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Effects of Molinate on Growth of Five Freshwater Species of Phytoplankton

C. Sabater, J. M. Carrasco

Pesticide Laboratory, Department of Biotechnology, E.T.S.I.A. Polytechnic University of Valencia, Camino de Vera, 14, 46022-Valencia, Spain

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The use of herbicides is increasing world-wide as the need for selective weed control becomes more important. Because there is considerable potential for the contamination of waterbodies with herbicides through spray drift, runoff and direct overspray, it is important to assess the adverse impacts these chemicals may have on nontarget organisms in aquatic ecosystems (Peterson *et al.* 1994). Algae have frequently been the subject of these investigations because of their importance as primary producers in freshwater systems (Jurgensen and Hoagland 1990).

The herbicide molinate (S-ethyl hexahydro-1H-azepine carbothioate) is widely used for the control of barnyard *grass* (*Echinochloa* spp.) in paddy rice fields. This thiocarbamate herbicide is usually applied to the water used to flood the rice paddies. This water flows through the irrigation system and drains into ponds, streams, lakes and other bodies of waters producing a temporarily high concentration of molinate, which can result in adverse effects on an ecosystem (Kuwatsuka 1983; Watanabe *et al.* 1984).

Studies on the metabolism of molinate by pure microbial cultures isolated from natural waters (Skryabin *et al.* 1978; Golovleva *et al.* 1981) and soils (Imai and Kuwatsuka 1986; Molinari *et al.* 1992) and its dissipation and persistence under field and laboratory conditions (Soderquist *et al.* 1977; Deuel *et al.* 1978; Thomas and Holt 1980; Ross and Sava 1986; Carrasco *et al.* 1992) have been reported but there are few data on the effects of molinate on freshwater algae.

In this study we determined the effective concentrations of molinate that caused 50% inhibition on growth, in pure cultures, of three wild species of phytoplankton, representatives of mediterranean wetlands, isolated from Lake Albufera (Valencia, Spain) and two laboratory strains.

MATERIALS AND METHODS

The chlorophyceae *Scenedesmus acutus* (Meyens), *Scenedesmus subspicatus* CCAP 276/22, *Chlorella vulgaris* Beijerinck and *Chlorella saccharophila* (Krüger) Migula and the cyanobacteria *Pseudanabaena galeata* (Böcher) were selected for the toxicity tests. *S. acutus* and *C. saccharophila* were isolated from samples collected

Correspondence to: C. Sabater

at Albufera lake in Valencia (Spain). *S.subspicatus* and *C. vulgaris* were kindly supplied by the Institute of Freshwater Ecology (Ambleside, UK) and by the Area of Environmental Toxicology (CISA-INIA, Spain), respectively. These four chlorophyceae were grown in a medium recommended by the OECD (1984). *P.galeata* was obtained from Dr. Romo (Valencia University) and was grown in the medium of Romo and Becares (1992). The stock cultures were maintained in a liquid medium at a temperature of 22 ± 2 °C and a light intensity of 1100 lux on a 12-hr light-dark cycle.

The molinate herbicide (S-ethyl hexahydro-1H-azepine carbothioate, 99% analytical standard) was supplied by "AGREVO, S.A.". Molinate (99% pure) is a clear liquid with an aromatic odour. Its solubility in water at 20 °C is 880 mg/l; its boiling point and vapour pressure are 202 °C/10 mmHg and 746 mPa (25 °C) and its K_{ow} is 760; molinate is miscible with acetone, ethanol, kerosene and xylene (Worthing and Hance 1991).

The inhibition test was carried out in accordance with the OECD (1984) protocol. The organisms were exposed to concentrations of molinate which ranged from 0.22 to 90.5 ppm, for 96 hours. Growth of cultures was measured by the turbidity at 750 nm wavelength using a spectrophotometer (Beckman DU^{R}-70; Ordög and Kuivasniemi 1989) at 24, 48, 72 and 96 hours after the start of the test. ANOVA and Student Newman-Keuls multiple range test (Reish and Oshida 1987) were used to determine significant ($P<0.05$) molinate effects. E_bC_{50} (O-96 hours) values with 95% confidence limits were determined by probit analysis (Abou-Setta *et al. 1986). In* this study, E_bC_{50} is the concentration of molinate, derived by the method of calculation “comparison of areas under growth curves”, which results in a 50% growth reduction relative to the control values, at 96 hours (OECD 1984). Details of culture methods and test protocols have been described earlier (Sabater and Carrasco 1996).

At the beginning of the assays, herbicide concentrations in test solutions were determined by gas chromatography; the results were analyzed statistically and used for calculating toxicities of molinate. For the extraction and determination of molinate the test cultures samples (25 ml) were acidified with HCl 1N and extracted with three 20 ml portions of n-hexane by shaking. The combined hexane extracts were dried, concentrated to 5 ml and analyzed with a Hewlett Packard model 5890 A gas chromatograph equipped with a nitrogen-phosphorus detector (NPD), a 10 m HP-5 column and a HP-3940A integrator. The oven temperature was 180 °C and injector and detector temperatures were both 220 °C (Pattchet *et al.* 1972). The recovery of molinate was in the range of 98% ± 5%.

RESULTS AND DISCUSSION

Table 1 shows molinate concentrations measured by GC (no nominal values) and regression equations of growth of five species for each herbicide treatment. The slope of each equation represents the average specific growth rate which decreased progressively with increasing concentration of molinate. The pH values of the culture media from 0 to 72 hours ranged from 7.9-9.8. In most of the phytoplankton

species the growth exhibited a broad optimum over the pH range of 7.0 to 10.0 (Richmond 1986).

Table 1. Exponential regression equations of *C. saccharophila, C. vulgaris, S. acutus, S. subspicatus* and *P.galeata growth,* for 96 hours.

Species	Concentrations (ppm) *	Regression equations **	R^2 ***
Chlorella saccharophila	0.0 ± 0.0 [a]	Ln Y = 9.68 + 0.073 X	0.96
	2.4 ± 0.1 [b]	Ln Y = 9.69 + 0.073 X	0.95
	4.9 ± 0.3	Ln Y = 9.56 + 0.072 X	0.97
	10.3 ± 0.5	Ln Y = 9.39 + 0.071 X	0.98
	18.8 ± 0.8	Ln Y = 9.33 + 0.067 X	0.98
	44.6 ± 2.0	Ln Y = 9.44 + 0.034 X	0.97
Chlorella vulgaris	0.0 ± 0.0 [a]	Ln Y = 9.52 + 0.065 X	0.94
	17.6 ± 0.3 [b]	Ln Y = 9.52 + 0.065 X	0.95
	24.4 ± 0.2	Ln Y = 9.55 + 0.063 X	0.95
	32.1 ± 1.1	Ln Y = 9.64 + 0.057 X	0.93
	50.2 ± 1.4	Ln Y = 9.74 + 0.049 X	0.89
Scenedesmus acutus	0.0 ± 0.0 [a]	Ln Y = 9.12 + 0.060 X	0.97
	0.22 ± 0.02 [b]	Ln Y = 9.11 + 0.060 X	0.97
	0.38 ± 0.04	Ln Y = 8.96 + 0.059 X	0.96
	0.85 ± 0.11	Ln Y = 8.80 + 0.054 X	0.97
	1.6 ± 0.10	Ln Y = 8.85 + 0.046 X	0.96
	3.2 ± 0.10	Ln Y = 9.29 + 0.016 X	0.98
Scenedesmus subspicatus	0.0 ± 0.0 [a]	Ln Y = 9.24 + 0.056 X	0.99
	0.37 ± 0.03 [b]	Ln Y = 9.26 + 0.056 X	0.99
	0.52 ± 0.03	Ln Y = 9.28 + 0.050 X	0.99
	0.69 ± 0.03	Ln Y = 9.20 + 0.042 X	0.98
	1.12 ± 0.08	Ln Y = 9.57 + 0.023 X	0.90
Pseudanabaena galeata	0.0 ± 0.0 [a]	Ln Y = 9.20 + 0.076 X	0.99
	1.7 ± 0.2 [b]	Ln Y = 9.22 + 0.076 X	0.99
	3.1 ± 0.1	Ln Y = 9.13 + 0.075 X	0.99
	6.4 ± 0.5	Ln Y = 9.07 + 0.074 X	0.99
	11.9 ± 0.5	Ln Y = 9.00 + 0.071 X	0.99
	26.9 ± 0.7	Ln Y = 9.02 + 0.065 X	0.99
	47.1 ± 0.9	Ln Y = 9.39 + 0.041 X	0.93

*: Concentrations of molinate that caused significant effects (P<0.05) on the algal growth with respect to control [a]. Mean of six values ± S.D.

**; Slopes are the average specific growth rate; Y=N°organisms/ml X=Time (Hours).
: Correlation coefficient

[b]: NOEC values [highest concentrations tested without significant effects (P<0.05) on the algal growth relative to control values].

The response of the five species to molinate was different (Table 1). At 44.6, 50.2, 3.2, 1.12 and 47.1 ppm of molinate the growth of *Chlorella saccharophila, Chlorella vulgaris, Scenedesmus acutus, Scenedesmus subspicatus* and *Pseudanabaena galeata* was strongly inhibited, respectively, after 96 hours. No growth was observed at 69.8 ppm for *Chlorella vulgaris* and 2.2 ppm for *Scenedesmus subspicatus.*

The difference in sensitivity between the chlorophyceas *Chlorella* and *Scenedesmus* was greater than between the chlorophyceas *Scenedesmus* and the cyanobacteria

Pseudanabaena. The growth of the most sensitive species, *S.acutus* and *S.subspicatus*, was reduced at molinate concentrations of 0.38 and 0.52 ppm, respectively while *C.saccharophila, C.vulgaris* and *P.galeata* needed doses of molinate much greater than *Scenedesmus*, between 3.1 and 24.4 ppm, to show similar inhibition effects. NOEC values for *C.. saccharophila, C. vulgaris* and *P. galeata* were 2.4, 17.6 and 1.7 ppm, respectively while 2.2 ppm was lethal for *Scenedesmus subspicatus* and 3.2 ppm strongly inhibited the growth of *Scenedesmus acutus*, during the duration of the assay.

96-hr E_bC_{50} values of molinate are shown in Table 2. The green algae seem to respond very differently to molinate; *C. saccharophila* and *C. vulgaris* were less sensitive to the herbicide with a 96-hr E_bC_{50} of 14.2 and 38.0 ppm, respectively while *S. acutus* and *S. subspicatus* were much more sensitive with a 96-hr E_bC_{50} of 0.87 ppm for the former and 0.63 ppm for the later *S. subspicatus* was more sensitive than *S. acutus* to molinate. The chlorophycea *Chlorella saccharophila* exhibited slightly less sensitivity to molinate than the cyanobacteria *Pseudanabaena*.

Table 2. E_bC_{50} (0-96 hours) values* of molinate for the five species.

	E_bC_{50}	95% confidence limits
C. saccharophila	14.2	12.4 - 16.1
C. vulgaris	38.0	36.8 - 39.3
S. acutus	0.87	0.80 - 0.93
S. subspicatus	0.63	0.61 - 0.65
P. galeata	13.1	11.0 - 15.3

*: ppm

Rajagopal *et al.* (1984) observed growth and N_2-fixation inhibition of cyanobacteria *Tolypothrix tenuis* and *Calothrix brevissima* by molinate at 0.1 ppm. Conversely, in our studies the growth of cyanobacteria *Pseudanabaena galeata was* unaffected by a molinate concentration of 1.7 ppm (Table 1). Molinate at 4-9 Kg/Ha reduced nitrogen fixation of *Nostoc muscorum, Anabaena variabilis* and *A. variabilis f. crassa* from 4.9-5.5 to 2.6-3.8 mg, which are dominant phytoplankton species in rice fields (Pokrovskaya and Maksudov 1976).

The chlorophyceas *Scenedesmus* and *Chlorella* with 96-hr EC_{50} values between 0.63 and 38.0 ppm (Table 2), have been less sensitive to molinate than the chlorophycea *Selenastrum capricornutum* with a 72-hr EC_{50} of 0.125 ppm (Hatakeyama *et al.* 1992).

Analyses of water from several streams have not shown concentrations of this herbicide in the range that is toxic to our algae and effects of this pesticide on natural algal communities are unlikely to occur in streams draining arable land. Carrasco *et al.* (1987) found levels below 0.09 ppm at different points in Lake Albufera during

the season of greatest contamination. Monitoring studies have documented concentrations up to 0.34 mg molinate/l in the Colusa Basin Drain (California), with exposures to herbicide lasting from 40 to 60 days (Finlayson and Faggella 1986). This value exceeded the 28-d LOEL value of 0.13 ppm (Finlayson and Faggella 1986) and the 21-d LC_{50} value of 0.18 ppm (Kawatsu 1977) for common carp. Concentrations of up to 42 µg/L and 24 µg/L have been reported in lower drainage channels from rice fields of the Murrumbidgee irrigation area of southern New South Wales, Australia (NSW Department of Water Resources, unpublished data) and in the Kokai River, Japan (Hatakeyama *et al.* 1992), respectively. These values are lower than toxicity values ranging from 0.63 to 38.0 ppm obtained for *Chlorella, Scenedesmus* and *Pseudoanabaena* (Table 2) and are below those considered lethal to many fishes.

Many studies have revealed the effects of molinate on other nontarget organisms such as aquatic invertebrates and fishes. Molinate 48-hr EC50 (immobilization) value for the cladoceran *Moina australiensis* Sars was estimated to be 2.4 mg/L (Julli and Krassoi 1995). The 96-hr LC_{50} value of molinate for the mysid *Neomysis meercedis* was 1.6 ppm (Brandt *et al.* 1993). Molinate 96-hr LC_{50} was 32.2 ppm for channel cattish (Brown *et al.* 1979) and 30 ppm for goldfish (Worthing and Hance 1991). Sanders and Hunn (1982) estimated a 96-hr LC_{50} value of molinate for rainbow trout of 1.2 ppm. Although the acute toxicity of molinate to common carp is low (96-hr LC_{50} value of 43.0 ppm; Nischiuchi and Yoshida 1972), molinate can produce anemia and eventual death in this species during chronic exposure (21-d LC_{50} value of 0.18 ppm; Kawatsu 1977).

The majority of short-term acute toxicity studies of other thiocarbamate herbicides like EPTC, tri-allate and thiobencarb have used fishes, the cladoceran *Daphnia magna* and some rotifers or mysids. EPTC and tri-allate showed a LC_{50} (96-hr) of 27 and 1.3 ppm respectively for the bluegill and 19 and 1.2 ppm respectively for rainbow trout (Worthing and Hance 1991). Tri-allate 48-hr LC_{50} for *Daphnia magna* was 0.43 ppm. Thiobencarb 24-hr LC_{50} for the rotifer *Brachionus calyciflorus* was 6.50 ppm (Fernandez-Casalderrey *et al.* 1992) and thiobencarb 96-hr LC_{50} for the mysid *Neomysis meercedis* was 0.28 ppm (Brandt *et al.* 1993). The 96-hr LC_{50} data of thiobencarb generated by Johnson and Finley (1980) for the fishes *Salmo gairdneri* and *Lepomis macrochirus* were 1.2 and 2.5 ppm, respectively.

The present study showed that two species of *Chlorella* were considerably more tolerant than two species of *Scenedesmus*. The cyanobacteria *Pseudanabaena* and the chlorophycea *Chlorella* responded similarly to molinate. *Scenedesmus subspicatus* was the most sensitive alga and *Chlorella vulgaris* the least sensitive. The results of the toxicity tests showed that thiobencarb (Sabater and Carrasco 1996) was more toxic than chlorsulfuron (Sabater and Carrasco 1997) and molinate. The green algae *C. saccharophila* and *C. vulgaris* were more resistant to these herbicides than *S. acutus* and *P. galeata.* Moreover, molinate was found to be less toxic to *Chlorella saccharophila, Chlorella vulgaris* and *Pseudanabaena galeata* than reported toxicity to some fishes and invertebrates.

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