Effects of three pesticides that differ in mode of action on the ecology of small indoor aquatic microcosms

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An evaluation of the effects of the insecticide chlorpyrifos, the herbicide atrazine and the fungicide carbendazim

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ABSTRACT

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In the current study, the usefulness of an 8 litre microcosm for the ecological risk assessment of pesticides is examined. Risk assessment studies were performed for three pesticides with different modes of action, i.e. insecticide (chlorpyrifos), fungicide (carbendazim) and herbicide (linuron) and compared to results from other, more larger scaled, experiments. When the recommendations workshops on the use of microcosm and mesocosm experiments in the ecological risk assessment of pesticides are used to evaluate the experimental design of the experiments, the test-system can be considered methodological correct. However, it should be noted that macrophytes and macroinvertebrates are absent (although snails were included in the three risk assessment studies) meaning that the test systems can not be used if major effects are expected on these organisms. Moreover, possible unrealistic fate of the chemical due to the absence of sediment is a demerit of these microcosms.

Keywords: Pesticides, ecological risk assessment, aquatic microcosms, chlorpyrifos, carbendazim, linuron

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Preface

This report is the result of a learning period performed as a part of the graduation program of Michiel Daam for Environmental Sciences and Health Sciences at the KUN. The work was supervised by Dr Paul Van den Brink from Alterra and co-supervised by Dr Jenny H.J. Copius Peereboom-Stegeman of the KUN. After finishing the learning period, graduation for both studies took place in August 2001. The research was also sponsored by the Dutch Ministry of Agriculture, Nature Management and Fisheries (DLO Research programme 359)

During the past years, numerous experiments have been conducted in micro- and mesocosms with the objective to set water quality criteria for pesticides or validate the procedures used to derive them. The size of the different artificial ecosystems that have been used vary to a great extent; from small aquaria in a laboratory up to artificial experimental ponds. Large experimental ecosystems correspond better to the natural situation and are consequently rather complex. On the other hand, experiments in smaller systems are easier to replicate and manipulate and are more useful in elucidating the chain of events following chemical stress than large systems (Leeuwangh et al., 1994).

On several European workshops on freshwater field tests (Monks wood workshop: SETAC-Europe, 1991); EWOFFT workshop: Crossland et al., 1992; HARAP workshop: Campbell et al., 1999; CLASSIC workshop: Gidding et al., 2002) the development of reliable, validated, more cost-effective smaller test systems was recommended. However, a small number of reliable experiments in small ecosystems have been conducted so far. In this study, experiments with an eight-litre test system were conducted to contribute to a better understanding of the use of small ecosystems in the risk assessment of pesticides.

Besides the authors, a large number of persons from Alterra contributed in conducting the experiments: Steven Crum and Arienne Matser (pesticide concentration analysis), Delia Van Dijk (nutrient concentration analysis), René Van Wijngaarden (zooplankton identification), Jos Sinkeldam (phytoplankton identification) and Leo Van der Pas (laboratory assistance). Moreover, Theo Brock assisted in the experimental design and read the report critically. They are all greatly acknowledged!

Definitions

BCF	Bio Concentration Factor
DT_{50}	half-life value for degradation
EC_{50}	concentration at which effects occur in 50% of the number of test organisms
Ecosystem	a functional unit consisting of a biotic community, the abiotic environment, and the mutual relations between community and environment; it is more or less self-supporting, and driven, directly or indirectly, by solar energy
First tier of aquatic risk assessment of pesticides:	first step in risk assessment of pesticides in which a pesticide is considered to be save if the PEC does not exceed the NEC
Higher tier of aquatic risk assessment of pesticides:	advanced tailor-made risk assessment procedure, in which ecologically more relevant data are included if the first tier indicates potential risks
KUN	Catholic University of Nijmegen, The Netherlands
LC_{50}	concentration at which mortality occurs in 50% of the number of test organisms
Mesocosm	man-made experimental ecosystem (i.e. tanks/ponds) with a volume of more than 15 m^3 or experimental streams more than 15 m length (Crossland *et al*., 1992)
Microcosm	man-made experimental ecosystem (i.e. tanks/ponds) with a volume of less than 15 m^3 water volume or experimental streams less than 15 m length (Crossland *et al.*, 1992)
NEC	No Effect Concentration, used in first tier risk assessment of pesticides, based on concentration-effect relationships studied with a limited number of "standard" species: an algae-, *Daphnia*- and fish-species
NOEC	highest concentration tested at which no effect is observed
$NOEC_{eco}$	NOEC for the most sensitive endpoint studied in the ecosystem
PEC	Predicted Environmental Concentration, generally calculated with the help of a computer model for a standard freshwater system on the basis of the recommended dose used for pest control and the amount of spray drift, drainage, run-off, atmospheric deposition and/ or accidental spills
UP	Uniform Principles (registration criteria for crop protection products according to the EU)
WU	Wageningen University, The Netherlands

Summary

In the current study, the usefulness of an 8 litre microcosm for the ecological risk assessment of pesticides is examined. In a first experiment, it was investigated what carbon source (CO_2, carbonate, or a combination) is most suitable to prevent a toxic rise in pH that was observed in a former study with these test systems. Thereafter, risk assessment studies were performed for three pesticides with different modes of action, i.e. insecticide (chlorpyrifos), fungicide (carbendazim) and herbicide (linuron).
In the IOC (InOrganic Carbon) experiment, CO_2 application alone or in combination with carbonate, resulted in a decrease in pH that negatively affected the zooplankton. The control and carbonate applied microcosms possessed a comparable and rather diverse zooplankton community after two weeks. Because carbonate application resulted in a higher buffer capacity (i.e. higher alkalinity) and a more "stable" zooplankton community compared to the untreated controls, carbonate was applied in the three experiments evaluating the effects of the pesticides.
In the study dealing with chlorpyrifos, effects were found at a concentration as low as 0.05 µg/L on structural (zooplankton community) as well as functional (e.g. pH) endpoints (NOEC = 0.005 µg/L). This concentration is lower than reported in other studies dealing with risk assessment of a single application of chlorpyrifos which is probably due to the absence of sediment and macrophytes and a consequent binding of chlorpyrifos, resulting in a prolonged exposure and therefore, more severe toxic effect and a lower NOEC.
Overall, the type and severity of the effects of carbendazim on the zooplankton community of the tested microcosms were comparable to other studies of carbendazim on model-ecosystems ($NOEC_{community}$ = 33 µg/L). However, in the other studies NOECs of 3.3 µg/L were found for one zooplankton species (*Bosmina* sp.) and on the macroinvertebrate community, with Oligochaeta, Turbellaria, Hirudinea and some Crustacea as the most sensitive groups. Since these groups except Crustacea were not represented in the tested microcosms, this could not be demonstrated in the current study. Possibilities to add these taxonomic groups into the test-system, will have to be investigated.
The NOEC of linuron in the current study was recorded at 5 µg/L. Comparable results were found in former microcosm studies dealing with a chronic treatment of linuron. The safety factors adopted by the EU in the UP obviously ensure adequate protection for the ecosystem in the case of single exposure to linuron.
When the recommendations from SETAC workshops on the use of microcosm and mesocosm experiments in the ecological risk assessment of pesticides are used to evaluate the experimental design of the experiments, the test-system can be considered methodological correct. However, it should be noted that macrophytes and macroinvertebrates are absent (although snails were included in the three risk assessment studies) meaning that the test systems can not be used if major effects are expected on these organisms. Moreover, possible unrealistic fate of the chemical due to the absence of sediment is a demerit of these microcosms.

1 Introduction

In the twentieth century, the use of pesticides expanded over years to gratify the increase in human population and the ability to raise profits of crops. The use of agricultural pesticides decreased in the last couple of years in the Netherlands (RIVM, 2001). However, the water quality standards of pesticides in the surface water as set by the Dutch government are still exceeded in 50 % of the sampled areas (RIVM, 2001; Nefyto, 2001).
Due to spray drift, drainage, run-off, atmospheric deposition and/ or accidental spills, pesticides frequently enter aquatic ecosystems (Capri and Trevisan, 1998). Since aquatic ecosystems include key species related to the target organisms of pesticides, undesirable side effects on aquatic plants and animals may ensue (Hill *et al.*, 1994). The consequences of pesticide exposure for aquatic ecosystems are therefore an important matter of concern in judging the acceptability of the use of pesticides.
In the first paragraph of this chapter, the registration for the placing of pesticides on the market as adopted by the European Union (EU, 1997) is presented. The relevance of the development of small model ecosystems is discussed in the second paragraph. In paragraph three, previous work on the microcosms used is presented. The aims of the present study are specified in the fourth paragraph. In the fifth paragraph, the toxicological data of the tested pesticides are scrutinised.

1.1 Tiered risk assessment approach

Obviously, the most realistic way to investigate the fate and effects of a pesticide is done under field conditions. Practical (i.e. time and costs) and ethical objections make it impossible to assess all pesticides in this way. Therefore, a tiered approach has been adopted for the admission of the pesticides on the market in Europe. In Table 1.1, the aquatic tiered risk assessment of pesticides is summarised.

Table 1.1. *EU criteria as set for the impact of pesticides on non-target aquatic species*

Tier	Criteria
First tier	Short-term PEC ≤ 0.01 LC50 or EC50 fish or daphnia
	Short-term PEC ≤ EC50 algae
	Long-term PEC ≤ 0.1 NOEC fish or Daphnia
	BCF ≤ 1000 for readily biodegradable active substances
	BCF ≤ 100 for not readily biodegradable active substances
Higher tier	Unless it is clearly established through an appropriate risk assessment that under field conditions no unacceptable impact on the viability of exposed species (predators) occurs – directly or indirectly – after use of the plant protection product according to the proposed conditions of use.

PEC: Predicted Environmental Concentration; LC50: Lethal Concentration 50%; EC50: Effect concentration 50%; NOEC: No Observed Effect Concentration; BCF: BioConcentration Factor

The first tier of aquatic risk assessment is based on the estimation of the PEC/NEC ratio. In this ratio, the concentration of the pesticide in surface water is predicted using model-simulation (Predicted Environmental Concentration) and compared with the expected No Effect Concentration (NEC). If the PEC/NEC ratio is smaller than one, no effect of the pesticide on the aquatic community is expected. The NEC is based on dose-response laboratory studies with a limited number of standard test species, i.e. algae, *Daphnia* and a fish. To protect sensitive species, the NEC is usually calculated by dividing the toxicity value of the most sensitive standard test species tested by an extrapolation factor (i.e. 100 or 10; see Table 1.1).
This first tier in the risk assessment procedure is rather simple and conservative because of the more or less worst case scenario to calculate the PEC, the safety factors used, the limited amount of species tested and the lack of ecological realism. Therefore, if the first tier indicates potential risks, European guidelines for the admittance of pesticides on the market offer the possibility to include ecologically more relevant data in an advanced risk assessment procedure, i.e. the second tier. This second tier does not consist of well-defined rules like the first one, but depends on the degree of uncertainty in the risk after the first tier. The requested additional information may range from a better estimation of the half-life of the chemical in water to additional experiments on ecosystem level (Campbell et al., 1999).

1.2 Usefulness of small model ecosystems

As mentioned in the former paragraph, the use of laboratory tests for the ecological risk assessment is questionable due to the little resemblance with the field situation. On the other hand, field studies are hampered by its complexity reducing reproducibility and making an experimental design impossible (Figure 1.1).

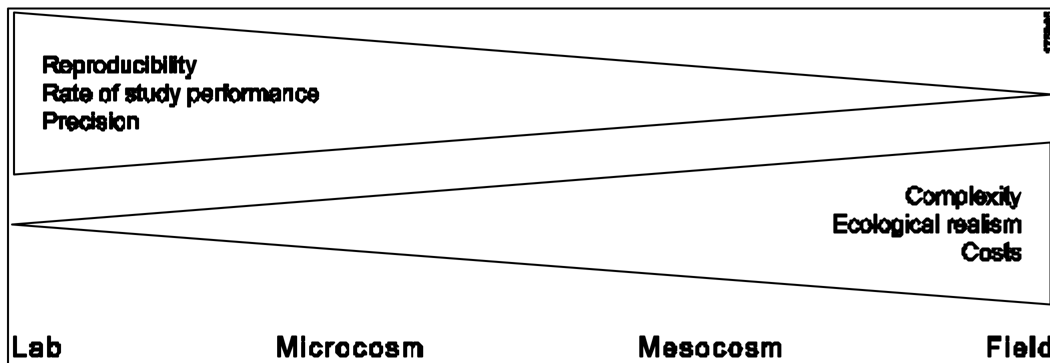

Fig. 1.1. *Microcosms and mesocosms provide a bridge between the lab and the field (Figure taken from Brock et al., 2000a)*

An alternative, frequently used, approach in pesticide aquatic risk assessment is the use of man-made experimental ecosystems: microcosms and mesocosms. The use of microcosms or mesocosms provides a bridge between laboratory and the field (Figure 1.1.), in terms of being manageable and allowing replication and hence an experimental set-up on the one side and providing realism in terms of ecological processes and exposure to the chemical on the other side (Brock *et al.*, 1995). The

difference between microcosms and mesocosms is their size and hence often their complexity (Van den Brink, 1999). A mesocosm has a volume of 15 m^3 or more, while a microcosm is defined as a model-ecosystem containing less than 15 m^3 (Crossland *et al*., 1992).

- Field tests should be seen in the context of a sequential (tiered) approach to hazard assessment. In general they only be required at a high tier (recommendation 2).
- Research studies should be carried out to investigate the effect of system dimensions on the fate of chemicals with different physicochemical properties (recommendation 1).
- The size of the system (for effect studies) can be chosen on the basis of the size and the kind of organisms identified as being most at risk (recommendation 12).
- Small-scale systems of 1 to 5 m^3 for studies on plankton and up to 25 m^3 for macroinvertebrates are more suitable for most freshwater field tests than are larger systems. In general, for cost reasons, the smallest possible system should be used to achieve the defined objective (recommendation 11).

Fig. 1.2. *Some recommendations towards future developments in freshwater pesticide tests by the EWOFFT (Crossland et al., 1992)*

It should be noted that 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. Since small model-ecosystems are easier to replicate and manipulate, they prove to be more useful in elucidating the chain of events following chemical stress than large test-systems (Leeuwangh *et al*., 1994). On the other hand, small microcosms are ecologically more realistic than laboratory studies. Consequently, with reference to Figure 1.1, they can be considered as a "bridge" between the lab and (large) microcosms and mesocosms.
On the European Workshop on Freshwater field tests (Crossland et al., 1992) in Potsdam (Germany) the development of reliable validated more cost-effective smaller test-systems was recommended (Figure 1.2). The HARAP workshop (Campbell et al., 1999) identified the fact that they can be run throughout the year as an extra advantages of semi-realistic laboratory microcosm tests over outdoor field tests. They also defined potential disadvantages: they do not contain normal densities of large organisms, long-term effects and recovery is difficult to study, the number of microhabitats present in the systems is limited and adequate sampling can be problematic. Only a small number of reliable small test-systems, however, have been conducted so far for pesticide risk assessment (Leeuwangh *et al*., 1994; Brock *et al*, 2000a; Brock *et al*., 2000b). This meets the need for validation of small test systems.

1.3 Validation of a 8 litre microcosm

In the current study, the usefulness of an 8-litre microcosm for the risk assessment of pesticides is investigated. Previous work by Jonker (2000) was focused on the possibility of developing a stable phyto- and zooplankton community in the test systems. The systems were filled with pool water and different amounts of nitrate

and phosphate were added to determine the most suitable level of nutrient supply. The experimental design of this pilot-study is summarised in Table 1.2.

Table 1.2. *The experimental design of the pilot experiments by Jonker (2000) on the microcosms*

	Experiment 1				Experiment 2			
Nutrient level	0	1	2	3	0	4	5	6
Pool water added (L)	8.5	8.5	8.5	8.5	6	6	6	6
Sinderhoeve water (L)	0	0	0	0	0.45	0.45	0.45	0.45
Medium (L)	0	0	0	0	2	2	2	2
NO_3 (mg N/ week)	0	3.5	7	14	0	0.23	0.45	0.9
PO_4 (mg/ week)	0	0.39	0.78	1.55	0	0.038	0.075	0.15
Light period		3:15 until 17:15				9:15 until 1:15		
Simocephalus (#)	40	40	40	40	35	35	35	35
D. magna (#)	0	0	0	0	10	10	10	10
Ostracoda (#)	0	0	0	0	10	10	10	10

During both experiments the pH raised above 11 in all systems that received nutrient additions. This high pH turned out to be lethal for the waterfleas *Daphnia magna* and *Simocephalus sp.*. Jonker (2000) discusses that a shortage of inorganic carbon is the most plausible explanation for this. Moreover, the variability of the biological parameters among the microcosms, i.e. chlorophyll-a and zooplankton, was high. This could be due to the low number of added zooplankton individuals. These problems will have to be solved before the use of the microcosms for ecological risk assessment of pesticides can be investigated.

1.4 Research questions

In order to continue the former work on microcosms, the following goals are formulated:

1. Development of a stable phyto- and zooplankton community in the microcosms.

In a first experiment, the ability to develop a stable phyto- and zooplankton community in the microcosms for at least two weeks is investigated. This means that the toxic drop in pH and the rather large variability in plankton community observed in the previous experiments will have to be prevented.
To this end, a carbon addition was applied to the microcosms as CO_2, $NaHCO_3$ or both to assess the preferable carbon source. The amount of nutrients applied was lowered and more zooplankton individuals were introduced compared to Jonker (2000) (for details see material and methods).

2. Assessment of the ecological threshold levels of three pesticides with different modes of action

To evaluate the usefulness of the microcosms for assessing the ecological risks of pesticides, the ecological threshold for three pesticides with different modes of action was determined. Chlorpyrifos (insecticide), carbendazim (fungicide) and linuron (herbicide) were chosen since reliable toxicity data is available from laboratory tests as well as macrophyte-dominated freshwater microcosms studies performed by our department (Chlorpyrifos; Van den Brink *et al.*, 1996; Van

Wijngaarden *et al.*, 1996; Van den Brink et al., 2002; Cuppen et al., 2002. Carbendazim; Cuppen *et al.*, 2000; Van den Brink *et al.*, 2000; Arts *et al.*, pers. comm. Linuron; Van den Brink *et al.*, 1997; Cuppen *et al.*, 1997; Kersting and Van Wijngaarden, 1999; Van Geest *et al.*, 1999) or other research groups (Slijkerman *et al.*, pers. comm.).

1.5 Relevant characteristics and toxicity data of the used pesticides

Some characteristics of the three pesticides are presented in Table 1.3. The acethylcholine-esterase inhibiting organophosphate chlorpyrifos was used as a model for insecticides. Since this insecticide has been studied extensively (see Brock *et al.*, 2000b for a review), a comparison with the current study could be made. Linuron was chosen as a model substance for the photosynthesis inhibiting herbicides, the type of herbicides most commonly used in the Netherlands (Van den Brink, 1999). Carbendazim, a benzimidazole fungicide, was evaluated because it is frequently used in the Netherlands. Semi-field experiments evaluating their effects on an ecosystem level were also available for both linuron as carbendazim.

Table 1.3. *Some characteristics of the pesticides evaluated as listed in the Pesticide manual (Tomlin, 1997. Table taken from Van den Brink, 1999)*

Pesticide group	Chlorpyrifos Insecticide	Linuron Herbicide	Carbendazim Fungicide
Chemical abstracts Name	0,0-diethyl 0-(3,5,6-trichloro-2-pyridinyl) phosphorothioate	N´-(3,4-dichlorophenyl)-N-methoxy-N-methylurea	Methyl 1H-benzimidazol-2-ylcarbamate
Log(Kow)	4.7	3.0	1.5 (pH=7)
Solubility (water, 25°C, mg/L)	1.4	63.8	7 (pH=8)
DT50 (days)	1.5-100	945	124 – >350
Mode of action	Cholinesterase inhibitor	Photosynthetic electron transport inhibitor	beta-tubilin synthesis inhibitor
Pest organisms	Coleoptera, Diptera, Homoptera, Lepidoptera	Grass, broad-leaved weeds, seedling perennial weeds	Micro-organisms

Table 1.4 summarises some relevant toxicity data of the three tested pesticides for aquatic standard test organisms. The $NOEC_{eco}$ for the pesticides as assessed in former model ecosystem studies are presented in Table 1.5.

Table 1.4. *Summary of relevant toxicity data of the pesticides for standard test organisms most susceptable to the chemicals (in µg/L)*

	Chlorpyrifios	Linuron	Carbendazim
LC50 Daphnia	0.2 (LC50, 48h; Van der Hoeven & Oldersma, 1989)	310 (LC50, 24h; Stepenson and Kane, 1984)	320 (LC50, 48h; Van Wijngaarden *et al.*, 1998)
NOEC Daphnia	0.1 (NOEC, 21d; Kersting and Van Wijngaarden, 1992)	-	10 (NOEC, 18d; Canton, 1976)
LC50 fish	4.7 (LC50, 96h; Van Wijngaarden *et al.*, 1993)	3200 (LC50, 96h; Crommentuijn *et al.*, 1997)	370 (LC50, 96h; Palawski and Knowles, 1986)
NOEC fish	-	-	-
EC50 algae	1000 (EC50, 72h; Van Donk *et al.*, 1992)	6 (EC50, 72h; Snel *et al.*, 1998)	340 (EC50, 48h; Canton, 1976)
NOEC algae	-	1.2 (NOEC, 72h; Snel *et al.*, 1998)	-

- : no data found

Table 1.5. *Summary of $NOEC_{ecosystem}$ and $LOEC_{ecosystem}$ of the tested pesticides in former model ecosystem studies (in µg/L)*

NOECecosystem	Chlorpyrifos	Linuron	Carbendazim
Single	0.1 – 0.9 (Van den Brink et al., 1996)	5 - 15 (Van Geest et al., 1999)	2.2 - 21 (Slijkerman et al., pers. comm.)
Chronic	0.01 – 0.05 (Van den Brink et al., 2002)	0.5 - 5 (Van den Brink et al., 1997)	3.3 - 33 (Cuppen et al., 2000)

2 Material and methods

2.1 Experimental set up

Each microcosm consisted of a glass chamber (diameter 24.5 cm; height 36 cm. Figure 2.1), filled with 8.5 litres of pool water taken from the pool north of the Alterra building (Wageningen, The Netherlands). The pool water was sieved over 0.75 mm before applying to the microcosms in order to exclude *Chaoborus* larvae, a known predator on zooplankton communities (Fedorenko, 1975; Black and Hairston, 1988).

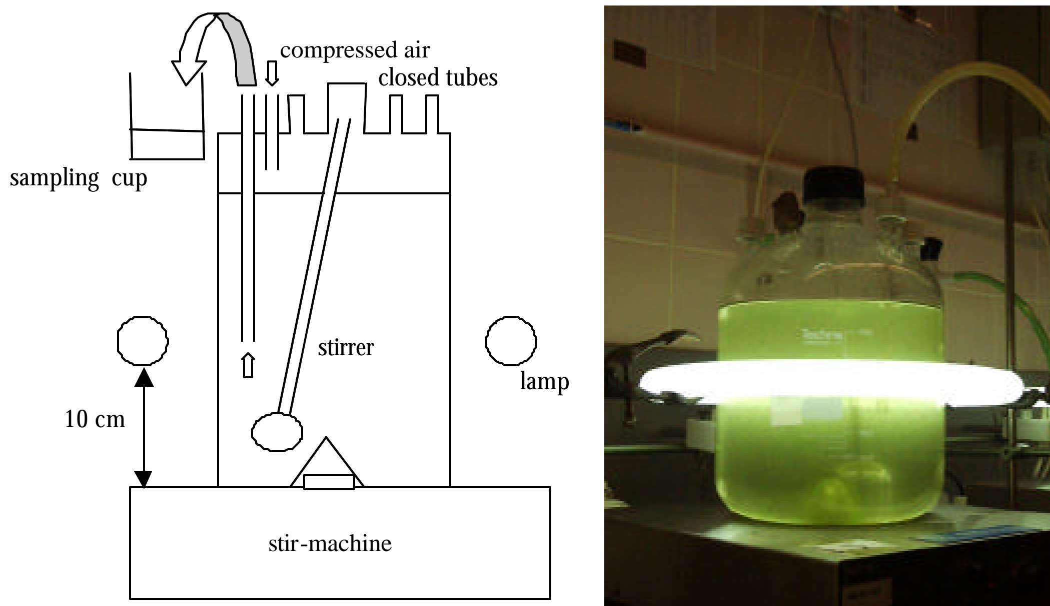

Fig. 2.1. *Schematic picture (left) and front view diagram (right) of test system*

The 12 microcosms were situated in a room disposed of daylight with a constant temperature of 21 ± 1 ºC (Figure 2.2). The systems were stirred for 5 minutes per 30 minutes at 20 rounds per minute (rpm) by means of a MCS-101L biological stirrer to achieve a water flow through the system. Around the microcosms, a fluorescent lamp (Philips TL'E 40W/33) was placed, 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 edge of the microcosms. The daily photoperiod was 14 hours (9.15 till 23.15).

Fig. 2.2. *The laboratory room accommodating the 12 test systems*

Besides an opening for the stirrer, the microcosms contained five smaller openings (Figure 2.1) of which four were closed with air-proof screw caps and one was connected to compressed air. To take water samples, one screw cap was replaced by one containing a rubber ring through which a glass pipette (diameter 10 mm) was put. By adding compressing air into the system, an overpressure arose, resulting in a water flow through the glass pipette into the sampling cup (Figure 2.1).

2.2 Experimental design

2.2.1 IOC experiment

In former experiments with the microcosms, pH raised above 11 resulting in a complete elimination of waterfleas, presumably due to a shortage of carbon (Jonker, 2000). Therefore, a prior IOC (InOrganic Carbon) experiment was performed to evaluate whether a phyto- and zooplankton community in the microcosms could be maintained by adding carbon to the microcosms. The inorganic carbon sources tested were carbon dioxide (CO_2), sodiumcarbonate ($NaHCO_3$) and a combination of these carbon sources (CO_2 + $NaHCO_3$).
Table 2.1 summarises the experimental design of the IOC experiment. On 5 July 2000 (day –5) the microcosms were filled with pool water, filtered over a 0.75 mm sieve. A day later, a first nutrient addition of 0.115 mg N (as $NaNO_3$) and 0.014 mg P (as KH_2PO_4) was applied to stimulate the growth of phytoplankton. During the rest of the experiment, this amount of nutrients was applied twice a week (Table 2.1). Zooplankton, derived from the pool north of the Alterra building (Wageningen, the Netherlands) was added to the microcosms 3 days before the start of the experiment, allowing equilibrium to develop in the microcosms.

Carbon was applied on 10 July 2000 (day 0), in 3 triplicate groups (CO_2, $NaHCO_3$ and CO_2 + $NaHCO_3$), while three other systems served as controls. Halve a litre CO_2 was administered from a CO_2-bottle (Hoekloos®) via a compressor and little rubber tubes. Carbonate applied microcosms received 0.96 mg $NaHCO_3$ on day zero and a similar amount was applied twice a week during the rest of the experiment to refill possible losses and to buffer nutrient applications.

Table 2.1. *Timing of additions and sampling, in days relative to the (start of the) treatment, during the four experiments*

	IOC	*Chlorpyrifos*	*Carbendazim*	*Linuron*
Introduction of 8.5 litres pool water	-5	-7	-7	-6
Pre-treatment nutrient addition(s)	-4	-7 and -4	-7, –5 and -3	-6 and -4
Addition of plankton	-3	-3	-5	-4
Addition of snails	-	-	-1 (*B. leachii*)	-3 (*L. palustrus*)
Nutrient application Measurement of all parameters	0	0	0	0
Addition of decomposition compartment	-	-	0	0
(Start of the) treatment	0	0	0	0
pH, temperature, O_2 (twice a day)	1-14	1-14	1-21	1-21
Chlorophyll-a, ammonium, nitrate, ortho-phosphate, conductivity, alkalinity (three time a week)	0, 2, 4, 7, 9, 11, 14	0, 2, 4, 7, 9, 11, 14	0, 2, 4, 9, 11, 14, 16, 18, 21	0, 2, 4, 9, 11, 14, 16, 18, 21
Nutrient application (twice a week)	3, 7, 10	3, 7, 10	3, 7, 10, 14, 17	3, 7, 10, 14, 17
Zooplankton	14	14	21	0, 3, 7, 14, 21
Phytoplankton	-	-	-	21
Decomposition	-	-	21	21
End of experiment	14	14	21	21

2.2.2 Chlorpyrifos

Table 2.1 also summarises the design of the experiment performed with the insecticide chlorpyrifos. A nutrient addition of N (0.115 mg, as $NaNO_3$), P (0.014 mg, as KH_2PO_4) and HCO_3 (0.7 mg, as $NaHCO_3$) was applied twice in the pre-treatment period and twice a week in the treatment period. With regard to the measured endpoints, the experimental set-up equals the set-up of the IOC experiment.
The treatment started on 10 July 2000. On this day, a single dose of chlorpyrifos (nominal concentrations: 0.005, 0.05, 0.5 and 5 μg/L), applied as Dursban®4E, were administered to two microcosms for each concentration and mixed by stirring (20 rpm for 5 minutes). Four microcosms were not dosed with chlorpyrifos and served as controls. At several moments during the experiment (day 0, 3, 7 and 14), water samples of 250 ml were taken at mid-depth (during stirring; 20 rpm for 5 minutes)

from each microcosm by means of a glass pipette and extracted with octadecyl (C-18, Bakerbond) solid phase extraction 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 2 successive portions of 2 ml hexane 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 GC-cups. The closed cups were stored at -22°C until analyses.
Chlorpyrifos was determined by splitless injection of 3 μl on a HP 5890 Gas Chromatograph. GLC operating parameters for the column: wide-bore WCOT fused silica capillary, coated with CP Sil 5CB, length 25 m, temp 240°C, nitrogen flow 10 ml/min; injection block: temp 250°C; detector: NPD, temp 280°C, hydrogen flow 3.5 ml/min; air 90 ml/min and auxiliary gas nitrogen 20 ml/min. Retention time of chlorpyrifos about 4 min. Detection limit 2.5 pg. Chlorpyrifos recovery from the water was 83.3 ± 8.4 (mean ± sd, n=6).

2.2.3 Carbendazim

The experimental set-up of this experiment is summarised in Table 2.1. In comparison to the former two experiments, the experimental period was prolonged to 21 days and snails (*Bithynia leachii*, 8 per microcosm) and a decomposition component (*Populus* leaves) were added to the systems. The snails were obtained from the agricultural area "de Veenkampen" (Wageningen, the Netherlands) and are moderately sensitive to carbendazim (NOEC 100 μg/L, Cuppen *et al.*, 2000).
On 25 September, a single initial dose of carbendazim (nominal levels: 3.3, 33, 100 and 1000 μg/L), applied as Derosal®, were administered to two microcosms for each concentration and mixed by stirring (20 rpm, 5 minutes). Four microcosms were not dosed and served as controls. Water samples (± 10 ml) were taken at several moments during the experiment (day 0, 3, 7, 14 and 21) at mid-depth during stirring with 20 rpm from each microcosm by means of a glass pipette. Samples were transferred to glass test tubes and stored at 4°C. At analyses, sub-samples were taken after mixing and directly analysed with high performance liquid chromatography (HPLC) using an external standard as described in Van Wijngaarden *et al.* (1998).

2.2.4 Linuron

As shown in Table 2.1, the experimental design of this experiment was comparable to the experiment dealing with carbendazim. In contrast, *Lymnea palustrus* (8 per microcosm) was used in this experiment since *Bithynia leachii* could not be attained in sufficient amounts because of season-influences.
Four Linuron doses were applied on 5 November 2000 as Afalon to two microcosms each (0.5, 5, 50 and 150 μg/L), while four microcosms served as controls. Water-samples were taken at several times of the experiment (day 0, 3, 7, 14 and 21) at mid-

depth during stirring with 20 rpm from each microcosm by means of a glass pipette. Samples with low linuron concentrations (control, 0.5 and 5 μg/L doses) were extracted with octadecyl (C-18, Bakerbond) solid phase extraction columns. The columns were conditioned with 5 ml methanol and 5 ml distilled water. After extraction of a certain volume of water, linuron was eluted from the column with 3 successive portions of 1 ml methanol and evaporated till 1 ml. The samples were then diluted with distilled water to a fixed volume of 5 ml and stored at 4°C. Water samples with a higher linuron concentration (50 and 150 μg/L) were analysed without previous treatment. Analysis was carried out with HPLC as described in Van Geest *et al.* (1999). Linuron recovery from water was 105.9 ± 5.8% (mean ± sd, n = 8).

2.3 Sampling and analyses of parameters

Water quality parameters

Dissolved oxygen (DO), pH and temperature were measured every working day in the morning, at the start of the photoperiod (9.15), and seven hours later (16.15). By measuring the difference in DO during the first seven hours of the light period, 90 till 95% of the 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 in paragraph 2.1. In this water sample, DO was measured first with a WTW oxygen meter (OXI 196) connected to a WTW oxygen probe (EO 196). Then, pH and temperature were measured with a WTW pH meter (pH 323) and a LF 91 temperature meter, respectively. The oxygen- and pH meters were verified every (sampling) day.
Alkalinity was measured 3 times (Monday, Wednesday and Friday) a week by tritrating a 25 ml water sample with 0.01 N HCl until pH 4.2. The exact titre of the HCl was determined using Borax (according to Van der Linden and Visser, 1969). Conductivity was measured with a WTW conductivity meter.
The nutrient concentrations were determined 3 times a week using the over glass fibre filters (Whatman GF/C) filtered samples of the chlorophyll-a measurement. Subsamples were transferred into centrifugal-tubes and stored (4° C) prior to analyses. At the end of the experiment, the samples were analysed 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).

Chlorophyl-a

Chlorophyll-a content was determined 3 times a week during the experimental period. To homogenise the water layer, microcosms were stirred for 30 seconds at 70 rpm. In order to prevent mass zooplankton losses, samples of 250 ml were taken only halve a minute after stirring. Water samples were filtered through glass fibre filters (Whatman, GF/C; 4.7 cm ∅), dried upon small aluminium dishes and analysed the same day.

Dried filters were transferred to numbered centrifugal-tubes with the side of the filter containing the chlorophyll-a pointing to the inside. The rest of the procedure was carried out in green light, since light with another wavelength influences the chlorophyll-a activity. After adding 10 ml 80% v/v ethanol (Merck), the tubes were closed, mixed 10 seconds on a vortex (full speed) and transferred to a preheated water bath for exactly 5 minutes. Thereafter, the tubes were cooled in ice, mixed another 10 seconds on the vortex (full speed) and spun for 5 minutes at 3000 rpm in a cooled centrifugal machine (Heraens, 6 ºC). The upper liquid was divided between two cuvets and the extinction at 750 nm (E_{750}) and 665 nm (E_{665}) was measured using a spectrophotometer. After adding a drop of 0.4 N HCl, the extinctions were measured again. The chlorophyll-a content was determined using the following formula:

$$\text{Chlor.-a } (\mu g/l) = 29{,}6 \times ((E665-E750)-(E665HCl - E750HCl)) \times (v/(V \times I))$$

v = volume extract in ml (=10 ml)
V = sample volume in liters (=0.25 liter)
I = Light distance in cuvet in cm (=1 cm)

Decomposition of particulate matter

In the carbendazim and linuron experiments, decomposition of particulate organic matter (POM) was studied using dried *Populus* leaves. The *Populus* leaves were acquired from previously leached (three times for two days) and dried (60 °C, 72 hours) leaves. Portions of approximately 0.4 grams were weighted and enclosed in stainless steel tea-eggs, leached for 3 days in distilled water and incubated at mid-height on day zero. At the end of the experiment (day 21), the content of the tea-eggs was gently washed in the corresponding microcosms to separate algae and invertebrates from POM. The leaf material was transferred to aluminium dishes to determine dry weight (105°C, 24 hours).

Phyto- and zooplankton

At the end of the experiment, the microcosms were stirred for 30 seconds at 70 rpm and emptied until a final volume of 6 litres. This was concentrated by passing the water through a 40 μm mesh net. Formol (Boom) was added until a final concentration of 4% v/v to preserve the samples.
In the linuron experiment, zooplankton was also sampled during the course of the experiment (Table 2.1). Water samples of 250 ml were transferred to glass cylinders after stirring the microcosms for 30 seconds at 70 rpm. Formol (Boom) was added (4%) to fix the samples, after which the cylinders were sealed with parafilm and decanted. After 2 days, the upper liquid was removed and the remainder was transferred to pre-weighted plastic bottles.
The micro-zooplankton was identified (to species level where possible) in a weighted subsample with a Zeiss inverted microscope. Since the density of macro-zooplankton was always relatively low, total samples were identified using a binocular microscope (Wild Heerburgg). Identification of phytoplankton was done in 10 counting fields of a subsample. Zooplankton- as well as phytoplankton data were eventually converted to number of individuals per litre. Moreover, total number of waterflaes were

counted on several moments during the experiment in a 250 ml watersample. Hereafter, this sample was returned to the cosm.

Snails

At the end of the carbendazim and linuron experiments, the (sub)lethal effects of the pesticides on *Bithynia leachii* and *Lymnea palustrus*, respectively, were determined. The sublethal effects were screened by evaluating their grazing activity, *i.e.* numbers of individuals grazing. Moreover, effects on *Bithynia leachii* were also measured by attempts to open the operculum with a pair of forceps. The subletal effects were screened by evaluating their grazing activity, i.e. numbers of individuals grazing and the number of individuals on the glass wall.

2.4 Statistics

The endpoints measured in the IOC experiment were evaluated using the Dunnet's test, i.e. every treatment was tested against the control. For the experiments performed with the pesticides, NOECs were calculated for all parameters using the Williams test, which assumes an increasing effect for an increasing dose. Analyses were performed with 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 valid.
The differences in structure of the zooplankton communities between the microcosms as sampled at the end of the experiments (and in addition the phytoplankton community of the linuron experiment) were visualised with the ordination technique called Principal Component Analysis (PCA) (Ter Braak, 1995; Van Wijngaarden *et al*., 1995). Ordination is able to express 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 which maximise the variation in species composition between sites, i.e. which 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 Figure 3.2 as an example). Samples with nearly identical species composition lie close together in the diagram, while samples that lie far apart have very different species composition. In the diagrams (biplots), species arrows point in the direction of higher values. Before analyses with CANOCO for windows (version 4. Ter Braak and Smilauer, 1998), the abundance data of the communities were $Ln(0.002x + 1)$ transformed, where x stands for the abundance value. This was done to down-weight high abundance values and approximate a normal distribution of the data (for rationale, see Van den Brink *et al*., 2000).
In the linuron experiment, the identifications of the zooplankton community during the course of the experiment were analysed by PRC (Principal Response Curves) using the CANOCO software package version 4 (Ter Braak and Smilauer, 1998). The

sample taken for zooplankton determination at the end of this experiment (i.e. 6 litres) is from a sampling point of view non-comparable with the samples taken during the course of the experiment (i.e. 250 ml). Therefore, the determined zooplankton community at the end of the experiment was not included in the analysis. PRC is based on the Redundancy Analysis ordination technique (RDA), the constrained form of Principal Component Analysis (Van den Brink and Ter Braak, 1999). The analysis result in a diagram showing the sampling day on the x-axis and the first Principal Component of the treatment effects on the y-axis (see Figure 3.16 as an example). This yield a diagram showing the deviations *in time* of the treatments compared to the control. 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.
For the IOC experiment Monte Carlo permutation tests were performed for the zooplankton data set, allowing the significance of the effects of every treatment to be tested against every other treatment.
For the other experiments this was not possible due to a lack of replication. For the experiments performed with the pesticides we 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).

3 Results

3.1 IOC experiment

Physico-chemical condition

During the entire experiment, the dissolved oxygen (DO) was rather stable in all microcosms and ranged from 8.4 to 13.0 with DO concentrations in the afternoon generally 0.6 to 1 mg/L higher than morning values. DO remained above 6 mg/L in all treatments, so anoxic conditions never occurred in the water column. Water temperatures fluctuated from 21.0 to 22.7 °C with temperatures in the afternoon higher than in the morning (mean difference 0.9 °C). No significant differences were found for water temperatures neither DO levels between the different treatments.
Application of CO_2, alone or in combination with HCO_3, resulted in a significant drop in pH to values as low as 6.1 ± 0.1. Although some recovery occurred, pH remained significantly lower than control and HCO_3 applied microcosms until the end of the experiment (Figure 3.1A).

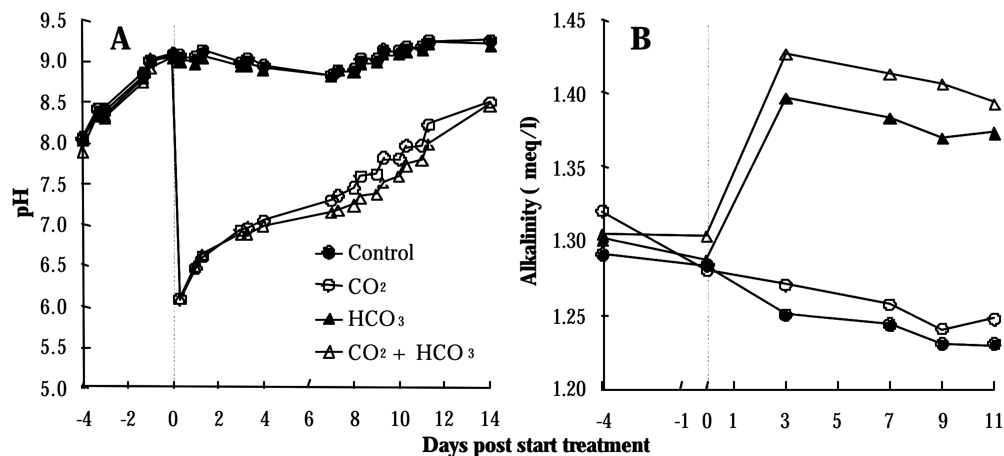

Fig. 3.1. *The level of pH (A) and alkalinity (B) at the different treatment levels during the course of the IOC experiment*

After the first carbonate application, alone or in combination with CO_2, alkalinity was increased compared to control and CO_2 treatments. This trend was maintained during the experimental period (Figure 3.1B). All carbon applications led to an increase in conductivity compared to the controls. Nutrient levels were low and no effect of any treatment was found (NH_4 < 0.1; NO_3 < 0.3; PO_4 < 0.02 mg/L).

Chlorophyll-a

Application of CO_2 and/ or HCO_3 did not lead to consecutive treatment effects on chlorophyll-a contents. However, chlorophyll-a values were increased on day three in microcosms applied with CO_2 either alone or in combination with HCO_3 compared to the controls ($p < 0.05$). On day 7 chlorophyll-a levels had returned to low control values (data not shown).

Zooplankton

At the end of the experiment, a total of 13 zooplankton taxa were identified in the microcosms. In order of decreasing abundance in terms of numbers of taxa, the community consisted of Rotatoria (*Lecane group lunaris, Lecane bulla, Keratella coclearis, Keratella quadrata* and *Lepadella patella),* Cladocera (*Chydorus spp., Daphnia magna, Daphnia galeata* and *Alona rectangula*), Copepoda (Cyclopoïda and nauplii), Insecta (*Cloeon spp.*) and Ostracoda (not identified at the species level).

A biplot of the principal component analysis (PCA) on the zooplankton data set is presented in Figure 3.2. The diagram summarizes the application effects in the data set, while still indicating the approximate species composition for all samples. Samples with nearly identical species composition lie close together, while samples with very different species composition lie far apart.

If an imaginary line is drawn through a species point and the origin of the plot, the relative abundance of this species in all samples can 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. The greater the distance between the projection of a sample and the origin, the more abundant this species is in this sample. If a sample point projects on the other side of the origin compared to the species point, numbers of this species are relative low in this sample.

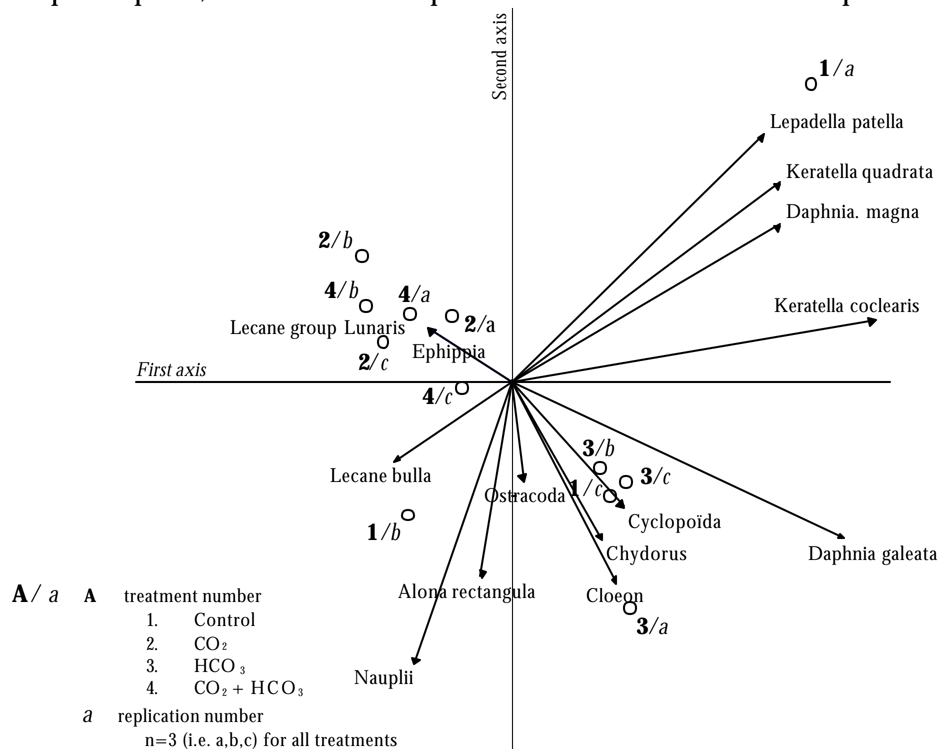

Fig. 3.2. *Ordination diagram (PCA) showing the differences in zooplankton community of (1) control microcosms and microcosms with application of (2) CO_2 (3) carbonate (4) CO_2 and carbonate. Of all variance, 49% is displayed on the horizontal axis and another 18% on the second axis*

The PCA-diagram indicates differences in zooplankton community composition between the different treatment groups (Figure 3.2). The clustering of the samples

from the CO_2 and $CO_2 + HCO_3$ applied microcosms in the upper left quadrant indicates differences in community composition of these microcosms relative to controls and HCO_3 applied microcosms. The permutation tests show that the zooplankton communities in CO_2 and $CO_2 + HCO_3$ microcosms were significantly different from HCO_3 applied microcosms but no significant difference was found compared to controls (Table 3.1). This is presumably due to the rather high variation within the controls, as the three replicates are positioned in three different quadrants of the PCA-diagram. Indeed, univariate analysis of zooplankton groups and taxa did not show significant differences between controls and any treatment (results not shown) but reveal significant decreases for *Keratella coclearis*, *Daphnia galeata* and nauplii in the CO_2 and $CO_2 + HCO_3$ microcosms compared to HCO_3-cosms (Table 3.2).

Table 3.1. *The results of the permutation tests for the different treatments. Since the power of the test was small due to the fact that only three replicates were used, p-values are set on 0.10*

Groups	P value
CTR vs CO_2	> 0.10
CTR vs HCO_3	>0.10
CTR vs $CO_2 + HCO_3$	>0.10
CO_2 vs HCO_3	0.10
CO_2 vs $CO_2 + HCO_3$	>0.10
HCO_3 vs $CO_2 + HCO_3$	0.10

Table 3.2. *Significant results of the univariate analyses of the zooplankton taxa and species in CTR-, CO_2- and $CO_2 + HCO_3$-cosms compared to HCO_3 cosms. Note that no differences between CTR- and HCO_3-cosms were found. All significant results are decreases in numbers compared to HCO_3-cosms*

Group/ species	CTR	CO_2	$CO_2 + HCO_3$
Rotatoria	-	< 0.01 ↓	-
Cladocera	-	< 0.05 ↓	< 0.05 ↓
Keratella cochlearis	-	< 0.05 ↓	< 0.05 ↓
Daphnia galeata	-	< 0.05 ↓	< 0.05 ↓
nauplii	-	< 0.05 ↓	-

In the diagram, several other species points are positioned far away from the left upper quadrant samples of CO_2 and $CO_2 + HCO_3$ microcosms, but no statistically significant decrease could be demonstrated. This is most likely due to low abundance values of these taxa in controls and HCO_3 applied microcosms. Therefore, univariate analyses were also performed for on zooplankton group level. This reveals that treatment with CO_2 in combination with HCO_3 resulted in a decrease in Cladocera, whilst treatment with CO_2 alone resulted in a decrease of Cladocera as well as Rotatoria (Table 3.2).

3.2 Chlorpyrifos

Chlorpyrifos concentrations

The mean nominal chlorpyrifos concentrations in the microcosms are presented in Table 3.3. These concentrations were within 10% from the target concentration for all applied doses.

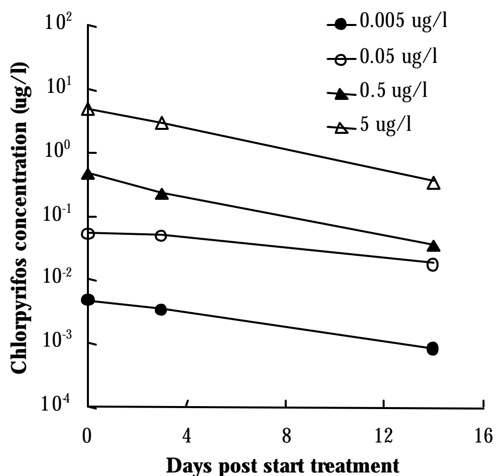

Fig. 3.3. *Chlorpyrifos concentrations in time*

Table 3.3. *Mean nominal Chlorpyrifos concentrations (µg/L) per dose.*

Target concentration (µg/L)	Nominal concentration (µg/L)
0.005	0.0048
0.05	0.053
0.5	0.47
5	4.92

Figure 3.3 shows the chlorpyrifos dynamics during the experimental period. No dose dependent differences in half-lives ($t_{½}$) were found for the applied doses (average 8 ± 3 days).

Physico-chemical condition

Like in the HCO_3 microcosms of the IOC experiment, the physico-chemical parameters measured in the control microcosms remained very stable during the course of the experiment. Oxygen-levels (10.0 ± 0.4), pH (10.0 ± 0.2), temperature (22.0 ± 0.8), alkalinity (0.9 ± 0.1), conductivity (285 ± 10.6) and nutrient concentrations (NH4 < 0.07; NO3 < 0.14; O-PO4 < 0.02 mg/L) were comparable to this former experiment.

Table 3.4 presents the NOECs as calculated by the Williams test for physico-chemical conditions. During the first week after the application of chlorpyrifos, oxygen levels were higher at all treatment levels but the lowest. Also the oxygen production was significantly increased at these concentrations ($p < 0.05$; Williams test).

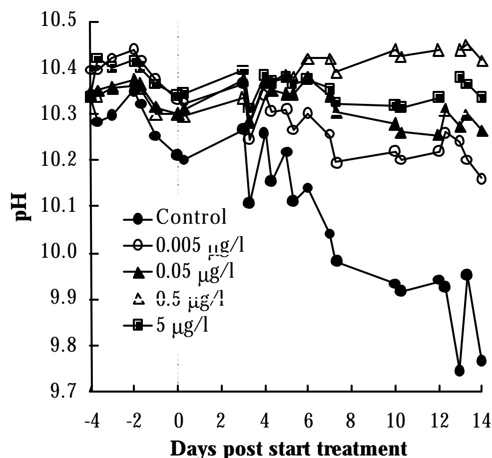

Fig. 3.4. *Effects of chlorpyrifos application on pH (A). Note the rise in pH for all applied doses after day .*

Table 3.4. *NOECs calculated for physico-chemical parameters*

Parameter	NOEC (µg/l)
pH morning	Control ↑
pH afternoon	Control ↑
O_2 morning	0.005 ↑
O_2 afternoon	0.005 ↑
O_2 production	0.005 ↑
Conductivity	0.05 ↑
Alkalinity	-
NH_4	-
NO_3	-
Ortho-PO_4	-

Chlorpyrifos had a prolonged effect on pH (Figure 3.4, Table 3.4). From 7 days post application onwards, pH was increased in all chlorpyrifos treatments. During the first 7 days, the lowest treatment level did not differ in pH from controls.
From 10 days post application onwards; conductivity was increased in the 0.5 and 5 µg/L applied microcosms. Application of chlorpyrifos had no significant treatment effect on the alkalinity or nutrient concentrations.

Chlorophyll-a

During the pre-treatment period, chlorophyll-a contents were rather stable in all microcosms. After application, chlorophyll-a values decreased in controls and the lowest chlorpyrifos application during the course of the experiment (Figure 3.5). In the higher chlorpyrifos concentrations, however, chlorophyll-a levels did not alter, leading to a concentration-dependent increase compared to controls (NOEC = 0.005 µg/L).

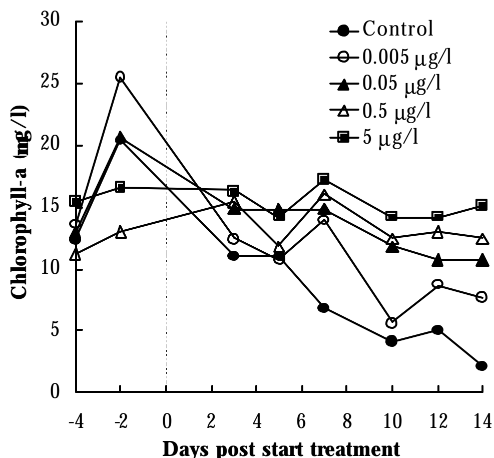

Fig. 3.5. *Effects of chlorpyrifos on the chlorophyll-a content*

Zooplankton

A total number of 21 invertebrate species were identified and there abundance determined. In terms of numbers of taxa, the most important taxonomic groups were Rotatoria (11), Cladocera (6), Copepoda (Cyclopoïda and nauplii), Insecta (1) and Ostracoda (not identified at the species level).

In Figure 3.6 A, the total number of waterfleas per litre is presented. Three days post application, waterfleas were absent in the two highest doses. In the 0.05 μg/L-applied microcosms, numbers of waterfleas were half of control values after three days and completely absent after one week. Numbers of waterfleas in the lowest application were comparable to control values throughout the experiment (NOEC = 0.005 μg/L).

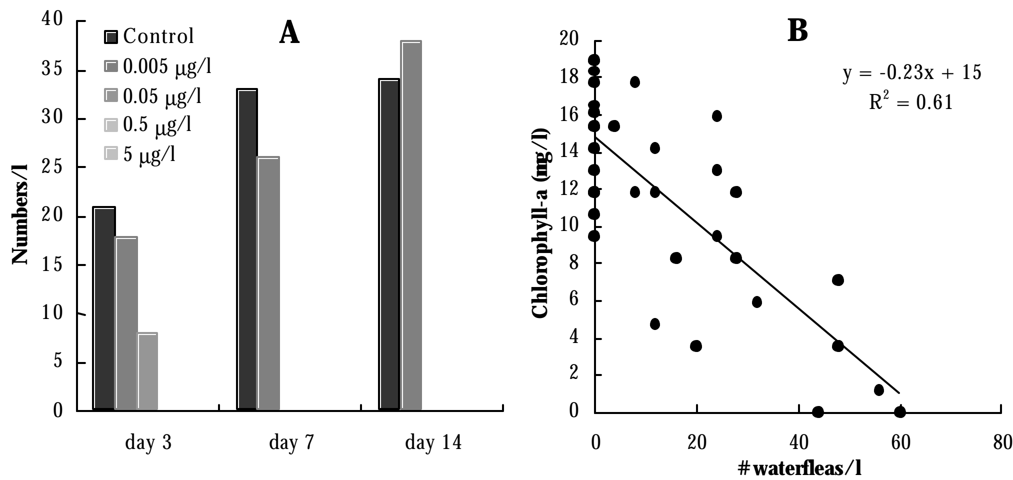

Fig. 3.6. *Number of waterfleas litre (A). Note that after 3 waterfleas were still observed in the 0.05 level. After 7 days, however, no waterfleas were found anymore in this level. Chlorophyll-a versus number of water-flies per litre (B)*

To test whether the increase in chlorophyll-a content could be (partly) attributed to the decrease in number of waterfleas, these parameters were correlated using simple linear regression (Figure 3.6 B). Indeed, a negative correlation between chlorophyll-a and number of waterfleas could be demonstrated ($R^2 = 0.61$, $p < 0.05$).

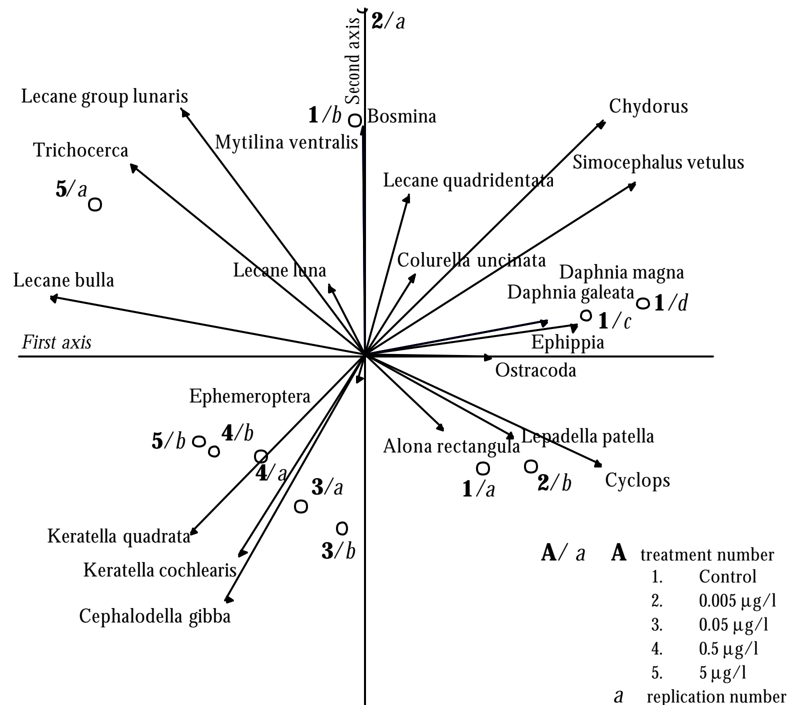

Fig. 3.7. *Ordination diagram (PCA) indicating effects of a single application of the insecticide chlorpyrifos on the zooplankton per treatment level. Of all variance, 36% is displayed on the horizontal axis and another 26% on the second one*

The PCA indicates pronounced treatment effects on the invertebrate data set (Figure 3.7, for interpretation of the diagram, see zooplankton section of the IOC experiment). The diagram reveals 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. The direction of the treatment vector in the diagram (Figure 3.7) is from the right to the left. Taxa less abundant in the treated microcosms are situated at the right and the unsusceptible and positively affected taxa at the left side of the diagram.

Table 3.5. *NOECs calculated for individual populations of the zooplankton community in microcosms treated with chlorpyrifos*

Species	NOEC (µg/l)
Lecane bulla	0.05 ↑
Trichocerca sp.	0.5 ↑
Lepadella patella	0.5 ↓
Cephalodella gibba	0.005 ↑
Chydorus sphaericus	0.005 ↓
Simocephalus vetulus	0.005 ↓

Numbers of *Chydorus sphaericus*, *Simocephalus vetulus* and *Lepadella patella* were significantly decreased at the higher treatment levels (Table 3.5). *Chydorus sphaericus*

and *Simocephalus vetulus* were even absent in the highest three concentrations. Taxa that occurred in significantly higher densities than in the controls were *Cephalodella gibba, Lecane bulla* and *Trichocerca*.
Overall, the Williams test on the PCA coordinates showed the treatment to have a significant effect on the invertebrate community at all chlorpyrifos applications but the lowest ($NOEC_{community}$ = 0.005 µg/L).

3.3 Carbendazim

Carbendazim concentrations

In Table 3.6, the target and nominal concentrations are enumerated. During the experimental phase (day 0 till 21) of the experiment, the average carbendazim concentrations of all treatment levels remained constant (see Figure 3.8). Therefore, the half-life for the disappearance of carbendazim from the water phase could not be calculated.

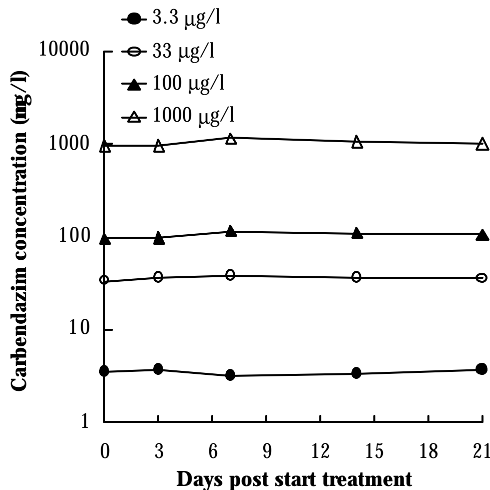

Fig. 3.8. *Mean carbendazim concentrations in the water phase per treatment level during the experimental period*

Table 3.6. *Target and nominal concentrations carbendazim*

Target concentration (µg/L)	Nominal concentration (µg/L)
3.3	3.5 ± 0.1
33	33.5 ± 1.6
100	97.8 ± 1.3
1000	976.5 ± 4.9

Physico-chemical conditions

During the pre-treatment period, the physico-chemical parameters were rather stable and comparable for all microcosms (results not provided). Application of carbendazim, however, had several effects on the DO-pH-alkalinity-conductivity syndrome (Table 3.7).
The highest carbendazim concentration led to an immediate and prolonged increase in DO production. Although DO levels were generally higher in the higher concentrations, no significant increase in DO could be demonstrated, which is probably due to the rather high variation in DO levels in the controls. The pH was significantly increased in the 1000 µg/L treated microcosms from the second week onwards and remained increased during the rest of the experiment (Figure 3.9, Table 3.7).

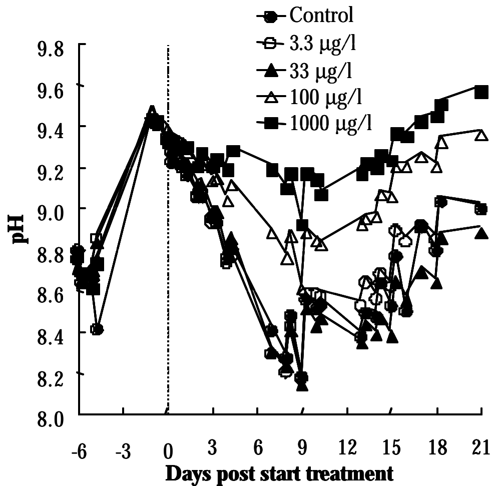

Fig. 3.9. *Effects of carbendazim on pH.*

Table 3.7. *NOECs calculated for physico-chemical parameters. Arrows indicate a significant increase (↑) or decrease(↓) compared to controls.*

Parameter	NOEC (µg/L)
pH morning	100 ↑
pH afternoon	100 ↑
O_2 morning	-
O_2 afternoon	-
O_2 production	100 ↑
Conductivity	3.3 ↓
Alkalinity	-
NH_4	-
NO_3	-
Ortho-PO_4	-

Corresponding with the increase in DO production and pH, all but the lowest treatments led to a decrease in conductivity between day 4 and 10 (Table 3.7). This effect, however, had a very small magnitude (1% difference between control and treatments) at all treatment levels. From two weeks onwards, however, no treatments were statistically different from controls. Carbendazim application had no significant treatment effects on either alkalinity or nutrient levels.

Chlorophyll-a

Application of the highest two carbendazim concentrations led to a time-dependent increase in chlorophyll-a content (Figure 3.10A). Four days post application, chlorophyll-a values in these treatment levels were twice and at the end of the experiment four times higher than in controls.

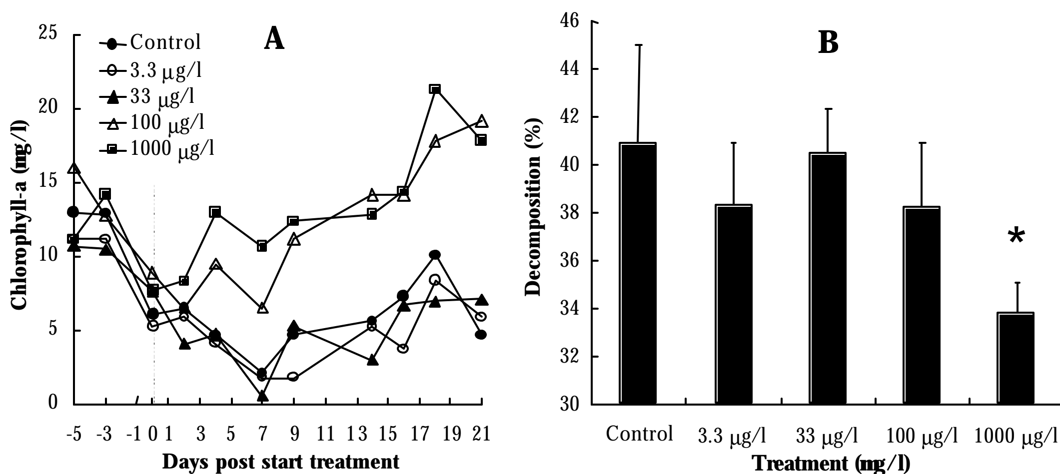

Fig. 3.10. *Chlorophyll-a concentration in time (A) and the decomposition of* Populus *leaves 21 days post application (B). Significant difference in % decomposition of* Populus *leaves are indicated by an asterisk,* $p < 0.05$

Decomposition

The relative amounts (%) of decomposition of *Populus* leaves after a decay period of three weeks are illustrated in Figure 3.10B. In controls and the lower carbendazim treatments, the residual dry weights amounted to approximately 60% of the initial dry weight. Application of the highest carbendazim concentration led to a significant decrease in breakdown compared to controls (Williams test, $p < 0.05$).

Zooplankton

A total number of 23 different zooplankton taxa were identified. In terms of the numbers of taxa, the control community was dominated by Rotifera (15), followed by Cladocera (4), Copepoda (Cyclopoïda and nauplii), Insecta (1) and Ostracoda (not identified at the species level).

Table 3.8. *Number of waterfleas in 250 ml*

Treatment/ Day	0	4	7	14	21
Control	2.9 ± 0.8	2.2 ± 1.7	12.0 ± 6.7	13.3 ± 3.9	6.0 ± 2.5
3,3 µg/L	2.5 ± 0.7	1.8 ± 1.4	10.3 ± 5.7	5.0 ± 3.5	8.4 ± 9.2
33 µg/L	2.0 ± 0	3.5 ± 0.7	10.5 ± 0.7	9.8 ± 2.8	5.5 ± 0.7
100 µg/L	1.8 ± 1.4	1.8 ± 1.4	1.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
1000 µg/L	2.5 ± 0.7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
NOEC	-	*100 µg/L*	*33 µg/L*	*CTR*	*33 µg/L*

The number of waterfleas in the microcosms revealed a clear concentration-dependent and time-related deviation of the 100 and 1000 µg/L treatments from the controls (Table 3.8). Waterfleas were totally absent in the highest concentration after 4 days. Two weeks post application, no waterfleas were counted in samples of both the 100 and the 1000 µg/L treated microcosms. Also, no individuals of any Cladocera species were identified in the 6 litres zooplankton samples taken at the end of the experiment from these microcosms (data not shown). Like for chlorpyrifos, a negative relation between chlorophyll-a and the number of waterfleas could be demonstrated ($R^2 = 0.32$, $p < 0.05$), indicating that the increase in chlorophyll-a can be (partly) contributed to the decreased abundance of waterfleas.
The PCA of the zooplankton dataset reveals a clear concentration-dependent deviation of the 100 and 1000 µg/L treatments from the controls (Figure 3.11). The visual differences were confirmed by the $NOEC_{community}$ calculation ($NOEC_{community}$ = 33 µg/L). Taxa negatively affected by the treatment are situated at the left side of the diagram, whilst insusceptible taxa are situated at the upper right quadrant (100 µg/L samples) and lower right quadrant (1000 µg/L samples). *Lepadella patella*, *Simocephalus vetulus*, *Grabtoleberes testudinalis*, *Alona rectangula* and *Ephippia* had a significant reduced abundance in the two highest treatments. Moreover, numbers of *Colurella uncinata* and *nauplii* were significantly lower at the highest treatment only (Table 3.9). *Branchionus angularis* occurred in higher densities at this treatment compared to controls. Total numbers of rotifers were only one third of controls ($p < 0.05$) in the highest applied dose and unaffected in the other applied carbendazim doses.

Table 3.9. *NOECs calculated for individual populations of the zooplankton community in microcosms treated with Cpf. Arrows indicate a significant increase (-) or decrease (¯) compared to controls*

species	$NOEC_{21\ days}$ (µg/L)
Branchionus angularis	100 ↑
Lepadella patella	33 ↓
Colurella uncinata	100 ↓
nauplii	100 ↓
Simocephalus vetulus	33 ↓
Grabtoleberes testudinalis	33 ↓
Alona rectangula	33 ↓
Ephippia	33 ↓

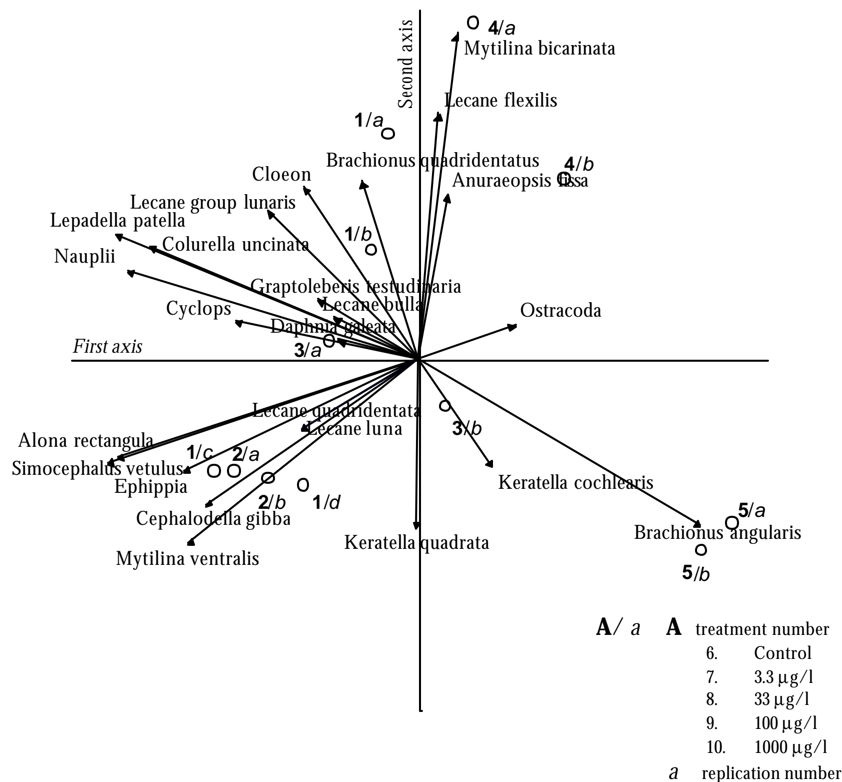

Fig. 3.11. *Ordination diagram (PCA) indicating effects of a single application of carbendazim on the zooplankton per treatment level. Of all variance, 37% is displayed on the horizontal axis and another 20% on the second one*

In summary, the zooplankton community revealed a dose-related response on the application of carbendazim. At the highest carbendazim dose, Cladocera and rotifers were eliminated respectively declined in numbers except *Brachionus angularis*. In the 100 µg/L applied microcosms Cladocera totally disappeared but rotifers were unaffected. The lower carbendazim applications did not lead to any significant treatment effects on the zooplankton community.

Macroinvertebrates; B. leachii

Application of carbendazim did not lead to a lethal effect on *Bithynia leachii*. Only one out of the initial eight snails was dead in the highest carbendazim treated microcosms with no dead individuals observed in the other microcosms.

Table 3.10. *Effect of carbendazim on the behavior of* B. leachii *expressed as the number of individuals on the glass walls, the number of individuals grazing and the ability to close the operculum (= operculum reflex) 21 days post application. Note that the NOEC is 100, 33 and 100 μg/L, respectively (Williams-test, $p < 0.05$)*

Parameter	Control	3.3 μg/l	33 μg/l	100 μg/l	1000 μg/l	*NOEC*
On the glass-wall	2.5 ± 0.6	2.5 ± 0.7	2.5 ± 0.7	2.5 ± 0.7	0.0 ± 0.0	*100 μg/l*
Grazing	7.3 ± 1.0	6.0 ± 0.0	7.5 ± 0.7	4.5 ± 0.7	0.0 ± 0.0	*33 μg/l*
Operculum reflex	7.8 ± 0.5	8.0 ± 0.0	8.0 ± 0.0	7.0 ± 1.4	5.0 ± 1.4	*100 μg/l*

However, carbendazim had a significant sublethal treatment effect at the 1000 μg/L and to a lesser extent at the 100 μg/L treatment levels (Table 3.10). The grazing behaviour was affected in the two highest carbendazim concentrations. In addition, in microcosms dosed with 1000 μg/L, the operculum reflex was decreased compared to control animals.

3.4 Linuron

Linuron concentrations

Figure 3.12 shows the linuron concentrations during the course of the experiment per treatment level. During the experiment, all linuron concentrations remained within 10% of the nominal concentrations (Figure 3.12; Table 3.11).

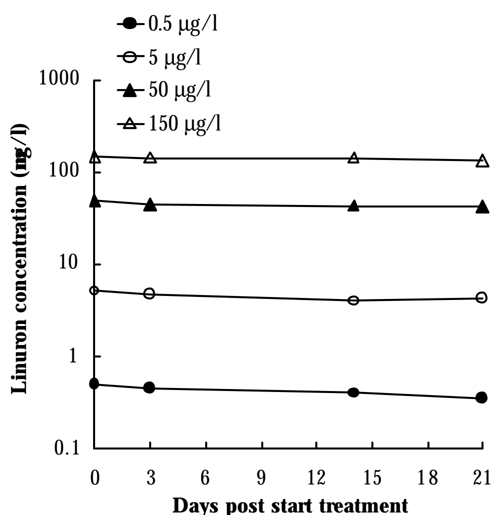

Fig. 3.12. *Mean linuron concentrations in the water for each treatment level*

Table 3.11. *Target and nominal concentrations of linuron*

Target concentration (μg/L)	Nominal concentration (μg/L)
0.5	0.49 ± 0.03
5	5.1 ± 0.0
50	49.5 ± 6.0
150	149.7 ± 0.5

Effects on primary producers

The PCA-biplot (Figure 3.13) 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*, *Epithemia* and *Closterium* individuals increased (Table 3.12). 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. The $NOEC_{phytoplankton\ community}$ as calculated by a Williams test on the PCA coordinates was 50 µg/L

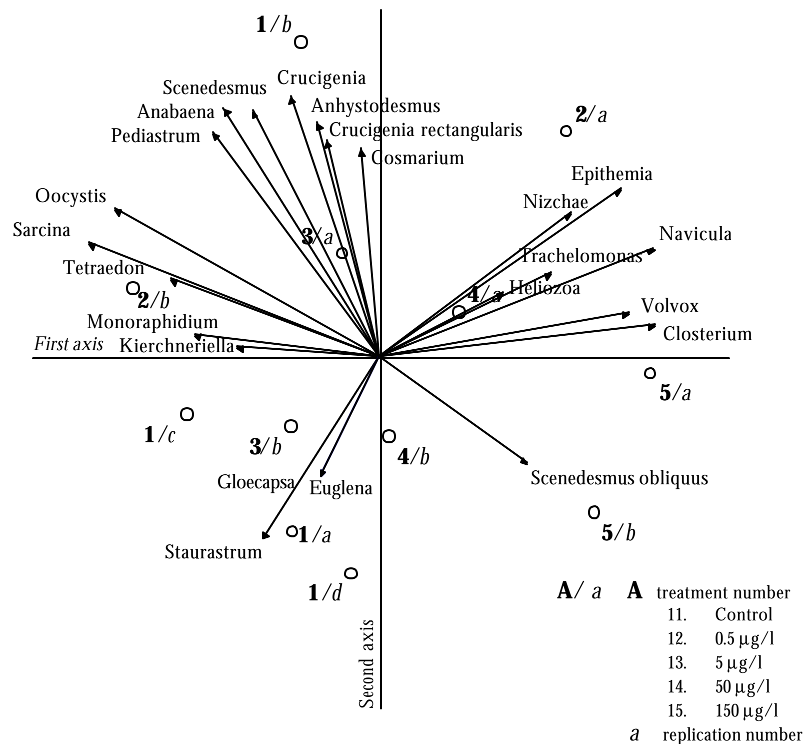

Fig. 3.13. *Ordination diagram (PCA) indicating effects of a single application of linuron on the phytoplankton per treatment level. Of all variance, 38% is displayed on the horizontal axis and another 20% on the vertical axis*

Table 3.12. *NOECs calculated for individual phytoplankton species in microcosms treated with linuron*

species	NOEC21 days (µg/L)
Navicula	5 ↑
Epithemia	CTR ↑
Scenedesmus	5 ↓
Monoraphidium	5 ↓
Closterium	50 ↑
Pediastrum	50 ↓
Tetraedon	50 ↓

The taxa *Scenedesmus*, and *Monoraphidium* were significantly less abundant in the highest two linuron applications compared to controls. In addition, *Pediastrum* and *Tetraedon* were significantly reduced at the highest application (Table 3.12). Reduction of total number of phytoplankton individuals was also most prominent in the 150 µg/L samples. In these samples, values were only one fourth of control levels (data not shown).

Chlorophyll-a

During the course of the experiment, chlorophyll-a content increased in controls and microcosms applied with the two lowest linuron concentrations (Figure 3.14). At the end of the experiment, chlorophyll-a levels were tripled in these microcosms compared to pre-treatment values.

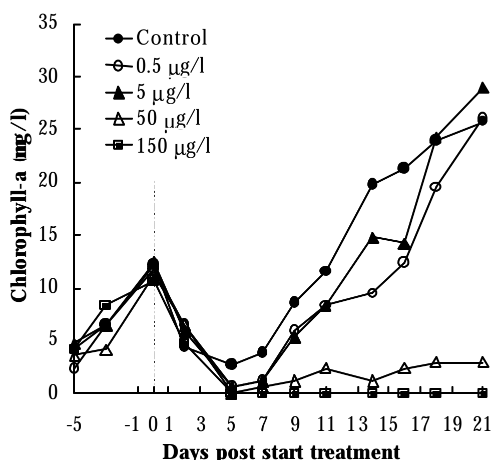

Fig. 3.14. *Chlorophyll-a content during the course of the linuron experiment*

Application of the higher linuron concentrations led to an absence in increase in chlorophyll-a levels. From 5 days post application onwards; chlorophyll-a totally disappeared in the highest treatment level. In the 50 µg/L applied microcosms, chlorophyll-a re-appeared one week after the treatment but did not regain normal values within the experimental period.

Physico-chemical conditions

During the pre-treatment period, the microcosms did not differ significantly in physico-chemical conditions (results not shown). After application of linuron,

however, treatment effects on the DO-pH-alkalinity-conductivity syndrome originated immediately and prolonged during the experiment (Figure 3.15A-C, Table 3.13).

Immediately after treatment, DO production was significantly decreased compared to controls at all linuron concentrations but the lowest (Figure 3.15A, Table 3.13). This resulted in a drop in DO in even the lowest concentration at the end of the dark period between day 14 and 17 although DO remained above 6 mg/L in all microcosms, so no anoxic conditions occurred. The DO levels in the afternoon were lower for all but the lowest treatment level for a prolonged period of time (NOEC = 0.5 µg/L, Day 0 through 15). At the end of the experimental period, all microcosms except those with the two highest linuron concentrations were not significantly different from controls.

Table 3.13. *NOECs as calculated by the Williams Test ($p < 0.05$) for physico-chemical parameters during and at the end of the experiment (last three measurements). Arrows indicate a significant increase (-) or decrease (¯) compared to controls*

	During the experimental period	At the end of the experiment
pH morning	0.5 ↓	5 ↓
pH afternoon	5 ↓	5 ↓
O_2 morning	Control ↓	5 ↓
O_2 afternoon	0.5 ↓	5 ↓
O_2 production	0.5 ↓	5 ↓
Conductivity	5 ↑	5 ↑
Alkalinity	50 ↑	-
Ammonium	5 ↑	5 ↑
Nitrate	5 ↑	5 ↑
Phosphate	5 ↑	5 ↑

The drop in oxygen concentration was accompanied with a drop in pH in the higher treatment levels (Figure 3.15B, Table 3.13). At the end of the experiment, the 5 µg/L microcosms regained normal “control” pH levels, whereas those with the highest concentrations remained decreased compared to controls.

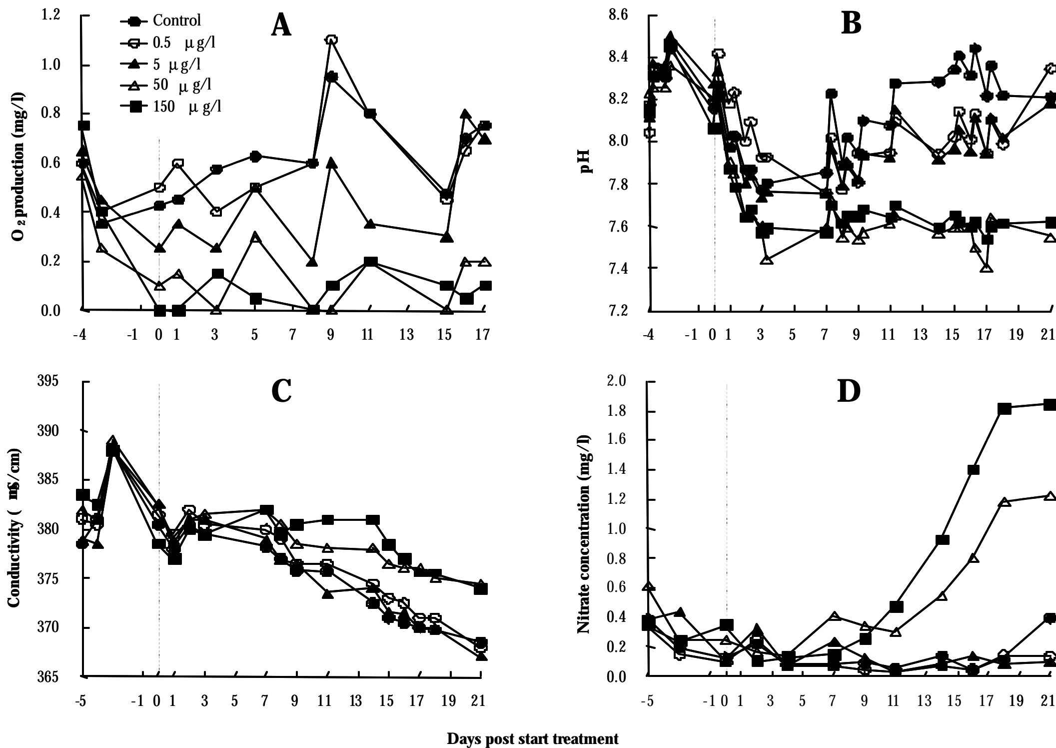

Fig. 3.15. *Dynamics of dissolved oxygen production (A), pH (B), conductivity (C) and nitrate concentration (D) during the experiment*

Corresponding with the decrease in pH and DO, application of 50 and 150 µg/L led an increase in alkalinity and conductivity (Figure 3.15C; Table 3.13). Although alkalinity was only increased during the second week post application, conductivity remained increased until the end of the experiment. The two highest linuron treatments led to increased levels of ammonium, nitrate and phosphate, compared to controls (Figure 3.15D, Table 3.13).

Zooplankton

A total number of 23 different taxa were identified. At the start of the experiment, the control microcosms were dominated by Rotifera (13 taxa), followed by Cladocera (6 taxa), Copepoda (Cyclopoïda and nauplii) and Ostracoda (no identification on taxon level). Insecta were represented by *Cloeon dipterum*. During the course of the experiment, abundance of Rotifera declined in the control microcosms and Cladocera became the dominant zooplankton group.

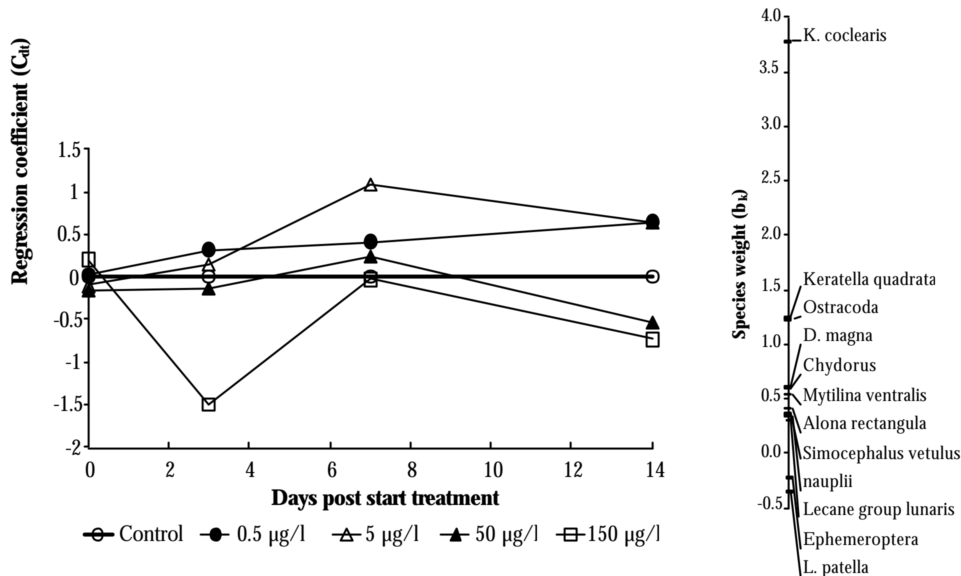

Fig. 3.16. *Principal Response Curves resulting from the analysis of the zooplankton data set indicating the effects of herbicide linuron on the zooplankton community during the experimental period. Of all variance, 60% could be attributed to sampling date, and is displayed on the horizontal axis, 18% could be attributed to treatment. Of the variance explained by treatment, 39% is displayed on the vertical axis. The lines represent the course of the treatment levels over time. The species weight (b_k) can be interpreted as the affinity of the taxon with the principal response curves. Taxa with a species weight between 0.25 and –0.25 are not shown*

In Figure 3.16, the effect of linuron application on the zooplankton community during the first two weeks of the experimental period is visualised. Since three weeks post application a bulk sample of 6 litres was taken, instead of the 250 ml samples during the course of the experiment, the zooplankton community as identified on that day is not included in the PRC. Taxa indicated with a positive species weight in this figure are expected to follow the response as given in the diagram, whereas taxa with negative weights are expected to show the opposite response. The more positive or negative the weight of a taxa, the stronger this response is.

In the PRC diagram (Figure 3.16), sixty percent of the total variance is explained by time, while 18% is explained by the treatment regime. Twenty-two percent of the total variance can thus be attributed to the differences between the replicates. The variance explained by time is displayed on the x-axis of the PRC diagram, while 39% of the variance explained by the treatment regime is displayed on the y-axis (significant part, $p < 0.05$).

Three days post application, the zooplankton composition of the 150 µg/L treatment clearly deviated from the control. As can be seen in Table 3.14, the two taxa with the highest weight in the PRC diagram (Figure 3.16), i.e. *Keratella coclearis* and *Keratella quadrata*, were most seriously affected (NOEC 50 µg/L). Seven days post application, the zooplankton community of the highest applied microcosms was comparable to control (Figure 3.16, Table 3.14), indicating recovery.

Table 3.14. *NOECs calculated for individual zooplankton species in microcosms treated with linuron*

species	days post start treatment	NOEC (µg/L)
Keratella coclearis	3	50 ↓
Keratella quadrata	3	50 ↓
Lecane bulla	21	CTR ↓
Simocephalus vetulus	21	50 ↓
Daphnia galeata	21	5 ↓
Daphnia magna	0, 3 and 21	5 ↓
Ephippia	21	5 ↓

However, after two weeks, the two highest linuron applications show a slight negative weight in the PRC diagram (Figure 3.16). The PCA diagram presented in Figure 3.17 summarises the treatment effects on the zooplankton community on day 21 in more detail.

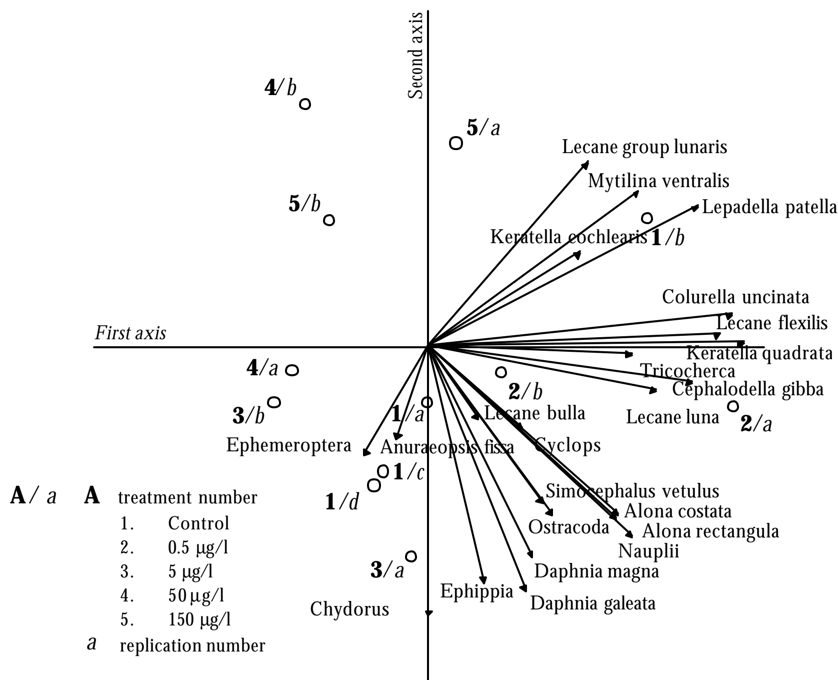

Fig. 3.17. *Ordination diagram (PCA) indicating effects of a single application of linuron on the zooplankton per treatment level on day 21. Of all variance, 35% is displayed on the horizontal axis and another 20% on the vertical axis*

The diagram reveals a decrease in abundance of especially Cladocera species for the highest two applications, as these sample points are positioned at the left upper

quadrant, and the Cladocera species points at the right under quadrant. These visual effects are consistent with the NOEC calculations (Table 3.14) and the observed decrease in counted waterfleas (data not shown), although no $NOEC_{community}$ could be calculated. Moreover, the numbers of counted waterfleas were correlated with the chlorophyll-a content, but in contrast to the chlorpyrifos and carbendazim cases the correlation is positive ($R^2 = 0.65$; $p < 0.05$. see Figure 3.18 A).

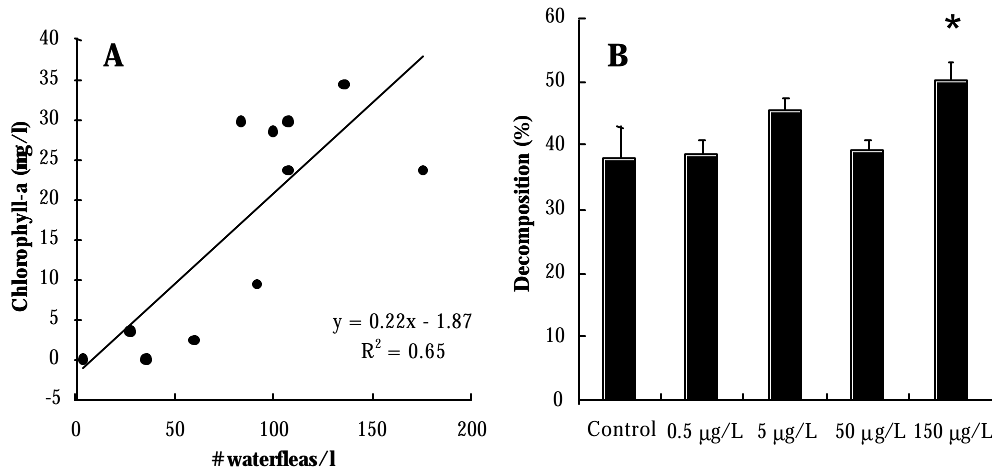

Fig. 3.18. *Correlation between the number of counted waterfleas and the chlorophyll-a content on day 21 (A) and the decomposition (%) per treatment level (B). Significant differences compared to control are marked with an asterisk*

Decomposition

The relative amounts (%) of breakdown of *Populus* leaves after a decay period of three weeks are presented in Figure 3.18B. The decomposition in controls and all but the highest linuron treatment was approximately 40%. Application of 150 µg/L led to a significant increase in decomposition compared to controls.

Macroinvertebrates; Lymnea palustrus

The application of linuron did not result in significant treatment effects on *L. palustrus*. At the end of the experiment, approximately six out of the initial eight snails were observed grazing. No animals died during the experiment.

4 Discussion

4.1 IOC experiment

CO_2 application, alone or in combination with carbonate, resulted in a drop in pH of 3 units to 6.1. No treatment effects of CO_2 on other water quality parameters were found. Chlorophyll-a content showed an increase during the first week post application in these microcosms compared to control and carbonate application alone (see Figure 3.3), what might be explained by CO_2 being the preferred carbon source for algae (Smith, 1996).
From laboratory bioassays (for a review see Locke, 1991) it is shown that low pH adversely affects survival, reproduction and growth rate of Cladocera. This explains that the zooplankton community was negatively affected by the decrease in pH after CO_2-application (see Tables 3.1 and 3.2). Therefore, it can be concluded that CO_2 application is not a useful tool to prevent an increase in pH. The pH in control and carbonate dosed cosms remained rather constant and below 11 (respectively 9.0-9.6 and 8.8-9.4).
Control and carbonate dosed microcosms possessed a comparable zooplankton community after two weeks. The control replicates, however, showed a larger variability in zooplankton community composition compared to carbonate applied microcosms (see Figure 3.2). Apparently, carbonate had a stabilizing effect that holds an advantage in pesticide risk assessment studies because it enhances the chance of demonstrating subtle effects on the zooplankton community.

4.2 Chlorpyrifos

Fate of chlorpyrifos

Irrespective of the applied dose, the rate of disappearance of chlorpyrifos was rather low. After three days less than 70%, and after two weeks less than 10% of the doses were detected in the water of all test systems. Mean half-lives in the water were eight days (± 2 days), which is high compared to other data on chlorpyrifos concentrations in the water (for a review; see Racke, 1993 and Giesy *et al.*, 1999). The relatively slow disappearance is probably due to the absence of macrophytes and sediment, which are known to sorb a large part of chlorpyrifos from the water (Crum and Brock, 1994). Indeed, Leeuwangh *et al.* (1994) found a half-live of one day in microcosms containing sediment and of comparable size (volume 7.5 litres) as the systems used in this study.

Effects on zooplankton

There was a rapid response of the Cladocera populations to the application of chlorpyrifos in the microcosms. Four days post application, no Cladocera were found in the two highest doses and in the 0.05 μg/L treated microcosms numbers were half of those in the controls. From day 7 onwards, Cladocera were only found

in controls and the lowest dose (see Figure 3.6). The impact of chlorpyrifos was more severe in this study than would be expected from laboratory data: $EC_{50,96h}$ for *D. galeata* is 0.3 μg/L, $EC_{50,96h}$ for *S. vetulus* is 0.4 μg/L, $LC_{50,48h}$ for *D. magna* is 1 μg/L, $NOEC_{21d}$ for *D. magna* is 0.1 μg/L (Van Wijngaarden *et al.*, 1993, Kersting and Van Wijngaarden, 1992). Moreover, also in microcosm and mesocosm experiments, effects of chlorpyrifos on zooplankton are not noted at concentrations as low as 0.05 μg/L (for a review; see Brock *et al.*, 2000b), when a single dose is evaluated. Using a chronic exposure, however, Van den Brink *et al.*(1995) reported effects of chlorpyrifos on *D. galeata* in microcosms at 0.1 μg/L. Van den Brink et al (2002) reported effects of a chronic exposure of 0.01 μg/L on the small cladoceran species *Bosmina longirostris*. In our study also the small cladoceran species *C. sphaericus* showed a pronounced response. So although a single application of chlorpyrifos is evaluated in this study, its results is more comparable to those evaluating chronic exposure. The most plausible explanation for this is the slower breakdown of chlorpyrifos and the consequently prolonged exposure to the toxic compound in the present study compared to other studies (Giesy *et al.*, 1999).

At concentrations higher than 0.005 μg/L, all cladoceran species disappeared within the experimental period. In these microcosms, the number of some insensitive rotifers, i.e. *Lecane bulla*, *Trichocerca sp.* and *Cephalodella gibba*, increased. The ordination diagram (see Figure 3.7) shows that, although *L. patella* (NOEC = 0.5 μg/L) was less abundant in the insecticide applied microcosms, high numbers of rotifers as a total were related to (high) chlorpyrifos application. An increase in rotifers abundance in model-ecosystems has been reported more often after insecticide application (Van den Brink *et al.*, 1995; Jak *et al.*, 1998; Brock *et al.*, 1992; see Brock *et al.*, 2000b for a review).

Overall, it can be concluded that the order of sensitivity for chlorpyrifos of zooplankton is small Cladocera > large Cladocera > rotifers.

Effects of chlorpyrifos on primary producers

From day 7 post application till the end of the experiment, chlorophyll-a content was increased in the two highest applied doses. The occurrence of algal blooms in the form of phytoplankton is frequently reported as an indirect effect in insecticide-stressed aquatic systems (Van Donk *et al.*, 1995; review by Brock *et al.*, 2000b). This increase can be explained by the decrease in grazing pressure of (the disappearance of) Crustacea. Indeed, a negative correlation was found for chlorophyll-a and number of Cladocera (Figure 3.6). The increase in phytoplankton biomass could not be counteracted by the increase in less sensitive rotifers because they are known to be less effective grazers (Jak *et al.*, 1998).

Surprisingly, a significant increase in chlorophyll-a was found for the lowest treatment level although no decrease in total number of Cladocera was noted. This may be a result to a decrease in filtration rate occurring at lower concentrations compared to immobility and death (see Hartgers et al., 1999 for an example on lindane)

Effects on water quality parameters

Oxygen concentrations and pH are both expressions of the metabolism of an ecosytem. Alkalinity and conductivity, in addition to pH and oxygen, are related to

the processes of anabolism and catabolism (Kersting and Van den Brink, 1997). Therefore, as in this study, the effects of a pesticide on the ecosystem metabolism are generally expressed with dissolved oxygen (DO), pH, alkalinity and conductivity, collectively called the “DO-pH-alkalinity-conductivity syndrome”.

In most studies no effects on physico-chemical characteristics after application of chlorpyrifos are found (Leeuwangh, 1994). Oxygen concentrations in the water, for example, are measured in many studies but the number of studies with significant effects on the oxygen concentration is limited (Brock *et al.*, 2000b). In the present study however, a pronounced increase in pH, DO, DO-production and conductivity was noted.

Most conducted pesticide hazard assessment studies are performed in rather complex model-ecosystems that include species-rich communities dominated by macrophytes as primary producers, in order to be representative for natural field aquatic environments (Brock and Budde, 1994). However, this hampers the demonstration of effects on ecosystem metabolism since functional redundancy is so effective in these systems that functions and consequently physico-chemical characteristics can be adequately maintained even after major changes in structure (Levine, 1989; Leeuwangh *et al.*, 1994).

In macrophyte-dominated testsystems, the change of demonstrating an effect on the DO-pH-alkalinity syndrome as a result of a shift in phytoplankton biomass is futher counteracted by the relatively low biomass of phytoplankton in comparison with the standing stock of macrophytes (Cuppen *et al.*, 2000). Indeed, in a study by Butcher *et al.*(1977) in artificial ponds without macrophytes, mean dissolved oxygen increased with chlorpyrifos concentration (4, 10 and 1000 μg/L). The excess of oxygen and the decrease of CO_2 were closely associated with the periods of algal blooms.

Another factor that hampers the demonstration of an effect on ecosystem metabolism in complex micro- and mesocosms is diffusion of oxygen from (oxygen saturated) water, resulting in unnoticed extra oxygen production (Cuppen *et al.*, 2000). In line with this, Kersting and Van den Brink (1997) were able to show a decrease of gross primary production after quantification of the diffusion of oxygen across the air/water interface. The glass-chambers used in the present study were closed so substantial diffusion of oxygen was excluded.

Ecological effect chain

The direct and indirect effects of chlorpyrifos on ecosystem structure and functioning are visualised in Figure 4.1. The decline in efficient (large) grazers provoked an increase in chlorophyll-a level. This resulted in a higher metabolic rate manifested by an increase of DO, DO production and pH.

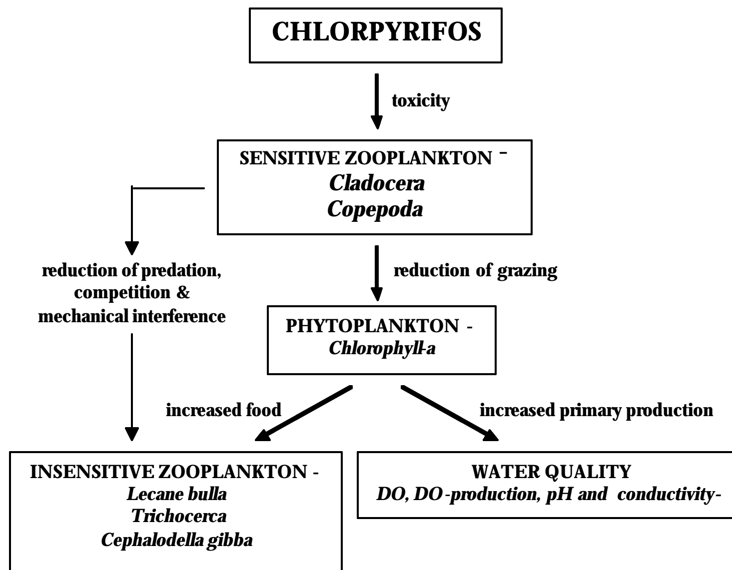

Fig. 4.1 *Schematic overview of the direct and indirect effects of chlorpyrifos on the ecosystem structure and functioning*

Ecological risk assessment

According to the Dutch ecotoxicological hazard assessment procedure for pesticides the acceptable concentration should be ≤ 0.01 EC_{50} or ≤ 0.1 NOEC for the most sensitive test species (see introduction). The most sensitive standard test species to chlorpyrifos is *D. pulex*, with an LC_{50} (48h) of 0.2 μg/L (Van der Hoeven and Oldersma, 1989), resulting in a UP criterion of 0.002 μg/L. The Maximal Permissable Concentration (MPC) as included in the Fourth Memorandum Water Management (NW4) is 0.003 μg/L.
In the present study effects of chlorpyrifos on the structure of the zooplankton community were found at a concentration as low as 0.05 μg/L. Although effects on functional endpoints were found at a concentration 0.005 μg/L, it seems reasonable to set the overall safe threshold level of our study at 0.05 μg/L. Besides pH, the observed effects on functional endpoints were occasional; no effects in two consecutive sampling moments were found and therefore not considered significant. Moreover, former experiments by Jonker (2000) with the microcosms made clear that pH is a extremely sensitive parameter. It is therefore questionable if the effects on pH found at 0.005 μg/L and the absence of effects on other endpoints at this dose should be incorporated in the overall safe threshold level. Although from a fate perspective the present study was performed under worst-case conditions, the resulting $NOEC_{eco}$ (NOEC of the most sensitive endpoint) of 0.005 μg/L does not exceed the UP criterion nor the MPC.

4.3 Carbendazim

Despite the wide application of fungicides like carbendazim, published information on their effects in micro- and mesocosms is very scarce. The only reported studies dealing with the effects of carbendazim in model ecosystems known to the authors was done in 600 L indoor macrophyte-dominated freshwater microcosms (Cuppen *et al.*, 2000; Van den Brink *et al.*, 2000), 3000 L outdoor plankton dominated microcosms (Slijkerman *et al.*, pers. comm.) and 60 m^3 outdoor mesocosms (Arts et al., pers. comm.). The latter experiment, however, only evaluated the effects and recovery of invertebrate community after a relatively high dose of carbendazim (300 µg/L). Therefore, a comparison of the results found in this experiment can only be made with the two microcosm studies and relevant laboratory toxicity data on carbendazim (van Wijngaarden *et al.*, 1998; Palawski and Knowles, 1986; Canton, 1976).

Fate of carbendazim

Carbendazim was found to be very persistent in the water layer. No breakdown of the compound was observed during the three-week experimental period for any concentration. Surprisingly, carbendazim concentrations rather seemed to increase (3-10%) than decrease. Both Van den Brink *et al.*(2000) and Slijkerman *et al.*(pers. comm.) reported a $t_{½}$ of 6 till 25 weeks, and $t_{½}$ was found to increase with the dose applied. The fact that in this study no breakdown of carbendazim, not even in the lowest dose, was observed might be due to the absence of sediment and macrophytes and associated microbial community.

Effects on zooplankton

Both univariate and multivariate tests revealed a significant influence of carbendazim on the zooplankton community in the 100 and 1000 µg/L treated microcosms (NOEC=33 µg/L). The standard test species for zooplankton, *D. magna* ($NOEC_{reproduction}$ 26 µg/L, Van Wijngaarden *et al.*, 1998) proves to be a sensitive representative of the zooplankton community in the used microcosms.
The order of susceptibility was Cladocera > sensitive rotifers > Copepoda. As shown in Table 3.8, total number of waterfleas follows a dose-response relation in time. The NOECs observed in a bioassay with *D. magna* by Van den Brink *et al.*(2000) resemble the NOECs for total number of waterfleas in this microcosm study. All Cladocera-species, i.e. *Simocephalus vetulus*; *Grabtoleberis testudinella* and *Alona rectangula*, and their eggs (Ephippia) were equally sensitive to carbendazim (NOEC 33 µg/L). In the studies of van den Brink *et al.*(2000) and Slijkerman *et al.*(pers. comm.), differences were found between the different Cladocera species in sensitivity to carbendazim. *D. galeata* (syn. *D. longispina*) turned out to be rather insensitive (NOEC > 1000 µg/L), although the other Cladocera present (*S. vetulus* and *A. exigua*) were as sensitive as the Cladocera in this study (NOEC 33 µg/L). In the study of Slijkerman *et al.*(pers. comm.) the small cladoceran species *Bosmina* sp. was even affected at 21 µg/L.
In both microcosms experiments, Copepoda were found to be more sensitive than rotifers. However, in the study of Van den Brink *et al.*(2000) this only became apparent at 3 weeks post application since the effect on cyclopoida resulted from a decrease in the numbers of their immature stage, Nauplius, rather than of direct

toxicity. Indeed, a decrease in Nauplius larvae was found in this study (NOEC 100 μg/L), but the experimental period was probably too short (i.e. 3 weeks) to be able to show a prolonged effect on mature Cyclopoida.
In our study, the rotifer *Branchionus angularis*, showed to be insensitive to carbendazim and was even increased in abundance at the highest dose (data not shown). This could be the result of an improved competitive position relative to other, sensitive, zooplankton taxa. In the other microcosm studies other rotifers, *Testidunella* and *Polyarthra,* were found to increase for the same reason.

Effects of carbendazim on primary producers
Chlorophyll-a showed an increase at the two highest concentrations (NOEC 33 μg/L) from day 4. Reduced grazing pressure due to the decline or elimination of Cladocera is most probably responsible for this increased chlorophyll-a of the phytoplankton. Elevated chlorophyll-a levels were also found in the higher treatments of the microcosm study by Van den Brink *et al* (2000), Slijkerman *et al.*(pers. comm.) reported a slight increase in chlorophyll-a at the highest treatment level (221 μg/l). In the plankton-dominated microcosms of this study, this increase was much more apparent and consistent during the experimental period. Apparently, the absence of nutrient-competing macrophytes or the much higher biomass of phytoplankton enhanced the demonstration of a clearer (indirect) effect on chlorophyll-a after the decline in grazers.

Effects on water quality parameters
The increase in the growth of planktonic algae resulted in an increase in conductivity, pH and oxygen production as discussed for chlorpyrifos. However, no increase in oxygen content was observed. In the macrophyte-dominated microcosms, no effects on oxygen levels were observed (Cuppen *et al.*, 2000). The authors of that study attribute this to the fact that the water was saturated with respect to dissolved oxygen, so a possible movement of oxygen to the air could have caused unnoticed extra oxygen production. In the microcosms used in this study, oxygen levels were near saturation too (8.8 ± 0.7; saturation is 100% at 9.4 mg/L) so this could have masked a possible increase.

Effects of carbendazim on decomposition
Although they can be really important in aquatic ecosystems, micro-organisms (bacteria, fungi, protozoa) are seldom identified and counted in pesticide studies because methods to examine these organisms are laborious and require specialised techniques (Brock and Budde, 1994; Cuppen *et al.*, 2000). Also in this study, no special attention was attributed to this.
In the highest applied dose, the decomposition was (significantly) decreased with 17% compared to controls. Therefore, some populations of micro-organisms apparently were affected, directly or indirectly, after exposure to 1000 μg/L carbendazim. However, no inhibitory effects on the sporulation of 18 species of aquatic hyphomycetes for carbendazim concentrations up to 5000 μg/L, nor on the germination of conidia of six species of hyphomecetes for carbendazim concentrations up to 1000 μg/L, have been reported (Chandrashekar and Kaveriappa, 1994).

Cuppen *et al.* (2000) reported a comparable decrease in decomposition (i.e. 15%) 4 weeks after application of the same dose carbendazim. They proposed a possible role of 'worm-like' taxa in altering the microbial community composition and activity and herewith the decomposition of *Populus* leaves. In this study, however, no 'worm-like' taxa were present. It might be possible that the shifts in water quality, phyto- and zooplankton community resulted in a change in the microbial community and hence the rate of decomposition.

Effect on Bithynia leachi

Carbendazim had an impact on the behaviour of the macroinvertebrate used in this experiment, *Bithynia leachi* (see Table 3.5). An increase in the number of individuals on the glass wall and a disruption of the operculum reflex was observed in the highest applied dose. Grazing was even affected at 100 μg/L carbendazim. In line with this, Cuppen *et al.* (2000) report a NOEC based on number of individuals caught on artificial substrate of 100 μg/L for this species.

Ecological effect chain and final conclusions

In Figure 4.2, the direct and indirect effects of carbendazim are visualised in a schematic overview. In short, carbendazim treatment affected the structure of the aquatic ecosystems directly by reducing the abundance of many species of zooplankton (mainly Cladocera) and indirectly by removing the grazing pressure on phytoplanktonic algae. This resulted in an increase in pH and oxygen production.

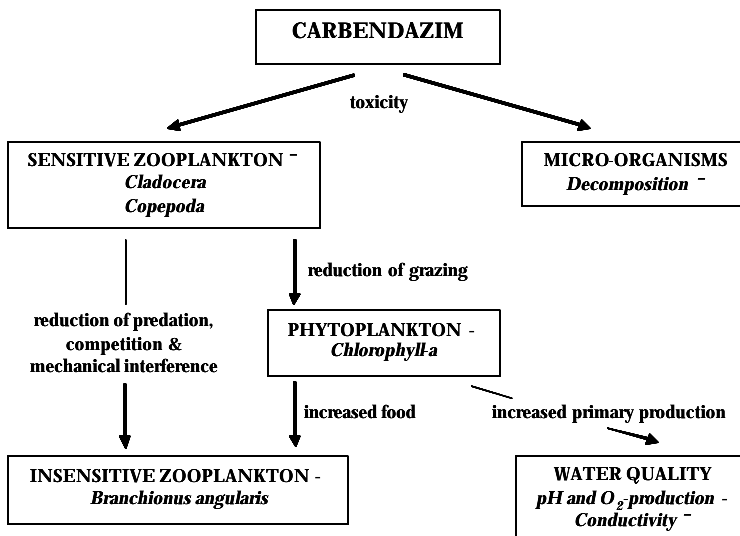

Fig. 4.2 *Schematic overview of the direct and indirect effects of carbendazim on the ecosystem structure and functioning*

Overall, the kind and severity of the effects of carbendazim on the zooplankton community of the tested microcosms is comparable to the two known reported study of carbendazim on model-ecosystems that evaluates low concentrations (Cuppen *et al.*, 2000; Van den Brink *et al*, 2000; Slijkerman et al., pers. comm.). In the study of Van den Brink *et al.* (2000) and this study 33 μg/L can be regarded as a safe concentration ($NOEC_{community}$) for zooplankton, while Slijkerman *et al.* (pers. comm.) reported effects on the small cladoceran *Bosmina* sp. at this concentration. However,

in the macrophyte dominated test-systems NOECs of 3.3 μg/L were found for the macroinvertebrate community, with Oligochaeta, Turbellaria, Hirudinea and some Crustacea as the most sensitive groups. Since these groups were not represented in the tested microcosms, effects on these organisms could not be demonstrated in the current study.

4.4 Linuron

Fate of Linuron

Linuron proved to be very persistent in the waterlayer. No decrease in herbicide-concentrations was found during the experimental period. This is in contrast to the half-lives of linuron reported in experimental ditches by Crum *et al.* (1998. $t_{½}$ 7.2 to 11.8 days), in indoor microcosms by Van den Brink *et al.* (1997. $t_{½}$ 11 to 49 days) and in small enclosures Stephenson and Kane (1984. $T_{½}$ 16-40 days).

Higher losses of linuron from the water by evaporation and sorption to macrophytes or sediment in the referred studies are unlikely to be a plausible cause, since linuron has a very low vapour pressure (1.5 x 10^{-5} mm Hg at 20°C) and sorption to e.g. clay particles is very limited (El-Dib and Aly, 1976b). Consequently, differences in degradation are most likely to be responsible for the observed difference in fate of linuron. With regard to degradation, El-Dib and Aly (1976a) showed that hydrolysis does not play a significant role in nature. With reference to biodegradation El-Dib and Aly (1976c) found that in raw river water this did not occur but after addition of an inoculum of *Bacillus cereus*, breakdown was noted, resulting in half-life values of 1-10 days. The rather simple design of the tested microcosms in this study (e.g. no macrophytes and sediment) may lack suitable habitats for microorganisms like *B. cereus* resulting in a small biodegradation of linuron.

Effects on primary producers

The ordination diagram (Figure 3.13) reveals that most taxa of the phytoplankton were negatively affected by linuron application. These effects are most likely direct effects via the inhibition of photosynthesis by linuron. A decrease in abundance of *Tetraedon*, *Pediastrum* and *Scenedesmus* species has previously been reported (Snel *et al.*, 1998; Van Geest *et al.*, 1999).

Diatoms, *Navicula* and *Epithemia*, showed an increase in numbers after the two highest linuron applications. Apparently, these taxa are insensitive to the linuron concentrations applied and could take advantage of the increase in available nutrients (ammonium, nitrate as well as phosphate increased as a consequence of a decrease in phytoplankton; see next paragraph).

Overall, phytoplankton taxa decreased in such numbers that chlorophyll-a content decreased in the two highest applied doses. This study is the first to show a significant decrease in phytoplankton after linuron treatment. The low initial biomass of the chlorophyll-a in other studies as a consequence of dominance by macrophytes is likely to be the reason of the absence of this phenomenon.

Effects on water quality parameters

The general effect chain of herbicides is a disruption of the functioning of the primary producers (e.g. by inhibiting of photosynthesis), resulting in a decrease in DO and pH (Stephenson and Kane, 1984; Van Geest *et al*., 1999; Van den Brink *et al*., 1997). In line with these findings, a prominent decrease in DO, net DO production and pH was observed, with a lowest NOEC of 0.5 µg/L for the evening DO.

Moreover, the decrease in primary production led to an increase in available nutrients, measured as ammonium, nitrate and phosphate. Van den Brink *et al.* (1997) also observed an increase in nitrate after application of linuron.

Effects on zooplankton

The EC_{50} of *Daphnia galeata* for linuron (360 µg/L; Stephenson and Kane, 1984) is considerably higher than the concentrations used in the current experiment. In line with this, the zooplankton communities in the microcosms revealed no immediate responses to the application of linuron. However, on day 4 a significant effect of the highest applied linuron concentration on the zooplankton community was found, mainly caused by a decline in *K. coclearis* and *K. quadrata*, indicating a possible role of direct toxicity. A more plausible explanation for the decrease in Rotatoria is the complete disappearance of chlorophyll-a from day 4 onwards at this dose. In agreement with the results of Cuppen *et al.* (1997), Cladocera numbers did not change in abundance in this period. They found a decrease in Rotatoria and no effect on Cladocera after application of linuron. They stated that this could be attributed to a decrease in planktonic and epiphytic algae and the increase in the flagellate *Chlamydomonas* since rotifers are filter feeding, making them less efficient grazers. Since no information of the phytoplankton community on day 4 is available, this can not be verified in the present study.

In addition to decreases in the rotifers *K. coclearis*, *K. quadrata* and *L. bulla,* Cladocera numbers were lower in the two highest applied doses three weeks post application. Numbers of *D. galeata*, *D. magna* and their eggs (ephippia*)* were lower in the two highest doses and numbers of *S. vetulus* were lower in the highest dose applied. In other micro- and mesocosm studies (e.g. Jenkins and Buikema, 1990; Cuppen *et al*., 1997) no negative effect on Cladocera are noted. As mentioned before, the EC_{50} of the most sensitive test-species is higher than the doses applied in the current study. The most plausible explanation for the decline in Cladocera is the absence of chlorophyll-a content in the highest treated microcosms, and the very low levels in the 50 µg/L applied microcosms (< 3 µg/L from day 4 onwards). Indeed, the number of Cladocera was positively correlated to the chlorophyll-a content on day 21. Moreover, 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. Starr *et al.*(1999) found a reduction in the reproductive success of a planktonic copepod (*C. finmarchicus*) after a monospecific diet of a *Navicula* species.

Effects of linuron on decomposition

Application of 150 µg/L resulted in a significant decrease in decomposition of *Populus* leaves (see Figure 3.25) despite a decrease in oxygen concentration. In a

review by Brock *et al.* (2000a), no effect on decomposition rate has been reported for herbicides. In the current study, a tremendous change in zooplankton- and phytoplankton community and ecosystem metabolism was observed in the 150 μg/L applied microcosms (see former paragraphs). These major shifts might have resulted in a change in microbial community composition and consequently the rate of decomposition.

Effects on Lymnaea palustrus

No direct effects of linuron were observed on the grazing behaviour of the included macro-invertebrate *Lymnaea palustrus*. Indeed, the LC_{50} of some macroinvertebrates, such as *Dugesia tigrina* (10 mg/L), *Lymnaea* (70 mg/L) and *Tubifex* (10 mg/L) are too high to expect direct effect on macro-invertebrates (Maier-Bode and Härtel, 1981).
The relatively small secondary effect of linuron observed in another microcosm study (Cuppen *et al.*, 1997), i.e. decrease in numbers of *Physella acuta* and eggs of *Lymnaea stagnalis*, were not indicated in the present study. Presumably, the numbers of individuals and the presence of epiphyton on the glass edges of the test-systems (personal observation) mask a possible subtle effect.

Ecological effect chain and final conclusions

An overview of the overall ecological impact of a single application of linuron on the structure and functioning of the test-systems is visualized in Figure 4.3. As a primary effect of linuron application, numbers of phytoplankton and consequently chlorophyll-a content were decreased due to inhibition of the photosynthesis (Snel *et al.*, 1998). This resulted in a decrease in dissolved oxygen, dissolved oxygen primary production, pH and an increase in nutrients (i.e. ammonium, nitrate and phosphate). The decrease in invertebrates can be explained by the decrease in chlorophyll-a (number of Cladocera is positively related to the chlorophyll-a content, Figure 3.18).

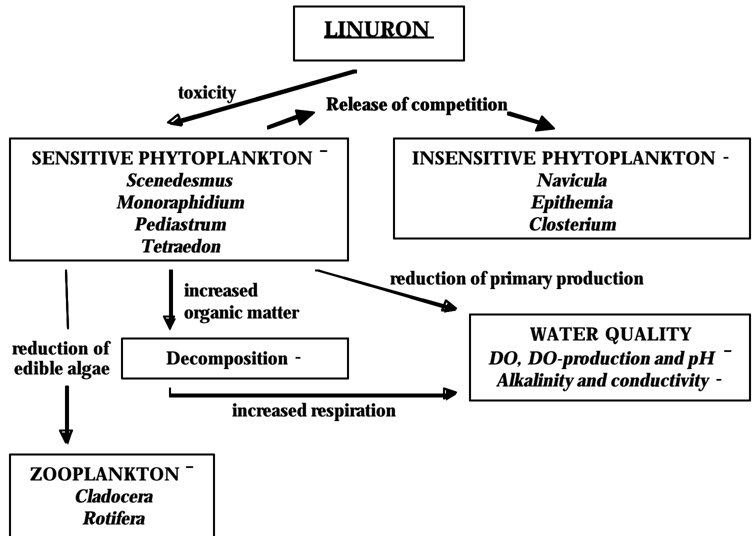

Fig. 4.3 *Schematic overview of the direct and indirect effects of linuron on the ecosystem structure and functioning*

The UP criterion for linuron is based on *Scenedesmus subspicatus*, the most sensitive standard algae, with an EC_{50} of 16 μg/L (Brock et al., 2000a). The UP criterion is

therefore 1.6 μg/L (0.1 x EC_{50} most sensitive test species). The MPC for freshwater is 0.25 μg/L, as reported by Crommentuijn *et al.* (1997). The $NOEC_{eco}$ of linuron in this study was recorded at 0.5 μg/L, which is in line with the NOEC (0.5 μg/L) found by Van den Brink *et al.* (1997) and Cuppen *et al.* (1997). The safety factors adopted by the EU in the UP obviously ensure adequate protection for the ecosystem in the case of single dose of linuron.

4.5 Methodology of the used microcosms

Comparing test-systems with each other is hampered by major differences in experimental design of test-systems, like size, endpoints and species composition. This meets the need for methodological guidelines to reduce the likelihood that poorly designed studies will be carried out, and that these may fail to address properly the issues of concern. It is, however, incorrect to strive for identical test-systems since temporal and spatial influences create heterogeneity even in the field situation. Moreover, different research questions desire a different design of the studies (Campbell *et al.*, 1999).
Methodological guidance for microcosm studies should therefore be sufficiently flexible to allow development of protocols for specific studies on a case-by-case scientific basis. SETAC-Europe (1991) gives a Guidance Document to indicate the types of information that can be gained from different kinds of mesocosm studies. Figure 4.4 gives an overview of some major recommendations based on four “topic areas” of this workshop taking into account that they should apply to *microcosms*. This means that some recommendations are rewritten.
When the recommendations mentioned in Figure 4.4 are used to test the procedure of the performed experiments, one can conclude that overall the test-system is methodological correct. However, remarks have to be made about the flora and fauna composition and the absence of sediment. The microcosms were phyto- and zooplankton dominated, excluding macrophytes and macroinvertebrates. The size of the test-systems makes it impossible to maintain a macrophyte community with various macroinvertebrate taxa. Insect larvae could not be included since they would eliminate the zooplankton community and annelids often require sediment. In the latter experiments with carbendazim and linuron, a snail species (*B. leachii* and *L. palustrus*, respectively) was introduced to represent the gastropods. The absence of macrophytes and macroinvertebrates will limit the use of the microcosms in pesticide risk assessment if major effects are expected on these groups of organisms (see discussion carbendazim).

1. Design, composition and characterisation

- During the establishing period, necessary action must be taken to ensure that, at the time of treatment, microcosms are similar in biological and physico-chemical characteristics (applied testwater and zooplankton were stirred before adding).
- The microcosm should develop or have a flora and fauna consistent with the study objectives and, where appropriate, should be representative of natural field aquatic environments (partly: many phyto- and zooplankton taxa but no macrophytes, annelids and insect larvae and gastropods). *
- Microcosms must contain a sediment layer (not present!). **
- If natural water is used, adequate precautions must be taken to minimise the possibility of undesirable organisms (test water filtered over 0,75 mm to prevent *Chaoborus* to enter).
- The microcosm should develop or have a flora and fauna consistent with the study objectives (indeed phyto- and zooplankton dominated).
- Addition of nutrients may be needed to maintain mesotrophic systems (application of P, N, and HCO_3).

2. Statistical design and treatment

- Microcosms must be randomly assigned to treatments (done).
- Statistical analyses will depend upon the experimental design, but two principal approaches can be adopted: fitting a dose-response model (regression) and/ or pairwise comparison with the control (analysis of variance [ANOVA]) (both were used).
- For short term studies (up to 1 month) a single application is recommended (application of tested pesticides was indeed as a single dose).

3. Endpoints and sampling

- Short-term studies can be used to measure acute effects on the taxa of interest.
- The sampling strategy for invertebrates must take account of the fact that their distribution is often patchy, particularly in small microcosms where edge-effects can be significant (systems were stirred before taking the samples).

4. Data handling

- Essentially, data collected and reported during a microcosm study will fall into three broad categories; system description, fate data and effect data.
- The data from microcosm studies should be interpreted in the light of all available information, not only that obtained within the study (a comparison is made with bigger microcosms and mesocosms).
- Care should be taken in the use of semi-quantitative data; they should not be dismissed as having no value (semi-quantitative data of snails were taken into account).

Fig. 4.4 *Some major recommendations for testing-procedures for pesticides in freshwater static microcosms after SETAC-Europe (1991). In brackets, the proposals are applied to the test-system used in this study to determine the methodological correctness of this system*
** The test-systems consisted of a rather simple plankton dominated community*
*** The absence of sediment is the major lack of the microcosms used in this study*

Moreover, the absence of sediment is obviously a demerit of these microcosms. A design without sediment was chosen because it turned out to be difficult already to develop a stable phyto- and zooplankton community for two weeks in the microcosms without sediment and the systems are stirred. A first pilot-experiment (n=1) is already done in which sediment was added to the system. Although it took more than a week to obtain clear water, it turned out to be possible to keep waterfleas alive in a rather stable system (data not shown). However, the absence of sediment does not mean the systems are not a useful tool in pesticide risk assessment. Although they may not fully represent the Dutch ditches, the microcosms can be used as a tool to find the concentration range to be tested in larger microcosm or mesocosm studies. Furthermore, as the concentration for sediment binding pesticides is higher in the present systems, they represent a worst-case and could consequently be used as a screening tool for the need of additional higher tier studies.

In Table 4.1, the NOEC's of former microcosm studies by our department dealing with the risk assessment of chlorpyrifos, carbendazim and linuron are compared with the NOEC's found in the current study.
Due to the absence of sediment and macrophytes, the fate of chlorpyrifos differed from earlier studies resulting in a prolonged exposure and consequently larger toxic effects and a lower NOEC. In a microcosms study with chronic exposure to chlorpyrifos and lindane, a NOEC of 0.01 µg/L was found (Van den Brink *et* al., 2002), which is very comparable to this study. With reference to carbendazim, sensitive macroinvertebrates were not present in large numbers nor taxa compared to other microcosm studies, resulting in a higher NOEC in the present study for carbendazim. The Cladocera species Bosmina sp., that showed a significant, negative response to 21 µg/L carbendazim in the study of Slijkerman *et al.*(pers. comm.) appeared to be more sensitive than the Cladocera species used in this study. The NOEC for linuron in this study matched the NOEC found by Van den Brink *et al.*(1997) and Cuppen *et al.*(1997). However, in the study performed in macrophyte-dominated microcosm by Van den Brink *et al.*(1997) and Cuppen *et al.*(1997) a NOEC of 0.5 µg/L after a chronic exposure was noted. They found the most severe effects on several phytoplankton species 3 and 4 weeks after the start of the treatment. The authors explained this late response by assuming that these species could survive until their energy reserves were depleted. The present study last 3 weeks only so the autotrophic organisms dosed with 0.5 µg/L could presumably survive this time period by using their energy reserves and therefore only effects at DO levels were found. Experiments with a longer follow-up may lead to a more dramatic change in functional and structural endpoints than shorter experiments.

Table 4.1 *The NOEC's and LOECs(in µg/L) as a result of a single and chronic application found in former microcosm studies and in the current study*

	Single	Chronic	This study
Chlorpyrifos	0.1 – 0.9 (Van den Brink *et al.*, 1996)	0.01 – 0.05 (Van den Brink *et al.*, 2002)	0.005 – 0.05
Carbendazim	2.2 – 21 (Slijkerman *et al.*, pers. comm.)	3.3 – 33 (Cuppen *et al.*, 2000)	33 – 100
Linuron	5 – 15 (Van Geest *et al.*, 1999)	0.5 – 5 (Van den Brink *et al.*, 1997; Cuppen *et al.*, 1997)	0.5 – 5

Overall, it can be concluded that the tested microcosm are useful in the risk assessment of pesticides. Due to differences in experimental design, however, the NOEC's in the present study were not always consistent with the results of former experiments in (larger) microcosms. As mentioned before, efforts may have to be made to add to the microcosms Moreover, possibilities to add (sensitive taxa of) macroinvertebrates will have to be investigated depending on the pesticide tested. The small size of the test systems, however, will not allow the development of a very rich macro-invertebrate community. Therefore, a pesticide risk assessment study should be conducted in bigger microcosms or mesocosms if major effects are expected on these macroinvertebrates or macrophytes.

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