Aquatic Toxicology, 23 (1992) 181–192

AQTOX 00528

Effects of Dursban® 4E and its carrier on three algal species during exponential and P-limited growth

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(Received 25 October 1991; revision received 20 January 1992; accepted 20 May 1992)

To study the direct effects of the insecticide Dursban® 4E (a.i. chlorpyrifos) on freshwater algae, toxicity tests were performed with three different species (diatom: *Cyclotella* sp., green alga: *Selenastrum capricornutum* and cyanobacterium: *Synechoccocus leopoliensis*). The tests were carried out with cells growing under non-limited and phosphorus-limited conditions, using free cells and cells immobilized in alginate beads. The carrier of Dursban 4E was tested separately.

Dursban 4E had no appreciable effect on the growth of non-limited algae at concentrations relevant for field situations. For P-limited algae, however, significant and dissimilar effects were found. Striking was the growth stimulation of the P-limited green alga at low concentrations of Dursban 4E (from 0.03 mg l^{-1}), while the P-limited diatom was inhibited from 0.1 mg l^{-1}. No marked effect was observed for the P-limited cyanobacterium. The carrier substances in Dursban 4E were responsible for the stimulating effect on the green alga.

Shifts in algal species composition and increase in biomass – previously observed in freshwater test ecosystems after treatment with Dursban 4E – may be partly the result of direct species-specific effects of the carrier compounds in Dursban 4E on algal growth. The algal cells immobilized in alginate beads reacted in a rather similar way as the free cells to the application of Dursban 4E and its carrier. This property, combined with other advantages of the beads, such as simple handling and the possibility for exposition of algal cells to toxic substances under field conditions, make the beads suitable for biotest purposes.

Key words: Dursban; Toxicity; Chlorpyrifos; Algae; Immobilization; Adjuvants

INTRODUCTION

A research project was initiated in 1989 in the Netherlands to study the ecotoxicological effects of the pesticide Dursban® 4E (active ingredient chlorpyrifos) on aquatic biotopes. Chlorpyrifos, an organophosphorus compound with an anticholinesterase mode of action, is a broad-spectrum insecticide used for crop protection in agriculture

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and horticulture. The compound may enter surface waters by runoff, spray drift or accidental spills (Miles et al., 1976; Wan, 1989; Cowgill et al., 1991). In the Netherlands a nominal concentration of 0.087 mg l^{-1} Dursban 4E is considered a worst-case level in shallow ditches that drain land where Dursban 4E is applied to control insect pests (Brock et al., 1992). In indoor model ecosystems treated with Dursban 4E, an increase in algal biomass has been observed. Brock et al. (in prep.) suggested that this was caused, at least partly, by a reduction in grazing.

Also in other studies (Hurlbert et al., 1972; Brazner et al., 1989; Butcher et al., 1977; Papst and Boyer, 1980; Siefert et al., 1989) algal blooms often followed treatment of enclosures and ponds with Dursban. Reduced grazing pressure was also here proposed as an important aspect of post-treatment increases in algae.

Studies by Butcher et al. (1977), however, tend to support the presence of additional factors. For instance, in some treated ponds filamentous algae, which were not grazable by zoo-plankton, increased significantly compared with the development in the untreated ponds. Also, while all the tested concentrations of the insecticide were sufficient to suppress macrozooplankton populations during the experiments, a good correlation was found between the concentration of the insecticide and the size of the bloom. These observations suggest possible direct effects of Dursban on algal growth.

To study these direct effects, toxicity tests were performed with three freshwater algal species (a diatom, a green-alga and a cyanobacterium) growing under different environmental conditions. The effects of the carrier substances (adjuvants) of Dursban 4E were tested separately. All tests were performed with algal clone cultures growing under non-limited and phosphorus-limited conditions. Free cells and alginate-immobilized cells were used respectively in the experiments.

P-limited cells were tested to determine if Dursban 4E can stimulate the growth rate of algae by phosphorus resulting from degradation of the compound. In addition the effects on free and immobilized cells were compared. The encapsulation of algal cells in an alginate matrix provides the possibility for exposition of algal cells to toxic effluents under field conditions. Hence, they may be suitable for biotest purposes (Bozeman et al., 1989).

METHODS

Insecticide

The insecticide Dursban® 4E is a emulsifiable formulation with 480 g l^{-1} chlorpyrifos [*O,O*-diethyl *O*-(3,5,6-trichloro-2-pyridyl) phosphorothioate] as active compound and 710 g l^{-1} of carrier substances. The composition of the formulation Dursban 4E is given in Table 1. The amount of phosphorus (soluble reactive P and total P) and nitrogen (Total N) in chlorpyrifos and the carrier were determined from diluted stocks of 1 and 10 mg l^{-1}, according to the standard methods used at the Norwegian Institute for Water Research (APHA, 1985). Dursban® 4E and Dursban® 4 Blank

(containing only the carrier substances) were supplied by DowElanco Europe. For physico-chemical properties of the insecticide see Marshall and Roberts (1978).

Free cell cultures

Three species of phytoplankton, including the diatom *Cyclotella* sp. NIVA BAC 8, the green alga *Selenastrum capricornutum* NIVA CHL 1 and the cyanobacterium *Synechoccocus leopoliensis* NIVA CYA 20, were selected for the toxicity tests. The species are kept in pure (non-axenic) clone cultures at the culture collection of the Norwegian Institute for Water Research (Skulberg and Skulberg, 1990). Inoculum cultures were incubated in an inorganic nutrient medium Z8 (Skulberg and Skulberg, 1990) diluted to 20% concentration in glass-distilled water at the same light and temperature conditions as during the tests. The illumination was provided by cool-white fluorescent tubes at 70 μE m^{-2} s^{-1} for the green alga and diatom and 30 μE m^{-2} s^{-1} for the cyanobacterium, all on a 14:10 h LD cycle at 20° C.

To obtain phosphorus-limited cultures, exponentially growing cells were inoculated into flasks containing medium without phosphorus. Cells were allowed to grow until they entered stationary phase. Cells from these cultures were inoculated in 1 L flasks, containing culture medium with a low phosphorus concentration (0.5 μgP l^{-1}), with an initial cell concentration of 10^3 cells ml^{-1}. A part of the exponentially growing cells in these cultures was used to produce P-limited immobilized algae (algae-alginate beads).

Immobilized cells (beads)

The method used for preparing beads of immobilized algal cells was based on the procedure described by Bozeman et al. (1989) and Abdel-Hamid et al. (1991). Sodium alginate (Sigma-No.A-7128) was dissolved in warm, distilled water to form a 4% w/v

TABLE 1

Composition of the organophosphorus insecticide Dursban® 4E. TP = Total P, SRP = Soluble reactive P, TN = Total N (b.d. = below detection level).

	Conc. (g l^{-1})	Weight (%)	TP	SRP (g l^{-1})	TN
Toxic compound					
Chlorpyrifos	480	40.3	55	b.d.	24
Carrier					
a.o. Trichlorethane, Xylene and Alkylbenzene sulphonate	710	59.7	<0.006	b.d.	24

solution. The solution was autoclaved for 13 min and cooled to room temperature. Selected volumes of the algal cultures (non limited and P-limited) were centrifuged and the algal pellets were transferred into the alginate solution. Aliquots of this mixture, after thorough stirring, were loaded into a 50 ml burette and extruded dropwise into a cool autoclaved solution of 0.03 M $CaCl_2$ (between 1 and 3°C). The burette system was carefully kept full of the alginate-algae mixture to assure a constant flow of about 5 drops s^{-1} (5 beads s^{-1}, approx. 16 beads ml^{-1}). The beads produced this way had a homogenous size and a normally distributed number of cells (Abdel-Hamid et al., 1991). The beads were kept in the cold $CaCl_2$-solution for at least 30 min to allow complete hardening of the alginate, washed several times with distilled water and stored in the dark at 4°C until use. The growth experiments were performed within two weeks after preparation of the beads. To determine the actual initial cell density in the beads, they were dissolved separately in 5% sodium hexametaphosphate and the algal cells were counted with a Coulter Multisizer after 15 min. The mean initial cell concentration chosen for the beads were 5000 (±360) cells/bead of *Cyclotella* sp., 25000 (±1150) cells/bead of *S. caprocornutum* and 36000 (±1430) cells/bead of *S. leopoliensis*.

Toxicity tests

The toxicity tests with the free algal cells growing at maximum growth rate and under P-limitation were started while the inoculum cultures were in the exponential growth phase. The tests were carried out on polystyrene microplates with 6x4 flat bottom wells of 3 ml capacity. The inoculum culture was diluted with growth medium (20% Z8) to give a cell density of 10^5 cells ml^{-1}. Each well on the plates was filled with 1.8 ml of the dilute culture. Separate stock solutions (320 and 100 mg l^{-1}) were prepared of both Dursban 4E (chlorpyrifos + carrier) and Dursban 4 Blank (only carrier). With these stock solutions serial dilutions were repeated to give 10 nominal concentrations in the range 32 mg l^{-1} to 0.001 mg l^{-1} of Dursban 4E and the carrier separately. The serial dilutions were performed according to the procedure described by Källqvist and Romstad (submitted). The tests were performed in duplicate and there were four controls (containing only growth medium and algae, no carrier or Dursban 4E).

The tests with the beads followed the same procedure as described for the free cells. One bead was placed in each well, containing medium and carrier, or medium and Dursban 4E, respectively. The control wells contained only growth medium.

The microplates were incubated on a reciprocal shaker under identical temperature and light conditions as the inoculum cultures.

The incubation time was restricted to the exponential growth phase of the control cultures. Since the maximum growth rates differed among the species, the duration of the test was adjusted accordingly to 3 days for the green alga, 5 days for the cyanobacterium and 6 days for the diatom. At the end of the test, the cell density in each

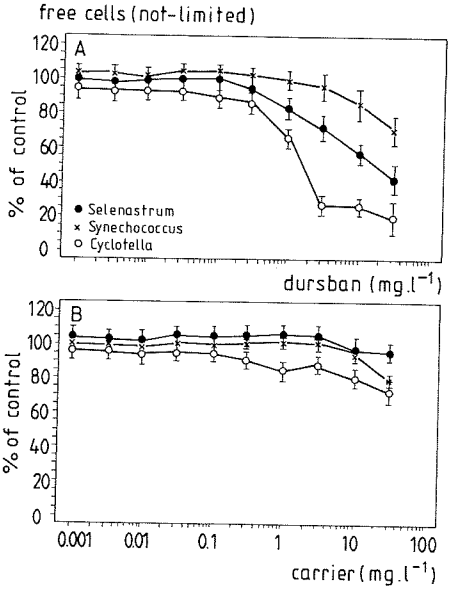

Fig. 1. The mean growth rate (+ max. and min. values) of the non-limited free algal cells at each Dursban 4E (A) and carrier (B) concentration is given as percentage of the mean growth rate of the control (see Table 2) and plotted versus the logarithm of concentration.

well was counted by a Coulter Multisizer. The average growth rate during the test was calculated as suggested by Nyholm and Källqvist (1989), from the initial and final cell densities. Mean growth rates of the controls with their 95% confidence intervals were calculated by a least squares linear regression analysis of log transformed data with more than one value of y per value of x (Sokal and Rohlf, 1969). The mean growth rate at each Dursban 4E and carrier concentration was calculated as percentage of the growth rates of the controls and plotted against the logarithm of concentration.

RESULTS

Response curves showing the effects of Dursban 4E and its carrier on the three algal clone cultures are shown in Figs. 1–4. The results obtained with the free cells are given in Figs. 1 and 2 and with the beads in Figs. 3 and 4. The effects of application of only the carrier are presented separately in Figs. 1b–4b. The mean growth rates of the controls with their 95% confidence intervals are listed in Table 2.

Effects on non-limited algae

The algae growing under optimal growth conditions demonstrated a significant growth inhibition ($p < 0.05$) only at high concentrations of Dursban 4E (> 0.32 mg l^{-1}

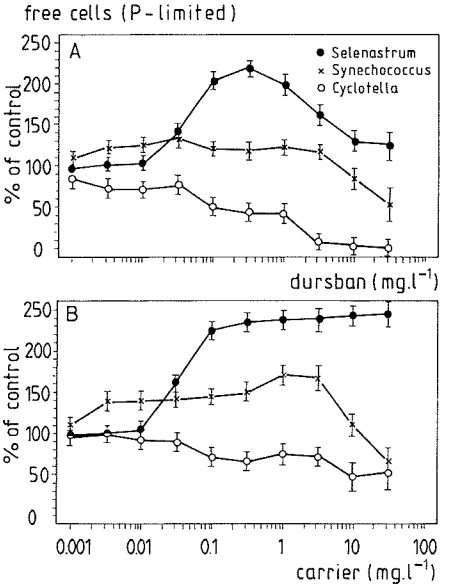

Fig. 2. The mean growth rate (+ max. and min. values) of the phosphorus-limited free algal cells at each Dursban 4E (A) and carrier (B) concentration is given as percentage of the mean growth rate of the control (see Table 2) and plotted versus the logarithm of concentration.

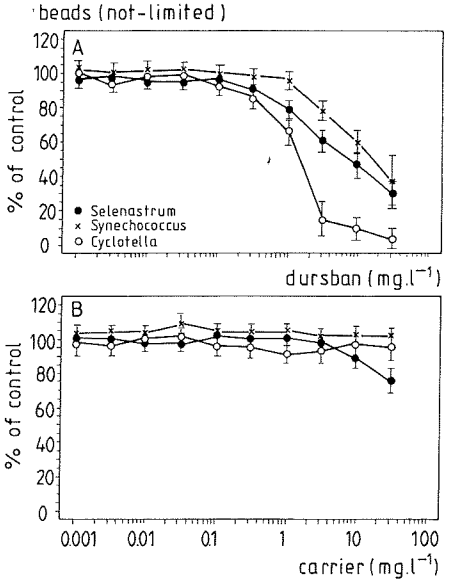

Fig. 3. The mean growth rate (+ max. and min. values) of the non-limited algal beads at each Dursban 4E (A) and carrier (B) concentration is given as percentage of the mean growth rate of the control (see Table 2) and plotted versus the logarithm of concentration.

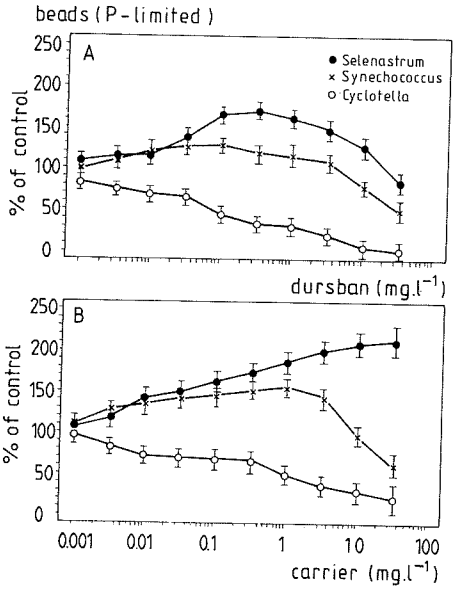

Fig. 4. The mean growth rate (+ max. and min. values) of the phosphorus-limited algal beads at each Dursban 4E (A) and carrier (B) concentration is given as percentage of the mean growth rate of the control (see Table 2) and plotted versus the logarithm of concentration.

for *Cyclotella*, > 1 mg l^{-1} for *Selenastrum* and >10.0 mg l^{-1} for *Synechococcus*) (Figs. 1a and 3a). Interpreting these results one has to keep in mind that the solubility of Dursban 4E in water is incomplete at concentrations > 2 mg l^{-1} Dursban 4E (pers. comm. K. Kersting) and also that these high concentrations are not realistic for field situations. For *Cyclotella* and *Selenastrum* the responses of the immobilized cells and

TABLE 2

Mean growth rates (μ, d^{-1}) of three algal species *Selenastrum capricornutum* (Sel), *Synechococcus leopoliensis* (Syn) and *Cyclotella* sp. (Cycl.) growing in microplates at 20°C under optimal conditions (+P) and P-limitation (−P). There were no additions of Dursban 4E or carrier and these growth rates are used as control growth rates in the toxicity tests. (The 95% confidence intervals are given in parentheses.)

		Sel	Syn	Cycl. (μ, d^{-1})
Free cells	+P	1.48 (±0.16)	1.31 (±0.28)	0.71 (±0.15)
	−P	0.47 (±0.05)	0.51 (±0.17)	0.21 (±0.08)
Beads	+P	0.77 (±0.12)	0.86 (±0.14)	0.52 (±0.09)
	−P	0.30 (±0.11)	0.33 (±0.06)	0.17 (±0.04)

the free cells were rather similar, but for *Synechococcus* cells the growth inhibition was stronger when encapsulated in beads.

Application of only the carrier had no significant effect on the growth of the three algal species. The control growth rates of the immobilized cells, however, were always lower than the rates measured for the free cells (Table 2).

Effects on P-limited algae

For the algae growing under P-limitation the responses to Dursban 4E application were more complex and very dissimilar for the different species (Figs. 2a and 4a). Significant growth stimulation ($p < 0.05$) was found for P-limited *Selenastrum* at concentrations > 0.03 mg l^{-1} Dursban 4E. Free cells reached an optimum of 220% (Fig. 2a) and beads of 170% (Fig. 4a) of the control at 0.32 mg l^{-1} Dursban 4E. A slight, but not significant, stimulation was observed for P-limited *Synechococcus* and significant inhibition of the growth rate was measured only at high concentrations of Dursban 4E (> 10 mg l^{-1}). Free cells and beads of *Synechococcus* demonstrated the same responses. No growth stimulation was found for the P-limited Cyclotella. Both the free and immobilized cells reacted with an significant inhibition ($p < 0.05$) of the growth rate at concentrations > 0.1 mg l^{-1} Dursban 4E.

Application of only the carrier gave some remarkable results for the P-limited algae (Figs. 2b and 4b). For *Selenastrum* application of the carrier alone up to concentrations of 0.32 mg l^{-1}, gave nearly the same results as application of Dursban 4E (Figs. 2a and 4a). At higher concentrations of the carrier, *Selenastrum* was further stimulated in growth and no optimum was reached like with application of Dursban 4E. For *Synechococcus* a significant growth stimulation was found at concentrations > 0.10 mg l^{-1} carrier. This stimulation, however, was less pronounced than for *Selenastrum* and at concentrations > 10 mg l^{-1} even an inhibition was observed. Both species showed a more pronounced growth stimulation due to carrier addition for free cells than for immobilized ones.

Striking is the fact that the carrier had no stimulating effect on the growth of *Cyclotella* and only at high carrier concentrations inhibiting effects were found (> 1 mg l^{-1} for beads and > 10 mg l^{-1} for free cells).

The control growth rates of P-limited immobilized cells were lower than rates measured for P-limited free cells (Table 2).

DISCUSSION

The study revealed different effects of Dursban 4E and its carrier on the growth rates of the three algal species. Striking was the growth stimulation of the P-limited green alga at low concentrations of Dursban 4E (from 0.03 mg l^{-1}), while the P-limited diatom was inhibited by 0.1 mg l^{-1}. No marked effect was observed for the P-limited cyanobacterium.

The usual explanation for occurrence of algal blooms after pesticide treatment is the release from grazing caused by the decimation of herbivorous zooplankton which normally control algal populations (Hurlbert, 1975). Our results, however, suggest that also direct, stimulatory and inhibitory, effects on algal growth are involved.

Variable primary effects of Dursban on the growth of various freshwater phytoplankton species were also observed by Brown et al. (1976) in polyethylene mesocosms treated with Dursban 4E (1.2 to 240 $\mu g\ l^{-1}$). Birmingham and Colman (1976) found an increase in the growth rate of the cyanobacterium *Anabaena flos-aquae*, growing in nitrogen-free medium, in response to 100 $\mu g\ l^{-1}$ Dursban® M-3633. Also the green alga *Chlamydomonas reinhardtii* showed growth stimulation in response to 100 $\mu g\ l^{-1}$ Dursban, while significant decrease in growth rate was observed for the diatom *Navicula minima*. Only Butcher et al. (1977), however, tried to give an explanation for the direct growth stimulation of Dursban® 3M-3633 on some algal species. They stated that increase in phytoplankton after application of Dursban may be due to an increase in nutrients (especially phosphorus) resulting from degradation of the compound.

We found that the carrier of Dursban 4E, and not the toxic compound chlorpyrifos, was responsible for the increased growth rate of the P-limited green alga *Selenastrum*. This, despite the fact that chlorpyrifos had much higher P content than the carrier (Table 1). The carrier contained among others xylene and alkylbenzene sulphonate (Table 1). Contrary to *Selenastrum* and *Synechococcus* the P-limited diatom *Cyclotella* was not stimulated by the carrier and inhibited by Dursban 4E. Reduction in diatom diversity and colonization after Dursban application was observed by Nelson et al. (1976), and Walsh (1983) determined an EC_{50} of 1.2 $mg\ l^{-1}$ Dursban for the marine diatom *Skeletonema*.

The present study does not reveal the specific mechanisms of the observed stimulatory/toxic effects. The carrier contains surfactants and it is conceivable that these substances may exert their influence on the physiology of algal cells (Lewis, 1986). The experiments were performed with species-pure clone cultures, but were not free from bacteria. The P-limited algal cultures contained probably more bacteria than the exponential growing algal cultures, due to excretion of organic carbon by algae under nutrient stress (Jensen, 1984; Baines and Pace, 1991). To get more insight into the specific mode of action of the carrier substances, physiological studies with bacteria-free algal cultures are required.

The algal cells immobilized in alginate beads reacted in a rather similar way to the application of Dursban 4E and its carrier as the free cells. This similarity in response may imply possibilities of applying beads in biotests.

The stimulatory and inhibitory effects of Dursban 4E on the P-limited algal species tested, were found at concentrations considered to be worst-case levels (0.087 $mg\ l^{-1}$ Dursban 4E) in Dutch shallow ditches that drain land where Dursban 4E is applied (Brock et al., 1992).

Finally we conclude that shifts in algal species composition and biomass, observed

in freshwater test ecosystems after treatment with Dursban 4E, may be a direct effect of the carrier substances in Dursban 4E. Hence, not only by secondary effects, through mortality among crustaceans and insects, but also by primary effects on the algal community, Dursban 4E can affect the structure and functioning of the ecosystem.

ACKNOWLEDGEMENTS

We thank DowElanco Europe for providing us with Dursban 4E and Dursban 4E Blank. The authors are indepted to Dr. P. Leeuwangh, Dr. Th.C.M. Brock, Dr. K. Kersting, Prof. Dr. J.H. Koeman, Prof. Dr. W.J. Wolff and Dr. O. Skulberg for reviewing the manuscript. NIVA's Culture Collection of Algae distributed the clone cultures and thanks are due to R. Skulberg.

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