Bull. Environ. Contam. Toxicol. (2003) 70:723–730

DOI: 10.1007/s00128-003-0043-z

Bulletin of Environmental Contamination and Toxicology

Comparative Study of the Toxicity of Molinate for Freshwater Organisms

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Received: 21 February 2001/Accepted: 5 December 2002

Molinate has been widely used in many countries for the control of germinating broad-leaves and grassy weeds in rice paddy fields. The use of herbicides is increasing world-wide as the need for selective weed control becomes more important. In Spain, and especially in the Valencian Community, rice occupies a relevant position among other irrigated crops. Because there is considerable potential for the contamination of waterbodies with herbicides, it is important to assess the adverse impacts these chemicals may have on non-target organisms in aquatic ecosystems. There is some information about toxic effects of molinate on microalgae (Sabater and Carrasco 1998) and it is known that algal susceptibility to herbicides differs among species.

D. magna is an important component of aquatic systems. Cladocerans, especially *Daphnia* spp., are among the most favourable test animals in aquatic toxicology. The many advantages of daphnids, e.g., sensitivity to toxicants, parthenogenetic reproduction and the short reproductive cycle and life span, can hardly be found in combination in any other species (Bodar et al., 1988). Effects of pollutants on the reproduction of *Daphnia magna* have been reported in several studies (Buhl et al. 1993; Ferrando et al. 1996). In most cases reproduction was affected negatively by toxic agents. The intrinsic rate of natural increase (r) is a demographic parameter which expresses the growth potential of a population in an unlimited environment. Furthermore it incorporates information of survival and reproductive success of a population and has been used in ecological theory since 1930 (Barbour et al. 1989).

On the other hand, rates of filtration and ingestion of food particles by zooplankton have been observed to decrease following exposure to various pesticides (Villarroel et al. 1999). Feeding behavior changes may be used as rapid and sensitive indicator of toxic stress and may help to explain other observed changes in the survival and reproduction of nontarget organisms. Since molinate may be present in surface water, the objective of this study was to assess the effect of different molinate concentrations on the algae (*Nannochloris oculata*) and the cladoceran (*Daphnia magna*) to determine their relative sensitivities to the herbicide. The effect of molinate on the feeding behavior of *D. magna* using *N.*

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oculata as food was also evaluated.

MATERIAL AND METHODS

A laboratory strain of freshwater algae *Nannochloris oculata* reared in a nutrient medium (Bischoff and Bold 1983) was used in these experiments. The study was initiated when 100 mL of test solution in each of three replicate Erlenmeyer flasks was inoculated with enough algae to bring the population density in the solution to 5 x 10^5 cells/mL. The five treatment solutions (0.5, 1.0, 3.0, 4.0 and 9.0 mg/L) were made by adding appropriate amounts of molinate to the algal nutrient medium. Untreated algal medium was used as a control solution. Because acetone was required as a carrier for molinate, an additional control, with acetone (50 L/L), was included. All flasks were loosely capped with cotton and placed in an environmental chamber set at 22¡C and equipped with wide-spectrum lights (40w, cool white). Each day, every flask was gently shaken. Population densities were evaluated 0, 24, 48 and 72 hours after the test cultures were inoculated (Nyholm and K llqvist 1989). A hemacytometer was used to count algal cells. Maximum growth rates, max (day^{-1}), were calculated by the method of Nyholm and K llqvist (1989). EC50 values at 24, 48 and 72 h were estimated in terms of concentrations needed to inhibit 50% algal growth compared with control growth figures.

Daphnia magna organisms were obtained from continuous cultures maintained in our laboratory in 6-L aquaria at 22–1¡C, in dechlorinated tap water (total hardness, 181.8–18.8 mg/L as $CaCO_3$; pH 7.9–0.2; alkalinity, 4.1 mmol/L), 12hr:12hr light:dark photoperiod and a density of below 50 animals/L. The medium was renewed two times each week and the daphnids were fed daily with the algae *Nannochloris oculata*. This algae was also continuously cultivated in our laboratory using a nutrient medium (Bischoff and Bold 1983). Offspring were separated at regular intervals. Test animals were 6-24 h juveniles, taken from cultures 3-5 weeks old (ASTM 1988).

Acute toxicity test were carried out according to the OECD (1984) in order to calculate the 24 h-EC50 (immobilization) in *D. magna* exposed to molinate. Based on these results, neonates (< 24 h old) of *Daphnia magna* were exposed to five sublethal concentrations of molinate (3.77, 4.71, 6.28, 9.42 and 18.85 mg/L) in a 21-days, static-renewal life cycle study. Fifteen 60 mL glass beakers, filled with 50 mL of test solution, were used at each of the five pesticide concentrations, plus the blank control and the acetone control (5 L/L). One cladoceran was randomly assigned to each of the beakers. Water quality characteristics were constantly maintained by transferring the cladocerans to fresh test solutions or control water every day. The cladocerans were exposed to a wide-spectrum light intensity of 64 ft-c, with a 12:12 h light:dark photoperiod. Approximately 5 x 10^5 cells/mL of *N. oculata* were added daily to each beaker to feed the daphnids. The size, fecundity and survival of the *D. magna* were monitored for each of the 15 replicates in this 21-d life cycle study. After 21 d, the length of each adult, from the apex of the

helmet to the base of the tail spine, was measured to the nearest 0.01 mm. The intrinsic rate of natural increase, r, was calculated using the formula of Lotka (1913): $\sum lx.mx.e^{-r.x} = 1$; where lx is the proportion of individuals surviving to age x, mx is the age-specific fecundity (number of females produced per surviving female at age x), and x in days. Since r calculated after 21 days is indistinguishable from r estimated for the entire life-span, due to the great importance of early reproduction, all calculations were based on 21-day experiments.

Based on the results from the chronic tests, the same sublethal molinate concentrations were chosen for the feeding study. Daphnids were exposed to 3.77, 4.71, 6.28, 9.42 and 18.85 mg/L of molinate, plus the blank control and the acetone control (5 μL/L). Each treatment consisted of five replicates. Filtration and ingestion rates were used as measures of feeding behavior. Feeding experiments with *D. magna* were carried out in 60 mL glass beakers containing 50 mL of the medium and 10 daphnids (< 24 hr old). The vials were placed in a temperature-controlled room at 22–1¡C under darkness and static conditions (Villarroel et al. 1999). Test organisms were exposed to the test solution containing also food (*N. oculata* = $5x10^5$ cells/mL) for 5 hours, after which the final food concentration was measured using a hemocytometer. Filtration rate (F, μL/ind/hr) is defined as the volume of medium swept clear per unit of time and the ingestion rate (I, cells/ind/hr) as the number of cells consumed by an animal in a specific interval time. For these calculations, the equations from Gauld (1951) were used. The EC50 (concentration of toxicant that reduces feeding rate to 50%) was calculated using linear regression analysis.

The molinate used in these experiments was 99% pure as assayed by AGREVO Company (Spain). Stock solutions were prepared by dissolving the toxicant in acetone inmediately prior to each experiment. Previous experiments carried out in our laboratory (Ferrando et al. 1992) indicated that molinate concentration under the experimental conditions used was almost 99% of the original concentration after 24 h. Based on these observations the test solutions was renewed every day in the experiments carried out with *D. magna*.

The percentages of mortality from the *D. magna* acute test were calculated for each herbicide concentration after 24 h exposure and the 24h-EC50 calculated using an IBM computer program. Data from *N. oculata* and *D. magna* experiments were analyzed using analysis of variance (ANOVA) followed by Duncan test ($p<0.05$) with the SPSS+ computer program.

RESULTS AND DISCUSSION

Most of the algal populations were initially affected by exposure to the herbicide molinate. Herbicide concentrations at/or above 1 mg/L significantly reduced ($p<0.05$) algal densities after only 24 h exposure (Fig. 1). Molinate effect on *N. oculata* densities was greater after 72 h exposure to all the herbicide concentrations tested. The maximum specific growth rates (MSGRs) of algal populations (Table 1) were significantly reduced by molinate concentrations of

Table 1. Growth inhibition (%I) of the alga *N. oculata* when exposed to several concentrations of Molinate.

Molinate (mg/L)	MSGRs [a]	24 h	48 h	72 h
blank control	0.53 – 0.00	-	-	-
acetone control	0.50 – 0.02	6.0 – 0.3	4.0 – 0.6	0
0.5	0.36 – 0.02*	33.0–4.1	11.5–4.5	31.6–3.68
1.0	0.33 – 0.02*	53.6–6.1	15.0–2.8	36.3– 3.68
3.0	0.31 – 0.00*	63.6–2.6	37.0–0.0	41.0– 0.0
4.0	0.26 – 0.00*	74.6–4.5	46.0–3.7	50.0– 0.0
9.0	0.09 – 0.00*	69.6–4.6	65.6–5.2	82.0– 0.0

Means – SD (* $p < 0.05$). [a] Maximum specific growth rates (72 hr). *Note*. EC50 (72 hr) for maximum specific growth rates: 4.36– 0.01 mg/L

Table 2. Size and fecundity of *D. magna* exposed to several concentrations of Molinate in a 21-d life study.

Molinate (mg/L)	Length (cm)	Longevity (days)	Days to first brood	No. of young per adult	Brood size per adult	No. Broods	r
blank control	0.49–0.01	21.0–0	7.8–0.1	131.7–15.1	25.9–2.5	5.1–0.1	0.33–0.01
acetone control	0.48–0.007	21.0–0	8.2–0.4	127.8–4.0	24.9–0.7	4.9–0.5	0.31–0.01
3.77	0.44–0.005*	19.1–3.8	8.3–0.2*	119.7–9.4	24.6–0.7	4.9–0.3	0.29–0.03
4.71	0.46–0.01*	19.3–2.0	8.4–0.2*	73.5–14.6*	19.7–1.2*	3.7–0.9*	0.30–0.01*
6.28	0.37–0.01*	19.4–2.0	8.9–0.1*	55.3–12.4*	15.6–3.5*	3.4–0.5*	0.28–0.02*
9.42	-	10.3–2.8*	-	0*	0*	0*	0*
18.85	-	4.5–0.7*	-	0*	0*	0*	0*

Values are means – SD (* $p < 0.05$). *Note*: r is the intrinsic rate of natural increase

0.5 mg/L and higher, and the MSGR EC50 (72 h) was 4.36 mg/L. As indicated in Table 1, growth inhibition (% μI) increased with increasing herbicide concentration. *N. oculata* growth was inhibited by more than 80% after 72 h exposure to molinate at 9 mg/L when compared to the control.

Molinate 24 h EC50 value for *Daphnia magna* was calculated in our laboratory as 37.7 (±0.08 SD) mg/L. The influence of sublethal molinate concentrations on the survival of *D. magna* is shown in Table 2. Herbicide concentrations higher than 9.42 mg/L produced a significant decrease on survival after 21 days of exposure. At 9.42 and 18.85 mg/L concentration of the pesticide all daphnids died before they could reproduce. The effects of molinate on the fecundity and size of *D. magna* after 21 d of exposure are summarised in Table 2. Reproduction was significantly reduced at molinate concentrations equal to or higher than 4.71 mg/L, as a result of a decreasing number of broods and neonates per brood. Number of neonates born declined significantly ($p<0.05$) from 132 youngs (control) to 55 youngs at 6.28 mg/L, respectively. The onset of reproduction of *D. magna* was delayed at 3.77 mg/L of molinate (8.3 days) and higher concentrations. Populations of *D. magna* under control conditions and exposed to the acetone control had r values of 0.33 and 0.31 respectively (Table 2). At pesticide concentrations higher than 3.77 mg/L, r was significantly reduced from 0.33 in controls to 0.28 after exposure to 6.28 mg/L molinate (Table 2). A significant reduction in mean caparace length of 21 day old daphnids was detected at molinate concentration of 3.77 mg/L or more (Table 2). This parameter decreased from 0.49 cm (controls) to 0.37 cm at 6.28 mg/L of the herbicide.

The effect of molinate on filtration and ingestion rates of *D. magna* was investigated. Filtration rates were significantly ($p<0.05$) reduced from 512 L/animal/hr (control) to 444, 379, 391 and 161μL/animal/hr at 4.71, 6.28, 9.42 and 18.85 mg/L of herbicide, respectively. Ingestion rates were also reduced at those toxicant concentrations from 158.4 x 10^3 cells/ind/hr (control) to 142, 129, 132 and 66 x 10^3 cells/ind/hr. The EC50 values for filtration and ingestion rates on *D. magna* were 14.4 and 17.0 mg/L, respectively. This means that the concentrations of molinate needed to reduce feeding rates in this species in only 5 hours were much lower than the 24hr-LC50 from the acute test (37.7 mg/L). Feeding rates in daphnid acetone control did not differ significantly ($p>0.05$) from control values.

N. oculata was the most sensitive of the two species exposed to molinate. The MSGR EC50 value for the studies with *N. oculata* (4.36 mg/L) was smaller than the EC50 for *D. magna* (37.7 mg/L). Sabater et al. (1993) found that pesticide algal susceptibility differs among species. They reported 96 hr EC50 values for molinate and thiobencarb of 0.8 and 0.02 mg/L for the algae *Scenedesmus acutus*, respectively.

The herbicide molinate inhibited algal growth after 72 h exposure, the concentrations at which the algae population was affected were lower than those found in the present study with the cladoceran *D. magna* after 21 days.

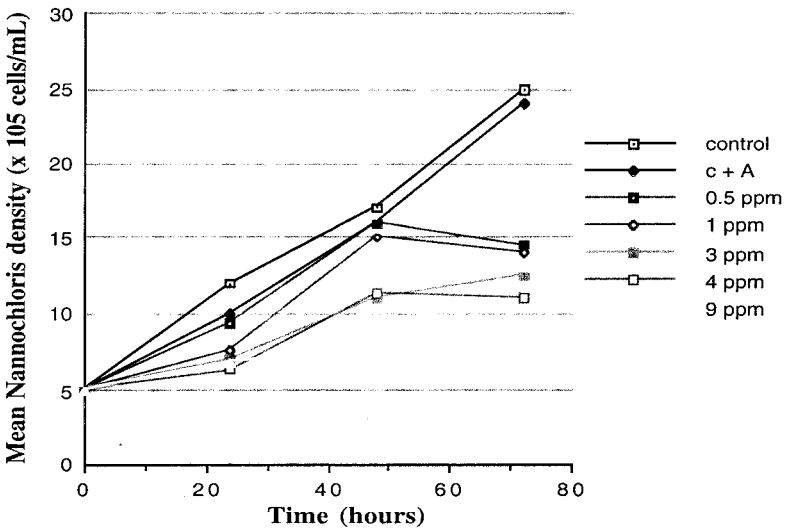

Figure 1. Mean *N. oculata* densities throughout the 72 hr test exposed to molinate.

Survival of *D. magna* was reduced at concentrations higher than 9.42 mg/L of molinate while reproduction was decreased at 4.71 mg/L of when exposure to toxicants continues throughout the entire life-cycle of the organism. Similarly, other investigators have reported that reproduction was a more sensitive index of chronic pesticide toxicity to *D. magna* than survival (Buhl et al. 1993). In contrast, other investigators have found that survival and reproduction were both equally sensitive indicators of chronic toxicant stress (Gersich and Milazzo 1988). In the present study, molinate produced a reduction in the reproduction capacity of *D. magna*. Similar results were reported by Day and Kaushik (1987) in *D. galeata mendotae* when exposed to 0.01 and 0.05 μg/L of fenvalerate. They observed a reduction in total young per female, mean brood size and number of broods, but they did not find any effect in the number of days to first reproduction with the pesticide levels tested. Julli and Krassoi (1995) reported a reduction in the number of young produced by parental *Daphnia magna* when animals were exposed to 0.29 mg/L of molinate and higher concentrations. In contrast, other investigators as Nebeker and Schuytema (1998) have found that survival and reproduction were equally affected in *Daphnia pulex* exposed to the herbicide diuron.

Molinate is a carbamate pesticide that affects invertebrate molting and fertility as reported by Cochran et al.(1997).Therefore, reproduction appears to be a sensitive chronic endpoint for molinate toxicity. The intrinsic rate of natural increase (r) is found to be a sensitive parameter of toxicity due to the effect of molinate on

reproduction and survival. Daniels and Allan (1981) found that cohorts of *Daphnia pulex* exposed to increased concentrations of dieldrin showed little reduction in r until a concentration of 5 g/L was reached. A reduction in the intrinsic rate (r) resulted as a consequence of chronic toxicant stress of fenvalerate on *D. magna* (Day and Kaushik 1987). A significant reduction in mean caparace length of 21-d old daphnids was also detected at herbicide levels equal and/or greater than 3.77 mg/L.

The effects of xenobiotics on feeding rates have been investigated by several workers. The filtration rate of *D. magna* was reduced after a 5 hr exposure to 0.1 mg/L tetradifon (Villarroel et al. 1999). The filtration of food by filter-feeding zooplankton requires movement of appendages and coordination of the nervous system. Therefore those toxicants that affect nervous system will cause loss of coordination and/or paralysis and will reduce rates of filtration. It seems that AChE activity of the cladoceran *Daphnia magna* is also affected by molinate exposure as Sanchez et al. (2001) reported.

Since this herbicide reduces the feeding rates of food by zooplankton after only 5 hours, it appears that constant exposure of these organisms to this toxicant over their entire life cycle may also reduce their ability to obtain adequate nutrition, and the production of young will decrease as we observed in the present study. Because of the above results, the use of molinate for aquatic weed control, would cause chronic damages to natural green algae populations and zooplankton crustaceans as *Daphnia magna* commonly present in aquatic environments proximal to direct applications. This would also affect the development of other natural populations of organisms closely connected, by means of its food relationships.

Acknowledgments. This work was supported by the grants IFD97-0963-C03-02 and AGF99-0286-CO3-02. M. Sanchez is recipient of a fellowship from Ministerio de Educacion y Ciencia, Spain.

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