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New Concept for Evaluating the Toxicity of Herbicides for Ecological Risk Assessment

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1. Introduction

The use of agricultural pesticides is increasing day by day for controlling pests and weeds in crop production, and among these pesticides, more than 65% of total amount are herbicides (USDA, 1998). Unfortunately, their exposure is usually not limited to the location where they are applied, and the pesticides reach aquatic environmental locations and compartments through various physical transport processes, such as spray drift, leaching, runoff or accidental spill, and affect the organisms living in the locations (Thurman et al., 1991; Squillace & Thurman, 1992). The ecotoxicological impact of pesticides has been usually measured by their effects on non-target organisms. Among the non-target aquatic organisms, aquatic plants received less attention for assessing the impact of pesticides, compared with the other aquatic organisms (i.e. algae, fish, daphnia etc.), however, aquatic plants play important roles in the environmental conditions of stagnant and flowing waters. They produce organic matter and oxygen, and provide food, shelter and substrate for a variety of aquatic organisms (Sand-Jensen, 1997), therefore, toxicity of herbicides to the non-target photosynthetic aquatic organisms is of concern. Peterson et al. (1997) showed that there can be several order of variation in sensitivity to herbicides between animals and aquatic plants. Huxley (1984) suggested that if one plant species becomes extinct from aquatic ecosystem, 10-30 other non-plant organisms may also become extinct. Therefore, it is of great important to understand the adverse effects by herbicides on non-target aquatic plants in the ecosystem. There is thus need of a convenient method to assay the toxicity of herbicides. For this purpose, test guidelines for ecotoxicology have been set in many countries (EU, US EPA, Japan, etc). For hazard prediction, two types of information are required: the exposure levels of non-target organisms to the chemicals, and the toxic effects of the chemicals on the non-target group under consideration. The expected environmental concentration (EEC) for the agricultural usage, which is a concentration calculated based on the input of the maximum proposed application rate, is used for the estimation of the exposure levels in an aquatic habitat (Boutin et al., 1993). The toxicity is expressed as the EC50 value, which causes 50% reduction of growth, and NOEC (no observed effect concentration), which is the maximum concentration that does not harm the test organisms. If the relationship between these two pieces of information suggests a hazard, the next step of risk assessment is to refine the assumptions to accurately predict risk. Although a short-term exposure test is required in the ecotoxicological test guidelines using an aquatic plant, duckweed, the results are not enough when considering environmentally because of the

three reasons: 1) the toxicity though a long-term exposure to a pesticide might be higher than that obtained by the short-term exposure test, 2) toxicological indexes does not indicate the lethality of the test material, and the recovery potential of the test species from the damage should be considered due to the rapid growth rate of duckweed, and 3) the exposure to several herbicides is usual in the aquatic environments, and the joint toxic effects could be affected by the combination of the chemicals.

2. Current toxicity test guidelines

In the past, the EPA relied extensively on the results of simulated and actual aquatic field studies to make final recommendations concerning risk to aquatic organisms for pesticides that trigger acute and/or chronic regulatory risk criteria (Urban & Cook, 1986). As a result of the controversy associated with the design and interpretation of the field studies in the 1980's and in the early 1990's, the EPA in 1992 decided to de-emphasize and limit its use of such studies in the aquatic risk assessment process. Instead, greater emphasis was placed on the traditional laboratory-derived toxicity test and comparison of the toxicity with EEC. Presently, the EPA requires field studies only under special circumstances, and post-registration monitoring studies are used to verify the mitigation of pesticides (Touart & Maciorowski, 1997).

In the ecological risk assessment for aquatic plants, most guidelines have focused their attention on short-term exposure toxicity, and the toxicity is usually expressed as the EC50 values (OECD, 2006; U.S. EPA, 1996). Then, the toxicity and environmental concentrations are compared to evaluate the magnitude and probabilities of the possible hazard. In the actual field, however, recovery of the reproduction capability of organisms after exposure to chemicals is another important factor that must be considered. Hughes et al. (1988) suggested that the determination of the EC50 alone does not indicate the lethality of the test material or the recovery potential of the test species. Due to the environmental significance, they recommended that if substantial inhibition is observed from a 4- or 5-day exposure to the test material, the long-term exposure toxicity and the recovery phase should be conducted, and the phytostatic and phytocidal concentrations should be determined as the primary responses, because the test procedure provides a better assessment of toxic effects on an aquatic plant population. In a study with an aquatic plant, *Lemna gibba,* the phytocidal concentrations were 2.6 to >36 times higher than the corresponding EC50 values depending on the type of the herbicides tested (Mohammad et al., 2006, 2010). In another study with *Scenedesmus quadricauda,* the EC50 of paraquat decreased with an increasing of the exposure period, and paraquat caused algistatic rather than algicidal effects at the higher concentration (Saenz et al., 2001). These findings suggest that it is important to establish a different model for understanding the potential impact of chemicals in aquatic ecosystems other than the model typically used, in which only short-term effects are considered.

In addition, misconceptions arise concerning the use of the toxicity tests. There has been some debate as to whether effects should be based on the EC50 value or the realistic exposure scenario of chemicals in the aquatic systems adjacent to the agricultural areas. To some cases, 50% reduction in growth is not considered ecologically significant due to the rapid growth rate of algae or duckweed, and the algistatic (phytostatic) and algicidal (phytocidal) concentrations are considered to be more relevant (Payne & Hall, 1979; Hughes et al., 1988). The choice of the effect parameters and calculations can impact the test results. The NOEC values based on several growth parameters varied by up to l0-fold for several

chemicals (Adams & Dobbs, 1984). The similar effect was seen between using a pigment content and a dry weight as the effect parameters (Sirois, 1990).
The way in which exposure is calculated also varies among regulatory agencies. An estimate of exposure is generally based on crop application rates. In the US, Canada and the UK guidelines, EEC in the aquatic environment is calculated from a hypothetical overspray of a water body at the maximum recommended label rate applications (Boutin et al., 1993; Holst & Ellwanger, 1982). In the calculation of the resulting concentrations in the water body, the US and Canada use 15 and 30 cm of water depth for forestry and agriculture, respectively, while in the UK, the concentration is calculated using a 100 cm-deep water body. The U.S. calculates its EEC value from 60% overspray, while Boutin et al. (1993) recommended a 100% overspray. Therefore, the concentration used to estimate hazard could be >10-fold difference among the guidelines.

3. Aquatic test organisms

Phytotoxicity data for aquatic plants have served a relatively minor role in regulatory decisions concerning the environmental hazard of most potential contaminants. A variety of phytotoxicity tests have been conducted with freshwater green algae, blue-green algae and diatoms (OECD, 2002; US EPA, 1996), and duckweed (OECD, 2006; US EPA, 1996). One of the important issues that needs to be resolved in toxicity testing is the great variability among organisms. Most aquatic toxicological research with chemicals has been conducted on algae as the standard organism, and the current scientific understanding concerning the phytotoxic effects of the contaminants is based mostly on results of algal test. The greatest limitation of these results is their uncertain environmental relevance due to the large variation among organisms in response of standard algal test species.
In addition, the interspecies variation of algae in sensitivity to a toxicant has been reported on many occasions. The sensitivities of different strains and geographical races of algae have varied as much as 200-fold (Blanck et al., 1984). Due to these differences in the algal sensitivity, there is an inability to extrapolate toxicity from one algal species to another. To improve this situation, a species battery approach needs to be used in laboratory phytotoxicity tests where several taxonomically different algae are exposed to the test substance. Swanson et al**.** (1991) provide a list of possible species.
Aquatic macrophytes are used less frequently than algae in the toxicity tests. Research with aquatic macrophytes has centered in the past on determining effective eradication techniques for nuisance growths of several species such as *Elodea canadensis* and *Ceratophyllum demersum* (Nichols, 1991). In addition, considerable research has been conducted to determine the usefulness of macrophytes as biomonitors of polluted environments (Haslam, 1982; Sortkjaer, 1984), and as bioremediative agents in wastewater treatment (Tripathi & Shukla, 1991). When macrophytes have been used in toxicity tests, the duckweeds *(Lemna* spp.) have been the species of choice, and they are often used as a representative species for all other vascular plants.
Although vascular aquatic plants are not as cosmopolitan as algae, *Lemna* sp. have been used as a test organism in various ecotoxicological test guidelines (ASTM, 1993; OECD, 2006; US EPA, 1996), and it has been reported that *Lemna* sp. is more sensitive than algae to some herbicides (Fairchild et al., 1997; Peterson et al., 1994; Mohammad et al., 2005). *Lemna* sp. is a relatively new bioindicator species, and commonly used in phytotoxicity tests because of its small size, high reproductive rate, ease of cultivation and ease of growth

measurement without specialized instruments (Wang, 1990). Due to unique in its floating structure, the exposure to herbicides can be both aerial and aquatic. There appears to be little difference in the sensitivities of the two more widely used species, *L. minor* and *L. gibba,* based on the results of Cowgill et al. (1991) and King & Coley (1985).

4. Effects of long-term exposure

4.1 Backgroud

Application of herbicides several times in the season are common in the actual field, therefore, non-target organisms are exposed to chemicals for longer periods than expected from their dissipation rates, and also in the case of slow degradation in the aquatic environment. It is believed that the toxic effects depend on both the duration and the concentration of the chemical. Davies et al. (2003) reported that the exposure to sulfosulfuron at 3.33 ppb for up to 21 days was tolerated by *Lemna* sp., but adverse effects were observed when the plants were exposed for 70 days at the same concentration. The toxicity tests for *Lemna* sp. are typically conducted for 7 days of exposure to pesticides, and toxicity usually evaluated by determining EC50 (OECD, 2006; US EPA, 1996). But, evaluation based on the short-term toxicity alone is not environmentally significant for risk assessment, because the organisms might be exposed to herbicides for longer periods as mentioned above. Therefore, it is necessary to examine long-term exposure effects on non-target organisms.

To obtain the basic information of toxicity to *Lemna gibba* of several herbicides with different mode of action, the short-term exposure tests were conducted (Mohammad et al., 2008). The herbicides used and their mode of action are listed in Table 1. The inhibitory effects were expressed by relative growth rate (RGR) at the seventh day of exposure compared with the control according to the equation (1) below.

$$\mathrm{RGR}(\%) = \frac{\text{Numberof new fronds in the test vessel at 7th day}}{\text{Numberof new fronds in the control vessel at 7th day}} \times 100 \qquad (1)$$

The frond number of *L. gibba* in the control cultures increased almost exponentially during exposure and the fronds remained green and healthy throughout the experiment. When herbicides were added, growth was affected depending on the type and concentration of the chemicals. Although growth was inhibited, no visible changes in appearance and no lethal effects were observed at any concentrations of any chemicals, except for paraquat. Higher concentrations of paraquat (100 and 1000 ppb) caused plant death with a bleaching effect. RGRs of *L. gibba* during exposure to herbicides are summarized in Fig. 1.

Five typical patterns were observed as follows: (1) cyhalofop-butyl and thiobencarb were relatively week. These chemicals inhibited growth moderately even at 1000 ppb, (2) atrazine showed moderate toxicity among the herbicides and inhibited growth completely at 1000 ppb, (3) simetryn, alachlor and diuron inhibited growth less than 16 % RGR at 100 ppb, (4) paraquat with 86% RGR in exposure at 10 ppb caused death at 100 ppb, and (5) bensulfuron-methyl and cyclosulfamuron showed higher toxicity with 24% RGR at 10 ppb and 48% RGR at 1 ppb, respectively.

Based on the results, long term exposure effects were examined for the representative herbicides, atrazine, alachlor, paraquat and cyclosulfamuron with different mode action (Mohammad et al., 2006, 2010).

Name	Chemical Family	CAS number	Mode of Action
Alachlor	Chloroacetamide	15972-60-8	Inhibition of very-long-chain fatty acid biosynthesis
Atrazine	Triazine	1912-24-9	Inhibition of photosynthesis at photosystem II
Bensulfuron-methyl	Sulfonylurea	83055-99-6	Inhibition of acetolactate synthase
Cyclosulfamuron	Sulfonylurea	136849-15-5	Inhibition of acetolactate synthase
Cyhalofop-butyl	Aryloxyphenoxy propionate	122008-85-9	Inhibition of acetyl CoA propionate carboxylase
Diuron	Urea	330-54-1	Inhibition of photosynthesis at photosystem II
Paraquat	Bipyridylium	1910-42-5	Photosystem-I-electron diversion
Simetryne	Triazine	1014-70-6	Inhibition of photosynthesis at photosystem II
Thiobencarb	Thiocarbamate	28249-77-6	Inhibition of very-long-chain fatty acid biosynthesis

Table 1. Herbicides used in this study

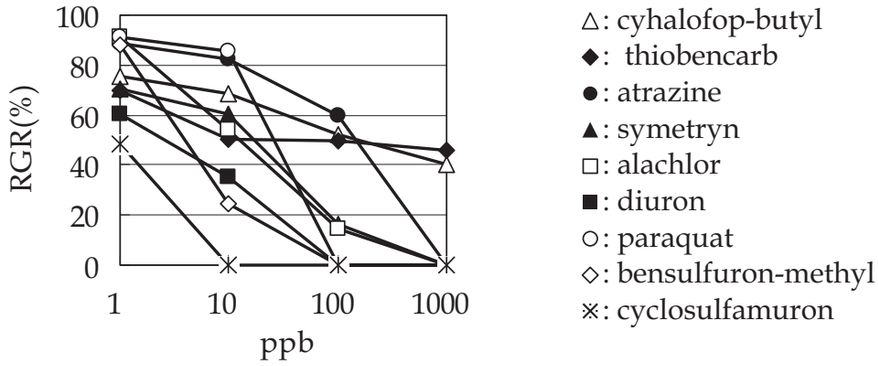


Fig. 1. Relative growth rate (RGR) of *Lemna gibba* with 7-day exposure to nine herbicides at 0, 1, 10, 100, and 1000 ppb.

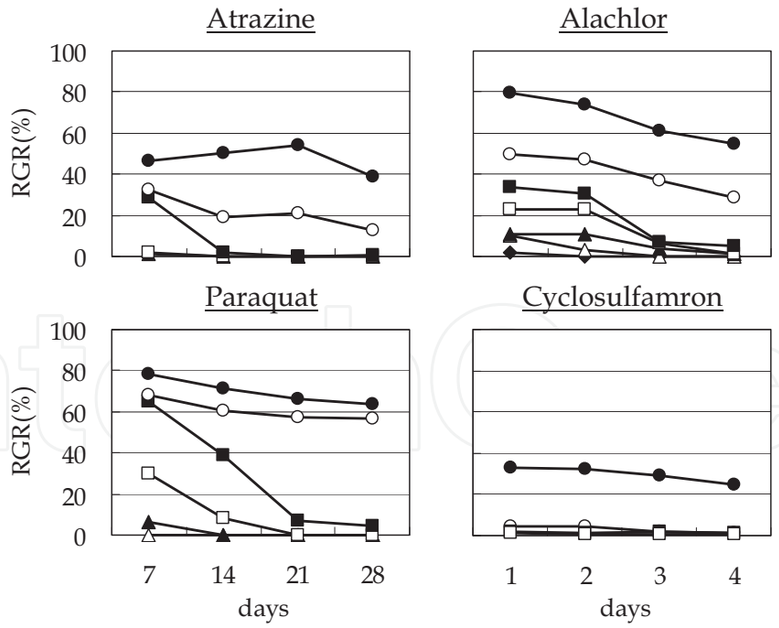
4.2 Long-term exposure experiment

The long-term toxicity to *L. gibba* was tested according to the draft OECD guidelines for the testing of chemicals (OECD, 2006). Fronds of *L. gibba* were collected from the pond in front of Lake Shinji Nature Museum, Izumo, Shimane prefecture, Japan. After collection, steps were taken to eliminate the contaminating organisms. A sample of plant materials was taken and the roots were cut off. The fronds were then shaken vigorously in clean water, followed by immersion in a 0.5% (v/v) sodium hypochlorite solution for 1 minute. The fronds were then rinsed with sterile water and placed on agar medium containing 1% saccharose to confirm the sterility. Visibly contamination-free fronds were then transferred to the same agar, and cultured for eight weeks. Sufficient colonies were transferred aseptically from the stock culture into fresh sterile medium and cultured for 10 days under the test condition before starting the test. *L. gibba* was cultivated using light conditions of 12:12 light:dark cycle, cool white fluorescent lighting at 85 $\mu E^{-2}s^{-1}$ and temperature conditions of 24 $\pm$ 2 ^{o}C.

Tests were conducted under static conditions using 9 fronds in each 100 mL test beaker containing 50mL growth medium. The beakers were covered by transparent wrapping paper with some pores for aeration. Stock solutions were prepared in either acetone or water, and different concentrations of test solution were prepared by mixing with 20X-APP growth medium based on OECD guidelines. The final concentration of acetone in the test solution was less than 0.01%. All stock solutions were prepared just before the experiments.
Frond numbers were counted at the third, fifth and seventh days of the test period. Inhibition of growth was estimated on the basis of frond number, which was calculated on the basis of frond area with a fraction of 0.2 compared with the standard mother frond. Each concentration was tested in triplicate. RGR was determined at the seventh day to evaluate the capacity of mother fronds to produce new ones.
The experiment was conducted with different exposure periods of 1, 2, 3 and 4 weeks at 200-3200 ppb for atrazine, 6.25-400 ppb for alachlor, 2.5-80 ppb for paraquat, and 1-100 ppb for cyclosulfamuron. Exposure to all chemicals were conducted under static-renewal conditions every 7 days.
Toxicity data, expressed as EC50, were determined by Ecotox-Statics 2.4 (Japanese Society of Environmental Toxicology). Multiple comparisons among the treatments in each week were analyzed by analysis of variance (ANOVA) with Duncan's test ($p>0.05$) using SPSS 12.0.

4.3 Long-term exposure effects of atrazine

When atrazine was tested at concencentrations of 0, 200, 400, 800, 1600 and 3200 ppb, the inhibition patterns with different exposure periods are shown in Fig. 2. Growth was



atrazine; ●:200, ○:400, ■:800, □:1600, ▲:3200 ppb, alachlor; ●:6.25, ○:12.5, ■:25, □:50, ▲:100, △:200, ◆:400 ppb, paraquat; ●:2.5, ○:5, ■:10, □:20, ▲:40, △:80 ppb, cyclosulfamron; ●:1, ○:10, ■:50, □:100 ppb

Fig. 2. Relative growth rate (RGR) of *Lemna gibba* fronds in exposure to herbicides for 28 days.

significantly inhibited ($p>0.05$) after a 7-day exposure at 200 ppb, and the comparable inhibition continued during 28 days of exposure. The RGR at 400 ppb slowly decreased during 28 days, from 32% to 12%. With exposure at 800 ppb, the RGR was 29% at 7 days but decreased to 2% at 14 days, and no growth was observed after 14 days of exposure. No growth was observed at 1600 or 3200 ppb after a 7-day exposure. There were no significant changes in the color of the fronds at any concentrations at any stages of exposure (Mohammad et al., 2010).

Atrazine disrupts photosynthesis, the most basic functionin the plant kingdom. It blocks the electron transport of photosynthesis, leading to a reduction in photosynthetic oxygen production and, finally, reducing the RGR. It has been assumed that chloroplast membranes can be damaged by this type of chemical (Corre et al., 1996).

4.4 Long-term exposure effects of alachlor

When the experiment was designed with alachlor at 0, 6.25, 12.5, 25, 50, 100, 200, 400, 800, 1600 and 3200 ppb for 7, 14, 21 and 28 day exposure, the results provide evidence that the growth of *L. gibba* was significantly affected at 6.25 ppb and almost stopped at 400 ppb for a 7-day exposure. There was a decrease in RGR as the exposure period and concentrations of alachlor increased (Fig. 2). A slowly decreasing tendency of RGR was observed at concentrations lower than the EC50, in which the RGR decreased from 80% to 55% at 6.25 ppb and from 50% to 30% at 12.5 ppb during a 28-day exposure. However, at concentrations higher than the EC50 level of 25 and 50 ppb, a rapidly decreasing tendency was observed for the RGR after 14 days (Mohammad et al., 2010).

The effects of 21-day exposure to alachlor on an algal community showed that a significant negative effect on algal biomass was observed at >10 pbb and approximately half the dominant algal taxa were affected, suggesting different sensitivity among algal species (Spawn et al., 1997). These researchers concluded that alachlor altered both algal community composition and biomass in agricultural streams. Therefore, it is necessary to examine the toxicity at the community level in duckweed for ecological risk assessment.

4.5 Long-term exposure effects of paraquat

Growth was significantly affected at 2.5 ppb and almost stopped at 40 ppb for a 7-day exposure (Fig. 2). The toxicological response varied after different exposure durations and concentrations of the compound paraquat. At the end of each contact test period of 28 days, there was some population growth with exposure to concentrations at <10 ppb, but the RGR decreased drastically at 10 ppb, which was lower than the EC50. The fronds were severely affected and appeared to be dead because of the bleaching effect at >20 ppb at the test duration of 28 days (Mohammad et al., 2010).

During photosynthesis, paraquat disrupts photosynthetic electron transfer by accepting electrons from photosystem I, and produces highly destructive superoxide radicals (Tomlin, 2000). Therefore, photosynthetic organisms are severely affected by exposure to paraquat and often die. In a previous study, it was found that freshwater algae generally died at paraquat concentrations between 0.25 and 0.5 ppm (Eisler, 1990), but the exposure period was not mentioned. A study with different exposure periods, 1, 2, 3, and 4 days, showed that the EC50 decreased from 0.89 to 0.22 ppm with an increasing exposure period with *Scenedesmus quadricauda* (Saenz et al., 2001).

4.6 Long-term exposure effects of cyclosulfamuron

Effects of exposure period (1, 2, 3, and 4 weeks) and concentration (1, 10, 50, and 100 ppb) on growth inhibition were examined using cyclosulfamuron. Growth was inhibited at 1 ppb, and completely stopped at 10 ppb in the first week of exposure (Fig 2). When the exposure period was prolonged by transferring the mother fronds once a week to new media, no change was observed in inhibition at 1 ppb even in the fourth week of exposure. But at higher concentrations (10-100 ppb), a bleaching effect was observed with longer exposure (3-4 weeks), during which color of fronds turned yellow to white. Sulfonylureas temporally inhibited the growth at less than the EEC of 3-20 ppb, and a longer exposure, beyond 21 days, caused severe damage, such as the death of fronds, at the EEC level (Mohammad et al., 2006).

Important points in risk assessment of sulfonylureas to *Lemna* sp. are presented in this study. Sulfonylureas are inhibiting the enzyme, acetolactete synthase, which is necessary in the first step for plants to synthesize the branched amino acids, valine, leucine and isoleucine (Brown, 1990; Schloss, 1994). Sulfonylureas inhibited only cell division on short-term exposure, but prolonged exposure resulted in lethality at the same concentration.

4.7 Conclusion

The toxic effects were affected by the exposure period and concentration, depending on the type of herbicide. All the tested herbicides showed stronger toxicity with the increasing exposure period than the toxicity of the standard exposure period suggested by guidelines. These characteristics of herbicides required a different model than typically used, where only short-term exposure is usually assumed, for understanding the potential impact of herbicides in aquatic ecosystems, e.g. comparison of toxicity at different concentrations with different exposure periods.

5. Recovery potential from damage

5.1 Background

In the actual field, recovery of the reproduction capability of *Lemna* sp. after exposure to herbicides is another important factor which must be considered. Hughes et al. (1988) suggested the importance of examining the recovery potential and determining phytostatic and phytocidal concentrations for a better assessment of toxic effects on an aquatic plant population. However, very few studies assessing the recovery potentials of *Lemna* sp. have been conducted. Nathalie et al. (2008) found on the algae *Scenedesmus vacuolatus* that the delay in recovery subsequent to S-metolachlor exposure contrasted with the fast recovery upon exposure to triazines and phenylureas (photosystem II inhibitors). While the effects following exposure to the photosynthesis inhibitors were readily reversible, exposure to herbicides that impaired cell division induced a delayed recovery. The results suggest that the mode of action of chemicals, the reversibility of their binding at the target site, and the degree of damage during exposure, all influence the potential recovery following exposure.

5.2 Recovery experiment

Recovery potential of *L. gibba* from the damage by several herbicides listed in Table 1 with different mode of action was examined (Mohammad et al., 2008). After each exposure period for 7 days was conducted according to the draft OECD guidelines (OECD, 2006), the nine mother fronds were collected from each beaker, washed in sterilized distilled water,

and transplanted to fresh medium for recovery. The tests were done under static conditions using 100 mL test beaker containing 50 mL growth medium. Frond numbers were counted at the third, fifth and seventh days of the recovery periods, and at the tenth day when the recovery was slow.

The effect of longer-term exposure on the recovery potential was examined for the herbicides, atrazine, alachlor, paraquat and cyclosulfamuron with different mode action (Mohammad et al., 2006, 2010). The basic test conditions were the same as those of the above mentioned experiment. After exposure for 1, 2, 3 and 4 weeks, the nine mother fronds were transplanted to fresh medium for recovery, and RGR was determined at the third, fifth and seventh days of the recovery periods. The phytostatic and phytocidal concentrations of the tested chemicals for *L. gibba* were determined according to the definition described by Hughes et al. (1988).

5.3 Recovery potential from damage by herbicides with different mode of action

When the fronds were transferred to fresh medium after 7 day-exposure for recovery, *L. gibba* started to grow again even in plots where they did not grow during the exposure period. RGRs of *L. gibba* during recovery are shown in Fig. 3. Patterns of RGRs in the recovery periods showed a tendency corresponding to the mode of action of the herbicides.

Cyhalofop-butyl and thiobencarb exhibited rapid recovery as well as the untreated control even at 1000 ppb and growth recovered to more than 70% RGR. Results from the recovery test with alachlor, having the same mode of action with thiobencarb, showed a slow recovery tendency for all the concentration tested. RGR in recovery for 1000ppb was 15% and for 100ppb was 32%. Triazine and urea herbicides showed moderate recovery. Although the growth was inhibited completely at 1000ppb in exposure (Fig. 1), 76% RGR was observed in recovery in case of atrazine. In case of simetryn, the RGR was >20% in recovery for 1000ppb. Therefore, the chemicals which act as inhibitors of photosystem II are moderately toxic to *L. gibba*, and moderate recovery (RGR, 76%) was observed with exposure for 7 days. Paraquat showed no recovery above the critical concentration. Recovery potential of *L. gibba* from inhibition by the sulfonylureas herbicides was greater than with other types of herbicides and recovery was possible even at 1000 ppb with 57 % RGR for bensulfuron-methyl and with 71% RGR at 10 days during the recovery period (data not shown) for cyclosulfamuron. In risk assessment, the expected environmental concentrations of the sulfonylureas were reported as 3-20 ppb (Peterson et al., 1994), which

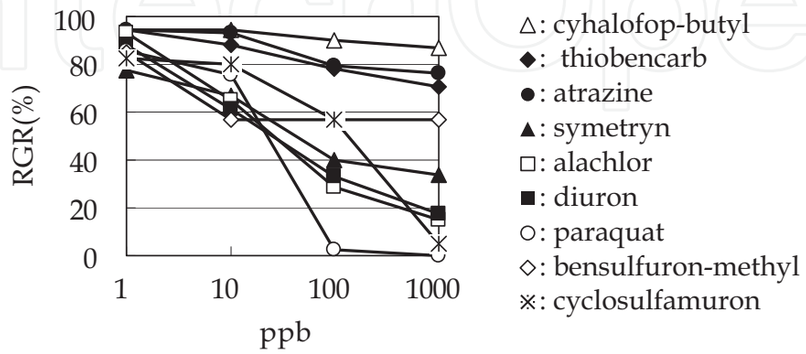


Fig. 3. Relative growth rate (RGR) of *Lemna* sp. In recovery in fresh medium after exposure to nine herbicides at 0, 1, 10, 100, and 1000 ppb for 7 days.

are greater than EC50 of *Lemna* sp. for some sulfonylureas, but recovery of growth is possible when the chemicals are dissipated by degradation in the environment.

5.4 Effect of long-term exposure on recovery potential from damage by atrazine

Fig. 4 shows the recovery after prolonged exposure of *L. gibba* at concentrations of 0, 200, 400, 800, 1600 and 3200 ppb. There was an apparent recovery of the population, even from no growth for 28 days at 3200 ppb. The RGRs were higher than those in the exposure for all concentrations tested (Fig. 2), although the significant difference was observed even at 200 ppb. The RGR in recovery depended on the concentration of atrazine in the exposure. The RGR decreased slightly after 7 days of exposure and were almost constant between 14 and 28 days of exposure. There were no significant changes in the color of the fronds at any concentrations at any stages of exposure and recovery. Under the experimental conditions, phytostatic concentrations of atrazine to *L. gibba* were 1600 and 800 ppb in the exposure periods of 14 and 28 days, respectively, and the phytocidal concentration was >3200 ppb for a 28-day exposure (Table 2) (Mohammad et al., 2010). At the highest concentration of 3200 ppb with exposure for 28 days, the RGR was 43% in recovery. The results suggest that even after a 28-day exposure to atrazine at an EEC level of 2667 ppb (Peterson et al., 1994), *L. gibba* might have capability to re-grow in the environment after the removal of atrazine by degradation.

5.5 Effect of long-term exposure on recovery potential from damage by alachlor

Recovery of alachlor after different exposure period and concentrations in fresh growth medium was according to the exposure duration and concentrations. The RGR was almost the same for 28 days when *L. gibba* was exposed at <12.5 ppb, but it constantly decreased at >12.5 ppb with exposure for longer than 14 days. The growth of populations exposed to 400 ppb for 14 days indicated a phytostatic response, while populations exposed for 21 days at ≥200 ppb showed a phytocidal response. The phytostatic concentration of alachlor to *L. gibba* was 400 ppb for a 14-day exposure, and the phytocidal concentration was >400 ppb within 14 days of exposure, but it decreased to 200 ppb for 21- and 28-day exposures (Fig. 4, Table 2) (Mohammad et al., 2010).

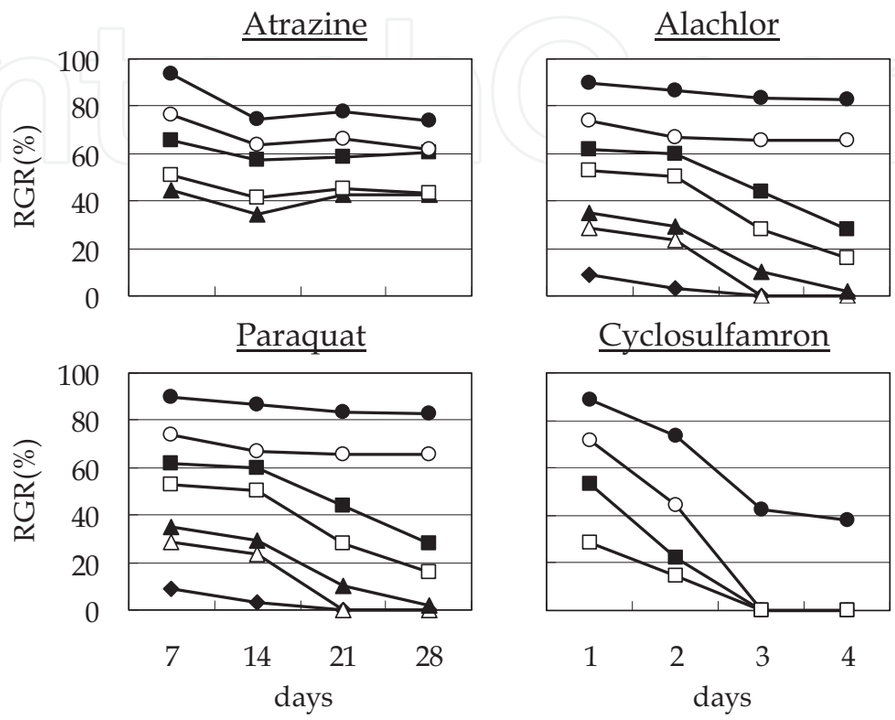
Alachlor interferes with metabolism and inhibits the synthesis of fatty acids (Weisshaar et al., 1993; Couderchet & Boger, 1993). It has nearly the same scenario as sulfonylurea, which disrupts amino acid biosynthesis and affects processes essential to all photosynthetic organisms (Moberg & Cross, 1990). Mohammad et al. (2006) showed that short exposures to cyclosulfamuron at higher concentrations caused longer lag periods for the initiation of growth in recovery, and a longer exposure period caused a slower growth rate without the lag period. The same tendency was observed in the case of alachlor (data not shown).

A recovery study with an algal community after exposure to alachlor at >10 ppb for 21 days showed that some algal taxa recovered after exposure, while others took longer or did not recover (Spawn et al., 1997). Therefore, it is assumed that recovery of other species of duckweed should be examined as there are differences in recovery among algal species.

5.6 Effect of long-term exposure on recovery potential from damage by paraquat

The RGR was higher in recovery than in exposure, but the difference was slight. Recovery was smooth at <10 ppb within a 14-day exposure, but it decreased when the exposure was longer than 21 days. The RGR constantly decreased at concentrations >20 ppb, and no

recovery was observed because of death in the case of an exposure longer than 7 days. The phytostatic concentration of paraquat was not determined because all phytostatic fronds could not grow in the recovery period. The phytocidal concentration decreased with exposure period from 80 ppb for a 7-day exposure to 20 ppb for 21- and 28-day exposures (Fig. 4, Table 2) (Mohammad et al., 2010).



atrazine; ●:200, ○:400, ■:800, □:1600, ▲:3200 ppb, alachlor; ●:6.25, ○:12.5, ■:25, □:50, ▲:100, △:200, ◆:400 ppb, paraquat; ●:2.5, ○:5, ■:10, □:20, ▲:40, △:80 ppb, cyclosulfamron; ●:1, ○:10, ■:50, □:100 ppb

Fig. 4. Relative growth rate (RGR) of *Lemma gibba* fronds in recovery in fresh medium after the exposure to herbicides.

Chemicals	Phytostatic concentrations (ppb)				Phytocidal concentrations (ppb)			
	Exposure period (days)				Exposure period (days)			
	7	14	21	28	7	14	21	28
Atrazine	1600	1600	800	800	>3200	>3200	>3200	>3200
Alachlor	>400	400	nd[a]	nd	>400	>400	200	200
Paraquat	nd	nd	nd	nd	80	40	20	20
Cyclosulfamuron	100	50	nd	nd	>100	>100	10	10

Table 2. Phytostatic and phytocidal concentrations of atrazine, alachlor, paraquat and cyclosulfamuron for *Lemna gibba* in different exposure periods. [a] not determined

Another study with different exposure periods, 1, 2, 3, and 4 days, showed that *Scenedesmus quadricauda* could recover its reproduction capability even with exposure at the maximum concentration in their test, 3.2 ppm, for 4 days, but the EC50 decreased from 0.89 to 0.22 ppm with an increasing exposure period (Saenz et al., 2001). Those researchers also explained that an extended lag phase was required for recovery, and that it was more extended for the population exposed at 3.2 ppm. However, they did not check the lethal concentrations with a longer exposure to paraquat. Similar results were also obtained in our study, as the RGR varied from 72% to 13% in recovery from a concentration of 10 ppb with exposure periods from 7 to 28 days. Therefore, growth was possible in recovery with exposure at 10 ppb, but the recovery potential drastically decreased with a longer exposure. Phytocidal concentrations were 80 and 20 ppb for exposure for 7 and 28 days, respectively. This shows remarkable variation in the sensitivity between duckweed and algae to paraquat.

5.7 Effect of long-term exposure on recovery potential from damage by cyclosulfamron

In the recovery period after different length of exposure, RGR decreased with longer exposure as shown in Fig. 4. Reproduction was observed within two weeks of exposure at 100 ppb, but no recovery occurred after exposure for three weeks at more than 10 ppb. Cyclosufamuron, with an EC50 of 0.91 ppb, was phytostatic at 100 and 50 ppb for 7- and 14-day exposures, respectively, indicating no lethal effects at more than 50 times the concentration of EC50 within an exposure period of 14 days. In the case of exposure for longer than 21 days, however, it exhibited phytocidal activity at 10 ppb (Table 2). The results suggest that the recovery potential of *L. gibba* drastically changes depending on both chemical concentration and exposure period. *L. gibba* can reproduce again at the same rate as that before the exposure if cyclosufamuron is removed within two weeks, even after complete inhibition at 100 ppb (Mohammad et al., 2010).

5.8 Conclusion

Recent studies demonstrated that a longer period of exposure caused more serious adverse effects on *Lemna* sp. and the exposure period could affect on recovery. When the relationship of RGRs between exposure and recovery periods was examined, the RGR in recovery from the damage by atrazine was not affected much by the RGR in exposure. In case of alachlor and paraquat, the RGR in recovery was dependent on the RGR in exposure. For cyclosulfamuron, RGR decreased along with exposure period, therefore, the potential for recovery was dependent on the exposure period (Mohammad et al., 2010). When considering phytostatic and phytocidal scenario, the phytostatic and phytocidal concentrations decreased with exposure period. In some cases, phytocidal concentration became lower than the EC50 value when exposure was prolonged. Therefore, incorporation of the both concentrations associated with the exposure period would be important for ecotoxicological risk assessment of herbicides.

6. Combined effect of herbicides

6.1 Background

It is common to find combinations of several herbicides in the surface water in agricultural areas, with the exact type of substance depending on the dominant crops in the area.

Herbicides in the environment rarely occur alone. In a large US monitoring program, more than 50% of all stream samples contained 5 or more pesticides, and about 15% contained more than 10 compounds (Gilliom et al., 1999). Therefore, herbicide toxicity in natural ecosystems is not generally the result from exposure to a single toxicant, but rather exposure to mixture of toxicants. Therefore, mixture toxicity has been a subject of ecotoxicological interest for several decades.

A small number of studies have reported the potential threat to macrophytes exposed to pesticide mixtures in aquatic model ecosystems (Fairchild et al., 1994; Lytle & Lytle, 2002, 2005; Wendt-Rasch et al., 2004). Recent laboratory studies with the standard OECD plant species *Lemna minor* addressed the joint toxicity of pesticides by applying the two common models of mixture toxicity: concentration addition (CA) and independent action (IA) (Belz et al., 2008; Cedergreen et al., 2007a, b; Munkegaard et al., 2008). The concept of CA is based on the assumption that any component of a mixture can be replaced by another without altering the overall effect of the mixture, and applied for toxicants with similar molecular target sites (Loewe & Muischnek, 1926). The concept of IA is based on the assumption that all mixture components independently contribute to a given effect by different modes of action (Bliss, 1939). However, how the joint toxicity of such combinations of pesticides should be estimated is still a matter of debate in the case of considering effects of long term exposure and recovery potential.

In this section, the results of our recent study are presented on the combined effects of the mixtures of herbicides with dissimilar modes of action. Based on the results of our previous studies (Mohammad et al., 2006, 2010), we selected three combinations: paraquar + atrazine, paraquat + alachlor, and paraquat + cyclosulfamuron. Because the combinations of the herbicides with different modes of action were used, the expected joint effects were calculated based on the IA model from the individual effects, and the actual joint effects were evaluated by comparing with the expected effects. Deviation from the prediction is thus an indication of antagonism (weaker effects) or synergism (stronger effects). The mixture effects were also evaluated on a basis of the effects of long term exposure and recovery potential from the damage.

6.2 Herbicides' combined effects experiment

The long-term toxicity to *L. gibba* and the recovery potential from the damage was tested. The experiments of the mixture of herbicides were conducted for 7, 14, 21 and 28 days exposure, followed by a 7-day recovery test in a fresh medium for each length of the exposure. The experimental conditions, test procedures, measurement of the number of fronds, determination of RGR, phytostatic and phytocidal concentrations were the same as described in the previous sections. The concentrations for mixture were set based on the results of our previous study as follows (Mohammad et al., 2006, 2010). In order to observe the combined effects during the long-term exposure to chemicals, the concentrations were basically set at lower than the EC50 values.

The EC50 of paraquat was found to be 31 ppb, but during a 28-day exposure, RGR drastically decreased at <10 ppb, and the bleaching effect was observed at >20 ppb. Recovery was smooth at <10 ppb within a 14-day exposure, but it decreased when the exposure was longer than 21 days. Therefore, the concentrations for paraquat were set at 2.5, 5 and 10 ppb.

The EC50 of atrazine was found to be 89 ppb, and its phytotostatic concentrations were 1600 and 800 ppb for exposure periods of 14 and 28 days, respectively, and no phytocidal effects were observed up to 3200 ppb for a 28-day exposure. Therefore, the concentration of atrazine for mixture with paraquat was set at 100 ppb, which is near the EC50 value of atrazine.

The EC50 of alachlor was found to be 31 ppb, and RGR slowly decreased during the exposure at concentrations lower than EC50 level of 6.25 and 12.5 ppb, but rapidly decreased at 25 and 50 ppb for 14 days of exposure. Therefore, the concentration of alachlor was set at 10 ppb for mixture with paraquat.

The EC50 of cyclosulfamuron was found to be 0.91 ppb for a 7-day exposure, and the phytostatic concentrations were 100 and 50 ppb, for a 7- and 14-day exposures, respectively, and the phytocidal activity was 10 ppb when the exposure was longer than 21 days. Considering the results of our study with cyclosulfamuron, the concentration of cyclosulfamuron for mixture with paraquat was set at 0.15ppb.

The expected RGR in exposure to combined herbicides was calculated based on the RGR values in exposure to each herbicide according to the equation (2) below.

$$\begin{aligned} &\text{Expected RGR}(\%) \text{ in exposure to compounds A and B mixture} \\ &= \text{RGR}(\%) \text{ in exposure to compound A x RGR}(\%) \text{ in exposure to compound B} / 100 \end{aligned} \quad (2)$$

The expected RGR in recovery from the damage by combined herbicides was calculated based on the RGR values in recovery from the damage by each herbicide according to the equation (3) below.

$$\begin{aligned} &\text{Expected RGR}(\%) \text{ in recovery for compounds A and B mixture} \\ &= \text{RGR}(\%) \text{ in recovery for compound A x RGR}(\%) \text{ in recovery for compound B} / 100 \end{aligned} \quad (3)$$

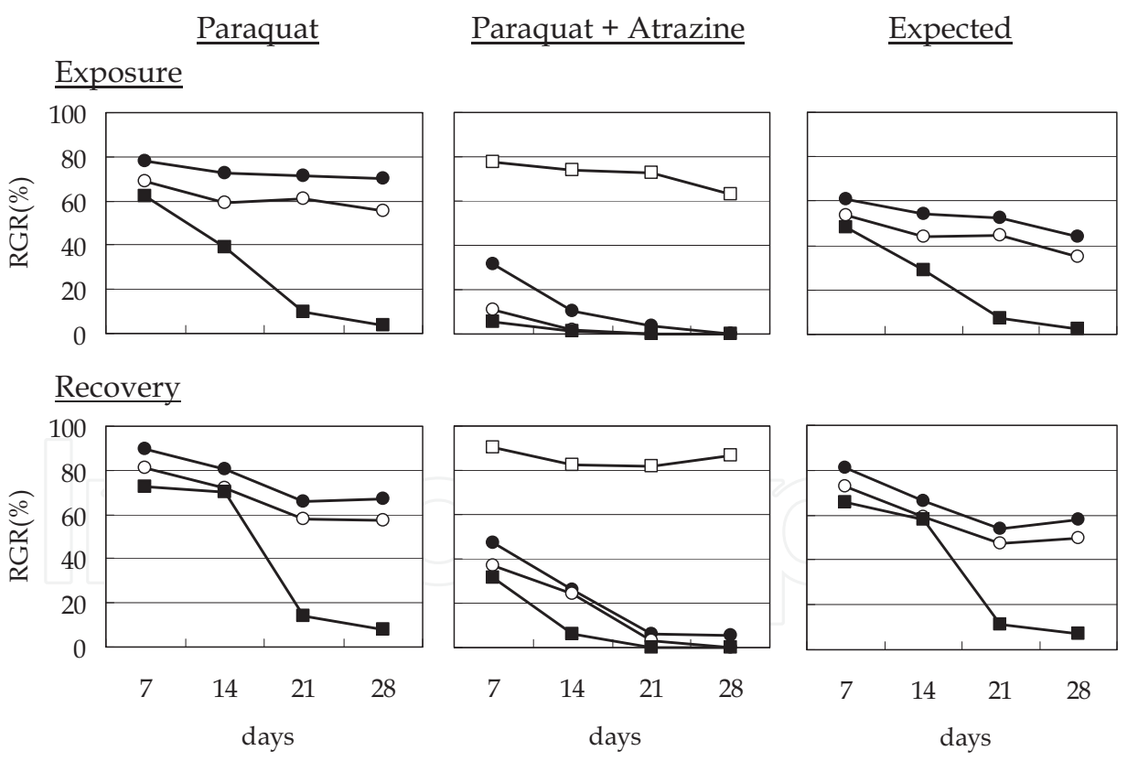
6.3 Mixture effects of paraquat and atrazine

The influence of mixtures of paraquat and atrazine on the growth and recovery potential of *L. gibba* are shown in Fig. 5. When *L. gibba* was exposed to mixtures of paraquat and atrazine, the growth inhibition increased with all the mixture concentrations, compared with individual paraquat and atrazine. The RGR value decreased from 78% to 32% for 7 days exposure, and 70% to 0% for 28 days exposure by the addition of 100 ppb of atrazine to 2.5 ppb of paraquat. The expected RGR calculated from the RGR for each herbicide was larger than the observed RGR, therefore, stronger synergistic effects than the expected ones were indicated. At the highest test concentration of mixture (paraquat 10 ppb + atrazine 100 ppb), the RGR decreased 62% to 5% for 7 days exposure, but there was no change in colour of any fronds at the end of the exposure period.

In the recovery phase, the reproduction was very slow in the case of mixture compared with the individual herbicide. The RGR in recovery ranged from 90% to 73% after a 7-day exposure to only paraquat at from 2.5 to 10 ppb, whereas the RGR was from 47% to 32% when 100 ppb of atrazine was added. The RGR in recovery in atrazine alone at 100 ppb was above 82% even after exposure for 28 days, and the observed RGR in recovery for the mixed herbicides were smaller than the corresponding expected RGR, suggesting that there was the synergistic effect also in the recovery phase.

Although no growth was observed at the mixture of 10 ppb of paraquat and 100 ppb of atrazine after 21-day exposure, there was no phytocidal effect in appearance. Phytostatic effect was found at the mixture of 5 and 10 ppb of paraquat and 100 ppb of atrazine for 21-day exposure, and at the mixture of 2.5 ppb of paraquat and 100 ppb of atrazine for 28-day exposure. The phytostatic concentration of atrazine was 800 ppb for the same period of exposure (Table 2), therefore, atrazine showed eight times stronger phytostatic effects by adding paraquat at 10 ppb. On the other hand, paraquat did not show any phytostatic effect at this concentration, but paraquat showed this type of character when mixed with atrazine. This is an interesting phenomenon in this combination.

Atrazine is a common contaminant of surface waters, as a result of agricultural non point surface and subsurface runoff, and is usually detected in levels from less than 0.5 ppb (Albanis et al., 1995; Squillace & Thurman, 1992) up to 100 ppb (Thurman et al., 1992). The toxic effects of a mixture of atrazine and metolachlor were examined in unialgal cultures of *Chlorella fusca var-fusca* using a bioassay system. In concentrations lower than the EC50, the combination resulted in reduced toxicity (antagonism) in comparison with the toxicity caused by the sum of toxic actions of the same levels of concentration from single chemicals (Kotrikla et al., 1999). Another study analyzed the toxicity of two mixtures (atrazine and the insecticide chlorpyrifos; atrazine and the fungicide chlorothalonil) to the marine



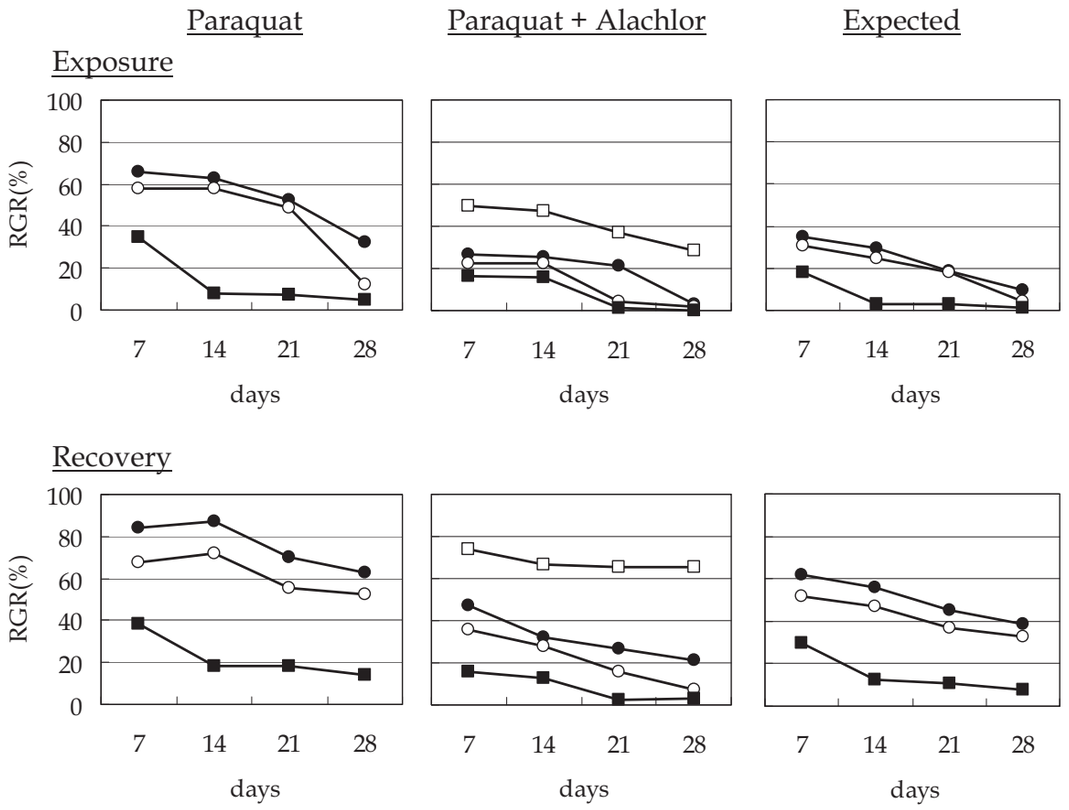
paraquat; ●:2.5, ○:5, ■:10, atrazine alone; □:100 ppb

Fig. 5. Relative growth rate (RGR) of Lemna giba in exposure and in recovery in fresh medium after the exposure to paraquat with and without atrazine. Expexted RGR in exposure and in recovery were calculated as (paraquat exposure RGR) x (atrazine exposure RGR)/100, and (paraquat recovery RGR) x (atrazine recovery RGR)/100, respectively.

phytoplankton species *Dunaliella tertiolecta* (Chlorophyta). Atrazine and chlorpyrifos in mixture displayed additive toxicity, whereas atrazine and chlorothalonil in mixture had a synergistic effect. The toxicity of atrazine and chlorothalonil combined was approximately 2 times greater than that of the individual chemicals (DeLorenzo & Serrano, 2003).
Our study using *L. gibba* showed that the sensitivity increased in presence of atrazine with paraquat for 7 to 28-day exposure. Fig. 5 shows that there are large difference between the expected and actual inhibition scenario in both exposure and recovery phases The results suggest the importance of examining combined effects of herbicides for ecotoxicological risk assessment.

6.4 Mixture effects of paraquat and alachlor

After 7 days of exposure, RGR significantly decreased from 70% to 29% by adding 10 ppb alachlor with the lowest paraquat concentration of 2.5 ppb (Fig. 6). The RGR in exposure to only alachlor for the same period was 50%, therefore, the observed RGR value was almost the same as the expected RGR (35%). When the exposure prolonged up to 28 days and the concentration of paraquat increased up to 10 ppb, the RGR decreased from 34% to 3% and



paraquat; ●:2.5, ○:5, ■:10, alachor alone; □:100 ppb

Fig. 6. Relative growth rate (RGR) of *Lemna gibba* fronds in exposure and in recovery in fresh medium after the exposure to paraquat with and without alachor. Expexted RGR in exposure and in recovery were calculated as (paraquet exposure RGR) x (alachor exposure RGR)/100, and (paraquet recovery RGR) x (alachor recovery RGR)/100, respectively.

from 36% to 17%, respectively, by adding alachlor. These decreases of RGR were also comparable to the expected RGR. The results indicated that the effects of a mixture of paraquat and alachlor could be predicted from the individual toxicity. Although the RGR becomes 0% at the highest mixture concentration, no discoloration of frond was seen.

Higher RGR values were observed in recovery than those in exposure for all selected mixture concentrations, even from complete inhibition at the highest mixture of 10 ppb of paraquat and 10ppb of alachlor, but the recovery was slow compared with each corresponding individual concentration of paraquat and alachlor. RGR was 47% at the lowest mixture concentration of 2.5 ppb of paraquat and 10 ppb of alachlor for 7 days exposure, while 84% and 74% RGR was observed in the individual corresponding concentrations of paraquat and alachlor, respectively. Moreover, although there was not a marked difference between the expected and actual RGRs in the exposure phase, in the recovery phase, the actual RGR was lower than the expected RGR at all combinations of the mixture. Therefore, the mixture of paraquat and alachlor showed stronger synergistic effects on *L. gibba* in recovery than in exposure.

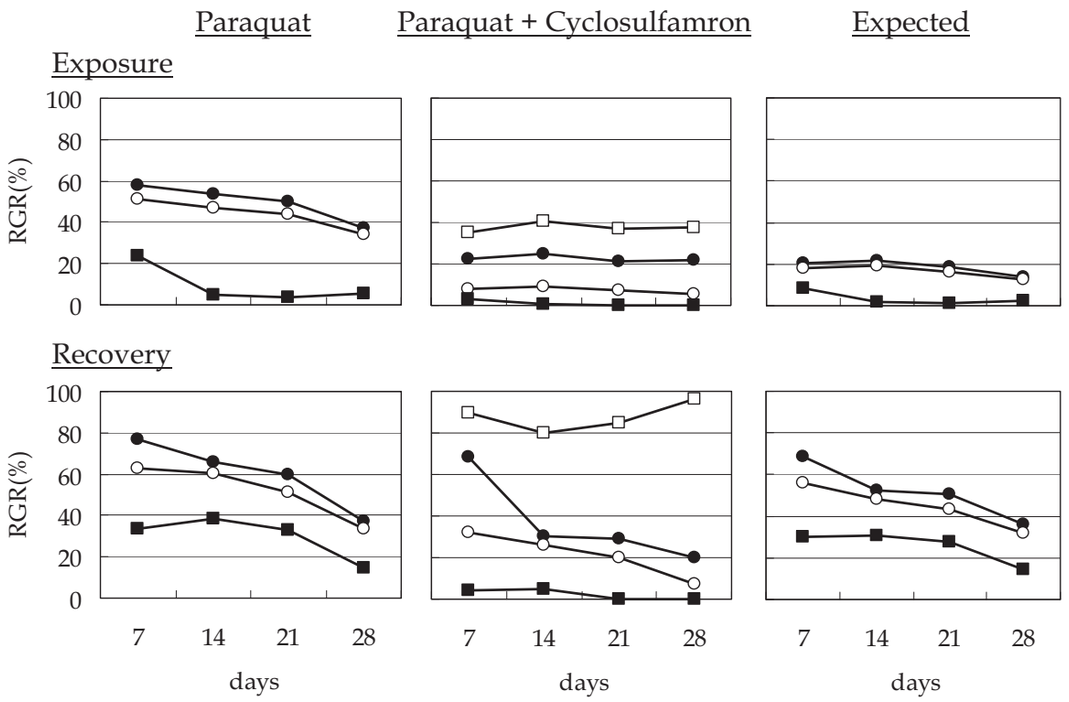
6.5 Mixture effects of paraquat and cyclosulfamuron

The RGR in exposure was significantly affected by all the selected mixture of paraquat and cyclosulfamuron (Fig. 7). RGR decreased from 58% to 23% by adding 0.15 ppb of cyclosulfamuron to 2.5 ppb of paraquat for 7 days exposure. When the exposure was prolonged up to 28 days, the RGR did not change as in the case of individual herbicide. The RGR at this set of concentration were similar to the corresponding expected RGR, while at 5 ppb of paraquat, lower RGR values were observed compared with the expected RGR values throughout the exposure period. With the highest mixture concentrations of 10 ppb paraquat and 0.15 ppb cyclosulfamuron, the RGR became 0% and strong discoloration (chlorosis) of total fronds was seen after 21 days exposure.

At the end of each test period, the fronds were transferred to fresh medium to observe the recovery potential of individual and mixture of chemical treatment up to 7 days. The recovery of fronds, which were exposed to individual chemical, was clearly possible, as RGR in recovery was 77% and 90% after individual exposure for 7 days to paraquat at 2.5 ppb and cyclosulfamuron at 0.15 ppb, respectively. And the mixture of the herbicides at these concentrations showed 67% RGR which was the almost same as the expected RGR (69%). But when the exposure period or concentration increased, the actual RGR were lower than the corresponding expected RGR. Bleached fronds at the height mixture concentration, exposed for 21 and 28 days, were supposed to be dead because no growth was observed in recovery. Therefore, the phytocidal concentration of the mixture was found to be a mixture of 10 ppb of paraquat and 0.15 ppb of cyclosulfamuron for 21 and 28 days exposure, and phytostatic scenario was identified for 14 days exposure at the same concentrations. Phytocidal concentrations of individual paraquat and cyclosulfamuron were 20 ppb and 10 ppb, respectively, for the same period of exposure (Table 2). Therefore, the results showed that a mixture of paraquat and cyclosulfamuron at lower concentration than their phytocidal level caused phytocidal effects on *L. gibba*. The results also showed that the mixture toxicity of paraquat and cyclosulfamuron not only increased sensitivity of *Lemna gibba,* but also lethal if exposure prolonged beyond 14 days with concentration of paraquat 10 ppb and cyclosulfamuron 0.15 ppb, which showed nether phytostatic nor phytocidal effect when act individually.

Deneer et al. (2000) found that the concentration addition was quite common for narcotic acting compounds. They demonstrated in experiments with 50 nonreactive chemicals that

even chemicals present at very low concentrations, equivalent to 0.01, 0.005, and 0.0025 toxic units, contributed to the overall toxicity.



paraquat; ●:2.5, ○:5, ■:10, cyclosulfamron alone; □:0.15 ppb

Fig. 7. Relative growth rate (RGR) of *Lemna gibba* fronds in exposure and in recovery in fresh medium after the exposure to paraquat with and without cyclosulfamron. Expexted RGR in exposure and in recovery were calculated as (paraquet exposure RGR) x (cyclosulfamron exposure RGR)/100, and (paraquet recovery RGR) x (cyclosulfamron recovery RGR)/100, respectively.

6.6 Conclusion

When focusing on ecotoxicological studies and risk assessments of mixture effects of herbicides on aquatic plants, synergism is the most important effect to protect against, since it can not be predicted and results in an increase of toxicological effects, as it is the worst interaction between components of mixtures. If one, two or more chemicals were present at low levels in the same ecosystems, each of them would be poorly deleterious to non-target species if considered separately, but their addition increase significantly the ecotoxicological risk by the accumulation of low level risks. Therefore, increased toxicity due to the synergistic nature of the herbicides could results in detrimental effects to primary producers at concentrations lower than expected from the individual toxicity. The joint effects of the herbicides to *L. gibba* presented in this section suggested that they often appeared stronger than the expected ones, therefore, they could not be predicted from the standard toxicity test using a single herbicide. In addition, the effects of long-term exposure to herbicides and recovery potential of duckweed were also affected by their combination, indicating further understanding of mechanisms how mixtures of herbicides affect non-target aquatic species is necessary.

7. Future perspective

Current scientific knowledge concerning the phytotoxicities of potential contaminants is based largely on results from laboratory toxicity tests for a few freshwater green algal species. The available results are used sometimes, with little scientific justification, as surrogates for other types of aquatic plants and organisms. Our knowledge in regard to how different organisms respond to herbicides is simply insufficient to be able to speculate about cause and effect scenarios. In addition to the regulatory testing that needs to be developed, there is a need for complimentary research that will expand our knowledge beyond the level given by these standard regulatory tests. The ultimate goal of any phytotoxicity test should be to provide results for a battery of relevant surrogate species. As a result, a composite picture can be obtained to estimate the short- and long-term influence of contaminants on the condition of an exposed plant community and ecosystem. With this in mind, there is a need not only to increase use of the available phytotoxicity test methods but also to continue to develop their ability to provide useful results.

Our results presented that the relative risk of a variety of scenarios of exposure and recovery with an aquatic vascular plant *Lemna* sp. exposed to individual and mixtures of herbicides are significant from both regulatory and research perspectives. To address actual environmental situations, the application of this approach would be a good solution for a better understanding of the ecological significance of the end points used in toxicity testing and how they are interpreted and applied in ecological risk assessment.

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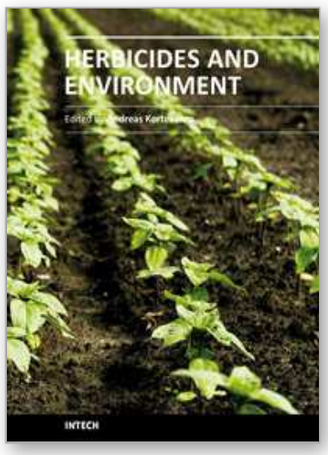

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Herbicides are much more than just weed killers. They may exhibit beneficial or adverse effects on other organisms. Given their toxicological, environmental but also agricultural relevance, herbicides are an interesting field of activity not only for scientists working in the field of agriculture. It seems that the investigation of herbicide-induced effects on weeds, crop plants, ecosystems, microorganisms, and higher organism requires a multidisciplinary approach. Some important aspects regarding the multisided impacts of herbicides on the living world are highlighted in this book. I am sure that the readers will find a lot of helpful information, even if they are only slightly interested in the topic.

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