Bull. Environ. Contam. Toxicol. (2006) 76:256–263

DOI: 10.1007/s00128-006-0915-0

Bulletin of Environmental Contamination and Toxicology

Recovery of *Lemna* sp. after Exposure to Sulfonylurea Herbicides

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Received: 19 September 2005/Accepted: 28 November 2005

Pesticides have been widely used for controlling pests and diseases in crop production. However, it is of great interest to understand their adverse effects on non-target organisms in the environment. Aquatic plant toxicity tests have been frequently conducted to determine the potential impact of contaminants on primary producers and to assess environmental risks. Among aquatic organisms to be tested, *Lemna* sp. is a relatively new bioindicator species suggested by US EPA (1996) and OECD (2002), and commonly used in phytotoxicity tests because of its small size, high reproductive rate, and easy cultivation in laboratories (Wang 1990). This organism is relevant to many aquatic environments, including ponds, lakes, streams, and can be used for effluents.

For the environmental risk assessment of pesticides, most studies have focused on their toxic effects, e.g. EC50, to determine the potential impact, but recovery after exposure of chemicals is another important factor to be considered, although there are few studies on the subject (Lawrence 2004). In addition to recovery potential, the exposure period of pesticides also should be considered to determine the potential impact. Many researchers and guidelines have focused their attention on short-term exposure toxicity to *Lemna* sp. (US EPA 1996; OECD 2002), therefore, little is known about long-term toxicity to this aquatic organism. The question is, if the exposure period is long, how much will it affect toxicity and recovery potential of the aquatic plant? Recent studies demonstrated that a longer period of exposure caused more serious adverse effects on *Lemna* sp. (Davies et al. 2003; Cedergreen et al. 2005). Factors believed to affect the aquatic organism are closely related to both the duration and concentration of pesticides. Biological properties of the organism that should be evaluated are sensitivity to chemicals and the ability to recover after exposure.

Sulfonylurea (SU) herbicides have been used for chemical weed control because of their exceptionally low application rates and favorable environmental and toxicological properties (Pimentel et al. 1991). In our previous study, sensitivity of *Lemna* sp. to eight SU was examined according to the OECD guideline (OECD 2002), in which EC50 values were determined through a 7 day exposure test (Mohammad et al. 2005). In addition to information on their toxicity, it is

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important to assess the recovery potential and long-term toxic effect of SU on the growth and reproduction potential of *Lemna* sp.

In this study, we assessed the recovery of *Lemna* sp. after 7 day exposure to eight SU herbicides, and examined the effect of different concentrations and exposure periods (1, 2, 3, and 4 weeks) on recovery using cyclosulfamuron, which was the most toxic SU to *Lemna sp.* used in this study.

MATERIALS AND METHODS

The eight SU herbicides used in this study were the same as those used in the previous study (Mohammad et al. 2005, Table 1). Stock solutions were prepared by dissolution with acetone and different concentrations of test solution were prepared by mixing with 20X-APP growth medium (OECD 2002). The final concentration of acetone in the test solution was less than 0.01%. All stock solutions were prepared just before the experiments.

Table 1. Sulfonylurea herbicides used in this study.

Common name	chemical name and CAS number	EC50 (ppb) *
Bensulfuron-methyl	methyl 2-[[[[[(4,6-dimethoxypyrimidin-2-yl)-amino]carbonyl]amino]sulfonyl]methyl]-benzoate, 83055-99-6	2.23
Pyrazosulfuron-ethyl	ethyl 5-[[[[(4,6-dimethoxy-2-pyrimidinyl)-amino]carbonyl]amino]sulfonyl]-1-methyl-1*H*-pyrazole-4-carboxylate, 93697-74-6	3.49
Imazosulfuron	2-chloro-*N*-[[(4,6-dimethoxy-2-pyrimidinyl)-amino]carbonyl]imidazo[1,2-*a*]pyridine-3-sulfonamide, 122548-33-8	1.46
Cyclosulfamuron	*N*-[[[2-(cyclopropylcarbonyl)phenyl]amino]-sulfonyl]-*N*-(4,6-dimethoxypyrimidin-2-yl)-urea, 136849-15-5	0.91
Flazasulfuron	*N*-[[(4,6-dimethoxy-2-pyrimidinyl)amino]-carbonyl]-3-(trifluoromethyl)-2-pyridine-sulfonamide, 104040-78-0	1.66
Ethoxysulfuron	2-ethoxyphenyl [[(4,6-dimethoxy-2-pyrimidinyl)-amino]carbonyl]sulfamate, 126801-58-9	1.72
Thifensulfuron-methyl	methyl 3-[[[[(4-methoxy-6-methyl-1,3,5-triazin 2-yl)amino]carbonyl]amino]sulfonyl]-2-thiophenecarboxylate, 79277-27-3	7.16
Nicosulfuron	2-[[[[(4,6-dimethoxy-2-pyrimidinyl)amino]-carbonyl]amino]sulfonyl]-*N,N*-dimethyl-3-pyridinecarboxamide, 111991-09-4	14.5

* Mohammad et al. (2005)

The procedures for collection and purification of *Lemna* sp. used were described previously (Mohammad et al. 2005). The fronds were transferred aseptically from the stock culture into fresh sterile medium and cultured for 10 days under test conditions before starting the test.

Lemna sp. was tested according to the draft OECD guidelines for the testing of chemicals (2002), using the same conditions described previously (Mohammad et al. 2005). The first experiment was conducted with all SU as a 7 day exposure at 0, 1, 10, 100 and 1000ppb followed by a 7-10 day recovery in fresh medium. The tests were done under static conditions using nine fronds initially in each 100 mL test beaker containing 50mL growth medium. The beakers were covered by transparent wrapping paper with some pores for aeration. After each exposure period, the nine mother fronds were collected, washed in sterilized distilled water, and transplanted to fresh medium for recovery. Frond numbers were counted at the third, fifth and seventh days of the exposure and recovery periods, and at the tenth day when the recovery was slow. Inhibition and recovery of growth were estimated on the basis of frond number and area. Each concentration was tested in triplicate.

The second experiment was conducted using the most toxic of the tested herbicides, cyclosulfamuron, with different exposure periods of 1, 2, 3 and 4 weeks at 0, 1, 10, 50 and 100 ppb, followed by a recovery test in fresh medium for 7-28 days. The basic test conditions were the same as those of the first experiment.

To evaluate the capacity of mother fronds to produce new ones, the relative growth rate (RGR) was determined at the seventh day compared with the control according to the equation below.

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

RESULTS AND DISCUSSION

The effect of each of the eight SU on frond reproduction on seven day exposure showed that growth was inhibited with the increasing concentration of chemical. In the case of cyclosulfamuron (EC50 0.91 ppb), new frond development stopped at 10 ppb as shown in Figure. 1a. There were no visible changes in appearance at any concentrations and the fronds were green and looked alive. When *Lemna* sp. was transferred to fresh medium after exposure, development of new fronds was observed for all SU even at 1000 ppb. But, initiation of new frond development was delayed as the exposure concentration increased. The growth recovery of *Lemna* sp. after cyclosulfamuron exposure of is shown in Figure. 1b as an example. At 10, 100, and 1000 ppb the delay periods were 3, 5, and 7 days, respectively, but the growth rates were almost the same as the

control. These delays in growth may be due to the period required for chemical dilution and/or detoxification in the plants.

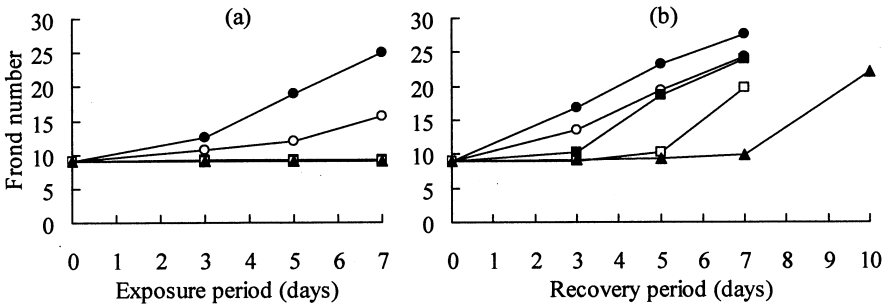

Figure 1. Frond growth of *Lemna* sp. for (a) 7 day exposure period and (b) recovery period in fresh medium after the exposure of cyclosulfamuron at 0 (●), 1 (○), 10 (■), 100 (□), and 1000 (▲) ppb.

Figure 2a and 2b summarize the relative growth rate (RGR) for all SU in the exposure and recovery periods, respectively. SU with lower EC50 values caused lower RGR at 10 ppb exposure, and frond reproduction was completely stopped at higher concentrations (100 and 1000 ppb) for all SU. New frond production was observed in the recovery test for all eight SU tested. RGR of six SU were more than 50% at 100 ppb, and for four SU at 1000 ppb.

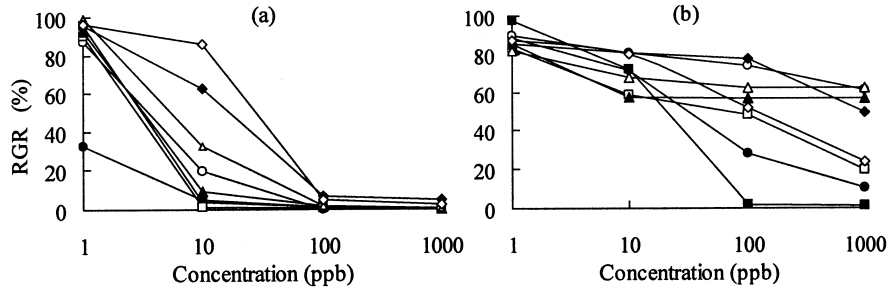

Figure 2. Relative growth rate (RGR) of *Lemna* sp. fronds at 7 day (a) exposure to cyclosulfamuron (●), imazosulfuron (○), flazasulfuron (■), ethoxysulfuron (□), bensulfuron-methyl (▲), pyrazosulfuron-ethyl (△), thifensulfuron-methyl (◆), nicosulfuron (◇), and (b) recovery period in fresh medium after the exposure. Development of new fronds was observed at 10 day during the recovery period after exposure of flazasulfuron at 100 and 1000 ppb.

There was no correlation between RGR in exposure and recovery (Fig. 3): $y = 10.7 \log x + 22.7$, $R^2 = 0.385$, where x and y are RGR in exposure at 10 ppb and recovery after exposure at 100 ppb, respectively. When ethoxysulfuron and

nicosulfuron are removed, the correlation is stronger ($y = 23.0 \log x - 9.77$, $R^2 = 0.793$). The differences between RGR during exposure and recovery may be due to differing metabolic activity for different chemicals with *Lemna* sp.

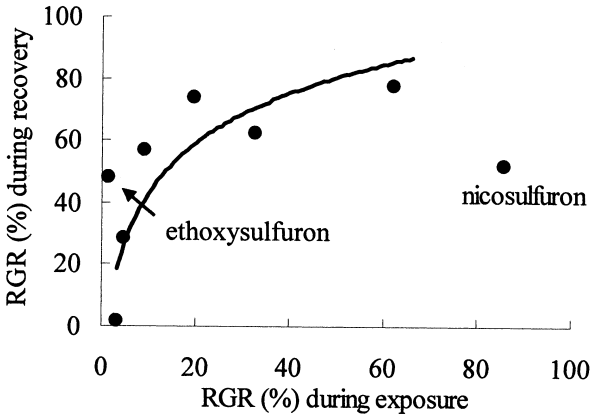

Figure 3. Comparison of relative growth rate (RGR) of *Lemna* sp. at 7 days of exposure at 10 ppb and recovery after exposure at 100 ppb to 8 sulfonylurea herbicides. The correlation curve was made without ethoxysulfuron and nicosulfuron data points.

Although growth was completely inhibited for 7 days, no lethal effect was observed. In risk assessment, the expected environmental concentrations of SU were reported as 3 – 20 ppb (Peterson et al. 1994), which are greater than EC50 of *Lemna* sp. for some SU, but recovery of growth is possible when the chemicals are dissipated by degradation in the environment.

Effects of exposure period (1, 2, 3, and 4 weeks) and concentration (1, 10, 50, and 100 ppb) on growth inhibition and recovery were examined using cyclosulfamuron. Growth was inhibited at 1 ppb, and completely stopped at 10 ppb (Fig 1a) in the first week of exposure. 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 in the fourth week of exposure (Fig 4a). 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. Reflecting the change in appearance of the fronds, recovery of growth was not observed at 10 days of recovery after exposure to more than 10 ppb in the four week exposure (Fig 4b). Inhibition of growth during recovery at 1 ppb was also much stronger with longer exposure period. In the case of exposure for one week, higher concentrations of chemicals caused longer lag periods for the initiation of growth recovery (Fig 1b). On the other hand, the longer exposure period caused slower growth rate without the lag period (Fig 4b). It is supposed that the longer exposure to SU results in the irreversible damage for *Lemna* sp.

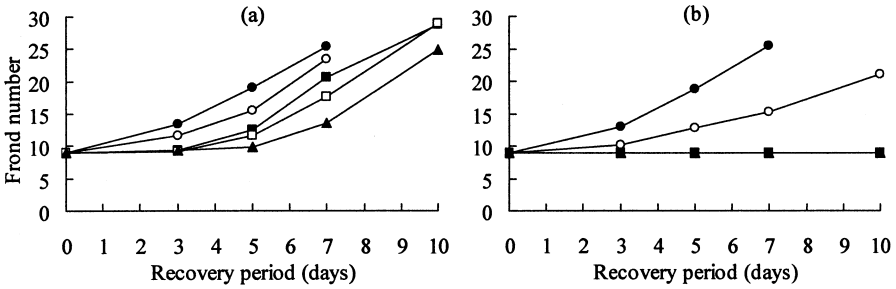

Figure 4 Frond growth of *Lemna* sp. for (a) 4 weeks exposure period and (b) recovery period in fresh medium after exposure to cyclosulfamuron at 0 (●), 1 (○), 10 (■), 50 (□), and 100 (▲) ppb.

Figure 5a and 5b summarize RGR at different exposure periods and concentrations of cyclosulfamuron for the exposure period and for the recovery period after exposure, respectively. RGR was constant within the exposure period of four weeks. In the recovery period, however, RGR decreased with longer exposure. 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.

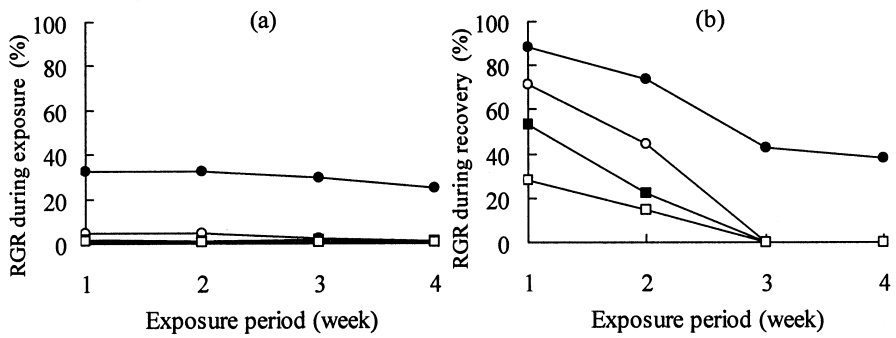

Figure 5. Relative Growth Rate (RGR) of *Lemna* sp. fronds at 7 days, (a) for different exposure periods to cyclosulfamuron at 1 (●), 10 (○), 50 (■), and 100 ppb (□), and (b) during the recovery period in fresh medium after different periods of exposure.

Important points in risk assessment of SU to *Lemna* sp. are presented in this paper. SU 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). Cell division stops quickly and is then followed by a relatively slow death. SU inhibit growth but typically do not result in lethality or permanent cell damage on short-term exposure. This characteristic of SU required a different model than typically used for

understanding the potential impact in aquatic ecosystems, where lethality is usually assumed, e.g. comparison of EC50 with expected environmental concentrations. As shown in this study, *Lemna* sp. can reproduce again at the same rate as that before the exposure if SU is removed within two weeks, even after complete inhibition at 100 ppb. In addition to the recovery potential, effects of the exposure period should be considered in risk assessment. Longer exposure beyond three weeks caused no recovery at 10 ppb, and retarded growth at 1 ppb. As SU are mainly used only in the beginning of rice cultivation and rapidly degraded in paddy soils, duration of SU exposure to aquatic environment is supposed to be limited (Okamoto et al. 1998). Therefore, the recovery potential after exposure should be examined in addition to determining EC50, and expected concentration and period of exposure in the environment should be evaluated for the risk assessment. Considering our results and the expected environmental concentrations of 3-20 ppb (Peterson et al. 1994), it is concluded that cyclosulfamuron does not pose a significant risk to *Lemna* sp. for up to two weeks of exposure at expected environmental concentrations.

Verdisson et al. (2001) examined toxicity of the fungicide pyrimethanil to *Lemna minor*, and observed recovery of vegetative growth of *L. minor* four days after treatment with low concentrations (5-25 ppb) and after five days with higher concentrations (50-100 ppb) during the course of six-day exposure experiments. The authors assumed that this recovery may be due to fungicide detoxification in the plant. Although they did not examine the effects of longer periods of exposure, it is supposed that *L. minor* can recover even in the presence of the fungicide. It would be important to take into account the fate of chemicals in the organism, such as uptake, transport, mode of action, metabolism, excretion, etc. for better understanding of the mechanisms of toxicity and recovery. Based on the toxicokinetic information, the reliable risk assessment would be possible.

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