Ecological and statistical evaluation of effects of pesticides in freshwater model ecosystems

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Stellingen

1. Insecticiden, herbiciden en fungiciden kunnen symptomen van eutrofiëring veroorzaken.
   Dit proefschrift.

2. Het ecologisch risico van individuele bestrijdingsmiddelen in oppervlaktewater is acceptabel als voldaan wordt aan de criteria voor waterorganismen zoals beschreven in de Europese regelgeving volgens de Uniforme Beginselen.
   Dit proefschrift, Lahr et al., 1998. STOWA Rapport 98-30; Van Wijngaarden et al., 1998. STOWA Rapport 98-31.

3. Multivariate technieken vormen, mits goed toegepast, een essentiële aanvulling op gangbare univariate statistische technieken om semi-veldexperimenten te analyseren.
   Dit proefschrift.

4. Semi-veldexperimenten zijn een waardevol hulpmiddel voor het vaststellen van de ecologische risico's van contaminanten.
   Dit proefschrift.

5. Voor het ontwikkelen van effectmodellen zijn gegevens over de levenscyclus van soorten van vitaal belang. Beschrijvend ecologisch onderzoek verdient meer waardering en aandacht bij academische instellingen.

6. De landbouwkundige praktijk van de gewasbescherming heeft emissie van verscheidene bestrijdingsmiddelen naar het oppervlaktewater tot gevolg. Daarom is voor een adequate risico-evaluatie naast de stofgerichte benadering ook een teeltgerichte benadering nodig.

7. Alhoewel de NOEC voor de evaluatie van laboratoriumtoetsen van beperkte waarde is, is deze parameter goed bruikbaar bij de evaluatie van semi-veldexperimenten.
Chapman et al., 1996. Ecotoxicology, 5: 169-186, 1996; dit proefschrift.

8. De discussie over welke ecologische effecten aanvaardbaar zijn bij landbouwkundig gebruik van bestrijdingsmiddelen, is niet alleen een wetenschappelijke maar vooral een politieke.

9. De beursgang van Ajax vertoont overeenkomsten met de grasmat waarop zij speelt.

10. Het is voor de wijnliefhebber prettig dat de geneeskunde steeds meer de gezonde kanten van de geneugtes des levens belicht.
Lemeshow et al., 1998. American Journal of Epidemiology 148: 298-306.

11. Meten is (z)weten maar zonder dit wordt modelleren fantaseren.

Stellingen behorende bij het proefschrift:
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Voor 'ons pap en ons mam'

Contents

Chapter 1.	General Introduction	1
Chapter 2.	Effects of the insecticide Dursban®4E (active ingredient chlorpyrifos) in outdoor experimental ditches: II. Invertebrate community responses and recovery	13
Chapter 3.	Sensitivity of macrophyte-dominated freshwater microcosms to chronic levels of the herbicide linuron I. Primary producers II. Community metabolism and invertebrates	25
Chapter 4.	Impact of the fungicide carbendazim in freshwater microcosms I. Water quality, breakdown of particulate organic matter and responses of macroinvertebrates II. Zooplankton, primary producers and final conclusions	49
Chapter 5.	Principal Response Curves: analysis of time-dependent multivariate responses of a biological community to stress	91
Chapter 6.	Multivariate analysis of stress in experimental ecosystems by Principal Response Curves and similarity analysis	115
Chapter 7.	Ordination of responses to toxic stress in experimental ecosystems	133
Chapter 8.	Summary and concluding remarks	139
	Samenvatting en slotopmerkingen	147
Curriculum vitae		161
List of publications		163
Dankwoord		165

Chapter 1

General introduction

The twentieth century has seen a rapid increase in the human population. To satisfy consumption needs, intensive agriculture was stimulated. The use of agrochemicals (fertilisers, pesticides) was greatly expanded to increase crop productivity in a cost-effective way. It soon became apparent, however, that intensive use of agrochemicals also caused environmental problems (e.g. Carson, 1962).

Agricultural pesticides are, as the name indicates, chemicals deliberately released into the environment to control pests that harm crops. This mode of application implies that they may reach non-target areas. Aquatic ecosystems, for instance, have been reported to become contaminated by pesticides due to spray drift, drainage, run-off, atmospheric deposition and/or accidental spills (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 (Hurlbert, 1975; Hill et al., 1994). Consequently, authorities have set criteria to protect aquatic life from pesticide stress. Recently, the European Union adopted the Uniform Principles, a registration procedure for the placing of plant protection products on the European market in which also water quality criteria are incorporated (EU, 1997; see also Table 1). These new water quality criteria are among the main reasons why several traditional products are now banned (or will be in the near future), and many new active ingredients are not allowed on the European market, or only in highly restricted circumstances (CTB, 1997). The current registration procedures, however, are often debated because of the economic consequences of unduly strict, and the ecological consequences of unduly lenient environmental risk assessment criteria.

Tiered risk assessment approach

Ideally, when assessing the ecological risks of a new pesticide, one investigates its fate and effects under realistic field conditions, taking into account normal agricultural practice and the spatial and temporal variability of the ecosystems potentially under stress. The time, costs and logistics necessary for this approach, however, make it impossible to evaluate all active ingredients and formulated products in this way. Therefore, a tiered approach has been adopted in Europe. The first, relatively simple, tier of aquatic risk assessment is based on the estimation of a PEC/NEC ratio. In this ratio, the calculated concentration of the pesticide in surface water (Predicted Environmental Concentration; PEC) is compared with the expected No Effect Concentration (NEC). If the PEC does not exceed the NEC, no effects of the pesticide on the aquatic community are expected.

Table 1. EU criteria as set for the impact of pesticides on non-target species.

Tier	*Compartment* and organisms	Criteria
First tier	*Terrestrial*	
	Birds and other terrestrial non-target invertebrates	short-term PEC ≤ 0.1 x LD50 long-term PEC ≤ 0.2 x NOEC BCF ≤ 1 unless..
	Honeybees	maximum application rate ≤ 50 x LD50 unless..
	Beneficial arthropods	maximum application rate may not cause effects or death for more than 30% of test organisms in a laboratory test unless..
	Earthworms	short-term PEC ≤ 0.1 x LD50 long-term PEC ≤ 0.2 x NOEC unless..
	Soil micro-organisms	maximum application rate may not cause inhibition of nitrogen or carbon mineralization of larger than 25% after 100 days in the laboratory unless..
	Surface water	
	Fish, *Daphnia* and algae	Short-term PEC ≤ 0.01 LC50 or EC50 fish or *Daphnia* Short-term PEC ≤ 0.1 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
Second 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; LD50: Lethal Dose 50%; NOEC: No Observed Effect Concentration; BCF: BioConcentration Factor; LC50: Lethal Concentration 50%; EC50: Effect Concentration 50%

The first tier PEC is generally calculated with the help of a computer model for a standard freshwater system (stagnant; water depth 30 cm) on the basis of the recommended dose used for pest control and the percentage of expected drift (or input from another entry route; Capri and Trevisan, 1998). The NEC is based on concentration-

effect relationships studied in the laboratory with a limited number of "standard" species, viz., an alga, *Daphnia* and fish (Figure 1). These species have been chosen because of their ease of handling and rearing in the laboratory. Their test procedures are highly protocolized and well described in, for instance, OECD guidelines (Organisation for Economic Co-operation and Development; OECD, 1993). The standard test species are regarded as convenient surrogates for sensitive indigenous species of aquatic ecosystems, despite a general awareness of the uncertainty associated with the extrapolation from one species to another. To protect sensitive indigenous aquatic populations, the NEC is usually calculated by multiplying the toxicity value of the most sensitive standard test species by an assessment factor (usually a factor of 1/100 for acute EC50s or a factor of 1/10 for chronic NOECs; for more details see Table 1).

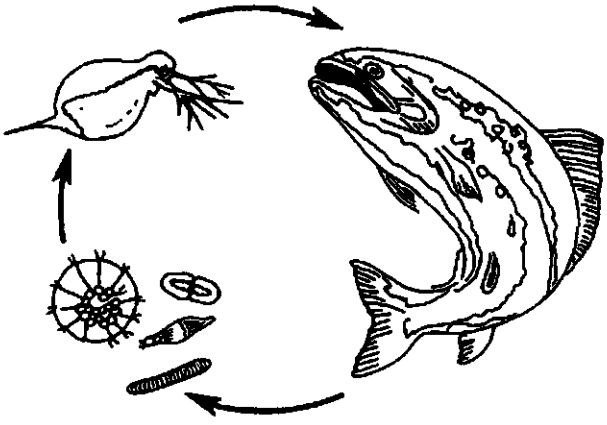


Figure 1. Standard test species as used for the aquatic risk assessment of pesticides.

The above-mentioned first tier in the risk assessment procedure is considered conservative, partly because of the higher dissipation rate and the generally lower bioavailability of pesticides in the field compared with the standardised test conditions in the laboratory, and partly because of the more or less worst case conditions adopted in the standard scenario to calculate the PEC. 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. This advanced risk assessment procedure can be regarded as the second tier, the "unless" procedure described in Table 1. This second tier does not consist of well-defined rules like the first one, but has to be tailor-made, depending on the degree of uncertainty in the risk remaining after the first tier. The requested additional information may range from, for instance, more information on the susceptibility of indigenous species to a better estimation of the half-life of the chemical in water. Experiments on an ecosystem level are

frequently requested and performed to demonstrate that the actual risks of a particular pesticide are acceptable when used under normal agricultural practice.

Need for validation and use of microcosms and mesocosms

The use of laboratory tests for the ecological risk assessment of pesticides is often disputed because of its lack of ecological realism (e.g. Kimball and Levin, 1985). To test whether the risk assessment procedure as described above protects the aquatic environment, the representativeness of the standard test species and the values of the assessment factors used should be validated. Ideally, the effects of pesticides should be evaluated at ecosystem level under natural conditions. This can be done by monitoring the dynamics of communities in impacted water bodies under field conditions and comparing them with a reference site. However, non-impacted reference sites are rarely available, and obscuring variables often make these field-observations difficult to interpret in terms of causal effects.

Another approach is the use of man-made experimental ecosystems: microcosms or mesocosms. Microcosms and mesocosm are made up of parts of natural ecosystems. They are brought together in a container (for instance an aquarium or a concrete tank) and are left to develop into a system that is complex enough to serve as a model for a natural ecosystem in terms of structure and function. The use of microcosms or mesocosms provides a bridge between the laboratory and the field, 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 also their complexity. The summary and recommendation document of the EWOFFT (Crossland et al., 1994) defined microcosms as experimental tanks/ponds with a water volume of less than 15 m^3 or experimental streams less than 15 m in length, while mesocosms are systems that are larger than 15 m^3 or 15 meter, respectively. It is very important that the dimensions, and hence often the complexity, of the test system meet the requirements needed to solve the research question. Thus, if scaled properly, the use of microcosms or mesocosms can provide the best of both worlds; if their dimensions are not adjusted to the research needs, it can suffer the disadvantages of both.

Pesticide studies in aquatic microcosms and mesocosms have frequently been used for both regulatory and scientific purposes (Hill et al., 1994; Graney et al., 1994). A framework of criteria and relevant endpoints for an acceptable aquatic microcosm or mesocosm study for pesticide registration is provided by several guidance documents (Touart, 1988; SETAC-RESOLVE, 1992; SETAC-Europe, 1992). However, the question "What should be measured to indicate the magnitude and duration of ecosystem responses to pesticide stress?" is not fully answered by these guidance documents,

because of the enormous complexity and variability of freshwater ecosystems. Nevertheless, it is generally recognised that a flexible framework can serve regulatory purposes, and that it is particularly the uncertainties arising from the earlier tiers of risk assessment which should guide the appropriate set of measurement endpoints in a microcosm or mesocosm study.

The need for interpretative tools

In recent years, the evaluation and interpretation of microcosm and mesocosm studies are frequently disputed between representatives of the industry and regulatory bodies. Questions like "How should these studies be evaluated?" and "What constitutes an acceptable effect?" are often raised.

A difficult stage in the evaluation of microcosm and mesocosm experiments is the analysis and interpretation of its results. The sampling of the various communities (e.g. zooplankton, phytoplankton, macroinvertebrates) in time results in large data sets comprising the dynamics of many species. These data sets are not easily analysed for treatment effects. Data from microcosm and mesocosm experiments are usually analysed with the same methods as those used for the analysis of standard laboratory test results. Normally, univariate methods like a statistical test (NOEC calculation) or regression analysis (EC50 calculation) is performed for each taxon. Because of the variability and/or low abundance of the majority of taxa, a satisfactory evaluation of treatment effects is only possible for a limited number of taxa (Van Wijngaarden et al., 1996). It has been recognised that in contrast to univariate methods, multivariate methods may enable researchers to use all information present (Crane et al., 1997). These methods are able to analyse treatment effects at a higher taxonomic level, viz., the community level. Over the last few years, various attempts have been made to analyse microcosm and mesocosm data at the community level (e.g. Leeuwangh, 1994; Van Wijngaarden et al., 1995; Shaw and Manning, 1996). The techniques used, however, are relatively complex and often not well understood. Also, the interpretation of the results they produce, for instance in the form of biplots, is often not straightforward, making the findings difficult to interpret.

Aims of the thesis

Four specific aims are addressed in this thesis.

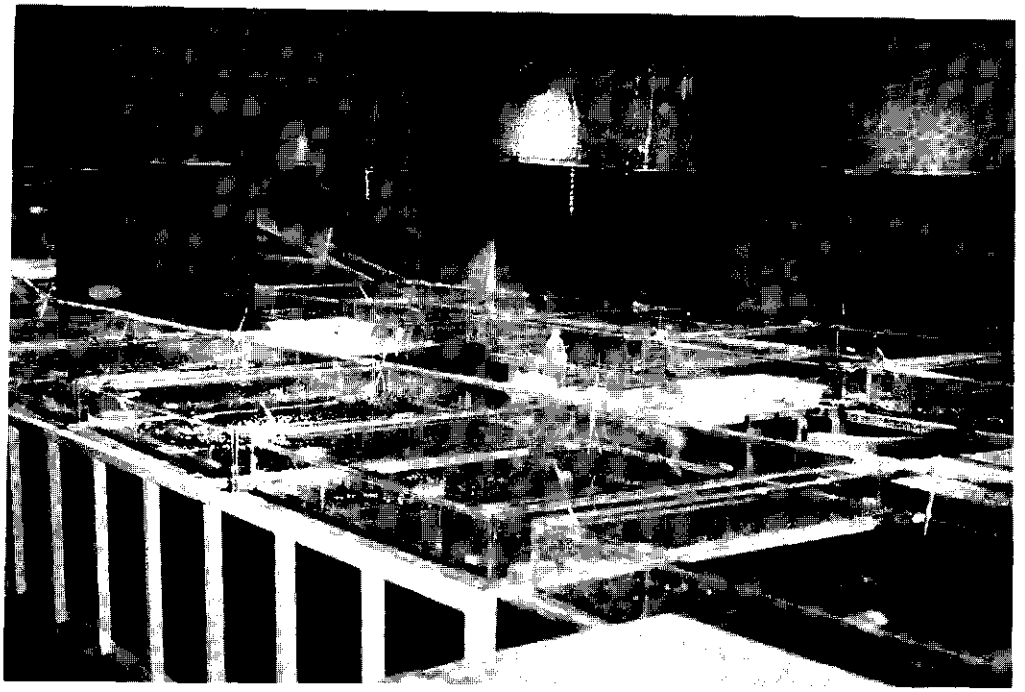
1 To validate the assessment factors of the first tier of aquatic risk assessment for pesticides using three chemicals with different modes of action (insecticide, herbicide, fungicide) as benchmark compounds.
2 To gain insight into long-term community responses and the factors determining the recovery of affected populations after a single application of an insecticide in experimental ditches.

3 To evaluate long-term responses in terms of ecosystem structure and functioning to chronic exposure to a herbicide and fungicide in aquatic microcosms.
4 To develop a technique that facilitates the interpretation of the results of microcosm and mesocosm experiments at the community level.

Test systems and substances used for this thesis

Two different test systems were used for the experiments described in this thesis, namely microcosms and mesocosms. Both test systems were chosen to mimic macrophyte-dominated drainage ditches, because this is the type of ecosystem that is expected to suffer most from pesticide contamination in the Netherlands. The microcosms are relatively small systems (1 m^3), situated in a laboratory of the Wageningen Agricultural University, the Netherlands (Figure 2A; Brock et al., 1992). The advantage of these indoor systems over the outdoor mesocosms is that they allow a relatively large level of control over the abiotic environment (e.g. temperature, light regime) and that they are relatively easy to handle in terms of construction and sampling. The disadvantage of these systems over outdoor systems is that it is impossible to maintain some populations in these systems and that they do not allow the researcher to study the recovery of all affected populations (e.g. emergent insects do not have the opportunity to re-enter an indoor microcosm after emergence). The large mesocosms (60 m^3; Figure 2B; Drent and Kersting, 1993) are situated at the experimental research station "De Sinderhoeve" in Renkum, the Netherlands. They were used for the study focussing on long term community responses and recovery of affected populations, while the indoor microcosms were used when the direct and indirect effects of chemicals and their threshold levels were the main objectives of the study.

The first three aims of this thesis were addressed by evaluating the effects of three pesticides, with different modes of action, with the help of microcosms and mesocosms. For this purpose an insecticide, a herbicide and a fungicide were chosen. The acethylcholine-esterase inhibiting organophosphate chlorpyrifos was used as a model substance for insecticides. Although this insecticide has been used in several other studies, at the time of the start of the experiment it had hardly been studied at realistic low concentrations and/or in great detail (Van Wijngaarden et al., 1998b). Linuron was chosen as a model substance for the photosynthesis inhibiting herbicides, the type of herbicides most commonly used in the Netherlands (NEFYTO, 1996). Carbendazim, a benzimidazole fungicide, was evaluated because it is frequently used in the Netherlands (NEFYTO, 1996), but reliable data on its toxicity and effects on aquatic ecosystems were largely lacking. Some physicochemical characteristics of the substances are shown in Table 2.



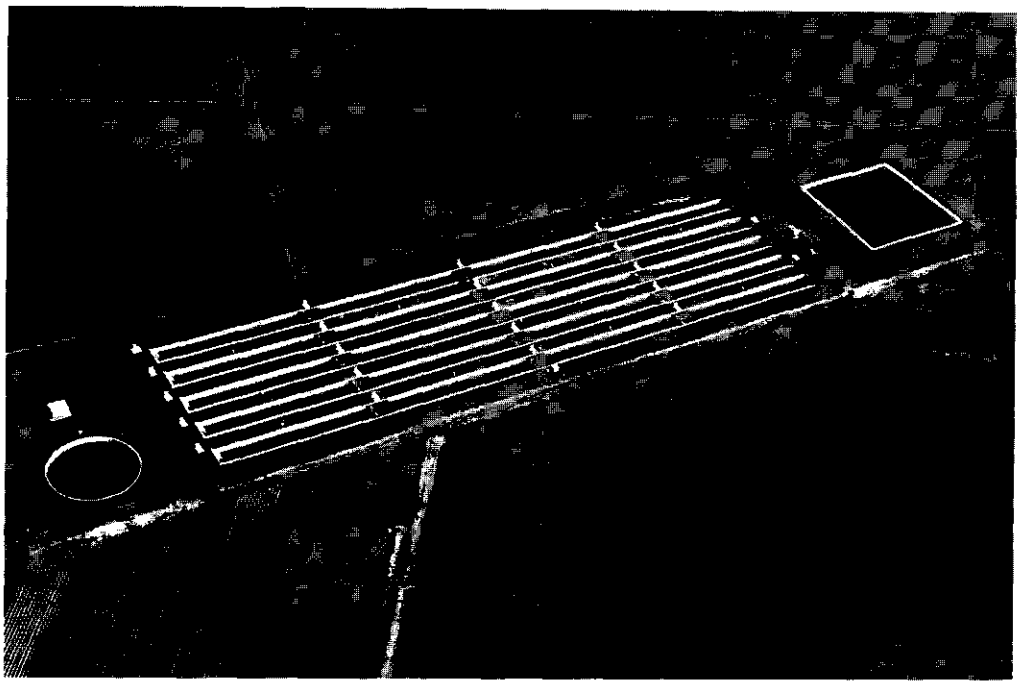


Figure 2. Overview of microcosms (top) and mesocosms (bottom) used.

Table 2. Some characteristics of the pesticides evaluated as listed in the Pesticide manual (Tomlin, 1997).

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 3 presents some relevant toxicity data of the three compounds for aquatic standard test organisms.

Table 3. Summary of relevant toxicity data of the pesticides for standard test organisms most susceptible to the chemicals (in µg/L).

	Chlorpyrifos	Linuron	Carbendazim
LC50 *Daphnia*	1 (LC50,48h; Kersting and Van Wijngaarden, 1992)	310 (LC50,24h; Stepenson and Kane, 1984)	320 (LC50,48h; Van Wijngaarden et al., 1998a)
NOEC *Daphnia*	0.1 (NOEC,21d; Kersting and Van Wijngaarden, 1992)	-	10 (NOEC,18d; Canton et al., 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

Outline of the thesis

Chapter 2 evaluates the long-term effects of a single application of an insecticide (chlorpyrifos) on the invertebrate community of freshwater outdoor experimental ditches. Effects on invertebrates are discussed at community level, with an interpretation at species level where necessary. Special attention is given to the relation between recovery patterns of taxa and their life-history characteristics.

Chapter 3 describes the response of indoor freshwater microcosms to chronic treatment with the herbicide linuron. Section I discusses the effects on primary producers and the risk assessment of linuron. The second section deals with the effects on community metabolism and invertebrates and discusses the ecological effect chain.

Chapter 4 discusses the effects of the fungicide carbendazim on the ecology of indoor freshwater microcosms. Section I. discusses the risk assessment and effects on water quality parameters and macro-invertebrates, while section II deals with the effects on zooplankton and primary producers and the overall ecological effect chain.

Chapter 5 presents a novel multivariate method, designed especially for the analysis of data from microcosm and mesocosm experiments. This new method, Principal Response Curves, is fully described and compared with two other methods used in ecotoxicology.

Chapter 6 shows that the Principal Response Curves technique is able to reveal not only the dominant response pattern present in a data set, but also more subdominant ones. This is discussed with the help of data sets from a multi-stress experiment. The results are compared with those of two similarity indices.

Chapter 7 discusses the applicability of a multivariate technique commonly used in ecology, Correspondence Analysis, for the analysis of results from microcosm and mesocosm experiments. Its results are compared, with the help of an example data set, with those of the Principal Response Curves and Redundancy Analysis.

Chapter 8 presents a summary of the thesis and discusses the contents of the other chapters in relation to the aims set in the present chapter. Some concluding remarks are made and suggestions for future research are given.

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Chapter 2

Effects of the insecticide Dursban®4E (active ingredient chlorpyrifos) in outdoor experimental ditches:

II. Invertebrate community responses and recovery

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EFFECTS OF THE INSECTICIDE DURSBAN® 4E (ACTIVE INGREDIENT CHLORPYRIFOS) IN OUTDOOR EXPERIMENTAL DITCHES: II. INVERTEBRATE COMMUNITY RESPONSES AND RECOVERY

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Abstract—This article describes the long-term effects on the macroinvertebrate and zooplankton community in outdoor experimental ditches after a single application of the insecticide chlorpyrifos. Nominal concentrations of 0.1, 0.9, 6, and 44 μg/L of chlorpyrifos were applied to two mesocosms each, while four served as controls. Both macroinvertebrates and zooplankton were sampled from 4 weeks before to 55 weeks after treatment. The macroinvertebrate and zooplankton data sets were combined into one data set and analyzed using the multivariate ordination technique "redundancy analysis." The method provided a clear description of the effects on the invertebrate community in time while still showing the effects at the species level. Crustacea and Insecta showed a rapid, concentration-dependent decrease in numbers after insecticide application (direct effects). An increase in gastropods and Oligochaeta was found, suggesting indirect effects. The start of recovery of the invertebrate populations affected was found to depend not only on the susceptibility of the taxa but also on ecological characteristics, such as the length of the life cycle. A no-observed-effect concentration of 0.1 μg/L could be derived both at the species and the community level. Safe concentrations, based on no-observed-short-term-effect levels for some characteristic indigenous taxa susceptible to chlorpyrifos, also appeared to protect the total invertebrate community in the long term. The invertebrate community at all treatment levels was considered to have recovered after 24 weeks posttreatment.

Keywords—Mesocosms Invertebrate community Multivariate ordination techniques Recovery NOEC

INTRODUCTION

Model ecosystems that mimic freshwater ecosystems are often used to assess the potential ecotoxicological hazards of pesticides [1–3]. A major advantage of these experimental freshwater ecosystems is their simulation of realistic pesticide exposures to aquatic organisms in a complex ecosystem. Thus, effects on and recovery of a wide array of species can be studied while allowing interactions between the various populations of a community.

This article is the second of a series of three dealing with the impact of a single application of the insecticide Dursban® 4E (active ingredient chlorpyrifos) on the ecology of outdoor mesocosms. The studies presented in this series were initiated in order to evaluate the significance of standard laboratory tests for predicting effects of a pesticide in aquatic ecosystems. The first article compared acute toxicity to indigenous species in the laboratory with short-term effects in the mesocosms. It also proposed a safe concentration for the mesocosms based on short-term effects observed in these systems [4]. The third article will deal with the effects of the insecticide on ecosystem functioning and oxygen metabolism in particular.

The aims of this article are to describe long-term effects of a single application of the insecticide chlorpyrifos on invertebrate populations and the invertebrate community of outdoor experimental ditches, to evaluate the rate of recovery of susceptible populations and the invertebrate community, and to set safe threshold values for susceptible indigenous populations and the invertebrate community.

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MATERIALS AND METHODS

Experimental design

On May 8, 1990, the organophosphorus insecticide Dursban 4E was applied once by means of a spray boom to eight outdoor experimental drainage ditches (mesocosms). Four dose levels were applied to two mesocosms each, while four other systems served as controls. Each mesocosm had the following characteristics: length, 40 m; width at water surface, 3.4 m; water volume, 60 m^3; and mean water depth, 0.5 m. Details of the construction and equipment of the mesocosms can be found in Drent and Kersting [5]. The aquatic community in the mesocosms resembled that of macrophyte-dominated drainage ditches.

The nominal concentrations of the active ingredient chlorpyrifos, calculated from the amounts of insecticide sprayed and the water volume of the mesocosms, were 0.1, 0.9, 6, and 44 μg/L. These concentrations are related to agricultural application in the sense that the lowest treatment level is considered a safe standard concentration, while the highest corresponds to a "realistic worst case" scenario. Common agricultural application of chlorpyrifos in the Netherlands results in predicted environmental concentrations (PECs) of 0 to 64 μg/L (authors' calculations). Detailed information on the experimental design can be found in the first article of this series [4].

Invertebrate community sampling and analysis

The invertebrate data set. From week −4 through week 56 the zooplankton and macroinvertebrate communities were sampled 15 times. These communities were sampled in both macrophyte-dominated and macrophyte-free locations. The sampled

individuals were identified in the laboratory, to species level if possible. The sampling and identification methods are described in detail in part I [4].

To evaluate the effects of the insecticide at the level of the invertebrate community, all zooplankton and macroinvertebrate data sets had to be combined into one. Abundances of macrozooplankton (>300 μm) in macrophyte-free and macrophyte-dominated locations were lumped. The lumped data set was then used to calculate average numbers for each mesocosm. The averages (numbers per liter) of the macrozooplankton and the data set of the microzooplankton were combined into a single zooplankton data set. As was described in detail in part I, the macroinvertebrates were sampled in both macrophyte-free and macrophyte-dominated locations by means of artificial substrates [4]. Samples of the two locations were also lumped and average numbers calculated. Abundance data for zooplankton (numbers per liter) and macroinvertebrates (numbers per substrata) were ln $(10x + 1)$-transformed (for the rationale of this transformation see Van den Brink et al. [6]) and subsequently standardized. The following formula was used for standardization:

$$\text{abundance values data set Macroinv}_{\text{standardized}} = \sqrt{\frac{tss_{\text{data set Zoopl}}}{tss_{\text{data set Macroinv.}}}} * \text{abundance values data set Macroinv.}$$

where *tss* is the total sum of squares of the corresponding macroinvertebrates (Macroinv) and zooplankton (Zoopl) data sets. This standardization was needed to make both data sets equally important in terms of amount of variance. In our case, the "square root term" in the formula resulted in a factor of 0.98. As a consequence, the log-transformed abundance values of the macroinvertebrate data set were multiplied by 0.98. All statistical analyses were performed using the invertebrate data set thus obtained.

Multivariate analysis of treatment effects. Effects at the community level can be analyzed by means of multivariate regression techniques such as principal component analysis (PCA) [7] and redundancy analysis (RDA). Redundancy analysis is the constrained form of PCA and has the advantage of allowing effects of explanatory variables to be expressed and can be combined with a Monte Carlo permutation test for statistical analysis [8]. These techniques have a limited and comprehensible output, even when starting with complex and large data sets. They provide a clear overview of temporal and treatment effects on a community and can indicate recovery of this community [8].

In the present study, the responses and recovery in time of the invertebrate community after the Dursban 4E treatment were analyzed using RDA. The sampling periods, comprising weeks −4 through 24 and weeks 42 through 55 (before and after the winter season, respectively), were analyzed separately.

Principal component analysis and RDA are based on a linear response model. This means that they calculate a linear regression line from the abundance data of all samples. This regression line represents a fraction of the total variance in the data set and is presented in a diagram as the first axis (see Fig. 3). A second regression line is extracted from the remaining variance, representing the second axis of the diagram. In extracting the regression lines, PCA takes into account all variance of a data set. In contrast to PCA, RDA is constrained to the fraction of the total variance that is explained by the explanatory variables. These explanatory variables are fixed upon the analysis a priori.

Table 1. Macrophyte biomass used as covariable in the redundancy analysis for weeks −4 through 24. The mean biomass values for the sampling weeks −2 and 13 are shown

Replicate number	Macrophyte biomass (kg dry wt./m²)				
	Control mesocosms	0.1 μg/L Treatment mesocosm	0.9 μg/L Treatment mesocosm	6 μg/L Treatment mesocosm	44 μg/L Treatment mesocosm
1	0.26	0.22	0.26	0.27	0.24
2	0.26	0.26	0.14	0.26	0.28
3	0.24	—	—	—	—
4	0.26	—	—	—	—

The percentage of the total variance of the data set explained by the explanatory variables is called the sum of all canonical eigenvalues. The axes in an RDA (e.g., Fig. 3) represent a percentage of this sum. The higher these percentages, the more variation is explained by the axes. Values of about 30 to 40% are quite common in ecological applications [9]. For more theoretical background information and technical details see Ter Braak [9–11]. Specific details on the application of RDA to the results of model ecosystem experiments are given in Van Wijngaarden et al. [8].

Redundancy analysis was performed using the CANOCO computer program, version 3.14 [10]. In the RDA, the factors "treatment" and "sampling week," plus their interaction, were used as combined dummy explanatory variables since we wanted to focus on the relevant variance of the invertebrate data set (i.e., only that variance which can be attributed to time or treatment). Since macrophytes play an important role in structuring the aquatic invertebrate community and since the macrophyte biomass at the time of application is an important factor influencing the fate and effects of Dursban 4E in aquatic ecosystems [12], macrophyte biomass was used as a covariable to correct for possible systematic differences between the mesocosms. In order to obtain a good macrophyte biomass estimate for the period comprising weeks −4 through 24, the mean of the macrophyte biomasses sampled in weeks −2 and 13 was used as a covariable. Macrophyte biomass for these sampling weeks was estimated (in kilograms dry weight per m²) by sampling the macrophytes in five 1-m² plots in each mesocosm. The macrophyte biomasses of the mesocosms, used as covariables, are given in Table 1. Only one mesocosm showed a deviant biomass, one replicate of the 0.9-μg/L treatment. Because no macrophyte biomass estimations were available for the period consisting of weeks 42 through 55, covariables were used only in the first analysis (weeks −4 through 24). Within CANOCO, we opted for scaling 1 (euclidean distances) since dummy explanatory variables were used [13]. Apart from this, the default options were chosen.

To check whether treatment-related differences shown in the RDA diagrams were statistically significant, Monte Carlo permutation tests, incorporated in CANOCO, were carried out. General concepts of Monte Carlo permutation testing, combined with ordination, have been described in Ter Braak et al. [10,14,15]. The permutation tests used in the present study have been described in Van Wijngaarden et al. [8].

Before testing, treatment levels were log-transformed. We did so because dose–response curves are intrinsically sigmoid [16], and this allowed us to fit the dose–response curve as closely as possible to the linear response model in the RDA. Because of the limited options for permutation, permutation testing of

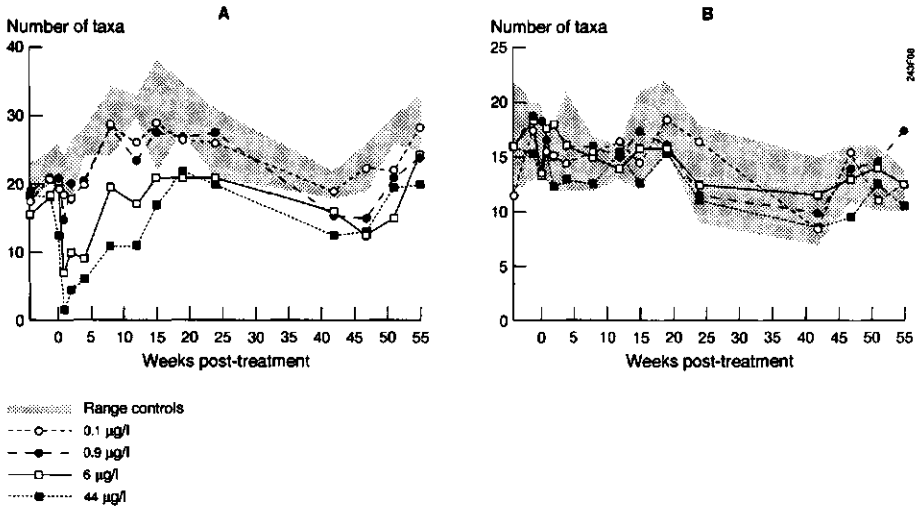

Fig. 1. Dynamics of numbers of arthropod (**A**) and nonarthropod (**B**) taxa. Shaded areas represent the minimum and maximum numbers collected in the control mesocosms. The lines represent the average number of taxa collected per treatment.

each treatment separately against the controls was useless. Therefore, all treatments were tested jointly with controls. The tests were performed for each sampling week, with ln $(20x + 1)$-transformed nominal concentrations as log–dose, where x is the nominal concentration (for the rationale see Van den Brink et al. [6]).

No-observed-effect concentration calculations. To study effects on and recovery of separate taxa, univariate analyses were performed on the 19 most discriminant species of the RDA analysis of the period comprising weeks −4 through 24. These analyses used the Williams test [17], which assumes an increasing effect for an increasing dose. This test allowed us to establish a no-observed-effect concentration (NOEC) ($p < 0.05$) for each sampling week for each taxon. The Williams test was performed using the Community Analysis computer program, version 3.5 [18].

Before the $NOEC_{community}$ could be obtained, a variable had to be calculated that best summarized the community variance. Redundancy analysis is not suitable for providing this variable because it uses explanatory variables, which are a priori-related to the toxicant. In PCA, however, the entire unconstrained variance of the data set is taken into account. Therefore, PCA was used to calculate the first principal component, which is the single variable that summarizes the community variation best; it is a linear combination of the species data, not a priori-related to the toxicant. Principal component analysis was performed on the invertebrate data set for each sampling week using the CANOCO computer program. When the principal component of the samples (coordinates of the first PCA axis) was analyzed with the Williams test, we tested whether these coordinates represented the treatment regime. These analyses resulted in an $NOEC_{community}$ for each sampling week.

Analysis of functional groups. It may be questioned whether effects on individual species are reflected in the properties of the community. We therefore evaluated effects on functional feeding groups of macroinvertebrate taxa. Five groups can be distinguished: shredders, scrapers, predators, collector filterfeeders, and collector gatherers [19,20]. Zooplankton was excluded since no information on functional groups was available. The original macroinvertebrate data set was used for the analysis. All abundance values of taxa belonging to the same functional group were added up; if a taxon belonged to two or three functional groups, its abundance value was divided evenly over these functional groups. From these summations, the relative share of each functional group could be calculated. These calculations were done for three periods: weeks −4 through −1, weeks 1 through 4, and weeks 47 through 51.

RESULTS

General sampling results for the invertebrate community

A total of 189 taxa were identified and their abundance determined (59 zooplankton and 130 macroinvertebrate taxa). In terms of the numbers of taxa, the most important taxonomic groups were Insecta (103), Rotatoria (36), Crustacea (22), and Gastropoda (15).

In the first week after insecticide treatment the number of arthropod taxa decreased substantially at the two highest treatment levels (Fig. 1A), unlike the number of nonarthropod taxa (Fig. 1B).

Before treatment, no differences in functional group composition or in absolute numbers of macroinvertebrate individuals sampled were observed between treatments (Fig. 2A). Compared to the controls, numbers of macroinvertebrates were significantly lower at the 0.9-µg/L treatment level and higher (Fig. 2B). At these treatment levels, ratios of the functional groups had shifted; shares of collector gatherers decreased and shares of collector filterers increased (Fig. 2B). One year after treatment the relative share of collector gatherers and scrapers was found to have decreased in all treatments (Fig. 2C). At all treatment levels except the highest, the share of shredders had increased in the year after treatment (Fig. 2C).

Multivariate and univariate analysis

Sampling period of weeks −4 through 24. The RDA diagram (Fig. 3) summarizes the treatment effects in the data set while

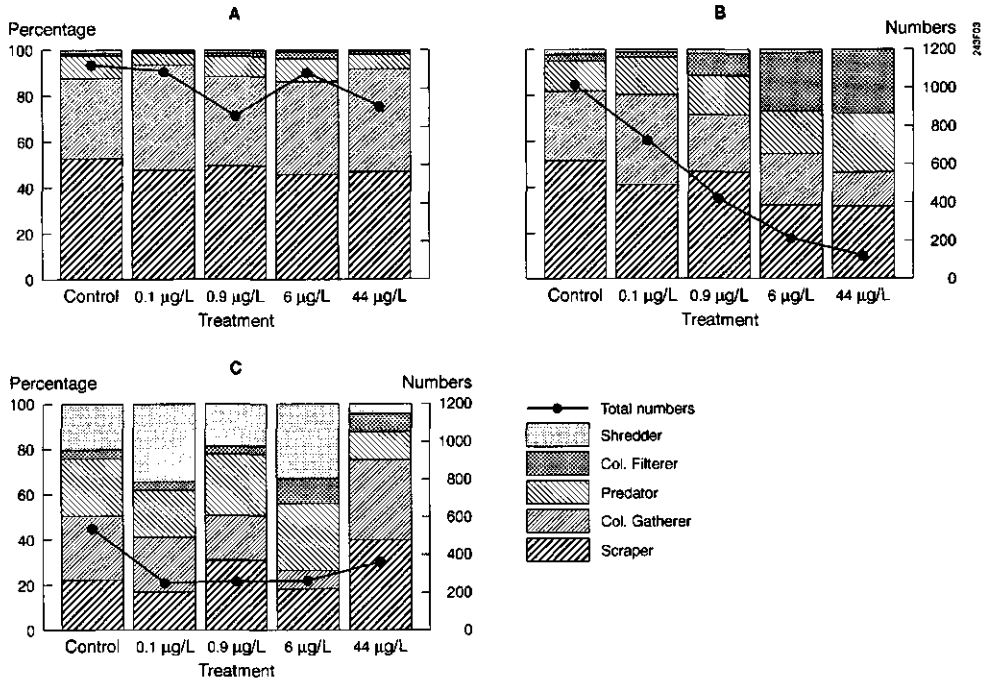

Fig. 2. Relative shares (%) of the macroinvertebrate individuals sampled for the functional feeding groups averaged over three periods. (A) Before treatment (weeks −4 and −1). (B) Weeks 1 through 4. (C) Weeks 47 through 51. The absolute numbers of sampled individuals per treatment are also indicated.

still showing the approximate species composition for all samples. In the diagram, 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. In the diagram, the species *Cloeon dipterum* is relatively abundant in all control samples and (almost) absent from the samples of weeks 1, 2, and 4 for the highest treatment level. To limit the number of taxa shown in the diagram, only the 45 most discriminant taxa in each analysis are presented. The 45 most discriminant taxa are defined as the 45 taxa with the highest fractions of variance explained by the axes.

The RDA indicated pronounced effects of the insecticide application on the invertebrates (Fig. 3). The diagram reveals a dose–effect relationship; the magnitude of the effect of the treatment decreases in the order 44 > 6 > 0.9 > 0.1 µg/L ≈ controls. The clustering of all pretreatment samples indicates minor differences between the mesocosms at the start of the experiment. The shift of the control samples from the left to the right indicates a time vector in this direction. The line representing the 0.1-µg/L treatment level is situated closest to the control line and most closely resembles its pattern. All week 24 samples of the treated mesocosms are situated close to the corresponding control samples, indicating that differences at 24 weeks posttreatment were minor. This suggests recovery of the invertebrate community in all treated mesocosms. The direction of the treatment vector is from the upper left quadrant to the lower right quadrant (Fig. 3). Those taxa affected negatively by the treatment are situated in the upper left quadrant and above the line representing the control treatment. Insusceptible and positively affected taxa are situated below this line. The treatment resulted in a decrease in the numbers of most arthropods, especially ephemeropterans, dipterans, coleopterans, zygopterans, trichopterans, megalopterans, amphipods, cladocerans, copepods, and ostracods. Nonarthropods showing a decreasing tendency included Ciliata (mainly *Halteria* sp.), and the mollusks Sphaeriidae and *Armiger crista*. The RDA diagram indicates a positive correlation between the numbers of gastropods (*Bithynia tentaculata* and *Radix peregra*), the leech *Erpobdella octoculata*, and oligochaetes on the one hand and treatment levels on the other.

No-observed-effect concentrations are presented for those taxa that showed a consistent response, i.e., a significant response on two or more consecutive sampling weeks (Table 2). Negative effects were most pronounced from weeks 0.1 through 4. Most taxa recovered within 24 weeks. *Caenis horaria* and *Gammarus pulex* failed to recover fully within the first 24 weeks posttreatment. The statistical analysis indicates that *Oligochaeta* spp. and *Stylaria lacustris* were significantly more abundant in the high treatment levels than in the controls (Table 2). *Cloeon dipterum* showed a decrease in numbers in the 0.9-, 6-, and 44-

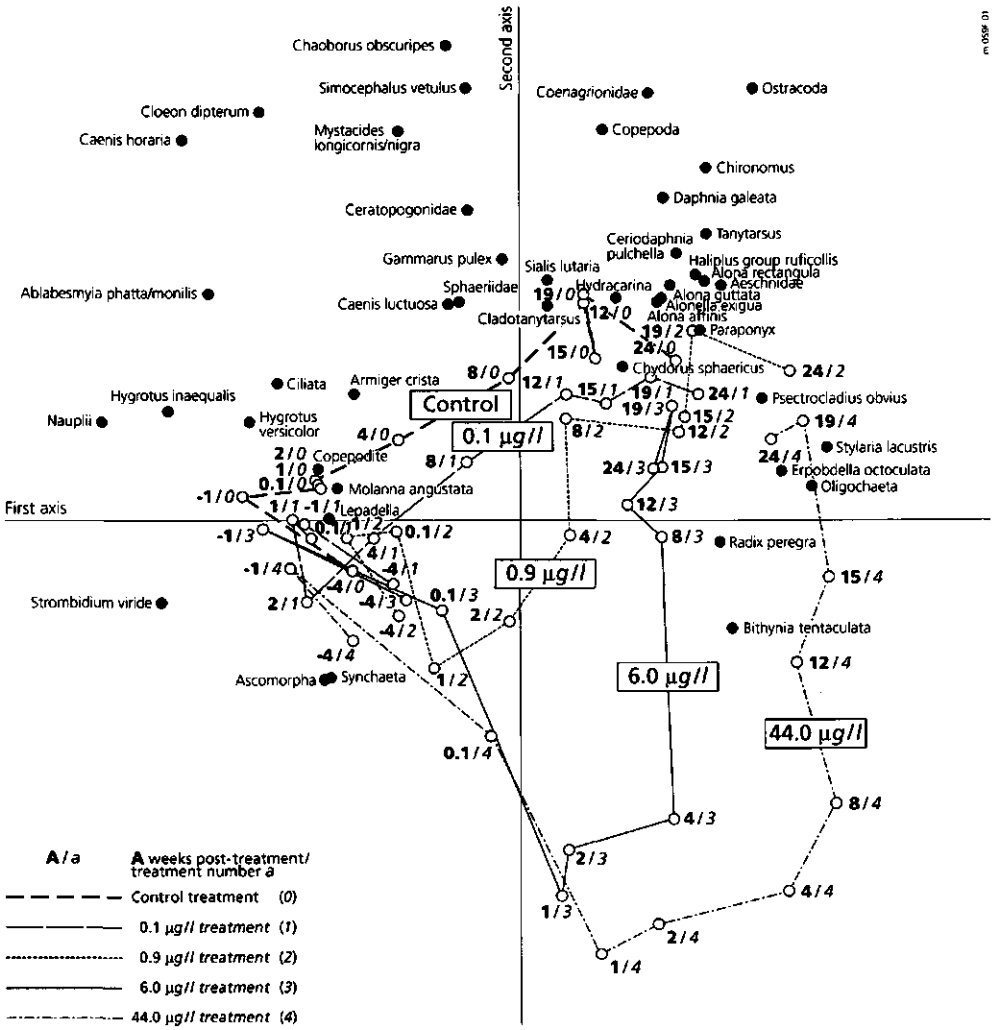

Fig. 3. Ordination diagram (redundancy analysis [RDA]) indicating effects of a single application of the insecticide chlorpyrifos on zooplankton and macroinvertebrates. The sampling period covered weeks −4 through 24. Sampling week and treatment level, as well as their interactions, were taken as explanatory variables, macrophyte biomass was taken as a covariable. The lines represent the course of the treatment levels in time. Of all variance, 55% can be attributed to the explanatory variables. Of this explained variance, 37% is displayed in the diagram. Only those 45 species most discriminant for the diagram are shown.

µg/L treatments compared to the controls. The 0.9- and 6-µg/L treatments returned to control abundance values within 8 weeks posttreatment; the 44-µg/L treatment, within 15 weeks. In contrast to *C. dipterum*, *C. horaria* failed to return to control abundance values in the 6- and 44-µg/L treatments within 24 weeks (Table 2 and Fig. 4).

Sampling period of weeks 42 through 55. The RDA over the sampling period of weeks 42 through 55 indicates treatment-related differences in species composition (Fig. 5), with the effect of the treatment decreasing in the order 44 ≈ 6 ≈ 0.9 > 0.1 µg/L ≈ controls. The direction of the treatment vector in the RDA diagram (Fig. 5) is from the top to the bottom. The direction of the time vector is from left to right. Taxa less abundant in the treated mesocosms are situated at the top, and the insusceptible and positively affected taxa are situated at the bottom. *Gammarus pulex*, *C. horaria*, and Coenagronidae spp. occurred in significantly lower numbers at the highest treatment levels (Table 2). *Gammarus pulex* was almost absent from the 0.9-µg/L and completely absent from the 6- and 44-µg/L mesocosms in week 55 (Fig. 6). Taxa that occurred in higher densities than in the controls (though no significant differences could be demonstrated) included the *Agrypnia/Dasystegia/Phryganea* complex and *Bithynia tentaculata* (see Fig. 5 and Table 2).

Monte Carlo permutation and $NOEC_{community}$. No significant differences between the invertebrate communities could be demonstrated before treatment (Table 3). After insecticide application, the permutation tests showed the treatment to have

Table 2. Results of the Williams test ($p \leq 0.05$) of the discriminant taxa of the redundancy analysis. The no-observed-effect concentration (NOEC) of each taxon is given per sampling week. Only those taxa that showed a significant response in two consecutive sampling weeks are presented

Taxon	Effect[a]	Sampling week[b] −4	−1	0.1	1	2	4	8	12	15	19	24	42	47	51	55
Annelida																
Oligochaeta	+	>	>	>	>	>	0.9	6	>	>	>	0.9	>	>	>	>
Stylaria lacustris	+	>	>	>	>	>	0.9	0.9	>	>	>	>	>	>	>	>
Arthropods																
Crustacea																
Simocephalus vetulus	−	>	>	0.9	0.9	0.9	0.9	6	>	>	>	>	>	>	>	>
Daphnia galeata	−	n.p.	n.p.	n.p.	>	>	0.1	0.1	6	6	>	>	n.p.	n.p.	>	>
Ostracoda	−	n.p.	L!	6	0.9	6	6	0.9	6	6	6	>	n.p.	L!	>	>
Copepoda (mature stages)	−	>	>	>	L!	6	>	0.9	>	>	>	>	>	>	>	>
Copepoda (nauplii)	−	>	>	0.9	0.9	0.9	0.9	6	>	n.p.	>	n.p.	n.p.	>	>	>
Gammarus pulex	−	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	0.1	L!	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Insecta																
Caenis horaria	−	>	>	6	0.9	0.9	0.1	0.9	6	0.9	6	0.9	>	0.1	L!	0.9
Caenis luctuosa	−	n.p.	n.p.	6	n.p.	0.9	0.1	0.9	>	>	n.p.	n.p.	n.p.	n.p.	L!	L!
Cloeon dipterum	−	>	>	0.9	0.1	0.1	0.1	6	>	>	>	>	>	>	>	>
Coenagrionidae	−	>	>	>	6	6	6	>	>	>	>	>	>	>	0.1	0.1
Sialis lutaria	−	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	0.1	n.p.	0.1	0.1	n.p.	0.1	n.p.	L!	n.p.
Hygrotus versicolor	−	n.p.	n.p.	>	0.9	0.9	n.p.	6	0.9	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.
Mystacides longicornis and *M. nigra*	−	>	>	0.9	0.9	L!	0.1	>	6	6	6	>	n.p.	>	>	n.p.
Ablabesmyia phatta and *A. monilis*	−	>	>	6	0.9	0.9	0.9	6	6	>	0.9	n.p.	n.p.	0.1	0.9	n.p.
Ceratopogonidae	−	>	>	>	0.9	>	L!	6	6	>	>	>	n.p.	n.p.	>	n.p.
Chaoborus obscuripes	−	L!	>	0.1	0.9	0.1	0.9	6	6	6	>	6	>	>	>	>
Chironomus	−	n.p.	n.p.	n.p.	>	n.p.	>	>	6	6	>	>	n.p.	n.p.	n.p.	>

[a]+ indicates a significant increase in numbers in treated mesocosms relative to controls; − indicates a significant decrease.
[b]L! indicates an NOEC < 0.1 μg/L; n.p. indicates that the Williams test was not performed because of the absence of the taxon from two or more controls (this criterion was used only when the effect of the treatment was negative); > indicates an NOEC of >44 μg/L.

a significant effect on the invertebrate community until week 24. After week 42 the effect became significant again. However, when *G. pulex* was omitted, no significant effects could be demonstrated after 24 weeks posttreatment. The lowest $NOEC_{community}$ found was 0.1 μg/L (Table 3).

DISCUSSION

Overall ecological effects

The application of the higher treatment levels of chlorpyrifos in our mesocosms resulted in a pronounced decrease in the number of arthropod species (Fig. 1A,B) and in a reduction of all arthropod populations abundant at the time of application (Table 2). The RDA diagram (Fig. 3) can be seen as a mean response pattern of all susceptible arthropod populations, suggesting a concentration-dependent negative effect during the first week after treatment and (the start of) recovery within 24 weeks. However, caution should be exercised in the interpretation of locations of taxa in the RDA diagram in terms of susceptibility to chlorpyrifos only. Seasonal aspects, such as natural succession, should also be taken into account. For example, the most susceptible species according to laboratory tests, *G. pulex* [21], was not collected in most mesocosms (in-

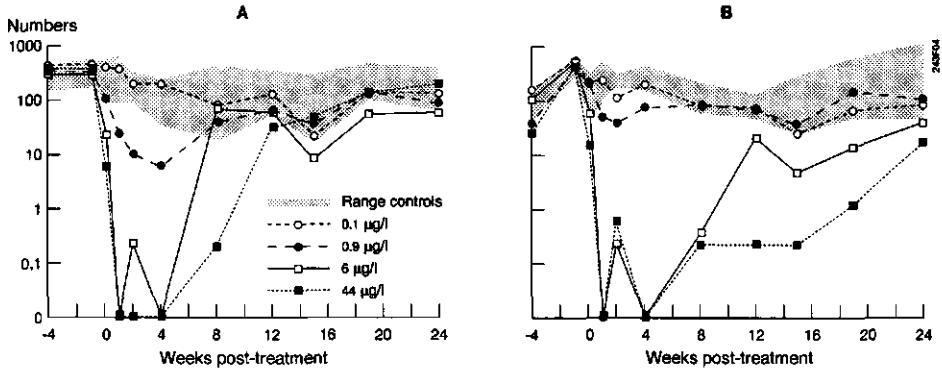

Fig. 4. Dynamics of numbers of the ephemeropterans *Cloeon dipterum* (**A**) and *Caenis horaria* (**B**). Shaded areas represent the minimum and maximum numbers collected in the control mesocosms. The lines represent the average numbers collected per treatment.

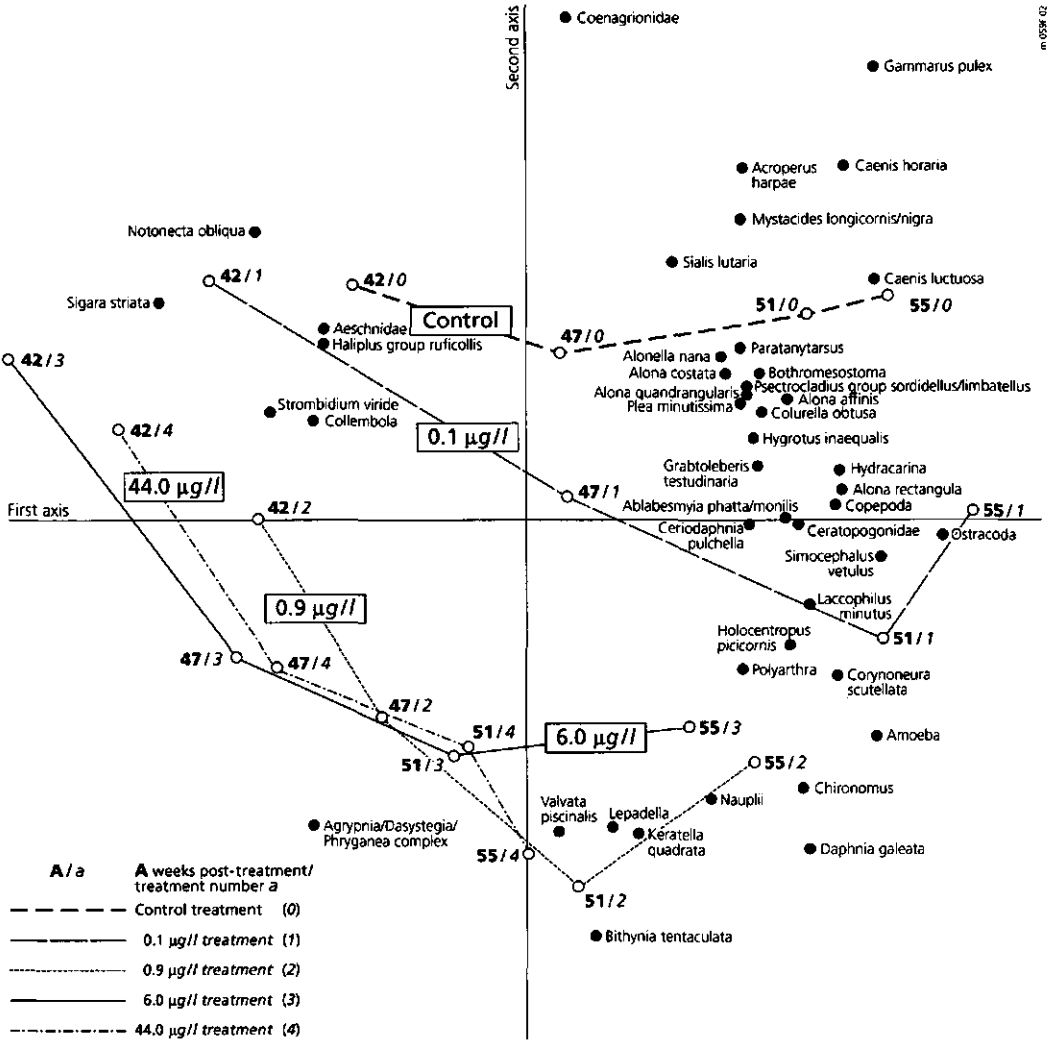

Fig. 5. Ordination diagram (redundancy analysis [RDA]) indicating effects of a single application of the insecticide chlorpyrifos on zooplankton and macroinvertebrates. The sampling period covered weeks 42 through 55. Sampling week and treatment level, as well as their interactions, were taken as explanatory variables. The lines represent the course of the treatment levels in time. Of all variance, 49% can be attributed to the explanatory variables. Of this explained variance, 40% is displayed in the diagram. Only those 45 species most discriminant for the diagram are shown.

cluding controls) at the time of treatment (Table 2). Numbers increased in the controls and 0.1-µg/L mesocosms in the course of the experiment and failed to do so at the two highest treatment levels (Fig. 6). This is why the position of this species in the RDA diagram is not really extreme (in view of the treatment level), in contrast to relatively less or equally susceptible species, such as *C. dipterum* and *Chaoborus obscuripes* [21], that were abundant at the time of insecticide treatment. Nevertheless, the significantly lower numbers of *G. pulex* in the 0.9-, 6-, and 44-µg/L treatments compared to the controls (Fig. 6 and Table 2) can be explained from its susceptibility to chlorpyrifos (96-h lethal concentration [LC50] of 0.07 µg/L [21]). In general, the negative effects on arthropod populations observed in our study are in accordance with results of single-species toxicity tests [21] and other aquatic model ecosystem studies performed with chlorpyrifos [12,22–31].

The loss of arthropod populations in the first week post-treatment (direct effects) did not result in many detectable indirect effects on other invertebrate populations. Significant effects on nonarthropod populations of zooplankton (Rotatoria, Ciliata) could not be demonstrated. Of the macroinvertebrates, only the oligochaete worms (Oligochaeta spp., *S. lacustris*; Table 2) showed significant increases in abundance. A similar indirect effect of chlorpyrifos on *S. lacustris* was observed in one of our experiments in indoor microcosms and was explained by the increased supply of food in the form of periphytic algae

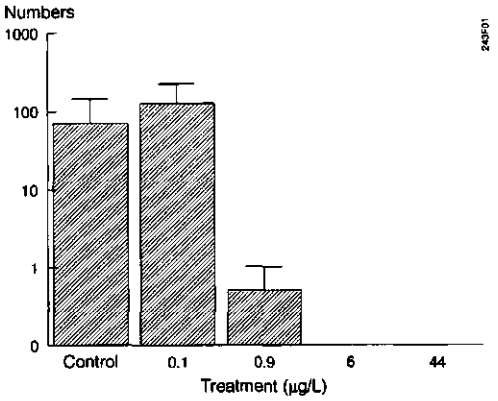

Fig. 6. Number of *Gammarus pulex* (average ± SD) per treatment level sampled in week 55.

after the loss of arthropod grazers [28]. In the three indoor microcosm experiments performed within the same research program, in which the communities of drainage ditches were simulated [27,32], indirect effects of a nominal chlorpyrifos treatment of 35 µg/L were much more diverse than those of the highest treatment level (44 µg/L) in the mesocosms. In the microcosms it was the invertebrate populations of Rotatoria, Turbellaria, Hirudinea, Oligochaeta, Mollusca, and Isopoda which showed indirect effects. The structure of the community in the indoor microcosms, however, was less complex than that of the outdoor mesocosms. Apparently, a structurally more diverse and complex ecosystem includes more redundant populations and feedback mechanisms, so indirect effects are harder to detect.

An understanding of the trophic structure of the community in the mesocosms is important in assessing the impact of chlorpyrifos stress. Before treatment, differences between the treatment levels in the distribution of macroinvertebrate individuals over functional groups were found to be small (Fig. 2A). Many of the susceptible arthropod taxa found appeared to be generalists rather than specialists with regard to their food habits [20]. In addition, the susceptible populations of Insecta in particular comprised all functional groups. Nevertheless, the share of collector gatherers showed a dose-dependent decrease in the first month after treatment due to the loss of susceptible taxa such as *C. horaria* and *C. dipterum* (Figs. 2 and 4). At the same time, the share of collector filterers increased, partly due to the (nonsignificant) increase in numbers of the snail *B. tentaculata* and the significant increase in oligochaete worms at the two highest treatment levels (Figs. 3 and 2B). Both functional groups use fine particulate organic matter (FPOM) as their food resource [20], so the loss of collector gatherers can explain the increase in collector filterers. One year after chlorpyrifos treatment, consistent differences between treatments in the relative shares of collector gatherers and collector filterers could no longer be demonstrated (Fig. 2C). In all treatments except the 44-µg/L mesocosms, the share of shredders was relatively high compared with the previous periods. This can be attributed to the increased abundance of the amphipod *G. pulex* in the controls and 0.1-µg/L treatment and of the isopod *Asellus aquaticus* in the 0.9- and 6-µg/L treatments (results not shown). Given that in the 44-µg/L treatment shredders were almost absent 1 year after chlorpyrifos treatment and that the most important shredders in freshwater ecosystems are usually Arthropods, this functional feeding group should be considered at least potentially susceptible to insecticide contamination (low redundancy). This is in accordance with observations of a decrease in shredder populations and breakdown of plant litter in microcosms treated with 35 µg/L chlorpyrifos [27,32] and with observations by Wallace et al. [33], who reported similar effects in a headwater stream treated with methoxychlor.

Recovery

In this article we consider a susceptible population to be recovered from chlorpyrifos stress when, over a prolonged period of time, significant differences in abundance between control and treated mesocosms can no longer be demonstrated.

In considering the recovery of Arthropods at the species level, it is convenient to distinguish between populations of Crustacea, which complete their life cycle strictly in water, and populations of Insecta, which usually have distinct aquatic and terrestrial life phases. Of the Crustacea in our mesocosms, representatives of Cladocera (*Simocephalus vetulus, Daphnia galeata*), Ostracoda, and Copepoda (including nauplii) showed a relatively rapid recovery within 12 to 24 weeks, even at the highest treatment level (Table 2). The relatively rapid recovery of microcrustaceans can be explained by their short life cycle and/or high reproductive capacity. In addition, pesticide-insensitive resting stages may be of importance (e.g., ephippia of daphnids). These properties allow a rapid development to normal population densities starting from a few surviving individuals or viable diaspores or after a few propagules happen to enter the treated systems after the insecticide concentration has dropped to below critical threshold levels. The lack of recovery of *G. pulex*, even a year after chlorpyrifos application (Fig. 6 and Table 2), can be explained by the fact that this species has

Table 3. *p* values calculated with the Monte Carlo permutation tests and no-observed-effect concentration ($NOEC_{community}$) values calculated by the Williams test for two data sets, the total invertebrate data set and the total invertebrate data set except for *Gammarus pulex*

Technique and data set	Sampling week[a]														
	−4	−1	0.1	1	2	4	8	12	15	19	24	42	47	51	55
Monte Carlo permutation (*p* value)															
Invertebrates	>	>	0.01	0.01	0.01	0.01	0.01	0.01	0.03	0.01	>	>	0.03	0.02	0.01
Invertebrates without *G. pulex*	>	>	0.01	0.01	0.01	0.01	0.01	0.01	0.05	0.02	>	>	>	>	>
Williams test ($NOEC_{community}$)															
Invertebrates	>	>	>	0.1	0.1	0.1	6	>	6	6	>	>	6	0.1	>
Invertebrates without *G. pulex*	>	>	>	0.1	0.1	0.1	6	>	>	6	>	>	>	>	>

[a]> indicates *p* values >0.05 and NOECs >44 µg/L.

Table 4. Reported no-observed-effect concentration ($NOEC_{ecosystem/community}$) values for chlorpyrifos in freshwater model ecosystems

NOEC (μg/L)	Type of system	Dose regime	Reference
<0.5	Lotic, outdoor, mesocosms	Chronic (100 d)	[29]
<0.1	Lentic, indoor, microcosms	Chronic (50 d)	[6]
<0.1	Lotic, outdoor, mesocosms	Chronic (21 d)	[39]
<0.5	Lentic, outdoor, mesocosms	Acute	[26]
<0.5	Lentic, indoor, microcosms	Acute	[30]
<1.7	Lentic, outdoor, microcosms	Acute	[24]
0.1	Lotic, outdoor, mesocosms	Acute (6 h)	[31]
0.1	Lentic, outdoor, mesocosms	Acute	This study

no resistant life stages and by the complete extermination of the population at the higher treatment levels. In addition, recolonization of this strictly aquatic amphipod was apparently restricted by the lack of connections between the mesocosms.

In aquatic insects, which are characterized by an adult terrestrial life stage and an ability to fly, the isolated position of the mesocosms probably does not restrict recolonization. It can be argued that the generation time is one of the important factors influencing rate of recovery in insects. For example, *C. dipterum* and *C. horaria* are more or less equally susceptible to chlorpyrifos (with 96-h effective concentration [EC50] values of 0.2 μg/L and 0.5 μg/L, respectively [21]), yet *C. dipterum* showed a rapid recovery, while *C. horaria* showed a delayed recovery (Table 2 and Fig. 4). *Cloeon dipterum* produces two or more generations per year [34] and can thus recover rapidly. *Caenis horaria*, however, produces only one generation per year [34] and consequently has a much smaller "time window" for recovery. Even 1 year after treatment this taxon was less abundant at the highest treatment levels compared to the controls (Table 2 and Fig. 4B). In the RDA diagram the species therefore occupies an extreme position for this treatment level (Fig. 5, upper right quadrant). Delayed recovery due to a relatively long generation time could also have occurred in *Mystacides longicornis* and *M. nigra*, Coenagrionidae, *Caenis luctuosa*, and *Sialis lutaria*, all of which situated in the upper right quadrant of Figure 5. Coenagrionidae and *S. lutaria* are reported to have one generation every 1 or 2 years [35,36]. *Mystacides longicornis*, *M. nigra*, and *C. luctuosa* have two generations per year, but the second generation is smaller in number than the first [37,38]. Caution should be exercised, however, in interpreting the position of these taxa in the RDA plot in terms of effects and recovery. At the end of the experiment, these taxa occurred in low numbers in the controls as well (mean abundance, <10 individuals).

The above examples of recovery at the species level show that the start of recovery cannot be predicted by simply calculating the time when the concentration of the insecticide becomes less than the laboratory NOEC or EC10 for the species concerned. Life-cycle characteristics must also be taken into account. This makes recovery at the species level hard to predict. Hence, toxicity and ecological data at the species level are needed to explain, and eventually predict, recovery.

At the level of the invertebrate community as a whole, results of the Monte Carlo permutation test suggest a recovery at the start of the winter season (Table 2 and Fig. 3). However, when all taxa are taken into account, the effect becomes significant again in week 47 (the following spring). When *G. pulex* is not taken into account, the treated mesocosms remain indistinguishable from the controls. Since the lack of recovery of this amphipod can be regarded as an artefact due to the isolated position of the mesocosms, the invertebrate community of the treated mesocosms was judged to have (potentially) recovered after 24 weeks.

Safe threshold levels

Although occasionally an NOEC <0.1 μg/L was calculated for some taxa in our mesocosm study, it seems reasonable to set the overall safe threshold level for susceptible species at 0.1 μg/L (Table 2). In the case of *C. obscuripes* and Ostracoda, the occasional NOECs <0.1 μg/L occurred during the pretreatment period, indicating systematic differences between mesocosms not related to the treatment or perhaps type I errors. In the case of *C. luctuosa*, an NOEC <0.1 μg/L was calculated for two consecutive sampling weeks (weeks 51 and 55; Table 1). This species, however, was present in very low numbers only. As a consequence, absence data are less indicative of the effects of chlorpyrifos. Furthermore, the most severe direct effects were expected in weeks 0 through 4, when chlorpyrifos concentrations were highest [4]. The occasional NOECs <0.1 μg/L found for Copepoda, Ceratopogonidae, and *Mystacides* spp. in this initial period might be a result of a type I error or indeed a direct effect of chlorpyrifos. In any case, the power of these NOEC values is limited because they were not always consistent with those of the preceding and subsequent sampling weeks. However, we are well aware that by leaving these incidental NOECs out of consideration, small transient effects on these taxa may be overlooked.

At the level of the invertebrate community, the chlorpyrifos treatment resulted in a concentration-dependent response. Figure 3 and the Williams test performed on the coordinates of the first PCA axis allowed us to determine an $NOEC_{community}$ value of 0.1 μg/L. In part I of the present series of articles, a short-term NOEC of 0.1 μg/L was reported for susceptible indigenous species [4]. The present mesocosm study thus shows safe concentrations based on no observed short-term effects on susceptible taxa to be adequate for protection of these taxa and the invertebrate community in the long term.

Several other model ecosystem studies have attempted to set safe threshold levels for chlorpyrifos at the community or ecosystem level (Table 4). In most of these studies, however, even the lowest concentrations tested showed effects, which meant that NOECs could not be determined. This seems to be a general problem in most of the microcosm and mesocosm studies that have been performed with pesticides. Hence, if one aims at better estimates of safe threshold values for ecosystems, it will be necessary to include lower test concentrations in future model ecosystem experiments.

The safe threshold value for chlorpyrifos of 0.1 μg/L that

we found in our mesocosm study corresponds with the outdoor model stream experiment of Pusey et al. [31]; both experiments were characterized by an acute exposure regime (Table 4). In our study, chlorpyrifos concentrations declined relatively fast after the single application [4].

Two studies in which chronic exposure concentrations of chlorpyrifos were maintained for 50 and 21 d [6,39] found that a level of 0.1 μg/L resulted in significant effects. Thus, in estimating safe threshold levels for ecosystems it seems wise to differentiate between acute and chronic exposure regimes. In the case of chlorpyrifos in Dutch drainage ditches, an acute exposure regime is more realistic because of the limited number of applications to agricultural crops and the relatively rapid decrease in bioavailability (rapid hydrolysis and sorption to organic matter) [40].

Evaluation of data analysis

Ordination was found to be a powerful tool for evaluating effects at the community level in ecotoxicological experiments [8]. In the present study the RDA ordination technique provided a clear description of the effects at the invertebrate community in time while still showing the effects at the species level. The great advantage of RDA over other multivariate techniques used in ecotoxicology [41] is that species and samples are analyzed simultaneously, so a "feedback" toward the species level is relatively easy. The RDA diagram allows hypotheses about ecological interactions to be made.

Another advantage of the implementation of ordination in ecotoxicology is the ability of statistical testing for the significance of effects at the community level [8]. The Monte Carlo permutation test has the advantage of testing all variance of a community. This test, however, has low power when few replicates per treatment are used. Testing the coordinates of the first PCA axis with the Williams test has the benefit of providing a $NOEC_{community}$, but it takes only a fraction of the total variance into account.

CONCLUSIONS

The chlorpyrifos treatment resulted in a reduction in those arthropod invertebrate taxa which were abundant at the time of application. Based on long-term observations, NOECs of 0.1 μg/L could be determined for the most susceptible species in the mesocosms and for the invertebrate community. This safe threshold level is similar to that established in the first part of this series [4], suggesting that, in the case of a single application, safe concentrations based on short-term observations are sufficient to protect communities in the long term. When a taxon starts to recover depends not only on the actual chlorpyrifos concentrations but also on its life-cycle characteristics and on infrastructural aspects of the ecosystem (e.g., the degree of isolation). The RDA ordination technique provided a clear description of the effects on the invertebrate community.

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Chapter 3

Sensitivity of macrophyte-dominated freshwater microcosms to chronic levels of the herbicide linuron

I. Primary producers

Paul J. Van den Brink, Elizabeth M. Hartgers, Uli Fettweis, Steven J.H. Crum, Ellen Van Donk and Theo C.M. Brock. *Ecotoxicology and Environmental Safety, Vol. 38: 13-24, 1997*

II. Community metabolism and invertebrates

Jan G.M. Cuppen, Paul J. Van den Brink, Hannelore Van der Woude, Nathalie Zwaardemaker and Theo C.M. Brock. *Ecotoxicology and Environmental Safety, Vol. 38: 25-35, 1997*

Sensitivity of Macrophyte-Dominated Freshwater Microcosms to Chronic Levels of the Herbicide Linuron

I. Primary Producers

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Effects of chronic concentrations of linuron (0, 0.5, 5, 15, 50, and 150 μg/L) were studied in indoor, macrophyte dominated, freshwater microcosms. The concentrations were kept at a constant level for 4 weeks. This paper is the first in a series of two and summarizes the course of the linuron concentrations in time and its effects on macrophytes, periphyton, and phytoplankton. These endpoints were studied from 3 weeks before the start of the treatment until 11 weeks after the start. The degradation of linuron in the water was lower at higher treatment levels, probably due to a decrease in pH. Linuron treatment resulted in a decrease in biomass of the macrophyte *Elodea nuttallii* and a clear decrease in abundance of the algae *Cocconeis, Chroomonas,* and *Phormidium foveolarum*. It was found that *Cocconeis* first decreased in biovolume and after 2 weeks also in abundance. The alga *Chlamydomonas* increased in abundance at the two highest doses, resulting in higher chlorophyll-a levels. The NOECs of 0.5 μg/L for the inhibition of the growth and photosynthesis of *Elodea nuttallii,* the abundance of *Cocconeis* and *Chroomonas,* and the oxygen and pH levels were the lowest recorded in the microcosms. The safety factors adopted by the EU in the Uniform Principles appeared to ensure adequate protection for the ecosystem in the case of chronic exposure to linuron. © 1997 Academic Press

INTRODUCTION

Pesticides used for crop protection may enter adjacent freshwater ecosystems by, e.g., spray drift, leaching, run-off, or accidental spills. To prevent adverse side effects of pesticides on aquatic ecosystems, authorities have set criteria that have to be met before these pesticides are allowed on the market. Recently, the member states of the European Union adopted the Uniform Principles (Council Directive 94/43/EEC), concerning the marketing of crop protection products (EU, 1994). This directive states that the predicted environmental concentration of a pesticide in surface water should not exceed 0.01 times the acute EC_{50} or 0.1 times the chronic NOEC of the most susceptible standard test species (algae, *Daphnia,* fish).

Although it has been disputed whether results of standard single species toxicity tests can be extrapolated to the wide array of indigenous species present in aquatic ecosystems (Kimball and Levin, 1985; Cairns, 1986), the justification of the use of these standardized tests is the lack of cost-effective alternatives (Van Leeuwen *et al.,* 1994). In addition, the current hazard assessment procedure, based on single species tests, most probably ensures adequate protection to freshwater communities if an appropriate safety factor is used (Persoone and Janssen, 1994; La Point and Perry, 1989).

Whether the safety factors proposed in the Uniform Principles are adequate, however, should be validated experimentally for compounds with a different mode of action (e.g., insecticides, herbicides, fungicides) and for different exposure regimes (acute, pulsed, chronic). This can be done with the help of freshwater model ecosystems, such as microcosms. Although smaller and less complex than real-world freshwater ecosystems, microcosms provide the opportunity to perform ecosystem-level research in replicable test systems under conditions that are manageable in terms of costs and logistics (Giddings, 1980).

The aim of the present paper is to validate the safety factors proposed by the Uniform Principles in the case of a chronic exposure regime to a herbicide. Although several model ecosystem experiments have been performed with herbicides (for a review see Brock and Budde, 1994) remarkably few have involved the testing of chronic levels with a lowest test concentration which did not result in an effect. To current knowledge, only experiments with the triazine herbicides atrazine and simazine have so far met these criteria (Jüttner *et al.,* 1995; Van den Brink *et al.,* 1995; Brockway *et al.,* 1984; Lynch *et al.,* 1985; Jenkins and Buikema, 1990).

In the present study, the phenylureum compound linuron was used as another model substance for photosynthesis inhibiting herbicides. This paper is the first in a series of two, and

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summarizes the effects of chronic levels of linuron on macrophytes, periphyton, and phytoplankton. The second paper will discuss the observed effects on the invertebrates and on community metabolism (Cuppen *et al.*, submitted). The discussion section of the present paper focuses on the hazard assessment of linuron in freshwater ecosystems, since primary producers are the most sensitive structural endpoints in herbicide stressed ecosystems. The second paper will focus on indirect effects of linuron on invertebrate populations and on the ecological effect chain of linuron in the microcosms.

MATERIALS AND METHODS

Experimental Design

On 15 February 1994, the herbicide Afalon (active ingredient linuron) was applied to 10 microcosms, in five duplicate doses, while two other systems served as controls. The concentration of linuron in the water of the microcosms was kept at a constant level for 4 weeks, followed by a posttreatment period in which linuron was no longer applied. The microcosms were situated in a climate room (constant temperature: 19 ± 2°C). Each microcosm consisted of a glass aquarium (length 1.1 m, width 1.1 m, height 0.7 m, water volume 600 L), filled with a 10-cm layer of lake sediment and a 50-cm water column. High pressure metal halide lamps (Philips HPI-T, 400W) were used to provide artificial daylight, resulting in a light intensity of approximately 120 $\mu E/m^2.s$ at the water surface. The daily photoperiod was 14 hr. The microcosms intended to model the community of Dutch drainage ditches. Details of the construction and layout of the microcosms can be found in Brock *et al.* (1992).

In the preparatory phase of the experiment, plankton and sediment dwelling macroinvertebrates were introduced into the microcosms, together with the natural sediment and well water. In addition, the macrophyte *Elodea nuttallii* and several populations of macroinvertebrates, characteristic of Dutch ditches, were deliberately introduced. A nutrient addition of P (initial concentration: 0.05 mg/L) and N (0.30 mg/L) was also applied in this period. Over an acclimatization period of 3 months, a biocoenosis was allowed to develop in the microcosms. Meanwhile, all microcosms were interconnected by tubes (internal diameter 2.6 cm) and the water was circulated using a pump with a flow rate of 3.5 L/min to achieve similarity between the communities in the systems. Before the start of the experiment the microcosms were disconnected.

To assess the effects of the herbicide on community structure in the microcosms, the dynamics of primary producers and invertebrates were studied. Physicochemical conditions were monitored to detect changes in the functioning of the overall ecosystem metabolism. The period before linuron application is referred to as the pretreatment period, while the period of 4 weeks of constant chronic exposure is called the treatment period and the period of the 7 following weeks, i.e., Weeks 5 through 11 after the start of the application, is called the posttreatment period.

Linuron Application and Analysis

The treatment started on 15 February 1994. On this day, the initial doses of linuron (nominal levels: 0.5, 5, 15, 50, and 150 $\mu g/L$), applied as Afalon, were distributed evenly over the water surface of two microcosms for each concentration and mixed by stirring.

During the treatment period, linuron was added twice a week to compensate for losses, which were calculated from the actual linuron concentrations measured. To promote even distribution of the pesticide, water from 5 cm above the bottom of the microcosm was pumped up and released above the water surface during the whole experiment. At several moments during the experiment, duplicate water samples (200 to 400 ml) were taken at mid-depth from each microcosm by means of a glass pipette. Water samples containing low linuron concentrations (control, 0.5 and 5 $\mu g/L$ doses) were extracted with octadecyl (C-18) solid phase extraction columns. The extraction columns were conditioned with 5 ml methanol and 5 ml distilled water. After extraction of a certain volume of water, the linuron was eluted from the column with three successive portions of 500 μl acetonitrile. The samples were then diluted with distilled water to a fixed volume of 5 ml. Water samples with a higher linuron concentration (15, 50, and 150 $\mu g/L$) were analyzed without previous treatment.

Analysis of the water samples was carried out with high performance liquid chromatography (HPLC). The HPLC system used consisted of a Waters model 510 pump, a Perkin–Elmer ISS100 autosampler, and a Perkin–Elmer LC-90 UV spectrophotometric detector. The mobile phase (water:acetonitrile = 60:40, v:v) was set at a flow rate of 1 ml/min. The column used was a Merck lichrosorb RP-18 (length 125 mm, width 4 mm) provided with a guard column of the same origin, while the oven temperature was adjusted to 40°C. Detection of the linuron samples was carried out at a wavelength of 254 nm. Under these circumstances, a retention time of 7 min was found for the linuron peak. Calculated concentrations were based on external standards. Linuron recovery from water was 100.7 ± 0.9% (mean ± SD, n = 6).

Endpoints

The sampling and measurement techniques of the studied endpoints are briefly described below. For a more detailed description of the methods and their sampling frequency, the reader is referred to Table 1 and the references cited in this table.

The phytoplankton community was sampled by taking several depth-integrated water samples by means of perspex tubes. A 1-L sample was stained with lugol and concentrated after sedimentation for 6 days. The concentrated sample was pre-

TABLE 1
Summary of Methods Used for the Sampling of the Indigenous Populations of Primary Producers in the Microcosms

Community	Unit	Sampling weeks	References
Phytoplankton			
Species composition	numbers/L	−1, 0, . . . 4, 6, 8, 10	Van Donk *et al.* (1995)
Chlorophyll-a	μg/L	−3, −2, . . . 11	Van Donk *et al.* (1995)
Periphyton			
Species composition	numbers/cm^2	−1, 2, 4, 6, 8, 10	Brock *et al.* (1995)
Chlorophyll-a (on glass slides)	μg/dm^2	−2, −1, . . . 6, 8, 10	Brock *et al.* (1995)
Chlorophyll-a (on *Elodea nuttallii*)	mg/g d.w.	−1, 0, . . . 6, 8, 10	Brock *et al.* (1995)
Biovolume per cell *Cocconeis*	μm^3	−1, 2	See text
Neuston			
Species composition	qualitative	6, 7, . . . 11	See text
Chlorophyll-a	μg/m^2	6, 7, . . . 11	See text
Elodea nuttallii	g d.w./m^2	11	Brock *et al.* (1995)

Note. . . . Indicates that samples were taken weekly. For a detailed description of methods see references.

served with formalin and cell counts were made. Chlorophyll-a estimations were obtained by concentrating the seston of another 1-L water sample over a filter (mesh size: 1.2 μm). Extraction of the pigments was performed using the method described by Moed and Hallegraeff (1978).

Periphyton was sampled from glass slides that served as artificial substratum. The slides were positioned in a frame at a fixed depth of approximately 10 cm below the water surface, and were incubated for 8 weeks. On each sampling day, six glass slides were used to study the taxa composition of the periphytic algae.

For chlorophyll-a analysis, another six slides were brushed visually clean and the periphyton removed was collected in tap water. The chlorophyll-a content of the water-periphyton solution was analyzed as described above. At intervals, 10 top 10-cm shoots of *Elodea nuttallii* were sampled from each microcosm to quantify the loosely attached periphyton associated with this macrophyte. The shoots of each system were collected in a 250-ml bottle, filled with 100 ml tap water, and shaken at 200 RPM for 5 min. Subsequently, the *Elodea* material was sorted out and the remaining water–periphyton solution was analyzed for chlorophyll-a as described above. In addition, the amount of *Elodea* in grams of dry weight was estimated for each sample. This allowed the quantity of loosely attached periphytic algae to be expressed as μg chlorophyll-a per gram dry weight of the macrophyte.

At the end of the posttreatment period, standing stock estimations of *E. nuttallii* were made by harvesting all macrophytes found in each microcosm. All harvested plants were divided into *E. nuttallii* and other macrophyte taxa and dried (105°C, 48 hr).

To evaluate the short-term effects of linuron on the biovolume of the most dominant taxon in the periphyton, the length distribution of the Bacillariophyceae (Diatom) taxon *Cocconeis* sp. was determined in Week -1 and 2. For each sample, the lengths of 50 individuals were measured under a microscope (magnification 400×). The biovolume per cell was calculated assuming that a *Cocconeis* cell is a box with an elliptic upper surface. The ratios of length, width, and height were chosen as 1:0.75:0.1.

In the posttreatment period a neustonic bloom of algae occurred at the two highest doses. Therefore, from Week 5 after the start of the application, the neuston was sampled by means of a representative subsample taken with a petri dish (surface: 64 cm^2) at the water surface in each microcosm. The species composition of the neuston was investigated qualitatively by mixing a little of the surface layer in water, followed by examination under the microscope. Chlorophyll-a estimations were done as described above.

Bioassay with the Macrophyte Species Elodea nuttallii

The direct effects of linuron on the growth of the macrophyte *E. nuttallii* were studied by means of a bioassay. In each microcosm, 4 g wet weight of *E. nuttallii* shoots were allowed to attach in a plastic beaker filled with sediment. Before the shoots were weighed, they were gently blotted dry with a tissue. The beaker was transferred to a transparent cage (length 10 cm, width 10 cm, height 50 cm), with one side consisting of gauze (mesh size: 55 μm). The cage was placed in the microcosm, at 45 cm below water level, and sufficient exchange of water was achieved by regularly raising the cage. The initial amount of *Elodea* in g dry weight was determined by drying four extra portions of 4 g wet-weight (105°C, 48 hr) and weighing them. The mean dry weight of these portions was 0.35 g. The bioassays lasted from the start of the application through Week 3 after the start, when the dry weight of *Elodea* was established. These data allowed the relative growth to be calculated for each microcosm {(biomass in Week 3 − biomass at start) / biomass at start}.

Laboratory Test Performed with Chlamydomonas reinhardtii

Adaptation of algae to herbicides is a frequently reported phenomenon (e.g., Kasai and Hanazato, 1995; Paterson and

Wright, 1987). To investigate the possible adaptation of planktonic algae to linuron, a laboratory test was performed with *Chlamydomonas reinhardtii* (Chlorophyceae). This taxon was chosen since a bloom of Chlamydomonas was observed in the linuron-treated microcosms. Two samples of a laboratory culture of *C. reinhardtii* were taken. One sample was cultivated in 20% Z8 medium, the other in 20% Z8 medium to which 150 μg/L linuron was added. After cultivation for 5 days, a single species toxicity test was performed with both strains. In a climate-room (T = 20°C) triplicate samples of both strains were exposed to 0, 15, 50, 150, and 500 μg/L linuron. The test units were 50-ml beakers, intended cell density at start: 5000 cells/ml. The beakers were placed on a shaking machine to avoid attachment of the algae to the glass walls. At day 0 (immediately after incubation) and day 3 after the start of the experiment, the total algal biovolume in μm^3/ml was measured with a coulter counter (Coulter Multisizer II) using two samples from each beaker. These data allowed the relative growth of the algae to be calculated for each beaker {(biovolume day_3 – biovolume day_0) / biovolume day_0}.

Data Analysis

NOEC calculations at taxon level ($p \leq 0.05$) were made using the Williams test (ANOVA) (Williams, 1972), which assumes an increasing effect for an increasing dose. These analyses were performed with the computer program Community Analysis, version 3.5 (Hommen *et al.*, 1994).

The effects on the phytoplankton community in time were described with the ordination technique called redundancy analysis (RDA). RDA was performed using the CANOCO computer program, version 3.14 (Ter Braak, 1988, 1990). RDA is a constrained multivariate regression technique, which allows effects of explanatory variables to be expressed. The explanatory, dummy, variables used in this study were treatment and sampling date, plus their interaction. In this way, only the variance of interest, i.e., the variance which can be attributed to the explanatory variables, is analyzed. The analysis was based on abundance data from the phytoplankton data set. For the theoretical background of this multivariate technique see Ter Braak (1987). The methodology of application and interpretation of RDA in mesocosm and microcosm studies has been described by Van Wijngaarden *et al.* (1995) and Van den Brink *et al.* (1996). Before analysis with the Williams test or CANOCO, the abundance data of the phytoplankton were $Ln(0.002x + 1)$ transformed. The rationale behind this transformation is discussed in Van den Brink *et al.* (1995).

The EC_{50} for the effects on biomass and relative growth inhibition of *Elodea nuttallii* in the bioassay was calculated using the general logistic model:

$$y = \frac{c}{1 + e^{-b(\mathrm{Ln}(x)-a)}}$$

where y is expected relative growth; a is Ln of the concentration at which the affected endpoint is the mean of the level in the controls and the value corresponding with 100% effect; b is slope parameter; c is expected biomass in control microcosms; x is exposure concentration.

If all observations consist of positive values (as in the case of biomass), "a" represents the EC_{50}. If the effect parameter was not biomass but relative growth, the EC_{50} was defined as outlined in Fig. 1. The model was programmed in GENSTAT, version 5.3.1, (Payne and Lane, 1987). A Poisson distribution of the relative growth data was assumed.

The results of the laboratory test with *Chlamydomonas reinhardtii* were analyzed with ANOVA ($P \leq 0.05$), also programmed in GENSTAT.

RESULTS

Linuron Concentrations

The mean linuron concentrations in the microcosms during the treatment period came within 10% from the target concentration at the lowest dose and within 5% at the other doses (Table 2, Fig. 2A). Compared to the higher doses, more linuron per unit volume had to be added at the lower doses to compensate for the losses during the treatment period (Table 2). This indicates a relatively faster disappearance of linuron from the water phase of the microcosms treated with the lower doses. This phenomenon also occurred in the posttreatment period: the higher the dose level, the relatively slower the decrease in linuron concentration (Fig. 2B). For the posttreatment period, the half-life for the disappearance of linuron from the water-phase ($t_{1/2}$) ranged from 11 days for the 0.5 μg/L dose to 49 days for the 150 μg/L dose (Fig. 2B).

Bioassay and Final Harvest Elodea nuttallii

In the bioassay, *Elodea nuttallii* had a significantly lower biomass at all doses except the lowest (NOEC = 0.5 μg/L, Table 3). During the 3 weeks, the plant biomass increased from

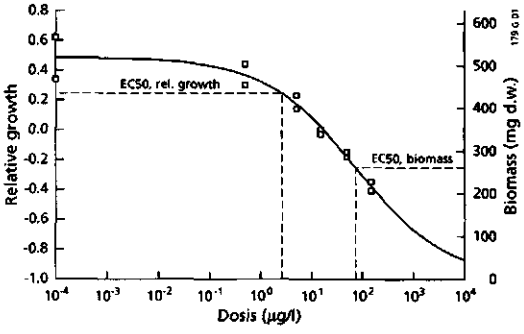

FIG. 1. The measured biomass and relative growth of *Elodea nuttallii* in the bioassay incubated in the microcosms and the model for EC_{50} calculations.

TABLE 2

Mean Linuron Concentrations (μg/L) per Replicate in the Treatment Period, Calculated by an Area Under the Curve Method (Van Wijngaarden *et al.*, 1996) and the Relative Amount of Linuron Added to Keep the Concentrations Constant during the Treatment Period Expressed as a Percentage of the Amount Added at the Start of the Treatment (Target Concentration)

Target concentration (μg/L)	Mean concentration (μg/L)	Amount of linuron added (%)
0.5	0.45, 0.46	178, 178
5	4.93, 4.92	117, 104
15	14.7, 14.7	87, 92
50	50.4, 50.9	57, 58
150	151, 156	60, 50

350 to 518 mg in the controls, while no growth occurred at the 15 μg/L dose, and the highest dose (150 μg/L) resulted in a reduction in biomass to 42% of the initial weight. The EC_{50} values, using biomass and relative growth as endpoints for *E. nuttallii*, were calculated as 75 μg/L (95% confidence interval: 45–124) and 2.5 μg/L (95% c.i.: 1.1–6.0, Fig. 1), respectively.

The results with regard to the standing stock of *E. nuttallii* in the microcosms (determined at the end of the posttreatment period) are presented in Table 3. Compared to the control microcosms, a nonsignificant increase in biomass of *E. nuttallii* was observed in the microcosms treated with the lowest two doses, and a significant decrease in those with the two highest doses (Table 3, NOEC = 15 μg/L). The highest dose resulted in an almost complete destruction of the standing stock of *E. nuttallii*, while the second highest dose reduced its final biomass to 47% (Table 3).

Phytoplankton

The RDA-biplot (Fig. 3) can be seen as a summary of the total phytoplankton data set. Most of the variation expressed on the first axis can be attributed to changes in species composition over time, those expressed on the second axis to the treatment. In the diagram, 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 points on this imaginary line. The samples whose projection on the "species line" is 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 that sample. If a sample point projects on the other side of the origin, compared to the species point, numbers of this species are relatively low in that sample. In the diagram, the taxon *Cocconeis* sp. is relatively abundant in all control samples and (almost) absent from the samples at the highest treatment level. To limit the number of taxa presented in the diagram, only the 13 taxa most discriminant for the analysis have been included.

The biplot indicates that after herbicide application the 150 μg/L samples, and to a lesser extent the 50 μg/L samples, diverged from the controls. The clustering of all pretreatment samples indicates minor differences in species composition between the microcosms at the start of the experiment. Only *Chlamydomonas* sp. exhibited a clear positive correlation with the highest treatment, with nearly all other taxa having a clear negative correlation. The dynamics in time of the four most discriminating taxa for the diagram are given in Fig. 4.

During the experiment, the taxon *Chlamydomonas* sp. increased its abundance significantly at the two highest doses, compared to the controls (Fig. 4A, Table 4). This taxon was also more abundant in the treated microcosms before herbicide application (Table 4), but revealed considerably lower numbers (Fig. 4A). The taxa *Cocconeis* sp. and *Phormidium foveolarum* demonstrated, compared to control values, significantly lower abundance values at the highest dose during both the treatment and posttreatment periods (Figs. 4B and 4D, Table 4). *Chroomonas* sp. exhibited a more pronounced response during the treatment period (NOEC = 0.5 μg/L), but not in the posttreatment period (NOEC > 150 μg/L; Fig. 4C, Table 4).

Periphyton and Neuston

The periphyton community was dominated by *Cocconeis* sp. and *Chlamydomonas* sp. As was the case in the phytoplankton samples, *Chlamydomonas* sp. became dominant at the two highest doses (Fig. 5A). In the posttreatment period, *Cocconeis* sp. demonstrated a concentration-dependent decrease at all doses except the lowest (Table 4, Fig. 5B).

At the highest dose, the individuals of *Cocconeis* sp. had a significantly lower biovolume per cell in Week 2 compared to control values (Table 5). Before application no significant differences could be found (Table 5).

The analysis of the species composition of the neuston indicated a *Chlamydomonas* sp. dominance at the two highest doses and a dominance of *Nostoc linckia* in the control and at the 0.5 μg/L dose, again revealing *Chlamydomonas* sp. as the dominant taxon at the highest doses. *Nostoc linckia* exhibited a negative correlation with the treatment in the phytoplankton samples (Fig. 3).

Chlorophyll-a

The chlorophyll-a content of the phytoplankton, periphyton and neuston samples increased after the start of application at the highest dose (Table 6). The chlorophyll-a values of the phytoplankton doubled at the highest dose compared to the controls (Table 6). In the posttreatment period, the neuston chlorophyll-a levels were even 15 times higher at the highest dose compared to the controls. At the 50 μg/L dose, only the

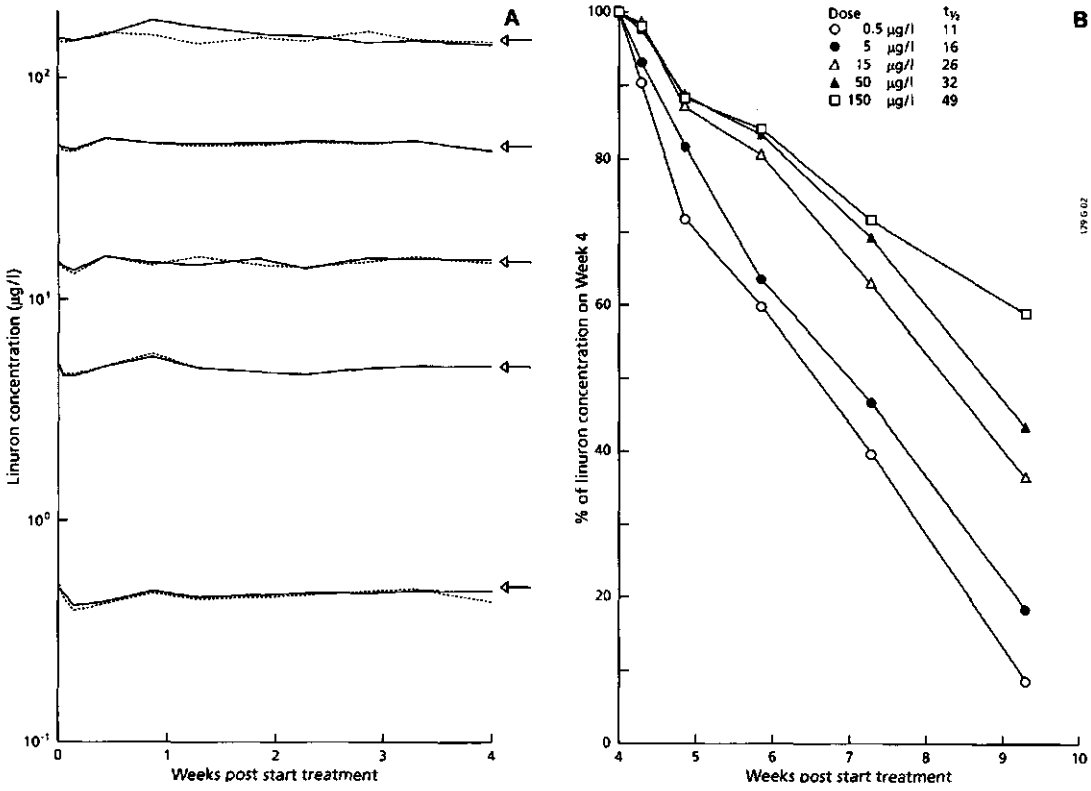

FIG. 2. Dynamics in linuron concentration per dose. (A) The linuron concentration per replicate in time for the treatment period. The arrows represent the target concentrations. (B) The decrease in linuron concentration per treatment level in the posttreatment period as a percentage of the actual linuron concentration at the end of the treatment period and the half lifetime for the disappearance of linuron from the water-phase per treatment level ($t_{1/2}$).

chlorophyll-a content of the periphyton on glass slides was significantly elevated (Table 6).

Laboratory Test with Chlamydomonas reinhardtii

The strain of *Chlamydomonas reinhardtii* originating from the culture previously treated with 150 µg/L linuron had a significantly larger relative growth when exposed to linuron concentrations of 150 and 500 µg/L than the strain previously cultivated in a linuron-free medium (Fig. 6). Relative growth did not differ significantly between the strains when they were exposed to 0, 15, and 50 µg/L linuron (Fig. 6).

DISCUSSION

Fate of Linuron

A concentration-dependent rate of disappearance of linuron from the water of the microcosms was found (Fig. 2B). The calculated half-life for the disappearance of linuron ($t_{1/2}$) in-

TABLE 3

Biomass Results of *Elodea nuttallii* from the Bioassay, Performed during the First Three Weeks of the Treatment Period, (Initial Biomass 350 mg d.w.) and Biomass of the Standing Stock of *E. nuttallii* at the End of the Experiment (Week 11)

Treatment level	Control	0.5 µg/L	5 µg/L	15 µg/L	50 µg/L	150 µg/L
Bioassay (mg d.w.)	518	481	416*	344*	292*	217*
Standing stock (g d.w./m^2)	91	118	112	90	43*	5*

Note. Significant differences related to the controls (Williams test, $P \leq 0.05$) are indicated by an asterisk.

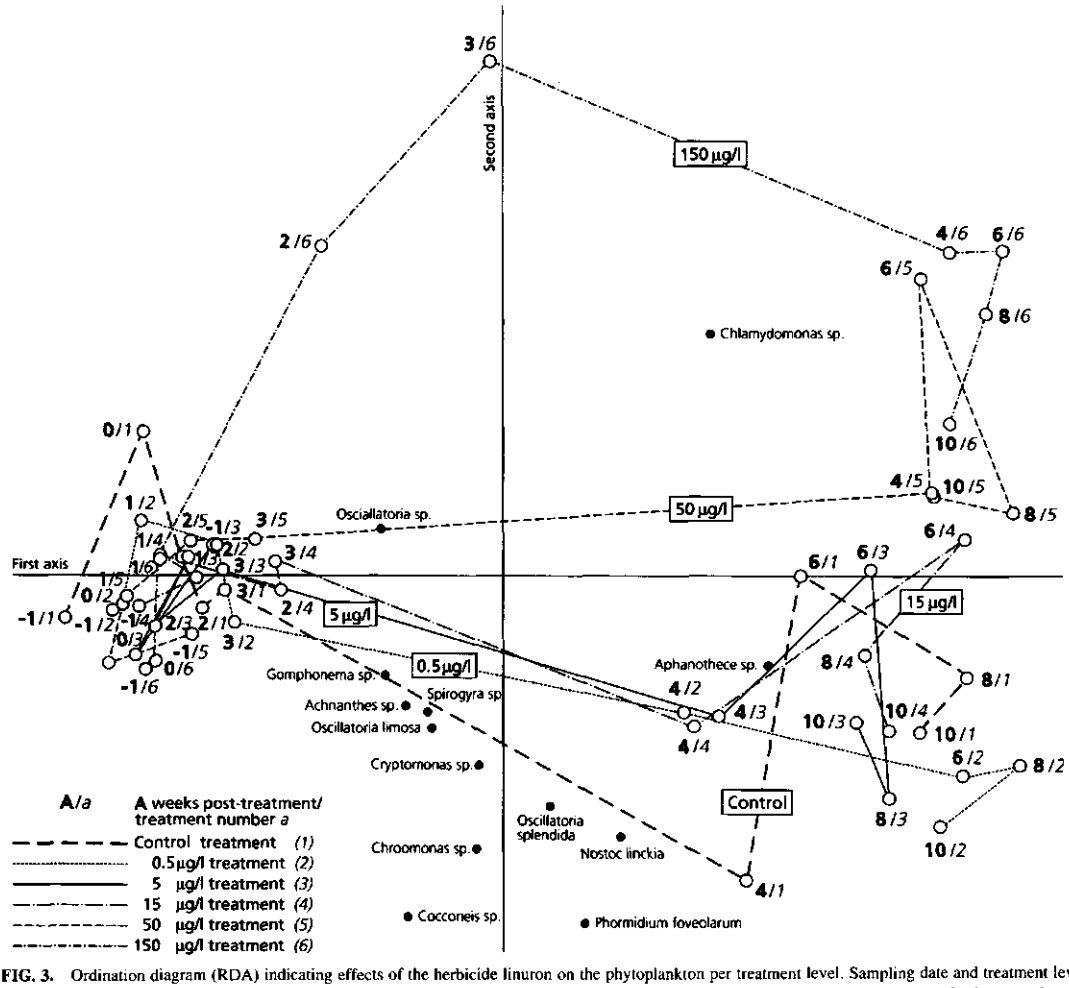

FIG. 3. Ordination diagram (RDA) indicating effects of the herbicide linuron on the phytoplankton per treatment level. Sampling date and treatment level, and their interactions, were taken as explanatory variables. The first number of the labels of the sample-points refer to the sampling week, the second to the treatment number (0 = control treatment, 5 = 150 µg/L dose). The lines represent the course of the treatment levels in time. 78% of all variance could be attributed to the explanatory variables. Of this explained variance, 61% is displayed in the diagram. Only the 13 most discriminating species for the diagram are shown.

creased with the dose (Fig. 2B). An explanation for this phenomenon is the difference in pH regime between the different treatments. Cserhati *et al.* (1976) found a significantly slower hydrolysis of linuron at pH 6 and 8 than at pH 4 and 10. Mean pH values during the treatment and posttreatment periods at the 0.5, 5, 15, 50, and 150 µg/L doses were 9.9, 9.5, 9.0, 8.1, and 7.8, respectively (see part 2, Cuppen *et al.*, submitted). The higher the dose, the lower the mean pH and hence the slower the hydrolysis of linuron. In the current study, linuron thus indicated a self-maintaining tendency by reducing photosynthesis and hence pH levels.

Effects on Elodea nuttallii

At the end of the experiment, the biomass of *E. nuttallii* was significantly reduced at the 50 and 150 µg/L treatments (Table 3). This is likely to be a result of the inhibition of photosynthesis by linuron. Since the long-term EC_{50} in the microcosms based on inhibition of photosynthesis was 8.4 µg/L (Snel *et al.*, submitted), effects at the 15 µg/L dose were expected. *E. nuttallii* may already have (partly) recovered from linuron stress due to the decrease in linuron concentrations during the posttreatment period. This recovery can take place relatively fast;

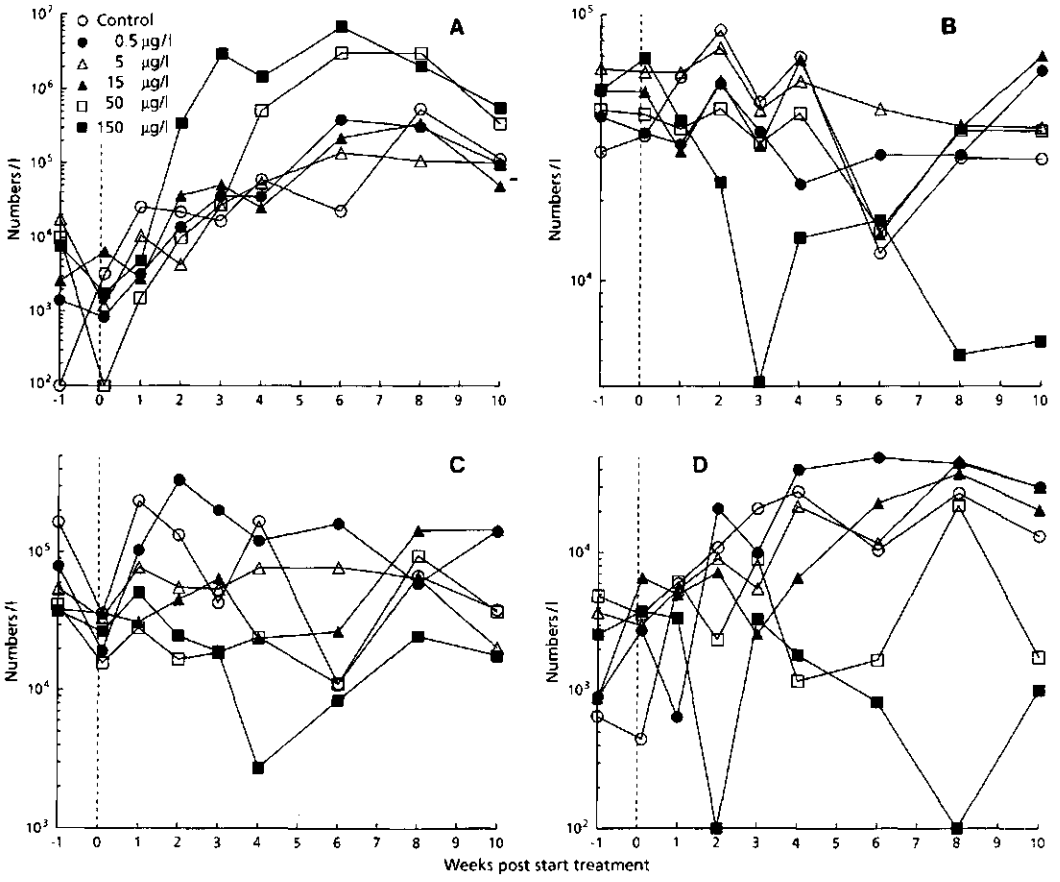

FIG. 4. Dynamics in numbers of four phytoplankton taxa. (A–D) The geometric means of the counted numbers per treatment level, of *Chlamydomonas* (A), *Cocconeis* (B), *Chroomonas* (C), and *Phormidium foveolarum* (D), are shown.

Snel *et al.* (submitted) found that the inhibition of photosynthesis disappeared within 6 hr when linuron-stressed *E. nuttallii* shoots were placed in a linuron-free medium.

Recovery of *E. nuttallii* from linuron stress in the posttreatment period, however, cannot be the sole explanation of the differences between the bioassay and the final harvest. During the posttreatment period, the concentration of linuron at the 15 µg/L dose was always higher than 5 µg/L. This concentration caused significant effects in the bioassay (NOEC = 0.5 µg/L, Table 3). Since *Elodea*, used in the bioassay, were introduced as shoots, part of their reserves was needed to form roots. It may be that this caused a more sensitive response of these shoots, when compared with the *Elodea* population in the microcosm, that was in its established phase at the moment of herbicide application.

The bioassay resulted in an EC_{50} of 75 µg/L when biomass

TABLE 4

NOECs (µg/L) as Calculated by the Williams Test ($P \leq 0.05$) for the Abundances of the Most Dominant Phytoplankton and Periphyton Taxa for Three Periods: The Pretreatment (Week -3 through -1), Treatment (Week 0 through 4) and Posttreatment (Week 5 through 11) Period

	Pretreatment	Treatment	Posttreatment
Phytoplankton			
Chlamydomonas	0.5↑	50↑	15↑
Cocconeis	—	50↓	50↓
Chroomonas	—	0.5↓	—
Phormidium foveolarum	—	50↓	50↓
Periphyton			
Chlamydomonas	—	50↑	15↑
Cocconeis	—	15↓	0.5↓

Note. A "—" indicates a NOEC > 150 µg/L.

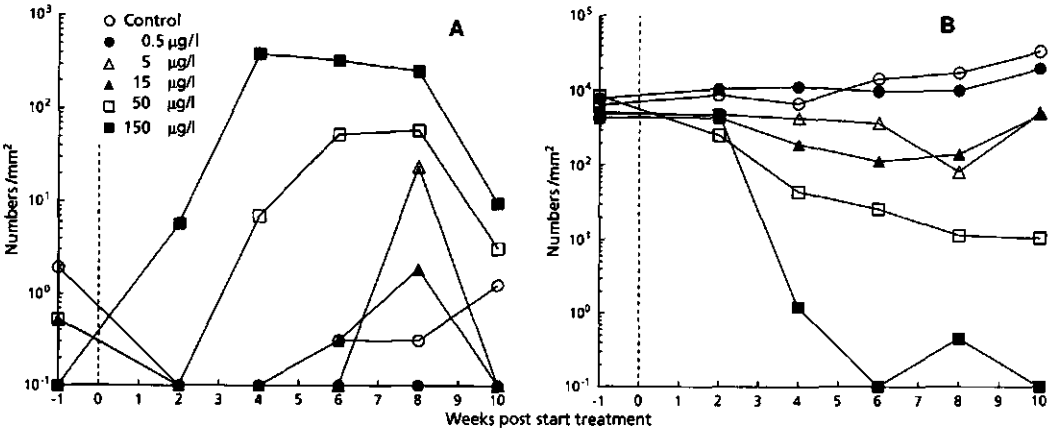

FIG. 5. Dynamics in numbers of two periphyton taxa on glass slides. (A and B) The geometric means of the counted numbers per treatment level of *Chlamydomonas* sp. (A) and *Cocconeis* sp. (B) are shown.

was taken as the endpoint. This EC_{50} value is in agreement with the effects of linuron on the biomass of other macrophyte species, as reported by Kemp *et al.* (1985). They reported EC_{50} values for the macrophyte species *Potamogeton perfoliatus* and *Myriophyllum spicatum* of 25 and 135 µg/L, respectively.

The NOEC and EC_{50} values reported for the inhibition of the relative growth at 0.5 and 2.5 µg/L are more in proportion with the inhibition of photosynthesis and changes in pH and oxygen metabolism. This was expected, since these endpoints are highly correlated.

Effects on Algae

Figure 3 reveals that most taxa of the phytoplankton were negatively affected by the linuron treatment. These effects are most likely direct effects caused by the inhibition of photosynthesis by linuron. Only *Chlamydomonas* sp. indicated an increase in abundance after the linuron treatment. The increase in numbers of *Chlamydomonas* sp. (Fig. 4A) can be explained by (i) the increase in available nutrients (nitrate increased as a consequence of the decrease in macrophyte biomass, Cuppen *et al.*, submitted) and (ii) the ability of this taxon to adapt to linuron (Fig. 6). This taxon increased to such numbers at the highest doses that the chlorophyll-a content of the phytoplankton, periphyton, and neuston samples after the start of treatment was significantly higher than in controls (Table 6).

Linuron addition resulted in a decrease in numbers of *Cocconeis* sp. on glass slides at doses of 5 µg/L and higher. Although the effects appeared after 2 weeks (Fig. 5B), this is considered to be a primary effect. An explanation for the delayed decrease in numbers can be derived from the effects on the biovolume per cell of *Cocconeis* sp. Since the mean biovolume per cell of *Cocconeis* decreased soon after herbicide application at the highest dose (Table 5), it can be assumed that the growth of *Cocconeis* cells responded fast to the inhibition of photosynthesis. The algae could still survive on their storage energy. Apparently, this storage energy was exhausted after 2 weeks and the population density then diminished.

TABLE 5

Mean Biovolume per Cell (in µm³ ± STD) of *Cocconeis* sp. per Dose, as Sampled on Glass Slides for Two Sampling Periods, Week-1 and 2

Treatment (µg/L)	Week -1	Week 2
0	255 ± 3	271 ± 51
0.5	185 ± 18	227 ± 50
5	272 ± 91	237 ± 33
15	232 ± 5	277 ± 46
50	193 ± 10	316 ± 13
150	187 ± 44	121 ± 14*

Note. An asterisk indicates a significant treatment related difference compared to control values (Williams test, $P \leq 0.05$).

Comparison of Results with Other Micro/Mesocosm Studies

Many authors have reported effects of herbicides on primary producers and functional endpoints in freshwater ecosystems (for a review see Brock and Budde (1994) and Kersting (1994)). The general effect chain described is a disruption of the functioning of the primary producers (e.g., by inhibition of photosynthesis), followed by a decrease in dissolved oxygen and pH levels. Furthermore, a decrease in population densities of algae and/or macrophytes has often been observed. In the current study we found the same effect chain, but also an increase in the alga *Chlamydomonas*.

Stephenson and Kane (1984) performed the only other microcosm experiment with linuron known to the authors. They reported adverse effects of linuron on macrophytes and a reduction in oxygen and pH levels, but not an increase in the chlorophyll-a content of the phytoplankton or periphyton, as

TABLE 6
Chlorophyll-a Results per Treatment Level Averaged over Three Periods: The Pretreatment (Week -3 through -1), Treatment (Week 1 through 4), and Posttreatment (Week 5 through 11) Period

Compartment	Period	Control	0.5 μg/L	5 μg/L	15 μg/L	50 μg/L	150 μg/L
Phytoplankton	Pretreatment	2.6	1.8	2.2	2.7	2.5	3.3
(μg/L)	Treatment	4.2	2.6	2.7	2.2	1.8	6.7*
	Posttreatment	9.4	4.9	8.0	2.6	8.5	16.5
Periphyton	Pretreatment	12.4	6.9	6.6	6.6	6.8	2.0*
(glass slides,	Treatment	5.2	5.5	7.8	13.1	11.9	26.1*
μg/dm^2)	Posttreatment	10.5	13.0	13.4	15.6	32.3*	60.7*
Periphyton	Pretreatment	0.19	0.11	0.15	0.17	0.15	0.16
(*Elodea*,	Treatment	0.14	0.13	0.15	0.08	0.09	0.12
mg/g d.w.)	Posttreatment	0.37	0.32	0.49	0.39	0.37	0.93*
Neuston							
(μg/dm^2)	Posttreatment	13.1	5.5	4.2	1.2	55.1	219.3*

Note. Significant differences with control values are indicated by an asterisk (Williams test, $P \leq 0.05$).

found in the current study. Most probably, the concentration they tested (1000 μg/L) was too high for the algae to adapt to.

Adverse effects of other herbicides on macrophytes and functional parameters, followed by an increase in chlorophyll-a, were found by Hodgson and Linda (1984). They reported a decline in macrophytes and an increase in the chlorophyll-a content of the phytoplankton and periphyton after a nominal dose of 200 μg/L diquat. A decline of macrophytes followed by a decrease in oxygen and pH levels and an increase in total algal densities was also found by Draxl *et al.* (1991), Scorgie (1980), and Peichl *et al.* (1985), for the herbicides diquat, cyanatryn, and atrazine, respectively, although their studies were supported by fewer observations. Thus, although adaption of algae to herbicides has often been reported (e.g., Kasai and Hanazato (1995), Paterson and Wright (1987), DeNoyelles *et al.* (1989), and Molander and Blanck (1992)), an actual increase in algal densities, after a decline of macrophytes, has only been reported in a few experiments. The reason might be the relatively high concentrations normally tested in micro- and mesocosm experiments, completely suppressing the algae.

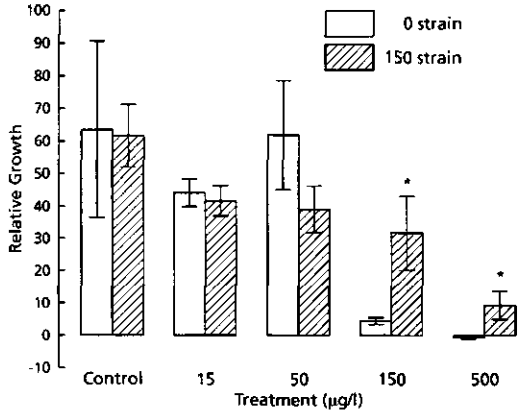

FIG. 6. Relative growth of two strains of *Chlamydomonas reinhardtii*, one strain originated from a linuron-free medium and one strain from a culture previously treated with 150 μg/L linuron. The two strains were exposed to 0, 15, 50, 150, and 500 μg/L linuron for 3 days. Significant differences between strains (ANOVA, $P \leq 0.05$) are indicated by an asterisk.

Hazard Assessment

One of the aims of this experiment was to validate the safety factors recently proposed by the European Union in their Uniform Principles (EU, 1994). In the present experiment, the hazard assessment was based on the direct effects of the herbicide on the efficiency of photosynthesis, on growth inhibition, and on densities of primary producers, as well as on oxygen and pH metabolism (Fig. 7). These endpoints are all related to linuron's photosynthesis-inhibiting properties, and appeared to be more sensitive than responses of invertebrates (see part 2, Cuppen *et al.*, submitted).

The lowest NOEC observed in this study was 0.5 μg/L (Fig. 7). This NOEC could be calculated for the abundance values of *Cocconeis* sp. and *Chroomonas* sp. (Fig. 4), the inhibition of growth and photosynthesis of *E. nuttallii*, pH values, and DO concentrations (Cuppen *et al.*, submitted). The EC_{50} of the most susceptible standard laboratory test species is 6 μg/L (Snel *et al.*, submitted), so the safety factors of 0.01 and 0.1 (to be multiplied by the lowest acute EC_{50} and chronic NOEC of the standard test species, respectively) appeared to ensure adequate protection for the community of the microcosms in the case of a chronic exposure regime to linuron.

CONCLUSIONS

In this study, chronic linuron exposure resulted in an "eutrophication-like" effect chain. The microcosms treated with 50 μg/L or more shifted from macrophyte-dominated to algae-dominated systems.

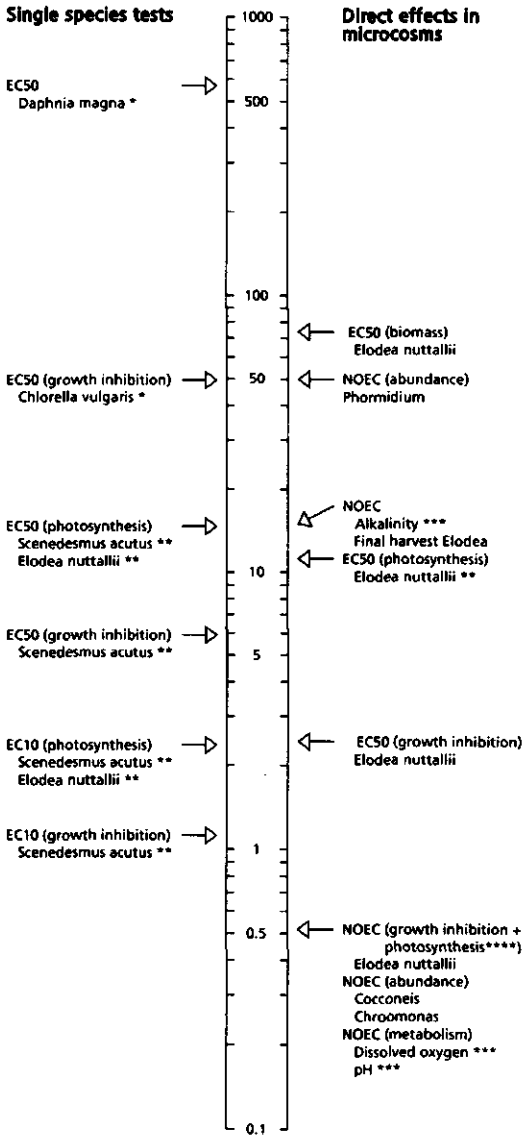

FIG. 7. Summary of laboratory tests results with linuron and direct effects found in the microcosms. *Stephenson and Kane, 1984; **Snel *et al.*, submitted; ***Cuppen *et al.*, submitted; ****Own calculations (Williams test, $P \leq 0.05$) on data from Snel *et al.*, submitted.

The hazard assessment indicated that the safety factors (0.01 times the acute EC_{50} or 0.1 times the chronic NOEC) adopted in the Uniform Principles sufficed to protect the freshwater community in the case of chronic exposure to linuron.

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Sensitivity of Macrophyte-Dominated Freshwater Microcosms to Chronic Levels of the Herbicide Linuron

II. Community Metabolism and Invertebrates

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Effects of a chronic application of the herbicide Afalon (active ingredient linuron) on physicochemical conditions, decomposition of plant litter, and densities of zooplankton and macroinvertebrates were studied in indoor microcosms intended to model drainage ditches. For 28 days, concentrations of 0, 0.5, 5, 15, 50, and 150 μg/L linuron were maintained, each in two replicates. The microcosms were dominated by the macrophyte *Elodea nuttallii*. The functional response of the ecosystem is discussed in relation to shifts in community structure. Treatment effects of linuron on community metabolism, as a direct effect of the inhibition of the photosynthesis of macrophytes and algae, resulted in a decrease in dissolved oxygen and pH, and an increase in alkalinity and conductivity (NOEC 0.5 μg/L). During the posttreatment period, differences between controls and highest dose fell gradually, but were still significant 7 weeks after the start of linuron application. Decomposition of particulate organic material in litter bags was not affected, despite decreases in DO. The negative effect of linuron on several algae (cryptophytes, diatoms) and the positive effect on the green alga *Chlamydomonas* resulted in a decrease of several Rotatoria and an increase in Copepoda, and, to a lesser extent, Cladocera. The complete disappearance of the macrophyte *E. nuttallii* in the 150 μg/L microcosms and a 50% reduction of its biomass in the 50 μg/L microcosms reduced the numbers of the snail *Physella acuta*, which normally inhabits macrophytes. Artificial substrates indicated a significant increase in the isopod *Asellus aquaticus* in the 50 and 150 μg/L microcosms during the posttreatment period. This, however, was counteracted by a significant decrease in *A. aquaticus* at the final harvest. Changes in the ecosystem structure (decline in macrophyte biomass) made the artificial substrates more attractive. © 1997 Academic Press

INTRODUCTION

Herbicides are often used in agriculture to reduce or destroy weeds, mainly to avoid competition for nutrients and light between crops and weeds. An undesirable side-effect of the use of these herbicides is that they may enter freshwater ecosystems by spray drift, leaching, run-off, and/or accidental spills. Contamination of surface waters with herbicides has been reported to have direct toxic effects on populations of phytoplankton, epiphyton, and macrophytes. In addition, when these primary producers are affected, indirect effects on ecosystem functioning and animal populations can also be expected (for a review see Brock and Budde (1994) and Kersting (1994)).

In the present study the herbicide linuron (applied as Afalon) was added to microcosms that intended to model stagnant macrophyte-dominated freshwater ecosystems. Within the scope of the present study, a previous paper (part I) dealt with the responses of the primary producers and with the hazard assessment of this herbicide in freshwater ecosystems (Van den Brink *et al.*, submitted).

The first aim of the present paper (part II) is to describe effects of a chronic application of the herbicide linuron on functional aspects such as oxygen metabolism and decomposition. Inhibition of the photosynthesis by linuron and effects on populations of primary producers (Snel *et al.*, submitted; Van den Brink *et al.*, submitted) are hypothesized to cause lower dissolved oxygen concentrations (DO) in the microcosms. A reduced primary productivity will also affect pH, alkalinity, and conductivity. These endpoints have been repeatedly reported as sensitive indicators of metabolic effects of toxicants (Stephenson and Kane, 1984; Brock *et al.*, 1993; Kersting, 1994). DO, pH, alkalinity, and conductivity are often found to be highly correlated, and treatment effects on these functional endpoints can be regarded as a stress syndrome (Giddings, 1982).

A second aim of this paper is to describe the effects of linuron on the secondary producers (zooplankton and macroinvertebrates). A priori, the authors did not expect direct effects on invertebrates, since the EC_{50} of *Daphnia longispina* for linuron (360 μg/L; Stephenson and Kane, 1984) is considerably higher than the concentrations used in the current experiment. Similarly, the LC_{50} values for some macroinvertebrates, such as *Dugesia tigrina* (10 mg/L), *Lymnaea* (70 mg/L), and *Tubifex* (10 mg/L) are too high to expect direct effects (Maier-

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Bode and Härtel, 1981). Indirect effects of herbicides on zooplankton and macroinvertebrate populations due to shifts in populations of primary producers, however, appear to be difficult to predict (Hurlbert, 1975; DeNoyelles *et al.*, 1989; Brock and Budde, 1994). A priori, the authors hypothesized either a decrease in zooplankton populations due to an expected decrease in phytoplankton or a shift in dominance from herbivorous taxa to detritivorous taxa. As regards macroinvertebrates, a shift in dominance from herbivores to detritivores was expected to result from the increase in organic matter due to the death of algae and macrophytes (*Elodea nuttallii*). The loss of the structure provided by the macrophytes was also expected to lead to a dominance of benthic organisms instead of epiphytic organisms.

The third aim of the present paper is to present a synthesis of the impact of herbicide application on the structure and functioning of the freshwater microcosms by incorporating the conclusions of the preceding paper.

MATERIALS AND METHODS

Experimental Design of the Microcosm Study

The construction and properties of the indoor microcosms (length and width 110 cm, depth 70 cm, water depth 50 cm, sediment depth 10 cm), the conditions in the climate room (temperature 20°C, photoperiod 14 hr), and the experimental design have been described in detail by Van den Brink *et al.* (submitted). Two of the 12 available microcosms served as controls. Of the remaining 10, two microcosms each were loaded with, respectively, 0.5, 5, 15, 50, and 150 μg/L linuron. The linuron concentration in the water column was kept constant for 28 days by adjusting it twice a week. The dynamics of the linuron concentration in the water has been described by Van den Brink *et al.* (submitted). All microcosms were investigated over a period of 14 weeks: a pre-treatment period of 3 weeks, a treatment period of 4 weeks, and a posttreatment (restoration) period of 7 weeks.

Water Sampling and Water Chemistry Analysis

Dissolved oxygen was measured with a WTW oxygen meter (Oxi 196) and a WTW oxygen probe (EO 196) at a depth of 10 cm at 1-week intervals from Week −3 through Week 11. Oxygen was measured in the morning, at the start of the photoperiod, and in the evening, just before the illumination was switched off. Conductivity and pH were measured with, respectively, a WTW conductivity meter and a Metrohm Herisau pH meter at weekly intervals from Week −3 through Week 11. During the same period, alkalinity was measured weekly in 100-ml samples taken at a depth of 10 cm (titration with 0.05 *N* HCl until pH 4.2).

Depth-integrated water samples from at least five localities well distributed over each microcosm were taken with a perspex corer (length 40 cm) in Weeks −3, −1, 0.2, 2, 4, 6, 8, and 10 for nutrient analysis. Subsamples of each day and microcosm were pooled and a portion of the well-mixed sample was filtered through prewashed glass fiber filters (Whatman GF/C). Part of the filtered water was transferred to 100 ml iodated polyethylene bottles and stored (−20°C). At the end of the experiment the samples were defrosted and analyzed for ammonium, nitrate, and orthophosphate using a Skalar 5100 Autoanalyser. Another part of the sample was stored (−20°C) after addition of 1.5 ml HCl. At the end of the experiment the samples were analyzed for Na^+, K^+, and Ca^{2+} using an atomic absorption spectrophotometer.

Decomposition Experiment

Decomposition of particulate organic matter (POM) was studied by means of the litter bag technique (Brock *et al.*, 1982). The POM used consisted of *E. nuttallii* shoots and *Populus x canadensis* leaves.

The *Populus* leaves had been leached three times for 2 days to remove the more easily soluble humic compounds. To allow storage of this material, it was dried in an oven for 72 hr at 60°C. Subsamples of *Elodea* and *Populus* were subsequently dried at 105°C for 24 hr to establish the 60°C/105°C dry weight ratio.

A 2 g dry weight (dried at 60°C) portion of *Elodea* or *Populus* was enclosed in each litter bag. The litter bags consisted of a glass petri dish (diameter 11.6 cm) closed with a cover of stainless steel wire (mesh size 0.7 × 0.7 mm), in which two holes (0.5 cm) had been punched to allow the passage of most invertebrates. The materials used are known to be inert to pesticides. In each model ecosystem two litter bags of each plant type were incubated at the sediment surface for a period of 2 weeks. Whenever a set of litter bags was retrieved on a sampling day, a new set was incubated. At the end of each 2-week decay period, the litter bags were gently washed in the overlying water of each microcosm to remove adhering sediment particles. The contents of the two bags of each plant type from each microcosm were then transferred to a white tray to separate invertebrates from the decomposing material. The plant material was transferred to aluminum foil to determine dry weight (24 hr, 105°C). After identification and counting, the invertebrates were released into their original microcosms.

Sampling of Zooplankton

Zooplankton was sampled from each model ecosystem at 2-week intervals with a perspex corer with a length of 40 cm and a diameter of 4 cm. Several subsamples were collected, regularly distributed over the microcosms, until a 5-L sample was obtained. The total sample from each microcosm was passed over a 55-μm mesh net. This was done over the microcosm to minimize the loss of water. The concentrated sample was fixed with 4 ml 35% formol and supplemented to 100 ml. Zooplankton was identified and counted with a Nikon inverted microscope at a magnification of 100× in a sedimentation cuvet. The density of zooplankton was always relatively

low, which is why total samples were counted. Numbers of zooplankton were divided by five to get the numbers per liter.

Sampling of Macroinvertebrates

Macroinvertebrates were sampled from each model ecosystem at 2-week intervals by means of artificial substrates and litter bags, as described above. In each system three multiplates and two pebble baskets served as artificial sample substrates (for a detailed description see Brock *et al.*, 1992).

On each sampling day, the artificial substrates were gently retrieved from each system, using a net to prevent the escape of swimming invertebrates. Pebble baskets were first washed in a container to remove invertebrates from the substrate. Subsequently, the macroinvertebrates present on the substrates and in the litter bags were collected by handpicking, identified and counted alive, and then released again into the model ecosystems. Data from artificial substrates and litter bags were pooled for further analysis.

At the end of the experiment (Week 13) the macroinvertebrates were quantitatively sampled. First, all macrophytes and artificial substrates were removed (carefully washed to remove all adherent invertebrates), after which the water level in the microcosms was reduced to a depth of 10 cm by means of a siphon. Escape of macroinvertebrates was prevented by leading the water over a net. Escaped organisms were reintroduced into the microcosms. The water in the microcosm was then gently stirred to achieve a random distribution of the macroinvertebrates. A corer with a surface of 30 × 30 cm was placed in each microcosm and the fauna in it was sampled with a small net. Animals were collected by handpicking and identified alive (Turbellaria) or fixed in 4% formol (Oligochaeta) or 70% ethanol (remainder of macroinvertebrates).

Data Analysis

Before analysis, the abundance values of the zooplankton and macroinvertebrates were ln $(10x + 1)$ and ln $(2x + 1)$ transformed, respectively, where x stands for the abundance value. A rationale for these transformations is given in Van den Brink *et al.* (1995).

NOEC calculations at parameter or species level were obtained using the Williams test (ANOVA) (Williams, 1972). The analyses were performed with the Community Analysis computer program (Hommen *et al.*, 1994).

The response of the communities to the linuron treatment was analyzed using the Redundancy Analysis (RDA) ordination technique. For the theoretical background and technical details of these techniques, see Ter Braak (1987, 1988, 1990). Specific details of the application of RDA in model ecosystem experiments have been described by Van Wijngaarden *et al.* (1995) and Van den Brink *et al.* (1996). RDA was used to obtain an overview of the combined effects of time and treatment at the community level. This technique produces a diagram which summarizes the data set, while still indicating species composition for all samples (see, for example, Fig. 3).

RESULTS

Physicochemical Conditions

Figures 1A–1C present the results of the measurements on the DO-pH-alkalinity-conductivity syndrome. After the start of the treatment, DO and pH levels were lower compared to controls at all linuron concentrations except the lowest (Figs. 1A and 1B, Table 1). DO remained above 6 mg/L in all treatments, even at the end of the dark period, when DO levels were generally about 1.0 to 1.5 units lower (results not provided), so that anoxic conditions never occurred in the water column. During the posttreatment period, the 5 and 15 μg/L microcosms regained normal DO and pH levels, whereas those with the two highest concentrations did not reach normal levels within the experimental period.

Corresponding with the decrease in DO and pH, the higher treatment levels led to increases in alkalinity and conductivity (Fig. 1C, Table 1), compared to the controls. At the end of the experimental period, all microcosms except those with the two highest linuron concentrations were not significantly different from control levels, mainly due to a rise in alkalinity levels in the controls.

Application of linuron had no significant treatment effect on the nutrients ammonium and phosphate, while a significant effect was observed for nitrate at the highest linuron concentration from Week 6 onwards (Fig. 1D). In the 150 μg/L microcosms the minerals Na^+, K^+, and Ca^{2+} revealed different responses to the linuron application, with a significant positive effect for calcium and potassium, and no effect for sodium.

Decomposition

The application of linuron did not result in significant treatment effects on the decomposition of particulate organic matter (*Populus* leaves and shoots of *E. nuttallii*) in litter bags (Fig. 2). The residual dry weights of *Populus* after decay periods of 2 weeks amounted to 60–70% of the initial dry weight. The residual dry weights of *Elodea* were distinctly lower and amounted to 50% in Week −1 and to approximately 40% in all other periods.

Zooplankton

The dominant species in the zooplankton samples belonged to the groups of Cladocera, Copepoda, and Rotatoria, while Ostracoda occurred in low numbers. The Cladocera were dominated by *Daphnia longispina, Simocephalus vetulus, Graptoleberis testudinaria,* and *Cladocera* spp. The Copepoda were represented by the genera *Macrocyclops, Eudiaptomus,* and *Canthocamptus,* while nauplii were dominant. Rotatoria were the most diverse, with *Synchaeta pectinata, Polyarthra remata,* and *Mytilina bicarinata* as the most numerous species. *Cipridiopsis vidua* was the only representative of the Ostracoda.

A biplot of the redundancy analysis on the zooplankton data set is given in Fig. 3. The diagram summarizes the treatment

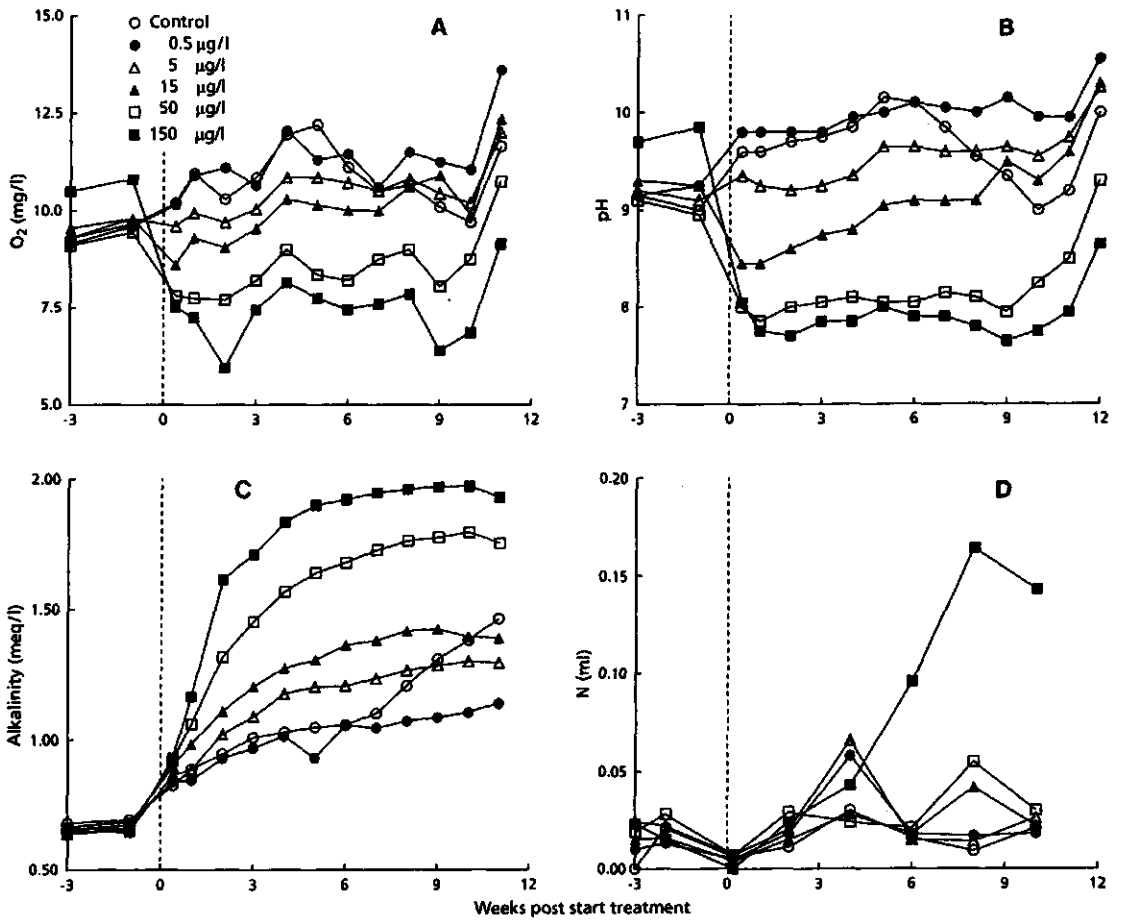

FIG. 1. Dynamics of evening values of dissolved oxygen (A), pH (B), alkalinity (C) (mean per treatment level, $n = 2$), measured at a depth of 10 cm in microcosms treated with a chronic dose of linuron, as well as dynamics in nitrate concentration in the overlying water (D).

effects in the data set, while still indicating the approximate species composition for all samples. The lines represent the course of the various treatment levels in time, so the variation between the replicates has been excluded from the analysis. In the diagram, treatment levels with nearly identical courses of species composition in time lie close together, while treatment levels with very different species compositions lie far apart. For further explanation of the diagram, the reader is referred to Van den Brink *et al.* (submitted). The variation expressed on the first axis reflects the changes in species composition and abundance in time (the treatment levels move from left to right in time). The clustering of all pretreatment samples (upper left quadrant) indicates minor differences between the microcosms at the start of the experiment. The second axis partly represents the treatment effects. After the start of the linuron application the samples from the 150 µg/L treatment, and, less clearly, the 50 and 15 µg/L treatments, are positioned at the bottom of the diagram, indicating differences in community composition of these microcosms relative to the controls. *Synchaeta pectinata, Polyarthra remata, Graptoleberis testudinaria,* and *Daphnia longispina* decreased considerably with time, while most other taxa exhibited increases. In general, the number of taxa increased in the course of the experiment, though most "new" taxa occurred only in low numbers. The copepod taxa *Macrocyclops* and nauplii revealed the strongest positive correlation to the treatment, the rotifers *Synchaeta pectinata* and *Polyarthra remata* the strongest negative one.

Responses of the main zooplankton groups are presented in Fig. 4 and Table 1. Significant treatment effects of Cladocera could be demonstrated in the posttreatment period in Weeks 7

TABLE 1

NOECs as Calculated by the Williams Test ($P \leq 0.05$) for Physicochemical Conditions and Abundances of Zooplankton and Macroinvertebrates for Three Periods: The Pretreatment (Week −3 through −1), Treatment (Week 1 through 4), and Posttreatment (Week 5 through 11) Period

	Pretreatment	Treatment	Posttreatment
Physicochemical			
Dissolved oxygen	50↑	0.5↓	15↓
pH	50↑	0.5↓	15↓
Conductivity	—	5↑	5↑
Alkalinity	—	5↑	5↑
Nitrate	—	—	15↑
Zooplankton			
Cladocera	—	—	15↑
Copepoda	0.5↓	—	50↑
Rotatoria	50↓	5↓	50↓
Ostracoda	—	—	15↓
Macroinvertebrates			
Physella acuta	—	50↓	50↓
Asellus aquaticus	—	—	15↑
Dugesia	—	—	15↑
Bithynia	—	—	—

Note. A "—" indicates a NOEC > 150 μg/L.

and 9, when a NOEC of 15 μg/L linuron was observed due to an increase in numbers at the highest concentrations. Copepods demonstrated a significant increase at 150 μg/L linuron during Weeks 5–9 in the posttreatment period. The results for the Copepoda were mainly determined by the changes in abundance of nauplii and, to a much lesser extent, of *Macrocyclops*.

Rotatoria were most numerous in the pretreatment period (65 individuals per liter), declined seriously during the treatment period, especially at the three highest linuron concentrations, and remained at these levels during the posttreatment period (Fig. 4, Table 1).

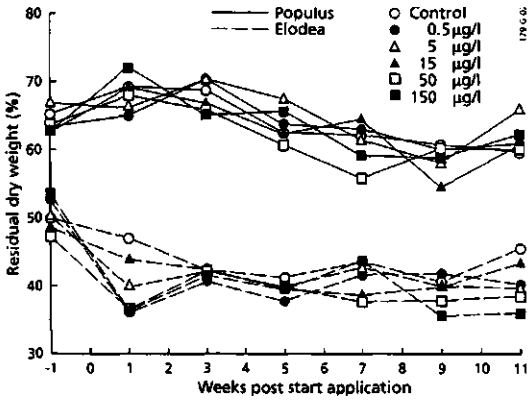

FIG. 2. Relative amounts of residual dry weight (mean per treatment level, $n = 2$) of decomposing *Populus* leaves and shoots of *Elodea nuttallii* in litter bags after decay-periods of 2 weeks in microcosms treated with a chronic dose of linuron.

Macroinvertebrates

The macroinvertebrates in the model ecosystems were dominated by snails, crustaceans, triclads, and oligochaetes, while leeches and nemerteans were less abundant. Insects, with exception of the phantom midge *Chaoborus obscuripes*, were very scarce, probably because of their inability to reproduce or oviposit in the climate room after their emergence in the preexperimental acclimatization period.

Herbivorous snails, mainly living on the macrophytes, were dominated by *Physella acuta* and *Lymnaea stagnalis*. The bottom and vegetation dwellers with more differentiated feeding habits were dominated by *Bithynia tentaculata* and *B. leachi*. Other snail species were less abundant. All crustaceans in the microcosms were shredders; they included the amphipod *Gammarus pulex* and the isopods *Asellus aquaticus*, *Proasellus meridianus*, and *P. coxalis*. *Dugesia tigrina* was the most abundant carnivorous triclad, while *D. lugubris* occurred in much lower numbers. The vegetation inhabiting herbivorous Oligochaeta consisted of *Stylaria lacustris* and *Chaetogaster* spec., while the detritivorous *Dero digitata* and Tubificidae were the most numerous benthic taxa. Carnivorous leeches, belonging to the genera *Erpobdella*, *Glossiphonia*, and *Alboglossiphonia* occurred in low densities.

A biplot of the redundancy analysis on the macroinvertebrates data set is given in Fig. 5. The variation expressed on the first axis is mainly related to treatment with linuron, while the second axis is related to changes in species abundances with time. *D. lugubris* indicated a positive correlation with the treatment, but even before the start of the treatment its abundance was highest in the 150 μg/L microcosms. Adult *Physella acuta* and their offspring exhibited a negative correlation with the treatment, but even in the pretreatment period this gastropod occurred in high numbers in the 0.5 μg/L microcosms. The 150 μg/L microcosms, and to a lesser extent those with 50 μg/L, demonstrated the most pronounced deviations from the other treatments and controls at the end of the experiment. On the second axis the snail *Anisus vortex* revealed a strong decline with time, while juvenile *Bithynia*, *Dugesia tigrina*, and *Chaetogaster* indicated an increase in numbers.

The development in time of the most discriminative populations in the RDA-diagram is presented in Fig. 6. Significant negative treatment effects (Table 1) were only found for *P. acuta* at the highest linuron concentration from Week 3 onwards (Fig. 6A). This negative treatment effect is in accordance with the results of the final sampling (Table 2). A significant positive treatment effect was found for *Asellus aquaticus* (Fig. 6B) and *Dugesia* (Fig. 6C), while *Bithynia* revealed a positive trend (Fig. 6D). The significant treatment effects were usually only found for the highest or two highest linuron concentrations. In most cases, these effects were not confirmed by the final sampling in Week 13; in fact, a significant negative

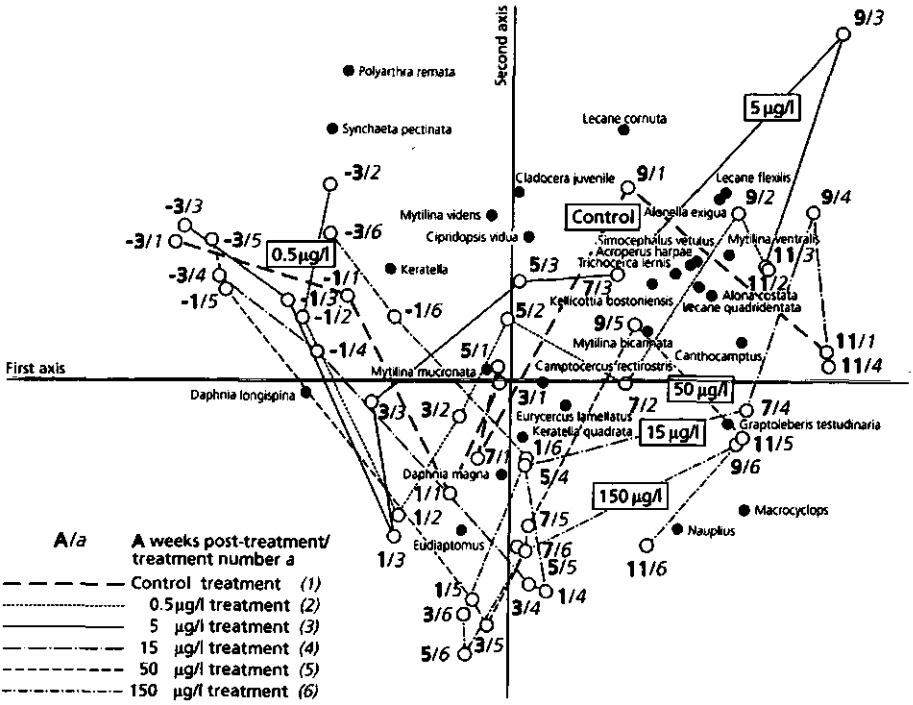

FIG. 3. Ordination diagram (RDA) indicating effects of a chronic application of the herbicide linuron on the zooplankton per treatment level. Sampling date (first digit) and treatment level (second digit), and their interactions were taken as explanatory variables. The lines represent the course of the treatment levels in time. 76% of all variance can be attributed to the explanatory variables. 57% of the explained variance is displayed in the diagram.

effect was found for *A. aquaticus* (Table 2), contrasting with the results of the artificial substrates.

DISCUSSION

Effects on Ecosystem Functioning

The first response of the microcosms to the addition of linuron was the almost immediate inhibition of the photosynthetic efficiency of primary producers (Snel *et al.*, submitted). This response is in accordance with the well-known inhibition of the photosynthesis of aquatic macrophytes and algae by triazine and phenylureum herbicides (Kemp *et al.*, 1985; DeNoyelles *et al.*, 1989). Corresponding with the inhibition of the photosynthesis, the dissolved oxygen concentration and the pH in the overlying water decreased, while alkalinity and conductivity increased (Figs. 1A–1C). Stephenson and Kane (1984) observed the same effects of a single dose of 1000 μg/L linuron in an enclosure in a small open pond. Other herbicides, such as diquat (Hodgson and Linda, 1984; Draxl *et al.*, 1991) and atrazine (DeNoyelles *et al.*, 1989) were found to result in similar effects on dissolved oxygen concentration, pH, conductivity, and alkalinity, at least temporarily. In the current study, the magnitude of the response was strongly dependent on the linuron concentration applied (NOEC 0.5 μg/L). Over the 4-week treatment period, a more or less stable difference between the different treatment levels was established. The differences for these four variables gradually diminished over the 7-week posttreatment period, but at the end of the experiment, the microcosms with the two highest linuron treatment levels still differed significantly from the controls. The restoration of these variables indicates that, at least at the lower linuron concentrations, the community metabolism regains its "normal" value in the posttreatment period, particularly in those systems in which macrophyte biomass recovered. The slow restoration of DO, pH, alkalinity, and conductivity in the 50 and 150 μg/L microcosms can be explained by the significant decreases in the biomass of *E. nuttallii*, of 50% and 100%, respectively (Van den Brink *et al.*, submitted). In (control) microcosms, macrophytes formed the bulk of the biomass and can be considered the most important primary producers. The dissolved oxygen, pH, alkalinity, and conductivity syndrome proved to be a very good indicator of the direct effects of linuron. Other herbicides such as atrazine (Neugebaur *et al.*, 1990; Lay *et al.*, 1984), diquat (Strange, 1976), and 2,4-D DMA (Boyle, 1980) have similar effects on this syndrome (see Kersting (1994) for a review).

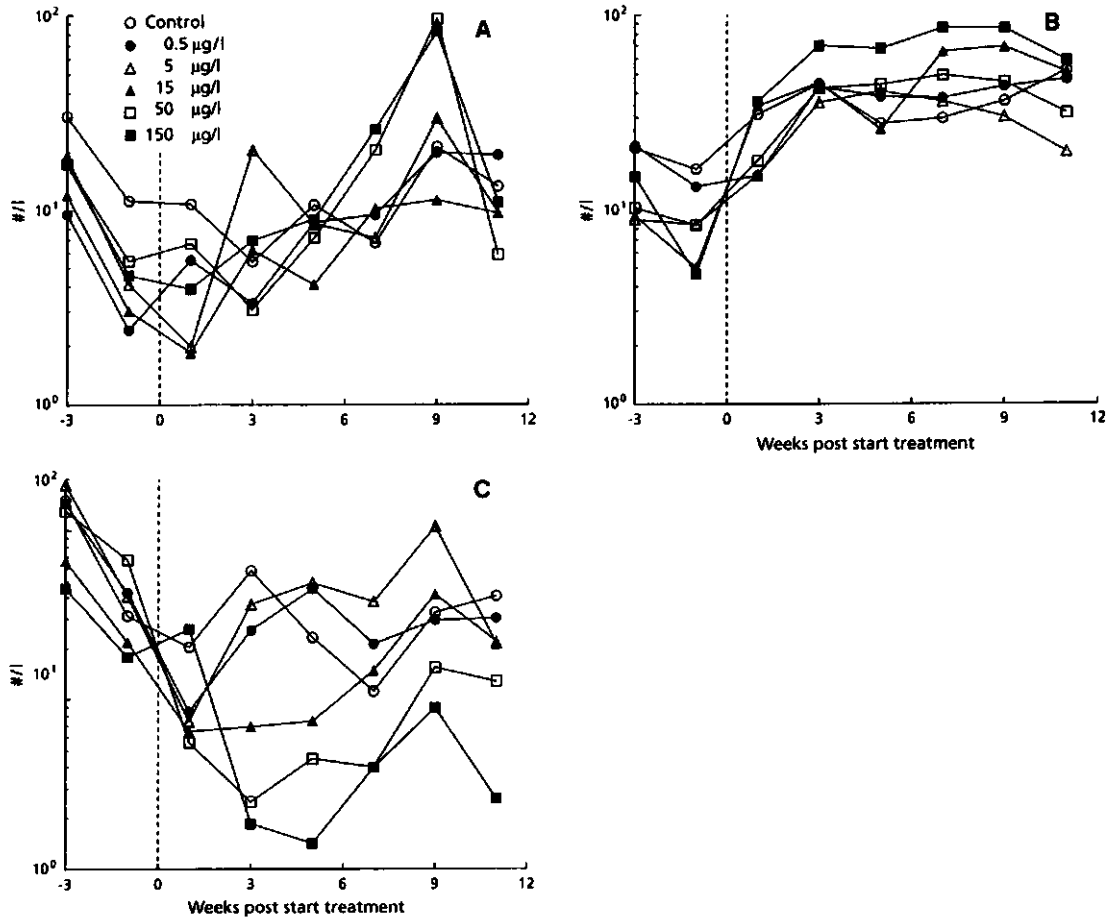

FIG. 4. Dynamics in numbers of three zooplankton taxa. (A–C) The geometric means of the counted numbers per treatment level, of Cladocera, Copepoda, and Rotatoria, respectively.

Despite the lower oxygen concentrations in the overlying water, the decomposition rate of *Populus* leaves and shoots of *E. nuttallii* was not influenced by linuron application (Fig. 2). The dissolved oxygen concentrations above the substrate in all microcosms were probably high enough to prevent anaerobic conditions and an inhibition of microbial activity. In accordance with these results, no evidence could be found in the literature of an inhibitory effect of linuron at concentrations below 150 µg/L on microbial communities in freshwater ecosystems. Despite an increased attractiveness of the artificial substrates for shredders like Amphipoda and Isopoda in the 150 µg/L microcosms (probably due to macrophyte decline), this did not result in an increased decomposition of particulate organic matter in the litter bags. This suggests that the processing of macroscopic detritus, either from terrestrial or aquatic origin, was mainly performed by microorganisms and to a lesser extent by macroinvertebrates. Brock *et al.* (1993) found a relatively small, though significant, decrease in the decomposition rate of *Elodea* in microcosms in which Amphipoda and Isopoda had been eliminated by the insecticide chlorpyrifos. They also suggested the relative unimportance of shredders in comparison with microorganisms.

Responses of Invertebrates

As expected, the zooplankton communities in the microcosms revealed no immediate responses to the application of linuron, though the decline of Rotatoria was relatively fast, indicating a possible role of direct toxicity. A likely explanation for the decrease in Rotatoria, however, is the decrease in the planktonic and epiphytic algae *Chroomonas*, *Phormidium*,

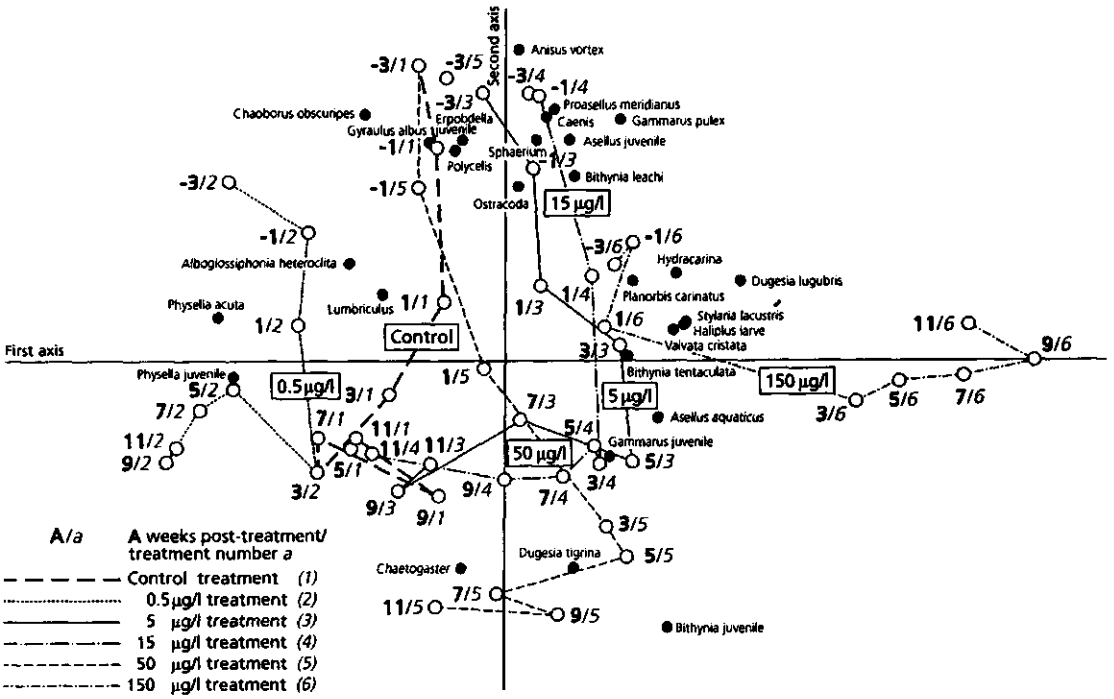

FIG. 5. Ordination diagram (RDA) indicating effects of a chronic application of the herbicide linuron on the macroinvertebrates per treatment level. Sampling date (first digit) and treatment level (second digit), and their interactions were taken as explanatory variables. The lines represent the course of the treatment levels in time. 56% of all variance can be attributed to the explanatory variables. 43% of the explained variance is displayed in the diagram.

and *Cocconeis* and the increase in the flagellate *Chlamydomonas* (Van den Brink *et al.*, submitted). During the treatment and posttreatment periods the zooplankton communities changed from Rotatoria-dominated to Copepoda-dominated (especially nauplii). Perhaps the selective feeding strategy of nauplii and *Macrocyclops* makes them better grazers of *Chlamydomonas* in microcosms than the filter-feeding rotifers. Cladocera and Ostracoda hardly changed in abundance (Figs. 3 and 4). In agreement with the results reported, e.g., Gunkel (1983), Hamilton *et al.* (1989), and Jenkins and Buikema (1990), the size of the dominant zooplankton species increased slightly in response to the treatment with linuron.

As expected, the macroinvertebrates did not indicate a direct response to the linuron application either. The secondary effects of linuron on macroinvertebrate communities were relatively small. The destruction of *E. nuttallii* biomass at the highest linuron concentration (Van den Brink *et al.*, submitted) caused a severe decline of the snail *Physella acuta* (Fig. 6A) and its offspring and in the number of egg cases of *Lymnaea stagnalis* (NOEC 15 µg/L, results not provided). Both species, which normally feed on epiphyton on aquatic macrophytes, probably suffered from the lack of food and oviposition sites on the plants. No total elimination of these snails from the microcosms was observed, as the walls of the aquaria still offered feeding and oviposition sites. The artificial substrates in the microcosms seemed not to be extra attractive to these snails after the decline of *Elodea*, in contrast to the isopod *A. aquaticus* and the triclads *D. tigrina* and *D. lugubris*, whose numbers increased on the artificial substrates (Figs. 6B and 6C). At the end of the experiment, however, a significant decrease in numbers of *A. aquaticus* was observed with the help of an absolute sampling method (Table 2). This proves that results on invertebrate dynamics as measured by means of artificial substrates should be interpreted with caution in ecosystems whose structure changes as a result of macrophyte decline due to herbicides. The absolute decrease in *A. aquaticus* at the end of the experiment is most probably the combined result of habitat destruction (decline of macrophyte biomass due to the herbicide) and severe predation by the triclads. At the end of the experiment, triclads outnumbered the isopods and a decline of triclads would have been expected had the experiment continued.

Normal application of linuron on agricultural fields will not greatly affect species composition of invertebrates in adjacent

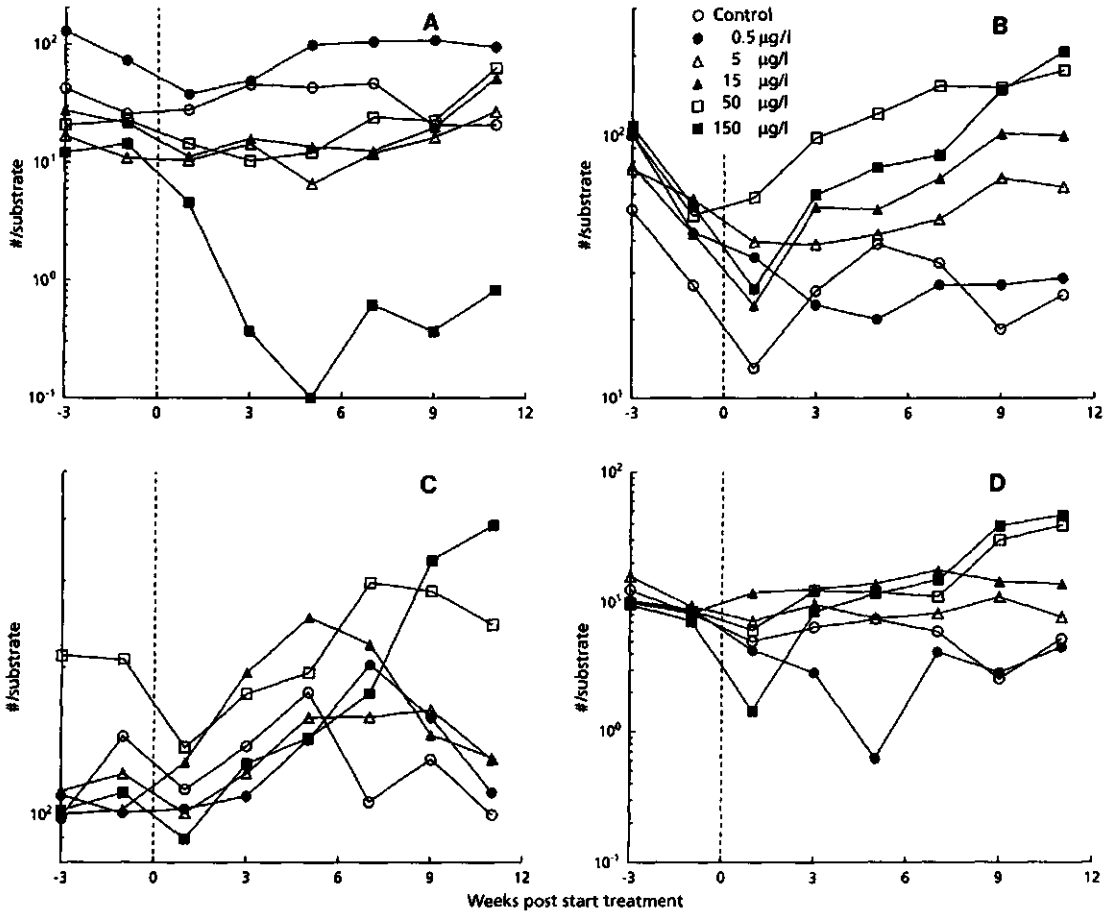

FIG. 6. Dynamics in numbers of four macroinvertebrate taxa. (A–D) The geometric means of the counted numbers per treatment level of the snail *Physella acuta*, the isopod *Asellus aquaticus*, the triclad genus *Dugesia* and the snail genus *Bithynia*, respectively.

aquatic ecosystems. It is only when the exposure concentrations are so high or longlasting that the macrophytes eventually die that this will have serious noxious effects on the invertebrate communities. It is very difficult to predict these secondary effects, as they will depend strongly on the structure of the system before application.

CONCLUSIONS

A synthesis of the overall ecological impact of a chronic application of linuron on the structure and functioning of the microcosms has been visualized in Fig. 7.

As a primary effect of linuron application, the photosynthesis of both algae and macrophytes was inhibited in the 5 µg/L microcosms and at higher concentrations (Snel *et al.*, submitted). This resulted in a decrease in dissolved oxygen and pH,

TABLE 2

Geometric Mean Numbers/m² of Some Species of Invertebrates at the End of the Experiment in Week 13 in Microcosms Treated with the Herbicide Linuron (n = 2)

Treatment level	Control	0.5 µg/L	5 µg/L	15 µg/L	50 µg/L	150 µg/L
A. aquaticus	323	247	385	257	676	61*
G. pulex	193	151	152	94	128	126
D. tigrina	788	930	649	91	1320	359
Bithynia	89	20	55	51	122	88
P. acuta	72	653	100	120	111	9
Dero	1846	2612	490	649	1667	1775

* Indicates a significant difference (Williams test, $P \leq 0.05$).

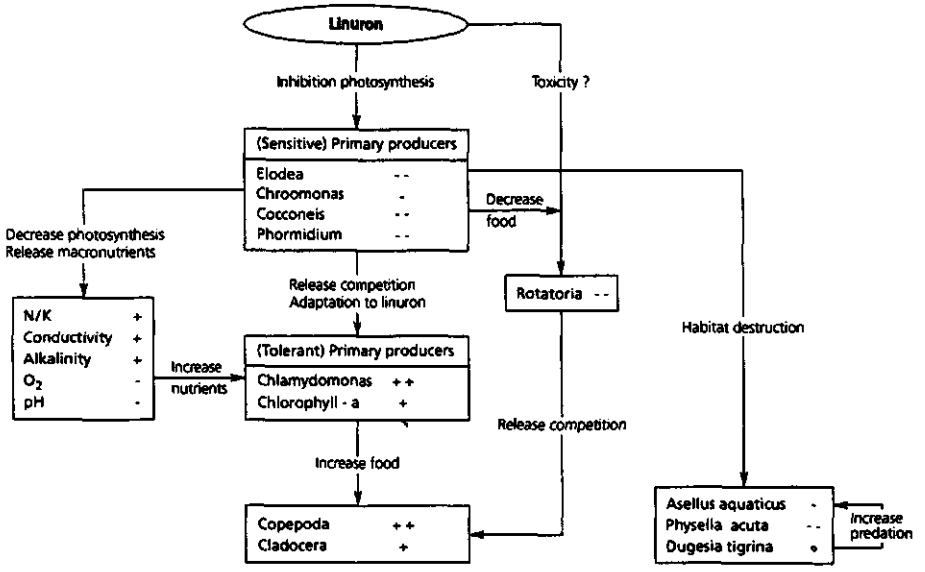

FIG. 7. Schematic overview of the nature and route of effects of a chronic high dose (150 μg/L) of linuron on ecosystem structure and function of *Elodea*-dominated microcosms (+, increase; –, decrease).

and an increase in conductivity and alkalinity. The decrease in dissolved oxygen, however, was moderate and did not result in anaerobic conditions in the water column, nor did it affect decay rates of *Populus* and *Elodea* in litter bags or invertebrate species composition. A decrease in invertebrates with a high oxygen demand due to oxygen depletion was reported by Murphy and Barrett (1990). The decreased pH caused a concentration-dependent degradation rate of linuron, with slower degradation at the highest linuron concentration (Van den Brink *et al.*, submitted).

The small, but significant increase in nitrate (and potassium) in the 150 μg/L microcosms can be explained by a reduction of the biomass of the macrophyte *E. nuttallii*, and to a lesser extent by the decline of several planktonic and epiphytic algae. The increase in these nutrients probably resulted in a higher chlorophyll-a concentration, caused by the significant increase in the more tolerant green alga *Chlamydomonas* (Van den Brink *et al.*, submitted). A decrease in the small rotifers in the 50 and 150 μg/L microcosms, perhaps as a combined effect of direct toxicity of linuron and an indirect effect of the decline of several planktonic and epiphytic algae (e.g., the cryptophyte *Chroomonas* and the diatom *Cocconeis*), resulted in an increase in zooplankters such as copepods, especially nauplii, and, to a lesser extent, cladocerans. The increase in these zooplankters was probably also caused by the increase in *Chlamydomonas*, a readily edible chlorophyte (Mitchell *et al.*, 1992).

The decrease in biomass of *E. nuttallii* and its associated periphyton in the 50 and 150 μg/L microcosms negatively affected the grazing snails *P. acuta* and *Lymnaea*. The decline of isopods, especially *A. aquaticus*, at the highest linuron concentrations was only found at the end of the experiment. Prior to that, a positive response was found for this species, probably due to an increased attractiveness of the artificial substrates in the otherwise nearly empty microcosms. No pronounced shift from herbivorous feeding strategies to more detritivorous feeding strategies was found, despite the decline of the macrophytes at the highest linuron concentrations. An increase in detritivorous macroinvertebrates, due to an increase in detritus as a result of macrophyte decay, has been reported by Stephenson and Mackie (1986), Feind *et al.* (1988), and Murphy and Barrett (1990).

The application of linuron had no significant effect on the decomposition of *Populus* leaves and *Elodea*, suggesting an uninfluenced microbial community.

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Chapter 4

Impact of the fungicide carbendazim in freshwater microcosms

I. Water quality, breakdown of particulate organic matter and responses of macroinvertebrates

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II. Zooplankton, primary producers and final conclusions

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Impact of the fungicide carbendazim in freshwater microcosms

I. Water quality, breakdown of particulate organic matter and responses of macroinvertebrates

Abstract

Effects of chronic application of the fungicide Derosal® (active ingredient carbendazim) were studied in indoor macrophyte-dominated freshwater microcosms. The concentrations (0, 3.3, 33, 100, 330 and 1000 µg/L) were kept at a constant level for four weeks.

This paper is the first of a series of two, and describes the fate of carbendazim and its effects on water quality parameters, breakdown of POM, and responses of macroinvertebrates. Carbendazim proved very persistent in the water layer. Values for $t_{½}$ varied between 6 and 25 weeks, and decreased with the treatment level. Significant effects on water quality parameters (DO, pH, alkalinity, conductivity) could not be demonstrated. After four weeks of incubation, the breakdown of *Populus* leaves was significantly slower at the two highest carbendazim concentrations.

The macroinvertebrate community was seriously affected by carbendazim application, with Oligochaeta, Turbellaria, Hirudinea and some Crustacea as the most sensitive groups. The snail *Bithynia* decreased in numbers, but other gastropods increased in numbers.

Safety factors as proposed by the European Union (Uniform Principles) for the risk assessment of pesticides, to be multiplied with toxicity data of the standard test species (*Daphnia*, fish, algae), appeared to ensure adequate protection of sensitive populations present in the microcosms.

Introduction

Fungicides are a widely used type of pesticide. In the Netherlands, 4,102 tons of fungicides were used in 1996, which amounts 38% of the total pesticide use (Brouwer, 1998). Despite the wide application of fungicides, published information on their effects in (model) ecosystems is very scarce. Brock and Budde (1994) reported only two studies dealing with the effects of fungicides on freshwater ecosystems. In general, little information exists on the sensitivity of indigenous freshwater organisms to fungicides. This is of particular importance because it is questionable whether the taxa expected to be susceptible to fungicides are fully represented by the standard aquatic test species (*Daphnia*, algae, fish). In this study we tried to obtain a better understanding of the effects of fungicides on freshwater ecosystems, by taking carbendazim as a model substance. The fungicide carbendazim is a prohibitor of nucleus division and an inhibitor of the activity of the enzyme acetylcholinesterase (Van

Gemerden, 1992). Benzimidazoles as carbendazim are widely applied in agriculture and veterinary medicine as fungicides and anthelminthic drugs (Davidse, 1987).

In our microcosm experiment, we applied the fungicide Derosal® (active ingredient carbendazim) to ten microcosms in a chronic exposure regime of four weeks. The concentrations applied (0.01, 0.1, 0.3, 1 and 3 times the lowest L(E)C50) were derived from the, at that time, available acute LC50 and EC50 tests with standard test organisms (the alga *Chlorella*: 340 µg/L (Canton, 1976), the water-flee *Daphnia magna*: 460 µg/L (Canton, 1976) and the fish *Salmo gairdneri*: 370 µg/L (Palawski and Knowles, 1986)). According to the standard set by the Uniform Principles (EU, 1997) the concentration level of 0.01 * acute L(E)C50 or 0.1 * chronic NOEC should be a safe concentration for aquatic biota.

This paper is the first in a series of two on the microcosm experiment, and summarises the fate of carbendazim as well as its effects on water quality parameters, breakdown of particulate organic matter (POM) and macroinvertebrates. The validity of the risk assessment procedure according to the Uniform Principles will be discussed. The second paper (Van den Brink et al., subm.) deals with the effects of carbendazim on zooplankton and primary producers (macrophytes, phytoplankton and periphyton). The discussion section of that paper presents an overall discussion of the ecological impact of carbendazim.

A separate paper describes additional laboratory single-species toxicity tests with several taxa inhabiting the microcosms (Van Wijngaarden et al., 1998). The vertical distribution of carbendazim in the sediments of the microcosms has been modelled by Koelmans et al. (subm.).

Materials and methods

Microcosms and experimental design

Each microcosm consisted of a glass aquarium (length 110 cm, width 110 cm, height 70 cm, water volume 600 L), filled with a 10 cm layer of lake sediment and a 50 cm water column. The microcosms were situated in a climate room (constant temperature: 19 ± 2°C). High pressure metal halide lamps (Philips HPI-T, 400W) were used to provide artificial daylight, resulting in a light intensity of approximately 120 $\mu E/m^2.s$ at the water surface. The daily photoperiod was 14 h. The microcosms were intended to model the community of Dutch drainage ditches. Details of the construction and lay-out of the microcosms can be found in Brock et al. (1992).

In the preparatory phase of the experiment, plankton and sediment dwelling macroinvertebrates were introduced into the microcosms, together with natural sediment and well water. In addition, the macrophyte *Elodea nuttallii* and several populations of macroinvertebrates and zooplankters, characteristic for Dutch ditches, were deliberately introduced. Over an acclimatisation period of three months, a biocoenosis was allowed to

develop in the microcosms. Meanwhile, all microcosms were interconnected by tubes (internal diameter 2.6 cm) and the water was circulated using a pump with a flow rate of 3.5 L/min to achieve similarity between the communities in the systems. The microcosms were disconnected before the start of the experiment (week –4).

The fungicide Derosal® (active ingredient carbendazim) was applied to ten microcosms, in five duplicate doses, while two other systems served as controls. The concentration of carbendazim in the water of the microcosms was kept at a constant level for four weeks, followed by a post-treatment period in which carbendazim was no longer applied. The effects of the fungicide on community structure in the microcosms were assessed by studying the dynamics of primary producers and invertebrates. Physico-chemical conditions were monitored to detect changes in the functioning of the overall ecosystem metabolism. The experimental period before carbendazim application is referred to as the pre-treatment period, while the period of four weeks of constant chronic exposure is called the treatment period and the period of the seven remaining weeks, i.e. weeks 5 through 11 after the start of the application, is called the post-treatment period. A nutrient addition of P (initial concentration: 0.015 mg/L) and N (0.22 mg/L) was applied weekly to all test systems during the experimental period.

Carbendazim application and analysis

The treatment started on 23 January 1995. On this day, the initial doses of carbendazim (nominal levels: 3.3, 33, 100, 330 and 1000 µg/L), applied as Derosal®, were distributed evenly over the water surface of two microcosms for each concentration and mixed by stirring.

During the treatment period, more carbendazim was added on 5 occasions to compensate for losses calculated from the actual carbendazim concentrations measured. To promote even distribution of the pesticide, water from a level 5 cm above the bottom of the microcosm was pumped up and released over the water surface during the entire experiment.

To estimate the exposure concentrations, water samples were taken at several moments after the start of the experiment. Duplicate depth-integrated water samples were collected by simultaneously stirring and sucking with a 100 ml volumetric pipette, and transferred to glass bottles. After mixing, sub-samples were transferred into 2 ml HPLC vials for direct analysis of the carbendazim concentration as described in Van Wijngaarden et al. (1998).

Water quality parameters

The dissolved oxygen (DO)-pH-conductivity-alkalinity syndrome was measured in the morning, at the start of the photoperiod, and in the evening, just before the illumination was switched off, at approximately one-week intervals from week -3 through week 9. DO was measured with a WTW oxygen meter (Oxi 196) and a WTW oxygen probe at a depth of 10 cm. Conductivity and pH were measured with a WTW conductivity meter and a Metrohm Herisau pH meter, respectively. Alkalinity was measured in 100 mL samples taken at a depth of 10 cm (titration with 0.05 N HCl until pH 4.2).

During the same period, depth integrated water samples from at least five localities well distributed over each microcosm were taken with a perspex corer for analysis of nutrients. Subsamples for each day and microcosm were pooled and the well-mixed sample was filtered through pre-washed glass fibre filters (Whatman GF/C), transferred to 100 mL iodated polyethylene bottles and stored (-20 °C). At the end of the experiment, the samples were defrosted and analysed for ammonium, nitrate and orthophosphate using a Skalar 5100 Autoanalyser.

Decomposition experiment

Decomposition of particulate organic matter (POM) was studied by means of the litter bag technique (Brock et al., 1982). The POM used consisted of *Elodea nuttallii* shoots and *Populus x canadensis* leaves. The *Populus* leaves had been leached three times for two days to remove the more easily soluble humic compounds. To allow storage of this material it was dried in an oven for 72 hours at 60 °C. Samples of *Elodea* and *Populus* were subsequently dried at 105 °C for 24 h to establish the 60 °C/ 105 °C dry weight ratio.

In the short-term decomposition experiment, a portion of 2 g dry weight (dried at 60 °C) of *Elodea* and *Populus* was enclosed in each litter bag, consisting of a glass petri-dish (diameter 11.6 cm) closed with a cover of stainless steel wire (mesh size 0.7 x 0.7 mm), in which two holes (0.5 cm) were punched to allow most invertebrates to enter. The materials used are known to be inert to carbendazim. In each microcosm, two litter bags of each plant type were incubated at the sediment surface for a period of two weeks. Whenever a set of litter bags was retrieved on a sampling day, a new set was incubated. At the end of each two-week decomposition period, the litter bags were gently washed in the overlying water of the microcosm to remove adhering sediment particles. The contents of the two bags of each plant type from each microcosm were then transferred to a white tray to separate invertebrates from POM. The plant material was transferred to aluminium foil to determine dry weight (24 h; 105 °C).

In the long-term decomposition experiment 72 nylon litter bags with a mesh size of 0.5 mm (non-accessible to invertebrates), each with 2 g dry weight of *Populus* leaves, were introduced into the microcosm at a depth of 20 cm on the first day of application of

carbendazim. Six litter bags were introduced into every cosm. After decay periods of 2, 4 and 8 weeks, respectively, two litter bags were retrieved from each microcosm. The plant material from each microcosm was transferred to aluminium foil to determine dry weight (24 h; 105 °C).

Sampling of macroinvertebrates

Macroinvertebrates were sampled from each model ecosystem at two-weekly intervals by means of artificial substrates and the short-term litter bags described above. In each system, two multiplates and two pebble baskets served as artificial substrates (for a detailed description see Brock et al., 1992).

On each sampling day, the artificial substrates were gently retrieved from their system, using a net to prevent the escape of nekton and invertebrates. Pebble baskets were first washed in a container to remove invertebrates from the pebbles. Subsequently, the macroinvertebrates present on both substrates and the litter bags were collected by handpicking, after which they were identified and counted alive, then released again into the model ecosystems. Data from artificial substrates and litter bags were pooled for further analysis.

At the end of the experiment (week 11), the macroinvertebrates in the microcosms were sampled in a different way. First, after removal of all macrophytes and artificial substrates (carefully washed to remove all adherent invertebrates) the water level in the cosms was lowered to a depth of 10 cm by means of a siphon. Escape of invertebrates was prevented by pouring the water through a net (mesh size 0.5 mm). Escaped individuals were reintroduced into the microcosms. The water in the microcosms was then gently mixed to obtain a random distribution of the macroinvertebrates. One sample from each cosm was taken with a small net in a corer with a 30 x 30 cm surface. Animals were collected by handpicking and identified alive (Turbellaria) or fixed in 4% formol (Oligochaeta) or 70% ethanol (other invertebrates).

Bioassays with macroinvertebrates

Glass cages (50 x 8 x 8 cm), with one side made out of small-mesh gauze (55 µm) to enable sufficient exchange with the ambient water, were used to monitor the mortality and natality of the crustaceans *Gammarus pulex* and *Asellus aquaticus*, and the snail *Bithynia tentaculata*. In each microcosm, 20 normally sized individuals of each species were introduced into separate cages. The bottoms of the cages were situated 5 cm above the substrate. Cages were introduced immediately after the start of the treatment in the case of *Gammarus* and *Asellus*; *Bithynia* was introduced in week -3. *Populus* leaves were added as shelter and additional food after one week. During the first four days, the survival of *Asellus*

and *Gammarus* was scored daily; after that, survival and numbers of neonates were scored weekly; the survival of *Bithynia* was monitored at bi-weekly intervals.

Data analysis

Prior to analysis, the macroinvertebrate data were Ln(2x+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., 1995).

No Observed Effect Concentration (NOEC) calculations at parameter or taxon level were derived using the Williams test (ANOVA; Williams, 1972). The test assumes that the mean response of the variable is a monotonic function of the treatment, thus expecting increasing effects with increasing dose. The analyses were performed with the Community Analysis computer programme (Hommen et al., 1994), resulting in an overview of NOECs in each sampling week for the data analysed.

The effects of the carbendazim treatment on the community level of macroinvertebrates were analysed by the Principal Response Curves method (PRC), which is based on the Redundancy Analysis ordination technique, the constrained form of Principal Component Analysis (Van den Brink and Ter Braak,1999). The PRC method is a multivariate technique specially designed for the analysis of data from microcosm and mesocosm experiments. PRC results in a diagram showing the sampling weeks on the x-axis and the first Principal Component of the treatment effects on the y-axis (see Fig. 1 as an example). This yields a diagram showing the deviations in time of the treatments compared to the control. For instance, Figure 1 indicates, for the period after the start of the treatment, that the largest deviations from the control occurred in the 1000 and 330 µg/L treatments, while smaller ones were found in the 100 and 33 µg/L treatments. It also indicates minor differences with respect to the control for the 3.3 µg/L treatment. The species weights shown on the right side of the diagram can be interpreted as the weight of each species with the response given in the diagram. Thus the flatworm *Dugesia tigrina*, which has the highest weight with the diagram, is indicated to have decreased most strongly at the higher treatment levels. The negative weight of the juvenile *Lymnaea* snail with the diagram indicates that its numbers have increased in the higher level treatments. In quantitatively terms: multiplying the weight b_k of species *k* by the regression coefficient c_{dt} of a treatment *d* at a certain sampling date *t* yields the fitted change on a log-scale of this species compared to the control. In terms of abundance: taking the exponent of this quotient yields the relative abundance compared to the control. For instance, the relative abundance of *Dugesia tigrina* in the microcosms with the highest treatment level at week 1 is exp(-1.2*4) = 0.0082 times the abundance in the control. This means that the abundance of this species in the microcosms with the highest treatment level has decreased to 0.8% of its abundance in the control in week 1. For a complete description and discussion of the PRC method, the reader is referred to Van den

Brink and Ter Braak (1997, 1998, 1999). The PRC analysis was performed using the CANOCO software package, version 3.14 (Ter Braak, 1988, 1990).

The PRC diagrams can also be evaluated in terms of the fractions of variance explained by the factors time and treatment and which fraction of the variance which is explained by treatment is shown in the PRC diagram.

In the CANOCO computer program, Redundancy Analysis is accompanied by Monte Carlo permutation tests to assess the statistical significance of the effects of the explanatory variables on the species composition of the samples (Van den Brink et al., 1996, Verdonschot and Ter Braak, 1994). The significance of the PRC diagram was tested by Monte Carlo permutation of the microcosms, i.e., by permuting whole time series of microcosms in the partial redundancy analysis from which PRC is obtained, using an F-type test statistic based on the eigenvalue of the component (Van den Brink and Ter Braak, 1999).

Monte Carlo permutation tests were also performed for each sampling date, using the Ln-transformed nominal dose as the explanatory variable (for rationale see Van den Brink et al., 1996). This allowed the significance of the treatment regime to be tested for each sampling date.

Besides the overall significance of the treatment regime, we also wanted to know *which* treatments differed significantly from the control, so as to infer the No Observed Effect Concentration (NOEC) at the community level. This could not be done by testing each treatment level against the control, because there were too few permutation possibilities (Van den Brink and Ter Braak,1999). In the corresponding univariate case, however, the Williams test (Williams, 1972) could be applied to calculate a NOEC. The Williams test can be applied to a multivariate data set if the data set is reduced to a single variable. The first principal component of a Principal Component Analysis suits this purpose. The NOEC calculations were therefore done by applying the Williams test to the sample scores of the first principal component of each sampling date in turn (Van den Brink et al., 1996).

Results

Fate of carbendazim

During the treatment period, the Average Exposure Concentration (AEC) of all treatment levels deviated by less than 10 % from the nominal concentrations (Table 1). The SDs of the AEC were also relatively small (within 10% of the AEC). The disappearance of carbendazim from the water layer in weeks 4 through 10 was found to be concentration dependent; carbendazim disappeared faster at the higher concentration levels than at the lower ones (Table 1). The half-life for the disappearance of carbendazim from the waterphase in the post-treatment period was 25 weeks for the 3.3 µg/L treatment level and 6 weeks for the highest treatment level (Table 1). $t_{½}$ values for the other treatment levels lay in

between these two extremes. At the 3.3 µg/L treatment level, however, the standard deviation of the half-life appeared to be quite high.

Table 1. Average Exposure Concentrations (AEC) in µg/L during the treatment period (4 weeks) estimated by the area under the curve (Van Wijngaarden et al., 1996), and concentrations measured at the end of the experiment (week 10) in µg/L. The dissipation rate of carbendazim from the water layer for the post-treatment period is also given ($t_{½}$).

Nominal concentration (µg/L)	AEC (µg/L) during treatment period ± SD	Concentration (µg/L) at week 10 ± SD	$t_{½}$ (weeks) ± SD for the post-treatment period
3.3	3.1 ± 0.3	2.3 ± 0.2	25 ± 18
33	30.5 ± 1.7	22.0 ± 1.3	15 ± 2
100	92.1 ± 2.5	62.6 ± 1.3	10 ± 1
330	319.0 ± 9.1	196.0 ± *	9 ± 2
1000	985.0 ± 35.0	511.0 ± 2.5	6 ± 0

SD= standard deviation; * = One of the two samples was lost during analysis

Water quality parameters

During the entire experiment, the dissolved oxygen concentration (DO) was very stable and ranged from 7.3 to 10.5 mg/L. Evening DO concentrations were generally 1.0 - 1.5 mg/L higher than morning concentrations. pH was also rather stable, ranging from 7.9 to 10.0. Evening pH was generally slightly higher than morning pH. In the course of the experiment, pH increased slightly in all microcosms. Conductivity was rather stable and ranged from 148 to 206 µS/cm, with no differences between morning and evening values. In the course of the experiment, conductivity decreased slightly in all microcosms. Alkalinity ranged from 1.1 to 1.9 mL HCl (0.05 N), showing a steady decrease over time in all microcosms. No consistent significant treatment effects of carbendazim were found on the DO-pH-conductivity-alkalinity syndrome (Williams test).

No significant treatment effects of carbendazim were found for orthophosphate (range: 0 to 28 µg/L), ammonium (range: 11 to 56 µg N/L) or nitrate (not detectable).

Decomposition experiment

The relative amounts (%) of remaining biomass of *Elodea nuttallii* shoots and *Populus* leaves in the litter bags after decay periods of two weeks are presented in Tables 2A and 2B respectively. The application of carbendazim did not result in significant treatment effects in this short-term decomposition experiment. The residual dry weights of *Elodea* amounted to

approximately 50% of the initial dry weight. The residual dry weights of *Populus* were distinctly higher, amounting to approximately 67% of the initial dry weight.

Table 2. Residual dry weights of *Elodea* shoots (A) and *Populus* leaves (B) in the short-term decomposition experiments per treatment level expressed as % of the initial biomass. Macroinvertebrates were allowed to access the litter bags. The duration of the decay period was 2 weeks. Significant differences related to the controls (Williams test, $p < 0.05$) are indicated by an asterisk.

	Treatment					
Week	Control	3.3 µg/L	33 µg/L	100 µg/L	330 µg/L	1000 µg/L
A *Elodea nuttallii*						
-3	48.0	48.0	46.5	49.0	51.0	52.5
-1	46.5	45.5	43.5	43.0	41.0	51.0
1	48.5	52.5	51.5	46.5	53.0	53.5
3	48.5	48.0	44.5	46.5	45.5	45.5
5	49.5	47.5	46.0	45.0	44.5	46.6
7	52.0	53.5	49.5	50.5	52.5	55.5
9	43.0	49.5	45.0	42.5	42.5	50.5
B *Populus*						
-3	67.5	67.0	67.5	69.5	71.5	70.0
-1	68.0	72.0	71.5	67.5	73.0	68.0
1	67.5	68.0	66.5	69.5	69.0	71.5
3	65.0	67.0	64.5	68.5	68.0	64.0
5	64.0	65.0	65.5	66.5	67.0	66.5
7	71.0	66.5	62.5	66.5	67.5	68.5
9	65.5	67.0	59.0	59.0	68.5	69.5

The long-term decomposition of *Populus* leaves in the mesh bags that did not allow the entrance of large macroinvertebrates (Table 3) showed a significant negative treatment effect for the two highest carbendazim concentrations after an incubation of only 4 weeks. After 8 weeks, however, a non-significant decrease in breakdown rate was found for these treatment levels as well (Table 3).

Table 3. Residual dry weights of *Populus* leaves in the long-term decomposition experiments per treatment level expressed as % of the initial biomass. Large macroinvertebrates were not allowed to access the litter bags. The duration of the decay period was 2, 4 or 8 weeks. Significant differences related to the controls (Williams test, $p < 0.05$) are indicated by an asterisk.

	Treatment					
Week	Control	3.3 µg/L	33 µg/L	100 µg/L	330 µg/L	1000 µg/L
0 - 2	72.0	74.0	70.0	72.0	73.0	70.0
0 - 4	56.5	59.0	55.0	57.5	62.0*	63.0*
0 - 8	34.5	27.5	29.0	30.5	38.0	45.5

Macroinvertebrates

Over the experimental period, a total of 86 different macroinvertebrate taxa were identified in the microcosms. The community was dominated by snails, crustaceans, turbellarians and oligochaetes, while bivalves, nemerteans and leeches were less abundant. Insects were scarce because of their inability to reproduce or oviposit in the climate room after their emergence in the pre-experimental acclimatisation period. The number of taxa declined slightly during the investigation period.

In order of decreasing abundance, *Physella acuta*, *Segmentina nitida*, *Bathyomphalus contortus* and *Bithynia tentaculata* were the dominant snail species. In addition *Anisus vortex*, *Bithynia leachi*, *Lymnaea stagnalis*, *Physa fontinalis*, *Planorbarius corneus*, *Planorbis carinatus*, *Potamopyrgus antipodarum* and *Stagnicola palustris* frequently occurred in relatively high numbers. Other snail species were much less abundant. The crustaceans found comprised the Ostracoda and the shredders *Gammarus pulex*, *Asellus aquaticus*, *Proasellus coxalis* and *Proasellus meridianus*; these taxa occurred in high numbers. The turbellarians were dominated by the rhabdocoelan *Mesostoma lingua* and the triclad *Dugesia tigrina*, while *Dugesia lugubris* occurred frequently but in lower numbers. The vegetation-inhabiting *Stylaria lacustris* and the surface dwellers *Dero digitata* and Tubificidae were the dominant oligochaetes in the microcosms. Leeches, except for *Alboglossiphonia heteroclita*, were rare. Particularly in the pre-treatment period, the chironomid *Corynoneura* and the phantom midge *Chaoborus obscuripes* occurred in high numbers.

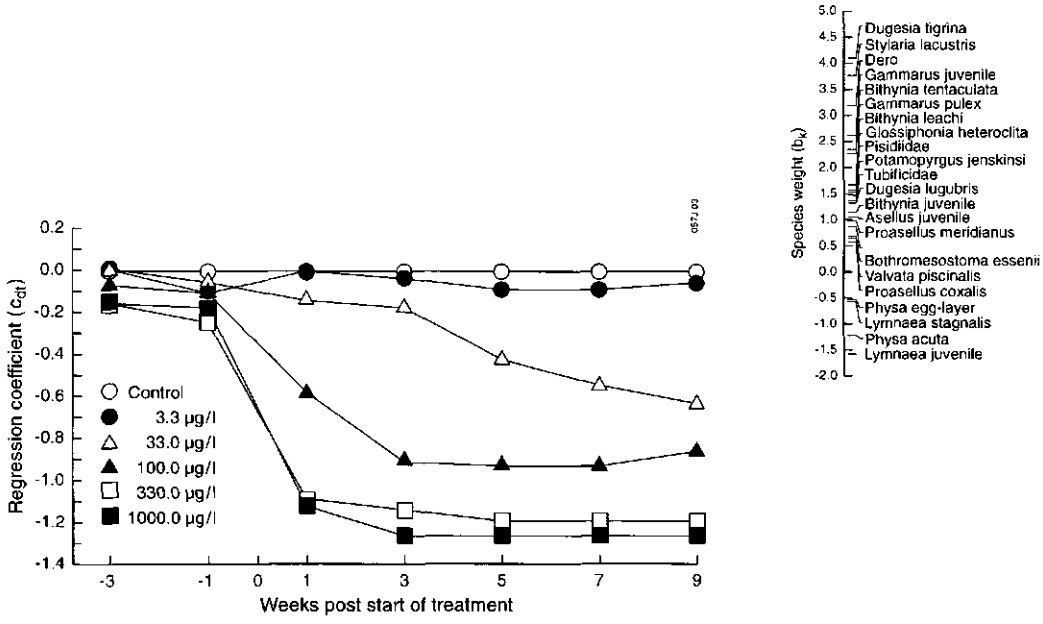

Figure 1. Principal Response Curves resulting from the analysis of the macroinvertebrate data set, indicating the effects of the fungicide carbendazim on the macroinvertebrate community. Of all variance, 30% could be attributed to sampling date; this is displayed on the horizontal axis. Forty-nine percent of all variance could be attributed to treatment. Of this variance, 44% is displayed on the vertical axis. The lines represent the course of the treatment levels in time. The species weight (b_k) can be interpreted as the affinity of the taxon with the Principal Response Curves. Taxa with a species weight between 0.25 and -0.25 are not shown.

The PRC of the macroinvertebrate data set shows that the 33, 100, 330 and 1000 µg/L treatments clearly deviated from the control after the start of the treatment (Fig. 1). These visual differences during the treatment and the post-treatment period are consistent with the results of the permutation tests. The PRC had an overall significance of $p < 0.01$, and the permutation tests indicated a significant treatment effect of $p < 0.01$ on all sampling dates after the start of the treatment. The Williams test, applied per sampling date on the first principal component, indicated a $NOEC_{community}$ of 3.3 µg/L for weeks 1, 5, 7 and 9.

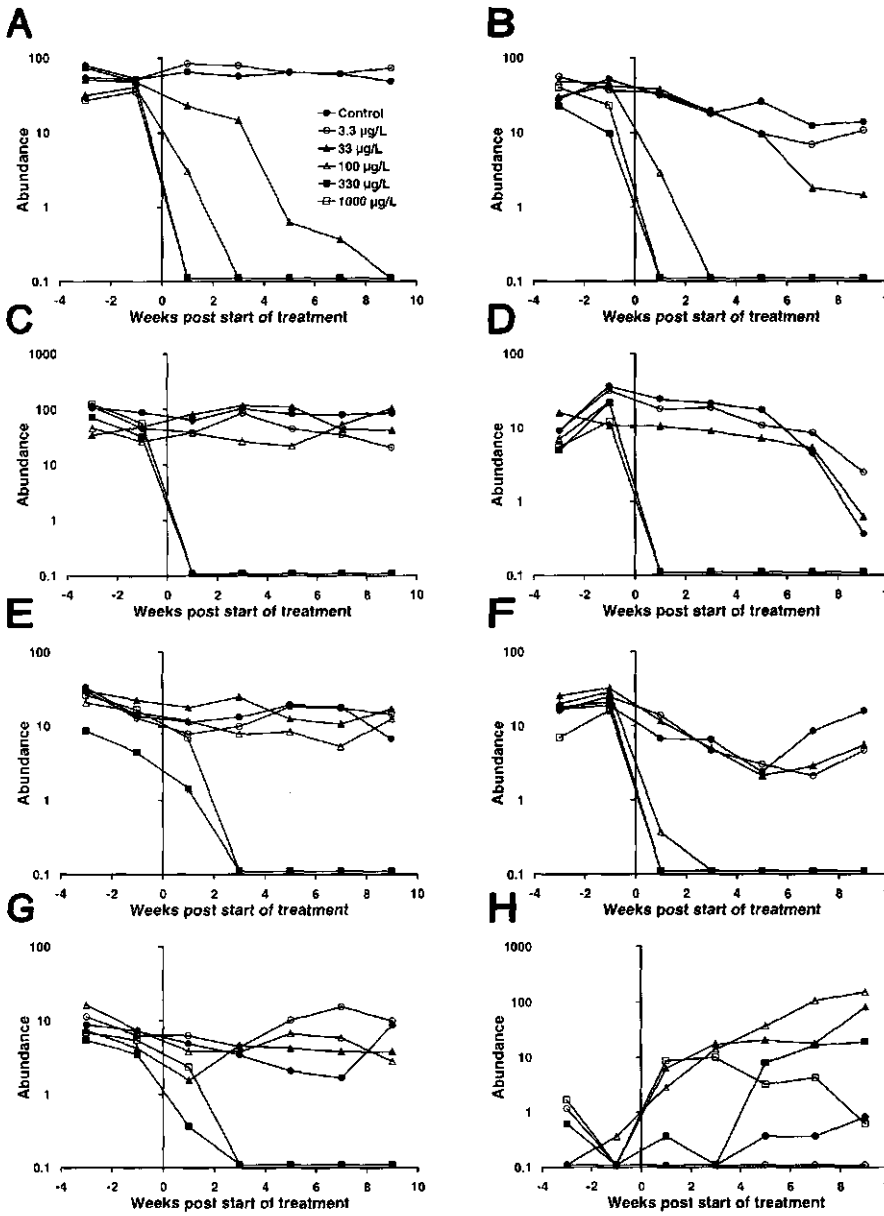

Figure 2. Dynamics in numbers of the 8 macroinvertebrate taxa, most important in the PRC analysis. Figures 2A through 2H show the geometric means of the numbers counted per treatment level, of *Dugesia tigrina* (A), *Dero* sp. (B), *Stylaria lacustris* (C), *Gammarus* juvenile (D), *Bithynia tentaculata* (E), *Gammarus pulex* (F), *Bithynia leachi* (G) and *Lymnaea* juvenile (H). For NOECs see Table 4. In the figures, absence is denoted by 0.1.

Thirty percent of the total variance is explained by time, while 49% is explained by the treatment regime. Twenty-one 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 (Fig. 1), while 44% of the variance explained by the treatment regime is displayed on the y-axis. The PRC diagram also reveals a more pronounced and faster response to the treatment with increasing doses of carbendazim. This is particularly seen in the 33 µg/L treatment, which though already significantly different from the control and 3.3 µg/L treatment from week 1 onwards, shows increasing deviation from the control as time progresses. The treatment with carbendazim had serious noxious effects on many macroinvertebrate taxa (species weights in Fig. 1). The dynamics of the 8 taxa with the highest weights in the PRC are given in Figures 2A through H. The most seriously affected taxa were the turbellarian *Dugesia tigrina* (Fig. 2A), the oligochaetes *Dero* (Fig. 2B) and *Stylaria lacustris* (Fig. 2C), the crustaceans *Gammarus* juvenile and *Gammarus pulex* (Fig. 2D and F) and the molluscs *Bithynia tentaculata* and *Bithynia leachi* (Fig. 2E and G). The taxon which increased most strongly at the higher treatment levels was that of the juveniles of the mollusc *Lymnaea* (Fig. 2H).

Table 4 shows the species for which a NOEC was calculated. Only NOECs calculated at least for two consecutive sampling dates were considered valid. The table also indicates if, and at what concentration, the taxa were eliminated. No specimens of *Dugesia tigrina*, *Dero* or *Stylaria lacustris* were found in the microcosms at concentrations of 33, 100 and 330 µg/L and higher, respectively, either during the treatment or during the post-treatment period (Table 4, Figure 2). However, at the final sampling (week 11) some individuals of *Stylaria* were found even at the highest carbendazim concentration, while *Dero* was then found at all concentrations except the highest. The hirudinean *Alboglossiphonia heteroclita* was eliminated, after the start of the treatment, at the two highest carbendazim concentrations. Of the Crustacea, the amphipods *Gammarus pulex* and juvenile *Gammarus* were eliminated from the microcosms at concentrations of 100 µg/L and higher. The isopod genera *Asellus* and *Proasellus* were significantly affected by carbendazim (NOEC 330 µg/L) but not eliminated, while no significant effects were found for Ostracoda. The snails of the genus *Bithynia* were the only gastropods which were negatively influenced by carbendazim; they were eliminated at the two highest concentrations. Juveniles were affected more severely than adults. The numbers of the herbivorous snails *Lymnaea stagnalis*, *Physella acuta*, *Physa fontinalis* and *Segmentina nitida* showed significantly higher abundance values in the post-treatment period in the microcosms with the higher treatment levels. No significant treatment effects were found for other macroinvertebrate taxa, mainly because they occurred in (too) low numbers or disappeared from all microcosms during the experiment, due to emergence (*Corynoneura*, *Chaoborus obscuripes*). Figs 3A through C show the dynamics for the taxa for which the lowest NOEC of < 3.3 µg/L was calculated. For *Nemertini* sp. an increase in abundance in the control microcosms was found for the post-treatment period (Fig 3A), for *Segmentina nitida* a decrease (Fig. 3C). For the Gastropod *Lymnaea stagnalis*

after the start of the treatment, a decrease in numbers was found for both the control and highest treated microcosms (Fig. 3B).

Table 4. NOECs calculated for individual populations of the macroinvertebrate community in microcosms treated with carbendazim. Only NOECs calculated for at least two consecutive sampling dates were considered.

Taxon	NOEC	On the base of	Conc. of elimination and week
Dugesia tigrina	3.3	Decrease	33 (week 9)
Dugesia lugubris	3.3	Decrease	33 (week 1)
Nemertini sp.	< 3.3	Decrease	100 (week 5)?
Dero sp.	3.3	Decrease	100 (week 3)
Stylaria lacustris	33	Decrease	330 (week 1)
Alboglossiphonia heteroclita	3.3	Decrease	330 (week 3)
Bithynia tentaculata	33	Decrease	330 (week 3)
Bithynia leachi	100	Decrease	330 (week 3)
Bithynia juvenile	33	Decrease	100 (week 3)?
Lymnaea stagnalis	< 3.3	Increase	
Lymnaea juvenile	3.3	Increase	
Physella acuta	3.3	Increase	
Physa fontinalis	3.3	Increase	
Segmentina nitida	< 3.3	Increase	
Asellus aquaticus	330	Decrease	not
Asellus juvenile	330	Decrease	not
Proasellus coxalis	330	Decrease	not
Proasellus meridianus	330	Decrease	not
Gammarus pulex	33	Decrease	100 (week 3)
Gammarus juvenile	3.3	Decrease	100 (week 1)

Bioassays with macroinvertebrates

The bioassays with *Bithynia tentaculata* showed nearly 100% survival at all treatment levels except the highest one, where all specimens were dead in week 9 (NOEC: 330 µg/L from week 3 onwards). *B. tentaculata* disappeared from the artificial substrates in the microcosms with the two highest concentrations (Fig. 2E).

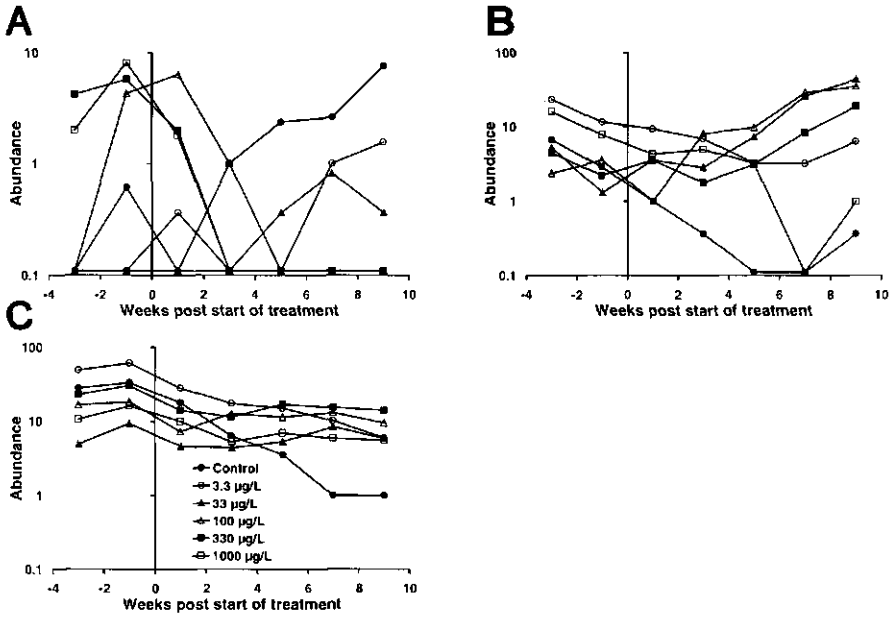

Figure 3. Dynamics in numbers of the 3 macroinvertebrate taxa for which a NOEC < 3.3 µg/L was calculated. Figures 3A through 3C show the geometric means of the numbers counted per treatment level, of Nemertini sp. (A), *Lymnaea stagnalis* (B) and *Segmentina nitida* (C). In the figures, absence is denoted by 0.1.

Table 5 lists the survival rates of adult *Gammarus pulex*. The lowest NOECs (< 3.3 µg/L) for the experiment were found between weeks 3 and 6. However, mortality in the controls (and other treatments) was higher than 25%, indicating the presence of another stressor besides carbendazim. *Gammarus pulex* was eliminated from the microcosms with the three highest carbendazim treatment levels within 3 weeks, showing increasing rates of disappearance. Neonates of *G. pulex* were found from week 3 onwards in the control and the 3.3 µg/L treatment.

Adult *Asellus aquaticus* showed better survival rates than *Gammarus pulex*, with a NOEC of 330 µg/l from week 3 onwards. Some specimens survived even at the highest carbendazim dose after 9 weeks. Neonates of *A. aquaticus* were found at all treatment levels from week 3 onwards, but their numbers were distinctly lower at the highest treatment level.

Table 5. Survival of *Gammarus pulex* in bioassays (n = 2) after treatment with carbendazim (µg/L), with corresponding NOECs. In each bioassay, 20 individuals of *G. pulex* were incubated.

	Treatment						
Day	Control	3.3 µg/L	33 µg/L	100 µg/L	330 µg/L	1000 µg/L	NOEC
0	20.0	20.0	20.0	20.0	20.0	20.0	
1	19.5	17.5	20.0	18.0	15.5	6.5	330
2	18.5	17.5	18.5	16.0	10.5	0.0	100
3	18.0	17.0	18.5	13.5	1.0	0.0	33
6	17.5	14.5	17.5	3.0	0.0	0.0	33
13	16.5	13.0	17.0	0.0	0.0	0.0	33
20	15.0	12.0	11.0	0.0	0.0	0.0	< 3.3
27	13.5	9.0	7.0	0.0	0.0	0.0	< 3.3
34	11.5	6.0	5.0	0.0	0.0	0.0	< 3.3
41	10.0	5.0	3.0	0.0	0.0	0.0	< 3.3
48	7.5	5.0	2.0	0.0	0.0	0.0	3.3
55	5.0	5.0	2.0	0.0	0.0	0.0	3.3
62	5.0	4.5	1.5	0.0	0.0	0.0	3.3

Discussion

Fate of carbendazim

The present study found carbendazim to be very persistent in the water layer. The $t_{½}$ varied between 6 and 25 weeks, and decreased with the treatment level (Table 1). This dependence of $t_{½}$ on treatment level is probably not a result of changes in the physico-chemical conditions in the microcosms, since no differences in pH, D.O., alkalinity and conductivity could be demonstrated between the different treatments. Since we know of no other study dealing with the fate of carbendazim in sediment/water systems, we cannot compare our results with other findings.

Effects on water quality parameters

Carbendazim treatment affected the structure of aquatic ecosystems indirectly, by stimulating the growth of phytoplanktonic algae and macrophytes as described in detail in the second paper on this experiment (Van den Brink et al., subm.). Despite these pronounced effects, no changes were found in water quality parameters. Several likely

causes for this can be identified. The fact that decomposition was not severely affected indicates the relatively low sensitivity of microbial communities to the carbendazim concentrations applied. The lack of effect on the DO-pH-conductivity-alkalinity syndrome was probably caused by the relatively low biomass of all phytoplankton, periphyton, zooplankton and macroinvertebrates together in comparison with the standing stock of the macrophyte *Elodea nuttallii* during the experiment. The abundance of *Elodea nuttallii* increased distinctly in all microcosms during the experiment, but the extra growth at the two highest treatment levels (significantly greater than in the controls) did not cause significant changes in the DO-pH-conductivity-alkalinity syndrome and apparently compensated the effects on this syndrome due to the loss of sensitive invertebrates.

Effects of carbendazim on decomposition

Despite the general importance of micro-organisms (bacteria, fungi, protozoa) in aquatic (model) ecosystems, they are seldom identified and counted in pesticide studies (Brock and Budde, 1994). Even in the present study of the fungicide carbendazim, presence and abundance of aquatic hyphomycetes were not investigated because methods to examine fungi are laborious and require specialized techniques. Secondly, the highest carbendazim concentration used in the present study (1000 µg/L) is lower than the concentration affecting the sporulation and germination of hyphomycetes. Chandrashekar and Kaveriappa (1994) reported 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 6 species of hyphomycetes for carbendazim concentrations up to 1000 µg/L. These data suggest that the carbendazim concentrations used in the present study probably not (seriously) affected growth, sporulation and germination of most populations of fungi present in the microcosms. On the basis of the decomposition experiments, however, we conclude that some populations of micro-organisms apparently were affected, but only after a prolonged exposure period (Table 2 and 3). In addition the higher biomass of *Elodea nuttallii* at the end of the experiment and in bioassays at the two highest concentrations, as described in detail in part II (Van den Brink et al., subm.) might be explained by a reduction of pathogens (e.g. fungal stress) due to carbendazim application.

Effects on macroinvertebrates

Benzimidazoles like carbendazim show varying degrees of effect on worm activity. Their effects on nematodes and earthworms cover a broad number of genera (Delp, 1987). In the present carbendazim microcosm experiment, "worm"-like taxonomic groups such as Oligochaeta, Turbellaria and Hirudinea, together with the amphipod *Gammarus pulex* and the snail *Bithynia tentaculata*, were found to be the most sensitive taxa. Van Wijngaarden et al. (1998) presented chronic $NOEC_{reproduction}$ values for *Dugesia lugubris*, *B. tentaculata*, *G. pulex*

and *Asellus aquaticus* of 3.4, 103, 30 and 300 µg/L respectively. Except for *Bithynia* and *G. pulex*, these values are in perfect accord with the NOECs found in our study (Table 4). For *Bithynia* the same NOEC was calculated from the bioassay but a slightly lower value was calculated for the free-living populations in the microcosms (33 µg/L). This could be a result of the behaviour of *B. tentaculata* which was observed in the bioassays. At increasing doses of carbendazim, the snails became less active, hardly fed and usually lay on the bottom of the cages with closed opercula. This means that in the microcosms, the individuals of *Bithynia* may have been unable to re-invade the artificial substrates after being removed from them during the previous sampling. For *Gammarus pulex*, very low NOECs (3.3 µg/L or lower) were found in the bioassays from day 20 onwards (Table 5). In the microcosms, however, the free-living adult population of *Gammarus pulex* was only affected in the 100 µg/L treatment levels and higher. This is in perfect accord with the results of Van Wijngaarden et al. (1998). In the bioassay, the mortality in the control (≥ 25%), indicated the presence of another stress factor besides carbendazim. This unknown stress factor could also have influenced the mortality in the treated cosms.

Comparison with other fungicides

Pentachlorophenol (PCP) is a fungicide (biocide) much in use for the preservation of wood, but in the Netherlands it is also used, like carbendazim, for the preservation of bulbs. It is the only chemical known to us that has fungicidal properties and has been tested on more taxa than standard species only and of which information is available in the free literature. Table 6 summarizes the lowest acute L(E)C50 and chronic NOEC values found for different taxa for PCP. It is striking that, like carbendazim, it affects not only fish and some crustaceans but also Annelida and Mollusca. Of course there is as yet no scientific explanation for this similarity of effects, and it is beyond this paper to discuss the possible causes, but it is clear that from a risk assessment point of view more information is needed on the effects of fungicides on non-standard test species. Such information could shed more light on the expected effects of fungicides on freshwater ecosystems.

Risk assessment

One of the aims of our experiment was to validate the safety factors proposed by the European Union in their Uniform Principles (EU, 1997). In the present experiment, the risk assessment was based on the direct effects of carbendazim on some macroinvertebrate species (this paper) and zooplankters as described in part II (Van den Brink et al., subm.).

A NOEC < 3.3 µg/L was calculated for the taxa Nemertini sp., *Lymnaea stagnalis* and *Segmentina nitida* (Table 4). Their dynamics are given in Fig. 3. For the latter two species (Figs 3B and C) the NOECs are based on higher abundance values in the treated cosms compared to the control ones (Table 4). It is clear from their dynamics that these NOECs are

based on a decrease in abundance in the control microcosms rather than on an increase in the treated cosms. Hence they have to be disregarded in the risk assessment. A NOEC < 3.3 µg/L was calculated for Nemertini sp. on the basis of a lower abundance in the treated cosms compared to the control cosms in weeks 3 and 5 (Table 4). Fig. 3A shows that these NOECs are based on an increase in numbers in the control cosms rather than a decrease in the treated cosms. Moreover the numbers collected were very low. Hence, this NOEC was also disregarded. We are, however, well aware that by disregarding these incidental NOECs, small transient effects on these taxa might be overlooked. But setting the lowest NOECs at 3.3 µg/L is supported by the calculated NOEC for the macroinvertebrate community as a whole ($NOEC_{community}$), which was 3.3 µg/L.

Table 6. Lowest acute L(E)C50 and chronic NOECs found in the literature for PCP.

Species (group)	Acute L(E)C50	Species	Chronic NOEC
Salmo gairdneri (Fish)	52 (LC)[A]	*Ceriodaphnia* (Crustacea)	< 4.1[2,C]
Scenedesmus (Alga)	80 (EC)[1,B]	*Ophryotrocha* (Annelida)	10[2,B]
Catastomus (Annelida)	85 (LC)[C]	*Pleuronectes* (Fish)	10[1,B]
Dreissena (Mollusca)	110 (LC)[B]	*Physa* (Mollusca)	< 26[2,C]
Pleuronectes (Fish)	140 (LC)[B]	*Lymnaea* (Mollusca)	50[2,B]

[1]: EC50 or NOEC based on inhibition of growth; [2]: NOEC based on inhibition of reproduction; [A]: Johnson and Finley (1980); [B]: Adema and Vink (1981); [C]: Hedtke et al. (1986)

The $NOEC_{community}$ and the NOECs for the different taxa based on decrease are given in Fig. 4, which also shows the results of laboratory tests with different taxa found in the literature. A NOEC of 3.3 µg/L was calculated for the macroinvertebrate community as a total and for the taxa *Dugesia tigrina*, *Dero*, *Alboglossiphonia heteroclita*, juveniles of *Gammarus pulex* and the cladoceran *Acroperus harpae* (Van den Brink et al., subm.). Values of EC50, 96h and chronic NOEC (25 days) of the most susceptible standard laboratory test species *Daphnia magna* are 87 and 26 µg/L respectively (Van Wijngaarden et al., 1998). The safety factors of 0.01 and 0.1 (to be multiplied by the lowest acute EC50 and chronic NOEC of the standard test species, respectively) thus appeared to ensure adequate protection for the microcosm community under chronic exposure to carbendazim.

It is debatable whether the standard test organisms (*Daphnia*, fish, algae) are sufficiently representative for the indigenous taxa belonging to non-arthropod taxa such as Turbellaria and Annelida, which suffered the severest effects in the microcosms. Though the aquatic communities were adequately protected on the basis of the criteria of the Uniform Principles, we would recommend adding non-arthropod macroinvertebrate species in an advanced tier of the risk assessment of fungicides. This is based on the assumption that the spectrum of effects of fungicides in aquatic ecosystems is comparable with that of

carbendazim. This is supported by the results of the laboratory tests with PCP performed with several standard and non-standard test species (Table 6).

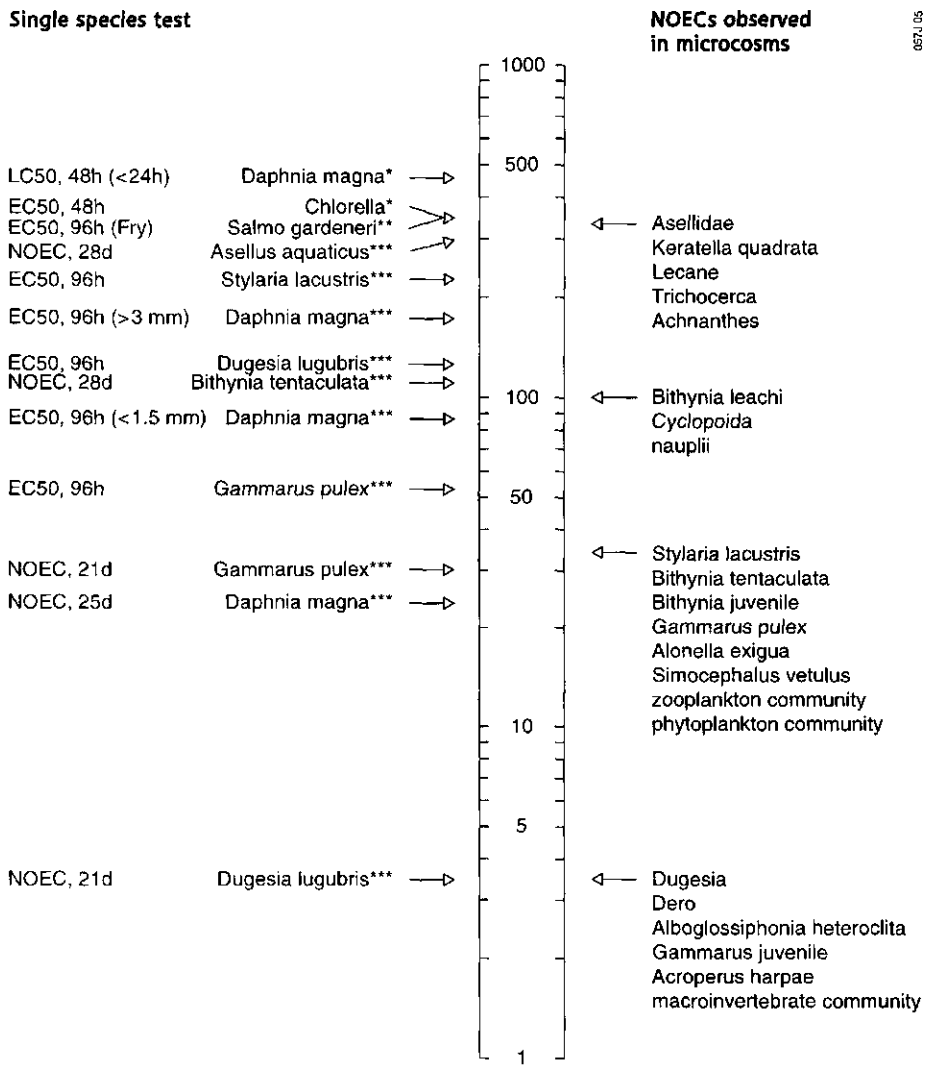

Figure 4. Summary of laboratory test results with carbendazim and direct effects observed in the microcosms. * = Canton, 1976; ** = Palawski and Knowles., 1986.; * = Van Wijngaarden et al., 1998.**

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Impact of the fungicide carbendazim in freshwater microcosms.

II. Zooplankton, primary producers and final conclusions

Abstract

Effects of chronic application of the fungicide Derosal® (active ingredient carbendazim) were studied in indoor macrophyte-dominated freshwater microcosms. The concentrations (0, 3.3, 33, 100, 330 and 1000 µg/L) were kept at a constant level for four weeks.

This paper is the second of a series of two; it describes the effects on zooplankton and primary producers and presents an overall discussion. The zooplankton community was negatively affected by the three highest treatment levels ($NOEC_{community}$ = 33 µg/L). At higher treatment levels Cladocera taxa were completely eliminated, while Copepod numbers were reduced. Rotatoria taxa decreased (*Keratella quadrata* and *Lecane* sp.) or increased in abundance (*Testudinella parva*) at the highest treatment level only.

Due to the reduced grazing pressure, the abundance of some phytoplankton taxa and the chlorophyll-a content of the phytoplankton increased at the three highest treatment levels ($NOEC_{community}$ = 33 µg/L). This effect was not observed for the periphyton, most probably because the reduced grazing pressure was compensated by the increased abundance of some snail species such as *Lymnaea stagnalis* and *Physella acuta*. At the end of the experimental period the biomass of the macrophyte *E. nuttallii* was significantly elevated at the two highest treatment levels. The slight reduction microbial activity, which was also indicated by long-term decomposition experiments, may have included the removal of pathogene organisms from this macrophyte.

Introduction

Fungicides are widely used in agriculture. As a consequence, drainage ditches and ponds may become contaminated by these chemicals. Nevertheless, the ecological effects of fungicides in freshwater ecosystems have hardly been investigated. In addition, taxa susceptible to fungicides may not be adequately represented among the standard aquatic test species (*Daphnia*, alga, fish) used in ecotoxicological risk assessment (Brock and Budde, 1994). To investigate the possible inadequacy of the current risk assessment procedure, and improve our understanding of the direct and indirect effects of fungicides on freshwater ecosystems, we performed a microcosm study was performed with carbendazim as the model substance.

This paper is the second in a series of two, and summarises the effects of carbendazim on zooplankton, periphyton, phytoplankton and macrophytes. The first paper

dealt with the fate of carbendazim in the waterlayer and its effects on water quality parameters, breakdown of particulate organic matter and macroinvertebrates (Cuppen et al., subm.). The discussion section of the present paper will present an overall discussion of the ecological impact of this fungicide.

Materials and methods

Experimental design

The indoor microcosms (length and width 110 cm; depth 70 cm; water depth 50 cm; sediment depth 10 cm) and the conditions in the climate room (constant temperature 19 ± 2°C; photoperiod 14 h) have been described in detail in part I (Cuppen et al., subm.). Two of the twelve microcosms served as controls. The other ten microcosms received a dose of the fungicide Derosal® (active ingredient carbendazim) at the start of sampling week 1. Concentrations of carbendazim were randomly allocated to the microcosms. Six sets of two microcosms were treated with 0, 3.3, 33, 100, 330 and 1000 µg/L carbendazim, respectively. The carbendazim concentration was kept constant for 28 days, the standard period of a chronic test, by adjusting of the concentration weekly. All microcosms were investigated for a period of 14 weeks: a pre-treatment period of three weeks, a treatment period of four weeks and a post-treatment (restoration) period of 7 weeks.

Zooplankton

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

On the first day of the treatment period, 25 individuals of *Daphnia magna* (≥ 1.5 mm) were put into a tube-like glass enclosure (length 40 cm, diameter 10 cm), with a bottom consisting of water permeable pumice stone. One such enclosure was placed in each of the microcosms, with its bottom at 35 cm below water level. Sufficient exchange of water was achieved by raising the enclosure daily. Twice a week, the daphnids were fed with a mixture of *Scenedesmus acutus* and *Scenedesmus subspicata*. On days 4, 7, 14, 21 and 28 after the start of the bioassay, the individuals were removed from the enclosure, counted and returned.

Table 1. Summary of methods used for the sampling of the investigated endpoints in the microcosms. (... indicates that samples were taken weekly). For a detailed description of methods, see references.

Community	Unit	Sampling weeks	References
Phytoplankton			
Species composition	numbers/L	-3, -1, ... 7, 9	Van Donk et al., 1995
Chlorophyll-a	µg/L	-3, -1, ... 7, 9	Van Donk et al., 1995
Periphyton			
Species composition	numbers/cm^2	-2, -1, 1, 3, 5, 7, 9	Brock et al., 1995
Chlorophyll-a (on glass slides)	µg/dm^2	-3, ... 1, 3, 5, 7, 9	Brock et al., 1995
Chlorophyll-a (on *Elodea nuttallii*)	mg/g d.w.	-3, 0, 1, 3, 5, 7, 9	Brock et al., 1995
Zooplankton			
Species composition	numbers/L	-3, -1, ... 7, 9	Cuppen et al., 1997

Phytoplankton

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

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

To measure (direct) effects of carbendazim application on phytoplankton growth in the microcosms, the green alga *Scenedesmus acutus* was immobilized in alginate beads as described by Van Donk et al. (1992). This encapsulation of algal cells in a matrix prevents algal cells from being washed out and grazed by zooplankton. At the same time they maintain their respiratory and photosynthetic activities (Van Donk et al., 1995). The initial cell volume per bead was 118 ± 19 µm^3 (mean ± STD; n=5). At the beginning of the treatment period, the beads were incubated in the microcosms in a glass petri dish (diameter 11.6 cm) closed with a cover of stainless steel wire (mesh size 0.7 x 0.7 mm). The petri dish was suspended 5 cm below the water surface. For the bioassay, samples were taken daily up to 7 days after the start of the bioassay. On each sampling date, the cell volume of two alginate beads was established as described by Van Donk et al. (1995). For each cosm and sampling date, the relative increase in biovolume could be calculated by dividing the increase in cell volume by the initial biovolume.

Periphyton

Periphyton was sampled from glass slides that served as artificial substrates. The slides were positioned in a frame at a fixed depth of approximately 10 cm below the water surface, and were incubated for eight weeks. On each sampling day, six glass slides were used to study the taxa composition of the periphytic algae (Table 1). For chlorophyll-a analysis, another six slides were brushed visually clean and the periphyton removed was collected in tap water. The chlorophyll-a content of the water-periphyton solution was analyzed as described above.

At regular intervals, the top 10 centimetres of 10 shoots of *Elodea nuttallii* were sampled from each microcosm to quantify the loosely attached periphyton associated with this macrophyte. The sampled shoots of each system were collected in a 250-mL bottle, filled with 100 mL tap water, and shaken at 200 RPM for five minutes. Subsequently, the *Elodea* material was removed and the remaining periphyton suspension was analyzed for chlorophyll-a as described above. In addition, the amount of *Elodea* in grams of dry weight was estimated for each sample. This allowed the quantity of loosely attached periphytic algae to be expressed as µg chlorophyll-a per gram dry weight of the macrophyte (Table 1).

Macrophytes

At the end of the post-treatment period, standing stock estimations of all macrophytes in each microcosm were made by harvesting. After harvesting the macrophytes were carefully rinsed to remove as much of the adhering periphyton, macroinvertebrates and sediment as possible. Subsequently, the individual plant species were dried (48 h; 105 °C) and ashed (4 h; 550°C) to assess the ash-free dry weight.

During the carbendazim treatment period (28 days), bioassays with the macrophytes *Elodea nuttallii* and *Lemna minor* and the filamentous alga *Oedogonium*, covering the whole period, were carried out. In each microcosm, 0.21 g fresh weight of *E. nuttallii* shoots (n=2) were allowed to attach in a plastic beaker filled with sediment. Before the shoots were weighed, they were gently blotted dry with a tissue. The beaker was transferred to a transparent enclosure (length 10 cm, width 10 cm, height 50 cm), with one side consisting of gauze (mesh size: 55 µm). The enclosure was placed in the microcosm, at 45 cm below water level, and sufficient exchange of water was achieved by regularly raising the cage. The bioassays lasted the entire treatment period (week 0 through week 4), after which the dry weight of *Elodea* was established in each enclosure. The bioassays with *L. minor* (initial fresh weight 0.023 g, n=2) and *Oedogonium* (initial fresh weight 0.026, n=2) were performed in enclosures of the same type, only without the beaker. *Lemna minor* was positioned at the water surface, the filamentous algae in the water column.

Data analysis

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

NOEC calculations at taxon level ($p \leq 0.05$) were done using the Williams test (ANOVA; Williams, 1972). NOECs were only considered valid when calculated for two consecutive sampling dates. Before univariate and multivariate analyses were performed, the abundance values of the zooplankton, phytoplankton and periphyton communities were, respectively, Ln(10x+1), Ln(0.001x+1) and Ln(x+1) transformed (for rationale see Van den Brink et al., 1995).

The LC10 and LC50 calculations on the results of the bioassays with *Daphnia magna* were done using the general logistic model:

$$y = \frac{c}{1 + \exp^{-b*(Ln(x)-a)}}$$

Where:
- y = expected number;
- a = Ln(EC50)
- b = slope parameter;
- c = expected number in control microcosms;
- x = exposure concentration.

The model was programmed in GENSTAT, version 5.3.1. (Payne and Lane, 1987). A Poisson distribution of the abundance data was assumed.

The effects of the carbendazim treatment on the zooplankton, phytoplankton and periphyton communities were analyzed by the Principal Response Curves method (PRC). The PRC method is a multivariate technique specially designed for the analysis of data from microcosm experiments. More information on PRC can be found in Van den Brink and Ter Braak, 1997; 1998; 1999. The statistical significance of treatment effects at a community level was also tested, using Monte Carlo permutation tests. The significance of the PRC diagram was tested by Monte Carlo permutation of the microcosms, i.e., by permuting entire time series in the partial redundancy analysis from which PRC is derived. Monte Carlo permutation tests were also performed per sampling date, allowing the significance of the effects of a treatment regime to be tested for each sampling date.

In addition to the overall significance of the effects of a treatment regime on a community, we also wanted to know *which* treatments differed significantly from the control, 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).

Results

Zooplankton

A total of 17 different zooplankton taxa were identified. The number of taxa in the control samples increased over time. The control community was dominated by Cyclopoida and Ostracoda, followed by Cladocera. Most taxa increased in abundance over time.

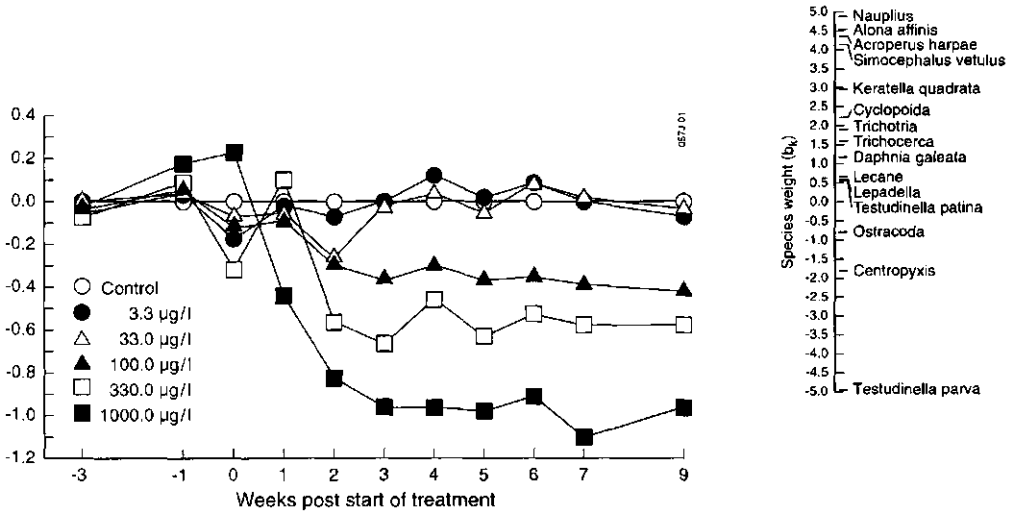

Figure 1. Principal Response Curves resulting from the analysis of the zooplankton data set, indicating the effects of the fungicide carbendazim on the zooplankton community. Of all variance, 25% could be attributed to sampling date, and is displayed on the horizontal axis, 51% could be attributed to treatment. Of the variance explained by treatment, 54% 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.

The PRC of the zooplankton data set reveals a clear concentration-dependent deviation of the 100, 330 and 1000 µg/L treatments from the controls (Fig. 1). The visual differences were confirmed by the permutation tests and $NOEC_{community}$ calculations (NOEC = 33 µg/L; Table 2). Twenty-five percent of total variance of the zooplankton data set was explained by the factor time, and is thus displayed on the horizontal axis of the PRC diagram (Fig. 1). Fifty-one percent of the total variance could be allocated to the treatment regime. Of this variance, 54% is displayed on the vertical axis (Fig. 1). *Acroperus harpae*, nauplius, *Alonella affinis* and *Simocephalus vetulus* have a high positive weight with the diagram (see species scores in Fig. 1.), while *Keratella quadrata*, Cyclopoida sp. and *Lecane* sp. have a lower positive one, and *Testudinella parva* has a

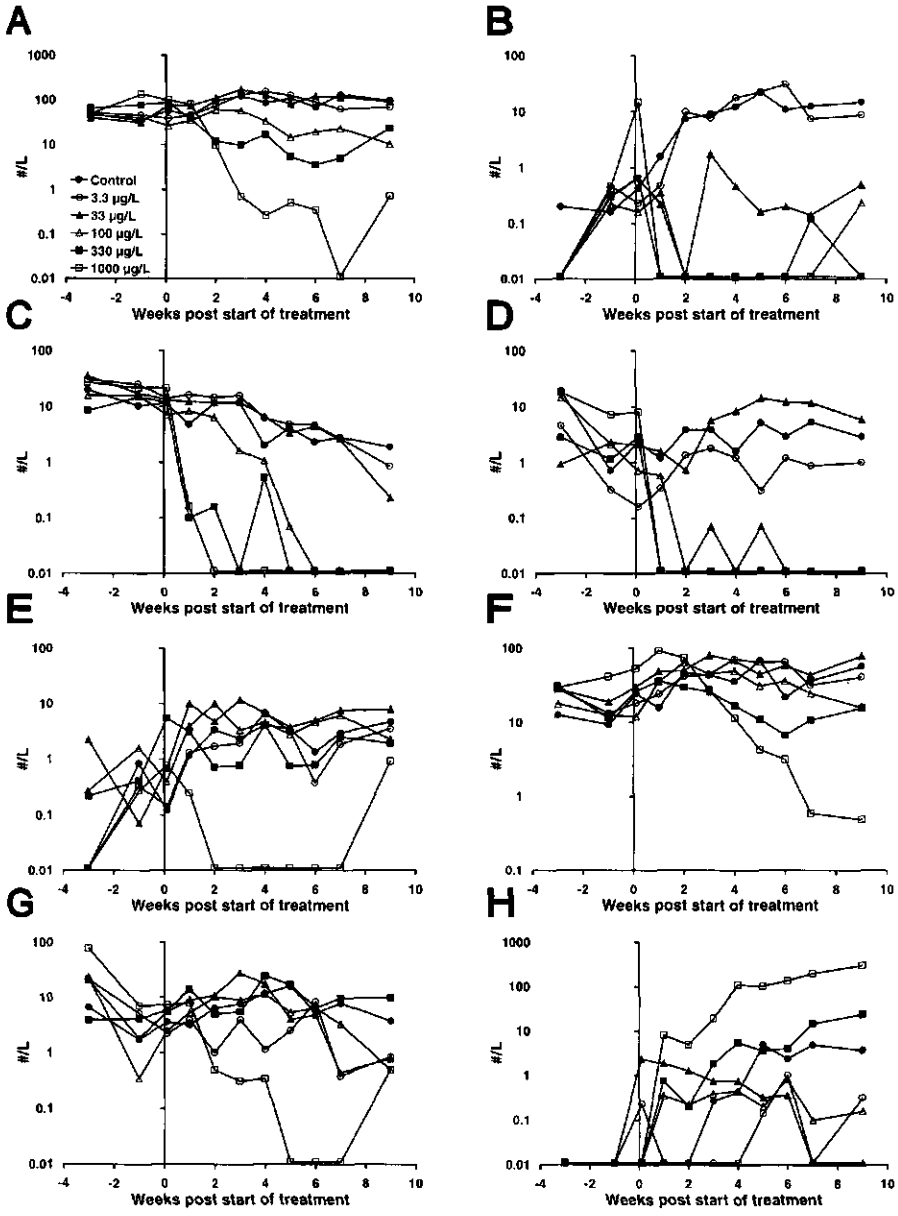

Figure 2. Dynamics in numbers of 8 zooplankton taxa found to b e most important in the PRC analysis. Figures 2A through 2H show the geometric means of the numbers, counted per treatment level, of nauplius (A), *Acroperus harpae* (B), *Alonella exigua* (C), *Simocephalus vetulus* (D), *Keratella quadrata* (E), Cyclopoida sp. (F), *Lecane* (G) and *Testudinella parva* (H). For NOECs see text. In the figures, absence is denoted by 0.01.

relatively high negative weight (Fig. 1). A positive weight with the diagram indicates a reduced abundance of the taxon in the treated microcosms, compared to the control, while a taxon with a negative weight has increased in numbers.

Table 2. Period of significant influence of carbendazim treatment (Monte Carlo permutation tests, $p < 0.05$), lowest $NOEC_{community}$ reported at least two times (Williams test on PCA co-ordinates, $p < 0.05$) and results on significance tests of the basic response patterns (Monte Carlo permutation tests) for the data sets analyzed.

Data set	Period of significant influence of treatment regime (in weeks)	Lowest NOEC reported on at least two sampling dates(in µg/L)	P-value for the PRC diagram
Zooplankton	1 through 9	33	≤ 0.001
Phytoplankton	3,4,6,9	33	≤ 0.001
Periphyton	-	> 1000	> 0.05

The dynamics of the 8 taxa which were most important in the PRC are shown in Fig. 2. Of these taxa, *Acroperus harpae* (Fig. 2B) was found to have the lowest NOEC: 3.3 µg/L. NOECs were considered valid when calculated for two consecutive sampling dates or more. The NOEC for the taxa *Simocephalus vetulus* (Fig. 2D) and *Alonella exigua* (Fig. 2C) was 33 µg/L, that for Cyclopoida sp. (Fig. 2F) and nauplius (Fig. 2A) 100 µg/L and that for *Keratella quadrata* (Fig. 2E), *Lecane* sp. (Fig. 2G), *Trichocerca* sp. and *Testudinella parva* (Fig. 2H) 330 µg/L. For all other taxa, a NOEC > 1000 µg/L was calculated.

In summary, the Cladocera taxa were completely eliminated at the higher treatment levels while copepod numbers were reduced. Rotatoria taxa decreased (*Keratella quadrata* and *Lecane* sp.) or increased in abundance (*Testudinella parva*) at the highest treatment level only (Fig. 2).

Table 3. Results of bioassay with *Daphnia magna* in NOEC, LC10 and LC50 values (µg/L with 95% confidence interval).

Day/ value	NOEC	LC10	LC50
4	100	65 (26 - 163)	113 (70 - 183)
7	33	15 (5 - 48)	52 (31 - 85)
14	3.3	22 (20 - 25)	39 (37 - 41)
21	33	25 (17 - 37)	41 (28 - 61)
28	33	20 (9 - 45)	37 (24 - 58)

The *Daphnia magna* bioassay resulted in acute (96h) NOEC, LC10 and LC50 values of 100, 65 and 113 µg/L respectively (Table 3; dynamics given in Fig. 3). The long-term (28 d) NOEC, LC10 and LC50 values were 33, 20 and 37 µg/L respectively.

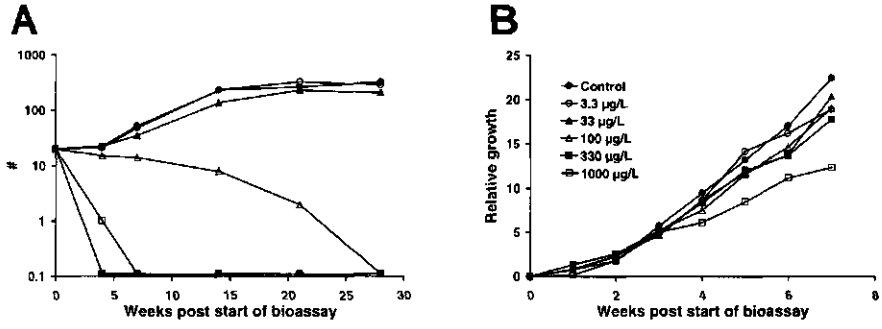

Figure 3. Results of the bioassays performed with *Daphnia magna* (A) and *Scenedesmus acutus* (B). For NOECs see text. In figure A, absence is denoted by 0.1.

Phytoplankton

A total of 54 different phytoplankton taxa were identified. Bacillariophyceae, Chlorophyta and Cryptophyta were the dominant groups, and *Achnanthes* sp., *Chilomonas* sp., *Chlamydomonas* sp., *Chroomonas* sp. *Cocconeis* sp., *Coenochloris* sp. and *Cryptomonas* sp. were the dominant taxa.

The PRC diagram of the phytoplankton deviated from the controls at the three highest treatment levels (Fig. 4). This deviation was also significant ($NOEC_{community}$ = 33 µg/L, Table 2). In the PRC diagram, the horizontal axis captures 24% of total variance (time), while the vertical axis displays a quarter of the variance which could be attributed to the treatment regime (40% of total variance). Fig. 5 summarizes the dynamics of the 5 most important phytoplankton taxa indicated by the PRC. *Stephanodiscus* sp. (Fig. 5A), *Cyclotella* sp. (Fig. 5B), *Cryptomonas* sp. (Fig. 5D), *Chlamydomonas* sp. (Fig. 5E) and *Monoraphidium* sp. were found to have a NOEC of 100 µg/L, while that for *Chilomonas* sp. and *Achnanthes* sp. was 330 µg/L. *Achnanthes* sp. was the only taxon whose NOEC was based on decreased numbers in the treated microcosms. For all other taxa, a NOEC > 1000 µg/L was calculated.

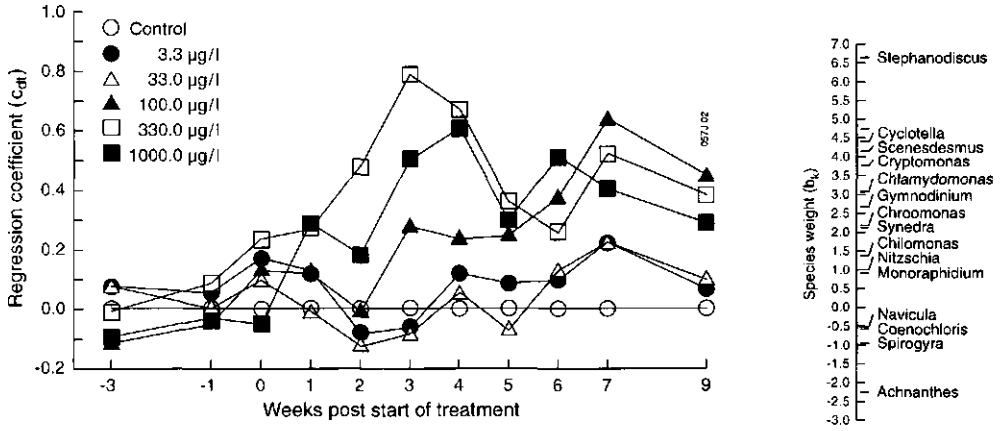

Figure 4. Principal Response Curves resulting from the analysis of the phytoplankton data set, indicating the effects of the fungicide carbendazim on the phytoplankton community. Of all variance, 24% could be attributed to sampling date, and is displayed on the horizontal axis, 40% could be attributed to treatment. Of the variance explained by treatment, 25% 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.

It was not only the abundance values of most phytoplankton taxa which increased at the higher treatment levels; chlorophyll-a also increased at the two highest levels (Fig. 6), but this increase was not significant. At the highest treatment level chlorophyll-a values increased for a short period, whereas at the 330 µg/L level it increased to lower values but for a prolonged period (Fig. 6).

In contrast to the increased abundance of *Scenedesmus* sp. (Fig. 5C) in the phytoplankton samples from the higher treatment levels, the bioassay showed a significantly reduced growth of *Scenedesmus acutus* at the highest treatment level (Fig. 3; NOEC = 330 µg/L).

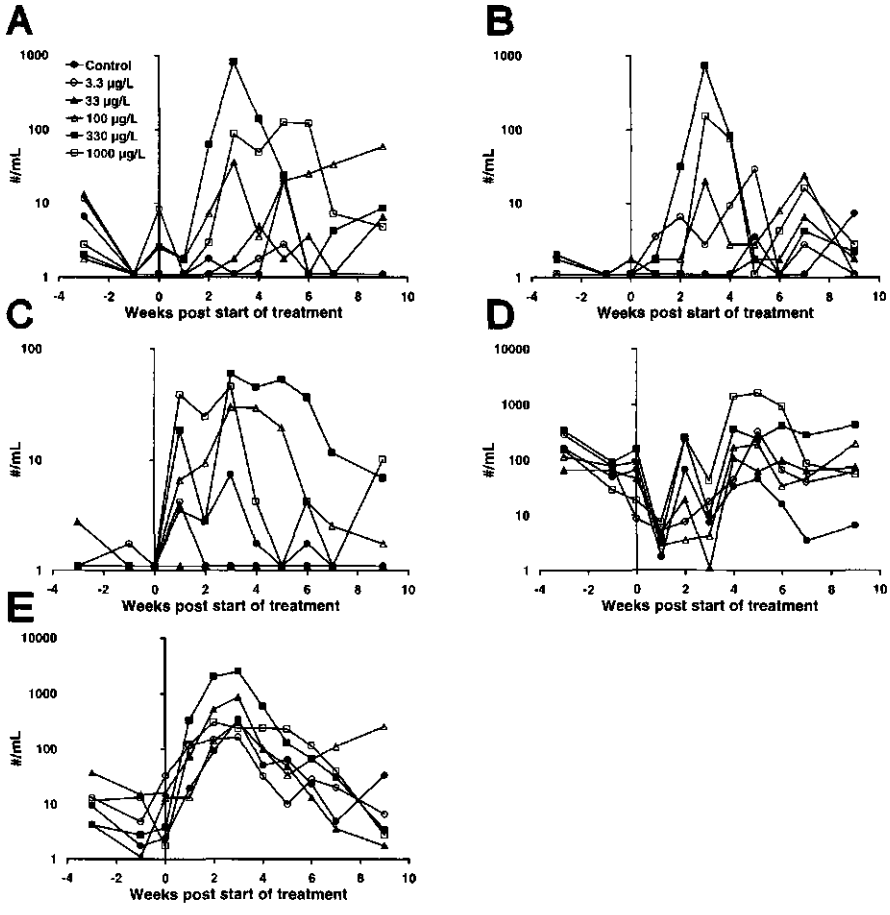

Figure 5. Dynamics in numbers of 5 phytoplankton taxa found to be most important in the PRC analysis. Figures 5A through 5E show the geometric means of the numbers, counted per treatment level, of *Stephanodiscus* sp. (A), *Cyclotella* sp. (B), *Scenedesmus* sp. (C), *Cryptomonas* sp. (D), *Chlamydomonas* sp. (E) and *Achnanthes* sp. (F). For NOECs see text. In the figures, absence is denoted by 1.

Periphyton

The PRC diagram of the periphyton community was not significant (Table 2) and is thus not shown. Nor could significant treatment effects be demonstrated with the Monte Carlo permutation tests, performed per sampling date.

In the control microcosms, the highest abundance among the periphyton were found for *Cocconeis placentula*, *Achnanthes* sp., followed by *Coleochaete* sp., *Epithemia* sp., *Oedogonium* sp. and *Spirogyra* sp. Only *Epithemia* sp. showed a significant treatment

effect (Williams test; Fig. 7). High numbers of this taxon were, however, only found in the controls and in the microcosms with the highest treatment level. In such cases, the results of the Williams test should be interpreted with caution, since it assumes an increasing effect with increasing dose.

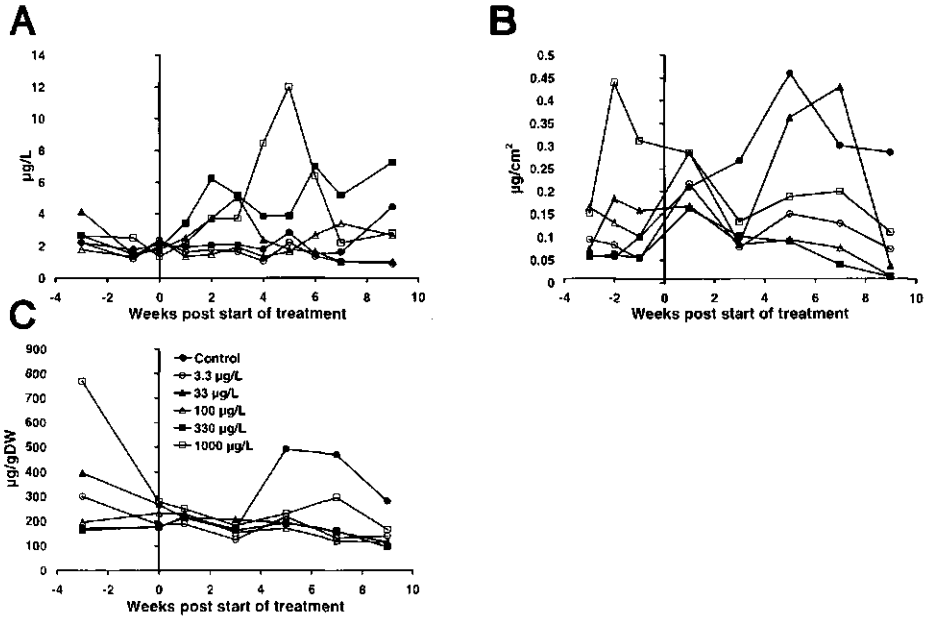

Figure 6. Results of chlorophyll-a measurements on phytoplankton (A), sampled on glass slides (B) and on *E. nuttallii* (C).

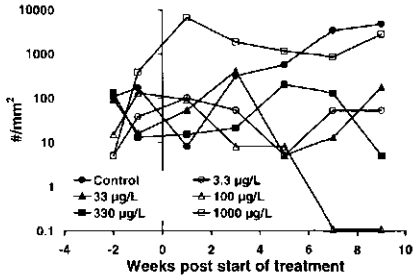

Figure 7. Dynamics in numbers (geometric means) of the only periphyton taxon, *Epithemia* sp., for which a NOEC could be calculated. For NOECs see text. In the figure, absence is denoted by 0.1.

Chlorophyll-a values of the periphyton on both glass slides, as well as on *Elodea nuttallii*, were relatively high in control microcosms in the post-treatment period (Fig. 6). Only for the chlorophyll-a levels on *E. nuttallii* could significant treatment effects be

calculated with the Williams test. Again, this result is difficult to interpret, because no clear dose-response relationship was observed.

Final harvest and bioassays with macrophytes

The results of the standing stock of the macrophytes in the microcosms (determined at the end of the post-treatment period) are presented in Table 4. No significant treatment effects were detected on a dry weight basis, but the total biomass of all macrophytes was clearly higher at the two highest carbendazim concentrations. *Elodea nuttallii*, the dominant macrophyte, which contributed more than 80% of the total biomass, showed the same trend, while filamentous algae (mainly *Oedogonium*) had a lower biomass at the three highest carbendazim concentrations. On the basis of ash-free dry-weight, the biomass of *E. nuttallii* was significantly higher at the two highest carbendazim concentrations (NOEC = 100 µg/L; Table 4).

Table 4. Mean macrophyte biomass (g dry weight) at the end of the experiment (week 10) per taxon and treatment level. Significant differences relative to controls (Williams test, $p < 0.05$) are indicated with an asterisk.

	Treatment					
Macrophyte	0 µg/L	3.3 µg/L	33 µg/L	100 µg/L	330 µg/L	1000 µg/L
Elodea	73.6	67.6	75.4	71.5	94.5	98.0
Elodea[1]	49.6	45.5	56.3	48.8	71.1*	67.7*
Oedogonium	15.2	20.8	12.3	9.3	8.7	8.7
Myriophyllum	0.6	2.4	3.8	3.9	6.1	2.0
Chara	0.8	3.4	0.2	2.8	3.3	1.7
Total biomass	90.1	94.1	91.8	87.5	112.5	110.4

[1] (g ash-free dry weight)

After three weeks of incubation in the treatment period, the biomasses of *Oedogonium* and *Lemna minor* in the bioassays did not show any significant treatment effects (Table 5). *Elodea nuttallii* biomass was significantly higher at the two highest carbendazim concentrations.

Table 5. Mean macrophyte biomass (g dry weight) at the end of the bioassay, per treatment level and species (incubation period 4 weeks). Significant differences relative to controls (Williams test, $p < 0.05$) are indicated with an asterisk.

	Treatment					
Macrophyte	0 µg/L	3.3 µg/L	33 µg/L	100 µg/L	330 µg/L	1000 µg/L
Elodea	0.165	0.190[a]	0.160	0.220	0.290*	0.285*
Lemna	0.017	0.016	0.013	0.013	0.017	0.017
Oedogonium	0.175	0.190	0.175	0.190	0.175	0.155

[a] one sample lost due to consumption by caterpillar *Cataclysta lemnata*

Discussion

Effects of carbendazim on zooplankton

Both the PRC diagram and the multivariate statistical tests revealed a significant influence of carbendazim on the zooplankton community at concentrations of 100 µg/L and higher (NOEC = 33 µg/L; Fig. 1 and Table 2). Of the standard test species, *Daphnia magna* ($NOEC_{reproduction}$ = 26 µg/L; Van Wijngaarden et al., 1998.) proved to be a sensitive representative of the zooplankton community in the microcosms.

At the species level, *Acroperus harpae* was the most sensitive representative of the zooplankton community (NOEC = 3.3 µg/L, Fig. 2). The order of susceptibility found was: Cladocera > Copepoda > sensitive rotifers (Fig. 2), assuming that all adverse effects were direct effects. The decreased abundance of adult Cyclopoida sp. is likely to be a result of a decrease in the numbers of their immature stage, nauplius, rather than of direct toxicity of carbendazim. This is supported by Van Wijngaarden et al. (1998) who recorded no effects on a copepod species (*Cyclops agilis*; highest concentration 3843 µg/L) after two days in the laboratory.

In our study, the rotifer *Testudinella parva* was insensitive to carbendazim and even increased its abundance at the higher treatment levels (significantly so at the highest level; Fig. 2). This could be the result of an improved competitive position relative to the other zooplankton taxa as regarded food supplies. The dynamics of especially nauplius and *Lecane* sp. indicate that some effects became manifest only after a certain period of time had elapsed. In the variously treated microcosms, these taxa showed the greatest deviation from the control after week 3 (Fig. 2). These delayed effects have also been reported for some macroinvertebrate taxa (Cuppen et al., subm.).

The LC50,96h values for *D. magna* as determined in the laboratory and the bioassay were 91 µg/L (Van Wijngaarden et al., 1998) and 113 µg/L respectively. The difference between these figures, a factor 1.2, is well within the range of the differences in LC50 values that have been reported for the same species by different laboratories (Rand

and Petrocelli, 1985). The long term toxicity was almost the same in both experiments ($NOEC_{reproduction}$ of 26 and 33 µg/L; Van Wijngaarden et al., 1998; and Table 3, respectively). Apparently, the bioavailability of carbendazim in the water layer of the microcosms was comparable to that under laboratory circumstances.

The results of the bioassay with *D. magna* makes clear that carbendazim acts very slowly. It was only after 7 days that the incipient LC50 was reached in the bioassay (Table 3). For *Daphnia magna*, Van Wijngaarden et al. (1998) also found large differences between EC50, 48h and EC50, 96h (1336 versus 186 µg/L) in the laboratory. The responses of many species in the microcosms (Cuppen et al., subm.), also indicate a slow mode of action of carbendazim. We therefore recommend the use of a test period of at least 96 hours in L(E)C50 tests of fungicides, as this will yield values which more closely resemble incipient values.

Effects of carbendazim on primary producers

Phytoplankton showed an increased abundance at the three highest concentrations (Fig. 4). At the taxon level, this increase was not always significant (e.g. *Scenedesmus*, Fig. 5), because it occurred at different sampling dates for different treatments (Fig. 5).

The overall increase in abundance of free-living *Scenedesmus* in the overlying water contrasts with the results of the bioassay performed with the same taxon. The bioassay revealed a significant inhibition of the growth of *Scenedesmus acutus* at the highest treatment level (Fig. 3), but in the microcosms *Scenedesmus* sp. showed higher abundance values at this level (Fig. 5). Since the grazing pressure of the zooplankton was reduced at the three highest treatment levels (Fig. 2) and *Scenedesmus* sp. increased in abundance in these microcosms (Fig. 5), the conclusion must be that grazing by the zooplankton controlled the abundance values in the controls and at the lower treatment levels. In the bioassay (alginate beads), however, grazing of *Scenedesmus* by zooplankton was prevented, so that the bioassay only showed the toxic effect of carbendazim. Reduced grazing pressure is most probably also responsible for the increased abundance of the other phytoplankton taxa (Fig. 5). The increased abundance of the phytoplankton also resulted in elevated chlorophyll-a levels at the higher treatment levels (Fig. 6).

The dynamics of the only taxon of the periphyton for which a NOEC could be calculated, *Epithemia* sp., indicated lower abundance values, relative to the control, for all but the highest treatment levels (Fig. 7). This taxon was only present in reasonable numbers in the controls and at the highest treatment level. The results of the Williams test, which assumes an increasing effect with increasing dose, should therefore be evaluated with caution. The significant effect on *Epithemia* sp. (NOEC < 3.3. µg/L calculated for weeks 7 and 9) is based on an increase in abundance in the controls. It was clearly not a

direct effect, since numbers at the highest treatment level did not drop after application (Fig. 7).

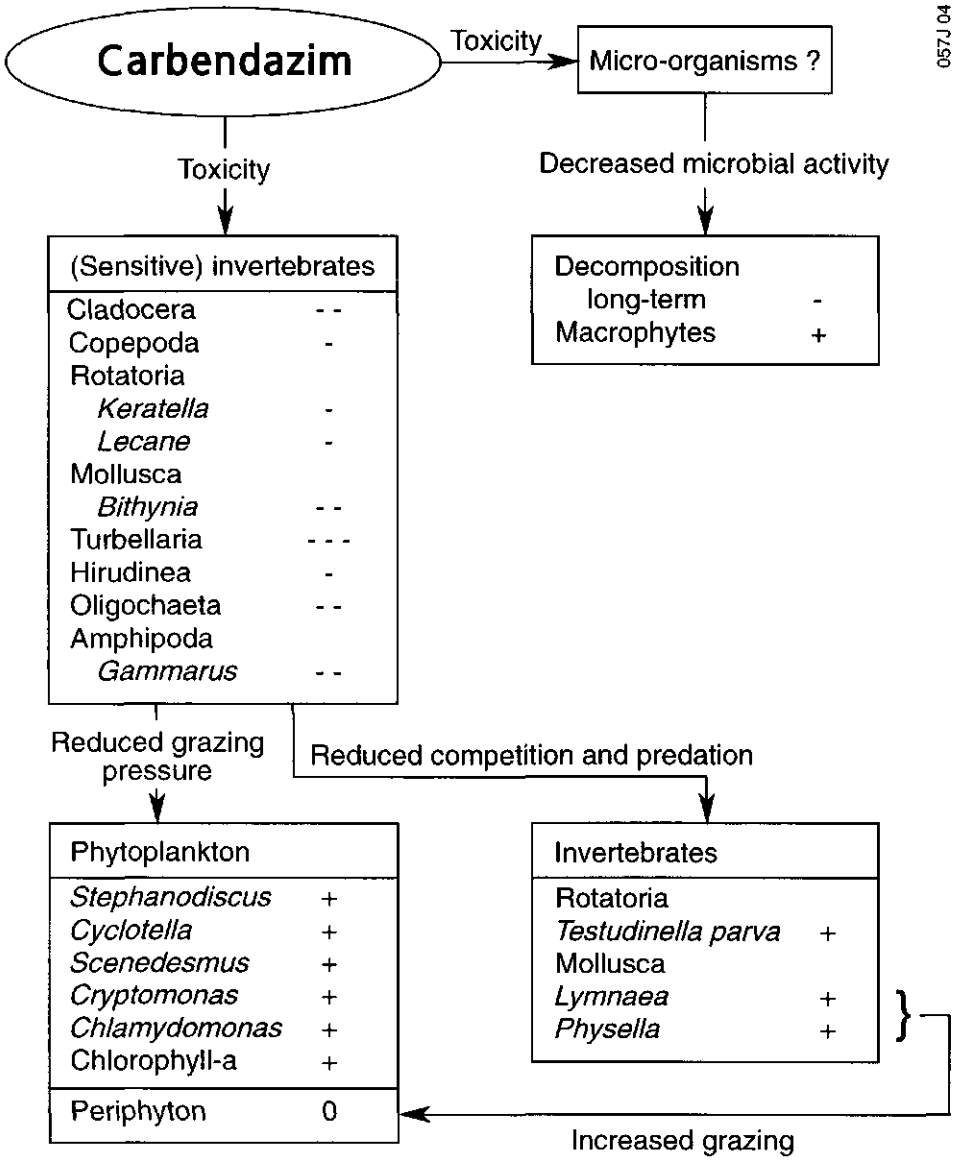

Figure 8. Schematic overview of the nature and route of effects of a chronic high dose (100 µg/L) of carbendazim on the ecosystem structure of *Elodea*-dominated microcosms (+, increase; -, decrease).

More or less similar responses were also found for the chlorophyll-a values on glass slides and on the macrophyte *E. nuttallii* (Fig. 6). Most probably, these differences are not *a priori* related to the toxicant.

The biomass of the macrophyte *E. nuttallii* was significantly elevated in the higher treatments at the end of the experimental period. The same effect was recorded for the bioassay (Table 5). A possible explanation is that the fungicide treatment removed some of the stress on this macrophyte from pathogene micro-organisms.

Ecological effect chain

The overall impact of chronic application of carbendazim on the structure and functioning of the microcosms is visualized in Fig. 8. Direct toxicity of carbendazim caused a serious decline or even elimination of many zooplankters (Cladocera, Copepoda, Rotatoria) and macroinvertebrates (Turbellaria, Oligochaeta, Hirudinea, Amphipoda, *Bithynia*). The reduction of the grazing pressure exerted especially the microcrustaceans, indirectly causing an increase in most phytoplankton species and consequently in chlorophyll-a levels. This reduced grazing pressure even outweighed the direct toxicity of carbendazim for *Scenedesmus* sp., which was in the bioassay (Fig. 3). In the plankton, only one taxon (*Testudinella parva*, Rotatoria) increased as result of the greater availability of food. The expected increase in periphyton and periphytic chlorophyll-a, due to the reduced grazing pressure from microcrustaceans, the oligocheates *Stylaria lacustris* and *Dero* and the snail *Bithynia*, was not observed. The reduced grazing pressure from these taxa was probably compensated by the increased abundance of some snail species such as *Lymnaea stagnalis* and *Physella acuta* (Cuppen et al., subm.).

In essence, carbendazim treatment affected the structure of the aquatic ecosystems directly by reducing the abundance of many species of zooplankters and macroinvertebrates, and indirectly by stimulating the growth of phytoplanktonic algae and macrophytes. Despite these pronounced effects changes in functional variables were not found. *Elodea nuttallii* showed a distinct increase in all microcosms during the experiment, but the extra growth at the two highest treatment levels (significantly increased with respect to controls) did not cause significant changes in the DO-pH-conductivity-alkalinity syndrome.

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Chapter 5

Principal Response Curves: analysis of time-dependent multivariate responses of a biological community to stress

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Principal Response Curves: analysis of time-dependent multivariate responses of a biological community to stress

Abstract

In this paper a novel multivariate method is proposed for the analysis of community response data from designed experiments repeatedly sampled in time. The long-term effects of the insecticide chlorpyrifos on the invertebrate community and the DO-pH-Alkalinity-Conductivity syndrome, in outdoor experimental ditches are used as example data. The new method, which we have baptized the Principal Response Curve method (PRC), is based on a Redundancy Analysis (RDA) adjusted for overall changes in community response over time, as observed in control test systems. This allows the method to focus on the time-dependent treatment effects. The principal component thereof is plotted against time, yielding a principal response curve of the community for each treatment. The PRC method distils the complexity of time-dependent, community-level effects of pollutants into a graphic form that can be appreciated more readily than the results of other, currently available, multivariate techniques. The PRC method also enables a quantitative interpretation of effects towards the species level.

Introduction

The industrialized world we live in today uses large amounts of chemicals, e.g. pesticides, which may enter aquatic ecosystems and have undesirable side-effects on the biological and functional properties of these ecosystems. At an appropriate stage of the assessment procedure, microcosm and mesocosm experiments may be carried out with these chemicals. Normally, these experiments result in large data sets comprising information about temporal changes in the structure and function of control and treated microcosms or mesocosms. A generally recognized disadvantage of these experiments is that they require great effort in sampling and measuring of endpoints, particularly for the identification of the biological populations, while data for only a few taxa or other endpoints appear to suffice for standard univariate statistical analysis (e.g. Van Wijngaarden et al., 1996).

Multivariate statistical analysis may be used to describe the effects of chemical stress at the community level. Van den Brink et al. (1996) proposed a method based on Redundancy Analysis (RDA), which is akin to the well-known method of Principal Components Analysis (PCA; Ter Braak, 1995; Jolliffe, 1986). The advantage of this and related multivariate methods over univariate methods is that they use and summarize all information on the investigated populations simultaneously, and in doing so evaluate the effects of contaminants at the community level. A serious drawback of multivariate methods is that the results are more abstract than those of univariate methods. In particular, results

are often reported as ordination diagrams or biplots, which are unfamiliar to most ecotoxicologists and legislators. Time-dependent multivariate responses often results in diagrams which are too cluttered to allow easy interpretation of the changes in treatment effects over time. In this paper we introduce a novel multivariate method, called Principal Response Curves, which overcomes these problems.

Redundancy analysis

Introduction

Redundancy analysis (RDA) can be considered as a constrained form of principal components analysis (PCA; Ter Braak, 1995; Jolliffe, 1986). We therefore first introduce PCA and then derive RDA from it.

With ecological data, multivariate methods are commonly used to find those factors that best explain the differences in species composition between samples. Of these, PCA, a type of factor analysis, is the most commonly used (Ter Braak, 1995; Jolliffe, 1986). PCA uses linear models similar to the linear model underlying regression analysis. A difference with regression analysis is that the explanatory variables are not measured (manifest) but latent. The values of the latent variable for the various samples are called sample scores, and the regression coefficients of the linear model are called species weights. The Rank 1 model (the model with one latent variable) of PCA can be written as

$$y_{ik} = \bar{y}_k + b_k x_i + \varepsilon_{ik}, \qquad (1)$$

with:

y_{ik} = the abundance of species k in sample i ($k = 1, ..., m$; $i = 1, ..., n$). As measure of abundance, we take ln (count + 1);

$\bar{y}_k$ = mean of y_{ik} for species k across all samples;

x_i = sample score of sample i on the latent variable (principal component);

b_k = species weight, *i.e.* the regression coefficient for species k with respect to the sample scores $\{x_i\}$;

ε_{ik} = the error term with mean zero and variance σ_k^2.

Some remarks on this model are in order. Because organisms multiply or die, count data are naturally modelled by proportional changes, *i.e.* by a multiplicative model. We analyzed the counts after taking the logarithm, so as to turn the multiplicative model for the counts into a linear model. Because the logarithm of zero is undefined, the counts were increased with the value 1 before the natural logarithm is taken. In the sequel, the term abundance refers to the so-transformed count. Finally, note that the term $b_k x_i$ in equation (1) expresses the deviation of species k in sample i from the mean $\bar{y}_k$. In terms of the

original counts, 1 - exp(b_k x_i) expresses the proportional change in count relative to the geometric mean count, exp($\bar{y}_k$).

The sample scores and species weights can be displayed in an ordination diagram as the first, horizontal axis (see Fig. 1). Together, the scores and weights explain a particular fraction of the total variance of the data set. A second latent variable can be extracted from the remaining variance, yielding a Rank 2 model. The second set of sample scores and species weights is displayed as the second, vertical axis of the ordination diagram. After extracting more and more latent variables, PCA eventually accounts for all the variance of a data set.

If, like in RDA, explanatory variables are manifest, *i.e.* fixed *a priori*, the total variance can be partitioned by multivariate regression analysis into explained variance and residual variance. RDA extracts, in contrast to PCA, information from the explained variance only; the axes of RDA (e.g. Fig. 1) represent a percentage of that variance. More formally, RDA can be defined in two equivalent ways: (1) RDA is a PCA that is applied to the fitted species data and (2) RDA is a PCA in which the sample scores are constrained to be linear combinations of the explanatory variables. Both definitions ensure that the ordination diagram displays only those differences between the samples that can be explained by the explanatory variables at hand. In this paper, the models are fitted by unweighted least-squares, disregarding possible differences in the residual variances $\{\sigma_k^2\}$. For more theoretical background information and technical details see Ter Braak (1994, 1995), Van Wijngaarden et al. (1995), Davis and Tso (1982) and Ter Braak and Looman (1994).

The use of RDA in the 1990 chlorpyrifos study

Invertebrate data set

The invertebrate data set used as an example in this paper has been described in detail by Van Wijngaarden et al. (1996) and Van den Brink et al. (1996). This data set was obtained from an experiment in outdoor experimental ditches. Twelve mesocosms were allocated at random to treatments; four served as controls and the remaining eight were treated once with the insecticide chlorpyrifos, applied as Dursban®4E, with nominal dose levels of 0.1, 0.9, 6 and 44 µg/L in two mesocosms each. The example data set comprises that of the invertebrates, which is a combination of macro-invertebrate and zooplankton data sets. Sampling was done 11 times, from Week -4 pre-treatment through Week 24 post treatment, giving in total 132 samples (12 mesocosms times 11 sampling dates) in the statistical analyses. A total of 189 different taxa were identified and counted in these samples. The responses and recovery of the invertebrate community after chlorpyrifos treatment, were analyzed in time using RDA, which was performed using the CANOCO computer program, version 3.14 (Ter Braak, 1988). The input data for RDA consisted of the 132 × 189 matrix of species data and a 132 × 55 matrix of explanatory variables.

These 55 explanatory variables are dummy variables indicating to which combination of the factors 'treatment' (5 levels) and 'sampling week' (11 levels) each sample belonged. The 5 × 11 dummy explanatory variables ensure, by way of the linear constraints in RDA, that the mesocosms that received the same treatment (the replicates), receive identical sample scores at each week. Equivalently, this RDA can be obtained from a PCA in which the data values are replaced by the treatment means. The RDA thus allowed us to focus on the variance in the invertebrate data set that can be attributed to time, treatment and their interaction. Within CANOCO, scaling 1 (Euclidean Distances) was used because of the presence of the dummy explanatory variables (Ter Braak, 1994). As in the previous section, the counts were ln-transformed prior to analysis. Apart from this, the default options were chosen.

The RDA summarized the effects on the invertebrate community in time, while still showing the effects at the species level (Fig. 1). In Figure 1 samples (open circles) with nearly identical species composition lie close together in the diagram, while samples with very different species composition lie far apart. To properly interpret the figure, we need the following rules. If an imaginary line is drawn through a species point (solid circles) and the origin of the plot, the relative abundances of this species in all samples can be derived by projecting the sample points onto 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 the species is in this sample. If a sample point projects on the other side of the origin, compared to the species point, numbers of the species in this sample are relatively low. Using these rules, we infer from the diagram that the species *Cloeon dipterum* is relatively abundant in all control samples, and (almost) absent in the samples for Weeks 1, 2 and 4 at the highest dose level. In general, Crustacea and Insecta showed a rapid, concentration-dependent decrease in numbers after insecticide application (direct effects). An increase in Gastropoda (e.g. *Bithynia tentaculata*) and Oligochaeta was found, suggesting indirect effects (Van Wijngaarden et al., 1996; Van den Brink et al., 1996).

In the CANOCO computer program, RDA is accompanied by Monte Carlo permutation tests to assess the statistical significance of the effects of the explanatory variables on the species composition of the samples (Van den Brink et al., 1996; Verdonschot and Ter Braak, 1994). In the 1990 chlorpyrifos study, Monte Carlo permutation tests were performed per sampling date, using the ln-transformed nominal dose as the explanatory variable (for rationale see Van den Brink et al., 1996). This allowed the significance of the treatment regime to be tested per sampling date. The results of the permutation tests indicated that the treatment regime had a significant effect ($p < 0.05$) on the invertebrate community in all post-treatment weeks up to week 24. At 24 weeks post-treatment, no effect could be demonstrated, suggesting recovery of the invertebrate community at all treatment levels.

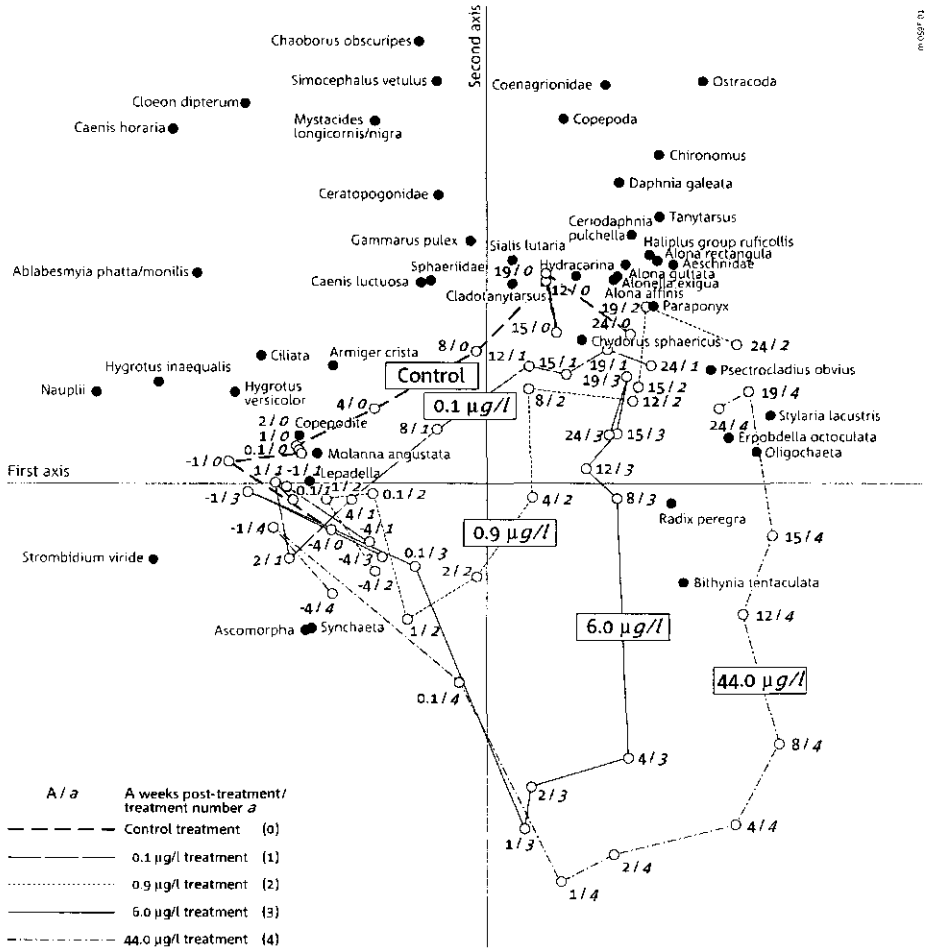

Figure 1: Ordination diagram (RDA) indicating effects of a single application of the insecticide chlorpyrifos on invertebrates. The sampling period covered Week -4 through Week 24. Sampling week and treatment level, as well as their interactions, were taken as explanatory variables. The lines represent the course of the treatment levels in time. Of all variance, 55% can be attributed to the explanatory variables. Of this explained variance, 37% is displayed in the diagram. Only those 45 (out of 189) taxa are shown that are most discriminating for the diagram.

Besides the overall significance of the treatment regime, we also wanted to know *which* treatments differed significantly from the control, so as to infer the No Observed Effect Concentration (NOEC) at the community level. This could not be done by testing every treatment against the control because there were too few permutation possibilities; testing 2 treated mesocosms against 4 controls only provides 15 permutation possibilities (6!/(2!4!)) and a corresponding lowest possible permutation-based *p*-value of 0.07 (1/15)

(Manly, 1990). In the corresponding univariate case, however, the Williams test (Williams, 1972) can be applied to calculate a NOEC. The Williams test can be applied to the multivariate data set if the data set is reduced to a single variable. The first principal component of a PCA suits this purpose. The NOEC calculations were therefore done by applying the Williams test to the sample scores of the first principal component of each sampling date in turn (Van den Brink et al., 1996). These analyses indicated a $NOEC_{community}$ of 0.1 µg/L for the invertebrates (Van den Brink et al., 1996).

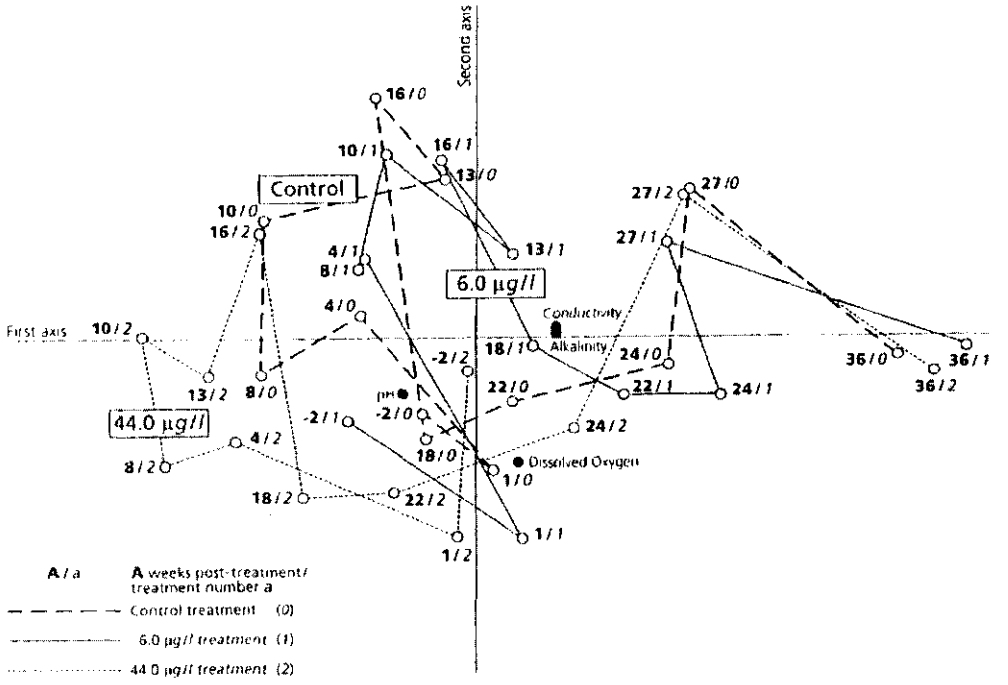

Figure 2. Ordination diagram (RDA) indicating effects of a single application of the insecticide chlorpyrifos on the DO-pH-Alkalinity-Conductivity syndrome for the 6 and 44 µg/L and control dose levels. The sampling period covered Week -2 through Week 36. Sampling week and treatment level, as well as their interactions, were taken as explanatory variables. The lines represent the course of the treatment levels in time. Of all variance, 80% can be attributed to the explanatory variables. Of this explained variance, 97% is displayed in the diagram.

Physico-chemical data set

The physico-chemical data set resulting from the above experiment has been described in detail by Kersting and Van den Brink (1997). In the mesocosms, continuous oxygen, pH and temperature measurements were made to determine the ecosystem metabolism. In addition, alkalinity and conductivity were determined weekly. The DO-pH-

Alkalinity-Conductivity Syndrome was analyzed with RDA and indicated a temporary effect of chlorpyrifos (Fig. 2). After being standardized to zero mean and unit variance, in this analysis the parameters of the syndrome played the part of the species. The RDA diagram was, however, very cluttered, so the interpretation was not as easy as that of Fig. 1. The analysis indicated higher pH and, though less pronounced, higher oxygen concentration, as well as lower alkalinity and conductivity for the highest chlorpyrifos concentration compared to the control treatment for the period Week 4 to roughly Week 10 post treatment (Fig. 2). These differences can be explained from the drop in gross primary production and oxygen consumption of the whole system in relation to the treatment. Oxygen consumption showed the largest decrease. These effects were explained as a decrease in the decomposition rate after the arthropods had been killed (Kersting and Van den Brink, 1997).

Proposed new analysis: Principal Response Curves

Introduction

RDA often results in very cluttered biplots, as can be seen in Fig. 2. The problem is that differences between treatments and controls do not stand out and that, because of the jagged trajectories, time is not expressed in a single direction in the diagram. To tackle these problems we focused on the differences between the species compositions of the treatments and that of the control at the corresponding time. This can be achieved by modelling the abundance of each particular species as a sum of three terms, namely its mean abundance in the control, a week-specific treatment effect and an error. Let, for species k of the invertebrate data set, τ_{dtk} be the treatment effect of treatment d in week t (k = 1, ..., 189; d = 0, ..., 4; t = -4, -1, 0.1, 1, 2, 4, 8, 12, 15, 19, 24). A raw estimate of the treatment effect is thus the mean difference between the abundance in the mesocosms of treatment d and that in the control mesocosms at each particular week. The response pattern of interest of each species k thus consist of the set of the 5 × 11 treatment effects $\{\tau_{dtk}\}$ for given k. By further modelling the response pattern τ_{dtk} for each species as a multiple (b_k) of one basic response pattern (c_{dt}), *i.e.* $\tau_{dtk} = b_k\, c_{dt}$, the statistical model for the abundance data becomes:

$$y_{d(j)tk} = \bar{y}_{0tk} + b_k\, c_{dt} + \varepsilon_{d(j)tk}, \qquad (2)$$

where $y_{d(j)tk}$ is the abundance of species k in replicate mesocosm j of treatment d at time t, $\bar{y}_{0tk}$ is the mean abundance of species k in week t in the control (d = 0) and $\varepsilon_{d(j)tk}$ is an error term with mean zero and variance σ_k^2. Note that c_{0t} = 0 for every t, because by definition τ_{0tk} = 0 for every t and k. The least-squares estimates of the coefficients $\{c_{dt}\}$ can be found by partial RDA, which is also known as reduced-rank regression with

concomitant regressors (Davis and Tso, 1982; Ter Braak and Looman, 1994). In CANOCO, these additional regressors are called covariables. There are two differences with the RDA of the previous section: (1) a 132 × 11 matrix of dummy covariables variables is specified indicating from which sampling week each sample is, and (2) the 11 explanatory dummy variables that indicate the control cosms, are deleted from the analysis so as to ensure that the treatment effects are expressed as deviations from the control, and $c_{0t} = 0$ for all t, as required above (reference coding). The desired estimates of the coefficients $\{c_{dt}\}$ are the canonical coefficients of the partial RDA so specified. For further mathematical and computational details and an illustration on a small artificial data set we refer to the Appendices I and II.

When the coefficients $\{c_{dt}\}$ are plotted against the sampling date (t), curves are obtained, one for each treatment, that can be interpreted as the principal response curves of the community (see Fig. 3 by way of example). The accompanying species weights (b_k) allow an interpretation at the species level: the weight b_k is the multiple by which the principal curves must be multiplied to obtain the fitted response curves of species k, because $\tau_{dtk} = b_k\ c_{dt}$. The higher the weight, the more pronounced the actual response pattern of the species is likely to follow the pattern in the PRC. Taxa with a high negative weight are inferred to show the opposite pattern, whereas taxa with near zero weight either show no response or a response that is unrelated to the pattern shown by the PRC. Note that, in terms of the original counts, $1 - \exp(b_k * c_{dt})$ expresses the proportional change of species k in treatment d and week t relative to its count in the control. More formally, the fitted value for the count in mesocosms with treatment d at week t is $\exp(b_k * c_{dt})$ times the geometric mean count in the controls.

The significance of the PRC diagram can be tested by performing a Monte Carlo permutation of the mesocosms, i.e. by permuting whole time series, in the partial RDA from which PRC is obtained, using an F-type test statistic based on the eigenvalue of the component. The null hypothesis of this test is that $\tau_{dtk} = 0$ for all t, d and k. The second axis of the RDA can be used to generate a second PRC diagram (see Appendix I). The significance of the second PRC can be tested by adding the sample scores samples of the first axis to the covariables.

Analyzed data sets

In order to make a direct comparison between the results of RDA and PRC, the invertebrate data set, the RDA results of which were displayed in Fig. 1, was also analyzed with PRC.

The physico-chemical data set (Fig. 2) was also analyzed with PRC, but the data set used for PRC was larger than that for the RDA displayed in Fig. 2. Kersting and Van den Brink (1997) deleted some sampling dates from those available to avoid overcluttering the RDA biplot. For the same reason they also left out the 0.1 and 0.9 µg/L treatments. For

the present paper, the whole data set, containing all available data from Week -4 through Week 24, was analyzed.

Monte Carlo permutation tests and $NOEC_{community}$ calculations were also performed for each data set.

Results

Invertebrate data set

The diagram of the first PRC of the invertebrate data set (Fig. 3) shows small variations in the pre-treatment period and larger concentration-dependent deviations from the control after the chlorpyrifos application. The higher the dose, the larger the deviations. Taxa indicated with a positive species weight in Fig. 3 are expected to decrease in abundance, relative to the controls, after treatment with the higher doses, whereas taxa with negative weights are expected to increase. In particular, in Fig. 3 *Caenis horaria* has the highest positive weight and is thus inferred to decrease most strongly in abundance after the chlorpyrifos treatment. In contrast, *Bithynia tentaculata* has a small negative weight, indicating a small increase in abundance due to the chlorpyrifos treatment. Note that the taxa with weights between -0.5 and +0.5 are not shown, because these are likely to show either a weak response or a response that is unrelated to that shown in Fig. 3. Eighty percent of the taxa with a species score of 0.5 or higher were arthropods (Fig. 3). None of the taxa with a negative weight belonged to Phylum Arthropoda.

For a quantitative evaluation of PRC, the quotient $\exp(b_k * c_{dt})$ can be calculated for each species k at treatment d and sampling date t. For example, the species weight of *C. horaria* is 4.75 (Fig. 3), so the fitted reduction for the highest treatment level in week 1 is $\exp(-1.4 * 4.75) = 0.0013$. The PRC analysis thus predicts that after the treatment *C. horaria* is reduced in the highest treatment to ca. 0.13% of its geometric mean count in the control. This is in good accordance with its actual response (see Van den Brink et al., 1996).

Table 1 shows that 22% of total variance can be attributed to time and is thus (implicitly) displayed on the horizontal axis, whereas 34% can be attributed to the treatment regime (including its interaction with time). Twenty-six percent of the latter variance is expressed on the vertical axis. On the basis of the Monte Carlo tests per sampling date, the treatment regime had a significant influence on the species composition from Week 0.1 through Week 19, so the ditches were considered to have recovered at Week 24. The lowest NOEC of 0.1 µg/L (Table 2) was reported for Weeks 1, 2 and 4. Whereas the first PRC was significant, the second PRC was not and is not shown.

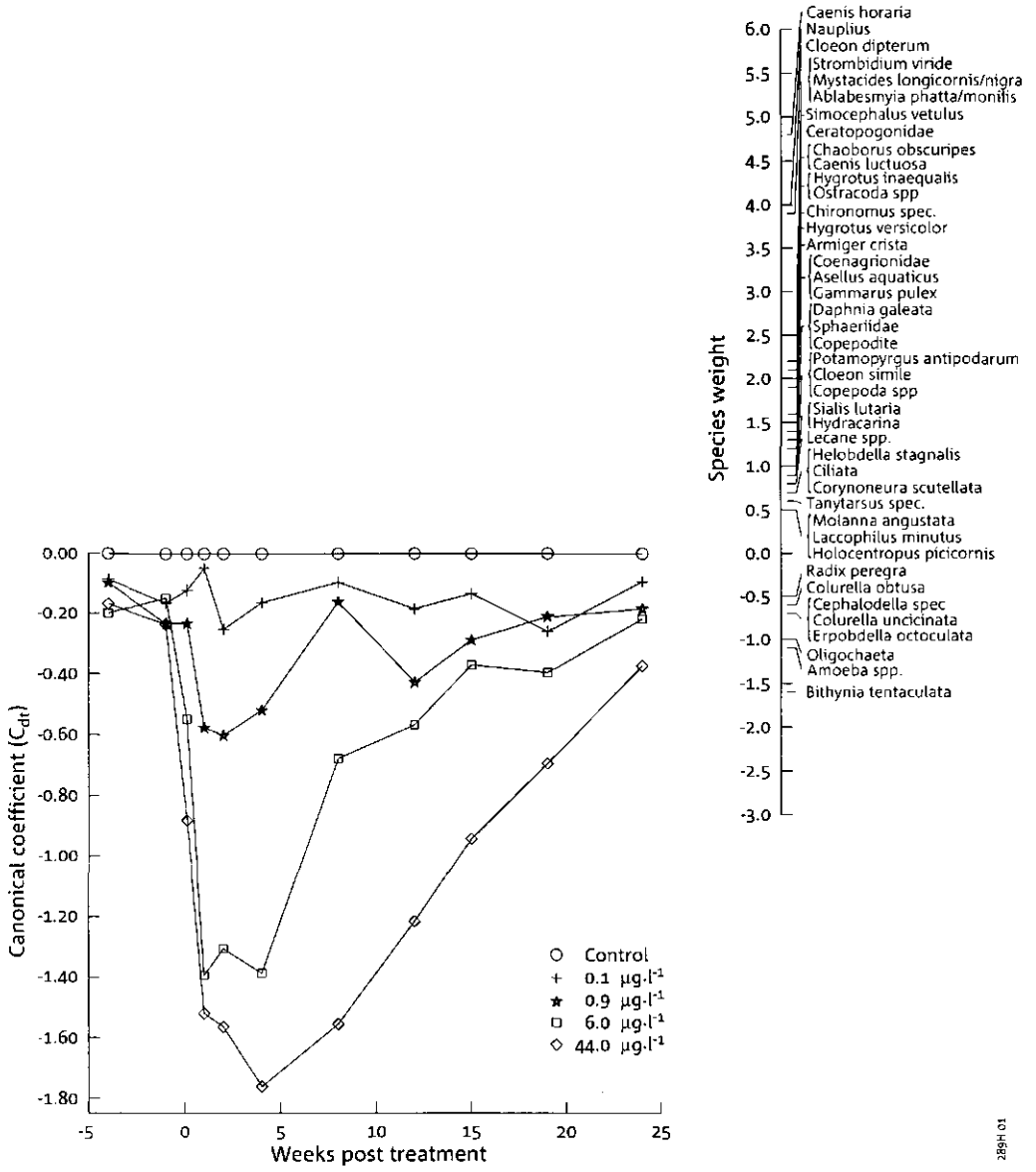

Figure 3. Principal Response Curves with species weights (see text) for the invertebrate data set, indicating the effects of a single application of the insecticide chlorpyrifos. For percentages variance accounted for and the significance level see Tables 1 and 2.

Table 1. Percentages of the total variance which can be attributed to time and treatment regime for the two analyzed data sets. The treatment component includes the interaction between treatment and time. The remaining fraction of the variance is residual variance. The Table also indicates which fraction of the variance explained by the treatment regime is captured by the first and second Principal Response Curves (PRC).

	% of variance accounted for by		% of variance explained by treatment regime captured by	
Data set	time	treatment regime	first PRC	second PRC
Invertebrates	22	34	26	9
Syndrome	47	23	83	10

DO-pH-Alkalinity-Conductivity syndrome

Fig. 4 shows a clear response of the DO-pH-Alkalinity-Conductivity syndrome at the highest treatment dose. This response, however, was not significant at the 5% level (Table 2). Nevertheless, the diagram of the first PRC, indicates an elevated pH and, less pronounced, elevated DO levels, as well as a lower conductivity and alkalinity at the highest treatment dose. These results are in accordance with findings by Kersting and Van den Brink (1997). The NOEC calculations showed significant differences at the highest dose for Weeks 2 and 3 only (NOEC = 6 µg/L, Table 2), whereas Fig. 4 indicates a longer period of difference. The second PRC was also not significant (Table 2).

Table 2. Period of significant influence of chlorpyrifos treatment (Monte Carlo permutation tests, $p < 0.05$), lowest $NOEC_{community}$ reported (Williams test on PCA co-ordinates, $p < 0.05$) and results of significance tests (Monte Carlo permutation tests) of the first and second Principal Response Curves (PRC) for the two data sets analyzed.

Data set	Period of significant influence of treatment regime (in weeks)	Lowest NOEC reported (in µg/L)	P-value for the first PRC	P-value for the second PRC
Invertebrates	0.1 - 19	0.1	0.005	> 0.05
Syndrome	2 - 3	6	> 0.05	> 0.05

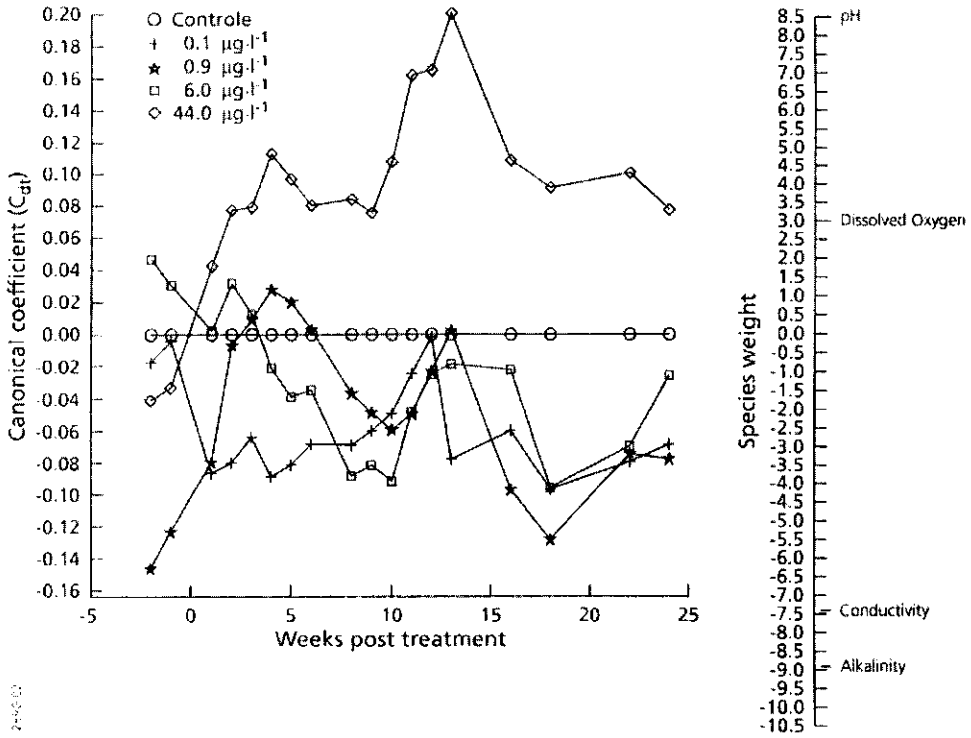

Figure 4. Principal Response Curves with species weights for the DO-pH-Alkalinity-Conductivity syndrome data set, indicating the effects of a single application of the insecticide chlorpyrifos. In this analysis the parameters of the syndrome played the role of the species. For percentages variance accounted for and the significance level see Tables 1 and 2.

Discussion

Comparison between RDA and the Principal Response Curves

Mesocosm experiments result in large data sets on the effects of pollutants on biological communities and how these change in time. The Principal Response Curve method that we propose in this paper to analyze such data sets, distils the complexity of time-dependent, community-level effects of pollutants to a graphic form that is easy comprehend. Moreover, the responses of individual species to the treatments can be inferred from the PRC curves.

The PRC method has distinctive advantages over the RDA method from which is derived. These advantages can be appreciated by comparing Figs. 3 and 4 (PRC) with Figs. 1 and 2 (RDA). Although the RDA diagrams show the changes in species composition between the treatments in time, they are difficult to interpret in terms of how the treatment effects develop in time and how they depend on the dose level. An important cause for this is that the trajectory for the control cosms in time may appear quite chaotic as is illustrated by Fig. 2. As a consequence, the treatment effects which are of the most interest, *i.e.* the deviations of the treatments from the control, do not stand out. The PRC methods overcomes these difficulties of interpretation by representing the time trajectory for the controls as a horizontal line (Figs. 3 and 4). This is achieved by taking the control treatment as the reference to which the other treatments are contrasted and by defining 'time' as the horizontal axis of the diagram. Notice that treatment effects displayed in Figs 1 and 2 could in principle be reexpressed in this new graphical form. The PRC method does better than this by accounting *a priori* for the development of the biological communities in the control cosms over time. This is why PRC is based on a partial RDA with, as covariables, indicator variables for the sampling dates. As a result, PRC shows in one glance insight into how the treatment effects develop over time (Figs. 3 and 4). With help of the species weights, the PRC can be used to infer about the response of individual species to the stressor.

Sometimes RDA and PRC represent essentially the same treatment effects. This happens when the time trajectory of the controls is approximately a straight line in the RDA diagram. For example, Fig. 1 is after a rotation over 45 degrees quite similar to Fig. 3. The taxa with a relatively high weight in the PRC are also discriminating in the RDA (Figs. 1 and 3). But even in this case, the PRC diagram is more easy to explain to non-experts than the RDA diagram.

We close this section with a cautionary note. A small species weight of a taxon in the PRC cannot be translated automatically into a low susceptibility of the taxa to the stressor. For instance, *Gammarus pulex* has a relatively low species weight in the PRC diagram (Fig. 3) but also happens to be the taxon which, as far as we know, is most susceptible to chlorpyrifos Van Wijngaarden et al., 1993). The reason for this discrepancy is that *G. pulex* has a very specific response pattern (see Van den Brink et al., 1996) that differs from Fig. 3. PRC shares this limitation with RDA and other multivariate techniques that search for global patterns in community data.

Comparison with other techniques used in mesocosm experiments

In this section we attempt to outline the similarities and dissimilarities of multivariate methods used in ecotoxicology, focusing on non-metric multidimensional scaling (MDS, incorporated in the PRIMER computer program; Clarke, 1993), non-metric clustering (NMC, incorporated in the Riffle computer program; Matthews and Hearne, 1991), and our

own approach of canonical ordination using RDA and PRC (incorporated in the CANOCO computer program; Ter Braak, 1994; 1988).

Multivariate analysis of ecotoxicological experiments consists of I) testing the statistical significance of treatment effects and II) estimation of the magnitude of treatment effects. Simultaneous multivariate analysis of many endpoints (many taxa) requires dimension reduction if one wishes to find patterns and display the results. CANOCO and PRIMER use ordination and thus reduce the data set to continuous variables, the axes of their ordination diagrams (e.g. Fig. 1). Riffle uses cluster analysis and thus reduces the data set to a nominal variable (indicating to which cluster a sample belongs). The display of results in reduced dimensions helps the analyst to grasp the essential information of the multivariate experiment. However, this often leaves the layman wondering what is actually being displayed. The dimension reduction often makes it difficult to explain what is actually estimated by these displays: the translation of the diagram into treatments effects is often nontrivial. This is the main problem we attempted to overcome with PRC.

Up to this point the similarities between the CANOCO and PRIMER approaches are evident. There are considerable differences also. CANOCO works on the original 'sample by species' abundance matrix, whereas PRIMER's MDS works on a derived 'sample by sample' matrix containing similarities or dissimilarities between samples (Clarke 1993; Clarke and Ainsworth; 1993). It is therefore difficult, although not impossible (Gower and Hand, 1996), to show individual taxa in an MDS diagram. As a consequence, a direct interpretation of treatment effects displayed in the diagram at the species level is not easily available. Also, since MDS does not use the original species data set, the fractions of the total variance of the data set which are explained by the axes cannot be calculated. These parameters are important for the evaluation of the merits of the analysis. It is an advantage of MDS, however, that it allows the user to choose a specific similarity or dissimilarity index (e.g. the Bray Curtis index), whereas PRC is restricted to Euclidean Distances (Ter Braak, 1988). This is, however, less restrictive for PRC than it looks, because there still is the possibility to transform the data prior to the analysis. For the invertebrate data sets of the 1990 chlorpyrifos study, the display of treatment effects provided by the MDS analysis (results not shown) was very similar to that of RDA, presented in Fig. 1.

A difference between RDA and PRC on the one hand, and MDS and NMC on the other, is that MDS and NMC are non-metric, whereas RDA and PRC are linear and metric. In theory, non-metric methods are better for dimension reduction and require fewer model assumptions. On the other hand, CANOCO allows the user to adjust the analysis for unwanted effects through covariables and to focus on the treatment effects, thereby counterbalancing its simplistic modelling. The linearity assumption should not be misunderstood: PRC is based on a linear method, but is well capable of showing non-linear treatment effects (see e.g. Figs. 3 and 4).

Multivariate methods are often divided into supervised and nonsupervised methods. Riffle is nonsupervised; it searches for a clustering of samples on the basis of the

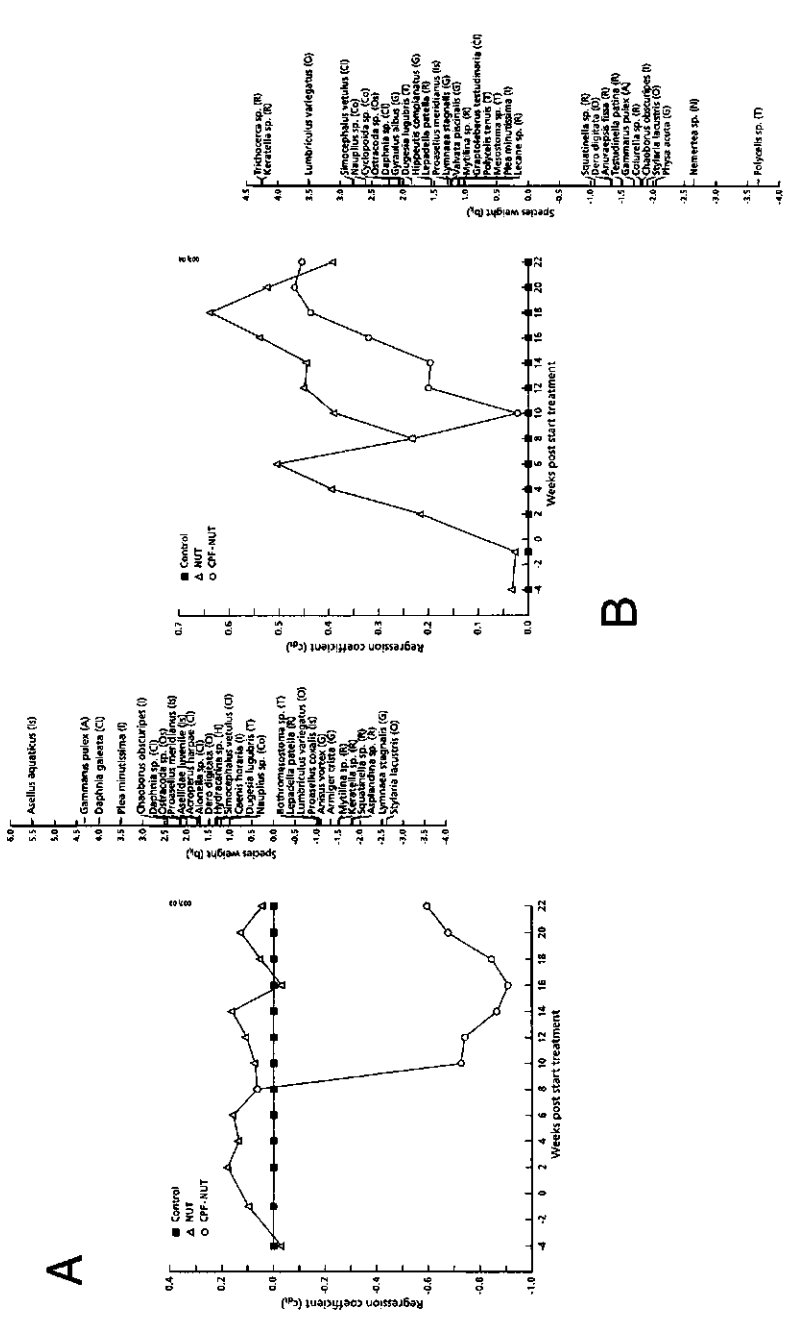

Figure 3. Principal Response Curves for the invertebrate data set, indicating the effects of nutrient additions (NUT) and the extra chlorpyrifos addition (CPF-NUT). The first PRC is given in A; the second PRC in B. See Table 2, for explained and displayed variance. Values deviating from the reference value of 0 indicate treatment effects. The species weight (b_k) can be interpreted as the affinity of the taxon with the principal response curves. Only the species with a weight of 1 or higher or −1 or lower with the diagrams are displayed. Explanation of the codes: A = Amphipoda; Cl = Cladocera; Co = Copepoda; G = Gastropoda; H = Hirudinea; I = Insecta; Is = Isopoda; N = Nemertea; O = Oligochaeta; Os = Ostracoda; R = Rotatoria; T = Turbellaria.

Table 2. Percentages of the total variance which can be attributed to time and treatment regime for the analyzed data sets. The treatment component includes the interaction between treatment and time. The remaining fraction of the variance is residual variance. The Table also indicates which fraction of the variance explained by the treatment regime is captured by the first and second Principal Response Curves (PRC)

Data set	% of variance accounted for by		% of variance explained by treatment regime captured by (p-value of PRC between brackets)	
	Time	Treatment regime	First PRC	Second PRC
Invertebrates	31	20	43 ($p \leq 0.01$)	15 ($p \leq 0.01$)
Phytoplankton	41	13	29 ($p \leq 0.01$)	26 ($p \leq 0.01$)

ponents to yield the joint fit of the change. The fitted change can be plotted against time to yield fitted response curves for individual species or species groups.

The significance of each principal component was tested by Monte Carlo permutation of the microcosms, i.e., by permuting whole time series in the partial redundancy analysis from which PRC is obtained, using an F-type test statistic based on the eigenvalue of the component (Van den Brink & Ter Braak, in press). In these tests, components that had already been extracted were added to the covariables. The PRC analyses and associated permutation tests were performed using the CANOCO software package, version 3.14 (Ter Braak, 1988, 1990).

We close this section with a technical remark. As described above, the PRC is the principal component of the treatment-by-species matrix of treatment effects. In Van den Brink & Ter Braak (in press), the PRC consists of the canonical coefficients of a partial redundancy analysis, in which the input data sets are the sample-by-species matrix of log-abundance values, the sample-by-week matrix of covariables, and the sample-by-(treatment in week) matrix of explanatory variables. It is known (Ter Braak & Looman, 1994) that the canonical coefficients of the explanatory variables in the redundancy analysis are the generalized principal components of the matrix of the regression coefficients, which in our context are the treatment effects. The heuristic introduction given above thus coincides with the PRC analysis by Van den Brink & Ter Braak (in press) in that they both apply principal component analysis with a particular weighting scheme. The weighting scheme becomes particularly important if treatments are not equi-replicated. In conclusion, the treatment scores $\{c_{dt}\}$ are both principal component scores and canonical coefficients of the treatments.

Tests of significance of treatment effects

To test on which sampling dates the treatments had significant effects on the biological communities, permutation tests were performed for each sampling date. Before week 9, the 8 treated cosms were tested against the 4 controls. After week 9, each of the two treatments was tested against the control and against the other treatment. The tests were carried out with the CA (Hommen et al., 1994) and CANOCO (Ter Braak, 1988) software packages. The permutation procedure in CA tests whether the similarity quotient is more below 1 than can be expected by chance, i.e. whether the quotient is significantly below 1. These tests were performed using the Bray-Curtis and Stander's index. The non-parametric method in CANOCO uses Monte Carlo permutation and the F-type test statistic of redundancy analysis (RDA). The reported P-values are based on 999 Monte Carlo permutations under the null model. Specific details for the application of Monte Carlo permutation in model ecosystem experiments have been described by Verdonschot and Ter Braak (1994), Van den Brink et al. (1996) and Van Wijngaarden et al. (1995).

Results

Invertebrate data set

Both the Bray-Curtis index and Stander's index quotients showed an effect of the treatments on the invertebrate communities after the second high nutrient addition and chlorpyrifos treatment in week 9 (Figure 2), with a higher effect size for the CPF-NUT

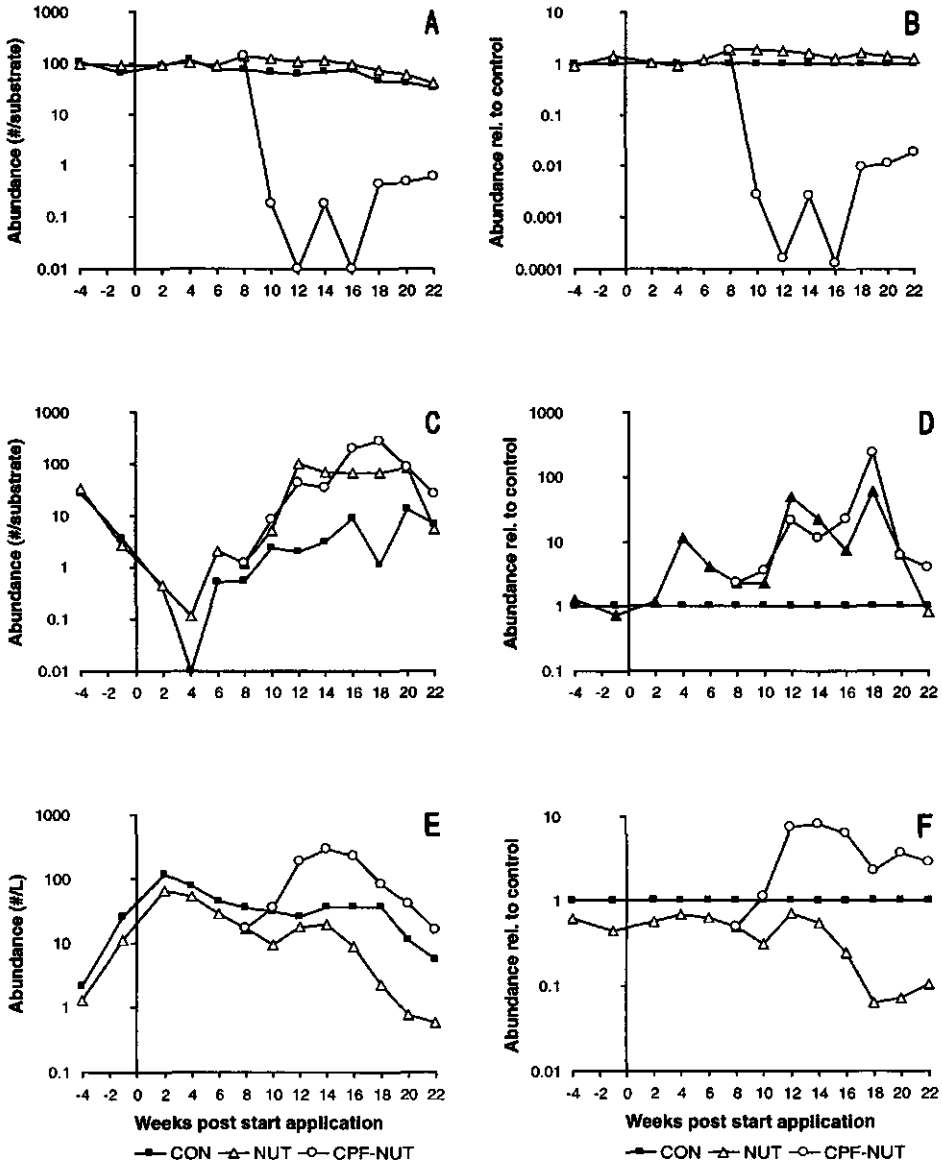

Figure 4. Dynamics in numbers of three invertebrate taxa. Figures 4A, 4C and 4E show the geometric means of the counted numbers per treatment, of *Asellus aquaticus*, *Stylaria lacustris* and *Keratella* sp. respectively. B, D and F shows their abundance relative to the control treatment, respectively.

treatment. The Bray–Curtis index showed a more stable pattern of effect sizes in time than Stander's index (Figure 2).

The PRC analysis indicated for the invertebrate data set that the overall variation among sampling dates was higher than that among treatments (Table 2). The first and second PRC components were statistically significant (Table 2), whereas the third component was not ($P>0.05$). The first PRC diagram (Figure 3A) shows that the curve for the CPF-NUT treatment drops abruptly after the chlorpyrifos addition (week 9), whereas the curve for the NUT treatment stays close to the zero line for the control during the whole sample period. In the second PRC (Figure 3B) the curve for the NUT treatment starts to deviate from the control after the first high nutrient dose, rises to a peak in week 6, drops and, after the second high nutrient dose in week 9, rises to a new peak in week 18. The curve for CPF-NUT in the period after week 9, starts at the zero level (week 10) and then roughly follows the trend in the NUT curve. The broad pattern that emerges is that the first PRC shows the dominant effect of the chlorpyrifos addition, whereas as the second PRC shows the subdominant nutrient effects.

Figure 4 shows the observed response patterns of three distinctive species. The first column of figures shows their (geometric) mean counts on a logarithmic scale, whereas the second column was obtained from the first one by plotting differences with respect to the controls. The decreases or increases shown by the species in the treatments compared with the level in the control, as displayed in the second column, constitute the treatment effects that the PRC method attempts to summarise. We now compare the response curves of the individual species with the response curves fitted by the first two PRCs.

Asellus aquaticus is the taxon with the highest weight with the first PRC, but a near zero weight with the second. Its observed response (Figure 4B) is, indeed, very similar in shape to the first PRC (Figure 3A). Quantitatively, the fitted reduction due to chlorpyrifos is also of the right order of magnitude, as we will now show. In week 10 the CPF-NUT score was about -0.8 (Figure 3A). The weight of *A. aquaticus* was 5.52, so that the fitted reduction was $\exp(-0.8 * 5.52) = 0.012$. The PRC analysis thus indicates that *A. aquaticus* is reduced to ca. 1% of its abundance in the control.

Keratella sp. has relatively higher weights with both PRCs. The fitted response pattern of *Keratella* sp.

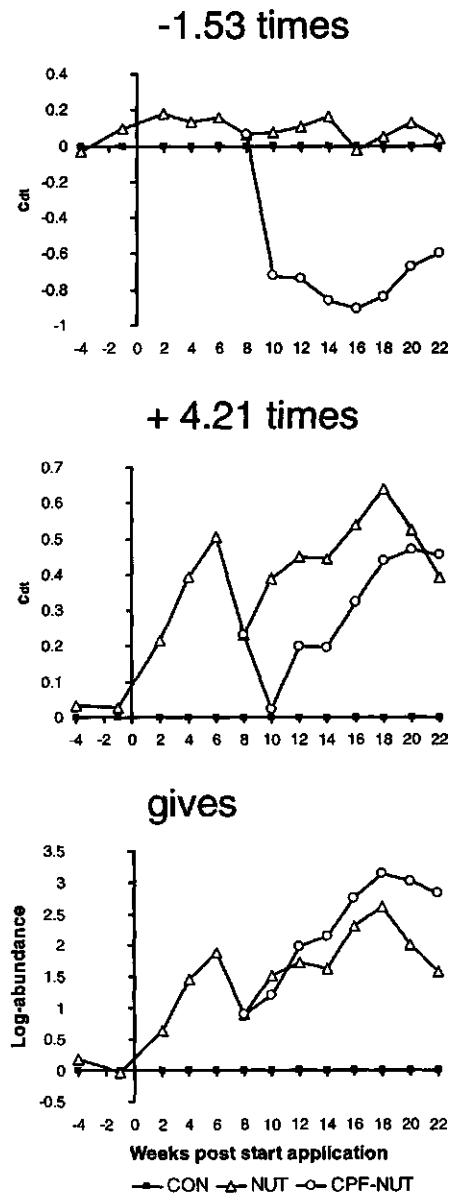

Figure 5. Outline of how the two PRC diagrams of Figure 3 must be combined so as to obtain the fitted response curves of *Keratella* sp., which has weights -1.53 and 4.21 on the first and second PRC, respectively.

can be obtained by multiplying these weights with the treatment scores in the corresponding PRCs and then summing the two products (Figure 5). The resulting fitted curves are similar to the observed response as given in Figure 4D, except for its response at the end of the experimental period (week 20 and 22). Quantitatively speaking, week 16, for example, shows a fitted change on a log scale of ca. 2.5 for both treatments (Figure 5). In terms of abundance, *Keratella* sp. was present with exp(2.5) = 12 times more individuals in the treatments than in the control, which roughly corresponds to the observed response.

The weighted summation of PRC curves for each individual taxon can be avoided, as is shown in Figure 6, where species have been positioned by their weights on the first and second PRCs (Figure 3). In this system of coordinates, species that lie on the same line through the origin have proportional response curves. Their fitted response curves thus have the same shape. Figure 6 shows the shape of the response curves for species situated on lines with 45° intervals and they give an impression of the type of shapes that can be obtained by combining PRCs. The four diagrams in the corners apply to species that have equal weights on the two PRCs (except for the sign). The PRC diagrams corresponding with the axes are precisely the PRCs of Figure 3. For example, the species positioned on the right side along the horizontal axis in Figure 6 (e.g., *Asellus aquaticus*) decreased due to chlorpyrifos, while those at the top of the vertical axis (e.g., *Trichocerca* sp.) increased due to the nutrient additions. Those at 45° (e.g., *Ostracoda*) decreased due to chlorpyrifos and increased due to nutrients, whereas those at the other end of the ray, i.e., at 225°, (e.g. *Stylaria lacustris*) show the opposite pattern. In this way, taxa can be grouped on the basis of similarity of response pattern.

The statistical significance of the effects of chlorpyrifos on the invertebrate community is demonstrated by the permutation tests per sampling date (comparison CPF-NUT versus NUT; Table 3). Using the F-type criterion and the Bray-Curtis index quotient, significant effects were found for the whole period after week 9, whereas Stander's index quotient indicated effects for only a few sampling dates (Table 3). The P-values obtained with the Bray–Curtis index quotient were, however, not always robust (weeks 10 and 12; Table 3).

The effects of the nutrient additions were significant from week 14 to week 20, as assessed on the basis of the similarity indices (NUT versus CON; Table 3). Using the F-type criterion, a few sampling dates showed significant effects (weeks 6, 16 and 20).

All tests indicate significant effects of the CPF-NUT treatment compared with the control (Table 3). Only for week 10 did the tests produce different results: the effect was judged to be significant using the F-type criterion only.

Phytoplankton data set

Both the Bray–Curtis and Stander's index showed minor effects of the first large nutrient addition on the phytoplankton community. After the second large nutrient addition, the effects were larger (weeks 10–14; Figure 7). There were no consistent differences between NUT and CPF-NUT.

As for the invertebrate data set, the overall variation among sampling dates was higher than among treatments (Table 2). Whereas the first and second PRC components were statistically significant, the third was not ($P > 0.05$). The percentage of variance explained by treatment was smaller in the phytoplankton data set than in the invertebrate data set. In the phytoplankton data set, the second PRC was about equally important as the first, as assessed on the basis of the percentage of variance accounted for. This means that the joint interpretation of the PRCs (Figures 8A and B) is even more important in the phytoplankton data set than it was in the invertebrate data set. Together, the two PRCs encompass 55% of the treatment variance, which is only slightly less than was the case with the invertebrate data set.

The first PRC indicates a deviation of the NUT treatment from the control for week 4. After the second high nutrient treatment and the chlorpyrifos application in week 9. Both treatments differed more consistently from the control, with a larger deviation for the CPF-NUT compared to the NUT treatment (Figure 8A). The second PRC shows opposite responses to both treatments after week 9. The differences between NUT and CPF-NUT shown by the first two PRCs are reduced when the second PRC is added to the first, and are enhanced when the second PRC is subtracted from the first, as shown in Figure 9 in the right upper and lower quadrants at 45° and 135°, respectively.

Figure 10 shows the observed dynamics of the three species that were in extreme positions in terms of their weight with the first PRC, namely *Volvox* sp., *Chroomonas* sp. and Algae sp. The lay-out of Figure 10 is the same as that of Figure 4. We now compare the response curves of these species with the

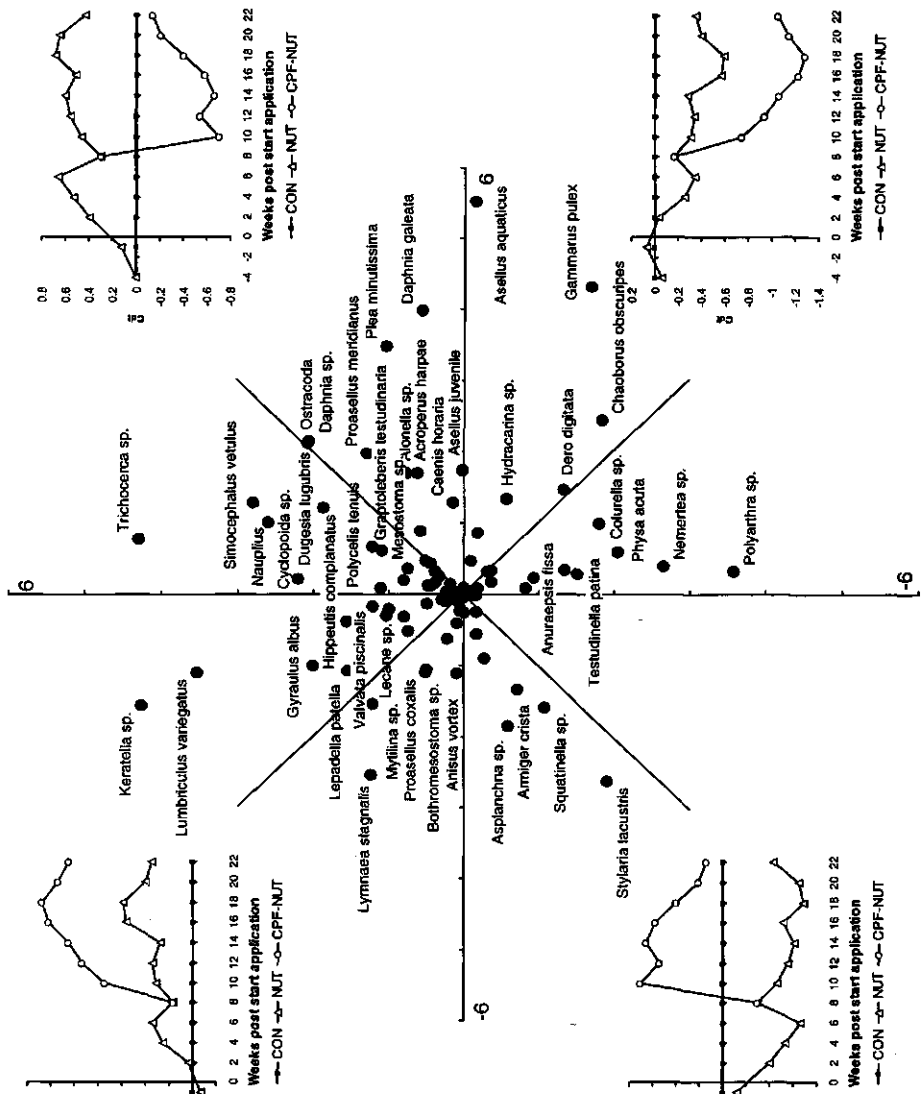

Figure 6. Two-dimensional plot of the weights of the invertebrate species on the first and second PRC, as given in Figure 3. The four diagrams in the corners apply to species that have equal weights on the two PRC's (except for the sign).

Table 3. Results of Monte Carlo permutation tests per sampling date; '–': calculation not applicable, F = $p < 0.05$ using the F-type criterion; $B = p < 0.05$ using the Bray–Curtis index quotient; S = $p < 0.05$ using Stander's index quotient; blanc = $P > 0.05$; * = when permutation procedure is repeated, sometimes a $p < 0.05$ is reported, sometimes $p > 0.05$. For abbreviations see Figure 1

Week	Invertebrates									Phytoplankton								
	NUT vs CON			CPF-NUT vs CON			CPF-NUT vs NUT			NUT vs CON			CPF-NUT vs CON			CPF-NUT vs NUT		
–4				–			–						–			–		
–1				–			–						–			–		
2				–			–						–			–		
4				–			–			F			–			–		
6	F		S	–			–						–			–		
8				–			–			F			–			–		
10				F			F	B*		F				B	S	F		
12				F	B	S*	F	B*		F	B	S	F	B	S*			
14		B	S	F	B	S	F	B	S	F	B	S		B	S*	F	B	S
16	F	B	S	F	B	S	F	B	S							F	B	S
18		B	S	F	B	S	F	B										
20	F	B	S	F	B	S	F	B										
22				F	B	S	F	B	S									

fitted curves that can be inferred from Figures 8 and 9. *Volvox* sp. increased in abundance in the NUT and CPF-NUT treatments, with a greater increase just after week 9 for the CPF-NUT treatment (Figures 10A and 10B). This response pattern is predicted well by Figure 9, where *Volvox* sp. had a high negative weight with the first PRC and a low weight with the second one. *Chroomonas* sp. had a high positive weight with the first PRC and near zero weight with the second (Figure 9), thus decreasing in abundance in both treatments, with a much larger decrease in the CPF-NUT treatment (Figures 10C and D). Algae sp. had high weights with both PRC diagrams. The predicted response curves of Algae sp. as indicated in Figure 9 at 45° are very much like the actual response curves (Figure 10F).

The effects of the prolonged nutrient additions and the single chlorpyrifos addition on the phytoplankton communities were both judged to be statistically significant during short sequences of sampling dates (Table 3). In this data set, the Bray–Curtis index and Stander's index quotients yielded similar test results, which sometimes differed from those based on the F-type criterion of RDA.

Discussion

Comparison of similarity analysis and PRC

Similarity analysis expresses the systematic differences among communities that are subjected to different treatments, in a single number. This number, defined as the quotient of the mean between-treatment and the mean within-treatment similarities, is a statistical measure of the total size of the treatment effects on the species. Similarity analysis results in a graph of the development of the size of the treatment effects with time, and is complemented by permutation tests that assess the statistical significance of the treatment effects. Although different in detail, the results obtained using the Bray–Curtis index and those obtained with Stander's index showed the same global pattern, namely, weak treatment effects before week 9 and strong treatment effects in the period after week 9 (Figures 2 and 7). This demonstrates the effect of the chlorpyrifos treatment and prolonged nutrient additions on both the invertebrate and the phytoplankton communities. The F-type statistic of RDA showed the same global pattern of effect sizes (Table 3).

In PRC, the principal components of the treatment effects on the species are each plotted against time. The first PRC diagram may sometimes resemble the graph from similarity analysis as, for example, in the phytoplankton data set. An important advantage is that PRC allows a direct interpretation down to the species

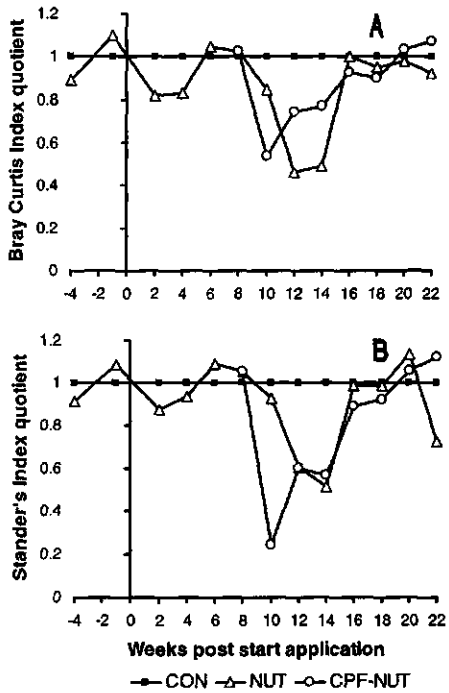

Figure 7. Size of the effects of nutrient additions (NUT) and extra the chlorpyrifos addition (CPF-NUT) in the phytoplankton data set as defined by the Bray-Curtis (A) and Stander's (B) Index quotient. For explanation see text and Figure 2.

level. In particular, PRC shows whether a particular species increased or decreased in the treated cosms relative to its abundance in the control cosms. In other words, PRC gives a signed difference (increase *vs.* decrease) whereas the similarity analysis provides an unsigned difference only, namely the total treatment size.

In both example data sets, PRC successfully reduced the treatment effects of the many species to two dimensions. The fact that more than one PRC was needed, demonstrates that the species reacted in qualitatively different ways to the treatments. When the PRCs are combined and displayed in a two-dimensional diagram of species weights, such as Figure 6, an overview is obtained of the main types of response curves that occur, and also of the species that react according to each particular type of response curve. For example, Figure 6 shows that all possible combinations of increase/decrease due to the nutrient additions (NUT) and increase/decrease due to both nutrient and chlorpyrifos additions (CPF-NUT) actually occurred. In addition, there are species that did not respond to nutrient additions but did decrease strongly when chlorpyrifos was added as well. These are the species that lie along the positive first axis. *Asellus aquaticus* shows this type of response most prominently.

In summary, both the PRC method and the similarity analysis provide endpoints for an evaluation of effects of toxicants at the community level. Both methods also allow the effects to be tested statistically. The main difference, however, is that the PRC method also allows the treatment effects to be evaluated directly down to the taxon level.

Comparison of effects as reported in Cuppen et al. (1995) versus PRC outcomes

The results reported by Cuppen et al. (1995) are summarized in Table 1. A qualitative interpretation of the PRC results of the invertebrates is also given, in the columns labelled 'PRC'. At first glance, no differences in interpretation between the reported effects on the invertebrates are apparent. This means that the PRC method succeeded in comprising the most important treatment effects of the invertebrate data set into two diagrams.

The increase in the chlorophyll-a content of the phytoplankton can of course not be compared with the abundance counts used in the example data set. For PRC, the entries in Table 1 are therefore empty as far as the chlorophyll-a content or biomass of the primary producers are concerned. In Van Donk et al. (1995), the only taxon reported to show a treatment effect was *Volvox* sp. (Van Donk et al., 1995). Although the chlorophyll-a content of the phytoplankton increased due to the nutrient additions and the chlorpyrifos application, PRC analyses indicated a decrease in abundance for most taxa that showed treatment-related effects (Figure 8A; e.g. *Chroomonas* sp.: Figure 10D). Only for *Volvox* sp. is an increase indicated (Table 1). Because of its size, this taxon evidently accounted for a considerable fraction of the chlorophyll-a content of the phytoplankton.

In conclusion, PRC not only revealed the same responses reported in Cuppen et al. (1995), but it also provided an overview of the effects at the community level.

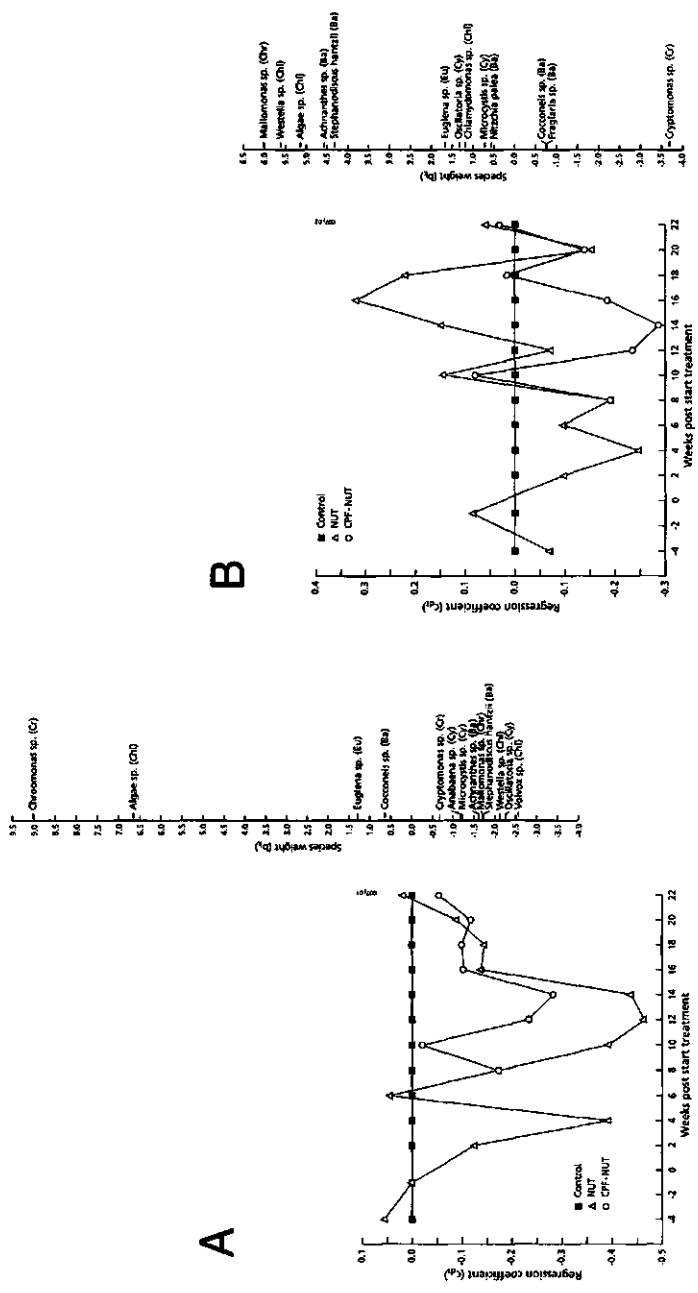

Figure 8. Principal Response Curves for the phytoplankton data set, indicating the effects of nutrient additions (NUT) and the extra chlorpyrifos addition (CPF-NUT). The first PRC is given in A; the second PRC in B. See Table 2, for explained and displayed variance, and Figure 3 for explanation. Only the species with a weight of 0.5 or higher or −0.5 or lower with the diagrams are displayed. Explanation of the codes: Ba = Bacillariophyceae; Chl = Chlorophyceae; Chr = Chrysophyceae; Cr = Cryptophyceae; Cy = Cyanophyta; Eu = Euglenophyta.

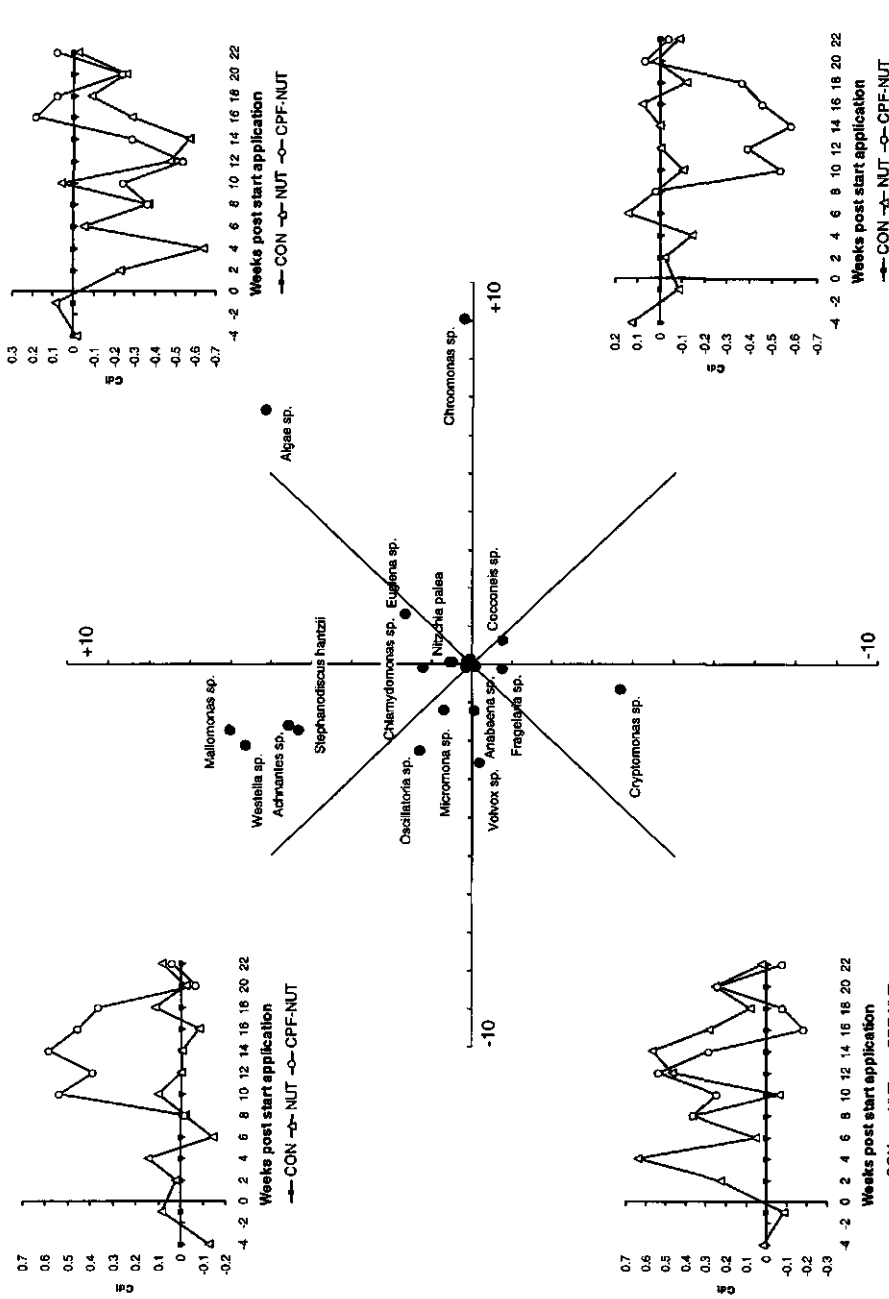

Figure 9. Two-dimensional plot of the weights of the phytoplankton species on the first and second PRC, as given in Figure 8. The four diagrams in the corners apply to species that have equal weights on the two PRC's (except for the sign).

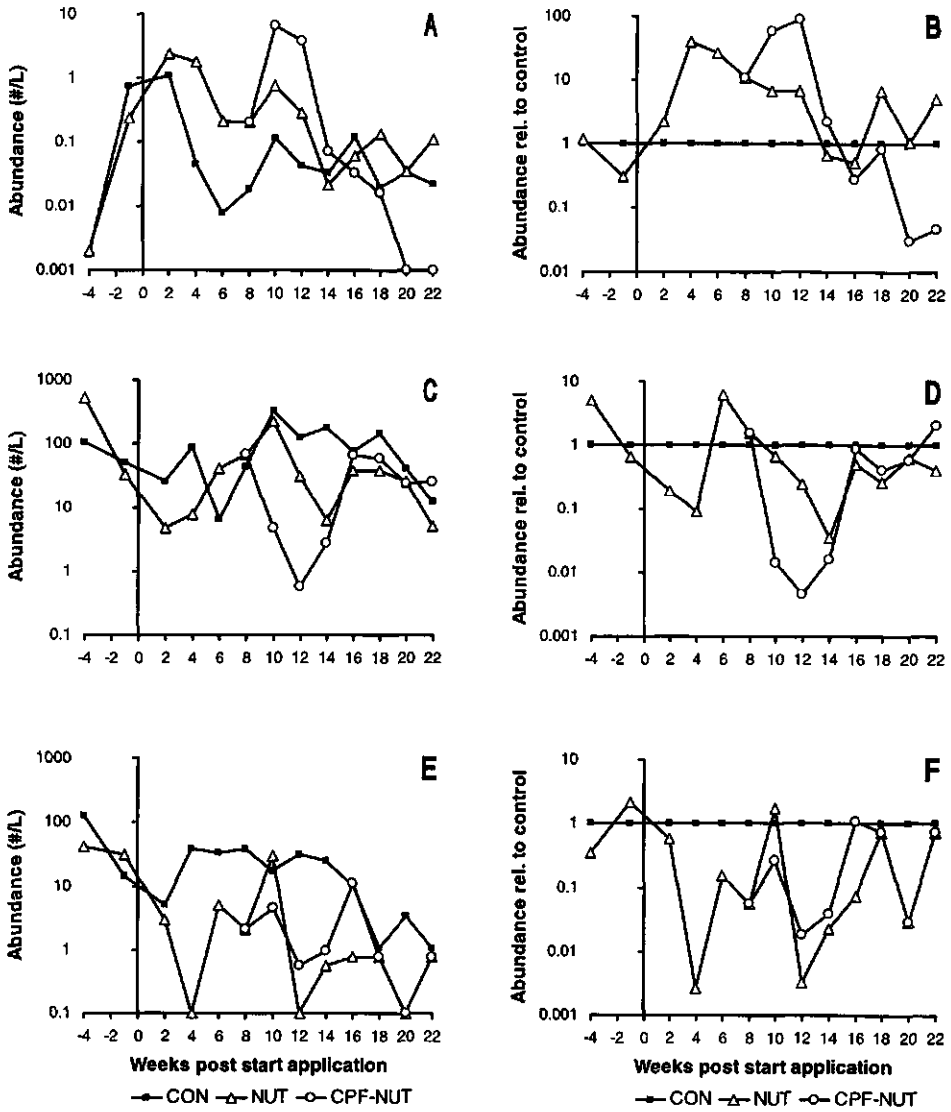

Figure 10. Dynamics in numbers of three phytoplankton taxa. A, C and E show the geometric means of the counted numbers per treatment, of *Volvox* sp., *Chroomonas* sp. and Algae sp. respectively. B, D and F shows their abundance relative to control, respectively.

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Chapter 7

Ordination of responses to toxic stress in experimental ecosystems

Paul J. Van den Brink and Cajo J.F. Ter Braak. *Toxicology and Ecotoxicology news, Vol. 4: 173-177, 1997*

Review

Ordination of Responses to Toxic Stress in Experimental Ecosystems

Paul J. Van den Brink and Cajo J.F. Ter Braak

Pesticides used for crop protection may enter adjacent freshwater ecosystems by spray drift, leaching, run-off or accidental spills. For a hazard assessment of these pesticides, microcosm and mesocosm experiments can be carried out. Although smaller and less complex than real-world freshwater ecosystems, microcosms provide the opportunity to perform ecosystem-level research in replicable test systems under conditions that are manageable in terms of costs and logistics [1]. These experiments, however, involve great effort in sampling, identification of the biological communities and measurements of parameters. Data of only the most common taxa appear suitable for univariate statistical analysis (e.g. ANOVA). By contrast, multivariate statistical analysis analyses all available data and describes the effects of chemical stress at the community level. In this paper we will discuss four different multivariate techniques.

Example data set

The example data set resulted from an experiment in microcosms, simulating the community of drainage ditches, and with the herbicide linuron as a stressor. The outline of the experiment is summarised in Textbox 1. The microcosms consisted of a glass aquarium (volume approximately 1 m^3) with a natural sediment layer of 10 cm and a water layer of 60 cm (photographs 1 and 2). During the preparatory phase of the microcosm experiment the macrophyte *Elodea nuttallii* and indigenous invertebrate and algal species were introduced. Zooplankton, macro-invertebrates, phytoplankton and periphyton were sampled and identified (bi)weekly from 1 week before the start of the experiment until 10 weeks after. During this period several physico-chemical parameters were also measured. The experiment and the phytoplankton data set, which is used in this paper as an example, are described and discussed in detail in Van den Brink *et al.* [2] and Cuppen *et al.* [3]. In the application of the first two methods (TWINSPAN and DCA), abundance values were averaged over replicates. The later methods used the original samples.

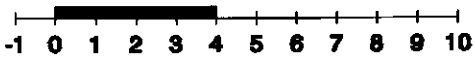
Textbox 1

Linuron experiment

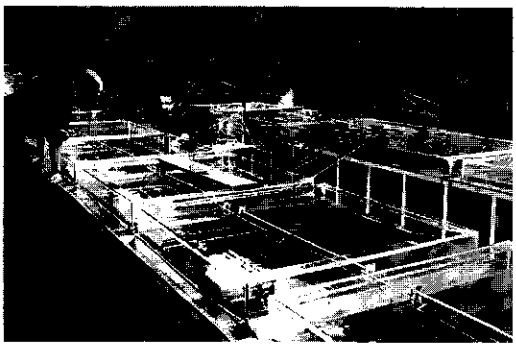
- 2 microcosms controls
  2 0.5 µg/L
  2 5 µg/l
  2 15 µg/l
  2 50 µg/l
  2 150 µg/l

  EC_{50} *Chlorella vulgaris* = 50 µg/L (Stephenson and Kane [13])

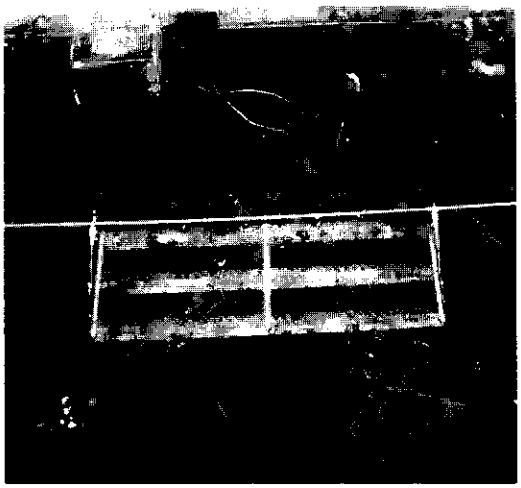
- Chronic exposure for 4 weeks



- Phytoplankton (51 taxa)
  Sampled from week -1 through 10



Photograph 1. *Overview of the climate room and the microcosms.*



Photograph 2. *Detailed view of macrophyte dominated microcosm.*

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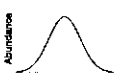
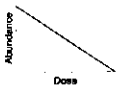
Textbox 2

Summary multivariate methods used

	Response model	
		
Clustering	TWINSPAN	
Unconstrained Ordination	DCA	(PCA)
Constrained Ordination	(DCCA)	RDA PRC

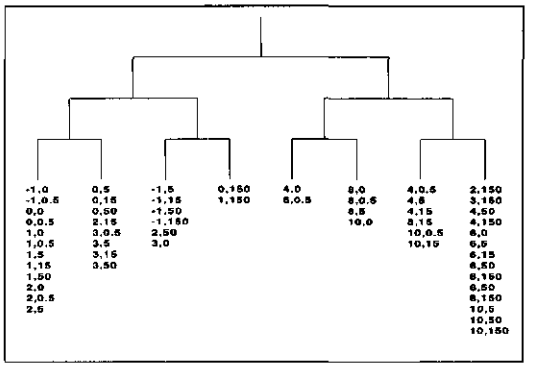


Figure 1. Dendrogram of the example data set using TWINSPAN. The sample codes, e.g. –1,0, are week number followed by dose.

TWINSPAN and DCA

TWINSPAN (Two-Way-INdicator-SPecies-ANalysis) [4] is a hierarchical divisive clustering method that is very popular among community ecologists. Starting with all samples in one group, it divides this group into two sub-groups. These sub-groups are then divided into sub-sub-groups, etc. Each division is based on Correspondence Analysis [5]. Correspondence Analysis has the desirable ability to detect clusters and to sequence sample data that arise from bell-shaped response curves (Textbox 2). Applied to the example data set, TWINSPAN yielded the dendrogram of Figure 1. The first division separates most early samples (weeks –1 to 3) from the later samples. Later divisions are more difficult to interpret, except for the final division on the right-hand side, which sets apart all samples from the 150 µg/L treatment taken in and after week 2 and all samples from the 50 µg/L treatment taken in and after week 4. This cluster contains an odd control sample. In its finer detail, the dendrogram thus suggests a treatment effect. It does not provide information on the magnitude and nature of the effects.

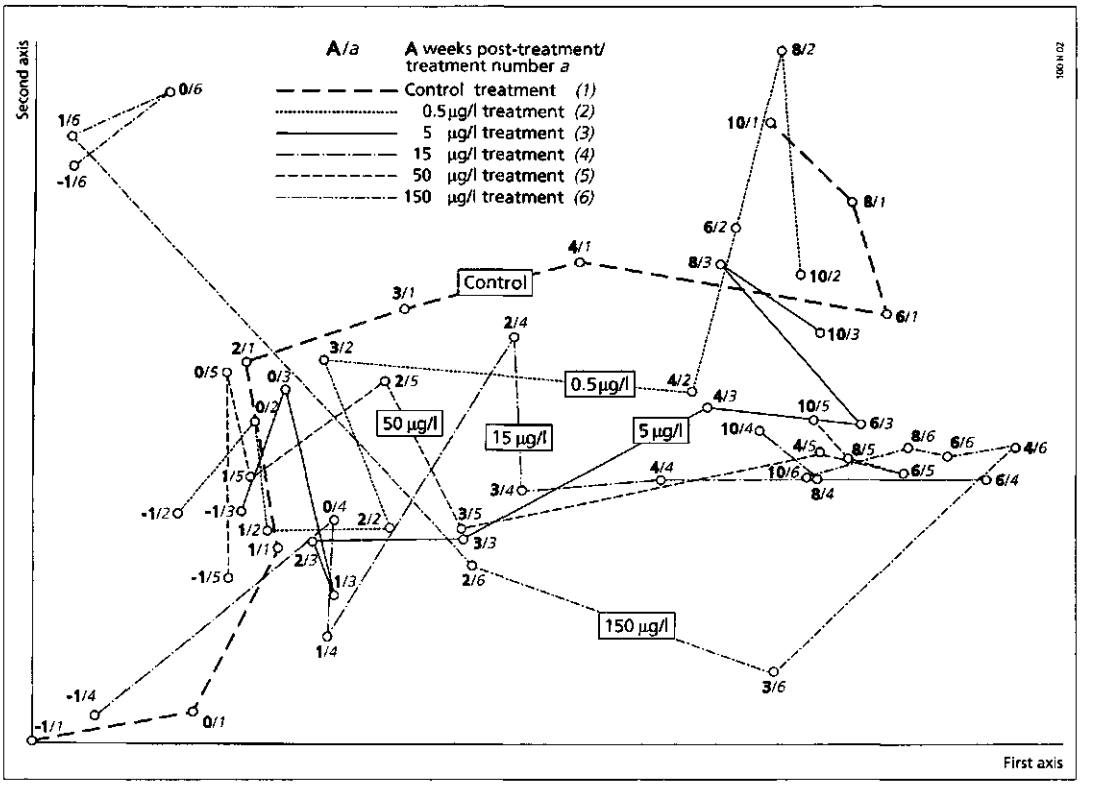


Figure 2. Ordination diagram resulting from a DCA on the example data set. The lines represent the course of the treatment levels in time. Of all variance, 18% is displayed on the horizontal axis and 7% on the vertical axis.

Detrended Correspondence Analysis (DCA), is an improved form of Correspondence Analysis. Incorporated in the computer programmes DECORANA [6] and CANOCO [7] it is among the most popular ordination methods used in ecology. It results in a diagram that represents the major pattern of between-sample variation (Figure 2). In the diagram, samples with nearly identical species composition lie close together, while samples with very different species composition lie far apart (for more explanation see Ter Braak, [8]). So, the samples taken at weeks –1, 0 and 1 in the highest treatment have a similar species composition, which differs from the species composition of all other samples. In an attempt to highlight the structure of the experimental design, time-trajectories are added to Figure 2. The trajectories for all treatments can hardly be separated from each other, so the treatment effects are difficult to discern from Figure 2. Usually the species are also presented in the diagram but in our study the species were placed too far outside the range of the samples to be shown.

RDA and PRC

Outside community ecology, Principal Component Analysis (PCA) is the most frequently used multivariate technique. In contrast to DCA, PCA is based on a linear response model. Redundancy Analysis (RDA) is the constrained form of PCA (Textbox 2). RDA constrains the ordination to that part of the total variance of a data set that is explained by a given set of explanatory variables. We used treatment, time and their interaction as explanatory variables. In this particular example, the same ordination could have been obtained by applying a PCA on abundance values averaged over replicates, as was done in DCA. Van Wijngaarden *et al.* [9] compare DCA, PCA and RDA and their usage in mesocosm

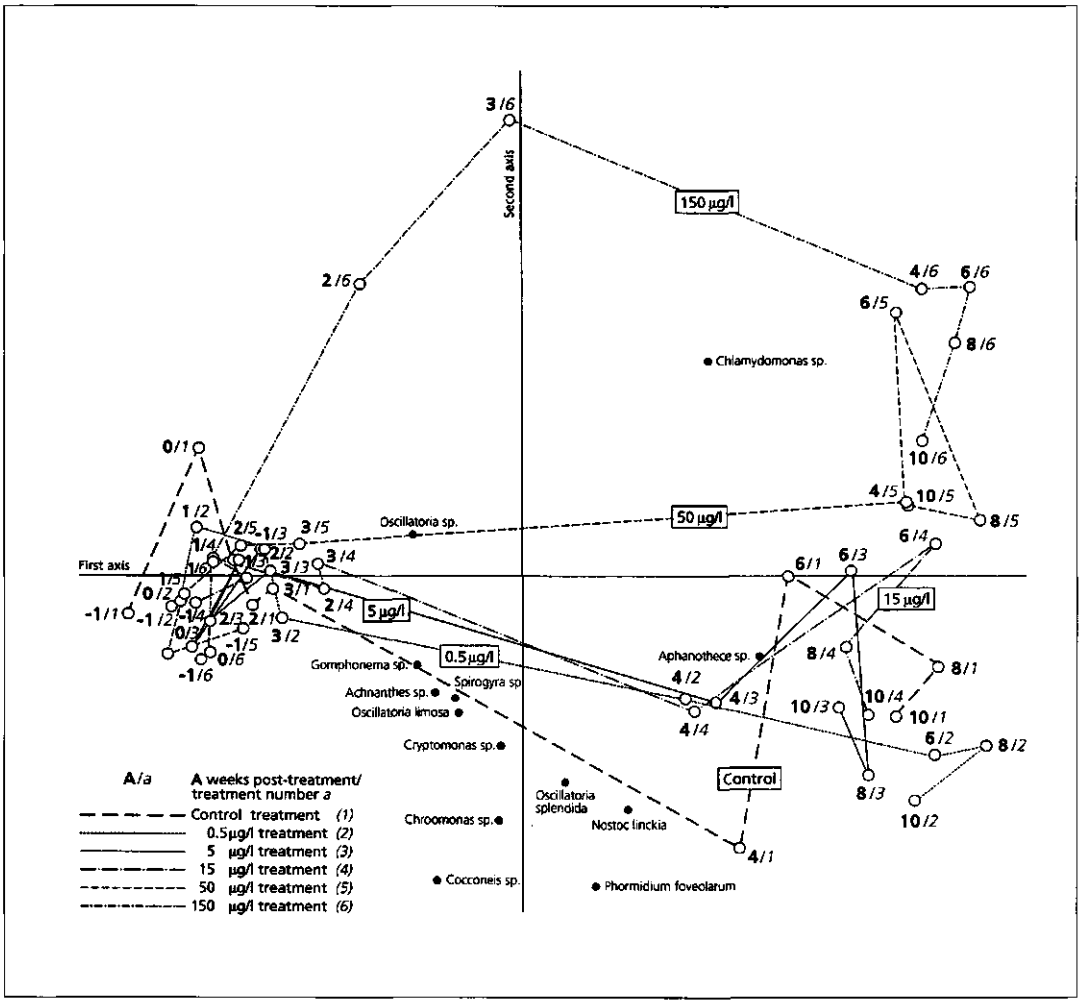


Figure 3. Ordination diagram (RDA) indicating effects of the herbicide linuron on the phytoplankton. Sampling week and treatment level, as well as their interactions, were taken as explanatory variables. The lines represent the course of the treatment levels in time. Of all variance, 78% could be attributed to the explanatory variables. Of this explained variance, 61% is explained in the diagram.

Textbox 3.

Principle Response Curves

- based on RDA
- Zooms in at differences in measurement endpoints between treatments and the control as reference
- Model used:

  $y_{b(i)tk} = y_{0tk} + b_k h_{bt} + \varepsilon_{b(i)tk}$

  with

  $y_{b(i)tk}$: abundance counts of taxon k at time t in replicate i of treament b.

  y_{0tk} : mean abundance in controls (0) for each sampling date

  h_{bt} : principal response of treatment b at time t (PRC)

  b_k : weight of species k with respect to $\{h_{bt}\}$

  $\varepsilon_{b(i)tk}$: error term
- In diagram horizontal axis : time
  vertical axis : h_{bt} and b_k

research in more detail. Van den Brink *et al.* [10] provide more examples of RDA.

Figure 3 displays the ordination with added time-trajectories resulting from RDA applied to the example data set. The horizontal axis shows mainly the changes in species composition in time. The vertical axis shows a difference compared to the control for the 150 µg/L treatment and less pronounced also for the 50 µg/L treatment. The samples taken at the start of the experiment are all placed close together. The placement of the species indicates higher abundance values in the highest treatments for *Chlamydomonas* sp. and lower abundance values for *Cocconeis* sp., *Chroomonas* sp. *Phormidium foveolarum*, etc.

Diagrams such as Figure 3 may become very cluttered (see for instance Kersting and Van den Brink, [11]). Even from Figure 3, it is hard to discern how the treatment effects change in time. To overcome these problems we recently developed a variant of RDA, called Principal Response Curves analysis (PRC [12]). PRC is designed to show optimally the major changes in treatment effects over time. The new method zooms in at differences in species-composition between the treatments and control at each particular timepoint. The model of PRC is outlined in Textbox 3. The result is a PRC diagram such as Figure 4. The horizontal axis denotes the week relative to the start of the treatment and the vertical axis the treatment effect, expressed as deviation from the control. The curves so obtained are called principal response curves. The curves for the 50 µg/L and 150 µg/L treatments each reach their maximum deviation from the control after four weeks, the maximum effect being much larger for the 150 µg/L treatment than for the 50 µg/L treatment. The lower doses do not show a strong effect. The scores of the taxa as shown on the right are weights expressing each taxon's affinity with the principal response curves. *Chlamydomonas* sp. has a high negative weight and thus occurred in higher abundance values at the two highest doses after start of the treatment. *Phormidium foveolarum* showed the opposite response since it has a positive weight with the diagram. The mean abundance

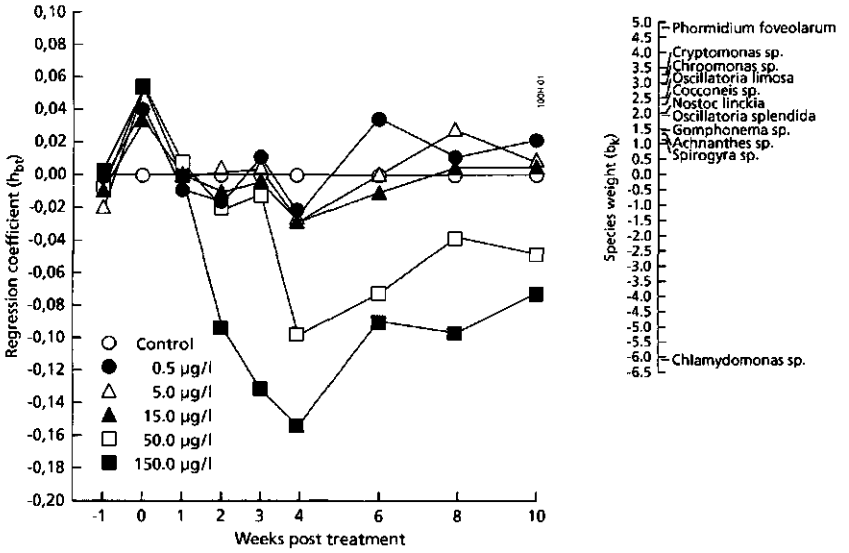


Figure 4. Principal response curves resulting from the analysis of the example data set, indicating the effects of the herbicide linuron on the phytoplankton community. Of all variance, 47% could be attributed to sampling date, and is displayed on the horizontal axis. Of all variance, 30% could be attributed to treatment. Of the variance explained by treatment, 23% is displayed on the vertical axis. The lines represent the course of the treatment levels in time. The species weight (b_k) can be interpreted as the affinity of the taxon with the principal response curves.

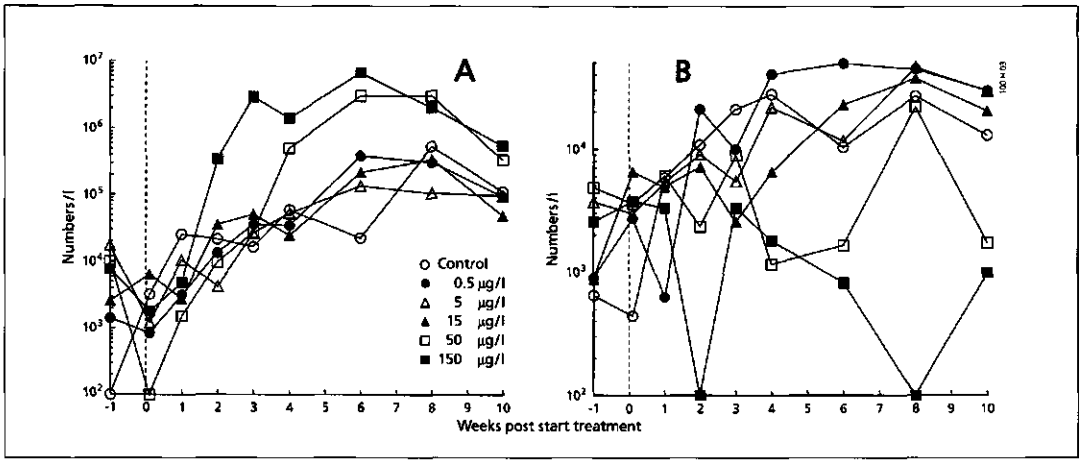


Figure 5. Dynamics in numbers of two phytoplankton taxa. Shown are the geometric means of the counted numbers per treatment level, of (A) Chlamydomonas *and (B)* Phormidium foveolarum.

patterns of both taxa are given in Figures 5A and 5B respectively. These patterns correspond very well with the treatment effects as indicated by the PRC diagram.

Conclusions

From this case study we conclude that RDA more clearly displayed the treatment effects than TWINSPAN and DCA. A possible explanation for this is that ecotoxicological dose–response relationships are usually logistic, which is better approximated by a linear model than by a unimodal model. In addition, Correspondence Analysis focuses on the relative abundances of taxa whereas RDA concentrates on the absolute abundances. An overall decrease in abundance may therefore go unnoticed in Correspondence Analysis.

The results of PRC were more easy to interpret than those of RDA. By filtering out the mean abundance pattern across time in the control, PRC focused on the deviation between treatment and associated control. The principal response curves displayed the major pattern in these deviations and were a good summary of response curves of individual taxa.

Noise is sometimes believed to be an overriding property of ecological data. Our most important conclusion is perhaps that proper statistical analysis revealed clear response patterns in noisy ecological data.

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Chapter 8

Summary and concluding remarks

Aquatic risk assessment of pesticides

The first tier in the aquatic risk assessment procedure consists of a comparison between a Predicted Environmental Concentration (PEC) with a No Effect Concentration (NEC). A requirement for registration is that the PEC should not exceed the NEC. The NEC is calculated from the toxicity of the pesticide for defined standard test species (viz. algae *Daphnia*, fish) and an assessment factor, which accounts for potential differences between standard test species and indigenous species. The assessment factors used are 100 (to be multiplied with the acute EC50 of *Daphnia* and fish) or 10 (to be multiplied with the chronic NOEC of fish or EC50 of algae). Because this approach lacks ecological realism, the first aim of the present thesis was to validate the assessment factors used in the first tier by evaluating three chemicals with different modes of action (insecticide, herbicide, fungicide) as benchmark compounds.

We compared the No Observed Effect Concentrations (NOECs), resulting from microcosm and mesocosm experiments using these compounds, with the NECs as used for the risk assessment procedure. Table 1 summarises the standards calculated from the first tier criteria set by the Uniform Principles (UP-standard), as well as the $NOEC_{ecosystem}$ for acute and chronic exposure regimes for the three substances. In addition, Table 1 lists the Dutch water quality standards. The assessment factors seem to protect the tested aquatic ecosystem against acute and chronic exposure to the insecticide chlorpyrifos and against chronic exposure to the herbicide linuron and the fungicide carbendazim (Table 1; chapters 2, 3 and 4). Dutch water quality standards for these three compounds were lower than the UP-standards and thus also seem to protect the aquatic ecosystems tested when exposed to individual compounds.

A comparison between the UP-standards and the Lowest Observed Effect Concentration at the ecosystem level ($LOEC_{ecosystem}$) indicates that when the NEC is exceeded by a factor of 10, effects cannot be excluded in the case of chronic exposure. In the case of a single application of the insecticide chlorpyrifos, however, the assessment factor can be considered overprotective; an assessment factor of 10 instead of 100 would also seem to suffice. Two extensive literature reviews on the impact of insecticides and herbicides on aquatic microcosms and mesocosms also demonstrate that the first tier criteria of the Uniform Principles are generally adequate to protect different aquatic ecosystems from pesticide stress (Lahr et al., 1998; Van Wijngaarden et al., 1998). For compounds such as fungicides, however, hardly any information could be found in the

open literature, so that validation of the assessment factors for these types of pesticide needs further attention.

Table 1. Derived UP-standards, Dutch water quality standards and $NOEC_{ecosystem}$ observed in semi-field studies for the insecticide chlorpyrifos, the herbicide linuron and the fungicide carbendazim (all concentrations in µg/L). UP-standards were calculated from criteria set by the first tier of aquatic risk assessment. For references to toxicity values see Table 3 in chapter 1 of this thesis.

	UP-standard		Dutch water	$NOEC_{ecosystem}$ / $LOEC_{ecosystem}$	
	Short-term	Long-term	quality standard	Acute exposure	Chronic exposure
Chlorpyrifos	0.01[a]	0.01[c]	0.003	0.1 / 0.9 (Chapter 2)	0.01[d] / 0.1[e]
Linuron	0.6[b*]	0.6[b*]	0.25	- / -	0.5 / 5 (Chapter 3)
Carbendazim	3.2[a]	1[c]	0.11	- / -	3.3 / 33 (Chapter 4)

* Dutch standard would be 0.1 µg/L (0.1 x NOEC of the standard test algae; Anonymous, 1995); - No data available; [a]: 0.01 x LC50 *Daphnia*; [b]: 0.1 x EC50 Algae; [c]: 0.1 x NOEC Daphnia; [d]: data from unpublished experiment, Van den Brink et al., in prep.; [e]: data from Van den Brink et al., 1995

Ecological effects and recovery

One of the aims of the present thesis was to gain insight into long-term community responses and into the factors determining the recovery of affected populations after a single application of an insecticide in experimental ditches. As was expected from its mode of action, application of chlorpyrifos resulted in large adverse effects on arthropod taxa (chapter 2). Because this experiment was performed in relatively large, outdoor systems, the recovery of the affected populations could be investigated. The recovery of populations of individual species was highly dependent on their life-cycle characteristics, such as the number of generations per year, the presence of resistant life stages and the ability to migrate from one system to another. In chapter 2 this is illustrated by the responses of two mayflies, cladocerans and an amphipod. The mayflies *Cloeon dipterum* and *Caenis horaria* do not have life stages resistant to chlorpyrifos, but are able to migrate from one ditch to another. They are also almost equally susceptible to chlorpyrifos in the laboratory but showed a very different recovery pattern. The former species recovered within 12 weeks at the highest treatment level, whereas the latter species took 24 weeks to recover fully. This can be explained from the difference in the number of generations per year. *C. dipterum* has many generations per year and thus recolonises the ditch repeatedly, thus recovering as soon as the concentration of chlorpyrifos allows this. *C. horaria*, however, produces only one generation per year, so that recovery can only take place when the next generation recolonises the ditch. Unlike mayflies, Cladocerans are not able to migrate actively from one ditch to the other. They did, however, show a very fast recovery at the higher concentration (Chapter 2). This is possible because they have a short generation

time and resistant life stages in the form of ephyppia. If a taxon is not able to recolonise an impacted system and does not have resistant life stages, the species can become extinct in isolated systems like the experimental ditches. This applies for the amphipod *Gammarus pulex*, which became extinct at the two highest concentrations and did not recover within the 55 week experimental period.

No significant effects on the invertebrate community, with the exception of Gammarus, were found from week 24 after insecticide application onwards, suggesting recovery.

As part of the third aim of the thesis, the long-term responses in ecosystem structure and functioning after chronic exposure to a herbicide and fungicide were studied in aquatic microcosms. The higher concentration of the photosynthesis-inhibiting herbicide linuron resulted in a decreased biomass of the macrophyte *Elodea nuttallii* and decreased abundance of most algal taxa (chapter 3). The dissolved oxygen and pH levels also decreased at lower pesticide concentrations as a consequence of inhibited photosynthesis. Although a decrease in the abundance of most algal taxa was observed after to the herbicide application, a net increase in chlorophyll-a was found for the phytoplankton, periphyton and neuston. This increase was completely caused by the green alga *Chlamydomonas* sp., which appeared to be relatively tolerant to linuron and also had the ability to develop a tolerance to relatively high concentrations within a week. As a result of this tolerance and the reduced competition for nutrients with macrophytes, the community in the microcosms shifted from macrophyte-dominated to an algae-dominated state, especially at the highest treatment level (150 µg/L). The Copepoda and Cladocera benefited from this increased food supply and showed elevated abundance values at the higher treatment levels. Some macrophyte-associated invertebrates decreased in abundance as a result of the decline of their habitat.

The fungicide carbendazim, which belongs to the bendimidazoles, is known to adversely affect micro-organisms and worms. This property explains its effects on the "worm-like" taxa of the Turbellaria and Oligochaeta, but could not explain its effects on invertebrate groups like Amphipoda, Gastropoda and Cladocera (chapter 4). Unlike the direct effects of chlorpyrifos and linuron, therefore those of carbendazim on freshwater populations could not be completely deduced from the latter's taxonomic relation with the pest organisms, carbendazim it is supposed to control. The fungicide appeared to have the mode of action of a biocide rather than a chemical with a specific mode of action. Due to the decline of many invertebrates and the concomitant reduction in grazing pressure, the chlorophyll-a level and the abundance values of some phytoplankton taxa increased at the two highest concentrations (330 and 1000 µg/L).

The "eutrophication-like" consequences of insecticide contamination have also often been reported and discussed in the literature (e.g. DeNoyelles et al., 1994, Cuppen et al., 1995). The increased abundance of algae due to a decrease in susceptible herbivores is a commonly reported consequence of insecticide contamination (Van Wijngaarden et al., 1998).

In the present thesis, the occurrence of herbicides in the aquatic ecosystem is regarded as an undesirable side effect of its use on land. However, herbicides are also deliberately released into aquatic ecosystems for the control of nuisance aquatic vegetation (Pieterse and Murphy, 1990). Aquatic weeds are most commonly removed using compounds with a mode of action specific to macrophytes. Since algae are relatively tolerant to these chemicals (Lahr et al., 1998), they may increase their biomass due to reduced competition for nutrients (Kobriaei and Whyte, 1996). Terrestrial weeds are, in the Netherlands, usually controlled by means of photosynthesis-inhibiting herbicides (NEFYTO, 1996). Although their mechanism is different, chapter 3 shows that prolonged exposure to the photosynthesis-inhibiting herbicide linuron may also result in a shift from macrophyte dominance to plankton dominance. The review published by Lahr et al. (1998) shows that this may be true for photosynthesis-inhibiting herbicides in general.

The effects of fungicides are largely unstudied, but chapter 4 indicates that fungicide contamination can also cause elevated algal densities. This means that all three pesticides can contribute to "eutrophication-like" effects, though the mechanisms differ. The significance of realistic concentrations of pesticides in causing symptoms of eutrophication in surface waters, however, largely remains to be investigated.

Tools to evaluate microcosm and mesocosm experiments

Semi-field experiments are usually evaluated at the taxon level. Since many species normally have low abundance values and/or show high variability (Van Wijngaarden et al., 1996), this approach has the great disadvantage that only a limited number of species can be properly analysed. This means that a substantial part of the information gathered is not used for the evaluation. This thesis presents a new multivariate tool for the analysis of treatment effects at the community level. Multivariate techniques have already been used for a long time in ecology to analyse the relation between communities and their environment. The most commonly used ordination technique is correspondence analysis, which is based on the bell-shaped unimodal model. This model fits in with the theory of the rise and fall in a preference of a species along an environmental gradient, described by their optimum and tolerance. Chapter 7 indicates why clustering and ordination based on correspondence analysis are not suitable for the analysis of the ecotoxicological data sets presented in this thesis. It argues that species normally have no optimum along the environmental axis of a stressor such as pesticides. Their response is more accurately described by a linear method; expected direct effects will increase with the concentration. On the basis of

laboratory tests, this relation between the endpoint and the concentration of stressor is assumed to be sigmoid, and it is argued that a linear response model is a good approximation of this.

Chapters 2 and 3 use Redundancy Analysis (RDA) to elucidate the effects of pesticides at the community level. RDA is the constrained version of the well-known ordination technique Principal Component Analysis (PCA) and is based on a linear response model (Jongman et al., 1995). In chapters 2 and 3 the analysis is constrained to the variance explained by treatment, time and their interaction. It was concluded that RDA successfully summarised the effects of a pesticide on a community in a single diagram, and is very useful especially when combined with Monte Carlo permutation tests for the determination of the significance of treatment effects. Kersting and Van den Brink (1997), however, found that output from RDA can sometimes result in very cluttered diagrams.

Chapter 5 presents a new method, termed the Principal Response Curves, which overcomes this problem. PRC is based on RDA and extracts the first principal component from the treatment variance, by excluding from the analysis the variance explained by time as well as differences between replicates. It results in an easy-to-read diagram, showing the deviations of all treatments from the control in time. In contrast to most other techniques, it also allows a quantitative interpretation down to the species level. Chapter 6 introduces the rank 2 model of PRC, this means that after the extraction of the first basic response pattern, a second pattern is extracted, which expresses the most important deviation from the first response present in the data set. The second pattern is of particular importance if no single dominant response pattern is present in a data set but several sub-dominant ones occur. In chapter 6 this is illustrated by an analysis of the invertebrate and phytoplankton data sets of a microcosm experiment with two stressors, the insecticide chlorpyrifos and nutrient additions. This example shows that PRC is also able to summarise several different response patterns in two diagrams.

Microcosm and mesocosm experiments are often said to be of limited value due to ecological variability and noise. From the experiments and statistical tools as described in this thesis we can conclude that despite the noise clear response patterns are revealed, if experiments are properly designed and analysed. Chapters 2, 3 and 4 illustrate that, even with a limited number of replicates, an ecological threshold level (e.g. $NOEC_{ecosystem}$) and an effect-chain covering different trophic levels can be obtained.

Suggestions for future research

In normal agricultural practice, protection of crops from pest organisms is not achieved by the application of a single compound; usually, several different compounds with different target organisms are used. Some pesticides are also administered repeatedly. The effects of combinations of pesticides on freshwater ecosystems are, however, largely unstudied (Hartgers et al., 1998). Therefore, it is important to develop criteria for the ecological risk

assessment of mixtures of compounds, using realistic pesticide treatment regimes for particular crops.

The problem of combination toxicity becomes even more complex when other substances used in agricultural areas, such as fertilisers, are taken into account. The combined effects of eutrophication and contaminant stress are largely unknown. It can be expected, however, that the trophic status of an ecosystem will alter the effects of pesticides (Chapter 6; Kramer et al., 1997).

The ecological effect chain resulting from the experiments with the herbicide linuron and fungicide carbendazim demonstrated that microcosm and mesocosm experiments with pesticides as stressors can be very useful tools to investigate trophic interactions in aquatic ecosystems. The results of these experiments are currently being used to build a food-web model (Traas et al., 1998a, 1998b). Such models are considered to hold great promise for an improved understanding of ecosystem functioning and may eventually provide the ability to predict effects of contaminants at ecosystem level (Health Council of the Netherlands, 1997). The greatest obstacles that have to be overcome are the lack of solid data on parameter values (data on for instance maximum growth rate) and the lack of validation. This means that the further development of food web models require not only laboratory research on parameters values but also semi-field research for the collection of validation data sets (Health Council of the Netherlands, 1997).

The modeling of direct effects and recovery patterns at the population level can be of great use for an assessment of the risks and a ranking of the effects of pesticides. For the future, modeling treatment effects and recovery patterns may be of great value as a research tool but also as a predictive tool. Models have the advantage that they allow integration of ecological and ecotoxicological knowledge, something that was largely absent from ecotoxicology until a few years ago. Development of these models will allow to a better evaluation of microcosm and mesocosm experiments performed for scientific or registration purposes.

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Hoofdstuk 8

Samenvatting en slotopmerkingen

Inleiding

De wereldbevolking nam in deze eeuw snel in omvang toe. Om aan de groeiende consumptiebehoeften te kunnen voldoen werd de intensieve landbouw gestimuleerd. Het gebruik van landbouwchemicaliën (kunstmest, bestrijdingsmiddelen) leidde tot een economisch efficiënte toename van de productie van gewassen. Snel bleek echter dat het intensieve gebruik van landbouwchemicaliën ook milieuproblemen met zich meebrengt (bijvoorbeeld Carson, 1962).

Landbouwbestrijdingsmiddelen dienen voor de onderdrukking van ongewenste doelorganismen (o.a. ziekteverwekkers, onkruiden, schadelijke insecten). Bij gebruik komen deze stoffen ook op niet-bedoelde plaatsen als bijvoorbeeld het oppervlaktewater terecht door overwaaiing, uitspoeling, afspoeling, atmosferische depositie en/of ongelukken (Capri en Trevisan, 1998). Hierbij kunnen effecten op soorten die aan doelorganismen verwante zijn, zoals insectenlarven, niet uitgesloten worden (Hurlbert, 1975; Hill et al., 1994).

Om het aquatisch leven te beschermen tegen de schadelijke neveneffecten van bestrijdingsmiddelen stelden de autoriteiten normen op. Recentelijk nam de Europese Unie een registratieprocedure aan voor het op de markt brengen van bestrijdingsmiddelen (de zogenaamde Uniforme Beginselen), die ook waterkwaliteitscriteria omvat (European Union, 1997; zie ook tabel 1). Deze nieuwe waterkwaliteitscriteria vormen de belangrijkste reden waarom diverse oude producten zijn verboden en waarom vele nieuwe bestrijdingsmiddelen niet, of beperkt, zijn toegelaten op de Europese markt (College, 1997). De toelatingsprocedure wordt echter bekritiseerd vanwege de economische gevolgen van te strenge, en de ecologische gevolgen van te liberale milieucriteria. Daarom is in de Uniforme Beginselen (UB) voor een getrapte risicobeoordeling gekozen.

Getrapte risicobeoordeling van bestrijdingsmiddelen

Voor de toelating van een nieuw bestrijdingsmiddel op de Europese markt moet een risico-evaluatie van de verwachte effecten op het milieu plaatsvinden. Voor een adequate risico-evaluatie voor het oppervlaktewater worden idealiter de lotgevallen en effecten onderzocht onder realistische veldomstandigheden, waarbij rekening wordt gehouden met de gangbare landbouwpraktijk en de variabiliteit van ecosystemen in tijd en ruimte. De tijd, kosten en logistiek die zo'n aanpak vergt maken het onmogelijk om ieder bestrijdingsmiddel op deze manier te evalueren. Daarom is in Europa gekozen voor een

getrapte risico-evaluatie. De eerste, relatief simpele trap bestaat uit een vergelijking van de verwachte concentratie in het veld met een concentratie die geacht wordt veilig te zijn voor het aquatisch ecosysteem. De tweede trap bestaat niet uit nauw gedefinieerde regels als de eerste trap, maar is maatwerk en hangt af van de onzekerheden in de voorspelde risico's na de eerste trap.

Tabel 1. Trappen en bijbehorende criteria die de EU opstelde voor de beoordeling van het risico van bestrijdingsmiddelen voor niet-doelorganismen (vis, *Daphnia*, alg) in het oppervlaktewater.

Fase in risicobeoordeling	Criteria*
eerste trap	korte-termijn PEC ≤ 0,01 LC50 of EC50 vis of *Daphnia* én korte-termijn PEC ≤ 0,1 EC50 alg én lange-termijn PEC ≤ 0,1 NOEC vis of *Daphnia* én BCF ≤ 1000 voor gemakkelijk biologisch afbreekbare stoffen én BCF ≤ 100 voor moeilijk biologisch afbreekbare stoffen,
tweede trap	tenzij een adequate risico-evaluatie duidelijk aantoont dat er geen onaanvaardbare effecten zijn op de levensvatbaarheid van blootgestelde soorten, rechtstreekse en indirecte (predatoren), na toepassing van het gewasbeschermingsmiddel volgens de voorgestelde gebruiksaanwijzing.

*: PEC: Predicted Environmental Concentration; voorspelde milieuconcentratie; LC50: Letale Concentratie 50%, concentratie van het bestrijdingsmiddel in het water waarbij 50% van de organismen sterft; EC50: Effect Concentratie 50%, concentratie waarbij 50% van de organismen een effect vertoont; NOEC: No Observed Effect Concentration; hoogst getoetste concentratie van een experiment waarbij geen significant effect aangetoond wordt; BCF: BioConcentratie Factor

Voor de eerste trap in de risicobeoordeling wordt gekeken of de voorspelde milieuconcentratie de veilige concentratie niet overschrijdt. De voorspelde milieuconcentratie voor het watercompartiment (Predicted Environmental Concentration; PEC) wordt berekend voor een stilstaand-zoetwatersysteem met een waterdiepte van 30 cm. Deze berekening wordt uitgevoerd met behulp van een model dat het gedrag en de lotgevallen van een bestrijdingsmiddel in het water beschrijft. Hiervoor worden gegevens over de aanbevolen hoeveelheid van het bestrijdingsmiddel en het percentage van drift van de stof naar de watergang, of een andere emissieroute, gebruikt (Capri en Trevisan, 1998). De veilige concentratie voor aquatische ecosystemen (No Effect Concentration; NEC) is gebaseerd op dosis-effectrelaties die in het laboratorium bepaald zijn voor een beperkt aantal standaardtoetssoorten, nl. een vis, een watervlo en een alg. Deze soorten zijn gekozen vanwege het gemak waarmee ze te kweken en te houden zijn in het laboratorium. De testprocedures zijn nauw omschreven in protocollen, zoals die van de

OECD (Organisation for Economic Co-operation and Development, 1993) richtlijnen. Aangezien extrapolatie van de gevoeligheid van de standaardtoetsorganismen naar inheemse soorten uiterst onzeker is wordt een veiligheidsfactor gebruikt om de verschillen in gevoeligheid tussen deze soorten te verdisconteren (normaal een factor 100 voor een acute EC50 en een factor 10 voor een chronische NOEC; zie Tabel 1 voor uitleg en meer details).

De hierboven beschreven eerste trap in de risico-evaluatie van bestrijdingsmiddelen kan worden beschouwd als conservatief, enerzijds omdat, vergeleken met de laboratorium-condities, de stof in het veld normaliter sneller verdwijnt en minder biobeschikbaar is, en anderzijds omdat min of meer "worstcase"-omstandigheden gebruikt worden voor het berekenen van de PEC. Daarom laten de Europese reglementen die de toelating van bestrijdingsmiddelen op de Europese markt behandelen, de mogelijkheid toe om ecologisch meer relevante data te gebruiken voor een nadere risico-evaluatie. Deze nadere evaluatie wordt beschouwd als een tweede trap in de risico-evaluatie van bestrijdingsmiddelen en bestaat uit de "tenzij-bepaling" van de UB beschreven in Tabel 1. De gevraagde extra informatie kan variëren van aanvullende data over de gevoeligheden van inheemse soorten tot een betere schatting van de halfwaardetijd van het middel. Experimenten op ecosysteemniveau worden vaak gevraagd en uitgevoerd om aan te tonen dat de werkelijke risico's van een bepaald middel aanvaardbaar zijn wanneer uitgegaan wordt van de normale landbouwpraktijk.

Behoefte aan validatie, het gebruik van microcosms en mesocosms.

Om te bepalen of de procedures het risico voor het aquatisch milieu voldoende inschatten, dienen de representativiteit van de standaardtoetsorganismen en de hoogte van de veiligheidsfactoren gevalideerd te worden. Idealiter worden hiervoor de effecten van bestrijdingsmiddelen onder natuurlijke omstandigheden op het ecosysteemniveau geëvalueerd. Dit kan gedaan worden door de dynamiek van gemeenschappen in een belaste situatie te volgen en die te vergelijken met een onbelaste referentie. Helaas zijn onbelaste situaties normaliter niet beschikbaar en zijn vele variabelen aanwezig die het interpreteren van de resultaten in termen van oorzaak en gevolg bemoeilijken. Een andere benadering is het gebruik van experimentele ecosystemen; ook wel microcosms en mesocosms genoemd. Microcosms en mesocosms zijn opgebouwd uit delen van natuurlijke ecosystemen, die bij elkaar gebracht zijn in een omhulsel, bijvoorbeeld een betonnen bak of een aquarium. In dit omhulsel kunnen de afzonderlijke onderdelen zich ontwikkelen tot een ecosysteem, complex genoeg in termen van functie en structuur om als vervanging te dienen voor een natuurlijk systeem. Het verschil tussen microcosms en mesocosms is hun grootte en daarmee vaak ook hun complexiteit. De EWOFFT (European Workshop On Freshwater Field Tests; Crossland et al., 1994) definieerde microcosms als experimentele systemen met een waterinhoud kleiner dan 15 m^3 of

experimentele goten korter dan 15 m, en mesocosms als systemen groter dan 15 m^3 en langer dan 15 m.

Het gebruik van microcosms en mesocosms vormt een brug tussen het laboratorium en het complexe veld. Microcosms en mesocosms zijn aan de ene kant hanteerbaar voor de onderzoekers en laten replicatie, en hiermee een experimentele opzet, toe en bieden aan de andere kant realisme voor ecologische processen en lotgevallen van de stof (Brock et al., 1995). Door replicatie is het mogelijk statistisch vast te stellen of een bepaalde concentratie van een stof een effect heeft. Hiermee kan worden bepaald of de veilige concentraties die zijn vastgesteld in de eerste trap, voldoende bescherming bieden.

Voor een goede risico-evaluatie van bestrijdingsmiddelen is het ook van belang de risico's van een normoverschrijding te bepalen. Naast het inschatten van de directe effecten is het inschatten van indirecte effecten op en eventueel herstel van populaties van belang. Terwijl duidelijk is dat directe effecten vrij goed voorspeld kunnen worden op grond van laboratoriumtoetsen (Van Wijngaarden et al., 1996), is het inschatten van de laatste twee effecten veel moeilijker. Daarom wordt in het onderzoek in de microcosms en mesocosms niet alleen gepoogd veiligheidsfactoren te valideren maar ook inzicht te krijgen in indirecte effecten en herstel van gevoelige populaties.

Behoefte aan instrumenten voor de interpretatie

Een moeilijke stap in de evaluatie van microcosm- en mesocosm-experimenten is het analyseren van de resultaten. Het bemonsteren van de verschillende gemeenschappen (bijvoorbeeld fytoplankton, zoöplankton, macro-evertebraten) levert grote datasets op, die de dynamiek van vele soorten in verschillende systemen omvatten. Deze datasets worden gewoonlijk met dezelfde univariate methodes geanalyseerd als de resultaten van laboratoriumtoetsen. Normaliter wordt voor iedere soort een NOEC berekend met een statistische test, of een EC50 met een regressieanalyse. Aangezien de meeste taxa in lage aantallen voorkomen en/of een hoge variabiliteit in aantal vertonen, kunnen meestal de effecten op slechts een beperkt aantal soorten op een bevredigende manier geëvalueerd worden (Van Wijngaarden et al., 1996).

In tegenstelling tot univariate technieken worden multivariate technieken bruikbaar geacht om alle informatie in een dataset te gebruiken (Crane et al., 1997). Deze methoden kunnen de effecten op een hoger niveau evalueren dan het soortniveau, namelijk het gemeenschapsniveau. De laatste jaren zijn verschillende pogingen gedaan om de data van microcosm- en mesocosm-experimenten op het gemeenschapsniveau te analyseren (bijvoorbeeld Leeuwangh, 1994; Van Wijngaarden et al., 1995; Shaw en Manning, 1996; Matthews et al., 1996). De technieken die zij gebruiken zijn echter relatief complex en moeilijk te begrijpen. Ook zijn de resultaten die zij geven, bijvoorbeeld in de vorm van ordinatiediagrammen, moeilijk en niet eenduidig te interpreteren.

De doelstellingen van het onderzoek

1 Het valideren van de veiligheidsfactoren die gebruikt worden in de eerste trap van de risicobeoordeling van bestrijdingsmiddelen. Dit gebeurt met behulp van microcosms en mesocosms en drie modelstoffen met verschillende werkingsmechanismen (insecticide, herbicide, fungicide).
2 Het verkrijgen van inzicht in de lange-termijnrespons van evertebraten na een eenmalige dosering van een insecticide, en het bepalen van factoren die het herstel van aangetaste soorten beïnvloeden.
3 Het evalueren van langetermijneffecten van een chronische blootstelling aan een herbicide en een fungicide op de structuur en functie van het aquatisch ecosysteem.
4 Het ontwikkelen van een multivariate techniek die het evalueren van data van microcosm- en mesocosm-experimenten op het gemeenschapsniveau vergemakkelijkt.

Modelecosystemen en bestrijdingsmiddelen die gebruikt zijn in dit proefschrift

Voor het onderzoek beschreven in dit proefschrift zijn microcosms en mesocosms gebruikt. Beide testsystemen bootsten macrofyt-gedomineerde afwateringssloten na, omdat dit type ecosysteem in Nederland veelvuldig voorkomt en vaak blootgesteld wordt aan bestrijdingsmiddelen. De microcosms bestaan uit aquaria van 1 m^3 en zijn gesitueerd in een laboratorium van de Landbouw Universiteit Wageningen (Brock et al., 1992). De mesocosms, ook wel proefsloten genoemd zijn systemen van 60 m^3 groot en liggen in de buitenlucht op het proefstation "De Sinderhoeve" te Renkum (Drent en Kersting, 1993). Het voordeel van microcosms boven de mesocosms is dat ze een grote controle over de abiotische omstandigheden (bijvoorbeeld lichtregime, temperatuur) toelaten. Ook zijn ze erg handzaam in termen van constructie en bemonstering. Het nadeel is dat sommige populaties waterorganismen in deze systemen niet te houden zijn en dat bestudering van herstel van aangetaste populaties hierdoor niet altijd mogelijk is. De mesocosms zijn vooral geschikt voor het bestuderen van het herstel van gevoelige populaties en de totale levensgemeenschap. De relatief kleine microcosms zijn geschikter om de directe en indirecte effecten van een bestrijdingsmiddel en haar ecologische drempelwaarden te onderzoeken.

Om te voldoen aan de doelstellingen van dit proefschrift zijn de effecten van drie bestrijdingsmiddelen, met verschillende werkingsmechanismen (insecticide, herbicide, fungicide), geëvalueerd in microcosms of mesocosms. Het insecticide chloorpyrifos is gekozen als modelstof voor de insecticiden. Deze stof behoort tot de organofosfaten en heeft een acetylcholine-esterase remmende werking. Dit insecticide is al gebruikt in eerdere studies, maar was nauwelijks bestudeerd met realistische concentraties en/of in voldoende detail (Van Wijngaarden et al.,1998b). Linuron is geselecteerd als modelstof voor de fotosyntheseremmende herbiciden, het type herbicide dat het meest gebruikt

wordt in Nederland (NEFYTO, 1996). Het fungicide carbendazim is gekozen omdat het in Nederland veel gebruikt wordt en er geen betrouwbare data beschikbaar waren over effecten op aquatische ecosystemen. In Tabel 2 zijn enkele relevante toxiciteitgegevens van de drie bestrijdingsmiddelen voor standaardtoetssoorten weergegeven.

Tabel 2. Samenvatting van de relevante toxiciteitdata van de bestrijdingsmiddelen voor standaardtoetsorganismen (in µg/L).

	Chloorpyrifos	Linuron	Carbendazim
LC50/EC50 *Daphnia*	1 (LC50, 48h)[a]	310 (LC50, 24h)[d]	320 (LC50, 48h)[g]
NOEC *Daphnia*	0,1 (NOEC, 21d)[a]	-	10 (NOEC, 18d)[h]
LC50/EC50 vis	4,7 (LC50, 96h)[b]	3200 (LC50, 96h)[e]	370 (LC50, 96h)[i]
NOEC vis	-	-	-
EC50 alg	> 1000 (EC50, 72h)[c]	6 (EC50, 72h)[f]	340 (EC50, 48h)[h]
NOEC alg	-	1,2 (NOEC, 72h)[f]	-

-: geen data; a = Kersting en Van Wijngaarden, 1992; b = Van Wijngaarden et al., 1993; c = Van Donk et al., 1992; d = Stephenson en Kane, 1984; e = Crommentuijn et al., 1997; f = Snel et al., 1998; g = Van Wijngaarden et al., 1998a; h = Canton, 1976; I = Palawski en Knowles, 1986

Resultaten van het onderzoek

Beoordeling van de aquatische risico-evaluatie van bestrijdingsmiddelen

De meest simpele methode om de veiligheidsfactoren die gebruikt worden in de UB (Tabel 1), te valideren is te bepalen of de normen die gesteld worden met behulp van deze factoren (de NEC die bepaald is in de eerste trap) geen effecten veroorzaken. Hiervoor werden in microcosms en mesocosms de concentraties bepaald waarvoor geen effecten aangetoond konden worden ($NOEC_{ecosysteem}$; Hoofdstukken 2, 3 en 4). Als de normen lager zijn dan de $NOEC_{ecosysteem}$ wordt in het veld ook geen effecten verwacht. Aan deze veronderstelling ligt wel ten grondslag dat de getoetste ecosystemen representatief zijn voor alle typen in het veld en dat statistisch niet-significante verschillen ook geen ecologische implicaties hebben.

In Tabel 3 zijn de normen, berekend volgens de criteria van de Uniforme Beginselen (UB-norm) en de $NOEC_{ecosysteem}$ voor een acute en chronische blootstelling voor de drie stoffen, samengevat. Tevens zijn de Nederlandse waterkwaliteitsnormen gegeven. Tabel 3 geeft aan dat de normen van de drie stoffen in alle gevallen lager of gelijk aan zijn aan de $NOEC_{ecosysteem}$ die is bepaald uit de microcosm- en mesocosm-experimenten. De UB-normen (en hiermee de veiligheidsfactoren waarop ze zijn gebaseerd) lijken het getoetste aquatisch ecosysteem te beschermen bij een acute en chronische blootstelling aan het insecticide chloorpyrifos en tevens bij een chronische

dosering van het herbicide linuron en het fungicide carbendazim (Hoofdstukken 2, 3 en 4). De waterkwaliteitnormen waren allemaal lager dan de UB-normen en bieden hiermee ook voldoende bescherming aan het getoetste aquatisch ecosysteem dat is blootgesteld aan individuele stoffen.

Een vergelijking van de laagst getoetste concentratie waarbij een effect optrad in de microcosms of mesocosms ($LOEC_{ecosysteem}$), met de UB-normen laat zien dat bij een overschrijding van de UB-norm van een factor 10, effecten niet uitgesloten kunnen worden bij een chronische blootstelling (Tabel 3; hoofdstuk 3 en 4). In het geval van een eenmalige dosering van het insecticide chloorpyrifos daarentegen, kan de UB-norm als te beschermend beschouwd worden: een tien maal zo hoge norm (veiligheidsfactor 10 in plaats van 100) lijkt ook bescherming te bieden (Tabel 3, hoofdstuk 2). Twee bestaande overzichts-rapporten over de effecten van insecticiden en herbiciden op aquatische microcosms en mesocosms lieten ook al zien dat normaliter de criteria van de eerste trap bescherming boden aan verschillende zoetwaterecosystemen tegen een blootstelling aan herbiciden en insecticiden (Lahr et al., 1998; Van Wijngaarden et al., 1998b). Voor andere stoffen zoals fungiciden kon nauwelijks enige informatie in de literatuur gevonden worden zodat de validatie van de veiligheidsfactoren voor deze groep nadere aandacht behoeft.

Tabel 3. Afgeleide UB-normen, Nederlandse waterkwaliteitnormen en NOEC- en $LOEC_{ecosysteem}$ die bepaald zijn vanuit (semi)veldexperimenten voor het insecticide chloorpyrifos, het herbicide linuron en het fungicide carbendazim (alle concentraties in µg/L). De UB-normen zijn berekend vanuit de criteria die zijn opgesteld in de eerste stap van de aquatische risicobeoordeling. De referenties van de toxiciteitwaardes zijn gegeven in Tabel 2.

	UB-norm		Nederlandse waterkwaliteit norm	$NOEC_{ecosysteem}$ / $LOEC_{ecosysteem}$	
	Acuut	Chronisch		Acute blootstelling	Chronische blootstelling
Chloorpyrifos	0,01[a]	0,01[c]	0,003	0,1 / 0,9 (Hoofdstuk 2)	0,01[d] / 0,1[e]
Linuron	0,6[b*]	0,6[b*]	0,25	- / -	0,5 / 5 (Hoofdstuk 3)
Carbendazim	3,2[a]	1[c]	0,11	- / -	3,3 / 33 (Hoofdstuk 4)

* Nederlandse norm is volgens het besluit milieutoelatingseisen bestrijdingsmiddelen (Besluit, 1995) 0,1 µg/L (0,1 x NOEC van de standaard alg); - geen data beschikbaar; [a]: 0,01 x LC50 *Daphnia*; [b]: 0,1 x EC50 Alg; [c]: 0,1 x NOEC Daphnia; [d]: data van een niet gepubliceerd experiment, Van den Brink et al., in voorbereiding; [e]: data uit Van den Brink et al., 1995

Ecologische effecten en herstel

De tweede doelstelling van dit proefschrift is inzicht te krijgen in de respons van gemeenschappen op de lange termijn en in de factoren die het herstel van aangetaste populaties bepalen na een eenmalige toediening van een insecticide in mesocosms (hoofdstuk 2). De chloorpyrifos bespuiting resulteerde, zoals verwacht vanuit het

werkingsmechanisme, in grote negatieve effecten op de taxa behorende tot de Arthropoda. Aangezien dit een experiment was in relatief grote, buiten gesitueerde systemen kon herstel van aangetaste populaties bestudeerd worden. Het herstel van individuele soorten was in grote mate afhankelijk van de karakteristieken van haar levenscyclus, zoals aantal generaties per jaar, het hebben van resistente levensstadia en de mogelijkheid om van de ene sloot naar de andere te migreren.

Dit is geïllustreerd in hoofdstuk 2 aan de hand van de respons van de larven van twee eendagsvliegen, de watervlooien en een vlokreeft. De larven van de twee eendagsvliegen *Cloeon dipterum* en *Caenis horaria* hebben geen levensstadia die ongevoelig voor chloorpyrifos zijn, maar kunnen van de ene sloot naar de andere migreren. In het laboratorium zijn ze beiden ongeveer even gevoelig voor het insecticide, maar ze hadden in de mesocosms een totaal verschillend herstelpatroon. *Cloeon* was al 12 weken na de belasting in de hoogste dosering hersteld, terwijl dit bij *Caenis* 24 weken duurde. Een verklaring hiervoor is het verschil in aantal generaties per jaar tussen de twee soorten. *Cloeon dipterum* heeft verschillende generaties per jaar en kan dus de sloot snel herkoloniseren wanneer de chloorpyrifosconcentratie dat toelaat. *Caenis horaria* daarentegen heeft maar één generatie per jaar en herstel kan pas plaatsvinden wanneer de volgende generatie het jaar erop de sloten herkoloniseert.

In tegenstelling tot eendagsvliegen zijn watervlooien niet in staat om actief van de ene sloot naar de andere te migreren. Desondanks vertoonden zij een zeer snel herstel in de hoogst belaste sloten. Dit zou kunnen komen omdat zij een korte generatietijd hebben alsook een levensstadium dat resistent is tegen chloorpyrifos, nl. wintereieren. Wanneer een soort een aangetast systeem niet kan herkoloniseren en ook geen ongevoelige levensstadia bezit kan een soort lokaal uitsterven in geïsoleerde systemen als de mesocosms. De vlokreeft *Gammarus pulex* bezit geen van beide eigenschappen en werd inderdaad niet meer aangetroffen in de twee hoogste doseringen tot aan het eind van de onderzoeksperiode, 55 weken na de toediening. Met uitzondering van deze vlokreeft werden 24 weken na de bespuiting geen statistisch significante effecten op de evertebratengemeenschap in de proefsloten meer aangetoond.

De derde doelstelling was om de langetermijneffecten van een herbicide en een fungicide op de structuur en functie van zoetwaterecosystemen te bepalen. In een microcosmstudie lieten de biomassa van de hogere waterplant *Elodea nuttallii* en de aantallen van vele soorten algen een duidelijke afname zien op de hogere behandelingsniveaus van het fotosyntheseremmende herbicide linuron (hoofdstuk 3). Het zuurstofgehalte en de pH van het water daalden ook in de lagere doseringen als gevolg van een afname in fotosynthetische activiteit. Ondanks dat vele algensoorten een afname in aantallen lieten zien, nam toch het chlorofyl-a van het fytoplankton, het perifyton en het neuston toe. Deze toename in chlorofyl-a was toe te wijzen aan één algentaxon, namelijk de groenalg *Chlamydomonas* sp. Dit taxon bleek relatief ongevoelig voor linuron en had de

eigenschap om binnen een week een tolerantie op te bouwen voor relatief hoge concentraties. Door zowel de relatieve ongevoeligheid voor het herbicide als de afgenomen concurrentie om nutriënten met waterplanten en gevoelige algen kon *Chlamydomonas* sp. snel in aantal toenemen. Hierdoor veranderde de levensgemeenschap van vooral de hoogst belaste microcosms van een waterplanten-gedomineerd naar een algen-gedomineerd systeem. De watervlooien (Cladocera) en roeipootkreeftjes (Copepoda) profiteerden van deze toename van voedsel en kwamen in hogere aantallen voor in de hoogste behandelingen vergeleken met de controle-microcosms. De evertebraten die hogere waterplanten als habitat gebruiken lieten als gevolg van de afname van plantenbiomassa ook een afname in aantallen zien.

Het fungicide carbendazim behoort tot de benzimidazolen en tast micro-organismen en wormen aan. Dit verklaart de effecten op wormachtige taxa behorende tot de vrijlevende platwormen (Turbellaria) en borstelwormen (Oligochaeta), maar dit kon niet de effecten op groepen evertebraten als vlokreeften (Amphipoda), slakken (Gastropoda) en watervlooien (Cladocera) verklaren (hoofdstuk 4). Dus in tegenstelling tot chloorpyrifos en linuron lieten de effecten van carbendazim zich niet geheel verklaren door de relatie van de aangetaste soorten met de doelorganismen van de stof. Het fungicide carbendazim bleek in de microcosms meer te werken als een biocide dan als een stof met een meer specifieke werking. Doordat vele evertebraten in aantal afnamen, nam ook de begrazing van het fytoplankton af en dit resulteerde bij de twee hoogste doseringen in een toename van enkele algensoorten en het chlorofyl-a-gehalte van het water.

In de literatuur zijn de "eutrofiërende" gevolgen van insecticiden en herbiciden in oppervlaktewater uitvoerig beschreven en bediscussieerd (Lahr et al., 1998; Van Wijngaarden et al., 1998b). De effecten van fungiciden zijn nauwelijks onderzocht, maar hoofdstuk 4 geeft aan dat ook een blootstelling aan een fungicide een verhoging van de algendichtheid tot gevolg kan hebben. Dus ook al verschillen de mechanismen, alle drie typen bestrijdingsmiddelen kunnen bijdragen aan symptomen van eutrofiëring. De bijdrage van realistische concentraties van deze bestrijdingsmiddelen aan dit fenomeen is helaas nog nauwelijks onderzocht.

Instrumenten voor de evaluatie van microcosm- en mesocosm-experimenten

De vierde doelstelling van dit proefschrift was een nieuw multivariaat instrument voor de analyse van behandelingseffecten op het gemeenschapsniveau te ontwikkelen. Deze technieken worden nog nauwelijks toegepast in de ecotoxicologie, terwijl ze in de ecologie al geruime tijd worden gebruikt voor de analyse van de structuur van gemeenschappen en hun relatie met het milieu. De meest gebruikte multivariate methode in de ecologie is de ordinatietechniek correspondentieanalyse. Deze is gebaseerd op een

unimodaal model. Soorten vertonen, daarentegen, in de regel geen unimodale respons langs de milieugradiënt van een toxische stof. Uit laboratoriumexperimenten weten we dat de relatie tussen de respons en de concentratie van een toxische stof meestal een sigmoïde verband vertoont. Een lineair model benaderd een sigmoïde curve beter dan een unimodaal model (hoofdstuk 7).

In hoofdstuk 2 en 3 is Redundantie Analyse (RDA) gebruikt om de effecten op gemeenschapsniveau te analyseren. RDA is de gedwongen vorm van de bekende ordinatie-techniek hoofdcomponentenanalyse (Principal Component Analysis, PCA) en is gebaseerd op een lineair model (Jongman et al., 1995). Gedwongen betekent dat de analyse beperkt wordt tot de variantie die geselecteerde milieuvariabelen verklaren. In hoofdstuk 2 en 3 is de analyse gedwongen tot de variantie die uitsluitend de behandeling, tijd en hun interactie verklaren. Uit de analyses blijkt dat RDA de effecten op een gemeenschap met succes in een diagram samenvat, en erg bruikbaar was in combinatie met permutatietoetsen voor de bepaling van de significantie van de behandelingseffecten. Kersting en Van den Brink (1997) vonden echter dat in hun geval RDA helaas leidde tot een moeilijk te interpreteren diagram.

In hoofdstuk 5 wordt een nieuwe methode, de Principal Response Curve methode (PRC), geïntroduceerd die bovengenoemd probleem van RDA oplost. PRC is gebaseerd op RDA en extraheert de eerste hoofdcomponent uit de variatie die verklaard wordt door de behandeling. Hierbij wordt het deel van de variatie dat door tijd en door verschillen tussen replica's verklaard wordt buiten beschouwing gelaten. Het gevolg is een diagram dat alleen de afwijkingen van de behandelingen ten opzichte van de controle in de tijd laat zien, het eerste responspatroon. In Figuur 3 van hoofdstuk 5 wordt de PRC gegeven van de evertebratengemeenschap van het experiment dat in hoofdstuk 2 is beschreven. De figuur laat niet alleen duidelijk de concentratie-afhankelijke afwijkingen van de behandelingen t.o.v. de controle op gemeenschapsniveau in de tijd zien, maar laat ook een directe interpretatie op soortniveau toe. Dit is een groot voordeel ten opzichte van vele andere methoden, bijvoorbeeld de veel gebruikte similariteitsindices (Hoofdstuk 6). In hoofdstuk 6 is het tweedimensionale model van PRC beschreven. Tweedimensionaal betekent dat na de extractie van het eerste responspatroon, een tweede wordt geëxtraheerd. Dit tweede responspatroon geeft de meest belangrijke afwijkingen ten opzichte van het eerste patroon weer. Het tweede responspatroon wordt van belang wanneer een dataset niet één dominant responspatroon bevat maar verschillende subdominante. In hoofdstuk 6 is dit geïllustreerd aan de hand van een evertebraten- en fytoplanktondataset van een experiment waarin twee stressoren werden toegediend; het insecticide chloorpyrifos en nutriënten. De eerste PRC van de evertebratengemeenschap laat duidelijk de respons op de toediening van chloorpyrifos zien, de tweede PRC duidelijk de respons als gevolg van de nutriëntentoedieningen (zie Figuur 3A en 3B van hoofdstuk 6). Dit hoofdstuk toont aan dat het met PRC mogelijk is om een aantal verschillende responspatronen samen te vatten in twee diagrammen.

Microcosm- en mesocosm-experimenten worden vaak vanwege hun ecologische variabiliteit en ruis van beperkte waarde beschouwd. De experimenten en statistische instrumenten, die zijn beschreven in dit proefschrift, tonen aan dat ondanks deze ruis duidelijke responspatronen verkregen kunnen worden als de experimenten maar goed opgezet en geanalyseerd worden. De hoofdstukken 2, 3 en 4 illustreren dat zelfs met een beperkt aantal replica's een ecologische effectketen en een ecologische drempelwaarde verkregen kunnen worden.

Suggesties voor toekomstig onderzoek

Bij de normale landbouwkundige praktijk is het voor de bescherming van het gewas meestal niet voldoende om één bestrijdingsmiddel te gebruiken, maar worden er verschillende bestrijdingsmiddelen toegepast voor de onderdrukking van de ongewenste organismen. Sommige bestrijdingsmiddelen worden ook herhaaldelijk toegediend in de tijd. De effecten van combinaties van bestrijdingsmiddelen op zoetwaterecosystemen zijn echter nog nauwelijks onderzocht (Hartgers et al., 1998). Daarom is het van belang criteria te ontwikkelen voor de ecologische risicobeoordeling van realistische mengsels van bestrijdingsmiddelen.

Het probleem van mengseltoxiciteit wordt zelfs groter wanneer ook andere agrochemicaliën, zoals kunstmest, in ogenschouw genomen worden. Er is weinig bekend van de combinatiewerking van vermesting en verontreiniging door bestrijdingsmiddelen. Wel is te verwachten dat de trofische status van een ecosysteem de effecten van bestrijdingsmiddelen zal beïnvloeden (hoofdstuk 6; Kramer et al., 1997).

De ecologische effectketens, die gevonden zijn in de experimenten uitgevoerd met linuron en carbendazim, geven aan dat microcosm- en mesocosm-experimenten een bruikbaar instrument zijn om de trofische interacties in het zoete water te onderzoeken. Op dit moment worden de resultaten van deze experimenten gebruikt voor de ontwikkeling van een voedselwebmodel (Traas et al., 1998a, 1998b). Verondersteld wordt dat deze modellen kunnen bijdragen aan een beter begrip van het functioneren van aquatische ecosystemen en uiteindelijk een instrument kunnen worden waarmee de ecologische effecten van stoffen voorspeld kunnen worden (Gezondheidsraad, 1997). De grootste obstakels hierbij zijn het gebrek aan betrouwbare parameterwaarden (bijvoorbeeld consumptie, sterfte) en het gebrek aan validatie van de modellen. Dit betekent dat voor een verdere ontwikkeling van deze voedselwebmodellen onderzoek naar parameterwaarden in het laboratorium en (semi)veldonderzoek voor het verzamelen van validatie-datasets van belang is (Gezondheidsraad, 1997).

Het modelleren van directe effecten en herstel op populatieniveau kan van groot belang zijn voor het bepalen van de risico's en het rangschikken van bestrijdingsmiddelen op basis van effecten. In de toekomst kunnen modellen die de behandelingseffecten en herstel-patronen beschrijven een grote rol spelen als onderzoeksinstrument en als

beleidsondersteunend instrument. Modellen hebben het voordeel dat zij een integratie van ecologische en ecotoxicologische kennis toelaten, iets wat tot een paar jaar geleden grotendeels afwezig was in de ecotoxicologie. De ontwikkeling van deze modellen stelt ons ook in staat om microcosm- en mesocosm-experimenten beter te analyseren.

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Curriculum vitae

Paul van den Brink was born in Oss, the Netherlands, on June 21, 1968. Between 1980 and 1986 he was enrolled at Titus Brandsma Lyceum (VWO) in Oss. After his graduation in 1986 he moved to Wageningen where he started to study Environmental Sciences at the Wageningen Agricultural University (WAU). In 1992 he graduated for his M.Sc. in Environmental Sciences. During this period, he conducted 5-months practical research periods at the Department of Aquatic Ecology and Water Quality Management (WAU) (supervisor: Drs. J.J.P. Gardeniers), at the DLO Winand Staring Centre in collaboration with the Department of Toxicology (supervisor: Ir. W.G.H. Lucassen) and at the Brawijaya University (UNIBRAW, Malang, Indonesia) (supervisor: Dr. J.H. van Weerd). From 1992 onwards he holds a position at the DLO Winand Staring Centre as a Scientific Researcher. Part of the research he conducted during this period is described in this thesis.

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