was significantly different between wild type and mutant (Figure 30).

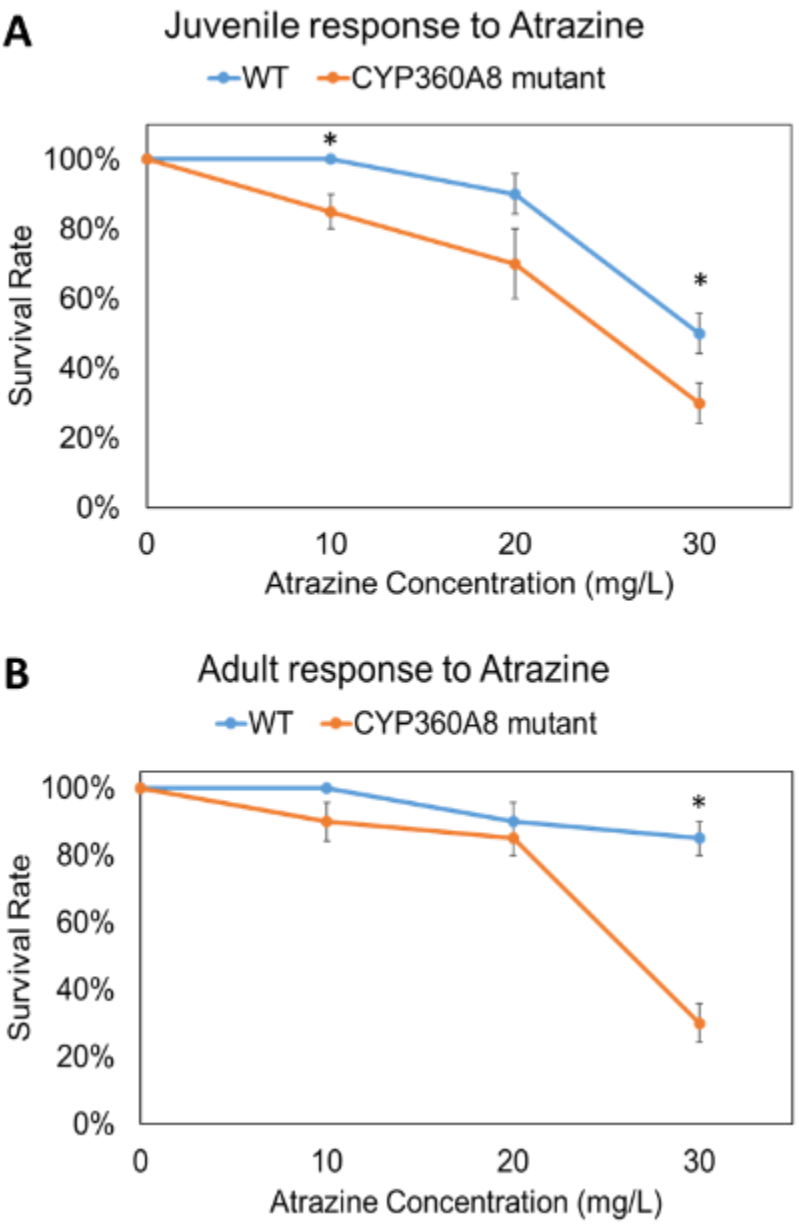

Figure 28. Acute immobilization test on wild type and *CYP360A8* mutant exposed to atrazine

(A) The survival rate of juvenile wild type and *CYP360A8* mutant after 48 h of atrazine exposure. Exposure starts on <24 h juvenile released from animal mother. (B) The survival rate of adult wildtype and *CYP360A8* mutant after 48 h of paraquat exposure. Exposure starts on 6 days old adults which already have 1st clutch eggs in the brood chamber.

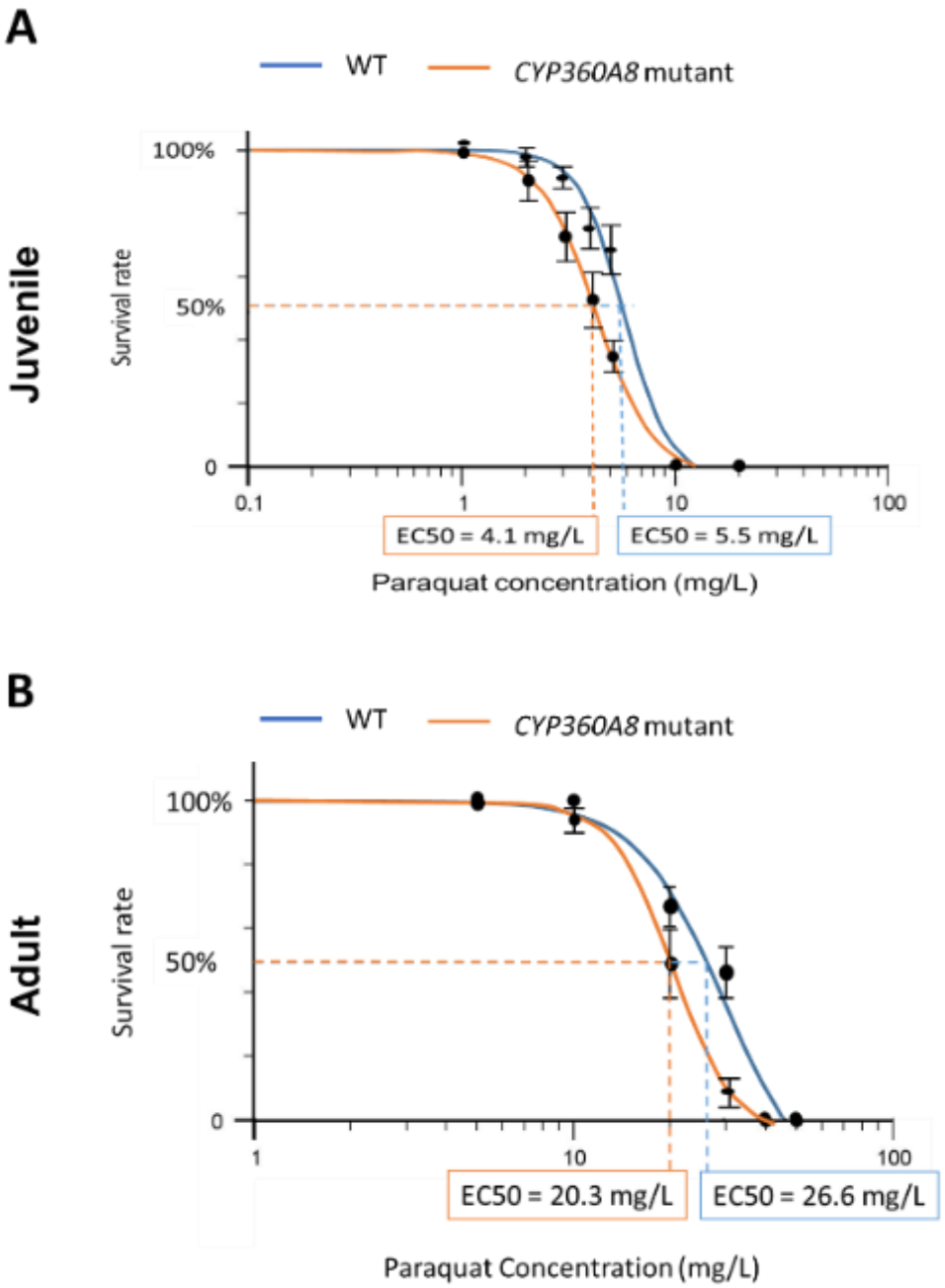

Figure 29. Acute immobilization test on wild type and *CYP360A8* mutant exposed to paraquat

(A) The survival rate of juvenile wild type and *CYP360A8* mutant after 48 h of paraquat exposure. Exposure starts on <24 h juvenile released from animal mother. Juvenile wild type EC50 = 5.5 mg/L; Juvenile mutant EC50 = 4.1 mg/L. Data are expressed at a mean survival rate (%) of 9 groups of tested animal containing 5 individual in each group. (B) The survival rate of adult wildtype and *CYP360A8* mutant after 48 h of paraquat exposure. Exposure starts on 6 days old adults which already have 1st clutch eggs in the brood chamber. Adult wild type EC50 = 26.6 mg/L; Adult mutant EC50 = 20.3 mg/L. Data are expressed at a mean survival rate (%) of 6 groups of tested animal containing 5 individual in each group.

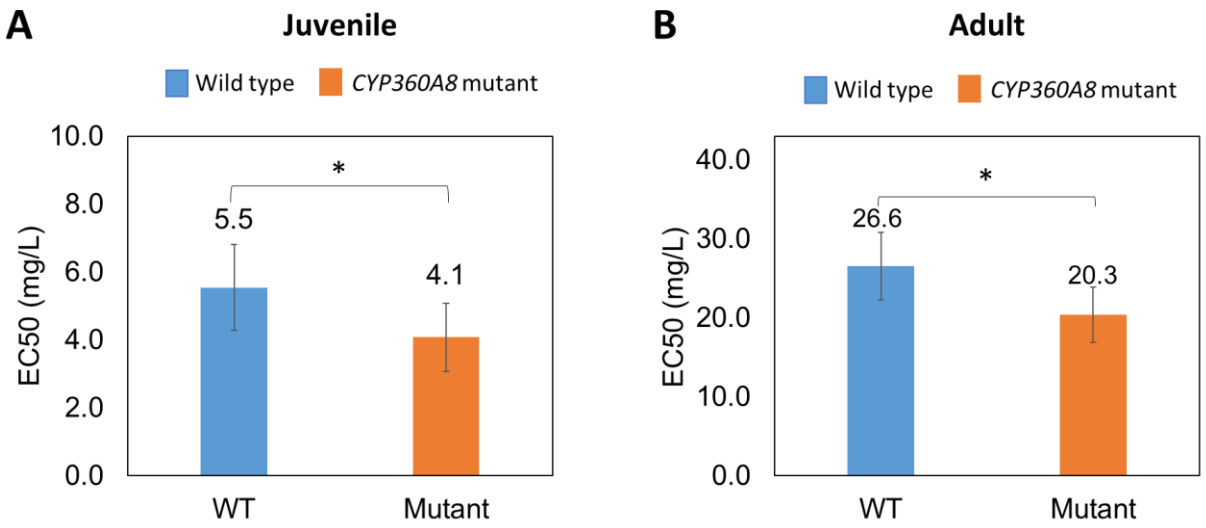

Figure 30. Paraquat EC_{50} on wild type and *CYP360A8* mutant presented with statistical significances.

Asterisk shows the significant difference (t-Test: two-sample assuming equal variances, $p < 0.05$). (A) EC_{50} of juvenile wild type and *CYP360A8* mutant after 48 h of paraquat exposure. Exposure starts on <24 h juvenile released from animal mother. Fold change of EC_{50} in juvenile is 1.36 fold. Experiment was performed on 9 groups of tested animal containing 5 individual in each group. (B) EC_{50} of adult wildtype and *CYP360A8* mutant after 48 h of paraquat exposure. Exposure starts on 6 days old adult which already have 1st clutch eggs in the brood chamber. Fold change of EC_{50} in adult is 1.30 fold. Experiment was performed on 6 groups of tested animal containing 5 individual in each group.

3.3.5. Exposure of atrazine and paraquat increases *CYP360A8* transcription level

To investigate the transcriptional response of *CYP360A8* to atrazine and paraquat in wild type and its mutant, I performed RT-qPCR with primers that amplify wild type *CYP360A8* transcripts. At 48 h after exposure, both juveniles and adults were subjected to this gene expression analyses.

For atrazine exposure, to prevent the possibility of lower quality of total RNAs in dead animals, I collected swimming daphniids exposed to 10 and 20 mg/L. As a positive control gene indicating transcriptional response to xenobiotics, I used the class Sigma glutathione S-transferase gene *GSTs1b* [84] (also known as *GST A4*) that increased its mRNA level in response to atrazine [69] and juvenile hormone agonists [68]. Significant upregulation could be found on WT juvenile on 20 mg/L, and slight upregulation among mutant juvenile (Figure 31). Same pattern was found in adult, although the fold change is higher than juvenile. In adult mutant, mRNA level didn't show any change even in 20 mg/L. As for *GSTs1b*, high fold change was found on both WT juvenile and adult, though juvenile mutant didn't show any change till 20 mg/L (Figure 31).

I did the same RT-qPCR analysis to daphniids exposed to paraquat. To prevent RNA degradation on dead daphniids, I used lower concentration than EC50 samples: 1 mg/L and 3 mg/L to the Day 0 juveniles; 5 mg/L and 10 mg/L to the Day 6 adult. In wild type, *CYP360A8* transcripts were increased in a dose-dependent manner up to 5 times and 2.5 times at juvenile and adult stages respectively (Figure 32) when exposed to paraquat. In the *CYP360A8* mutant, expression level of the wild type *CYP360A8* transcript was reduced by half as expected from monoallelic nature of this mutation. Induction level of *CYP360A8* was almost 3 times and 50 times lower than that of *GSTs1b* at juvenile and adult stage respectively (Figure 32).

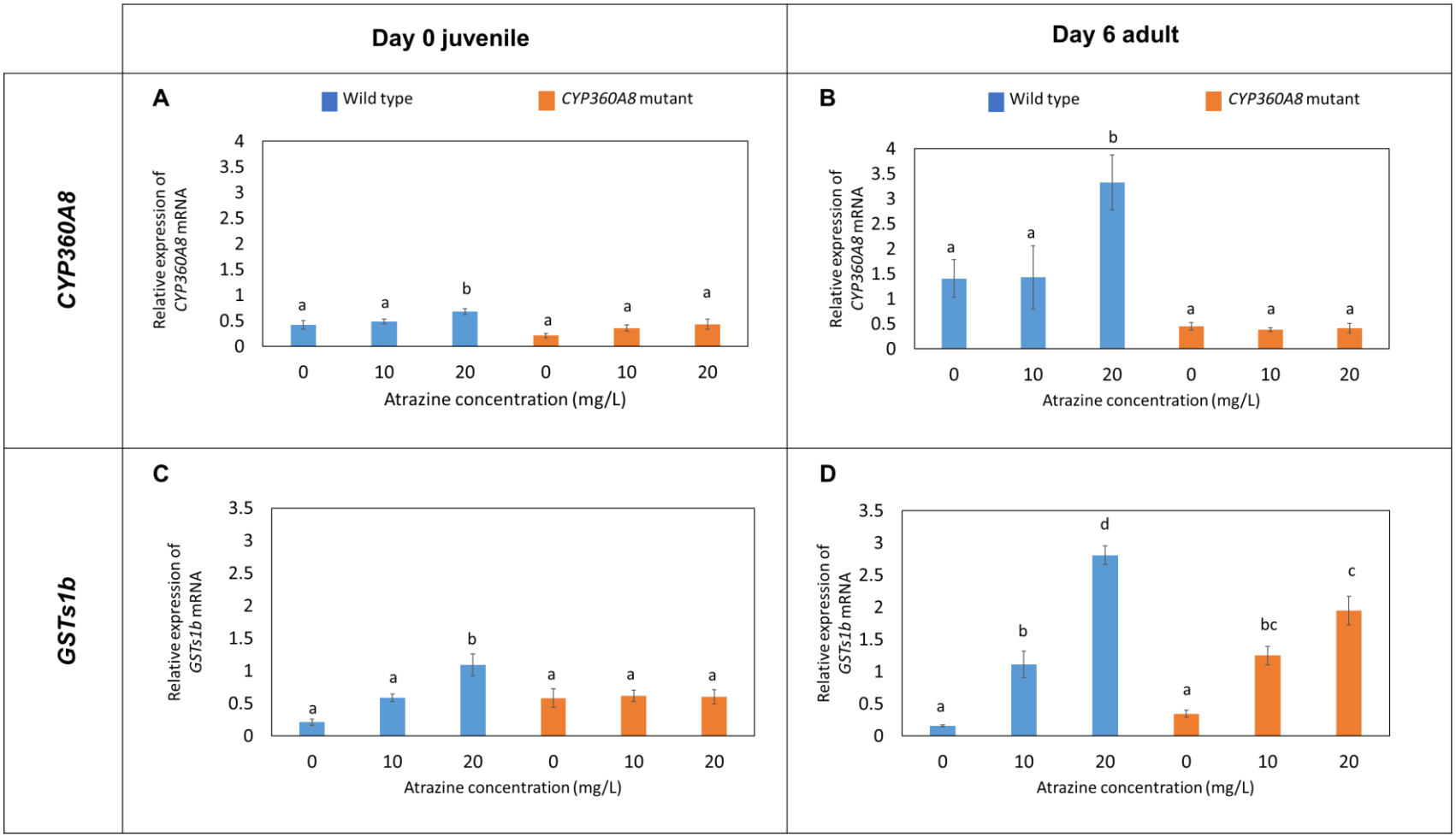

Figure 31. Relative expression of phase-I putative *CYP360A8* and phase-II putative *GSTs1b* normalized by L32 in wildtype and *CYP360A8* mutant *D. magna* at 48 h after atrazine exposure.

Data are presented as means ±SEM, n = 3/group (9 daphniids). Different alphabet shows the significant difference (ANOVA, Tukey HSD, $p < 0.05$). Data are expressed at relative expression to housekeeping gene of 60s ribosomal protein L32. All exposure is performed on the specific stage (day 0 and day 6) for 48 h.

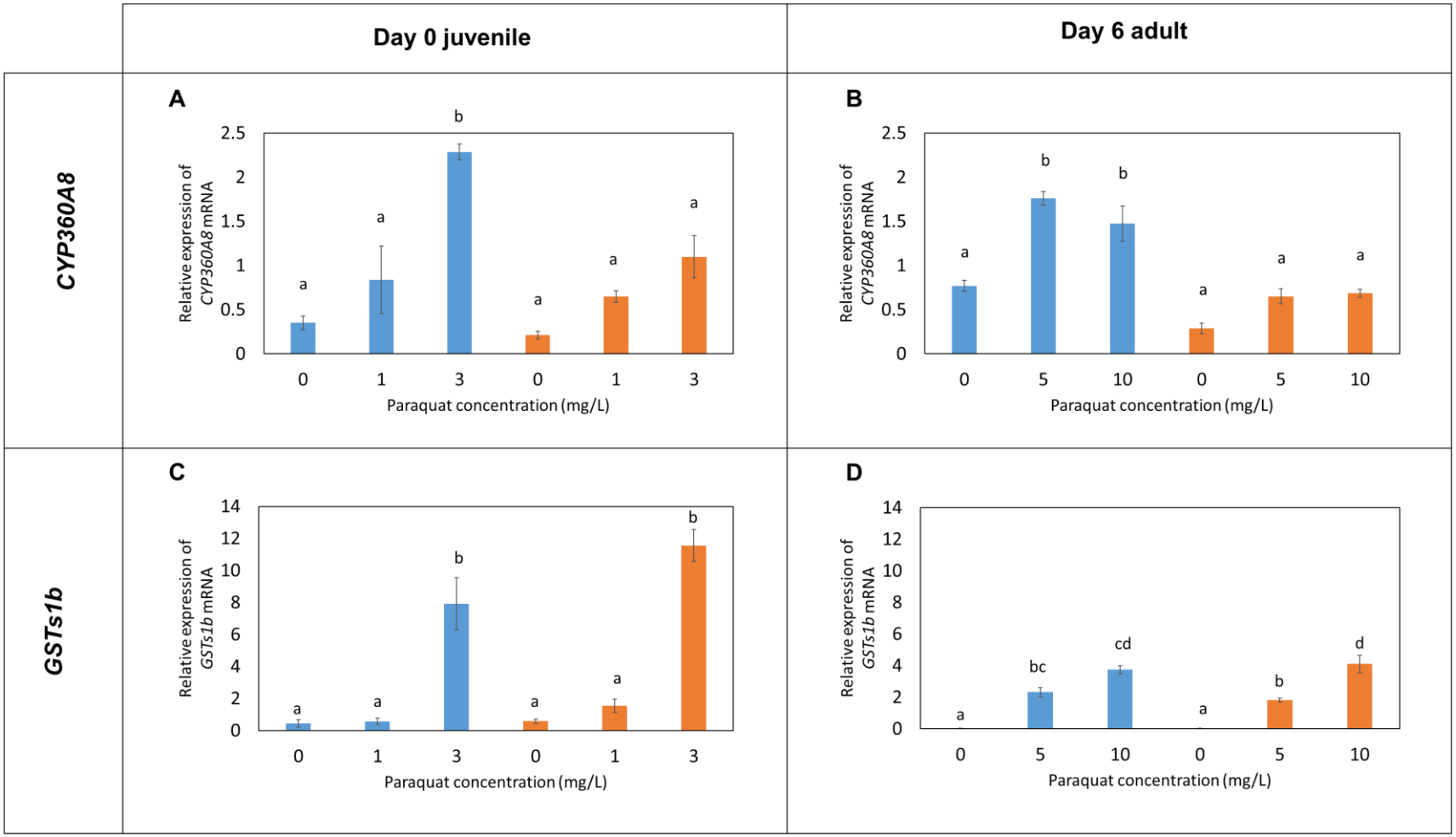

Figure 32. Relative expression of phase-I putative *CYP360A8* and phase-II putative *GSTs1b* normalized by L32 in wildtype and *CYP360A8* mutant *D. magna* at 48 h after paraquat exposure.

Data are presented as means ±SEM, n = 3/group (9 daphniids). Different alphabet shows the significant difference (ANOVA, Tukey HSD, $p < 0.05$). Data are expressed at relative expression to housekeeping gene of 60s ribosomal protein L32. All exposure is performed on the specific stage (day 0 and day 6) for 48 h.

3.4. Discussion

In this chapter, the objective is to identify a high abundant CYP gene which expressed higher in adult stage. As abundance of CYP is one of the important factors for xenobiotic metabolism [75], I analyzed transcriptome in adults and compared basal expression levels of CYP genes. As described in materials and methods, I chose the five abundantly expressed CYP genes, *CYP360A8* (clan 3), *CYP370A10* (clan 2), *CYP360A1* (clan 3), *CYP4AP4* (clan 4), and *CYP360A10* (clan 3) (Figure 20). The highest expressing *CYP360A8* was found to have increasing expression towards adulthood. Not only this gene expressed continuously, but also it has an immense expression starting from around ovulation time, a sign of maturity in *Daphnia* which may suggest an increased ability of metabolizing xenobiotics. Temporal change of CYP expression during development had already been reported in various organisms [85]. In human, for example, *CYP1A2* (the clan 2 gene related to caffeine N-demethylation) is more highly expressed in adult age, vice versa to *CYP3A7* (clan 3 gene, related to glucocorticoids drugs) which is not expressed in adult but only in fetal age [86]. In *Drosophila*, *CYP6G1* that is inducible by caffeine, shows higher expression in 3rd instar larvae compared to 1st instar [87]. Lower *CYP360A8* expression in juveniles might be due to an immature state of a tissue potentially such as fat body expressing this gene at juvenile stages because its expression increased when this animal matured. Analyzing localization of *CYP360A8* mRNA and protein would be important for understanding molecular mechanism of xenobiotic metabolism in a developmental context. The profile of *CYP360A8* mRNA level also in corresponds to the *in vitro* CYP activity by ethoxycoumarin O-deethylase (ECOD) assay [63] which may suggest that general CYP activity in *D. magna* comes from *CYP360A8*.

The highest expressed *CYP360A8* has never been characterized before in *Daphnia* and this study is the first one to examine its temporal expression. The ortholog in *Drosophila melanogaster*, *CYP6A13* (based on highest match by BLASTp in *Drosophila* genome database

https://flybase.org/blast/) was found to be induced by paraquat [88], [89] and alcohol [90]. The information on *CYP6A13* basal expression in *Drosophila* is lacking, so there is limited information on how this gene behaves in the first place. The fact that *D. magna CYP360A8* belongs to clan 3 (as well as its ortholog in *D. melanogaster*), which often considered as the important CYP clan in xenobiotic metabolism, supporting the speculation that *CYP360A8* is highly important in *D. magna* defense mechanism. As the *CYP360A8* characteristics and function is barely known in branchiopod *Daphnia* (as well as its ortholog in insect), I tried to find out how it contribute to xenobiotic metabolism by performing reverse genetic strategy.

CRISPR/Cas mediated mutagenesis in *CYP360A8* resulted in 5 bp nucleotide deletion, starting 30 bp after start codon. This resulted in possible loss of almost half of 20 hydrophobic amino acids in the N-terminal of the protein which is important for anchoring the CYP into the membrane of the endoplasmic reticulum (ER). A possible partial loss of highly conserved I-helix with threonine amino acid (second conserved motif, D**T**T) which plays important role in the catalytic process [80] may also happened due to the deletion resulted in frameshift mutation even before the first conserved motif. The third (**E**xL**R**) and fourth conserved motif (PxxFX**P**) which are important for stabilizing the overall structure of the protein [80] may also be disturbed. The disturbance of the highly conserved fifth motif (PFxxGxRx**C**xG) which carries cysteine and is a ligand to the heme iron in the active site of CYP [80], may cancel out the heme binding to the CYP, therefore cancelling the catalytic cycle needed to perform the detoxification reaction. Bringing these all together, this monoallelic mutation may result in partial loss of CYP attachment to ER membrane, unstable protein structure, and non-functional catalytic domain.

Using *CYP360A8* mutant, I was able to examine its sensitivity towards two kinds of common herbicides. Both atrazine and paraquat seemed to be more toxic to juveniles than adult. This may be consistent with the lower expression level in juveniles although we need to

consider differences between juveniles and adults such as the excretion system [91]. Although interestingly, both mutant juvenile and adult shows same level of sensitivity in 30 mg/L atrazine exposure. RT-qPCR result shows that 20 mg/L atrazine doesn't increase mRNA level in mutant, though it does in juvenile. This may suggest that in WT, *D. magna* has resistant to a very high concentration of atrazine. But once one of the allele is disturbed, it greatly affect adult sensitivity towards atrazine. As for juvenile that has always been more sensitive, the mutation doesn't affect the sensitivity as much as adult.

The planktonic crustacean *D. magna* lives in freshwater ecosystems where foreign chemicals accumulate and is always at risk of being exposed to toxic substances. I found that the *CYP360A8* gene, which is important for xenobiotic metabolism, is highly expressed even under unexposed condition and somewhat less responsive to chemicals in adults, suggesting the importance of constitutive *CYP360A8* expression for xenobiotic metabolism. This pattern is different from the phase II detoxification gene, *GSTs1b*, which relative expression is lower than *CYP360A8* especially in adult stage (also confirmed in RNA-seq data of adult wildtype). *GSTs1b* shows larger induction level than *CYP360A8*, showing a different mode of action of these two detoxification genes. Lower responsiveness of CYP genes has been reported in previous *Daphnia* transcriptome studies. For example, in response to the juvenile hormone agonist epofenonane, the expression of CYP-related genes was approximately 3-fold, whereas *GSTs1b* was 7-fold increased [68]. In addition, the expression of CYP genes in atrazine-exposed *D. magna* was increased by less than 3-fold in contrast to more than 4 fold increase in 2 GST genes expression [69]. These are different from the well-known chemical responses of CYP genes in the other organisms such as *Drosophila* [87], [92], [93] and mammals [94]. *Daphnia* may acquired a unique system for constitutive CYP expression to respond rapidly to chemicals in water during evolution, suggesting the importance for further studying xenobiotic metabolism in this ecologically important species.

Chapter 4. General Discussion and Conclusion

4.1. General Discussion

D. magna has been an interest of many researchers studying on how aquatic organism responds to xenobiotic, especially to the xenobiotics that enter the freshwater ecosystem. Its sensitivity to xenobiotics makes it possible to perform risk assessment to a very wide variety of chemicals. Either for detecting targeted xenobiotic or for understanding its defense to xenobiotics in natural setup, *D. magna* detoxification mechanism has been an interesting topic to be explored. Not only does *D. magna* is sensitive in responsing to xenobiotics, it also is the primary consumer in the trophic level in aquatic system, making it a good model for ecological risk assessment. The idea of performing risk assessment to single organism is widely used as it is convenient and easy to evaluate. But, as organisms exist together in the ecosystem, using this strategy can lead to risk underestimation in community level. With this idea, looking more into the relationship between xenobiotics, *Daphnia* as primary consumer and other trophic level may give a new perspective on risk assessment.

Examining *Daphnia* trophic level interaction and chemical stress was previously done, such as using in-vivo or microcosm experiments, and modelling [5], [95], [96]. Multi-factors in risk assessment leads to more comprehensive prediction on effects in all ecosystem level, as stressor is rarely in isolation in real environment [97]. In particular, this multi factor risk assessment for commonly used chemical has a high importance in today's community. Therefore, in this study, I decided to focus on herbicide atrazine which used enormously in agricultural application.

Atrazine has always been considered as a potential EDC. Therefore rather than performing acute toxicity test, investigating sub-lethal effect makes more sense to learn more on how it affects the hormonal system in living organism. Moreover, lethal effect is usually

reached in a very high concentration. In this study, I found out that atrazine in a environmentally relevant concentration of 150 µg/L affects *D. magna*'s reproduction directly (through medium) or indirectly (through its food). Previous study in *D. pulex* examine the effect of fungicide pyrimethanil when exposed directly (to medium), indirectly (to food, *Scenedesmus obliquus*), or both exposure [98]. This study shows, pyrimethanil effect the same way via food or medium, while presence of predator and temperature may hold more importance in controlling the reproduction of *D. pulex* [98]. My results suggest, in the case of atrazine, exposure via food may held importance than exposure via water, though it resulted in same phenotype. Bioaccumulation of atrazine in algae cells may amplify the severity compared to direct exposure. A previous study shows that exposure of 1 mg/L $nTiO_2$-5A in medium, resulted in accumulation of around 64 mg/kg *Daphnia*, while when the same amount is exposed to algae first, it ended up with $nTiO_2$ accumulation of more than 2000 mg/kg *Daphnia* [99].

In chapter 2, I tried to relate the discrepancy of phenotype between high non-viability in 1st clutch (compared to 3rd clutch) to the same amount of atrazine accumulated in embryos. Although the relation need to be confirmed, the maturity of the mother may be a good reasoning behind this (Figure 33). As confirmed by *in vitro* assay [63] that *D. magna* CYP activity increased over age, later also confirmed by *CYP360A8* expression over 12 days (Chapter 3), the aging of mother may change the phenotype of offspring. This can be referred as maternal effect. Maternal effects happens in *D. magna* in several aspects, for example the feeding rate of offspring is affected by feeding quantity of mother [100] and mother exposed with cyanobacterial toxins has increased tolerance over 3 generations [54]. Though, as far as I would like to refer to previous studies, I couldn't find any study explaining the vitality difference over clutch of the same generation in *D. magna*.

Several studies shows that vertebrate mostly metabolize atrazine by using CYP and minority is done by GST [101]–[103]. Information of atrazine metabolism on invertebrate,

including insect or crustacean though is lacking. This makes learning the xenobiotic impact on invertebrates challenging while in fact, insects and crustaceans are very prone to xenobiotic exposure as a result of human activities. Most studies in invertebrates only focused on CYP gene response (upregulation or downregulation) on xenobiotics exposure. Information of what CYP has role in atrazine degradation metabolism is still unknown, including in *Daphnia*. Though several studies have revealed upregulation of several CYP genes in response to xenobiotics in *Daphnia* [69], [104]–[106], there has been no information on basal expression of detoxification related genes. No CYP mutant has been established for understanding molecular mechanism of xenobiotic detoxification in *Daphnia*. This study may be the first to perform *in vivo* demonstration that links specific CYP gene function to chemical detoxification in the common ecotoxicology model organism *D. magna*.

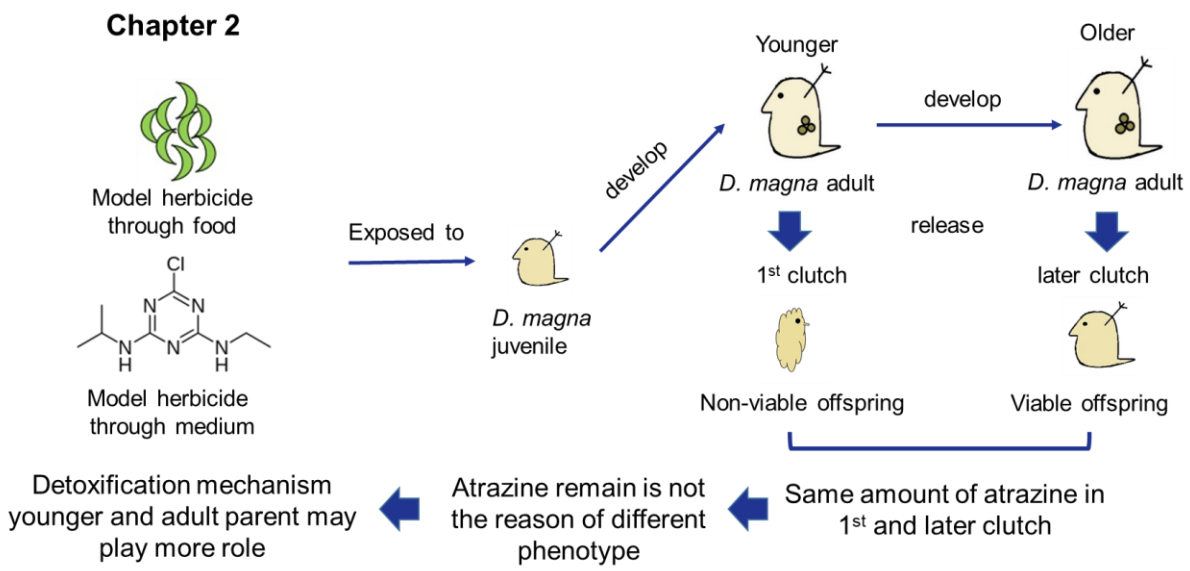

Figure 33. Summary of Chapter 2
Model herbicide atrazine affects *D. magna* in sublethal effect by producing of nonviable offspring in early clutch. Atrazine content was found to be similar among clutches, leads to hypothesis that detoxification difference between younger and older parent may have role in this phenotype

Referring back to the finding of older parent releases healthier offspring (Chapter 2), I decided to find a detoxification gene that highly abundance and expresses higher in adult stage and found *CYP360A8* to fill these criteria. This gene exhibited the highest level of mRNA expression among CYP genes. Abundance of CYPs is often linked to predictive models of drug clearance [75]. Developmental changes of the *CYP360A8* transcript level were in good agreement with temporal change of total CYP activity *in vivo* that has been assayed based on 7-ethoxycoumarin-O-dealkylation activity [63] and which may in accordance to the production of healthy offspring by older parent. This gene also belongs to the clan 3, a member of the CYP clan that is responsible for xenobiotic metabolism not only in vertebrates [71] but also in insects, represented by CYP6 families [107]–[109] that shows the closest similarity to *Daphnia CYP360A8* [66]. Furthermore, the mutant of this gene increased sensitivity to two herbicides, atrazine and paraquat, compared to wild type. Taken together, *CYP360A8* would be the major CYP that contributes to xenobiotic metabolism in this species.

If we interconnect the summary in Chapter 2 to the findings in Chapter 3, detoxification temporal change, which mostly is represented by *CYP360A8* as the highest expressed one, may results in better response of older parent to toxicants and the release of healthier offspring (Figure 34). I suggest that this continuously detoxification mechanism which maturate in older age is an important response of *Daphnia* as a crustacean living in a toxicant prone environment. As many chemicals may exist in the water, this may lead to some adjustment in *D. magna*'s detoxification mechanism to be continuously and highly active the whole life. This idea is supported with the tendency of the low induction level of most CYP genes in *D.magna* compared to CYP genes belong to the same CYP clan 3 in other species [93], [94], [110].

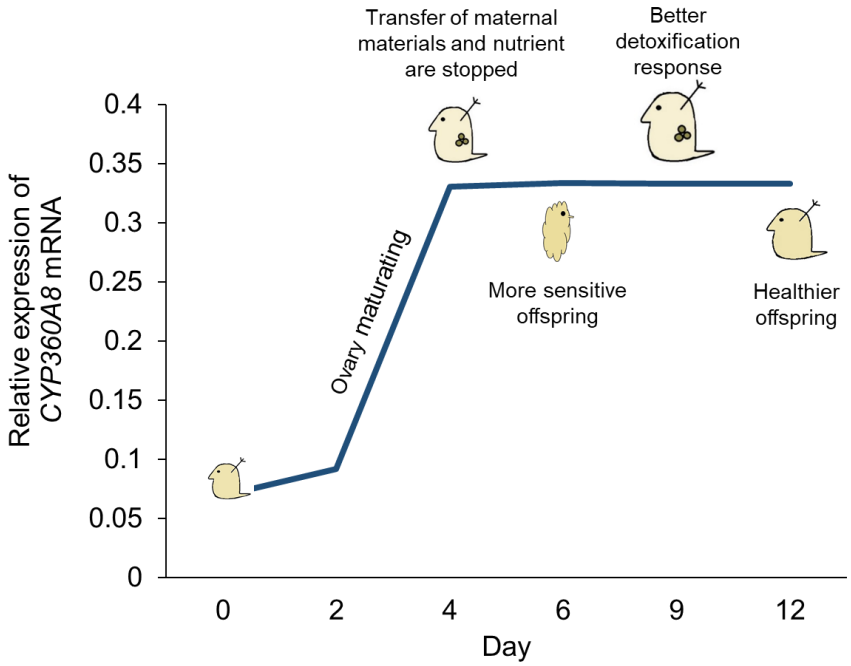

Figure 34. Suggested detoxification mechanism in relation to the maturing of *D. magna*

CYP360A8 temporal change may affect the detoxification mechanism in adult *D. magna* resulted in healthier offspring

For future perspective, in order to further understand xenobiotic metabolism in *D. magna*, it is necessary to investigate individual CYP genes involved in the metabolism of chemicals. A panel of CYP gene – xenobiotic relationship in this species can be established because information on chemical toxicity has been accumulated due to OECD test and genetic analysis has become possible. Some panels have already been established in human CYP genes [85], [111]. In this study, I produced a mono-allelic mutant of *CYP360A8*, but if it is not lethal, it may be necessary to produce animals with the biallelic mutation. Disruption of the CYP gene cluster might also be useful as reported in the other animals [71], [72]. In addition, there is a need to investigate the response of CYP mutants to a wide range of chemicals. Since the branchiopod crustacean *Daphnia* is closely related to insects [61], this approach could lead to the development of safe herbicide or insecticides that have no effect on crustaceans.

4.2. Conclusion

In this study, I identified 1) production of non-viable offspring as a sub-lethal effect of atrazine to *D. magna*, and 2) older parent produced less non-viable offspring. This leads me to focus in the detoxification mechanism in *D. magna* parent to find out if certain detoxification gene was highly active in adult stage. From this, I identified 1) *CYP360A8* as the highest expressed CYP gene and has increased expression in maturation time, which may lead to the phenotype described in Chapter 2, and 2) mutation of *CYP360A8* leads to higher sensitivity of *D. magna* towards herbicide atrazine and paraquat. The whole study demonstrates on how xenobiotic assessment in sub-lethal level continued by molecular analysis and reverse genetics strategy add new knowledge on xenobiotic metabolism of environmentally significant model organism, *D. magna*.

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List of Publications

- **Religia, P.**, Kato, Y., Fukushima, E. O., Matsuura, T., Muranaka, T., Watanabe, H. (2019). Atrazine exposed phytoplankton causes the production of non-viable offspring on *Daphnia magna*. Marine Environmental Research, 145 (March 2019), 177–183.
- **Religia, P.**, Nguyen, N.D., Nong, Q.D., Matsuura T., Kato, Y., Watanabe, H., Mutation of the cytochrome P450 *CYP360A8* gene increases sensitivity to paraquat in *Daphnia magna*. Environmental Toxicology and Chemistry.
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