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Effects of Chlorpyrifos, Carbendazim, and Linuron on the Ecology of a Small Indoor Aquatic Microcosm

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Abstract. To validate the use of small indoor microcosms for the risk assessment of pesticides, the fate and effects of chlorpyrifos, carbendazim, and linuron were studied in 8.5–liter indoor freshwater microcosms. Functional and structural responses to selected concentrations were evaluated and compared with responses observed in larger-scale model ecosystem studies. Overall, the microcosms adequately displayed the chain of effects resulting from the application, although they did not always predict the exact fate and responses that were observed in larger semifield studies. Because closed systems were used that did not contain sediment and macrophytes, pesticides were relatively persistent in the present study. Consequently, calculated toxicity values were generally more comparable with those reported in studies with long- than with short-term exposure. Carbendazim had a higher overall no-observed-effect concentration (NOEC) compared with experiments performed in larger systems because macroinvertebrate taxa, the most sensitive species group to this fungicide, were not abundant or diverse. Future refinements to the test system could include the addition of a sediment compartment and sensitive macroinvertebrate taxa. However, the simple design offers the potential to perform experiments under more controlled conditions than larger and, consequently, more complex model ecosystems, while maintaining relatively high ecologic realism compared with standard laboratory tests. Further implications for risk-assessment studies are discussed in an ecotoxicologic and methodologic context.

Microcosms and mesocosms have often been used as a higher-tier study for the ecologic risk assessment of pesticides. They provide a bridge between laboratory and the field in terms of being manageable and allowing replication; hence, they provide a robust experimental design as well as realism in terms of ecologic processes and exposure to the chemical(s) being studied (Brock *et al*. 2000a).

In recent years, the question of scale in ecologic studies has been widely recognized as a critical issue and underpins conceptual frameworks of how we understand ecologic phenomena (Flemer *et al*. 1997). Larger and consequently more complex model-ecosystems are not necessarily preferable over smaller ones. A research question can only be solved if the dimensions of the test system meet the requirements needed to solve this question. Larger and consequently more complex model ecosystems are ecologically more realistic than smaller systems. In contrast, smaller model ecosystems are easier to replicate and manipulate; therefore, they prove to be more useful in elucidating the chain of events occurring after chemical stress than large test-systems (Leeuwangh *et al*. 1994).

The development of reliable, validated, more cost-effective, smaller test systems was recommended by the European Workshop on Freshwater Field Tests (Crossland *et al*. 1992) and the Higher-tier Aquatic Risk Assessment for Pesticides (HARAP) workshop (Campbell *et al*. 1999). However, only a small number of reliable small test systems have been used so far for pesticide risk assessment (Leeuwangh *et al*. 1994; Brock *et al*. 2000a, 2000b).

In this study, the effects of the insecticide chlorpyrifos, the fungicide carbendazim, and the herbicide linuron on the ecology of an 8.5-L microcosm were evaluated. The fate and effects of the pesticides were compared with experiments performed in larger-scale model ecosystems and were examined in an ecotoxicologic and methodologic context. This was done to validate the use of small model ecosystems for the risk assessment of pesticides.

Materials and Methods

Experimental Design

Twelve microcosms were situated in a room devoid of daylight and maintained at 21°C ± 1°C. Each microcosm consisted of a glass chamber (diameter 24.5 cm and height 36 cm; Fig. 1), filled with 8.5

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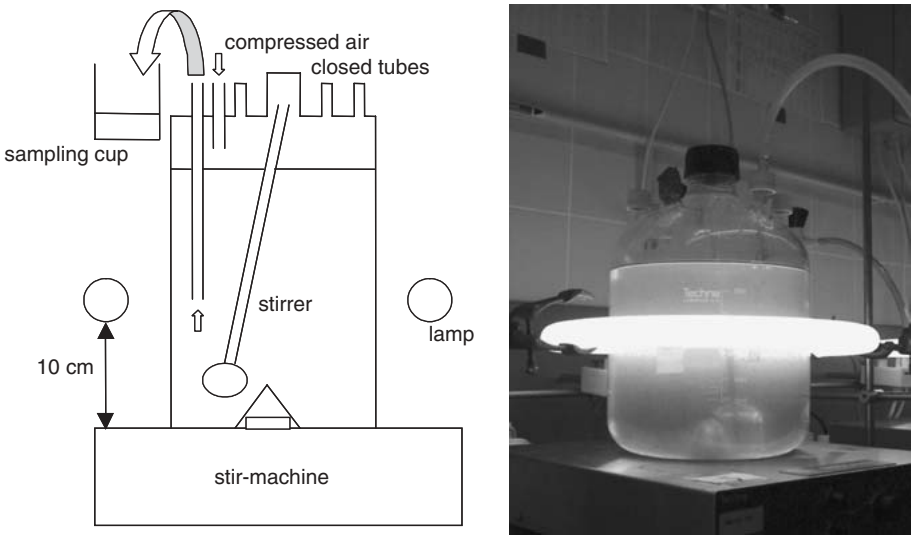

Fig. 1. Schematic representation (left) and overview (right) of the microcosm

L pond water obtained from a pond next to the building of the research institute Alterra. The pond water was sieved over a 0.75-mm mesh before adding it to microcosms; *Chaoborus* larvae, a known predator on zooplankton communities (Fedorenko 1975; Black and Hairston 1988), were herewith excluded.

The systems were stirred for 5 minutes every 30 minutes at 20 rpm by means of a MCS-101L biologic stirrer to achieve water movement in the system to prevent settling of planktonic algae. A fluorescent lamp (Philips TL'E 40W/33) was placed around the microcosms, resulting in a light intensity of approximately 45 $\mu E/m^2.s$ in the middle and 60 $\mu E/m^2.s$ at the edges of the chambers. The daily photoperiod was 14 hours (9:15 until 23:15).

Other than an opening for the stirrer, the microcosms contained five smaller openings, of which four were closed with air-tight screw caps, and one was connected to a compressed air line (Fig. 1). To take water samples, one of the screw caps was replaced by a cap having a rubber ring through which a glass pipette was inserted to 10 cm below the water surface. By adding compressed air into the system, water was forced through the glass pipette into a sampling cup.

During the preparatory phase of the experiments, additional plankton were introduced into the microcosms together with the pond water. Moreover, in the experiments with carbendazim and linuron, eight individuals of *Bithynia leachii* and *Lymnaea palustrus,* respectively, were added to control periphyton growth on the vessel walls. A nutrient addition of 0.115 mg N (as $NaNO_3$), 0.014 mg P (as KH_2PO_4), and 0.7 mg HCO_3 (as $NaHCO_3$) was applied twice during the pretreatment period and 2 times/week during the treatment period. Microcosms were allowed to stabilise for 1 week, which was defined as the pretreatment period, after which the systems were treated. The systems were monitored for several end points during an experimental period of 2 weeks for chlorpyrifos and 3 weeks for carbendazim and linuron.

Pesticide Application and Analysis

Chlorpyrifos was applied as Dursban 4E (nominal concentrations: 0.005, 0.05, 0.5, and 5 µg a.i./L) to two microcosms for each concentration, and four other systems served as controls. On days 0 (directly after application), 3, and 14, 250-ml water samples were taken at mid-depth and extracted with octadecyl (C-18, Bakerbond) solid phase extraction (SPE) columns. The columns were conditioned with 5 ml methanol and 5 ml distilled water. After extraction of a certain volume of water, the chlorpyrifos was eluted from the column with two successive portions of hexane, 2 ml, into glass test tubes. The samples were then evaporated by placing them in a heated water bath and supplying compressed air into the tubes. The residue was taken up in exactly 1.5 ml hexane. After shaking the samples thoroughly by hand, the hexane layer was transferred to gas chromatography cups and stored at –22°C until analysis. Chlorpyrifos concentrations were determined by splitless injection on a HP 5890 gas chromatograph (as described in Brock *et al*. 1992). The detection limit and recovery of chlorpyrifos were 0.01 µg/L and 83.3% ± 8.4% (mean ± SD, n = 6), respectively.

For the experiment with the fungicide derosal (active ingredient carbendazim), the treatment concentrations of 3.3, 33, 100, and 1000 µg a.i./L were applied to two microcosms each. Four other systems were used as untreated controls. Water samples were taken on days 0 (directly after application), 3, 7, 14, and 21; stored at 4°C; and analysed directly using high-performance liquid chromatography (HPLC) using external standard as described in Van Wijngaarden *et al*. (1998).

Linuron was applied to eight microcosms in four duplicate doses (0.5, 5, 50, and 150 µg/L), and four other systems served as controls. Water samples were taken on days 0 (directly after application), 3, 7, 14, and 21. Those taken from microcosms with the two lowest linuron applications were extracted according to the method described for chlorpyrifos. Linuron was eluted from the SPE columns with three successive aliquots of 1 ml methanol, diluted with distilled water to a fixed volume of 5 ml, and stored at 4°C. Water samples from the higher linuron concentrations (50 and 150 µg/L) were analysed without a concentration step. Analysis was carried out with HPLC as described in Cuppen *et al*. (1997). Linuron recovery from water was 105.9% ± 5.8% (mean ± SD, n = 8).

The half-life for the disappearance of the pesticides (DT_{50}) was calculated assuming first-order dissipation kinetics, *i.e.*, by linear regression using logarithmic-transformed measured pesticide concentrations.

DO-pH-Alkalinity-Conductivity Syndrome

Dissolved oxygen (DO), pH, and temperature were measured every working day at the start of the photoperiod and 7 hours later. By measuring the difference in DO during the first 7 hours of the light period, 90% to 95% of oxygen production can be determined (Beyers and Odum 1993). After stirring the microcosms for 5 minutes at 20 rpm, 50-ml water samples were taken with a glass pipette as described previously. In this water sample, DO was measured with a WTW

oxygen meter (OXI 196) connected to a WTW oxygen probe (EO 196). The pH and temperature were measured with a WTW pH meter (pH 323) and an LF 91 temperature meter, respectively. Alkalinity was measured 3 times/wk by titrating a 25-ml water sample with 0.01 N HCl until pH = 4.2. Conductivity was measured with a WTW conductivity meter.

Chlorophyll-a and Nutrients

Chlorophyll-a measurements were made three times/wk by filtering a 250-ml water sample over a Whatman GF/C glass fibre filter (mesh 1.2 μm). Extraction of the pigments was performed according to the method described by Moed and Hallegraeff (1978). Subsamples of the filtrate were transferred to centrifuge tubes and stored at 4°C before analyses were conducted for ammonium, nitrate, and orthophosphate using a Tecator 5042 detector connected to a Tecator 5027 sampler and a Tecator 5011 analyser (nitrate and ammonium) or Tecator 5010 analyser (orthophosphate).

Decomposition

In the experiments with carbendazim and linuron, decomposition of particulate organic matter (POM) was studied using dried *Populus* leaves. The *Populus* leaves were obtained from a stock of previously leached (three times for 2 days) and dried (60°C for 72 hours) leaves. Approximately 0.4 g pretreated leaves were weighed and placed in stainless steel tea strainers, then leached for 3 days in distilled water. One tea strainer was incubated in each microcosm at mid-height on the day of application. At the end of the experiment (day 21), the content of a tea strainer was gently washed in the corresponding microcosm to separate algae and invertebrates from POM. The leaf material was transferred to aluminium dishes to determine dry weight (105°C for 24 hours).

Zooplankton and Phytoplankton

Zooplankton samples were taken at the end of each experiment. After stirring the microcosms, 6-L samples were passed over a 40-μm mesh net. Formalin was added to a final concentration of 4% v/v to preserve the samples. After 2 days, the upper liquid was removed, and the remainder was transferred to preweighed plastic bottles. The microzooplankton were identified (to species level when possible) in a weighed subsample with an inverted microscope. For macrozooplankton, total samples were identified using a binocular microscope because their density was always relatively low. In the experiment with linuron, the phytoplankton community was also sampled at the end of the experiment. A 1-L sample was taken, stained with Lugol's solution, and concentrated after sedimentation for 6 days. The concentrated sample was preserved with formalin, and cell counts were made. This was done in 10 counting fields of a subsample. Zooplankton and phytoplankton data were expressed as number of individuals per liter.

Furthermore, on five occasions during the course of the experiment, the total numbers of Cladocerans were determined during the experiment in a 250-ml water sample. After counting, the water samples were returned to the corresponding microcosm.

Snails

At the end of the carbendazim and linuron experiments, the sublethal effects on *B. leachii* and *L. palustrus*, respectively, were determined. The sublethal effects were screened by evaluating the grazing activity, which was measured as numbers of individuals grazing. Effects on *B. leachii* were also determined by attempts to open the operculum with a pair of forceps.

Data Analysis

NOECs were calculated for all parameters using the parametric Williams test. The Williams test is an analysis of variance (ANOVA) test that assumes increasing effect for increasing dose. Abundance data were $\mathrm{Ln}(Ax + 1)$ transformed, where x stands for the abundance value, and $Ax = 2$ when taking the lowest abundance value >0 for x (*e.g.*, if the lowest number >0 is 1, $A = 2$; for rationale, see Van den Brink *et al*. 2000). This was done to down-weight high abundance values and approximate a normal distribution of the data. Analyses were performed using Community Analysis, version 3.5 (Hommen *et al*. 1994). Statistical significance was accepted at $P < 0.05$. Only NOECs calculated for at least two consecutive sampling dates were considered in the interpretation of the data.

The differences in the structure of the zooplankton communities between the microcosms as sampled at the end of the experiments and the phytoplankton community of the linuron experiment were visualised using principal component analysis (PCA) (Ter Braak 1995; Van Wijngaarden *et al*. 1995). Ordination expresses differences in species composition between samples without the use of measured environmental or explanatory variables. In such an analysis, ordination constructs imaginary, latent explanatory variables that maximise the variation in species' composition between sites, *i.e.*, those that best represent the underlying structure in the data set (Ter Braak 1995). The first latent variable is constructed in such a way that it explains the largest part of the total variance, the second one the largest part of the remaining variance, *etc*. The first two latent variables are normally used to construct an ordination diagram, of which they form the axes. Samples and species are represented in the diagram by points (or arrows) plotted at the scores (values) they have on the latent variables (See Fig. 5 as an example). Samples with nearly identical species composition lie close together in the diagram, whereas samples that lie far apart have very different species composition. In the diagrams (biplots), species arrows point in the direction of higher values. For examples on the application of ordination techniques in Ecotoxicology, please refer to Van den Brink *et al*. (2003). Before analyses with CANOnical Community Ordination (CANOCO) for Windows (version 4; Ter Braak and Smilauer 1998), the abundance data of the communities were $\ln(Ax + 1)$ transformed (see previously for rationale).

The NOEC at the community level for the phytoplankton community at the end of the linuron experiment, as well as the zooplankton communities at the end of the three experiments, were calculated by applying the Williams test to the sample scores of the first principal component (for rationale, see Van den Brink *et al*. 1996).

Results

Pesticide Concentrations

In all three experiments, the initial concentrations deviated by <10% of the nominal concentrations (Table 1). Linuron and carbendazim were very persistent; pesticide concentrations measured at the end of the experiment were similar to initial concentrations. Therefore, no DT_{50} for the disappearance of carbendazim and linuron could be estimated.

Chlorpyrifos concentrations decreased during the experimental period, and the rate of dissipation was dose dependent;

Table 1. Nominal concentrations (μg/L) and initial concentrations (μg/L) of pesticides for the experiments with chlorpyrifos, carbendazim, and linuron[a]

Pesticide	Nominal concentrations (μg/L) ± SD	Initial concentrations (μg/L) ± SD	Concentration at end of experiment (μg/L) ± SD	DT_{50} (d) ± SD[b]	Duration of experiment (d)
Chlorpyrifos	0.005	< DL	< DL	–	14
	0.05	0.053 ± 0.0057	0.018 ± 0.004	9.6 ± 0.0	
	0.5	0.47 ± 0.085	0.035 ± 0.006	6.1 ± 0.2	
	5	4.92 ± 0.47	0.35 ± 0.06	6.6 ± 0.2	
Carbendazim	3.3	3.5 ± 0.1	3.7 ± 0.1	–	21
	33	33.5 ± 1.6	36 ± 1.3	–	
	100	97.8 ± 1.3	107.5 ± 3.5	–	
	1000	976.5 ± 4.9	1003 ± 48.1	–	
Linuron	0.5	0.5 ± 0	0.4 ± 0.1	–	21
	5	5.1 ± 0	4.3 ± 0.2	–	
	50	45.1 ± 0.9	40.1 ± 1.5	–	
	150	146.6 ± 0.5	146.6 ± 0.5	–	

[a] Concentrations at the end of the experiments (μg/L) and the dissipation rate from the water layer (DT_{50}) are also noted. The detection limit (DL) was 0.01 pg/L.
[b] – implies that no DT_{50} could be calculated.
SD = standard deviation.

chlorpyrifos disappeared faster in the highest two concentrations compared with the 0.5 μg/L treatment (Table 1). The half-life was approximately 10 days for the 0.5 μg/L treatment level and 6 to 7 days for the two highest treatment levels. The chlorpyrifos concentration in the 0.005 μg/L dosed microcosms decreased below detection level quickly after application, so no half-life could be calculated.

Water-Quality Parameters

The overall effects of the pesticides on water-quality parameters are listed in Table 2. Application of chlorpyrifos led to an increase in DO and DO production (Fig. 2) in all but the lowest treatment and increased pH levels in all treatments. Significantly higher pH levels in all treatments were the result of decreased pH in the control microcosms from 10.3 to 9.7 during the course of the experiment. From 10 days after application onward, conductivity increased in the 0.5 and 5 μg/L microcosms. Application of chlorpyrifos had no significant treatment effect on alkalinity or nutrient concentrations.

The highest carbendazim treatment led to an immediate and prolonged increase in DO production (Fig. 3). Although DO levels were generally higher at this treatment level, this difference was not significant. Furthermore, pH increased in the highest and conductivity in all but the lowest concentration from 1 week after application onward. Carbendazim did not lead to significant treatment effects on alkalinity and nutrient levels (Table 2).

Effects on water quality were most prominent in the linuron experiment. Immediately after treatment, DO production (Fig. 4) decreased significantly compared with controls at all linuron concentrations but the lowest (Table 2). This resulted in decreased DO in even the lowest concentration at the end of the dark period between 14 and 17 days and application. The DO levels in the afternoon were lower in all but the lowest treatment for a prolonged period of time. DO remained >6.8 mg/L in all microcosms, so no anoxic conditions occurred. At the end of the experimental period, only the microcosms with the highest two linuron concentrations were still significantly different from controls. The decrease in DO was accompanied by a decrease in pH in the higher treatment levels. At the end of the experiment, the microcosms treated with 5 μg/L regained normal ''control'' pH levels, whereas those with higher concentrations remained lower than controls. Corresponding with the decrease in pH and DO, application of 50 and 150 μg/L led to an increase in conductivity and in the highest concentration to an increased alkalinity. Although alkalinity was only increased during the second week after application, conductivity remained increased until the end of the experiment. The two highest linuron treatments had an increase in concentrations of ammonium, nitrate, and phosphate compared with controls.

Zooplankton

In the bulk sample taken at the end of the chlorpyrifos experiment, a total number of 21 invertebrate taxa were identified and their abundance determined. In terms of numbers of taxa, the most important taxonomic groups were Rotatoria, Cladocera, Copepoda, Insecta, and Ostracoda (not identified at species level). Treatment-related dynamics in densities of Cladocera in the course of the experiment are presented in Fig. 2. In the controls and the 0.005 μg/L microcosms, densities of Cladocera increased in time, whereas Cladocera were eliminated in test systems treated with higher concentrations.

A biplot resulting from the PCA on the zooplankton data set is given in Fig. 5. The diagram summarises the treatment effects in the data set, yet still indicates the approximate species composition for all samples. Samples with nearly identical species compositions lie close together, whereas samples with very different species compositions lie far apart. If an imaginary line is drawn through a species point and the origin of the plot, the relative abundance of that species in all samples can

Table 2. NOECs and LOECs (in μg/L) found in the present study and studies with a single load and constant exposure of chlorpyrifos, carbendazim, and linuron[a]

	Chlorpyrifos			Carbendazim			Linuron		
End point/ pesticide load	Single peak	Constant	This study	Single peak	Constant	This study	Single peak	Constant	This study
Community metabolism									
DO	6–44↑	0.1–0.5↑	0.005–0.05↑	21–226	> 1000	> 1000	0.5–5	0.5–5↓	0.5–5↓
DO production	6–44↓	NM	0.005–0.05↑	NM	NM	100–1000↑	5–15	NM	0.5–5↓
pH	6–44↑	0.05–0.1↑	Ctr–0.005↑	>226	>1000	100–1000↑	0.5–5	0.5–5↓	0.5–5↓
EC	6–44↓	0.05–0.1↓	0.05–0.5↑	>226	>1000	3.3–33↓	NM	0.5–5↑	5–50↑
Alkalinity	6–44↓	0.01–0.05↓	> 5	NM	> 1000	> 1000	NM	0.5–5↑	50–150↑
Ammonium	NM	NM	> 5	NM	> 1000	> 1000	NM	>150	5–50↑
Nitrate	NM	NM	> 5	NM	> 1000	> 1000	NM	50–105↑	5–50↑
Phosphate	NM	>0.5	> 5	NM	> 1000	> 1000	NM	>150	5–50↑
Decomposition									
Breakdown of POM	NM	0.01–0.05↓	NM	NM	100–330↓	100–1000↓	NM	>150	50–150↑
Zooplankton									
Community	0.1–0.9↓	0.01–0.05↓	0.005–0.05↓	2.21–21↓	33–100↓	33–100↓	>50	5–15	5–50↓
Phytoplankton									
Community	NM	0.05–0.1↑	NM	NM	33–330↑	NM	>50	0.5–5↓	50–150↓
Chlorophyll-a	NM	0.05–0.1↑	0.05–0.5↑	21–226↑	33–330↑	33–100↑	>50	50–150↑	5–50↓
Overall NOEC	0.1–0.9	0.01–0.05	0.005–0.05	2.2–2.1	3.3–33	33–100	5–15	0.5–5	0.5–5
Study number	1	2		3	4		5	6	

[a] Arrows indicate a significant increase (↑) or decrease (↓) compared with controls. Study numbers refer to the studies as given under this table. NM = not measured.

1. Van Wijngaarden *et al*. 1996; Van den Brink *et al*. 1996; Kersting and Van den Brink 1997.
2. Cuppen *et al*. 2002; Van den Brink *et al*. 2002.
3. Slijkerman *et al*. 2004.
4. Cuppen *et al*. 2000; Van den Brink *et al*. 2000.
5. Kersting and Van Wijngaarden 1999; Van Geest *et al*. 1999.
6. Van den Brink *et al*. 1997; Cuppen *et al*. 1997.

be derived by perpendicularly projecting the sample point on this imaginary line. The samples projecting on the ''species line'' far away from the origin but on the same side of the origin as the species point contain relatively high numbers of this species. If a sample projects on the other side of the origin compared with the species point, numbers of this species are relatively low in this sample (Van den Brink *et al*. 2003).

The PCA of the chlorpyrifos zooplankton data set revealed treatment-related differences in species composition, with the effect of the treatment decreasing in the order 5 ≈ 0.5 ≈ 0.05 > 0.005 μg/L ≈ controls (Fig. 5). The direction of the treatment vector in the diagram is from the right to the left, meaning that taxa less abundant in the treated microcosms are situated at the right, and the unaffected and positively affected taxa are situated at the centre and left side of the diagram.

Numbers of *Chydorus sphaericus*, *Simocephalus vetulus*, and *Lepadella patella* were significantly decreased at the higher treatment levels (Table 3). *C. sphaericus* and *S. vetulus* were eliminated in the three highest concentrations. Taxa that occurred in significantly higher densities than in the controls were the rotifers *Cephalodella gibba, Lecane bulla*, and *Trichocerca*. Overall, the Williams test on the PCA coordinates showed a significant treatment effect on the invertebrate community at all chlorpyrifos applications but the lowest ($NOEC_{community}$ = 0.005 μg/L).

In the samples taken at the end of the experiment evaluating carbendazim, a total number of 23 different zooplankton taxa were identified. The control community was dominated by Rotifera, followed by Cladocera, Cyclopoida, Insecta, and Ostracoda (not identified at species level). Treatment-related dynamics in densities of Cladocera in the course of the experiment are presented in Fig. 3. In test systems treated with the two highest concentrations (100 and 1000 μg/L), Cladocera decreased and were even eliminated at the end of the experiment.

The PCA biplot of the zooplankton data set revealed a clear dose-related deviation of the 100 and 1000 μg/L carbendazim treatments compared with controls (Fig. 6). The visual differences were confirmed by the $NOEC_{community}$ calculation ($NOEC_{community}$ = 33 μg/L). Taxa negatively affected by the application are situated on the left side of the diagram, whereas unaffected taxa are situated at the upper right (100 μg/L samples) and lower right quadrants (1000 μg/L samples). In the two highest concentrations, Cladocera were completely eliminated and, consequently, abundances of *S. vetulus*, *Grabtoleberes testudinalis*, *Alona rectangular*, *Ephippia*, and the rotifer *L. patella* were significantly compared with controls. In addition, the highest carbendazim dose also had pronounced effects on rotifers. Total numbers of individuals were only one third of those in controls (Williams test, $P < 0.05$) and *Colurella uncinata* and *nauplii* abundances were significantly decreased (Table 3). *Branchionus angularis* occurred in higher densities at this treatment compared with controls.

In the experiment with linuron, a total number of 23 different taxa were identified dominated by Rotifera, followed by Cladocera, Copepoda (Cyclopoida and nauplii), and Ostracoda

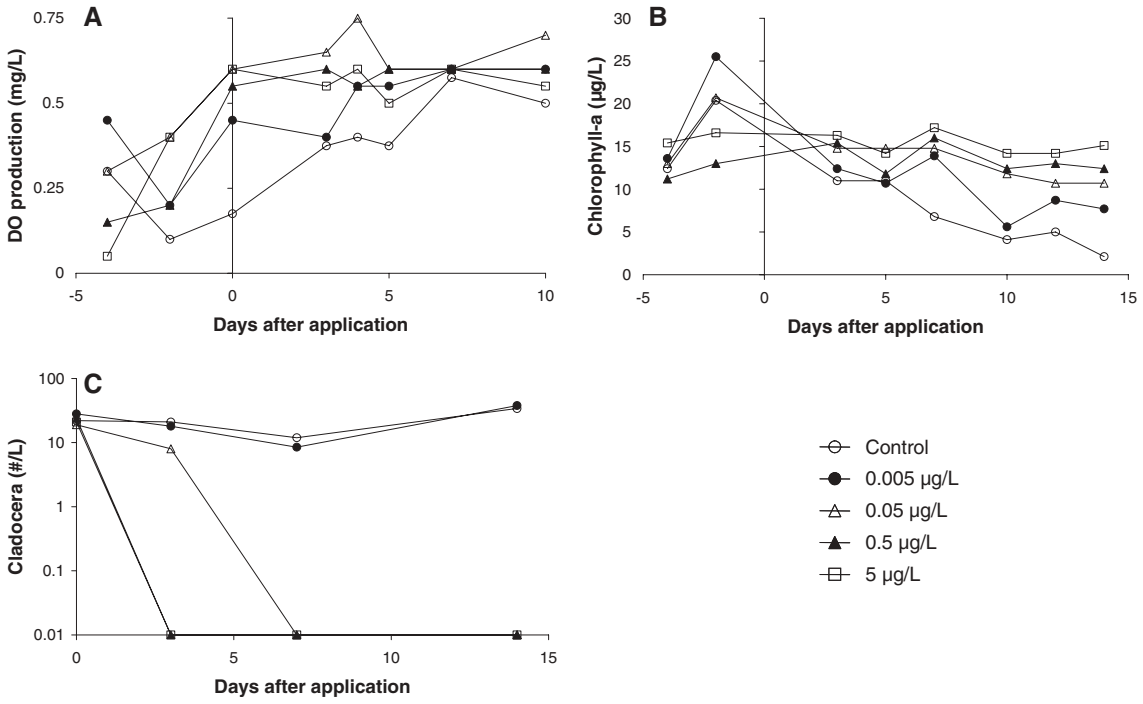

Fig. 2. Dynamics of DO production (**A**), chlorophyll-a (**B**), and Cladocera numbers (**C**) in the chlorpyrifos experiment

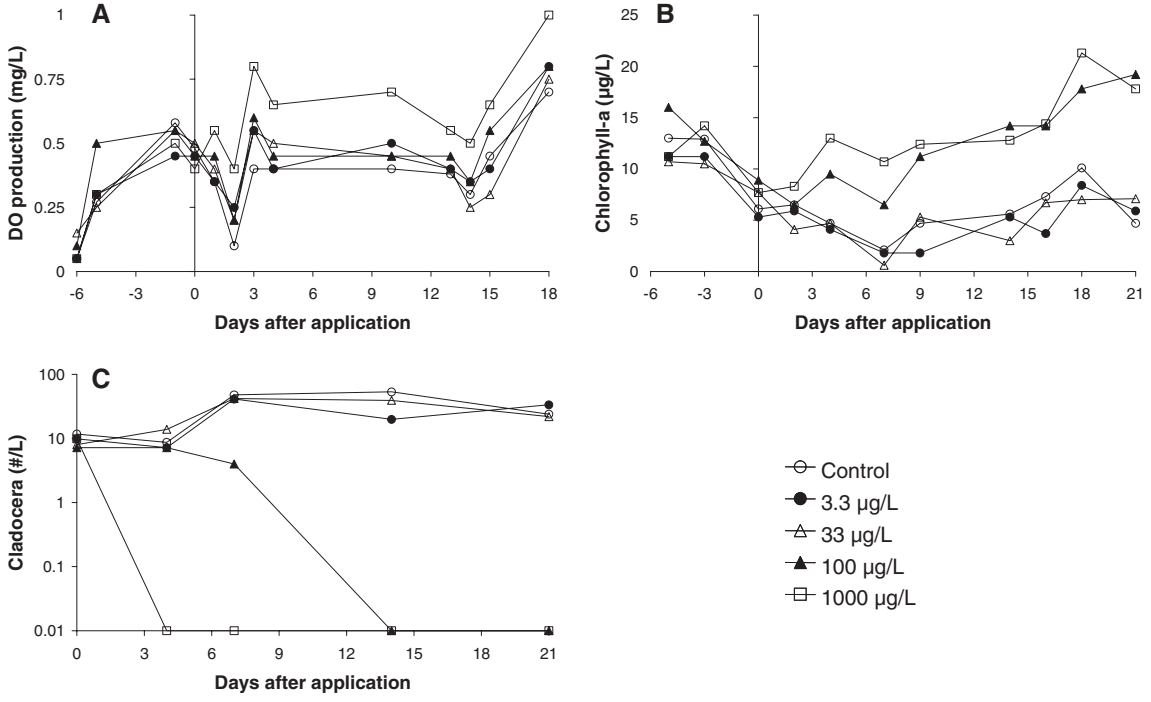

Fig. 3. Dynamics of DO production (**A**), chlorophyll-a (**B**), and Cladocera numbers (**C**) in the carbendazim experiment

(no identification on the taxon level). Total number of Cladocera as counted during the course of the experiment was lower in the two highest treatments only at the end of the experiment (Fig. 4).

The PCA diagram presented in Fig. 7 summarizes treatment effects on the zooplankton community on day 21. The diagram reveals a decrease in abundance of especially Cladocera species for the highest two applications because these sample points are positioned at the upper left quadrant and the Cladocera species points at the lower right quadrant. Although these visual effects could not be confirmed with $NOEC_{community}$ calculations (>150 µg/L), negative treatments

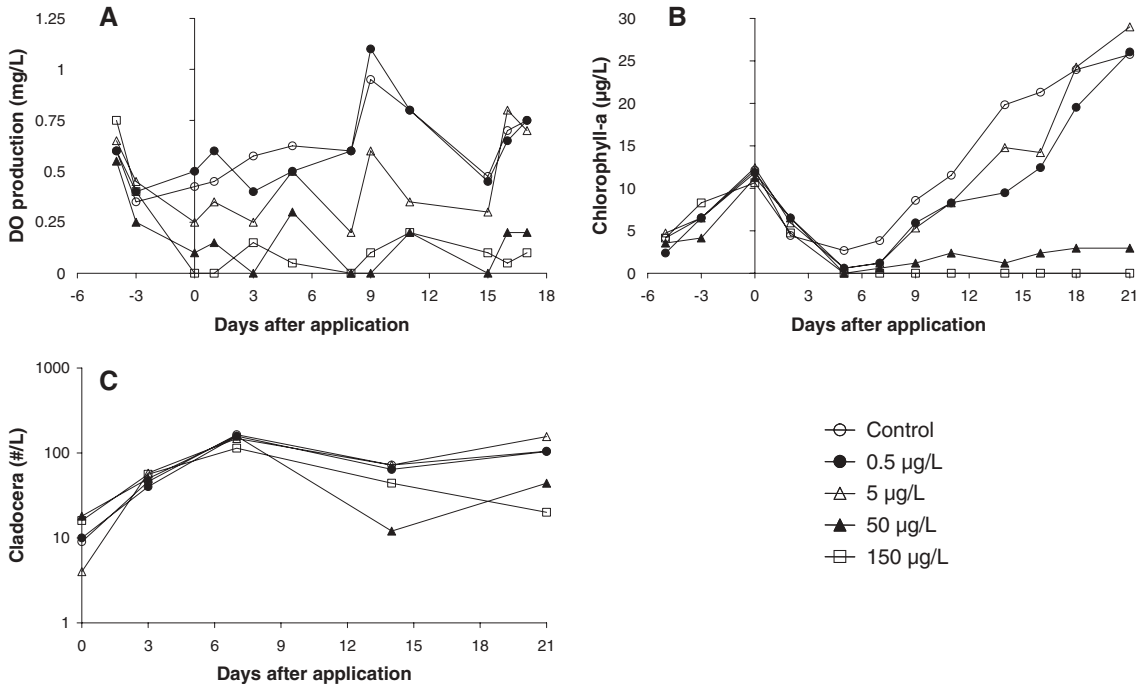

Fig. 4. Dynamics of DO production (**A**), chlorophyll-a (**B**), and Cladocera numbers (**C**) in the linuron experiment

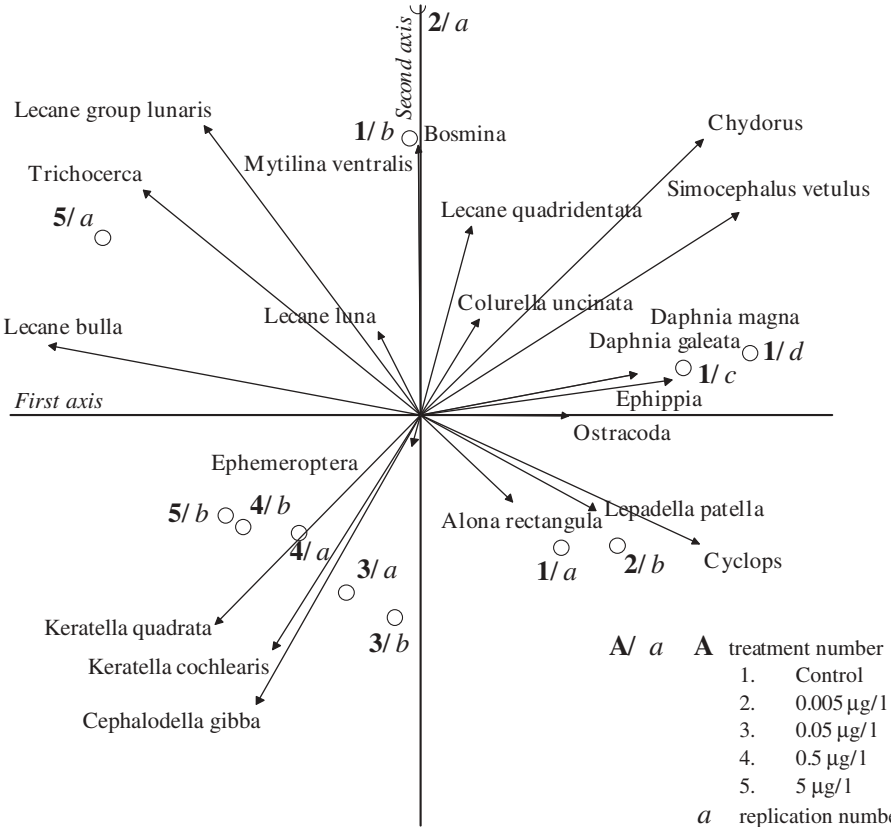

Fig. 5. Ordination diagram (PCA) indicating the effects of a single application of the insecticide chlorpyrifos on the zooplankton per treatment level. Of all variance, 36% is displayed on the horizontal axis and another 26% on the second axis

effects were found for several species (Table 3). Abundances of the Cladocerans *Daphnia galeata and D. magna*, together with *Ephippia*, were decreased in the highest two concentrations and *S. vetulus* in the highest only. *L. bulla* showed decreased numbers at all linuron applications on this day (Table 3).

Table 3. NOECs (µg/L) as calculated by Williams test ($P \leq 0.05$) for the abundances of zooplankton taxa for the three different experiments as well as phytoplankton taxa for the linuron experiment[a]

Chlorpyrifos zooplankton	Carbendazim zooplankton	Linuron zooplankton	Linuron phytoplankton
Decrease	Decrease	Decrease	Decrease
0.005 *Simocephalus*, *vetulus*, 0.005 *C. sphaericus*, 0.5 *L. patella*	33 *S. vetulus*, 33 *Graptoleberis testudinaria*, 33 *L. patella*, 33 *A. rectangula*, 33 *Ephippia*, 100 *Nauplii*, 100 *C. uncinata*	0 *L. bulla*, 5 *D. galeata*, 5 *D. magna*, 5 *Ephippia*, 50 *S. vetulus*	5 *Scenedesmus*, 5 *Monoraphidium*, 50 *Pediastrum*, 50 *Tetraedon*
Increase	Increase	Increase	Increase
0.005 *C. gibba*, 0.05 *L. bulla,* 0.5 *Trichocerca sp.*	100 *Brachionus angularis*	None	0 *Epithemia*, 5 *Navicula*, 50 *Closterium*

[a] All measurements were performed at the end of the experiments.

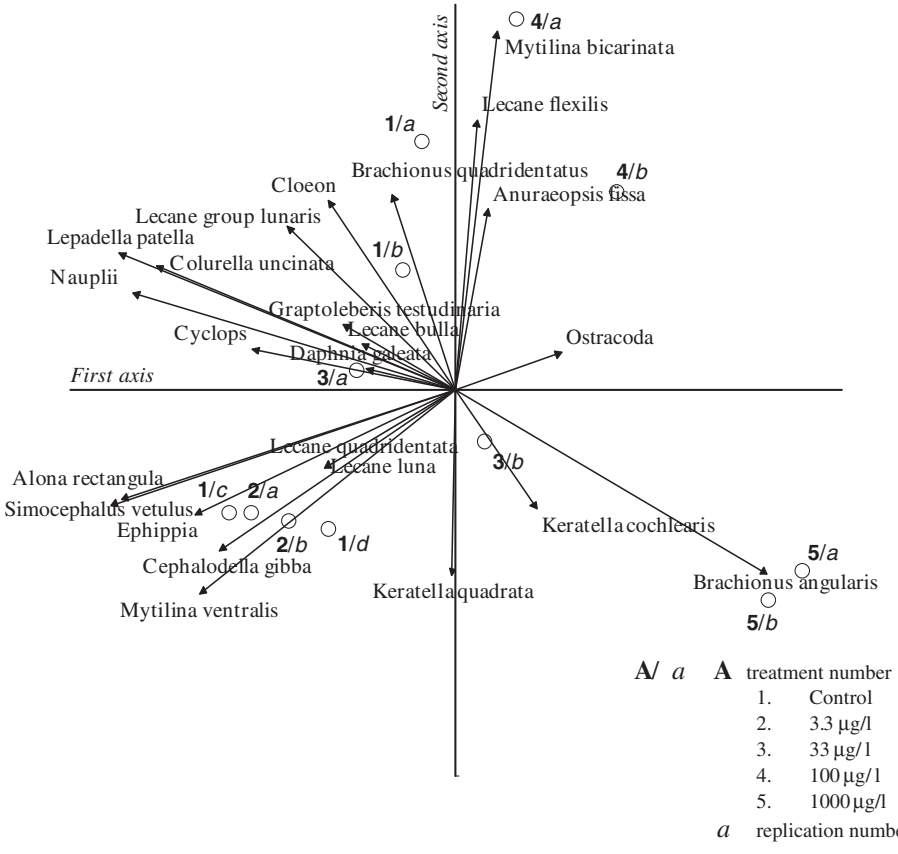

Fig. 6. Ordination diagram (PCA) indicating the effects of a single application of carbendazim on the zooplankton per treatment level. Of all variance, 37% is displayed on the horizontal axis and another 20% on the second axis

Phytoplankton

After the start of the treatment, chlorophyll-a values decreased in controls and the lowest chlorpyrifos application during the course of the experiment (Fig. 2). In the higher chlorpyrifos concentrations, chlorophyll-a levels did not alter, leading to a concentration-dependent increase compared with controls (NOEC = 0.005 µg/L; Table 2).

Application of the highest two carbendazim concentrations led to a time-related increase in chlorophyll-a content (Fig. 3). Four days after application, chlorophyll-a values in these treatment levels were two times and at the end of the experiment four times higher than in controls.

During the course of the linuron experiment, chlorophyll-a content increased in controls and microcosms applied with the two lowest linuron concentrations (Fig. 4). At the end of the experiment, chlorophyll-a levels were tripled in these microcosms compared with pretreatment values. Application of the two highest linuron concentrations led to a decrease in chlorophyll-a levels. Five days after treatment, no chlorophyll-a could be detected in samples from microcosms treated with these concentrations. In the 50 µg/L–treated microcosms, chlorophyll-a reappeared 1 week after treatment but did not regain normal values within the experimental period. Chlorophyll-a levels in the highest concentration remained nil until the end of the experiment.

The PCA-biplot (Fig. 8) visualises the overall effect of linuron on the phytoplankton community. The diagram reveals that the phytoplankton community of the 150 µg/L microcosms and, to a lesser extent, the 50 µg/L microcosms diverged from controls. Most species that were affected by linuron application decreased in numbers, although *Navicula*,

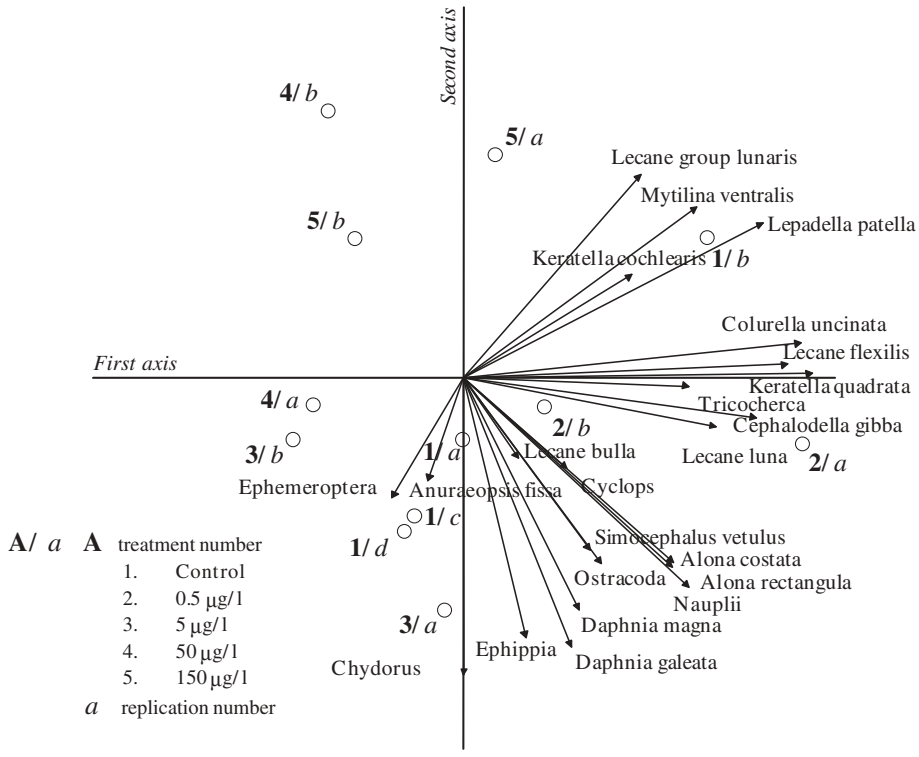

Fig. 7. Ordination diagram (PCA) indicating effects of a single application of linuron on the zooplankton per treatment level on day 21. Of all variance, 35% is displayed on the horizontal axis and another 20% on the vertical axis

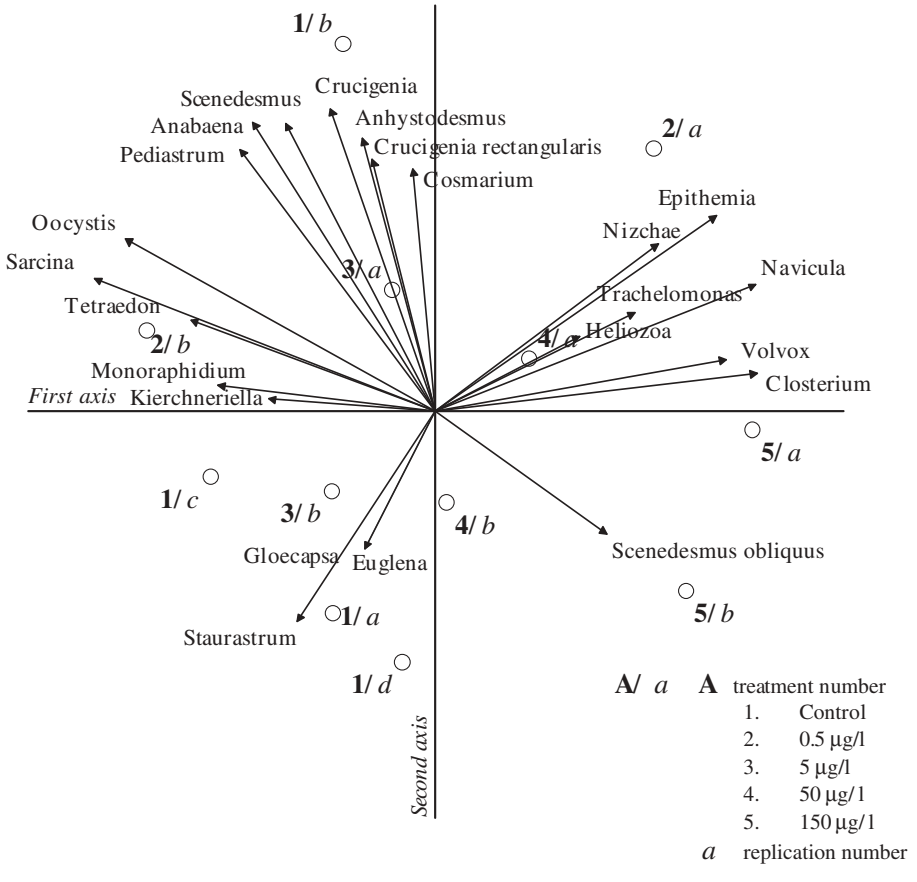

Fig. 8. Ordination diagram (PCA) indicating the effects of a single application of linuron on the phytoplankton per treatment level. Of all variance, 38% is displayed on the horizontal axis and another 20% on the vertical axis

Epithemia, and *Closterium* individuals increased significantly in abundance (Table 3). The direction of the treatment vector is from the left to the right part of the diagram, *i.e.*, those taxa negatively affected by the treatment are situated at the left and insusceptible and positively affected taxa are situated at the right side of the biplot (Fig. 8).

The $NOEC_{phytoplankton\ community}$, as calculated by a Williams test on the PCA coordinates, was 50 μg/L for linuron (Table 2). The taxa *Scenedesmus* and *Monoraphidium* were significantly less abundant in the highest two linuron applications compared with controls. In addition, *Pediastrum* and *Tetraedon* were significantly decreased at the highest application. Decrease in the total number of phytoplankton individuals was also most prominent in the 150 μg/L samples. In these samples, numbers were only one fourth of control levels.

Snails

Application of carbendazim did not result in lethal treatment effects on *B. leachii* at the concentrations applied. However, grazing behaviour was affected in the two highest carbendazim-concentration groups. In addition, in microcosms dosed with 1000 μg/L, the operculum reflex was decreased compared with control animals. The NOEC for the sublethal effects on *B. leachii* was therefore determined to be 33 μg/L (data not shown). The application of linuron did not result in any significant treatment effect on *L. palustrus*.

Decomposition Experiment

The residual dry weights of the *Populus* leaves in control and the lower-carbendazim treatment groups amounted to approximately 60% of the initial dry weight (data not shown). Application of 1000 μg/L led to a slight although significant decrease in decomposition compared with controls (Table 2).

The decomposition of *Populus* leaves in all but the highest linuron treatment groups were comparable with that in the carbendazim experiment: Residual dry weights were approximately 60% of initial values. The rate of decomposition in the highest-dosed microcosms was 10% higher than in controls (Table 2).

Discussion

Fate of the Pesticides

Chlorpyrifos was relatively persistent, and the DT_{50} varied between 10 days for the 0.05 μg/L treatment and 6 to 7 days for the higher treatments (Table 1). This dependence of DT_{50} on treatment level was most probably caused by higher pH values in the higher treatment levels because the stability of chlorpyrifos decreases rapidly as pH increases (Macalady and Wolfe 1983). The relatively slow disappearance of chlorpyrifos in the present study, compared with other microcosm studies (Racke 1993; Giesy *et al*. 1999), was presumably the result of the absence of macrophytes and sediment, which are known to substantially absorb chlorpyrifos from water (Crum and Brock 1994).

The concentrations of carbendazim and linuron remained constant during the experimental period for all doses applied (Table 1). In an indoor macrophyte-dominated microcosm experiment with carbendazim, Cuppen *et al*. (2000) found a DT_{50} between 6 and 25 weeks, which decreased with the treatment level. The investigators suggested that the dependence on treatment level was probably not the result of changes in physicochemical conditions because no differences were observed between the different treatments. It is therefore unlikely that the absence of breakdown in the present study was caused by water-quality parameters. A more plausible explanation was the fact that closed systems were used that did not contain sediment and macrophytes.

Van den Brink *et al*. (1997) and Cuppen *et al*. (1997) performed a microcosm study in indoor macrophyte-dominated systems with linuron and found a half-life of 11 days for 0.5 μg/L dosed up to 49 days for 150-μg/L dosed microcosms. They concluded that the difference in DT_{50} was caused by differences in pH regime between the different treatments. Cserhati *et al*. (1976) found a significant slower hydrolysis of linuron at pHs 6 and 8 compared with pHs 4 and 10. In the present study, pH ranged from 7.8 to 8.1 for the different treatments so, based on previously data, a DT_{50} of approximately 50 days could be expected, which is considerably longer than the duration of this study.

Effects on Ecosystem Structure and Functioning

The primary response of the chlorpyrifos application was a decrease in the number of zooplankton species and abundances (Cladocera in particular). The consequent decrease of grazing pressure indirectly caused an increase in chlorophyll-a levels, resulting in an increase in pH, DO and DO-production, and conductivity (Table 2; Figs. 2 and 5). However, a decrease in conductivity, caused by increased photosynthesis, was expected (Brock *et al*. 1993). The small increase in conductivity (280 to 300 μS/cm) after chlorpyrifos treatment can be explained by the release of dissolved substances that were until then part of the biomass of the zooplankton. All other direct and indirect effects are comparable with effects found in other microcosm studies with chlorpyrifos (Kersting and Van den Brink 1997) and other insecticides (see Brock *et al*. 2000b and Van Wijngaarden *et al*. 2004 for reviews). The order of susceptibility of the zooplankton groups was Cladocera > Copepoda and Ostracoda > Rotifera, which is in accordance with other model ecosystem studies (Van den Brink *et al*. 1996, 2002; Brock *et al*. 1992). Rotifera, which have low sensitivity to chlorpyrifos, have frequently been reported to increase in numbers in insecticide-stressed aquatic systems as a result of a lower competition and decreased grazing pressure caused by a decrease in Cladocerans (Brock *et al*. 2000b). In the current study, rotifer abundances were 30% higher in the 0.5 μg/L-applied microcosms and doubled in the two highest treatments. Although this increase was not significant because of a high variation in controls, a significant increase in numbers of the individual species *C. gibba*, *L. bulla*, and *Trichocerca* sp. was demonstrated for higher chlorpyrifos concentrations (Table 3).

Direct effects of carbendazim have been reported on zooplankton and macroinvertebrates in model ecosystem studies after single (Slijkerman *et al*. 2004) and chronic (Cuppen *et al*. 2000; Van den Brink *et al*. 2000) exposure. In line with this, the higher carbendazim applications in the present study had negative treatment effects on zooplankton and *B. leachii*.

Cladocera and Rotifera were found to be the most susceptible zooplankton groups (Figs. 3 and 6). In a former microcosm experiment, Copepoda were found to be more sensitive than Rotifers. This only became apparent 3 weeks after application because the effect on Cyclopoida resulted from a decrease in the numbers of their immature stage, nauplius, rather than from direct toxicity (Van den Brink *et al*. 2000). Indeed, a decrease in nauplius larvae was also found in the present study (NOEC 100 μg/L), but the experimental period was probably too short to show a prolonged effect on mature Cyclopoida.

The decrease in zooplankton abundance and the consequent increased growth of planktonic algae resulted in increased pH and oxygen production as discussed for chlorpyrifos. As observed in a macrophyte-dominated microcosm study with chronic carbendazim exposure, no increase in dissolved oxygen concentrations was observed (Cuppen *et al*. 2000). The investigators of that study attribute this to the fact that the water was saturated with dissolved oxygen, so possible movement of oxygen to air could have caused unnoticed additional oxygen production. Oxygen levels were near saturation in the present study too (8.8 ± 0.7; saturation 100% at 9.4 mg/L), so this could have masked a possible increase. Carbendazim application, however, led to decreased conductivity values in the two highest concentrations, most probably caused by the increase in chlorophyll-a (Fig. 3). Carbendazim had a significant treatment effect on decomposition, which may have resulted in decreased levels of breakdown products and, consequently, lower conductivity.

In microcosms treated with the highest carbendazim concentration, the operculum reflex of *B. leachii* was disrupted, and grazing was even affected at 100 μg/L carbendazim. In line with this, Cuppen *et al*. (2000) found a NOEC based on the number of individuals caught on artificial substrates of 100 μg/L for *B. leachii* and 33 μg/L for *B. tenticulata*. In a laboratory bioassay, Van Wijngaarden *et al*. (1998) reported a $NOEC_{reproduction}$ of 103 μg/L for the latter species.

The general effect chain of linuron application in the present study was the same as described for macrophyte-dominated freshwater microcosm studies with linuron (Van den Brink *et al*. 1997; Cuppen *et al*. 1997) and other herbicides (for a review, see Brock *et al*. 2000a). The primary effect of linuron is inhibition of the photosynthetic efficiency of the primary producers (Snel *et al*. 1998), leading to decreased DO and pH levels and increased alkalinity and conductivity (Table 2). A decrease in sensitive phytoplankton population densities caused an increase of the nonsensitive or rapidly adapted species *Ephitemia*, *Navicula* and *Closterium* because of decreased competition (Fig. 8). Increased levels of ammonium, nitrate, and ortho-phosphate, as a consequence of an overall decrease in primary production and the decomposition of the sensitive phytoplankton biomass, further stimulated the growth of these species.

The EC_{50} of *D. galeata* for linuron (360 μg/L; Stephenson and Kane 1984) is considerably higher than the concentrations used in the current experiment. Numbers of the Cladocerans *D. galeata*, *D. magna*, and *S. vetulus* and total numbers of Cladocera had significantly decreased 3 weeks after application (Table 3 and Fig. 4). Cladocerans are more efficient grazers than Rotifers (Canasta 1998), so they could survive the decreasing phytoplankton biomass during a longer period. The eventual decrease in *D. galeata*, *D. magna*, and *S. vetulus* can be further explained by the phytoplankton community composition as identified in the higher treatment level 3 weeks after application. The phytoplankton community was dominated by the diatoms *Epithemia* and *Navicula*, taxa that possess a tough cover, probably making them less edible for (young) Cladocera (Fig. 8). Indeed, Starr *et al*. (1999) found a decrease in the reproductive success of a planktonic copepod (*Cleanups finmarchicus*) after a monospecific diet of a *Navicula* species.

Despite the lower oxygen concentrations, the decomposition of *Populus* leaves was significantly higher in the highest-dosed microcosms. The DO concentrations above the substrate were probably high enough to prevent inhibition of microbial activity. In macrophyte-dominated freshwater microcosms treated with the same concentration of linuron, no effect on decomposition of *Populus* leaves was noted (Cuppen *et al*. 1997). However, increased decomposition rates after herbicide treatment have been reported for 2,4-D (Sherry 1994) because of changes in the microorganism species composition. Because microorganisms were not studied, we can not verify whether this also occurred in the present study. A possible explanation of the faster decomposition of POM might be that invertebrates increased their grazing of microorganisms associated with POM because of decreased food in the form of phytoplankton.

No treatment effects of linuron were observed on the grazing behaviour of *L. palustrus*. In line with this, the LC_{50} of some macroinvertebrates, such as *Dugesia tigrina* (10 mg/L), *Lymnaea* (70 mg/L), and *Tubifex* (10 mg/L), were too high to expect any treatment effects (Maier-Bode and Härtel 1981).

Comparison of Safety Thresholds with Other Microcosm Studies

In Table 2, the NOECs of former microcosm studies by our department dealing with the risk assessment of chlorpyrifos, carbendazim, and linuron were compared with the NOECs found in the current study. For chlorpyrifos, the overall NOEC was set at 0.005 μg/L, although a small effect on pH was observed in this study at this concentration (Table 2). This effect was dismissed because the differences were small (<0.5 unit) and not caused by an increase in pH in the treated systems but a decrease in the control systems. The $NOEC_{community}$ for the zooplankton community was therefore used as an overall NOEC. Also, for carbendazim, the zooplankton community proved to be the most sensitive end point and therefore its NOEC was also used as an overall NOEC. For the linuron experiment, two NOECs at the control level were calculated for day 21, at which point an increase in all systems of the algal species *Epithemia* and a decrease in the zooplankton species *L. bulla* (Table 3) occurred. The increase in the algal species *Epithemia* at the lowest concentration is probably not treatment related because no species are decreased at this concentration, and no effects on pH and DO were reported (Table 2). The decrease of *L. bulla* was only significant for one sampling date, and for all other sampling dates this species was not even present. We therefore set the overall NOEC of the linuron study at 0.5 μg/L (NOEC of DO and pH; Table 2).

With reference to chlorpyrifos, the absence of sediment and macrophytes resulted in a prolonged exposure. Therefore, although a single application of chlorpyrifos is evaluated in this

study, the effects are more comparable with those evaluating chronic exposure because of a lower dissipation compared with "normal" circumstances where sorption to sediment and macrophytes are present. Indeed, in this study, a lower NOEC for functional as well as structural end points was found compared with other microcosm studies with single chlorpyrifos application (Table 2). In a microcosm study with chronic exposure to chlorpyrifos and lindane, an overall NOEC of 0.01 μg/L was found (Van den Brink *et al*. 2002), which is comparable with this study.

In the experiment with carbendazim, macroinvertebrates were not present in large numbers, nor were taxa compared with those in other microcosm studies. Because the macroinvertebrate group, with *Oligochaeta*, *Turbellaria*, and *Hirudinea* as the most sensitive groups, is the most susceptible animal group to carbendazim (Cuppen *et al*. 2000; Van Wijngaarden *et al*. 1998), a higher overall NOEC was found in the present study compared with studies using more complex ecosystems (Table 2). The sensitivity of the zooplankton community was comparable with laboratory toxicity tests (NOEC *D. magna* = 26 μg/L, Van Wijngaarden *et al*. 1998) and microcosm studies with chronic carbendazim exposure (Cuppen *et al*. 2000). The microcosms used in the present study were more susceptible for effects on functional end points than reported in macrophyte-dominated microcosms (Table 2). This may have been the result of the absence of macrophytes and sediment decreasing the complexity of the ecosystem and the fact that the systems were closed.

The overall NOEC for linuron in this study was lower than the NOEC found by Van Geest *et al*. (1999; Table 4), who also evaluated a single application. However, it matched the NOEC as noted in a microcosm study with chronic exposure of linuron (Table 2), which may have been a result of the persistence of linuron in the present study (Table 1). The phytoplankton community in this study was a bit less sensitive than in the microcosm studies by Van den Brink *et al*. (1997) and Cuppen *et al*. (1997) (Table 2). These investigators found the most severe effects only after 4 weeks of exposure and explained this late response by assuming that phytoplankton species can survive until their energy reserves are depleted. The experimental period in the present study was only 3 weeks, so—presumably—the autotrophic organisms dosed with the lower concentrations could survive this time by using their energy reserves. As expected, chlorophyll-a levels were lowered in the higher-dosed microcosms (Table 2). In the microcosm study with chronic exposure, chlorophyll-a levels were increased because of a bloom of the insensitive *Chlamydomas*, a species not found in the present study. Effect on ecosystem functioning was comparable between our study and the study in the macrophyte-dominated microcosms (Table 2).

Implications for Risk Assessment and Final Conclusions

Because of differences in experimental design, our NOECs were not always consistent with the results of former experiments. The fate of pesticides was more comparable with constant than single peak exposure, mainly because of the lack of sediment. Including a sediment compartment would lead to a more realistic fate of the pesticide applied. In contrast, this would decrease the simplicity of the test system and hence the reproducibility and interpretability of the results.

In the carbendazim experiment, a higher overall NOEC was found because of the absence of macroinvertebrates, the most susceptible species group for carbendazim. Possibilities to include (sensitive taxa) of macroinvertebrates will have to be studied, although the small size of the test systems will not allow the development of a very rich macroinvertebrate community. Therefore, a pesticide risk-assessment study should be conducted in larger microcosms or mesocosms if major effects are expected on macroinvertebrates or macrophytes.

The general effect chains of chlorpyrifos, carbendazim, and linuron were the same as described in larger-scale microcosm studies. The simple design of the microcosm offers the possibility to perform experiments with a relatively high ecologic realism compared with laboratory tests under more controlled conditions and much more inexpensively than larger-scale model ecosystems. This makes these systems ideal for investigating ecologic processes and the chain of effects after stress, *e.g.*, pesticides. Recovery of a certain species can only be studied if the stressor does not lead to a complete disappearance of that species and the life cycle can be completed in water. Furthermore, these test systems are useful in selecting treatment concentrations for larger-scale model ecosystem studies.

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