Bull. Environ. Contam. Toxicol. (1996) 56:977-984

Bulletin of Environmental Contamination and Toxicology

Effects of Thiobencarb on the Growth of Three Species of Phytoplankton

C. Sabater, J. M. Carrasco

Laboratorio de Plaguicidas, Departamento de Biotecnología, E.T.S.I. Agrónomos, Universidad Politécnica de Valencia, Camino de Vera, 14, 46022 Valencia, Spain

Received: 25 July 1995/Accepted: 14 December 1995

Thiobencarb (*S*-4-chlorobenzyl diethylthiocarbamate) is a thiocarbamate herbicide widely used for weed control in paddy fields (Worthing and Hance 1991). Undergoes volatilisation, adsorption, chemical and microbiological transformations in the environment (Ishikawa et al. 1977; Ross and Sava 1986; Sabater 1994). However, their application for the plants protection can produce adverse effects on aquatic ecosystems in areas nearby agricultural fields. This could be the case of Albufera lake (Valencia, Spain) since the herbicide used to treat the crops in the area may well migrate into the lake, thus leading to contamination that could be dangerous to both its fauna and flora. This contamination is most likely to occur when water from the rice fields is drained into Albufera (Carrasco et al. 1987).

In order to evaluate the importance of this aquatic contamination it is necessary to resort to toxicity bioassays since chemical and physical tests are not sufficient to determine the true risks to the aquatic organisms (Tarzwell 1971). Nontarget microorganisms, like microalgae, are used in these bioassays. Modification of parameters such as growth, O_2 production and chlorophyll a content help assess the toxic effects of the chemicals after exposure.

Algal susceptibility to herbicides differs among species (Torres and O'Flaherty 1976). This fact has been shown using either single-species (Cullimore 1975; Maule and Wright 1984, Stratton 1984) or community level toxicity studies (DeNoyelles *et al.* 1982).

A limited amount of information on the toxicological aspects of thiobencarb in populations of phytoplankton, and specially on wild strains, is available (Zargar and Dar 1990; Bhunia et al. 1991; Kasai and Hatakeyama 1993). This paper describes the effects of the herbicide thiobencarb on the growth of pure cultures of three species of phytoplankton isolated from Albufera lake (Valencia, Spain).

Correspondence to: C. Sabater

MATERIALS AND METHODS

The species of phytoplankton used in unialgal assays were: *Scenedesmus acutus* (Meyens) (Chlorophyceae), *Chlorella succharophila* (Krüger) Migula (Chlorophyceae) and *Pseudanabaena galeata* (Böcher) (Cyanophyceae). *Scenedesmus* and *Chlorella* were isolated from samples collected at Albufera lake in Valencia (Spain). These species have been determined to be suitable for the study the effects of chemicals on algal growth (OECD 1988). *Pseudanabaena* was a gift from Dr. Romo (Valencia University); it is an abundant species in Albufera lake together with *Planktothrix agardhii* (Gom.) Anagn. Kom. and *Geitlerinema* sp. (Romo and Miracle 1993).

The stock cultures of each algal species were incubated in a liquid medium in an environmental chamber maintained at 22 ± 2 °C. These cultures were illuminated with daylight lamps (Sylvania GRO-LUX F30W/T8/GRO) with an intensity of nearly 1100 lux for 12 hr/day and shaken manually once a day. New anexic cultures were started each week.

Culture medium was prepared for each species according to its particular needs. The green algae *C. saccharophila* and *S. acutus* were grown in a medium recommended by the OECD (1988). *P. galeata* was grown in the medium of Romo and Becares (1992).

Thiobencarb (*S*-4-chlorobenzyl diethylthiocarbamate, 100% analytical standard) was obtained from "Industrias Químicas Argos, S.A."(Spain). The water solubility of thiobencarb is 30 ppm, at 20 °C; its melting and boiling point are 3.3 °C and 126-129 °C/0.008 mmHG, respectively; its vapour pressure is 2.2 Pa at 23 °C and its K_{ow} is 2360 (Worthing and Hance 1991).

The published OECD (1988) method was employed, in which pure cultures of the organisms were exposed to concentrations of thiobencarb ranged from 0.005 to 13.0 $mg \cdot L^{-1}$, for 96 hours. According to the OECD (1988) protocol, the bioassays were conducted with six replicates of controls (without herbicide) and three replicates at each test concentration of thiobencarb. Five concentrations arranged in a geometric series were chosen for each algal species after preliminary screening. Test cultures containing the desired concentrations of thiobencarb were prepared by diluting aliquots of a saturated solution obtained by mechanical dispersion. 250 ml Erlenmeyer flasks were used for the algal bioassays each containing 125 ml of the test solution (culture medium for the controls and culture medium + herbicide for the other replicates).

All flasks were inoculated with an initial algal density of 10^4 organisms/ml. The inoculum was obtained from a preculture which was incubated under test conditions and used when cells were exponentially growing.

The flasks were placed in a climatic chamber at 24 ± 2 °C under continuous illumination provided by six daylight lamps (Philips TLD 36W/84). Light intensity was suitable for the optimal growth of algae: 6400 lux for *S. actutus* and *C. saccharophila* (OECD 1988) and 3200 lux for *P. galeata* (Romo and Becares 1992). Each day, flasks were randomly moved in the climatic chamber to overcome any differences in illumination and were shaken manually twice a

day to keep the algae in suspension and to facilitate transfer of CO_2. The pH was measured at 0 and 72 hours.

At the beginning of the assays, thiobencarb concentrations in test cultures were determined by gas chromatography (Moon and Kuwatsuka 1984) and the results were analyzed statistically. These values were used for calculating toxicities of thiobencarb. The recovery of thiobencarb at 0 hours, after its addition into the medium, was in the range of 95 % ± 5 %.

For the extraction and determination of thiobencarb 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 first concentrated in a rotary evaporator to 10 ml and then under a stream of dry nitrogen at 40 °C to 5 ml. The extract solutions were injected into a Hewlett Packard model 5890 gas chromatograph equipped with a nitrogen-phosphorus detector (NPD), a HP-5 (10 m x 0.53 mm) column and a HP-3940A integrator. The operating temperatures of the detector, column and injection port were 250, 220 and 250°C respectively.

In each flask the turbidity at 750 nm was measured at 24, 48, 72 and 96 hours, after the start of the test (Ordög and Kuivasniemi 1989) and the cell concentration was calculated with a standard curve (Stein 1979). A spectrophotometer Beckman DU®-70 and a haemocytometer (Neubauer Improved "Superior"; 0.1 mm, 0.0025 mm^2) were used for turbidity and cell concentration measures.

In each bioassay, the mean cell concentrations calculated in above described form, at different concentrations of thiobencarb were plotted against time to generate growth curves.

A statistical analysis (ANOVA and Student Newman-Keuls test) was carried out to determine if there was significant differences between the treated and control cells (Reish and Oshida 1987). The percent inhibition of cell growth at each thiobencarb concentration was calculated by comparison of areas under growth curves for each herbicide concentration (OECD 1988); the percents inhibition were transformed into probit values and then represented on log doses of thiobencarb in order to calculate the E_bC_{50} (0-96 hours) values and their 95% confidence limits (Abou-Setta *et al.* 1986). In this study, E_bC_{50} is the concentration of thiobencarb which results in a 50% reduction in algal growth relative to the control values, at 96 hours.

RESULTS AND DISCUSSION

The NOEC values (96 hours) or the highest concentrations tested without significant effects ($P<0.05$) on the algal growth relative to control values were 0.92, 0.005 and 0.08 ppm in unialgal cultures of *Chlorella, Scenedesmus* and *Pseudanabaena,* respectively. The concentrations of thiobencarb that caused significant effects ($P<0.05$) on the algal growth ranged from 1.6-12.2 ppm for *C. saccharophila,* from 0.009-0.10 ppm for *S. acutus* and from 0.21-2.3 ppm for *P. galeata* (Table 1). These data are concentration values measured by GC (no nominal values). The pH values of the culture media from 0 to 72 hours ranged from 8.0-9.3 in all flasks.

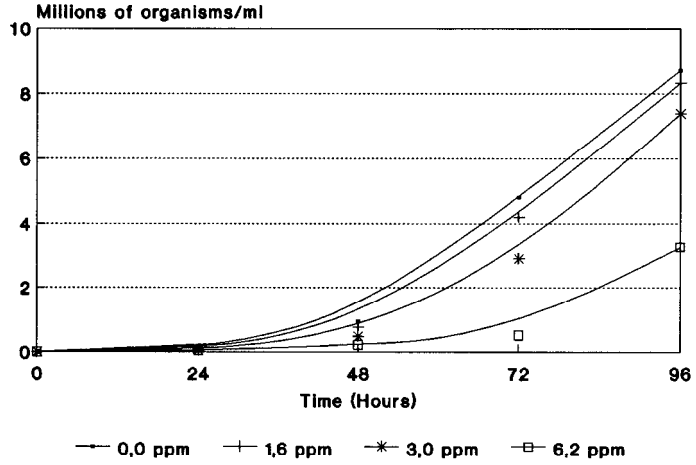

Figure 1. Effect of thiobencarb concentration on *Chlorella saccharophila* (Krüger) Migula growth.

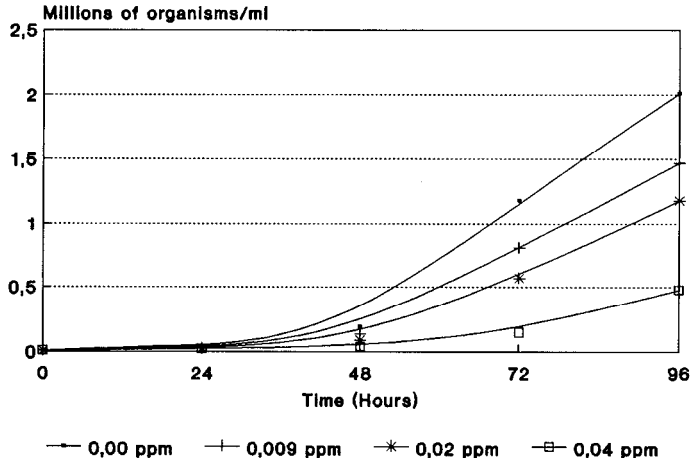

Figure 2. Effect of thiobencarb concentration on *Scenedesmus acutus* (Meyens) growth.

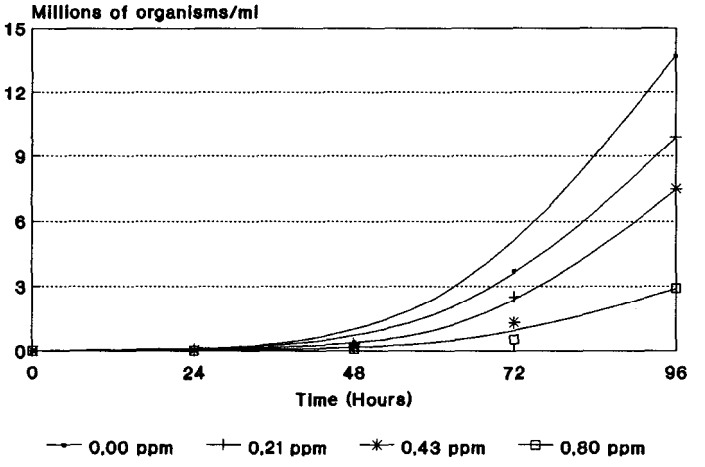

Figure 3. Effect of thiobencarb concentration on *Pseudanabaena galeata* (Böcher) growth.

Table 1. Initial thiobencarb concentrations (ppm).

Series	*C. saccharophila*	*S. acutus*	*P. galeata*
Series 1	0.92 ± 0.03	0.005 ± 0.001	0.08 ± 0.01
Series 2	1.6 ± 0.2	0.009 ± 0.001	0.21 ± 0.01
Series 3	3.0 ± 0.1	0.02 ± 0.01	0.43 ± 0.02
Series 4	6.2 ± 0.2	0.04 ± 0.01	0.80 ± 0.04
Series 5	12.2 ± 0.7	0.10 ± 0.01	2.3 ± 0.1

Mean of 6 values ± Standard Deviation.

Changes in growth curves of three algal species, given in terms of millions of organisms/ml, due to thiobencarb treatments, are shown in Figures 1, 2 and 3. As shown as in these figures, at 6.2, 0.04 and 0.8 ppm of thiobencarb the growth of *Chlorella* (Figure 1), *Scenedesntus* (Figure 2) and *Pseudanabaena* (Figure 3) was significantly decreased. Doses greater than 12.2 ppm for *Chlorella,* 0.10 ppm for *Scenedesmus* and 2.3 ppm for *Pseudanabaena,* completely inhibited algal growth during the duration of the assay.

There were obvious differences in the sensitivity to thiobencarb between the three species of algae. *Scenedesmus* was strongly inhibited by thiobencarb after 96 hours at concentrations of 0.04 ppm while *Chlorella* and *Pseudanabaena* showed no inhibition at 0.92 ppm and 0.08 ppm, respectively. *Chlorella* growth

was strongly inhibited (76%) at 6.2 ppm; whereas, at doses of 0.10 ppm for *Scenedesmus* and 2.3 ppm for *Pseudanabaena* any growth was observed.

E_bC_{50} values of thiobencarb were 4.0, 0.017 and 0.37 ppm for *C. saccharophila, S. acutus* and *P. galeata,* respectively (Table 2). The two green algae tested responded very differently to thiobencarb; *C. saccharophila* was less sensitive to the herbicide while S. *acutus* was more sensitive. Sensitivity of cyanophycea *Pseudanabaena* was intermediate between *Scenedesmus* and *Chlorella.*

Table 2. Probit regression equations * and E_bC_{50} (0-96 hours) values of thiobencarb for the three algal species.

	Probit regression equations	E_bC_{50}	(95% confidence limits) $mg \cdot L^{-1}$
C. saccharophila	Y = 2.91 + 1.522 X	4.0	(3.8 - 4.1)
S. acutus	Y = 8.75 + 0.924 X	0.017	(0.016 - 0.019)
P. galeata	Y = 6.05 + 1.072 X	0.37	(0.35 - 0.40)

* : Y = Probit of % inhibition; X = ln (mg of thiobencarb/liter).

The 96 hour E_bC_{50}, for thiobencarb exposure in the present study for *Chlorella saccharophila* was 4.0 ppm with 95% confidence limits of 3.8-4.1. These results are very close to the EC_{50} (0-72 hours) values reported by Kasai and Hatakeyama (1993) of 3.3 ppm for *Chlorella vulgaris* CCAP 211/11b with 95% confidence limits of 2.6-4.2 ppm and 3.8 ppm for *Chlorella vulgaris* NIES-227 with 95% confidence limits of 2.6-5.5 ppm.

Kasai and Hatakeyama (1993) reported a 72 hour EC_{50} value of thiobencarb of 0.039 for the chlorophycea *Selenastrum capricornutum* ATCC 22662 with 95% confidence limits of 0.022-0.069 ppm. Our results indicate that doses lower than 0.017 (0.016-0.019) ppm thiobencarb produce 50% inhibition of the growth of chlorophycea *Scenedesmus acutus.* The strain *Selenastrum capricornutum* NIES-35 is also more sensitive than S. *capricornutum* ATCC 22662 with a 72 hour EC_{50} of 0.020 (0.016-0.026) ppm (Kasai and Hatakeyama 1993).

P. galeata has been reported to be more sensitive to thiobencarb than other species of blue-green algae. Results obtained by several authors in studies with other species of cyanophyceas, using growth inhibition, chlorophyll *a* content and nitrogen fixation, were variable. Zargar and Dar (1990) found a negligible variation in the dry biomass yield, nitrogen content and chlorophyll *a* content at 35 ppm of thiobencarb in mixed culture of *Anabaena, Nosloc* and *Oscillatoria* for 3 weeks; at concentrations of 55 ppm a drastic reduction in these parameters occurred. Bhunia *et al.* (1991) showed that the growth of *Nostoc muscorum* was significantly decreased at thiobencarb concentrations of 2 and 4 ppm, but at 6 ppm severe reduction in growth occurred; thiobencarb at 8 ppm was found to be lethal after 8 days of exposure.

Carrasco *et al.* (1987) found levels of 0.005 ppm at the outfall of several channels in Lake Albufera during the season of greatest contamination; these

values are lower than toxicity values obtained on the three algal species in our assays (Table 2). Monitoring studies have documented concentrations up to 0.057 mg thiobencarb/l in the Colusa Basin Drain (California) (Finlayson and Faggella 1986), with exposures to herbicide lasting from 40 to 60 days. Concentrations of thiobencarb in the Colusa Basin Drain are also lower than toxicity values of 4.0 and 0.37 ppm obtained for *Chlorella* and *Pseudanabaena,* respectively, but exceeded the E_bC_{50} value of 0.017 ppm for *Scenedesmus*.

Many studies have revealed the effects of thiobencarb on other nontarget organisms such as aquatic invertebrates and fishes. Thiobencarb 24-hr LC_{50} was 6.50 ppm for the rotifer *Brachionus calyciflorus;* this rotifer is a more resistent species than studied freshwater algae and other aquatic invertebrates and fishes (Fernandez-Casalderrey *et al.* 1992). Thiobencarb 96-hr LC_{50} data generated by Johnson and Finley (1980) for the fishes *Salmo gairdneri* and *Lepomis macrochirus* were 1.2 and 2.5 ppm, respectively. Acute and chronic tests of thiobencarb on fishes *Cyprinodon variegatus* and *Mysidopsis bahia* resulted in 96-hr LC_{50} values of 1.4 and 0.90 ppm, respectively (Finlayson and Faggella 1986).

The majority of short-term acute toxicity studies under controlled conditions of another thiocarbamate herbicides like EPTC, tri-allate and molinate have used either fish or the cladoceran *Daphnia magna*. EPTC and tri-allate showed a LC_{50} (96-hr) of 27 and 1.3 ppm for the fish bluegill, respectively and 19 and 1.2 ppm for rainbow trout. Tri-allate 48-hr LC_{50} for *Daphnia magna* was 0.43 ppm. Molinate 96-hr LC_{50} was 1.3 ppm for rainbow trout and 30 ppm for goldfish (Worthing and Hance 1991).

The results of the present study indicate that algal susceptibility to thiobencarb differs among the three species studied. *Chlorella saccharophila* was less sensitive to thiobencarb while *Scenedesmus acutus* was more sensitive. Sensitivity of *Pseudanabaena galeata* was intermediate between the two green algae essayed. Moreover, thiobencarb was found to be less toxic to *Chlorella saccharophila* than reported toxicity to fishes.

Acknowledgments. This work was supported by a grant from Comisión Interministerial de Ciencia y Tecnología nº AMB 92-1277.

REFERENCES

Abou-Setta MM, Sorrell RW, Childers CC (1986) A computer program in basic for determining probit and log-probit or logit correlation for toxicology and biology. Bull Environ Contam Toxicol 36:242-249

Bhunia AK, Basu NK, Roy D, Chakrabarti A, Banerjee SK (1991) Growth, chlorophyll a content, nitrogen-fixing ability and certain metabolic activities of *Nostoc muscorum:* effect of methylparathion and benthiocarb. Bull Environ Contam Toxicol 47:43-50

Carrasco JM, Plantá M, Gomez-Casals V, Moragués V (1987) Pesticide residues in Lake Albufera, Valencia, Spain. J Assoc Off Anal Chem 70(4):752-753

Cullimore DR (1975) The *in vitro* sensitivity of some species of Chlorophyceae to a selected range of herbicides. Weed Research 15:401-406

DeNoyelles F, Kettle WD, Sinn DE (1982) The responses of plankton communities in expcrimental ponds to atrazine, the most heavely used pesticide in the United States. Ecology 63:1285-1293

Fernandez-Casalderrey A, Ferrando MD, Andreu-Moliner E (1992) Acute toxicity of several pesticides to rotifer (*Brachionus calyciflorus*). Bull Environ Contam Toxicol 48:14-17

Finlayson BJ, Fagella GA (1986) Comparison of laboratory and field observations of fish exposed to the herbicides molinate and thiobencarb. Transactions of the American Fisheries Society 115:882-890

Moon YH, Kuwatsuka S (1984) Properties and conditions of soils causing the dechlorination of the herbicide benthiocarb (thiobencarb) in flooded soils. J Pesticide Sci 9:745-754

Ishikawa K, Nakamura Y, Niki Y, Kuwatsuka S (1977) Photodegradation of benthiocarb herbicide. J Pesticide Sci 2:17-25

Johnson WW, Finley MT (1980) Handbook of acute toxicity of chemicals to fish and aquatic invertebrates. US Fish Widl Serv Resour Pub 137:1-98

Kasai F, Hatakeyama S (1993) Herbicide susceptibility in two green algae, *Chlorella vulgaris* and *Selenastrum capricornutum*. Chemosphere 27(5):899-904

Maule A, Wright SJL (1984) Herbicide effects on the population growth of some green algae and cyanobacteria. J Appl Bacteriol 57:369-379

OECD (1988) Algal inhibition test. Official Journal of the European Communities N° L 133:89-93

Ördog V, Kuivasniemi K (1989) Studies on the effect of cell division-inhibiting herbicides on unialgal and mixed algal cultures. Int Revue Ges Hydrobiol 74(2):221-226

Reish DL, Oshida P (1987) Manual of methods in aquatic environment research. Part 10, Short-Term static bioassays. FAO Fisheries Technical Paper 247. Food and Agriculture organization of the United Nations.

Rome S, Becares E (1992) Preservation of filamentous cyanobacteria cultures (*Pseudanabaena galeata* Böcher and *Geitlerinema amphibium* (Ag. ex Gom.) Anagn.) under low temperature. J Microbial 16(2):85-89

Romo S, Miracle R (1993) Long-term periodicity of *Planktothrix agardhii, Pseudanabaena galeata* and *Geitlerinema sp.* in a shallow hypertrophic lagoon, the Albufera of Valencia (Spain). Arch Hydrobiol 126(4):469-486

Ross LJ, Sava RJ (1986) Fate of thiobencarb and molinate in rice fields. J Environ Qual 15:220-25

Sabater C (1994) Degradación de plaguicidas en aguas contaminadas. Thesis Doctoral

Stein JR (1979) Handbook of phycological methods. Culture methods and growth measurements. Cambridge University Press, New York, 448 pp.

Stratton GW (1984) Effects of the herbicide atrazine and its degradation products, alone and in combination, on phototrophic microorganisms. Arch Environ Contam Toxicol 13:35-42

Tarzwell CM (1971) Bioassays to determine allowable waste concentrations in the aquatic environment. I Measurement of pollution effects on living organisms. Proc Royal Soc London B177:279

Tones AMR, O'Flaherty LM (1976) Influence of pesticides on *Chlorella, Chlorococcum, Stigeoclonium* (Chlorophyceae), *Tribonema, Vaucheria* (Xanthophyceae) and *Oscillatoria* (Cyanophyceae). Phycologia 15:25-36

Worthing CR, Hance RJ (1991) The pesticide manual (9th ed.). The british Crop Protection Council. Registered Office, 49 Downing Street, Farnham, Surrey GU9 7PH UK.

Zargar MY, Dar GH (1990) Effect of benthiocarb and butachlor on growth and nitrogen fixation by Cyanobacteria. Bull Environ Contam Toxicol 45:232-234