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Effects of a Mixture of Two Insecticides in Freshwater Microcosms: II. Responses of Plankton and Ecological Risk Assessment

PAUL J. VAN DEN BRINK,[1,*] ELIZABETH M. HARTGERS,[2,†] RONALD GYLSTRA,[2] FRED BRANSEN[2] AND THEO C.M. BROCK[1]

[1] *Alterra Green World Research, Wageningen University and Research centre, P.O. Box 47, 6700 AA Wageningen, The Netherlands*

[2] *Wageningen University, Aquatic Ecology and Water Quality Management Group, Wageningen University and Research centre, P.O. Box 8080, 6700 DD Wageningen, The Netherlands*

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Abstract. This paper reports on the chronic effects of a mixture of the insecticides chlorpyrifos and lindane in freshwater microcosms. Chronic treatment levels corresponding to concentrations of 0, 0.005, 0.01, 0.05, 0.1 and 0.5 times the LC50 of the most sensitive standard test organism were evaluated. The zooplankton community structure was altered from the 0.05 * LC50 treatment level upwards. Cladocerans were the most susceptible group, followed by Copepoda and Ostracoda. Rotifera increased in abundance at the higher treatment levels. Increased abundance of some phytoplankton taxa and increased chlorophyll-a levels were found at the two highest treatment levels, most probably a consequence of decreased grazing pressure. Threshold levels for the mixture, both at population and community/ecosystem level, corresponded well with those reported in the literature for the individual compounds. The overall risk assessment indicates no antagonistic or synergistic effects of the mixture at ecosystem level. It was found that the safety factors set by the Uniform Principles for individual compounds also ensure protection against chronic exposure to a mixture of insecticides at community level, though not always at species level.

Keywords: chlorpyrifos; ecological risk assessment; lindane; pesticides; plankton; semi-field

Introduction

Experiments evaluating the effects of pesticides in microcosms and mesocosms are frequently performed for scientific and/or regulatory purposes (Campbell et al., 1999; Boyle and Fairchild, 1997). The advantage of experiments with microcosms or mesocosms over field and laboratory experiments is that they provide the best of both worlds, in that they offer both an experimental set-up, including the option of replication, and ecological realism. These experiments are usually performed with a single pesticide as the stressor, mostly to determine ecological threshold levels and/or to investigate the ecological effect chain.

In normal agricultural practice, however, protection of crops from pest organisms is not achieved by the application of a single compound; usually, several

*To whom correspondence should be addressed:
E-mail: p.j.vandenbrink@alterra.wag-ur.nl
†Present address: RIKZ National Institute for Coastal and Marine Management, P.O. Box 20907, 2500 EX, The Hague, The Netherlands.

different compounds for different target organisms are used. Some pesticides are also administered repeatedly. The effects of combinations of pesticides on freshwater ecosystems are largely unstudied (Hartgers et al., 1998). Therefore, it is important to evaluate whether the first-tier risk assessment procedure for individual compounds (Uniform Principles, UP; European Union, 1997) is conservative enough to protect aquatic life from exposure to pesticide mixtures. The first experiment conducted in the present research project concerned the effects of three herbicides and has been described by Hartgers et al. (1998). The safety factors used in the UP were indeed found to protect the aquatic community against chronic exposure to these herbicides. The risk assessment of herbicides can, however, not be extrapolated to other types of pesticides because of differences in their toxicological mode of action and in the susceptibility of water organisms. Firstly, aquatic plants are expected to be the most susceptible group for herbicides, while arthropods are expected to be susceptible to insecticides. Secondly, the trophic level affected in the ecosystem (primary producers versus primary consumers) could cause different secondary effects within the food chain. Thirdly, the risk assessment of herbicides (growth inhibition) and insecticides (immobilisation or death) are based on different endpoints.

We, therefore, evaluated the effects of a mixture of two insecticides on the aquatic communities in freshwater microcosms. This is the second paper in a series of two. The first paper focussed on the fate of the insecticides and the responses of macroinvertebrates (Cuppen et al., 2002), whereas the present paper deals with the effects on plankton and with overall ecological risk assessment.

Materials and methods

Experimental set-up

The indoor microcosms (length and width 110 cm; depth 70 cm; water depth 50 cm; sediment depth 10 cm) and the conditions in the climate room (constant temperature $19 \pm 2\,^{\circ}C$; photoperiod 14 h) have been described in detail in Part I (Cuppen et al., 2002). The test systems were chosen to mimic plankton-dominated shallow freshwater ecosystems. Two of the twelve microcosms served as controls. The other ten microcosms received a dose of a mixture of the insecticides chlorpyrifos and lindane at the start of sampling week 1. Chlorpyrifos was applied as the commercial formulation Dursban®4E, lindane as Lindafor® flo. Concentrations of the insecticides were randomly allocated to the microcosms. Six sets of two microcosms were treated with 0, 0.005, 0.01, 0.05, 0.1 and 0.5 * LC50 of the most susceptible standard test species for each of the two insecticides. The lowest LC50 values available were 1 µg/L for chlorpyrifos (48h-LC50 *Daphnia magna*; Kersting and Van Wijngaarden, 1992) and 30 µg/L for lindane (96h-LC50, *Oncorhynchus mykiss*; Mayer and Ellersieck, 1986). The concentrations of both insecticides were kept constant over a period of 28 days, the standard period of a chronic test. To compensate for losses, extra insecticide dosages were added at least three times a week. Water loss due to sampling and evaporation was replenished weekly. All microcosms were investigated over a period of fourteen weeks: a three-week pre-treatment period, a four-week treatment period and a seven-week post-treatment (restoration) period.

Zooplankton

Zooplankton was sampled from each microcosm using a Perspex corer with a length of 40 cm and a diameter of 4 cm. Several sub-samples were collected, evenly distributed over the microcosms, until a 5 L sample had been obtained. The total sample from each microcosm was concentrated by means of a 55 µm mesh net and was preserved in formol. All cladoceran, copepod or ostracod individuals were counted. Per sample, at least 400 specimens of Rotifera were identified and counted with an inverted microscope. Numbers were converted to numbers per litre. For sampling frequencies and further details, see Table 1.

The direct effects of the mixture of insecticides on the water flea *Daphnia magna*, were examined by means of a bioassay. On the first day of the treatment period, 23–25 individuals of *D. magna* ($\geq$1.5 mm) were put into a glass tube enclosure (length 40 cm, diameter 10 cm) with a bottom consisting of water-permeable pumice stone. One of these enclosures was placed in each of the microcosms, with its bottom at 35 cm below the water surface. Exchange of water

Table 1. Summary of methods used to sample the investigated endpoints in the microcosms

Endpoint	Unit	Sampling Weeks	References
Physico-chemical			
pH, DO	—, mg/L	−3, . . . 7, 11	Cuppen et al. (2000)
Alkalinity, conductivity	meq/L, μS/cm	−3, . . . 7, 11	Cuppen et al. (2000)
Inorganic N, ortho-P	mg/L, μg/L	−3, . . . 7, 11	Cuppen et al. (2000)
Zooplankton			
Species composition	numbers/mL	−3, . . . 7, 11	Cuppen et al. (1997)
Phytoplankton			
Species composition	numbers/L	−2, . . . 7, 11	Van Donk et al. (1995)
Chlorophyll-a	μg/L	−3, . . . 7, 11	Van Donk et al. (1995)
Periphyton			
Chlorophyll-a	$\mu g/cm^2$	−3, . . . 7, 11	Brock et al. (1995)

(. . . indicates that samples were taken weekly). For a detailed description of methods, see references.

was achieved by raising the enclosure daily. On day 4 after the start of the bioassay, the individuals were removed from the enclosure and counted.

Phytoplankton and periphyton

The phytoplankton community was sampled by taking several depth-integrated water samples by means of Perspex tubes. A 1-L sample was stained with lugol and concentrated after sedimentation for 6 days. The concentrated sample was preserved with formol and cell counts were made using an inverted microscope (Table 1).

Chlorophyll-a estimations were obtained by concentrating the seston of another 1-L water sample over a Whatman GF/C glass fibre filter (mesh size: 1.2 μm). Extraction of the pigments was performed using the method described by Moed and Hallegraeff (1978) (Table 1).

Periphyton was sampled from glass slides that served as artificial substrates. The slides were horizontally positioned in a frame at a fixed depth of approximately 5 cm below the water surface, and were incubated for eight weeks. On each sampling day, six glass slides were used for chlorophyll-a analysis. They were brushed visually clean and the periphyton removed was collected in tap water. The chlorophyll-a content of the water–periphyton solution was analysed as described in the phytoplankton section (Table 1).

To measure (direct) effects of the insecticide application on phytoplankton growth in the microcosms, the green alga *Scenedesmus acutus* was immobilised in alginate beads as described by Van Donk et al. (1992). This encapsulation of algal cells in a matrix prevents algal cells from being washed out and grazed by zooplankton. At the same time, they maintain their respiratory and photosynthetic activities (Van Donk et al., 1995). The initial cell volume per bead was $118 \pm 19\ \mu m^3$ (mean ± SD; $n = 5$). At the beginning of the treatment period, the beads were incubated in the microcosms in a glass petri dish (diameter 11.6 cm) closed with a cover of stainless steel wire (mesh size 0.7 mm × 0.7 mm). The petri dish was suspended 10 cm below the water surface. The bioassays were performed weekly with an incubation period of 7 days. On each sampling date, a Coulter Multisizer II (100 μm capillary; Van Donk et al., 1995) established the cell volume of ten alginate beads.

To check whether the phytoplankton community in the microcosms was nutrient-limited or not, the growth potential of algae was tested. To this end, 400 mL of water from each of the cosms was filtered over a sieve (30 μm) to remove the zooplankters. The 400 mL sample was divided into four 100 mL portions, which were stored separately in 300 mL flasks. For each cosm, one flask was untreated, while the other three received either 0.5 mg/L P or 5 mg/L N or both. After an incubation period of 12 days at 20 °C (day/night: 16 h/8 h), the biovolume of the algae in the flasks was measured with the help of a Coulter Multisizer II.

Water quality parameters

Dissolved oxygen (DO), pH, alkalinity and conductivity were measured in order to detect possible changes in community metabolism (Table 1). In addition, levels of inorganic nitrogen and orthophosphate

were determined in the water phase with the help of a Skalar 5100 autoanalyser.

Data analysis

This section only gives a concise description of the methods of data analysis applied. A full description is given in Part I of the present series of papers (Cuppen et al., 2002)

NOEC calculations at taxon level ($p \leq 0.05$) were done using the Williams test (ANOVA; Williams, 1972). NOECs were only considered valid when calculated for two consecutive sampling dates. Before univariate and multivariate analyses were performed, the abundance values of the zooplankton and phytoplankton communities were, respectively, $\mathrm{Ln}(2x+1)$ and $\mathrm{Ln}(x+1)$ transformed.

The effects of the insecticide treatment on the zooplankton and phytoplankton communities were analysed by the principal response curves (PRC) method. The PRC method is a multivariate technique specially designed for the analysis of data from microcosm experiments. More information on PRC can be found in Van den Brink and Ter Braak (1998, 1999) and in the first paper of this series (Part I, Cuppen et al., 2002). The statistical significance of treatment effects at community level was also tested, using Monte Carlo permutation tests. The significance of the PRC diagram was tested by Monte Carlo permutation of the microcosms, i.e., by permuting entire time series in the partial redundancy analysis from which PRC is derived. Monte Carlo permutation tests were also performed per sampling date, allowing the significance of the effects of a treatment regime to be tested for each sampling date.

In addition to the overall significance of the effects of treatment regime on a community, we also wanted to know *which* treatments differed significantly from the controls, so as to infer the no observed effect concentration (NOEC) at the community level. The NOEC calculations were done by applying the Williams test to the sample scores of the first principal component of each sampling date in turn (For rationale, see Van den Brink et al., 1996).

The results of the bioassays performed with *Daphnia magna* and *Scenedesmus acutus* were analysed with the Williams test (ANOVA; Williams 1972). The results of the experiment on the growth potential of algae were also analysed, using a two-way ANOVA test.

Results

Zooplankton

A total of 28 different zooplankton taxa were identified. The Cladocera included 12 taxa, as did Rotifera. Three taxa belonging to the Copepoda were identified, whereas Ostracoda were not identified to a lower level. Copepod nauplii were the most abundant taxon in the control microcosms, followed by Ostracoda sp., *Daphnia galeata/magna* (mainly *D. galeata*), Cyclopoidea sp., *Bosmina longirostris*, *Simocephalus vetulus* and *Keratella quadrata.*

The PRC diagram indicates treatment effects at the three highest treatment levels, with an increasing effect at increasing dosage (Fig. 1). For the two highest treatment levels, there were differences in zooplankton community structure compared to controls for both the treatment and the post-treatment period, whereas the 0.05 * LC50 treatment level only showed differences for the treatment period. This is confirmed by the results of the Monte Carlo permutation tests and Williams tests on the first principal component of a principal component analysis (PCA). These tests show a significant difference in species composition between the control community and the communities at the two highest treatment levels from the start of the treatment onwards. The 0.05 * LC50 treatment community was only significantly different from those in the controls during weeks 2–4.

Taxa belonging to the Cladocera, Copepoda and Ostracoda have a positive weight in the PRC diagram, which means they decreased in abundance at the higher treatment levels (Fig. 1). Several Rotifera taxa show a negative affinity, which means an increase in abundance (Fig. 1). The actual abundance values of the taxa with a relatively high affinity in the diagram are given in Figs 2 and 3.

At the species level, the lowest NOEC of the 0.005 * LC50 treatment level was calculated for the cladoceran *Bosmina longirostris* (Table 2). For several other crustacean taxa a NOEC, based on decreased abundance values in treated systems, of the 0.01 or 0.05 * LC50 treatment level was calculated (Fig. 2). Three rotifer taxa showed increased abundance at the two highest treatment levels (Table 2; Fig. 3).

The bioassay performed with *Daphnia magna* resulted in 100% mortality at the highest treatment level and partial mortality in the other microcosms

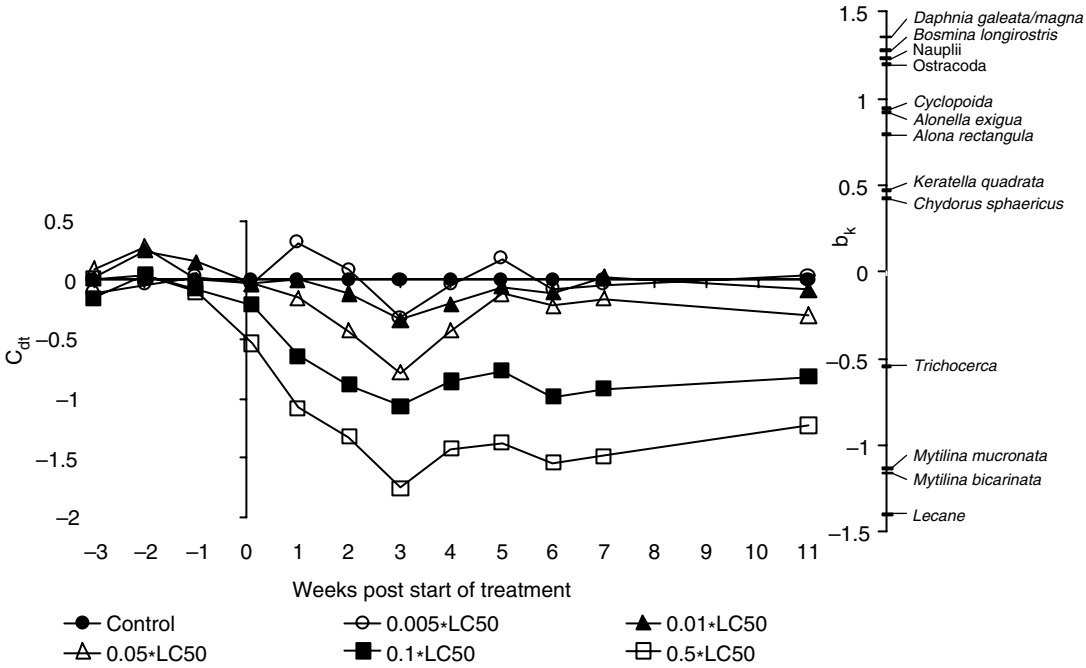

Figure 1. PRCs resulting from the analysis of the zooplankton data set, indicating the effects of the insecticide mixture on the zooplankton community. Of all variance, 33% could be attributed to sampling date; this is displayed on the horizontal axis. Forty-six percent of all variance could be attributed to treatment level. Of this variance, 56% is displayed on the vertical axis. The lines represent the course of the treatment levels in time. The species weight (b_k) can be interpreted as the affinity of the taxon with the PRCs. Taxa with a species weight between 0.25 and −0.25 are not shown. A Monte Carlo permutation test indicated that a significant part of the variance explained by treatment level is displayed in the diagram ($p < 0.01$). The treatment regime had a significant influence on the zooplankton community from week 0.1 onwards. The lowest $NOEC_{community}$ reported for at least two consecutive sampling dates was the 0.01 * LC50 treatment level of each of the insecticides in the mixture.

and the controls (up to 17%, Table 3; NOEC = 0.1 * LC50 treatment level).

Phytoplankton and periphyton

The PRC diagram of the phytoplankton is given in Fig. 4. The overall permutation test indicated that the PRC diagram only displays a moderately significant part of the treatment variance ($p = 0.10$), indicating an absence of large treatment-related effects, or the presence of several sub-dominant response patterns instead of one dominant one. The pre-treatment deviations of all treatment levels from the controls were very small. In week 2, all treatment levels showed a relatively large deviation from the controls. After week 2, a small and brief deviation was seen for the 0.1 * LC50 treatment level and a larger and prolonged deviation for the highest treatment level. The treatment regime had a significant influence on the phytoplankton community from week 4 onwards. Only the differences between the highest treatment level and the controls were significant ($p \leq 0.05$, Fig. 4).

Most of the taxa with a positive weight in the diagram belonged to the Chlorophyceae, while the two taxa with the highest scores were Dynophyta (Fig. 4). Most taxa with a negative weight were Cryptophyceae. *Oscillatoria splendida* (Cyanophyceae) had the highest negative score.

Cryptophyceae showed a significant decrease in the treated systems compared to the controls (Table 4). The lowest NOEC of the 0.005 * LC50 treatment level was calculated for the diatom *Nitzschia* sp., but this taxon had very low abundance values in the controls (between 0 and 24 #/mL) and its development in time was very erratic (results not shown). The other taxa of Table 4 showed a more stable course in time in the controls, with abundance values between 10 and

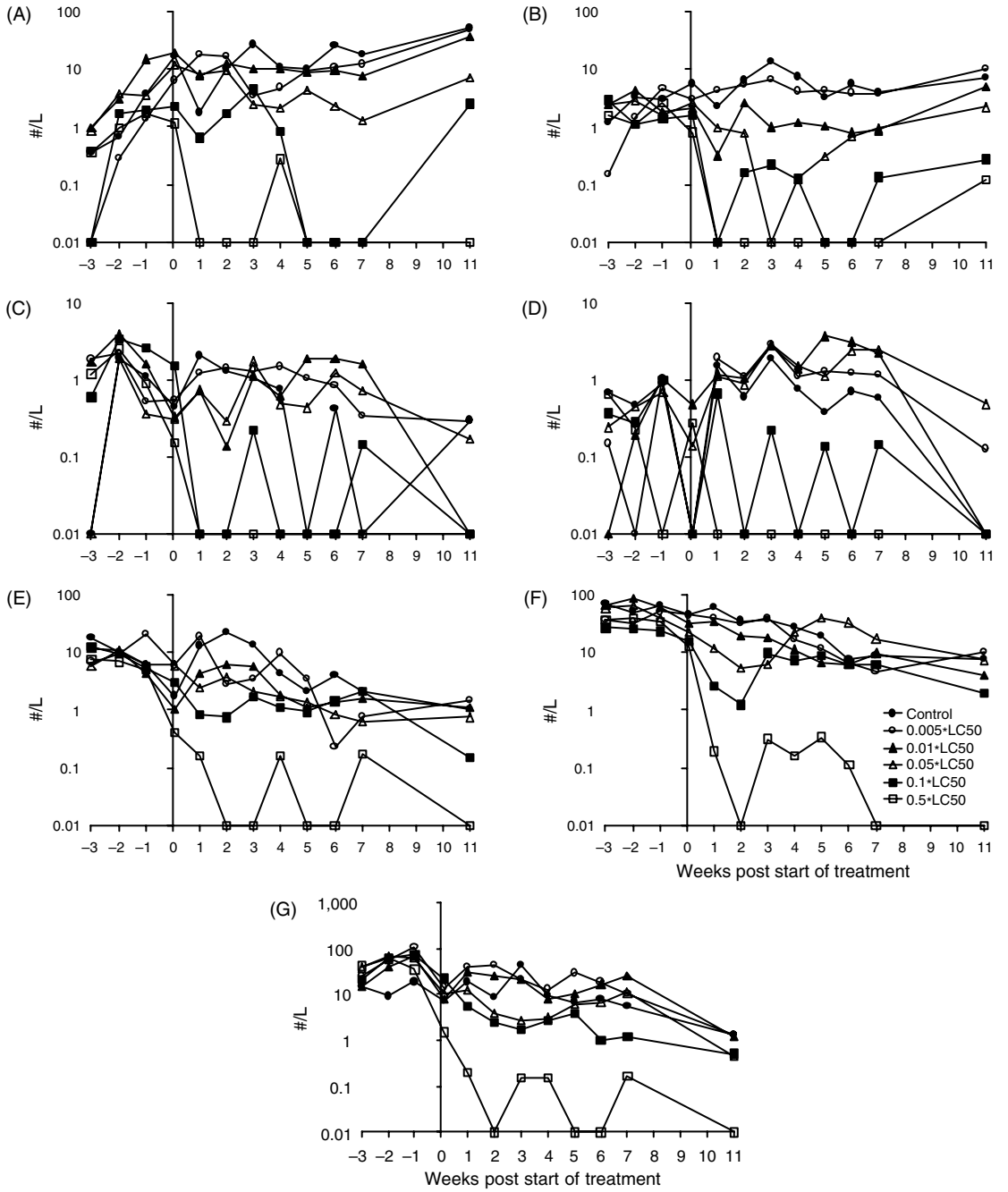

Figure 2. Changes in numbers of seven zooplankton taxa belonging to the Crustacea, expressed as the geometric means of the numbers counted per treatment level of *Daphnia galeata/magna* (A), *Bosmina longirostris* (B), *Alona rectangula* (C), *Alonella exigua* (D), Cyclopoidea sp. (E), nauplii (F) and Ostracoda sp. (G). For NOECs, see Table 2.

100 #/mL. A significant increase at the highest treatment level was found for two taxa, one belonging to the Dynophyta (*Gymnodinium* sp.), the other to the Chlorophyceae (Table 4).

Table 4 and Fig. 5 summarise the treatment effects on six different phytoplankton groups. The Cryptophyceae showed a significant decrease in numbers at the highest treatment level, especially

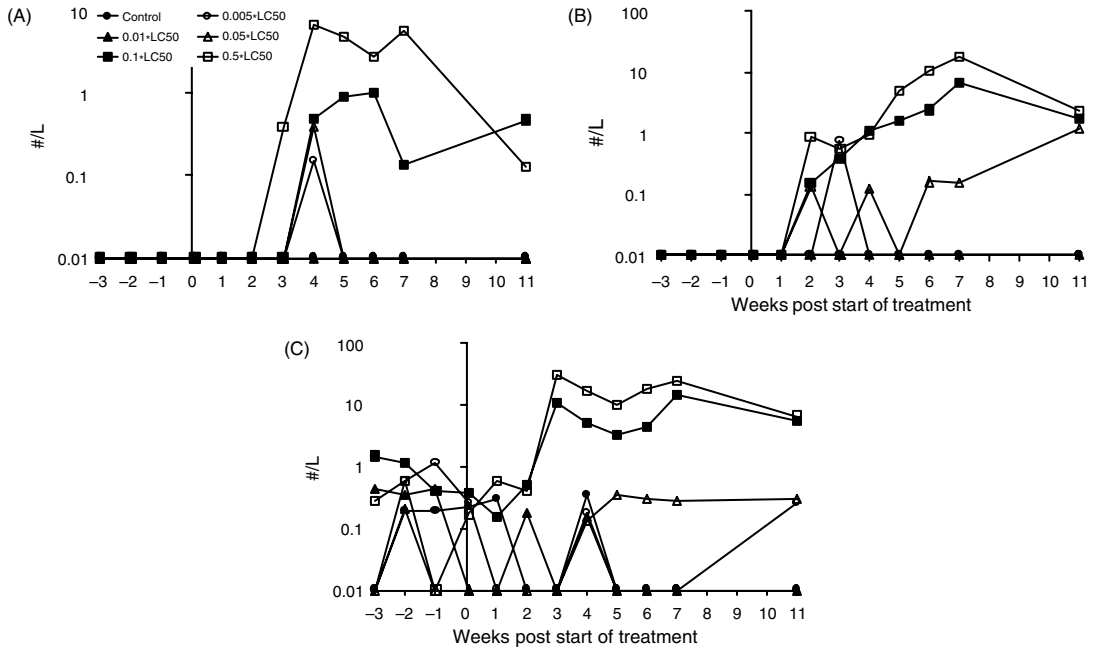

Figure 3. Changes in numbers of three zooplankton taxa belonging to the Rotifera, expressed as the geometric means of the numbers counted per treatment level of *Mytilina mucronata* (A), *Mytilina bicarinata* (B) and *Lecane* sp. (C). For NOECs, see Table 2.

Table 2. NOECs, plus direction of effect, as calculated by the Williams test ($p \leq 0.05$) from the abundance of zooplankton for at least two consecutive sampling dates

Taxon	NOEC
Cladocera	
Daphnia galeata/magna	0.01 * LC50 treatment level ↓
Bosmina longirostris	0.005 * LC50 treatment level ↓
Alona rectangula	0.05 * LC50 treatment level ↓
Copepoda	
Cyclopoidea sp.	0.05 * LC50 treatment level ↓
Nauplii	0.01 * LC50 treatment level ↓
Ostracoda	
Ostracoda sp.	0.05 * LC50 treatment level ↓
Rotifera	
Mytilina mucronata	0.1 * LC50 treatment level ↑
Mytilina bicarinata	0.05 * LC50 treatment level ↑
Lecane sp.	0.05 * LC50 treatment level ↑

during the post-treatment period (Table 4, Fig. 5A). Both the Chlorophyceae and the Dynophyta showed a significant increase in numbers at this treatment level during the post-treatment period (Table 4, Fig. 5B and C). The Cyanophyceae showed a non-significant increase in numbers at the highest treatment level during the treatment period (Table 4, Fig. 5E). The two other groups did not show treatment-related effects (Table 4, Figs 5D and F).

Table 3. Mortality (%) of *Daphnia magna* in bioassay on day 4, per replicate

Treatment level	Replicate A	Replicate B
Control	4	17
0.005 * LC50 treatment level	4	12
0.01 * LC50 treatment level	8	16
0.05 * LC50 treatment level	0	8
0.1 * LC50 treatment level	8	16
0.5 * LC50 treatment level	100	100

The NOEC is the 0.1 * LC50 treatment level.

The chlorophyll-a contents of the water layer and the glass slides were very low. The highest measured chlorophyll-a values were 19 µg/L for the water layer and 0.9 µg/cm^2 for the glass slides (Fig. 6). Unlike the phytoplankton community structure, no effects of the treatment could be demonstrated on the chlorophyll-a content of the phytoplankton. Only a non-significant increase was found for the treatment period, but in this period elevated values were also found in the control microcosms (Fig. 6A). The periphyton showed no consistent treatment effects either. Only in week 1 was an increased chlorophyll-a content found for the highest treatment level (Fig. 6B).

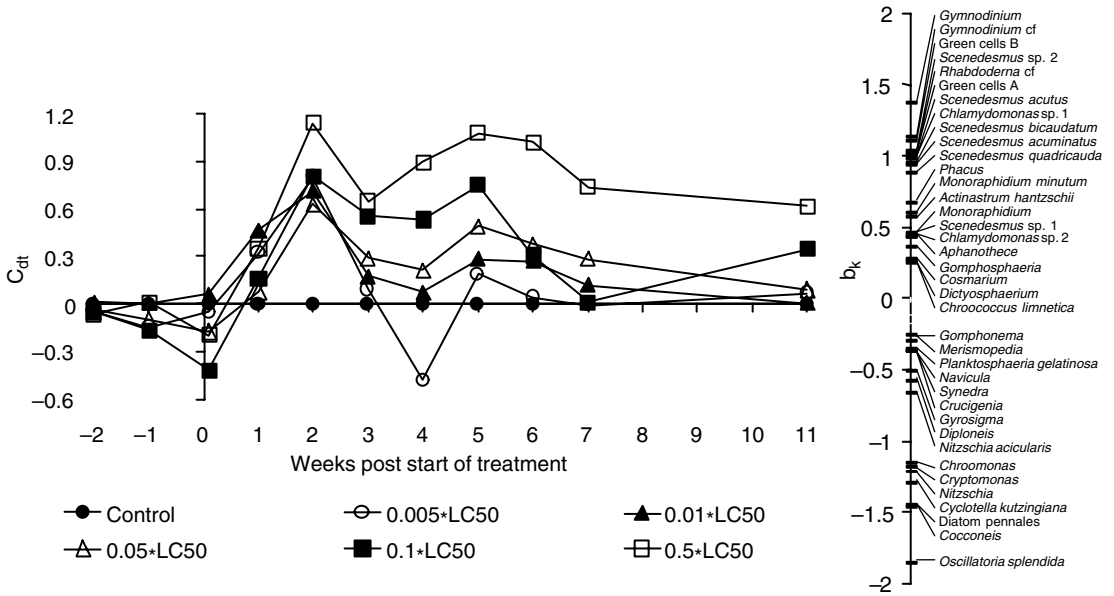

Figure 4. PRCs resulting from the analysis of the phytoplankton data set, indicating the effects of the insecticide mixture on the phytoplankton community. Of all variance, 32% could be attributed to sampling date; this is displayed on the horizontal axis. Thirty-four percent of all variance could be attributed to treatment. Of this variance, only 21% is displayed on the vertical axis. The lines represent the course of the treatment levels in time. The species weight (b_k) can be interpreted as the affinity of the taxon with the PRCs. Taxa with a species weight between 0.25 and −0.25 are not shown. A Monte Carlo permutation test indicated that the diagram only displays a moderately significant amount of the variance explained by treatment ($p = 0.10$). The treatment regime had a significant influence on the phytoplankton community from week 4 onwards. The lowest $NOEC_{community}$ reported for at least two consecutive sampling dates was the 0.1 * LC50 treatment level of each of the insecticides in the mixture.

Table 4. NOECs plus direction of effect, as calculated by the Williams test ($p \leq 0.05$) from the abundance of phytoplankton for at least two consecutive sampling dates at taxon level and for different groups

	NOEC
Taxon	
Nitzschia sp.	0.005 * LC50 treatment level ↓
Diatomeae pennales	0.05 * LC50 treatment level ↓
Cocconeis sp.	0.05 * LC50 treatment level ↓
Oscillatoria splendida	0.01 * LC50 treatment level ↓
Chroomonas sp.	0.1 * LC50 treatment level ↓
Gymnodinium sp.	0.1 * LC50 treatment level ↑
Chlorophyceae sp. 1	0.1 * LC50 treatment level ↑
Group	
Cryptophyceae	0.1 * LC50 treatment level ↓
Chlorophyceae	0.1 * LC50 treatment level ↑
Dynophyta	0.1 * LC50 treatment level ↑
Diatomeae	>0.5 * LC50 treatment level
Cyanophyceae	>0.5 * LC50 treatment level
Other	>0.5 * LC50 treatment level

The bioassay performed with the green alga *Scenedesmus acutus* resulted in a non-significant decrease in biovolume on the last two sampling dates for the highest treatment level (Fig. 7).

The algal growth potential experiment showed that the algal communities in the microcosms were both nitrogen- and phosphorus-limited. The biovolume was 18-fold higher in the flasks in which both nutrients had been added, compared to a control flask. The addition of nitrogen or phosphorous alone had no significant influence on the biovolume measured on day 12 (Fig. 8).

Water quality parameters

The parameters of the DO-pH-alkalinity-conductivity syndrome (Brock et al., 1993) indicated increased primary production, especially at the highest treatment level (Table 5). Figure. 9, however, shows that the magnitude of these differences was very small. Like pH, DO was slightly elevated at the highest treatment

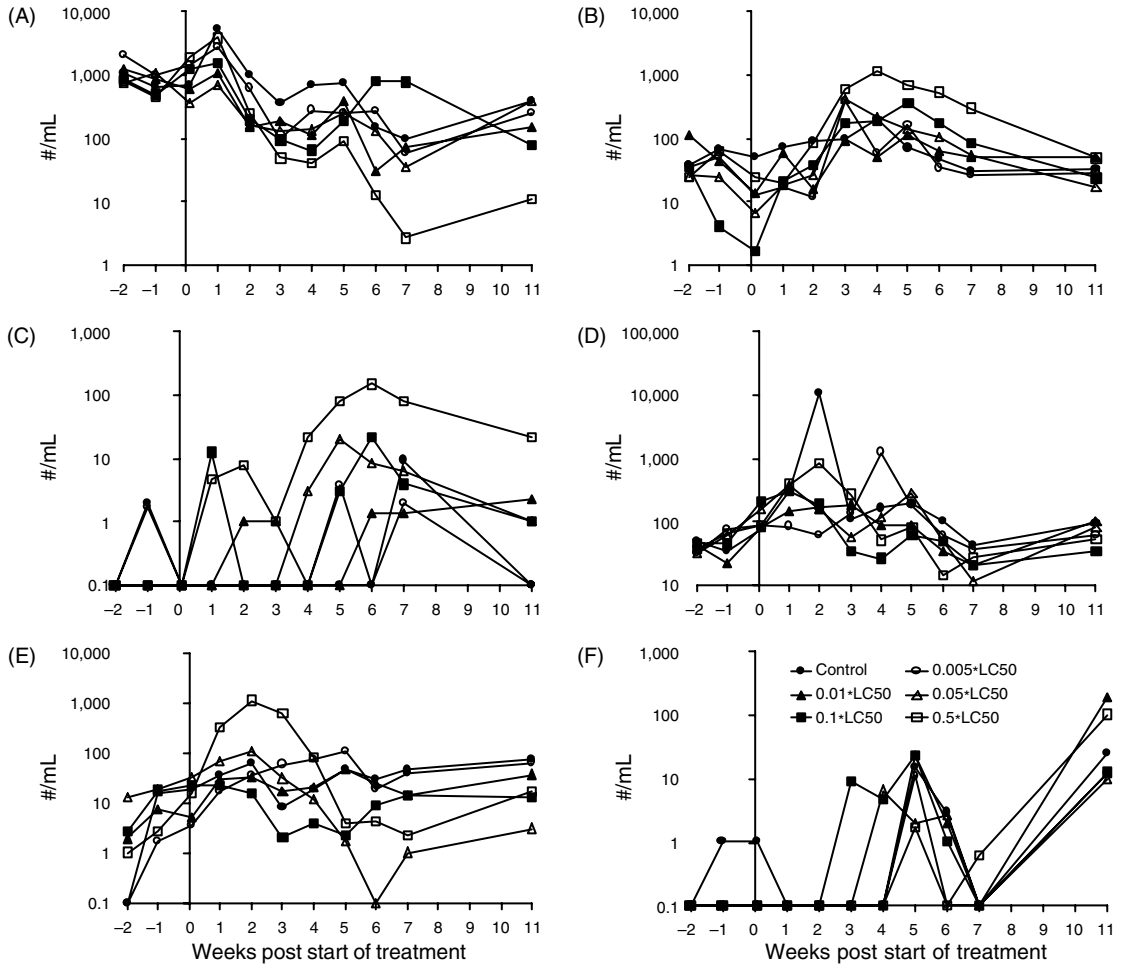

Figure 5. Changes in numbers of six phytoplankton groups, expressed as the geometric means of the numbers counted per treatment level of Cryptophyceae (A), Chlorophyceae (B), Dynophyta (C), Diatomeae (D), Cyanophyceae (E) and other taxa (F). For NOECs, see Table 5.

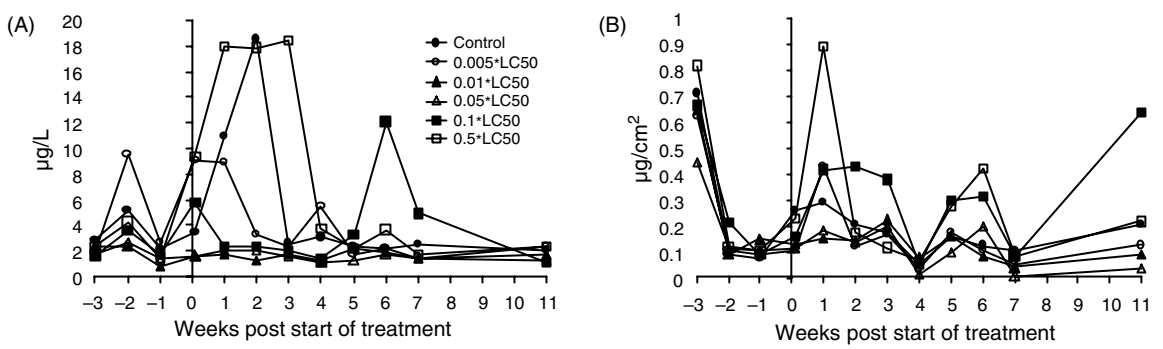

Figure 6. Changes in chlorophyll-a content of phytoplankton (A) and periphyton (B). For the chlorophyll-a of the periphyton, a NOEC of 0.1 * LC50 treatment level (increase) was calculated for week 1.

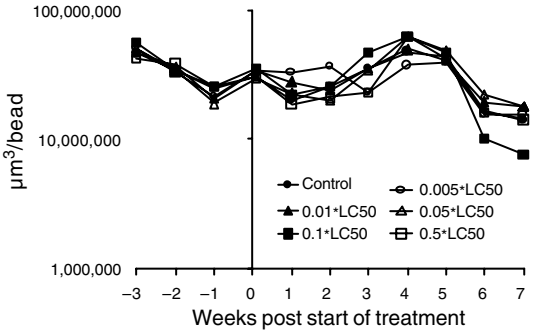

Figure 7. Results of the bioassays performed weekly with the algae *Scenedesmus acutus*. No statistical differences were found between the treatments ($p > 0.05$, Williams test).

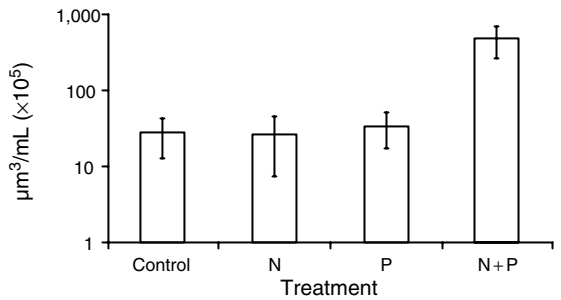

Figure 8. Results of the algal growth potential experiment. The volumes of the algae for the N + P treatment was significantly higher than that for all other treatments ($p < 0.001$; ANOVA). No other significant differences between treatments were found ($p > 0.05$).

Table 5. NOECs (in LC50 units of the most sensitive test species for each insecticide), plus direction of effect, as calculated by the Williams test ($p \leq 0.05$) from levels of physico-chemical parameters for at least two consecutive sampling dates

Endpoint	NOEC
Alkalinity	0.01 * LC50 treatment level ↓
Inorganic N	0.01 * LC50 treatment level ↓
Conductivity	0.05 * LC50 treatment level ↓
pH	0.05 * LC50 treatment level ↑
DO	0.1 * LC50 treatment level ↑
Ortho-P	>0.5 * LC50 treatment level

level in weeks 1 and 2. Around week 6, these parameters showed higher values compared to the controls at the two highest treatment levels (Figs 9A and B). Alkalinity values were generally lower at the highest treatment levels after the start of the treatment. During the post-treatment period, alkalinity was significant lower at the three highest treatment levels compared to the controls (Williams test, Table 5; Fig. 9C). At the end of the experiment, conductivity was reduced at the 0.01 * LC50 treatment level and higher, but the Williams test showed this difference to be significant only at the two highest treatment levels (Table 5; Fig. 9D).

Compared to the controls, the levels of inorganic nitrogen were significantly lower at the three highest treatment levels (Table 5). Figure 9E also shows that values of inorganic nitrogen were generally lower in all treated systems compared to the untreated ones. No effects of the insecticide applications on the orthophosphate levels were recorded (Table 5; Fig. 9F).

Discussion

Measured endpoints

Zooplankton. As expected from their mode of action, the application of the two insecticides resulted in a decrease in those zooplankton taxa belonging to the arthropods (Fig. 1). A major and long-term decrease in numbers was found at the two highest treatment levels, with a smaller, short-term decrease at the 0.05 * LC50 treatment level. Only for the cladoceran *Bosmina longirostris* were significant treatment effects recorded at the 0.01 * LC50 treatment level (Table 2).

The largest adverse effects were reported for Cladocera, followed by nauplii, Ostracoda and adult Copepoda (Fig. 2). Several taxa belonging to the Rotifera increased in abundance due to the treatment regime (Fig. 3). Table 6 lists the lowest acute EC50 values for the four groups, as found in the literature and the AQUIRE database (AQUIRE, 1998). These values show that the adverse effects on Cladocera are most likely to have resulted from the chlorpyrifos treatment and that the adverse effects on Copepoda and Ostracoda can be explained by the lindane treatment. This is in accordance with observations in microcosm and mesocosm experiments performed with the individual substances. According to the literature, the order of susceptibility of the zooplankton groups for chlorpyrifos is Cladocera > nauplii > Copepoda and Ostracoda > Rotifera (Van den Brink et al., 1996; Brock et al., 1992; Stay et al., 1989; Biever et al., 1994). Peither et al. (1996) reported an order of lindane susceptibility of

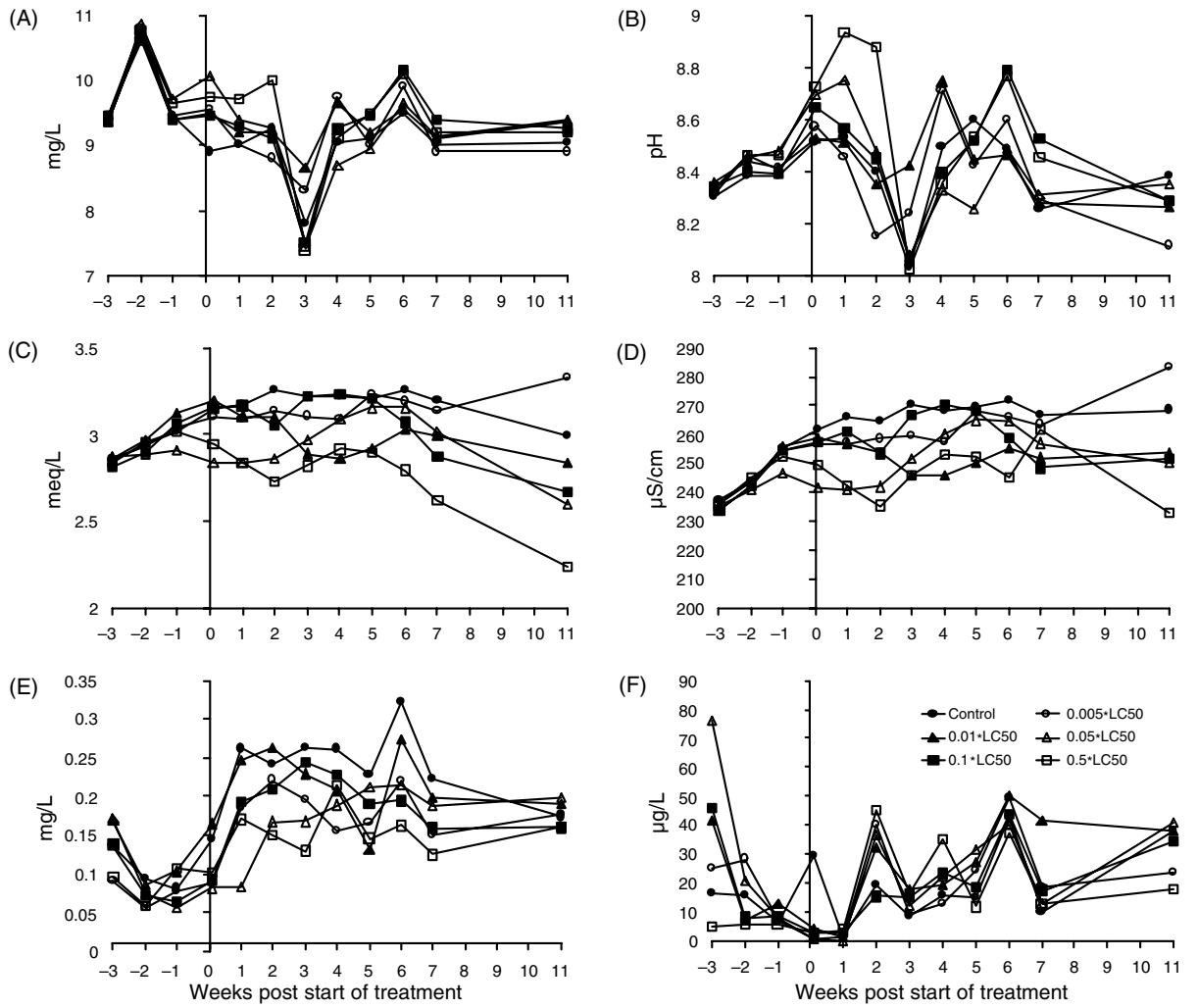

Figure 9. Changes in several physico-chemical parameters, expressed as the means of the parameter values per treatment level of DO (A), pH (B), alkalinity (C), conductivity (D), inorganic nitrogen (E) and orthophosphate (F). For NOECs, see Table 4.

Table 6. Lowest acute EC50 values (µg/L) found in the literature or AQUIRE for four zooplankton groups

Group	EC50 chlorpyrifos	EC50 lindane
Cladocera	0.08–0.13	460
Copepoda	1.4[a]	17[a]
Ostracoda	10	3.2
Rotifera	11900	—

AQUIRE data were used if no literature data were available (AQUIRE, 1998; Barron and Woodburn, 1995; Mayer and Ellesieck, 1986). The highest treatment level in the microcosms was 0.5 µg/L chlorpyrifos and 15 µg/L lindane—No data available.
[a]AQUIRE.

Copepoda > Cladocera ≥ Rotifera. No obvious synergistic or antagonistic effects of chlorpyrifos or lindane were found for the zooplankton.

The most sensitive cladoceran in our study was the small species *Bosmina longirostris*. These water fleas were adversely affected at concentration levels of 0.01–0.5 µg/L chlorpyrifos (Table 2). The chronic effects observed in the microcosms (at treatment levels of 0.01 or 0.05 µg/L and higher) were as expected from the lowest acute toxicity value of chlorpyrifos available for Cladocera. The effects of the mixture on cladocerans can thus be explained from their susceptibility to chlorpyrifos alone.

Our finding that the small cladoceran *B. longirostris* reacted more sensitively to the insecticide application than the larger ones is in contrast with the findings of Hanazato (1998), who reported increasing sensitivity of cladocerans to the insecticides carbaryl and fenthion with increasing body size. Our results indicate that this relation is not a general property of insecticides and cannot be transferred to other insecticides.

In the present study, the LOEC for nauplii was the 0.05 * LC50 treatment level. Assuming that lindane was the insecticide causing the adverse effects, this is equivalent to 1.5 μg/L lindane. Peither et al. (1996) reported small effects on nauplii after repeated administration of 2 μg/L lindane and large adverse effects at 6 μg/L and higher. This is in perfect accordance with the observed effects in the microcosms, which showed small and temporal effects at the 1.5 and 3 μg/L treatment levels and large, prolonged effects at the 15 μg/L treatment level.

Peither et al. (1996) also reported effects on adult Copepoda, but explained their decrease from the diminished nauplii abundance. In our case, however, effects on adult Copepoda were observed immediately after insecticide application, suggesting acute effects (Fig. 2F). Laboratory toxicity values confirm these effects (acute LC50 was approximately a factor of 10 higher than chronic LOEC in microcosms).

The increase in Rotifera is likely to be a result of the decreased competition with the Cladocera. Normally, Cladocera are superior to Rotifera in competition for food (Hanazato, 1998), and increased Rotifera abundance after elimination of Cladocera by insecticides is indeed a generally observed phenomenon (Brock et al., 2000).

The calculated NOEC of the bioassay with *Daphnia magna* was the 0.1 * LC50 treatment level (Table 3). This concentration is equivalent to 0.1 μg/L chlorpyrifos and 3 μg/L lindane. The 48h-LC50 values of *Daphnia magna* as determined in semi-static single species tests for chlorpyrifos and lindane are 1 μg/L (Kersting and Van Wijngaarden, 1992) and 460 μg/L (Mayer and Ellersieck, 1986), respectively. These LC50 values show that adverse effects are likely to be a result of chlorpyrifos exposure alone. Kersting and Van Wijngaarden (1992) reported a 48h-NOEC of 0.1 μg/L, which is in perfect accordance with the bioassay results. In view of its laboratory LC50 for 2 days of 1 μg/L, however, complete mortality was not expected at the highest (0.5 μg/L) chlorpyrifos treatment level. Recent laboratory studies indicate that in some cases, 50% mortality can be expected within 2 days at this concentration (48h-LC50 = 0.6 μg/L; Moore et al., 1998), which indicates that the complete mortality after 4 days is not a result of strong synergistic effects of the insecticides. In the first part of this series, Cuppen et al. (2002) also argued that the bioavailability of both chemicals was comparable to those in laboratory tests. Cuppen et al. (2002) found that the toxicity of the individual insecticides for *Gammarus* and Asellidae as determined in laboratory tests was comparable to that of the mixture observed in the microcosms.

Water quality parameters and algae. The water quality parameters indicated a small increase in photosynthetic activity after the start of the insecticide treatment for the two highest treatment levels (Fig. 9A and B). This is confirmed by the chlorophyll-a measurements of phytoplankton and periphyton, which showed a clear, though non-significant, increase. The most obvious explanation for this increase in algae biomass is reduced grazing pressure as a result of the decrease in Cladocera, especially at the two highest treatment levels (see Fig. 2). The nutrient limitations may have suppressed this effect at the intermediate treatment levels.

The highest treatment level resulted in a long-term change in the species composition of the phytoplankton community, the second highest in short-term and non-significant changes (Fig. 4). At the taxon level, most effects were found for the two highest treatment levels (Table 4). These changes in taxon composition are likely to be a result of the decreased grazing pressure. That competition for nutrients is an important factor can be deduced from the algal growth potential experiment, which showed that the phytoplankton community was both N- and P-limited. However, why certain groups decreased in numbers while others increased remains largely unexplained.

Ecological effect chain

The ecological effect chain observed in the microcosms that received the highest insecticide doses is summarised in Fig. 10. Many invertebrates were killed as a direct effect of the administration of the insecticides (see also Cuppen et al., 2002). This resulted in a decreased decomposition of *Populus* leaves in litter bags allowing access to

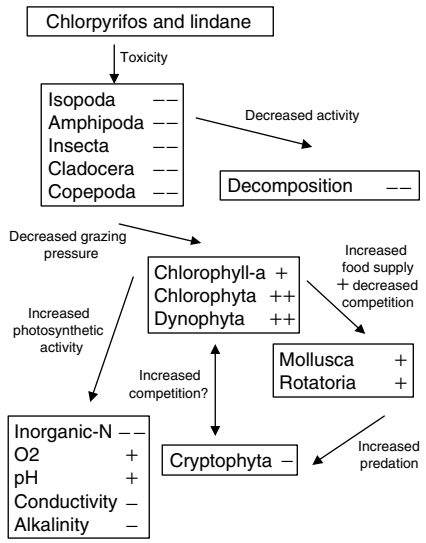

Figure 10. Schematic overview of the nature and route of effects of chronic high doses of chlorpyrifos and lindane on the ecosystem structure of plankton dominated microcosms (+, increase; −, decrease).

macroinvertebrates. The decrease in grazing pressure resulted in increased chlorophyll-a levels of the phytoplankton and periphyton and an altered species composition of the phytoplankton community. The increase in algal chlorophyll-a resulted in increased pH and DO levels and a decrease in the conductivity and alkalinity. These responses can be explained from increased photosynthetic activity (and hence increased glucose synthesis and oxygen production). The general increase in algal chlorophyll-a led to an increased biomass of some Mollusca and Rotifera taxa, which suppressed the increase in algae at the end of the experiment (Fig. 6). This ecological effect chain has often been reported for insecticides (Brock et al., 2000).

Overall risk assessment

The experiment described in the present paper only gives insight into the effects of the mixture of the insecticides, not the effects of the individual substances. It was, therefore, important for the interpretation of the results that a proper overview of the toxicological profile of the two test substances was available. Such an overview was found in the AQUIRE database (AQUIRE, 1998). Acute EC50 values were used because they were available for far more species than chronic NOEC values. All EC50 values with a test duration between 2 and 4 days were extracted from the database and imported into a spreadsheet. If more than one EC50 value was available for a species, the geometric mean was used. All available acute EC50s were used to calculate the species sensitivity percentile (e.g. Van Straalen and Denneman, 1989). To this end, the EC50s were plotted cumulatively against themselves, and scaled as percentages. This allowed 5th and 50th percentiles (i.e., with 5% or 50% of all E50 values being lower than this value) to be calculated, using a general logistic model described in Aldenberg and Slob (1993). These values were calculated per test substance for four different groups; arthropods, other invertebrates, fish and algae.

Figures 11A and B shows the cumulative distribution of the acute EC50s of the four groups of water organisms for the two test substances. For both chemicals, arthropods were found to be the most sensitive group, followed by fish, algae and other invertebrates. The sensitivity distribution of the algae was very narrow for both chemicals. This was expected, since it can be assumed that both insecticides will have a specific mode of action towards arthropods and fish and will act merely as an anaesthetic on algae and invertebrates other than arthropods. For the algae, this is confirmed by the 96h-EC50 of *Selenastrum capricornutum* for the anaesthetic mode of action of both insecticides, calculated from a QSAR formulated by Calamari et al. (1983). This QSAR yielded 96h-EC50 values of 120 and 2,950 µg/L for chlorpyrifos and lindane, respectively (Log (p) values taken from De Bruin et al. (1989). This is in the range of the EC50 values presented by the AQUIRE database for other algae (Fig. 11).

The calculated 5th and 50th percentiles for the species sensitivity are given in Table 7. The species sensitivity distribution (SSD) curves indicate possible effects of the three highest treatment levels on arthropods and, if they were included in the experiment, possible effects of the highest treatment level on fish. The highest chlorpyrifos dose (0.5 µg/L) corresponds with the 50th percentile of Arthropoda (0.71 µg/L; Table 7). The same was true for lindane (15 and 18 µg/L, respectively; Table 7).

As indicated by Fig. 11, only Arthropoda suffered direct effects in the microcosms. No direct effects on algae or non-arthropod invertebrates could be demonstrated, as had been expected, since their 5th

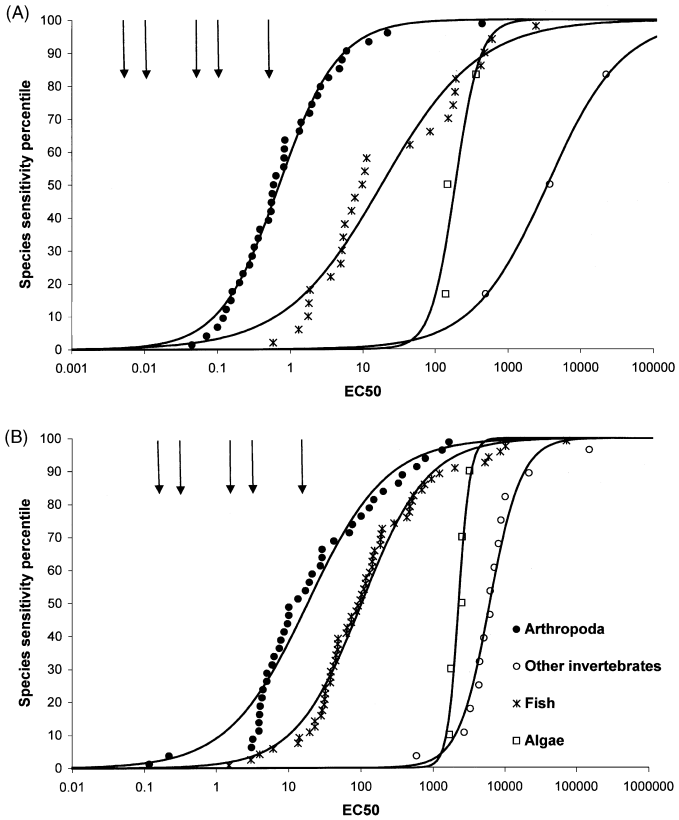

Figure 11. SSD curves for acute toxicity (individual points are EC50 values) for the insecticides chlorpyrifos (A) and lindane (B) to four groups of water organisms. Results of the logistic regression on these data are represented by lines. Data were obtained from AQUIRE (AQUIRE, 1998). Arrows indicate the concentrations tested.

Table 7. Fifth and 50th species sensitivity percentiles (based on acute EC50s; in μg/L with confidence intervals) of arthropods, other invertebrates, fish and algae for the insecticides chlorpyrifos and lindane

Group	5th percentile	50th percentile
Chlorpyrifos		
Arthropods	0.045 (0.036–0.055)	0.71 (0.65–0.76)
Fish	0.20 (0.14–0.30)	18 (16–21)
Algae	60 (45–82)	189 (171–210)
Other invertebrates	106 (43–258)	3509 (2529–4869)
Lindane		
Arthropods	0.45 (0.34–0.59)	18 (17–20)
Fish	4.5 (3.7–5.4)	102 (95–109)
Algae	1323 (1188–1474)	2272 (2184–2363)
Other invertebrates	1396 (1140–1710)	6151 (5765–6562)

percentile of the individual compounds was not exceeded by the highest concentration of the mixture in the microcosms (Table 7). Direct effects on Arthropoda in the microcosms due to chronic exposure became apparent at mixture concentrations below the 5th percentile of Arthropoda, based on the results of acute single species tests performed with individual substances (Table 7). This indicates that a lower percentile, based on acute, single compound, EC50 values, must be used to protect aquatic communities against chronic exposure to a mixture. Brock et al. (2000) suggested for microcosm and mesocosm experiments to use a factor of 10 for the extrapolation of ecological threshold levels based on an acute exposure to one for a chronic exposure. Van den Brink et al. (2002) suggest the same factor when acute EC50s are used to construct a SSD protective against chronic exposure. In this case, an extra factor

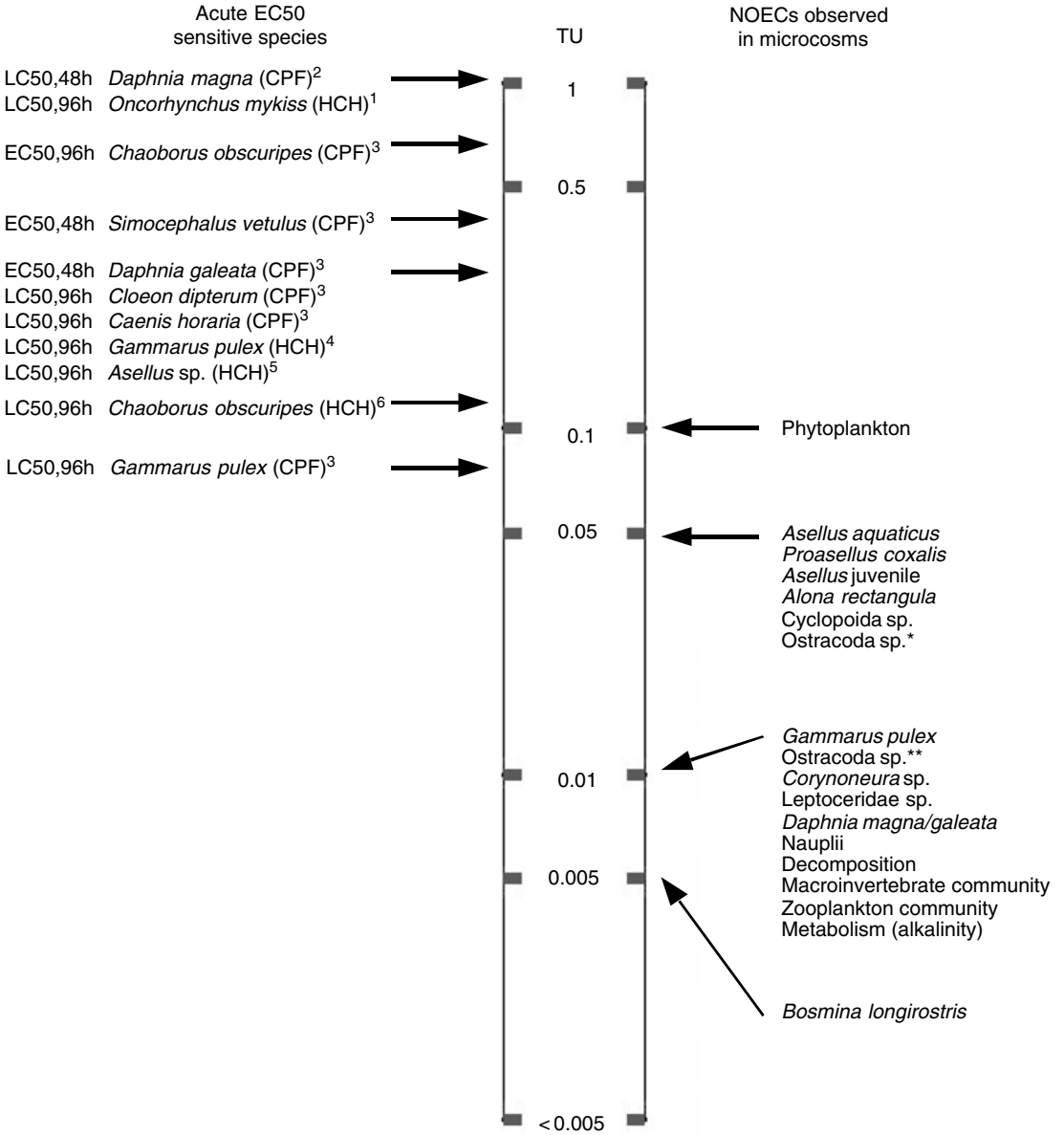

Figure 12. Summary of acute laboratory test results (in LC50 units of the most sensitive test species for each insecticide) with chlorpyrifos (CPF) and lindane (HCH) and direct effects observed in the microcosms ([1]Mayer and Ellersieck, 1986; [2]Kersting and Van Wijngaarden, 1992; [3]Van Wijngaarden et al., 1993; [4]Stephenson, 1983; [5]Johnson and Finley, 1980; [6]Bluzat and Seuge, 1979; *from zooplankton samples, **sampled with macroinvertebrates).

of 10 applied to the 5th percentile, based on acute EC50 values, would have yielded concentrations of 0.0045 µg/L chlorpyrifos and 0.045 µg/L lindane, both lower than the concentrations corresponding with the 0.005 * LC50 treatment level of the mixture (the NOEC of the most sensitive taxon in our microcosm experiment). This validation is in line with the weight of evidence provided by Versteeg et al. (1999).

The chronic treatment with the two insecticides, as applied to the microcosms, resulted in effects at the

level of the UP standards (0.01 * LC50 treatment level) for only one taxon, the water flea *Bosmina longirostris* (Fig. 12). At the treatment level just above the standard (0.05 * LC50 treatment level), considerable adverse effects on many invertebrates were found, at both population and community level. Figure 11 also shows that concentrations at the level of the standards (0.01 * EC50 of *Daphnia* or fish, equivalent to 0.01 µg/L for chlorpyrifos and 0.3 µg/L for lindane) should not be expected to yield acute effects on many taxa, but only effects on a few populations. The first-tier risk assessment procedure, as set by the UP, thus ensures protection at community level, but not always at species level, upon chronic exposure to a mixture of insecticides.

The overall $NOEC_{ecosystem}$ of 0.01 * LC50 treatment level for the mixture is equivalent to 0.01 µg/L chlorpyrifos and 0.3 µg/L lindane. We are aware of two experiments using chronic application of chlorpyrifos which allow the determination of a $NOEC_{ecosystem}$. Both experiments, that by Van den Brink et al. (1995) in stagnant water and that by Ward et al. (1995) in running water, gave a $NOEC_{ecosystem}$ of < 0.1 µg/L. They both found effects on arthropods caused by a chronic (between 21 and 56 days) concentration of 0.1 µg/L. Unfortunately, no studies evaluating lower concentrations are known to us, but the data available do not undermine the $NOEC_{ecosystem}$ of 0.01 µg/L found for chlorpyrifos in our study. For lindane, Peither et al. (1996) reported effects on stagnant experimental ecosystems at chronic concentrations of 2 µg/L and higher. Unfortunately, this was also the lowest concentration tested so no $NOEC_{ecosystem}$ could be determined. Mitchell et al. (1993) determined a $NOEC_{ecosystem}$ of 0.2 µg/L for running waters, with a $LOEC_{ecosystem}$ of 0.7 µg/L. All of these values supports our $NOEC_{ecosystem}$ of 0.01 * LC50, which is equivalent to 0.3 µg/L lindane. The $NOEC_{ecosystem}$ values obtained from other studies all refer to the effects of individual insecticides, and hence do not indicate synergistic or antagonistic effects of the mixture in microcosms at the ecosystem level.

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