



**MICHIEL ADRIAAN
DAAM**

**INFLUÊNCIA DE FACTORES CLIMÁTICOS E DA
COMPLEXIDADE DE MICROCOSMOS NO DESTINO E
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MICROCOSM COMPLEXITY ON THE FATE AND
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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Dr. António José Arsénia Nogueira, Professor Associado com Agregação do Departamento de Biologia da Universidade de Aveiro e Dr. Paulus Johannes van den Brink, Investigador Principal do Alterra, Wageningen University and Research centre, Holanda.

Apoio financeiro do POCTI no âmbito
do III Quadro Comunitário de Apoio.

Apoio financeiro da FCT e do FSE no
âmbito do III Quadro Comunitário de
Apoio através de uma bolsa de
doutoramento ref.
SFRH/BD/8213/2002.



Para a Ana e a Adriana

O júri

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Agradecimientos

Although there is only one name written on the cover of this manuscript, it was of course not all done by me... Therefore, I would like to acknowledge the following people.

First of all, my supervisors António Nogueira (University of Aveiro, Portugal) and Paul Van den Brink (Alterra, The Netherlands) for all their support throughout my PhD and their patience in the writing phase of this thesis. Paul, many thanks for the statistical analysis you did for me!

During the work at the Asian Institute of Technology (AIT, Thailand), I received support from many people. I would like to thank Dr. Amara for having me at AIT and Kriengkrai Satapornvanit for all his help in any case I needed. Pe Manot and Phongnakhorn Yang for running the nutrient analysis and Kuhn Pond for general laboratory assistance. I am indebted to all the co-workers of the hatchery for helping me cleaning and filling the microcosms with sediment and water from the AIT canal. Not the most favourable work, but never a single complain... Kuhn Tinwin from the Environmental Engineering department for assistance in the HPLC and GC work and the IREM Laboratory for providing me with the weather data from the meteorology station on AIT. Khob khun kap!!!

Prof. Wongat (Kasetsart University, Thailand) and Prof. Chitlom and his co-workers (University of Chiang Mai, Thailand) are acknowledged for an introduction in the identification of the Thai plankton and macroinvertebrates, respectively. Steven Crum (Alterra, The Netherlands) broke the Guinness World Records by always replying emails regarding the development of analytical methods within 24 hours: bedankt!

Yuwadee Peerapornpisal (University of Chiang Mai, Thailand), Nuansri Tayaputch (Department of Agriculture, Thailand), La-orsri Sanoamuang (Khon Kaen University, Thailand), Kriengkrai Satapornvanit (Kasetsart University, Thailand) and René Van Wijngaarden (Alterra, The Netherlands) are acknowledged for sending reprints of manuscripts.

I am grateful to António Rodrigues (New University of Lisbon) for allowing me to use his inversed microscope for plankton identifications. Last, but not least, I would like to thank my friends and family, especially my parents, for their support and patience. Ana and Adriana, my lovely ladies, this work is dedicated to you.

Palavras-chave

meio aquático, pesticida, risco de impacte, microcosmos.

Resumo

Apesar dos estudos de microcosmo e mesocosmo terem um papel importante no procedimento de registo dos pesticidas, a extensão pelos quais os resultados dos diferentes estudos de modelos de ecossistema poderem ser extrapolados para outros casos é ainda um assunto de debate. Esta tese pretende contribuir para a discussão sobre a influência local, temporal e dos factores metodológicos nos resultados dos estudos do microcosmo. Para este efeito, foram efectuados estudos do microcosmo com água doce em condições experimentais distintas e os efeitos do tratamento e o destino comparados com os reportados em experiências similares.

As experiências com pequenos microcosmos laboratoriais com aplicações únicas de clorpirifos, linurão e carbendazim nem sempre previram as respostas exactas, tal como observado em experiências com modelos de ecossistema em larga escala. Uma vez que os sistemas utilizados eram fechados e não continham sedimento nem macrófitas, os pesticidas eram mais persistentes e os valores de toxicidade calculados tornaram-se mais comparáveis com os estabelecidos nas experiências com exposição prolongada. As implicações e as recomendações para a metodologia de estudos de avaliação de risco aquático são discutidos na secção discussão geral.

Uma experiência do microcosmo na Tailândia, lidando com múltiplas aplicações de clorpirifos, conduziu à conclusão que o tempo de aplicação tem uma elevada influência nos efeitos do insecticida nas comunidades de água doce. Isto é explicado em relação às fases da população das comunidades de zooplâncton no momento da aplicação.

Os valores de toxicidade calculados nos estudos do microcosmo tropical depois de aplicações únicas de pesticida estavam dentro da gama (clorpirifos e carbendazim) ou mais elevado (linurão) que os reportados em estudos temperados. Assim, estes resultados suportam o uso de dados de toxicidade de estudos de ecossistemas modelo levados a cabo em zonas temperadas para a avaliação de risco ambiental em países tropicais.

Keywords

Aquatic, pesticide, risk assessment, microcosms.

Abstract

Although micro- and mesocosm studies play an important role in the registration procedure of pesticides, the extent by which the results of different model ecosystem studies may be extrapolated to one another is still a matter of debate. This thesis aims to contribute to the discussion concerning the influence of spatial, temporal and methodological factors on the outcome of microcosm studies. For this purpose, freshwater microcosm studies were carried out under different experimental conditions and fate and treatment effects compared with those reported in similar experiments.

Small indoor microcosm studies with single applications of chlorpyrifos, linuron and carbendazim did not always predict the exact responses as was observed in larger-scale model ecosystem studies. Since closed systems were used that did not contain sediment and macrophytes, pesticides were more persistent and calculated toxicity values were therefore generally more comparable with those reported in studies with long-term exposure. Implications and recommendations for the methodology of aquatic risk assessment studies are discussed in the general discussion section.

A microcosm study in Thailand dealing with multiple chlorpyrifos applications led to the conclusion that the time of application has a large influence on the effects of the insecticide on freshwater communities. This is explained in relation to the population phase of zooplankton communities at the time of application.

Threshold values calculated in tropical microcosm studies after single pesticide applications were well in range (chlorpyrifos and carbendazim) or higher (linuron) than those reported in temperate studies. These findings thus support the use of toxicity data from model ecosystem studies carried out in the temperate zone for the environmental risk assessment in tropical countries.

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CHAPTER 1

GENERAL INTRODUCTION

With the modernization and intensification of agricultural practices in the past century, the use of pesticides was initiated to increase productivity of yields. As a consequence, adjacent water courses surrounding agricultural fields were reported to become contaminated via spray drift, drainage and run-off and/ or accidental spills (Capri and Trevisan, 1998). To protect sensitive freshwater ecosystems against pesticide side-effects, jurisdictions of many countries have set water quality criteria and started to require a prospective Environmental Risk Assessment (ERA) before registration of a pesticide (e.g., EU: EU, 1997; US: Zeeman and Gilford, 1993; Canada: Environment Canada, 1997).

Tiered risk assessment approach

In the past decades, a wide array of aquatic toxicity tests have been developed and applied to determine side effects posed by chemicals like pesticides on freshwater community structure and functioning.

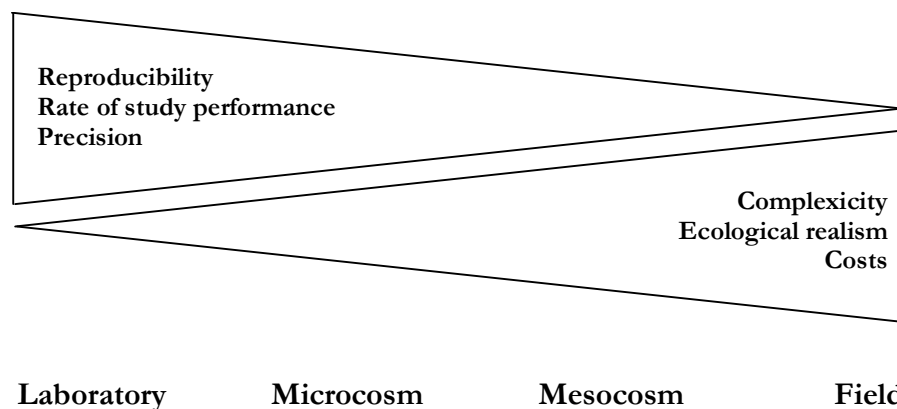


Figure 1.1 Different levels of ecotoxicological testing and their advantages and shortcomings; microcosms and mesocosms provide a bridge between the laboratory and the field (Picture redrawn from Brock et al., 2000a).

These tests range from relatively simple laboratory bioassays to large complex field studies (Figure 1.1). Evaluating the fate and effects under field conditions may be considered ideal for an ecological realism point of view. On the other hand, such studies are rather costly and the higher level of complexity implies that causal-effect relationships are more difficult to establish. Therefore, the use of tiers or steps in the process of criteria setting and ERA has frequently been recommended in risk assessments of pesticides (e.g., Suter et al., 1993; Campbell et al., 1999).

The initial use of conservative (lower or first-tier) assessment criteria allows substances that do not present a risk to be eliminated from the risk assessment early, thus allowing the focus of resources and expertise on more problematic substances. From lower to higher tiers, the exposure and effect estimates become more realistic and hence, the uncertainty in the extrapolation of effects is reduced (Solomon et al., accepted). In the EU, for example, the first-tier conservative ERA as laid down in the Uniform Principles for the registration of pesticides on the market is based on the ratio of the *toxicity* (NEC: No Effect Concentration) and the predicted *exposure* (PEC: Predicted Environmental Concentration. EU, 1997). The PEC is usually calculated using computer programs like TOXSWA (Adriaanse, 1996), using pesticide characteristics, the recommended pesticide dose and a simulated landscape scenario as input parameters. The NEC is based on toxicity threshold values from laboratory bioassays with a limited number of standard test species, usually *Daphnia*, an algae and a fish. Since the threshold value is normally a point estimate derived from a concentration-response series, an uncertainty factor (UF) is applied to protect against lower concentrations of response in the same organism as well as the possibility of higher sensitivity of other non-tested sensitive aquatic organisms. The UF in the EU is usually 100 for acute L(E)C50 values and 10 for chronic NOECs, whereas the U.S. EPA uses UFs from 1 to 20 depending on the response measured (EU, 1997; Solomon et al., accepted).

If this first-tier risk assessment indicates potential risks, ecologically more relevant approaches are needed in a higher-tier ERA to assure an adequate protection of aquatic life. Additional information needed depends on the uncertainty in the first-tier risk assessment and may thus range from a better estimation of the sensitivity of susceptible aquatic organisms to a more realistic calculation of the exposure to the chemical (Van den Brink, 1999).

The use of microcosms and mesocosms in ERA

Microcosm and mesocosm experiments have frequently been performed for a higher-tier risk assessment of pesticides (Campbell et al., 1999; see Brock et al., 2000a and Van Wijngaarden et al., 2005 for recent reviews). Microcosms and mesocosms provide a bridge between laboratory and the field, in that they allow replication and hence an experimental set-up on the one side and provide ecological realism on the other side (Figure 1.1; Brock *et al.*, 2000a). The difference between microcosms and mesocosms is their size and hence often their complexity (Van den Brink, 1999). Microcosms are man-made test systems with a water volume of less than 15 m³ or experimental streams less than 15 m in length, while mesocosms are defined as model ecosystems containing more than 15 m³ water or experimental streams longer than 15 m (Crossland *et al.*, 1992).

Unlike the first-tier laboratory test procedures, which are highly protocolized in for instance OECD and US EPA guidelines (OECD, 2006; US EPA, 1996), no defined general protocol exists for model ecosystems. The most standardized test systems are of the “generic” type, which do not mimic any natural system in particular, but rather bring together some basic components of ecosystems. Most aquatic model ecosystems used for effect evaluations of pesticides are of the “semi-realistic” type and are intended to mimic real ecosystems as discussed by Van Wijngaarden (2006). This author also stipulates that this type of test systems may be further classified according to the type of freshwater ecosystem they represent (e.g. tropical vs. temperate and plankton- vs. macrophyte-dominated), and whether they are situated indoors or outdoors. For outdoor model ecosystems, a distinction may be made between constructed systems, e.g. concrete or glass-fiber tanks, and enclosed parts of existing ecosystems, e.g. plastic bags or polycarbonate cylinders (Van Wijngaarden, 2006).

Due to this large diversity in microcosm and mesocosm test systems, toxic effects and recovery of freshwater communities in different model ecosystem studies testing the same compound may be different (e.g. chlorpyrifos: Brock et al., 1992 a, 1992b, 1993; Leeuwangh, 1994; Leeuwangh et al., 1994, Caquet et al., 2001, Van Wijngaarden et al., 2005). Thus, although model ecosystem approaches provide ecologically more realistic assessments, interpretation and extrapolation to other (model) ecosystem types may be problematic (Boxall et al., 2002). Evaluation and interpretation of these studies has become the subject of wide-ranging discussions (ECETOC, 1997; Campbell et al., 1999; Giddings et al., 2002; Van Wijngaarden, 2006).

For example, the issue of model ecosystems scale has been recognized as a critical issue and underpins conceptual frameworks of how we understand ecologic phenomena (Flemer et al. 1997). Although larger and consequently more complex model ecosystems may be ecologically more realistic than smaller systems, smaller model ecosystems are easier to replicate and are less costly (Figure 1.1). The development of reliable smaller test systems was therefore recommended by the European Workshop on Freshwater Field Tests (Crossland et al., 1994) and the Higher-tier Aquatic Risk Assessment for Pesticides (HARAP) workshop (Campbell et al. 1999). However, only a small number of reliable small test systems have been used so far for pesticide risk assessment (Leeuwangh et al. 1994; Brock et al. 2000a, 2000b).

Recently, the question to what extent ecotoxicological data can be extrapolated from one geographical region to another has been raised (Van Wijngaarden, 2006; Kwok et al., 2007; Brock et al., accepted). The utmost importance of this may be stressed by the fact that ecotoxicological research has mainly focused on temperate regions, while little is known about the fate and effects of chemicals like pesticides in tropical regions (Bourdeau et al., 1989; Castillo et al., 1997; Lam and Wu, 1999; Gopal, 2005; Racke, 2003). Consequently, many tropical water quality criteria rely on extrapolations from temperate data even though physical and chemical environmental parameters in the tropics can be very different (Jungbluth, 1996; Lacher and Goldstein, 1997; Kwok et al., 2007). There is thus an urgent need to validate whether ecotoxicological principles and data developed in the temperate zone are applicable to countries in the tropical zone.

Besides the above mentioned differences in experimental design of the test systems used and geographical (spatial) factors, variation in study outcomes may be due to temporal factors like seasonal (Willis et al., 2004; Van Wijngaarden et al., 2006) or successional (Hanazato and Yasumo, 1990) differences in the state of the freshwater communities at the time of the pesticide treatment. Thus, although model ecosystems studies play an important role in the registration procedure of pesticides (EU, 1997; Campbell et al., 1999), the extent by which the results of different model ecosystem studies may be extrapolated to one another is still largely unknown.

Aims of the thesis

This thesis aims to contribute to the discussion concerning the influence of spatial, temporal and test system scale factors on the outcome of model ecosystem studies. Four specific aims are distinguished:

- 1) To validate the use of small indoor test microcosms for the risk assessment of pesticides;
- 2) To gain insight in the fate and effects on ecosystem structure and functioning following single-peak pesticide stress under tropical semi-field conditions;
- 3) To evaluate the influence of repeated insecticide exposure on ecotoxicological effects in a tropical (model) ecosystem;
- 4) Ultimately, to quantify to which extent temperate toxicity data may be used for tropical ERA based on obtained results (under 2 and 3).

Benchmark compounds and model ecosystems

To the ends described in the previous section, case studies were performed with three pesticides with different modes of action, namely:

- i) the acetylcholinesterase inhibiting insecticide chlorpyrifos,
- ii) the beta-tubulin synthesis inhibiting fungicide carbendazim, and
- iii) the photosynthesis inhibiting herbicide linuron.

These compounds were chosen because they are used worldwide (FAO/WHO, 1995, 2005; Sørensen et al., 2005) and larger-scale model-ecosystem studies carried out in temperate countries are available as references (Table 1.1). Treatment levels in the experiments presented in this thesis were chosen based on (threshold) treatment levels in these reference studies to ensure the inclusion of a sufficiently low concentration and to facilitate comparison of obtained test results.

The pesticides were evaluated with three different test systems. Small 8.5-liter microcosms situated in a laboratory at Alterra (Wageningen, The Netherlands) were used to address the first aim (Figure 1.2). The microcosms were maintained under controlled conditions of 21 ± 1 °C with an artificial 14 hours daily photoperiod using fluorescent lamps resulting in a light intensity of approximately $45 \mu\text{E}/\text{m}^2\cdot\text{s}$ in the middle of the chambers. For the second and third aim of this thesis, circular and rectangular microcosms were set up outdoors at the

hatchery of AIT (Asian Institute of Technology) in Thailand (Figure 1.2). These freshwater (model) ecosystems were thus subject to the tropical climatically conditions in the period the experiments were performed as presented in figure 1.3.

Table 1.1 Toxicity data (in µg/L) from first-tier laboratory toxicity tests with standard test species and higher-tier model ecosystem studies carried out in temperate countries.

	Chlorpyrifos	Carbendazim	Linuron
<u>Laboratory toxicity tests</u>			
L(E)C ₅₀ <i>Daphnia</i>	1 (LC50, 48h)	320 (LC50, 48h)	310 (LC50, 24h)
NOEC <i>Daphnia</i>	0.1 (NOEC, 21d)	25.8 (NOEC, 25d)	-
Reference	Kersting and Van Wijngaarden, 1992	Van Wijngaarden et al., 1998	Stephenson and Kane, 1984
L(E)C ₅₀ algae	> 1000 (EC50, 72h)	340 (EC50, 48h)	6 (EC50, 72h)
NOEC algae	-	-	1.2 (NOEC, 72h)
Reference	Van Donk et al., 1992	Canton, 1976	Snel et al., 1998
L(E)C ₅₀ fish	4.7 (LC50, 96h)	370 (LC50, 96h)	3200 (LC50, 96h)
NOEC fish	-	-	-
Reference	Van Wijngaarden et al., 1993	Palawski and Knowles, 1986	Crommentuijn et al., 1997
<u>Model ecosystem studies*</u>			
(NOEC – LOEC)			
Single-peak exposure	0.1 – 0.9	2.2 – 21	<20 - 20
Test system	Outdoor macrophyte-dominated experimental ditches	Outdoor Microcosms**	Outdoor Microcosms***
Reference	Van Wijngaarden et al., 1996; Van den Brink et al., 1996; Kersting and Van den Brink, 1997	Slijkerman et al., 2004	Slijkerman et al., 2005
Chronic exposure	0.01 – 0.05	3.3 – 33	0.5 – 5
Test system	Indoor plankton-dominated microcosms	Indoor macrophyte-dominated microcosms	Indoor macrophyte-dominated microcosms
Reference	Cuppen et al., 2002; Van den Brink et al., 2002	Cuppen et al., 2000; Van den Brink et al., 2000	Van den Brink et al., 1997; Cuppen et al., 1997

* For more reference model ecosystem threshold values for the pesticides tested: see the corresponding chapters and the general discussion. ** Not mentioned whether macrophytes were present or not. *** Two bunches of the macrophyte *Elodea* sp. were deployed the day before dosing. Presence of other macrophytes not mentioned.



Figure 1.2 Overview of the microcosms used: small laboratory microcosms at Alterra (left), outdoor circular (top right) and rectangular (bottom right) microcosms in Thailand.

Climatically conditions during the microcosms experiments in Thailand

The tropical climate in Thailand is regulated primarily by the monsoon winds that produce three seasons: the cool, hot and rainy season. From May through mid-October the Southwest Monsoon brings warm moist air across Southeast Asia. The surface temperature of the land is higher than the arriving air mass, resulting in thunderstorm formations and the start of the rainy season (June – October). Rainfall is intense but of short duration, accompanied by much lightning and high winds. The rainfall has a moderating effect on the air temperature and direct sunlight is often blocked by cloud cover. Except immediately before and during thunderstorms, surface winds are very light during the wet monsoon. Beginning in July, rainfall of longer duration and greater regularity replaces the less dependable thunderstorm

precipitation. In mid-October the Southwest winds are replaced by winds from the Northeast, which brings cool, dry air from Central Asia across Thailand. The rains abruptly cease, and the cool season (November – February) follows. The air becomes clear and the direct sunlight warms the earth rapidly. The cool season is followed by the hot season (March – May). A gradual decrease in wind velocity begins in February, leading eventually to a period of air stagnation and high daytime temperatures. The hot season ends with the arrival of the Southwest Monsoon in May. This annual seasonal cycle occurs with great regularity (Heckman, 1979).

The experiments evaluating single and repeated chlorpyrifos applications were carried out from mid July till the end of October 2003. These experiments were thus carried out during the rainy season, with average daily temperatures of 29 ± 0.6 °C and a cumulative rainfall of 116 mm. Sunlight was often blocked by the relatively high cloud cover values (4.8 ± 1.7 octas), resulting in a radiation levels of 18.8 ± 2.5 mJ/m². Linuron and carbendazim were evaluated in Thailand between mid-January and the end of April 2005, so the experimental period covered a part of the cool season (January – February) and a part of the hot season (March - April). Average daily temperatures increased accordingly in the course of the experiments from 24 °C at the start to 33 °C by the end of the experiments (Figure 1.3). To compensate for the preceding unusual dry period, the cloud seeding technique described in European Patent Office (2004) was applied in the first semester of 2005 by the Thai government to artificially produce rain. Consequently, cloud cover showed a rather high variation and ranged from 1.4 to 6.7 octas, accompanied with low radiation levels for the time of the year (15.1 ± 2.7 mJ/m²).

Outline of the thesis in relation to the aims set

Chapter 2 evaluates the effects of a single application of chlorpyrifos (insecticide), carbendazim (fungicide) and linuron (herbicide) on the ecology of a small indoor microcosm. Treatment effects are compared with effects observed in larger scale model ecosystem studies and discussed in an ecotoxicological and methodological context (aim 1).

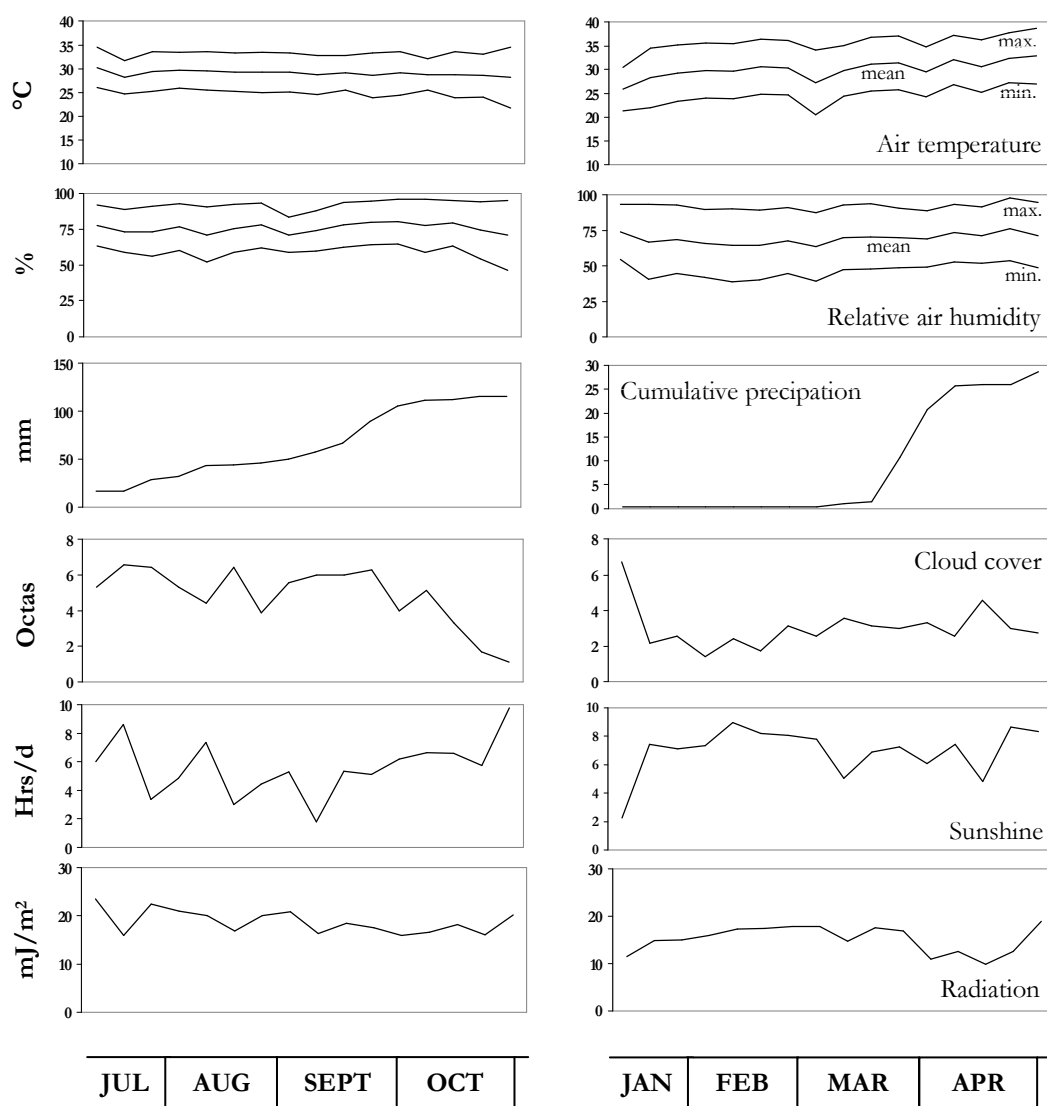


Figure 1.3 Meteorological conditions and physical/chemical water characteristics during the course of the experiments in 2003 (left) and 2005 (right). Data were obtained from the meteorological station at AIT.

Chapters 3, 5, 6 and 7 present the results of the microcosm studies in Thailand with single applications of chlorpyrifos, carbendazim and linuron. Direct and indirect effects on ecosystem structure and functioning are discussed. Study findings are compared with studies evaluating single applications of these pesticides in temperate regions to determine the influence of spatial factors on the toxic effect of chlorpyrifos on ecosystem functioning (aim 2).

Chapter 4 deals with the outcomes of a microcosm study in Thailand with multiple chlorpyrifos applications. The impact of temporal factors on the ecotoxicology of chlorpyrifos under tropical climatic conditions is discussed in the discussion section of this chapter (aim 3).

Chapter 8 discusses the contents of the other chapters and the extent by which temperate first-tier and higher-tier toxicity data may be used for tropical ERA (aim 4). Concluding remarks and suggestions for future research are also made in this chapter.

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CHAPTER 2

EFFECTS OF CHLORPYRIFOS, CARBENDAZIM AND LINURON ON THE ECOLOGY OF A SMALL INDOOR AQUATIC MICROCOSM

(Published in *Archives of Environmental Contamination and Toxicology* 53(1): 22-35.)

Abstract

To validate the use of small indoor microcosms for the risk assessment of pesticides, the fate and effects of chlorpyrifos, carbendazim and linuron were studied in 8.5-liter indoor freshwater microcosms. Functional and structural responses to selected concentrations were evaluated and compared to responses observed in larger-scale model ecosystem studies.

Overall, the microcosms described the chain of effects resulting from the application adequately, although they did not always predict the exact fate and responses that were observed in larger semi-field studies. Since closed systems were used that did not contain sediment and macrophytes, pesticides were relatively persistent in the present study. Consequently, calculated toxicity values were generally more comparable with those reported in studies with long-term than short-term exposure. Carbendazim had a higher overall NOEC compared to experiments performed in larger systems because macroinvertebrate taxa, the most sensitive species group to this fungicide, were not abundant or diverse.

Future refinements to the test system could include the addition of a sediment compartment and sensitive macroinvertebrate taxa. However, the simple design offers the potential to perform experiments under more controlled conditions than larger and consequently more complex model ecosystems, whilst maintaining relatively high ecological realism compared to standard laboratory tests. Further implications for risk assessment studies are discussed in an ecotoxicological and methodological context.

Introduction

Microcosms and mesocosms have often been used as a higher tier study for the ecological risk assessment of pesticides. They provide a bridge between laboratory and the field in terms of being manageable and allowing replication and hence a robust experimental design on the one

hand and by providing realism in terms of ecological processes and exposure to the chemical on the other (Brock et al. 2000a).

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

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

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

Materials and methods

Experimental design

Twelve microcosms were situated in a room disposed of daylight and a maintained temperature of 21 ± 1 °C. Each microcosm consisted of a glass chamber (diameter 24.5 cm; height 36 cm. Figure 2.1), filled with 8.5 litre of pond water, obtained from a pond next to the building of the research institute Alterra. The pond water was sieved over a 0.75 mm mesh before addition to the microcosms in order to exclude *Chaoborus* larvae, a known predator on zooplankton communities (Fedorenko 1975, Black and Hairston 1988).

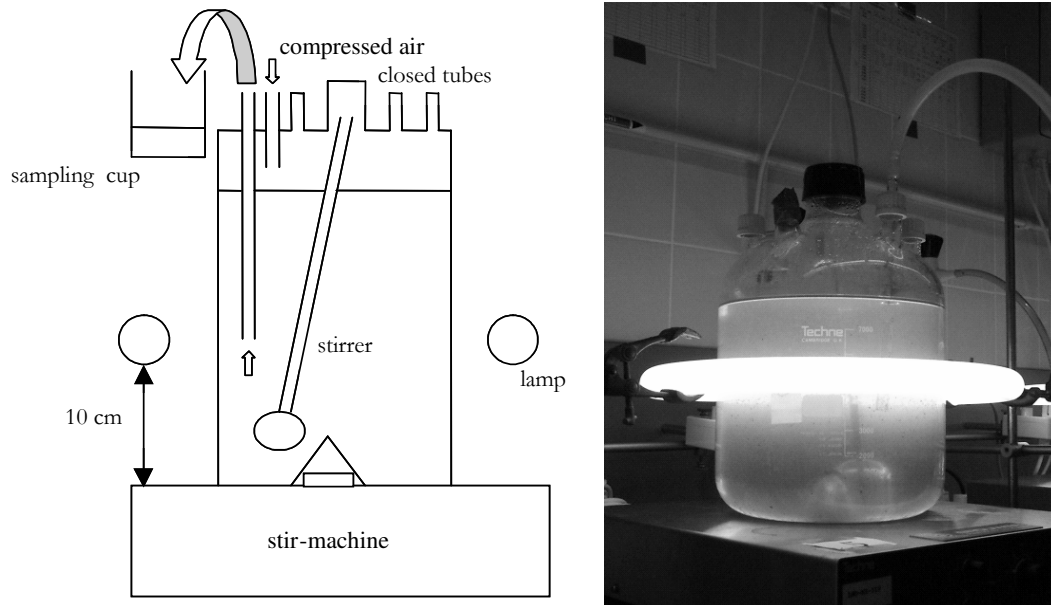


Figure 2.1 Schematic representation (left) and overview (right) of the microcosm.

The systems were stirred for 5 minutes every 30 minutes at 20 rpm by means of a MCS-101L biological stirrer to achieve water movement in the system in order to prevent settling out of planktonic algae. A fluorescent lamp (Philips TL'E 40W/33) was placed around the microcosms, resulting in a light intensity of approximately 45 $\mu\text{E}/\text{m}^2\cdot\text{s}$ in the middle, and 60 $\mu\text{E}/\text{m}^2\cdot\text{s}$ at the edge of the chambers. The daily photoperiod was 14 hours (9.15 till 23.15).

Besides an opening for the stirrer, the microcosms contained five smaller openings of which four were closed with air-tight screw caps and one was connected to a compressed air line (Figure 2.1). To take water samples, one of the screw cap was replaced by one containing a rubber ring through which a glass pipette was put to 10 cm below the water surface. By adding compressed air into the system, water was forced through the glass pipette into a sampling cup.

In the preparatory phase of the experiments, additional plankton was introduced into the microcosms, together with the pond water. Moreover, in the experiments with carbendazim and linuron, eight individuals of respectively *Bithynia leachii* and *Lymnaea palustris* were added to control periphyton growth on the vessel walls. A nutrient addition of 0.115 mg N (as NaNO_3), 0.014 mg P (as KH_2PO_4) and 0.7 mg HCO_3 (as NaHCO_3) was applied twice in the pre-treatment period and twice a week in the treatment period. Microcosms were allowed to stabilise for 1 week, which was defined as the pre-treatment period, after which the systems

were treated. The systems were monitored for several endpoints over an experimental period of two weeks for chlorpyrifos and three weeks for carbendazim and linuron.

Pesticide application and analysis

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

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

Linuron was applied to eight microcosms, in four duplicate doses (0.5, 5, 50, 150 µg/L), while four other systems served as controls. Water samples were taken on day 0 (directly after application), 3, 7, 14 and 21. Those taken from microcosms with the two lowest linuron applications were extracted according to the method as described for chlorpyrifos. Linuron was eluted from the SPE columns with three successive aliquots of 1 ml methanol, diluted with distilled water to a fixed volume of 5 ml and stored at 4°C. Water samples from the higher linuron concentrations (50 and 150 µg/L) were analysed without a concentration step.

Analysis was carried out with HPLC as described in Cuppen et al. (1997). Linuron recovery from water was $105.9 \pm 5.8\%$ (mean \pm sd, n = 8).

The half-life for the disappearance of the pesticides (DT50) was calculated assuming a first order dissipation kinetics, i.e. by linear regression using logarithmic transformed measured pesticide concentrations.

DO-pH-alkalinity-conductivity syndrome

Dissolved oxygen (DO), pH and temperature were measured every working day at the start of the photoperiod and seven hours later. By measuring the difference in DO during the first seven hours of the light period, 90 to 95% of the oxygen production can be determined (Beyers and Odum 1993). After stirring the microcosms for 5 minutes at 20 rpm, 50 ml water samples were taken with a glass pipette as described previously. In this water sample, DO was measured with a WTW oxygen meter (OXI 196) connected to a WTW oxygen probe (EO 196). The pH and temperature were measured with a WTW pH meter (pH 323) and a LF 91 temperature meter, respectively. Alkalinity was measured three times a week by titrating a 25-ml water sample with 0.01 N HCl until pH 4.2. Conductivity was measured with a WTW conductivity meter.

Chlorophyll-a and nutrients

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

Decomposition

In the experiments with carbendazim and linuron, decomposition of particulate organic matter (POM) was studied using dried *Populus* leaves. The *Populus* leaves were obtained from a stock

of previously leached (three times for two days) and dried (60 °C, 72 hours) leaves. Approximately 0.4 grams of pretreated leaves were weighed and placed in stainless steel tea strainers, then leached for 3 days in distilled water. One tea strainer was incubated in each microcosm at mid-height on the day of application. At the end of the experiment (day 21), the content of a tea strainer was gently washed in the corresponding microcosm to separate algae and invertebrates from POM. The leaf material was transferred to aluminium dishes to determine dry weight (105°C, 24 hours).

Zooplankton and phytoplankton

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

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

Snails

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

Data analysis

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

The differences in structure of the zooplankton communities between the microcosms as sampled at the end of the experiments and the phytoplankton community of the linuron experiment were visualised with the ordination technique called Principal Component Analysis (PCA) (Ter Braak 1995, Van Wijngaarden et al. 1995). Ordination is able to express differences in species composition between samples without the use of measured environmental or explanatory variables. In such an analysis, ordination constructs imaginary, latent explanatory variables which maximise the variation in species composition between sites, i.e. which best represent the underlying structure in the data set (Ter Braak 1995). The first latent variable is constructed in such a way that it explains the largest part of the total variance, the second one the largest part of the remaining variance etc. The first two latent variables are normally used to construct an ordination diagram of which they form the axes. Samples and species are represented in the diagram by points (or arrows) plotted at the scores (values) they have on the latent variables (See Figure 2.5 as an example). Samples with nearly identical species composition lie close together in the diagram, while samples that lie far apart have very different species composition. In the diagrams (biplots), species arrows point in the direction of higher values. For examples on the application of ordination techniques in ecotoxicology the reader is referred to Van den Brink et al. (2003). Before analyses with CANOCO for windows (version 4. Ter Braak and Smilauer 1998), the abundance data of the communities were $\ln(Ax + 1)$ transformed (see above for rationale).

The NOEC at community level for the phytoplankton community at the end of the linuron experiment, as well as the zooplankton communities at the end of the three experiments, were

calculated by applying the Williams test to the sample scores of the first principal component (for rationale, see Van den Brink et al. 1996).

Results

Pesticide concentrations

In all three experiments, the initial concentrations deviated by less than 10% of the nominal concentrations (Table 2.1). Linuron and carbendazim were very persistent; pesticide concentrations measured at the end of the experiment were similar to initial concentrations. Therefore, no DT50 for the disappearance of carbendazim and linuron could be estimated.

Chlorpyrifos concentrations decreased over the experimental period and the rate of dissipation was found to be dose dependent; chlorpyrifos disappeared faster in the highest two concentrations compared to the 0.05 µg/L treatment (Table 2.1). The half-life was approximately 10 days for the 0.05 µg/L treatment level and 6 to 7 days for the two highest treatment levels. The chlorpyrifos concentration in the 0.005 µg/L dosed microcosms dropped below detection level quickly after application so no half-life could be calculated.

Table 2.1 Nominal concentrations and initial concentrations of the pesticides for the experiments with chlorpyrifos, carbendazim and linuron. Concentrations at the end of the experiments and the dissipation rate from the water layer (DT50) are also noted. Presented concentrations are in µg/L. The detection limit (DL) for chlorpyrifos was 0.01 µg/L. A “-” implies that no DT50 could be calculated. SD = standard deviation.

Pesticide	Nominal Concentrations	Initial Concentrations mean ± SD	Concentration at end of experiment mean ± SD	DT50 (days) mean ± SD	Duration experiment (days)
Chlorpyrifos	0.005	< DL	< DL	-	14
	0.05	0.053 ± 0.0057	0.018 ± 0.004	9.6 ± 0.0	
	0.5	0.47 ± 0.085	0.035 ± 0.006	6.1 ± 0.2	
	5	4.92 ± 0.47	0.35 ± 0.06	6.6 ± 0.2	
Carbendazim	3.3	3.5 ± 0.1	3.7 ± 0.1	-	21
	33	33.5 ± 1.6	36 ± 1.3	-	
	100	97.8 ± 1.3	107.5 ± 3.5	-	
	1000	976.5 ± 4.9	1003 ± 48.1	-	
Linuron	0.5	0.5 ± 0	0.4 ± 0.1	-	21
	5	5.1 ± 0	4.3 ± 0.2	-	
	50	45.1 ± 0.9	40.1 ± 1.5	-	
	150	146.6 ± 0.5	146.6 ± 0.5	-	

The overall effects of the pesticides on the water quality parameters are presented in Table 2.2. Application of chlorpyrifos led to an increase in dissolved oxygen (DO) and DO production (Figure 2.2) in all but the lowest treatment and increased pH levels in all treatments. Significantly higher pH levels in all treatments were the result of a decrease in pH in the control microcosms from 10.3 to 9.7 during the course of the experiment. From 10 days post application onwards conductivity increased in the 0.5 and 5 $\mu\text{g/L}$ -applied microcosms. Application of chlorpyrifos had no significant treatment effect on alkalinity or nutrient concentrations.

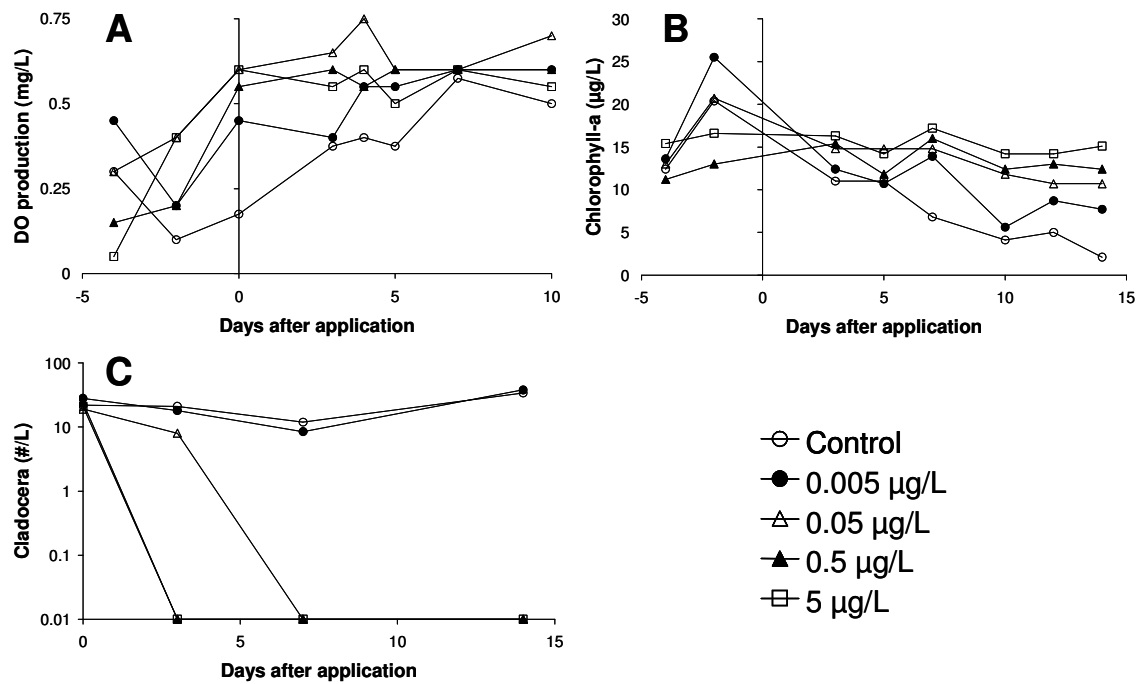


Figure 2.2 Dynamics in dissolved oxygen production (A), chlorophyll-a (B) and numbers of cladocera (C) of the chlorpyrifos experiment.

The highest carbendazim treatment led to an immediate and prolonged increase in DO production (Figure 2.3). Although DO levels were generally higher at this treatment level, this difference was not significant. Furthermore, pH increased in the highest and conductivity in all but the lowest concentration from one week post application onwards. Carbendazim did not lead to significant treatment effects on alkalinity and nutrient levels (Table 2.2).

Table 2.2 NOECs and LOECs (in µg/L) found in the present study and studies with a single load and constant exposure of chlorpyrifos, carbendazim and linuron. Arrows indicate a significant increase (↑) or decrease (↓) compared to controls. The study numbers refer to the studies as given under this table. nm = not measured.

Endpoint/ pesticide load	Chlorpyrifos			Carbendazim			Linuron		
	Single peak	Constant	This study	Single peak	Constant	This study	Single peak	Constant	This study
<u>Community metabolism</u>									
DO	6-44↑	0.1-0.5↑	0.005-0.05↑	21-226	>1000	> 1000	0.5-5	0.5-5↓	0.5-5↓
DO production	6-44↓	nm	0.005-0.05↑	nm	nm	100-1000↑	5-15	nm	0.5-5↓
pH	6-44↑	0.05-0.1↑	ctr-0.005↑	>226	>1000	100-1000↑	0.5-5	0.5-5↓	0.5-5↓
EC	6-44↓	0.05-0.1↓	0.05-0.5↑	>226	>1000	3.3-33↓	nm	0.5-5↑	5-50↑
Alkalinity	6-44↓	0.01-0.05↓	> 5	nm	>1000	> 1000	nm	0.5-5↑	50-150↑
Ammonium	nm	nm	> 5	nm	>1000	> 1000	nm	>150	5-50↑
Nitrate	nm	nm	> 5	nm	>1000	> 1000	nm	50-150↑	5-50↑
Phosphate	nm	>0.5	> 5	nm	>1000	> 1000	nm	>150	5-50↑
<u>Decomposition</u>									
Breakdown of POM	nm	0.01-0.05↓	nm	nm	100-330↓	100-1000↓	nm	>150	50-150↑
<u>Zooplankton</u>									
community	0.1-0.9↓	0.01-0.05↓	0.005-0.05↓	2.2-21↓	33-100↓	33-100↓	>50	5-15↓	5-50↓
<u>Phytoplankton</u>									
community	nm	0.05-0.1↑	nm	nm	33-330↑	nm	>50	0.5-5↓	50-150↓
chlorophyll-a	nm	0.05-0.1↑	0.05-0.5↑	21-226↑	33-330↑	33-100↑	>50	50-150↑	5-50↓
<u>Overall NOEC</u>	0.1-0.9	0.01-0.05	0.005-0.05	2.2-21	3.3-33	33-100	5-15	0.5-5	0.5-5
Study number *	1	2		3	4		5	6	

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

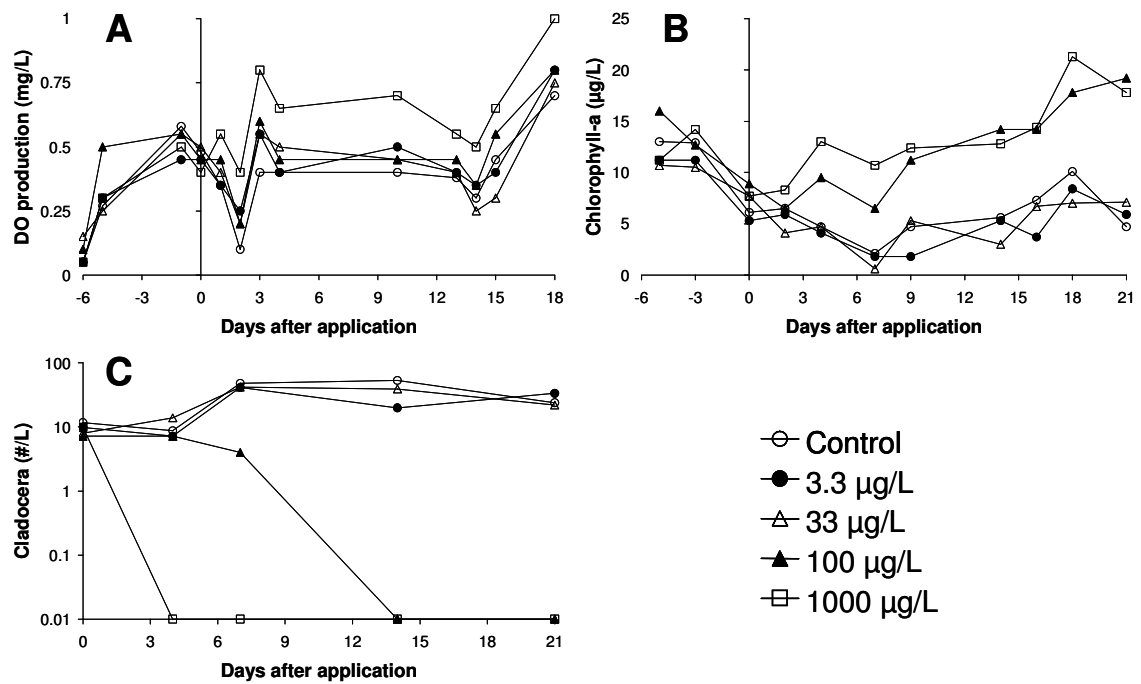


Figure 2.3 Dynamics in dissolved oxygen production (A), chlorophyll-a (B) and numbers of cladocera (C) of the carbendazim experiment.

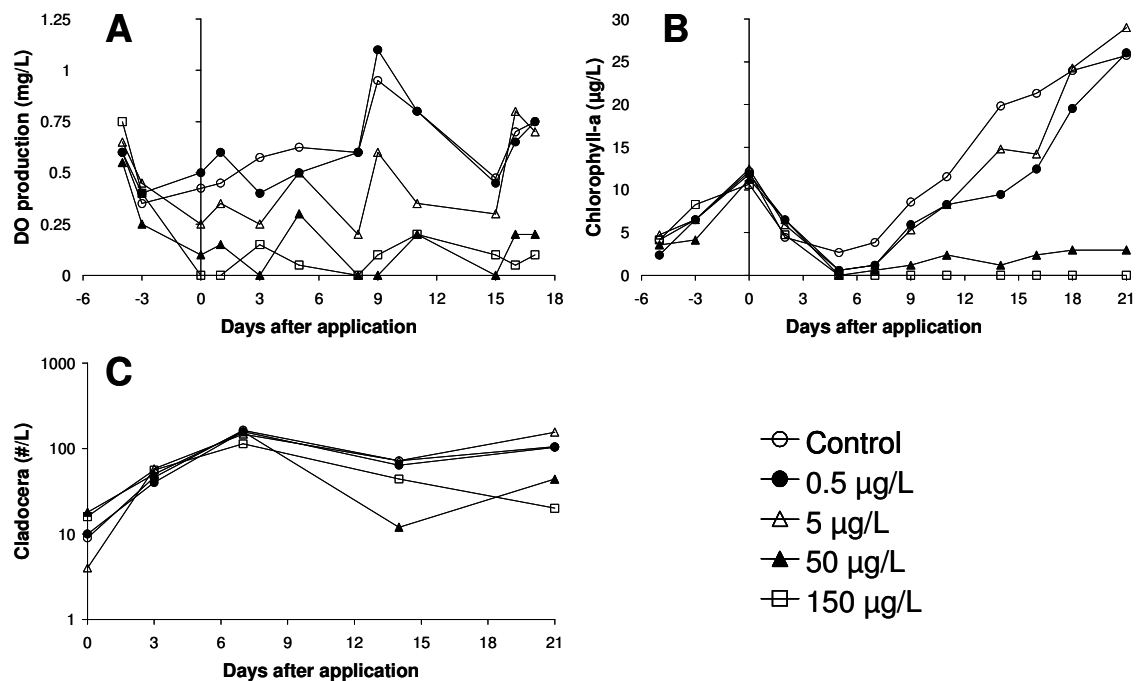


Figure 2.4 Dynamics in dissolved oxygen production (A), chlorophyll-a (B) and numbers of cladocera (C) of the linuron experiment.

Effects on water quality were most prominent in the linuron experiment. Immediately after treatment, DO production (Figure 2.4) was significantly decreased compared to controls at all linuron concentrations but the lowest (Table 2.2). This resulted in a drop in DO in even the lowest concentration at the end of the dark period between 14 and 17 days post application. The DO levels in the afternoon were lower in all but the lowest treatment for a prolonged period of time. DO remained above 6.8 mg/L in all microcosms, so no anoxic conditions occurred. At the end of the experimental period, only the microcosms with the highest two linuron concentrations were still significantly different from controls. The drop in DO was accompanied by a drop in pH in the higher treatment levels. At the end of the experiment, the microcosms treated with 5 µg/L regained normal “control” pH levels, whereas those with higher concentrations remained lower than in controls. Corresponding with the decrease in pH and DO, application of 50 and 150 µg/L led to an increase in conductivity and the highest concentration to an increased alkalinity. Although alkalinity was only increased during the second week post application, conductivity remained increased until the end of the experiment. The two highest linuron treatments resulted in an increase in concentrations of ammonium, nitrate and phosphate compared to controls.

Zooplankton

In the bulk sample taken at the end of the chlorpyrifos experiment, a total number of 21 invertebrate taxa were identified and their abundance determined. In terms of numbers of taxa, the most important taxonomic groups were Rotatoria, Cladocera, Copepoda, Insecta and Ostracoda (not identified at species level). Treatment-related dynamics in densities of Cladocera in the course of the experiment are presented in Figure 2.2. In the controls and 0.005 µg/L microcosms densities of Cladocera increased in time, while in test systems treated with higher concentrations Cladocera were eliminated.

A biplot resulting from the PCA on the zooplankton dataset is given in Figure 2.5. The diagram summarises the treatment effects in the dataset, while still indicating the approximate species composition for all samples. Samples with nearly identical species compositions lie close together, while samples with very different species compositions lie far apart. If an imaginary line is drawn through a species point and the origin of the plot, the relative abundance of that species in all samples can be derived by perpendicularly projecting the sample point on this imaginary line. The samples projecting on the “species line” far away

from the origin but on the same side of the origin as the species point contain relatively high numbers of this species. If a sample projects on the other side of the origin compared to the species point, numbers of this species are relatively low in this sample (Van den Brink et al. 2003).

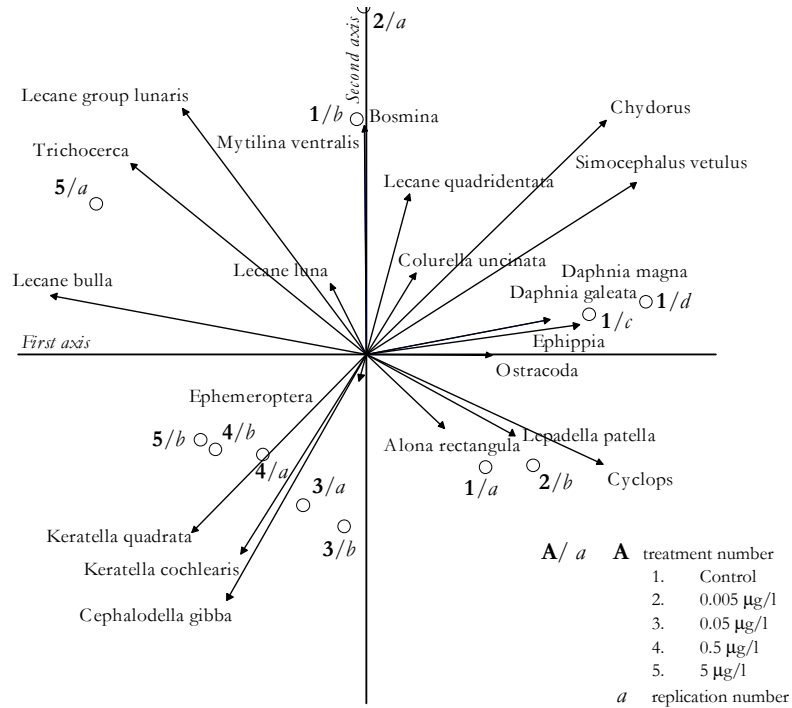


Figure 2.5 Ordination diagram (PCA) indicating effects of a single application of the insecticide chlorpyrifos on the zooplankton per treatment level. Of all variance, 36% is displayed on the horizontal axis and another 26% on the second one.

The PCA of the chlorpyrifos zooplankton dataset revealed treatment related differences in species composition, with the effect of the treatment decreasing in the order $5 \approx 0.5 \approx 0.05 > 0.005 \mu\text{g/L} \approx \text{controls}$ (Figure 2.5). The direction of the treatment vector in the diagram is from the right to the left, meaning that taxa less abundant in the treated microcosms are situated at the right and the unaffected and positively affected taxa at the centre and left side of the diagram.

Numbers of *Chydorus sphaericus*, *Simocephalus vetulus* and *Lepadella patella* were significantly decreased at the higher treatment levels (Table 2.3). *Chydorus sphaericus* and *Simocephalus vetulus* were eliminated in the three highest concentrations. Taxa that occurred in significantly higher densities than in the controls were the rotifers *Cephalodella gibba*, *Lecane bulla* and *Trichocerca*.

Overall, the Williams test on the PCA coordinates showed a significant treatment effect on the invertebrate community at all chlorpyrifos applications but the lowest (NOEC_{community} = 0.005 µg/L).

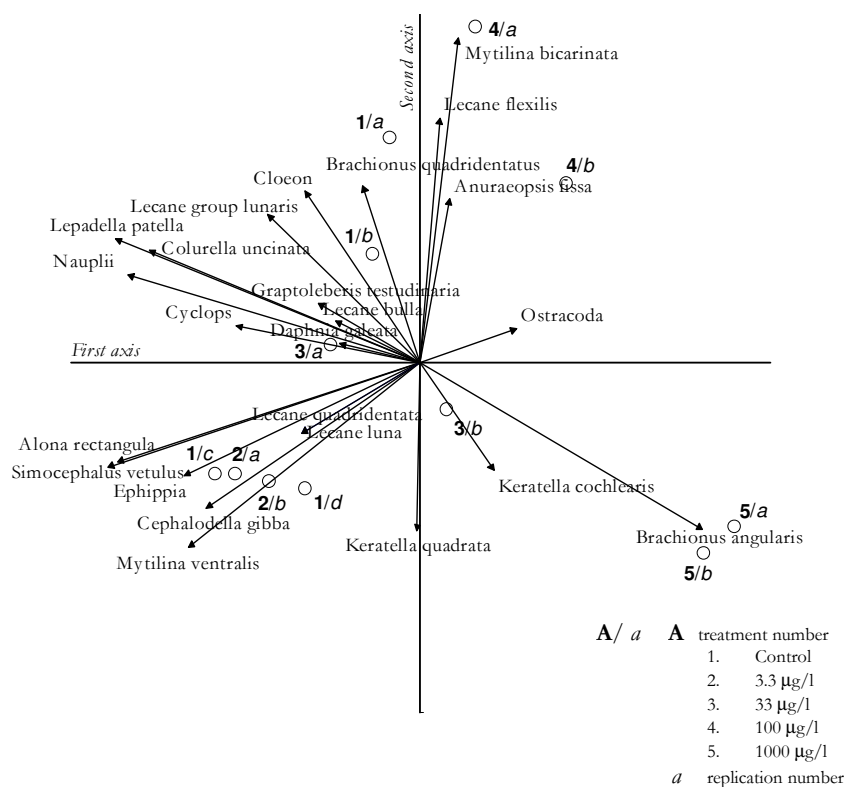


Figure 2.6 Ordination diagram (PCA) indicating effects of a single application of carbendazim on the zooplankton per treatment level. Of all variance, 37% is displayed on the horizontal axis and another 20% on the second one.

In the samples taken at the end of the experiment evaluating carbendazim, a total number of 23 different zooplankton taxa were identified. The control community was dominated by Rotifera, followed by Cladocera, Cyclopoida, Insecta and Ostracoda (not identified at species level). Treatment-related dynamics in densities of Cladocera in the course of the experiment are presented in Figure 2.3. In test systems treated with the two highest concentrations (100 and 1000 µg/L) Cladocera declined and were even eliminated at the end of the experiment.

The PCA biplot of the zooplankton dataset reveals a clear dose-related deviation of the 100 and 1000 µg/L carbendazim treatments from the controls (Figure 2.6). The visual differences were confirmed by the NOEC_{community} calculation (NOEC_{community} = 33 µg/L). Taxa negatively affected by the application are situated on the left side of the diagram, whilst

unaffected taxa are situated at the upper right (100 µg/L samples) and lower right quadrant (1000 µg/L samples). In the two highest concentrations, cladocerans were completely eliminated and, consequently, abundances of *Simocephalus vetulus*, *Graptoleberis testudinialis*, *Alona rectangular*, ephippia and also the rotifer *Lepadella patella* were significantly lower than in controls. In addition, the highest carbendazim dose also had pronounced effects on rotifers. Total numbers of individuals were only one third of those in the controls (Williams test, $p < 0.05$) and *Colurella uncinata* and nauplii abundances were significantly reduced (Table 2.3). *Branchionus angularis* occurred in higher densities at this treatment compared to controls.

Table 2.3 NOECs (µg/L) as calculated by the Williams Test ($P \leq 0.05$) for the abundances of zooplankton taxa for the three different experiments as well as phytoplankton taxa for the linuron experiment. All measurements were performed at the end of the experiments.

Chlorpyrifos	Carbendazim	Linuron	Linuron
Zooplankton	Zooplankton	Zooplankton	Phytoplankton
Decrease:	Decrease:	Decrease:	Decrease:
0.005 <i>Simocephalus vetulus</i>	33 <i>Simocephalus vetulus</i>	0 <i>Lecane bulla</i>	5 <i>Scenedesmus</i>
0.005 <i>Chydorus sphaericus</i>	33 <i>Graptoleberis testudinaria</i>	5 <i>Daphnia galeata</i>	5 <i>Monoraphidium</i>
0.5 <i>Lepadella patella</i>	33 <i>Lepadella patella</i>	5 <i>Daphnia magna</i>	50 <i>Pediastrum</i>
	33 <i>Alona rectangular</i>	5 Ephippia	50 <i>Tetraedon</i>
	33 Ephippia	50 <i>Simocephalus vetulus</i>	
	100 Nauplii		
	100 <i>Colurella uncinata</i>		
Increase:	Increase:	Increase:	Increase:
0.005 <i>Cephalodella gibba</i>	100 <i>Branchionus angularis</i>	None	0 <i>Epithemia</i>
0.05 <i>Lecane bulla</i>			5 <i>Navicula</i>
0.5 <i>Trichocerca sp.</i>			50 <i>Closterium</i>

In the experiment with linuron, a total number of 23 different taxa were identified dominated by Rotifera, followed by Cladocera, Copepoda (Cyclopoida and nauplii) and Ostracoda (no identification on taxon level). Total number of Cladocera as counted during the course of the experiment was lower in the two highest treatments only at the end of the experiment (Figure 2.4).

The PCA diagram presented in Figure 2.7 summarises the treatment effects on the zooplankton community on day 21. The diagram reveals a decrease in abundance of especially

Cladocera species for the highest two applications, as these sample points are positioned at the upper left quadrant, and the Cladocera species points at the lower right quadrant. Although these visual effects could not be confirmed with NOECcommunity calculations ($> 150 \mu\text{g/L}$), negative treatments effects were found for several species (Table 2.3). Abundances of the cladocerans *Daphnia galeata*, *Daphnia magna* together with ephippia were decreased in the highest two concentrations and *Simocephalus vetulus* in the highest only. *Lecane bulla* showed decreased numbers at all linuron applications on this day (Table 2.3).

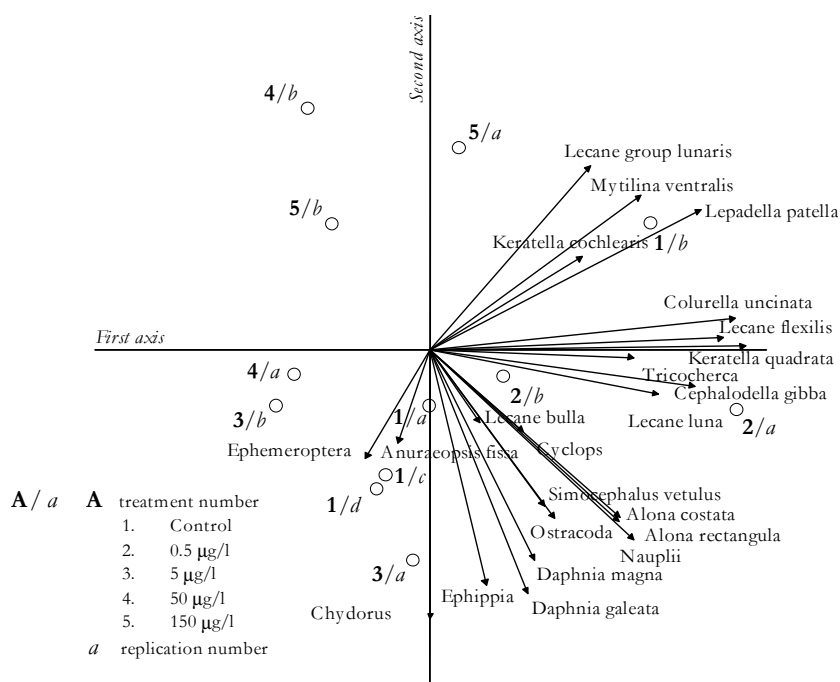


Figure 2.7 Ordination diagram (PCA) indicating effects of a single application of linuron on the zooplankton per treatment level on day 21. Of all variance, 35% is displayed on the horizontal axis and another 20% on the vertical axis.

Phytoplankton

After the start of the treatment, chlorophyll-a values decreased in controls and the lowest chlorpyrifos application during the course of the experiment (Figure 2.2). In the higher chlorpyrifos concentrations chlorophyll-a levels did not alter, leading to a concentration-dependent increase compared to controls (NOEC = 0.005 $\mu\text{g/L}$, Table 2.2).

Application of the highest two carbendazim concentrations led to a time-related increase in chlorophyll-a content (Figure 2.3). Four days post application, chlorophyll-a values in these treatment levels were twice and at the end of the experiment four times higher than in controls.

During the course of the linuron experiment, chlorophyll-a content increased in controls and microcosms applied with the two lowest linuron concentrations (Figure 2.4). At the end of the experiment, chlorophyll-a levels were tripled in these microcosms compared to pre-treatment values. Application of the two highest linuron concentrations led to a decrease in chlorophyll-a levels. Five days post treatment, no chlorophyll-a could be detected in samples from microcosms treated with these concentrations. In the 50 µg/L treated microcosms, chlorophyll-a re-appeared one week after the treatment but did not regain normal values within the experimental period. Chlorophyll-a levels in the highest concentration remained zero until the end of the experiment.

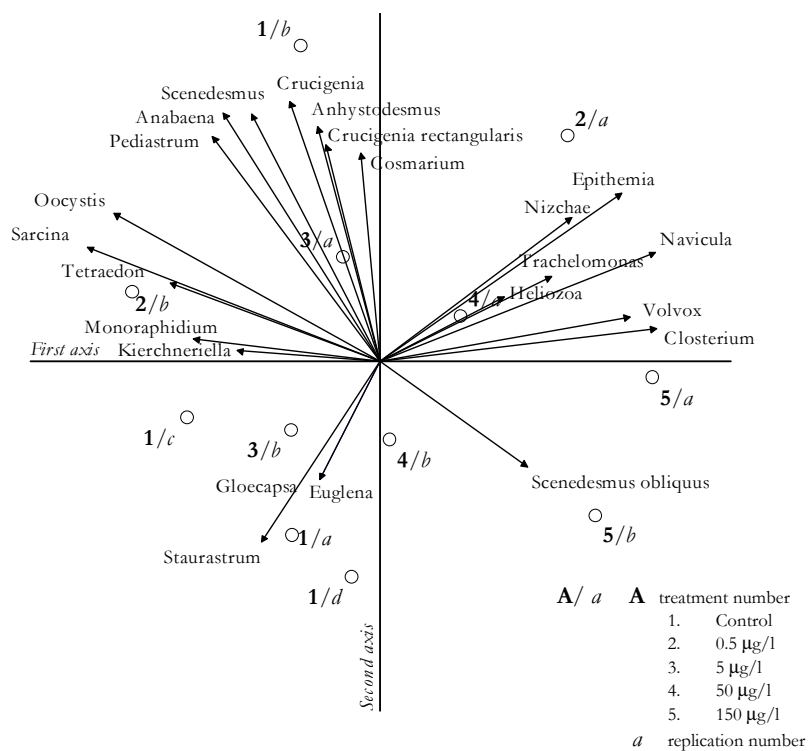


Figure 2.8 Ordination diagram (PCA) indicating effects of a single application of linuron on the phytoplankton per treatment level. Of all variance, 38% is displayed on the horizontal axis and another 20% on the vertical axis.

The PCA-biplot (Figure 2.8) visualises the overall effect of linuron on the phytoplankton community. The diagram reveals that the phytoplankton community of the 150- $\mu\text{g}/\text{L}$ microcosms and, to a lesser extent, the 50 $\mu\text{g}/\text{L}$ microcosms diverged from controls. Most species that were affected by linuron application decreased in numbers, although *Navicula*, *Epithemia* and *Closterium* individuals increased significantly in abundance (Table 2.3). The direction of the treatment vector is from the left to the right part of the diagram, i.e. those taxa negatively affected by the treatment are situated at the left and unsusceptible and positively affected taxa are situated at the right side of the biplot (Figure 2.8).

The NOECphytoplankton community as calculated by a Williams test on the PCA coordinates was 50 $\mu\text{g}/\text{L}$ for linuron (Table 2.2). The taxa *Scenedesmus* and *Monoraphidium* were significantly less abundant in the highest two linuron applications compared to controls. In addition, *Pediastrum* and *Tetraedon* were significantly reduced at the highest application. Reduction of total number of phytoplankton individuals was also most prominent in the 150 $\mu\text{g}/\text{L}$ samples. In these samples, numbers were only one quarter of control levels.

Snails

Application of carbendazim did not result in lethal treatment effects on *Bithynia leachii* at the concentrations applied. However, grazing behaviour was affected in the two highest carbendazim concentrations. In addition, in microcosms dosed with 1000 $\mu\text{g}/\text{L}$, the operculum reflex was decreased compared to control animals. The NOEC for the sublethal effects on *B. leachii* was therefore determined to be 33 $\mu\text{g}/\text{L}$ (Data not shown).

The application of linuron did not result in any significant treatment effect on *Lymnaea palustris*.

Decomposition experiment

The residual dry weights of the *Populus* leaves in control and the lower carbendazim treatments amounted to approximately 60% of the initial dry weight (data not shown). Application of 1000 $\mu\text{g}/\text{L}$ led to a slight though significant decrease in decomposition compared to controls (Table 2.2).

The decomposition of the *Populus* leaves in all but the highest linuron treatments were comparable with the carbendazim experiment: residual dry weights were approximately 60%

of initial values. The rate of decomposition in the highest dosed microcosms was 10% higher than in controls (Table 2.2).

Discussion

Fate of the pesticides

Chlorpyrifos was relatively persistent and the DT50 varied between 10 days for the 0.05 µg/L treatment and 6 to 7 days for the higher treatments (Table 2.1). This dependence of DT50 on treatment level is most probably due to higher pH values in the higher treatment levels, as the stability of chlorpyrifos decreases rapidly as pH increases (Macalady and Wolfe 1983). The relatively slow disappearance of chlorpyrifos in the present study compared to other microcosm studies (Racke 1993, Giesy et al. 1999) is presumably the result of the absence of macrophytes and sediment, which are known to substantially absorb chlorpyrifos from the water (Crum and Brock 1994).

The concentrations of carbendazim and linuron remained constant over the experimental period for all doses applied (Table 2.1). In an indoor macrophyte-dominated microcosm experiment with carbendazim, Cuppen et al. (2000) found a DT50 between 6 and 25 weeks, which decreased with the treatment level. The authors suggested that the dependence on treatment level was probably not the result of changes in physico-chemical conditions, since no differences were observed between the different treatments. It is therefore unlikely that the absence of breakdown in the present study was caused by water quality parameters. A more plausible explanation was the fact that closed systems were used that did not contain sediment and macrophytes.

Van den Brink et al. (1997) and Cuppen et al. (1997) performed a microcosm study in indoor macrophyte-dominated systems with linuron and found a half-life of 11 days for 0.5 µg/L dosed up to 49 days for 150 µg/L dosed microcosms. They concluded that the difference in DT50 was due to differences in pH regime between the different treatments as Cserhati et al. (1976) found a significant slower hydrolysis of linuron at pH 6 and 8 compared to pH 4 and 10. In the present study, the pH ranged from 7.8 to 8.1 for the different treatments so, based on the above, a DT50 of around 50 days could be expected, which is considerably longer than the duration of this study.

The primary response of the chlorpyrifos application was a decline in the number of zooplankton species and abundances (Cladocera in particular). The consequent reduction of the grazing pressure indirectly caused an increase in chlorophyll-a levels, resulting in an increase in pH, dissolved oxygen (DO), DO-production and conductivity (Table 2.2, Figure 2.2 and 2.5). A decrease in the conductivity due to increased photosynthesis was, however, expected (Brock et al. 1993). The small increase in conductivity (280 to 300 $\mu\text{S}/\text{cm}$) after chlorpyrifos treatment can be explained by the release of dissolved substances that were until then part of the biomass of the zooplankton. All other direct and indirect effects are comparable with effects found in other microcosm studies with chlorpyrifos (Kersting and Van den Brink 1997) and other insecticides (see Brock et al. 2000b and Van Wijngaarden et al. 2005 for reviews). The order of susceptibility of the zooplankton groups was Cladocera > Copepoda and Ostracoda > Rotifera, which is in accordance with other model ecosystem studies (Van den Brink et al. 1996, 2002, Brock et al. 1992). The Rotifera (which are of low sensitivity to chlorpyrifos) have frequently been reported to increase in numbers in insecticide-stressed aquatic systems as a result of a lower competition and grazing pressure caused by a decline in cladocerans (Brock et al. 2000b). In the current study, rotifer abundances were 30% higher in the 0.5 $\mu\text{g}/\text{L}$ applied microcosms and doubled in the highest two treatments. Though this increase was not significant due to a high variation in controls, a significant increase in numbers of the individual species *Cephalodella gibba*, *Lecane bulla* and *Trichocerca sp.* was demonstrated for higher chlorpyrifos concentrations (Table 2.3).

Direct effects of carbendazim have been reported on zooplankton and macroinvertebrates in model ecosystem studies after single (Slijkerman et al. 2004) and chronic (Cuppen et al. 2000, Van den Brink et al. 2000) exposure. In line with this, the higher carbendazim applications in the present study had negative treatment effects on zooplankton and *Bithynia leachii*. Cladocera and Rotifera were found to be the most susceptible zooplankton groups (Figure 2.3 and 2.6). In a former microcosm experiment, Copepoda were found to be more sensitive than rotifers. This only became apparent three weeks after application since the effect on Cyclopoida resulted from a decrease in the numbers of their immature stage, nauplius, rather than of direct toxicity (Van den Brink et al. 2000). Indeed, a decrease in nauplius larvae was also found in the present study (NOEC = 100 $\mu\text{g}/\text{L}$), but the experimental period was probably too short to show a prolonged effect on mature Cyclopoida.

The reduction in zooplankton abundance and the consequent increased growth of planktonic algae resulted in an increase in pH and oxygen production as discussed for chlorpyrifos. As observed in a macrophyte-dominated microcosm study with chronic carbendazim exposure, no increase in dissolved oxygen concentrations was observed (Cuppen et al. 2000). The authors of that study attribute this to the fact that the water was saturated with dissolved oxygen, so a possible movement of oxygen to the air could have caused unnoticed additional oxygen production. Oxygen levels were near saturation in the present study too (8.8 ± 0.7 ; saturation is 100% at 9.4 mg/L) so this could have masked a possible increase. Carbendazim application, however, led to decreased conductivity values in the two highest concentrations, most probably due to the increase in chlorophyll-a (Figure 2.3). Carbendazim had a significant treatment effect on decomposition, which may have resulted in decreased levels of breakdown products and, consequently, also contributed to a lower conductivity.

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

The general effect chain of linuron application in the present study was the same as described for macrophyte dominated freshwater microcosm studies with linuron (Van den Brink et al. 1997, Cuppen et al. 1997) and other herbicides (for a review see Brock et al. 2000a). The primary effect of linuron is an inhibition of the photosynthetic efficiency of the primary producers (Snel et al. 1998), leading to decreased dissolved oxygen and pH levels and an increase in alkalinity and conductivity (Table 2.2). A decrease in sensitive phytoplankton population densities caused an increase of the non-sensitive or rapidly adapted species *Ephitemia*, *Navicula* and *Closterium* by reduced competition (Figure 2.8). Increased levels of ammonium, nitrate and ortho-phosphate, as a consequence of an overall decrease in primary production and the decomposition of the sensitive phytoplankton biomass, further stimulated the growth of these species.

The EC₅₀ of *Daphnia galeata* for linuron (360 µg/L; Stephenson and Kane 1984) is considerably higher than the concentrations used in the current experiment. Numbers of the cladocerans *D. galeata*, *D. magna* and *S. vetulus* and total numbers of Cladocera had significantly decreased three weeks after application (Table 2.3, Figure 2.4). Cladocerans are more efficient

grazers than rotifers (Hanazato 1998) so they could survive the decreasing phytoplankton biomass over a longer period. The eventual decrease in *D. galeata*, *D. magna* and *S. vetulus* can be further explained by the phytoplankton community composition as identified in the higher treatment level three weeks post application. The phytoplankton community was dominated by the diatoms *Epithemia* and *Navicula*, taxa that possess a tough cover probably making them less edible for (young) Cladocera (Figure 2.8). Indeed, Starr et al. (1999) found a reduction in the reproductive success of a planktonic copepod (*Calanus finmarchicus*) after a monospecific diet of a *Navicula* species.

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

No treatment effects of linuron were observed on the grazing behaviour of *Lymnaea palustris*. In line with this, the LC50 of some macroinvertebrates, such as *Dugesia tigrina* (10 mg/L), *Lymnaea* (70 mg/L) and *Tubifex* (10 mg/L) are too high to expect any treatment effects (Maier-Bode and Härtel 1981).

Comparison of safety thresholds with other microcosm studies

In Table 2.2, the NOECs of former microcosm studies by our department dealing with the risk assessment of chlorpyrifos, carbendazim and linuron are compared with the NOECs found in the current study. For chlorpyrifos the overall NOEC was set at 0.005 µg/L, although a small effect on pH was observed in this study at this concentration (Table 2.2). This effect was dismissed because the differences were small (less than 0.5 unit) and not caused by an increase in pH in the treated systems but a decrease in the control systems. The NOECcommunity for the zooplankton community was therefore used as an overall NOEC.

Also for carbendazim the zooplankton community proved to be the most sensitive endpoint and therefore also its NOEC was used as an overall NOEC. For the linuron experiment two NOECs at the control level were calculated for day 21, an increase in all systems of the algal species *Epithemia* and a decrease for the zooplankton species *Lecane bulla* (Table 2.3). The increase in the algal species *Epithemia* at the lowest concentration is probably not treatment related because no species are decreased at this concentration and no effects on pH and DO were reported (Table 2.2). The decrease of *L. bulla* was only significant for one sampling date, for all other sampling dates this species was not even present. We therefore set the overall NOEC of the linuron study at 0.5 µg/L (NOEC of DO and pH; Table 2.2).

With reference to chlorpyrifos, the absence of sediment and macrophytes resulted in a prolonged exposure. So although a single application of chlorpyrifos is evaluated in this study, the effects are more comparable to those evaluating chronic exposure due to a lower dissipation compared to “normal” circumstances where sorption to sediment and macrophytes is present. Indeed in this study, a lower NOEC for functional as well as structural endpoints was found as compared to other microcosm studies with single chlorpyrifos application (Table 2.2). In a microcosm study with chronic exposure to chlorpyrifos and lindane, an overall NOEC of 0.01 µg/L was found (Van den Brink et al. 2002), which is comparable to this study.

In the experiment with carbendazim, macroinvertebrates were not present in large numbers nor taxa compared to other microcosm studies. Since macroinvertebrates, with *Oligochaeta*, *Turbellaria* and *Hirudinea* as the most sensitive groups, is the most susceptible animal group to carbendazim (Cuppen et al. 2000, Van Wijngaarden et al. 1998), a higher overall NOEC was found in the present study compared to studies using more complex ecosystems (Table 2.2). The sensitivity of the zooplankton community was comparable with laboratory toxicity tests (NOEC *Daphnia magna* = 26 µg/L, Van Wijngaarden et al. 1998) and microcosm studies with chronic carbendazim exposure (Cuppen et al. 2000). The microcosms used in the present study were more susceptible for effects on functional endpoints than reported in macrophyte-dominated microcosms (Table 2.2). This may have been the result of the absence of macrophytes and sediment reducing the complexity of the ecosystem and the fact that the systems were closed.

The overall NOEC for linuron in this study was lower than the NOEC found by Van Geest et al. (1999, Table 2.2), who also evaluated a single application. However, it matched the NOEC as noted in a microcosm study with chronic exposure of linuron (Table 2.2) which may be a

result of the persistence of linuron in the present study (Table 2.1). The phytoplankton community in this study was a bit less sensitive than in the microcosm study by Van den Brink et al. (1997) and Cuppen et al. (1997) (Table 2.2). They found the most severe effects only after 4 weeks of exposure and explained this late response by assuming that phytoplankton species can survive until their energy reserves are depleted. The experimental period in the present study was only 3 weeks so presumably the autotrophic organisms dosed with the lower concentrations could survive this time by using their energy reserves. As expected, chlorophyll-a levels were lowered in the higher dosed microcosms (Table 2.2). In the microcosm study with chronic exposure, chlorophyll-a levels were elevated due to a bloom of the insensitive *Chlamydomas*, a species not found in the present study. Effect on ecosystem functioning were comparable between our study and the study in the macrophyte-dominated microcosms (Table 2.2).

Implications for risk assessment and final conclusions

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

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

The general effect chains of chlorpyrifos, carbendazim and linuron were the same as described in larger scale microcosm studies. The simple design of the microcosm offers the possibility to perform experiments with a relatively high ecological realism when compared to laboratory tests under more controlled conditions and much cheaper than larger scale model ecosystems. This makes these systems ideal for investigating ecological processes and the chain of effects after stress, e.g. pesticides. Recovery of a certain species can only be studied if the stressor

does not lead to a complete disappearance of that species and the life-cycle can be completed in water. Furthermore, these test-systems are useful in selecting treatment concentrations for larger scale model ecosystem studies.

Acknowledgments

This study was supported by the Dutch Ministry of Agriculture, Nature and Food Safety (DLO/PO research programme 416). The authors are indebted to Steven Crum and Arienne Matser for pesticide analysis; René Van Wijngaarden and Jos Sinkeldam for assistance in respectively zooplankton and phytoplankton identification; Delia Van Dijk for nutrient analysis; Leo Van der Pas for laboratory assistance and Theo Brock for various valuable discussions and reviewing this paper.

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CHAPTER 3

FATE AND EFFECTS OF THE INSECTICIDE CHLORPYRIFOS IN OUTDOOR PLANKTON-DOMINATED MICROCOSMS IN THAILAND

(Submitted to *Environmental Toxicology and Chemistry*)

Abstract

The fate and effects of the insecticide chlorpyrifos were studied in plankton-dominated freshwater microcosms in Thailand. Single applications of 0.1, 1, 10 and 100 $\mu\text{g/L}$ were applied to two tanks each, while four other tanks were untreated to serve as controls. The aim of the study was to compare the fate and effects of chlorpyrifos under tropical conditions with those found in previous studies in temperate regions.

Disappearance rates of chlorpyrifos from the water column observed in this study were similar to those found in temperate regions. Insecticide accumulation in the sediment was relatively low, the major part being found in the top layer.

The application of chlorpyrifos led to significant changes in freshwater biological communities. Clam shrimps (Conchostraca) and the cladoceran *Moina micrura* were the most susceptible species (NOEC = 0.1 $\mu\text{g/L}$), macroinvertebrates the most sensitive community (NOEC = 0.1 $\mu\text{g/L}$). These results are in agreement with results from semi-field experiments with chlorpyrifos in temperate regions. The results of an in-situ bioassay were used to calculate a NOEC of 0.1 $\mu\text{g/L}$ and a 48h-LC50 of 0.6 $\mu\text{g/L}$ for *M. micrura*, values which are similar to toxicity values reported for *Daphnia magna* in studies in temperate regions.

Overall, these findings support the use of toxicity data from temperate regions for the risk assessment of low-persistent insecticides like chlorpyrifos for aquatic communities in tropical regions.

Introduction

The tropical climate in Thailand makes the country ideal for agriculture but also results in the occurrence of various pests that can cause significant damage to crops. Rapid economic and population growth has led to an intensification of agricultural practices and, consequently,

heavy use of pesticides to increase yields. Since 1960, following the “Green Revolution”, increasing amounts and varieties of chemicals have been introduced into the country, in particular those classified as organochlorines (OC), organophosphates (OP) and carbamates (Staring, 1984; Jungbluth, 2000).

Ecotoxicological studies to determine effects of pesticide stress on freshwater ecosystems have focused almost exclusively on countries and ecosystems in the temperate zone. Techniques and procedures developed for temperate regions are often applied to tropical countries, even though community compositions and environmental conditions there may be very different (Lacher and Goldstein, 1997). Also, very few studies have been performed on the fate of pesticides in tropical freshwater environments. The need for studies into the fate and effects of pesticides under tropical conditions has long been recognized (Bourdeau et al., 1988; Castillo et al., 1997; Racke, 2003).

Aquatic microcosms and mesocosms have often been used for the (higher-tier) risk assessment of pesticides. Compared to laboratory bioassays, they provide more ecological realism in terms of ecological processes and exposure to the chemical. At the same time, they allow replication and hence an experimental set-up, making these test systems ideal to assess the influence of climatic conditions on the fate and effects of pesticides.

The present study aimed to evaluate the fate and effects of the organophosphorus insecticide chlorpyrifos in outdoor microcosms under tropical conditions and to set safety threshold values for susceptible indigenous freshwater communities. Furthermore, since various model ecosystem studies with chlorpyrifos have been conducted in temperate regions (e.g. Van den Brink et al., 1996; Biever et al., 1994; Brazner et al., 1989; Pusey et al., 1994; Van Wijngaarden et al., 2005a; 2005b), we compared these threshold values with those reported in the literature. Ultimately, this study aimed to validate whether toxicity data obtained from experiments with chlorpyrifos in temperate regions can be used to ensure adequate protection of freshwater populations in tropical regions like Thailand.

Materials and methods

Experimental design

The experiment was performed in twelve outdoor microcosms at the hatchery of the Asian Institute of Technology (AIT), located 42 km north of Bangkok (Thailand). Each microcosm

consisted of a concrete tank (length 1 m, width 1 m, height 1.15 m, water volume 1000 L) coated with non-toxic epoxy paint. The tanks were filled with a 10-cm layer of sediment and a 1-m water column, taken from the canal surrounding AIT. The canal water was passed through a net (mesh size 0.1 mm) to avoid fish and prawns entering the systems. The microcosms were intended to model the community of Thai farm canals.

In the preparatory phase of the experiment, zooplankton and macroinvertebrates were collected from the AIT canal and introduced into the microcosms. Over an acclimation period of 6 weeks, a biocoenosis was allowed to develop in the microcosms. In this period, the water was circulated twice a week by exchanging 100 litres between the microcosms by means of a Perspex tube to achieve similarity between the communities in the systems. A nutrient addition of N (1.4 mg/L as urea) and P (0.35 mg/L as TSP) was added twice a week during the entire experimental period.

Chlorpyrifos application, sampling and analysis

On the day of application, the organophosphorus insecticide Dursban (active ingredient chlorpyrifos) was applied once to 8 microcosms, in 4 duplicate doses (nominal levels: 0.1, 1, 10 and 100 µg/L). Treatments were assigned randomly to the tanks, and applied by pouring a solution of Dursban into the tanks. The systems were gently stirred immediately after application to mix the compound with the water column while preventing an upflow of sediment particles. Four other systems only received water, and served as controls. Nominal chlorpyrifos concentrations were calculated from an analysis of subsamples of the treatment solutions and the water volume in the microcosms.

Depth-integrated water samples of approximately 10 L were collected in a glass container at various moments during the experiment, using a Perspex tube. Subsamples of 750 mL were transferred to glass bottles and shaken with 50 mL n-hexane (HPLC grade) for one hour. A part of the upper liquid was collected and transferred to GC vials for analysis by splitless injection of 3 µL on a HP 5890 Gas Chromatograph. GLC operating parameters were as follows: capillary column coated with HP-5, length 30 m, internal diameter 0.32 mm, film thickness 0.25 µm; initial oven temperature 70°C, increasing by 20°C/min to a final temperature of 280°C, which was kept constant for 10 min (total run time 20.5 min); detector: Electron Capture Detector; nitrogen flow: 1.5 mL/min. The detection limit for chlorpyrifos

was 0.07 µg/L and the recovery rate was $108 \pm 9\%$ (mean \pm s.d., $n = 9$). Correction for the recovery was made when calculating chlorpyrifos residues in water.

Sediment cores were collected by means of a Perspex tube (internal diameter 3.6 cm) and stored in a freezer until analysis. Chlorpyrifos was extracted from the upper 1.5 cm of a sediment core with 35 mL acetonitrile. The suspension was shaken for two hours. After centrifugation (2600 rpm; 5 min), subsamples of the liquid layer were transferred to GC vials and analysed directly on GC-ECD as described above for the water samples. Chlorpyrifos recovery from sediment samples was $93 \pm 2\%$ (mean \pm s.d., $n = 12$). Sediment chlorpyrifos residue levels were calculated based on dry weight (24 h; 105 °C) and corrected for recovery.

Stratification of the insecticide in the sediment compartment was studied in the microcosms dosed with the highest chlorpyrifos concentration. Sediment cores were divided into layers of 1.5 cm and extracted separately as described above.

Endpoints

Dissolved oxygen was measured approximately 10 cm below the water surface at 1-week intervals with a YSI 58 oxygen meter connected to a YSI 5739 probe. Together with the oxygen, conductivity and pH were measured with a WTW conductivity meter and a CONSORT pH meter, respectively. Alkalinity was measured at weekly intervals in a 100-mL subsample from the bulk sample described below, by titrating with 0.05 N HCl until pH 4.2.

The concentrations of ammonia, nitrate, nitrite, total nitrogen, ortho-phosphate and total phosphate were analysed at two-week intervals in a 1-L sample taken at approximately 10 cm below the water surface using the method described in APHA (1992).

At several moments during the experiment, a 10-L bulk water sample was collected in a bucket by taking several depth-integrated water samples, using a Perspex tube. From this bulk sample, a 1-L subsample was taken for phytoplankton chlorophyll-a and alkalinity analysis. The bucket was then partially emptied, leaving 5 L, which was passed through a zooplankton net (mesh size 60 µm) to examine treatment effects on the zooplankton community.

The concentrated zooplankton sample obtained from the 5-L sample as described above was fixed with formalin to a final concentration of 4%. Subsamples were counted with an inverted microscope and numbers were converted to numbers per litre of microcosm water.

On the day of chlorpyrifos application, a bioassay was performed by placing 25 individuals of *Moina micrura* into circular Perspex enclosures (length 30 cm, diameter 10 cm), with the

bottom and top covered by a nylon net. One enclosure was suspended in each microcosm, with its bottom at a fixed depth of approximately 20 cm. The numbers of individuals were counted 24 and 48 hours after the application to determine the direct effects of the pesticide. The chlorophyll-a content of the phytoplankton was sampled by filtering a known volume of microcosm water through a Whatman GF/C glass fibre filter (mesh size 1.2 μm) until the filter was saturated. Periphyton was sampled from glass slides that served as an artificial substratum. The slides were positioned in a glass frame that was suspended at approximately 10 cm below the water surface. Periphyton chlorophyll-a was sampled at two-week intervals by brushing five slides visually clean, after which the slides were re-introduced in their original microcosm. Pigments were extracted using the ethanol method described by Moed and Hallegraeff (1987). Macroinvertebrates were studied by means of pebble stone baskets that served as artificial substrates. Two baskets were incubated in each microcosm on top of the sediment. Macroinvertebrates were sampled every other week by gently retrieving the substrates using a net to prevent the escape of animals. The macroinvertebrates were collected by washing the artificial substrates in a container, after which they were identified and counted alive and subsequently released in their original microcosm. Since few studies on macroinvertebrate taxonomy have so far been made in Thailand, and expertise and knowledge are still limited (Dudgeon, 2003), identification was only made to class level, to prevent misidentification. The data from the two baskets were pooled for statistical analysis.

Statistics

NOECs were calculated for all parameters using the Williams test, which assumes an increasing effect with increasing dose (Williams, 1972). Abundance data were $\text{Ln}(Ax + 1)$ transformed, where x stands for the abundance value and Ax makes 2 by taking the lowest abundance value higher than zero for x. This was done to down-weight high abundance values and approximate a normal distribution of the data (for rationale, see Van den Brink et al., 2000). Analyses were performed with Community Analysis, version 4.3.05 (Hommen et al., 1994). Statistical significance was accepted at $p < 0.05$. Effects were considered to be consistent when found on two consecutive sampling dates.

The zooplankton and macroinvertebrate data sets were analysed by PRC (Principal Response Curves) using the CANOCO software package, version 4.5 (Ter Braak and Smilauer, 2002). PRC is based on the Redundancy Analysis ordination technique (RDA), the constrained form

of Principal Component Analysis. The analysis results in a diagram showing the sampling day on the x-axis and the first Principal Component of the treatment effects on the community on the y-axis (see Figure 3.3 for an example). This yields a diagram showing the deviations in time of the treatments compared to the control. In this way, PRC shows the most dominant community response to the treatment present in the data set. The species weights are shown in a separate diagram, and indicate the affinity the species have with this dominant response. The species with a high positive weight are indicated to show a response similar to that indicated by PRC, while those with a negative weight one that is opposite to the response indicated by PRC. Species with a near zero weight are indicated to show a response very dissimilar to that indicated by PRC or no response at all. 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 (Van den Brink and Ter Braak, 1999).

Permutation tests were performed per sampling date using Ln-transformed treatment levels as explanatory variables to determine the significance of the treatment regime per sampling date. The NOEC values at community level for the zooplankton and macroinvertebrate communities were calculated for each individual sampling date by applying the Williams test to the sample scores of the first principal component of each sampling date (for rationale, see Van den Brink et al., 1996). The LC50 calculations on the results of the in situ bioassay with *Moina micrura* were done according to Van den Brink et al. (2000).

Results

Chlorpyrifos concentrations

Chlorpyrifos concentrations in the water compartment decreased rapidly (Figure 3.1). After 1 week, approximately 25% and after two weeks less than 10% of the applied dose was found in the water column. The rapid disappearance of chlorpyrifos from the water was accompanied by a slight accumulation of chlorpyrifos in the sediment over time (Figure 3.1). In the first week post application, the top 1.5 cm of sediment accounted for approximately 90% of the total amount of chlorpyrifos detected in the sediment compartment (Figure 3.2). From two weeks post application onwards, relative amounts of chlorpyrifos in deeper layers of sediment increased, though the major part was always found in the top layer(s).

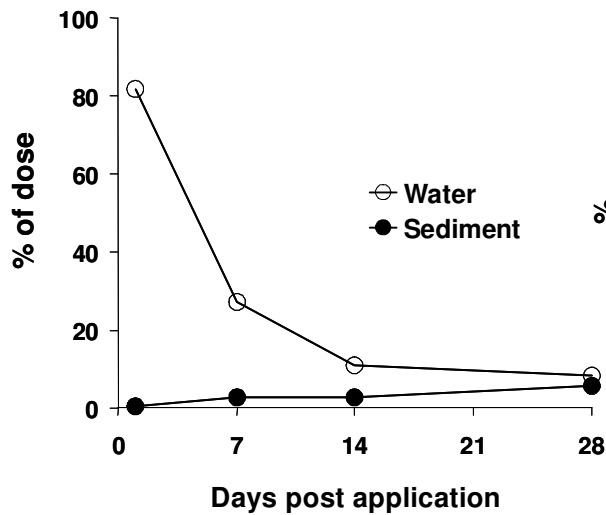


Figure 3.1 Dynamics of chlorpyrifos concentrations in water and sediment as a percentage of the dose applied in the 100 µg/L microcosms.

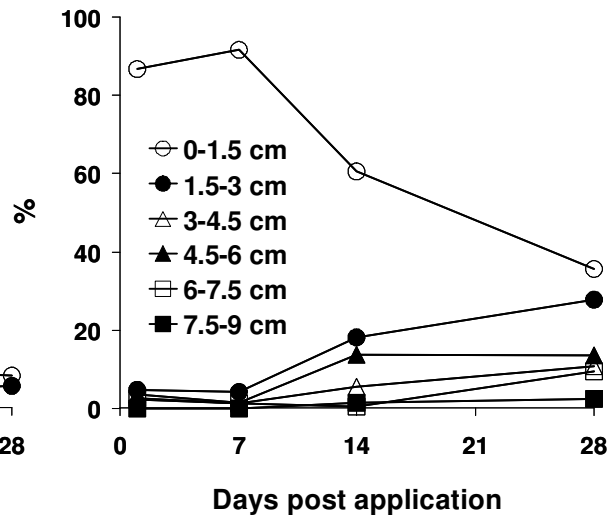


Figure 3.2 Relative contributions of the different sediment layers to the total amount of chlorpyrifos in all layers combined.

Zooplankton

In terms of abundances, the microcosms were dominated by Rotifera and Copepoda, followed by Cladocera and Ostracoda in the pre-treatment period. Rotifera were also the most diverse group, represented by 7 different taxa. The effects of the chlorpyrifos application on the zooplankton community are visualised in the PRC diagram presented in Figure 3.3. The diagram shows that the three highest treatment levels led to a deviation from the controls. Only the zooplankton communities in the 1 µg/L microcosms returned to a state resembling that of the controls within the experimental period. The diagram indicates that *Moina micrura* decreased in numbers due to the treatment, while *Filinia longiseta*, *Ceriodaphnia cornuta* and Calanoid copepods increased. The Monte Carlo permutation tests indicated significant treatment effects for all post-treatment sampling dates. The Williams test indicated a significant treatment effect for the two highest chlorpyrifos treatment levels only (NOEC_{community} = 1 µg/L).

The dynamics of the 4 most discriminating taxa from the PRC analysis are shown in Figure 3.4, whilst all taxa for which a NOEC was calculated are presented in Table 3.1. The most severely affected species was the cladoceran *Moina micrura*, which was completely eliminated by

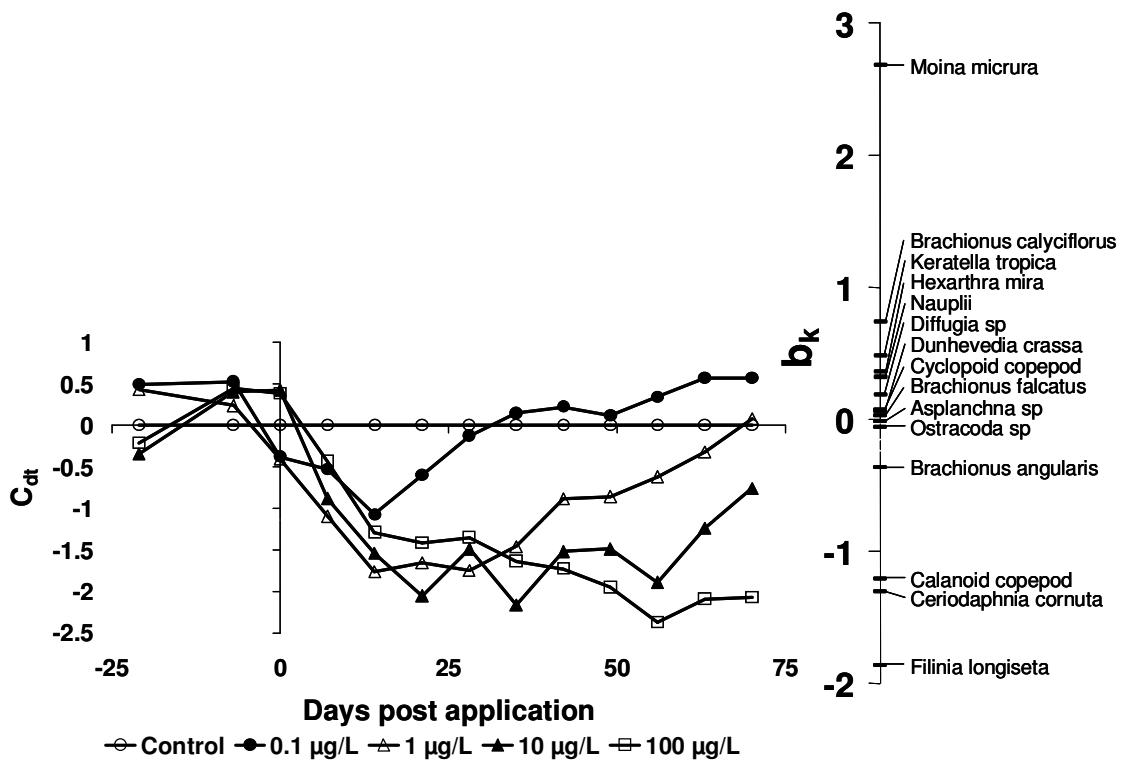


Figure 3.3 Principal response curves resulting from the analysis of the zooplankton data set, indicating the treatment effects of chlorpyrifos on the zooplankton community. Seventeen percent of all variance could be attributed to sampling date; this is displayed on the horizontal axis. Thirty-six percent of all variance could be attributed to treatment level. Of this variance, 31% 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. A Monte Carlo permutation test indicated that a significant part of the variance explained by treatment level is displayed in the diagram ($P = 0.011$).

the higher chlorpyrifos concentrations (Figure 3.4A; NOEC = 0.1 $\mu\text{g/L}$). Another cladoceran, *Ceriodaphnia cornuta*, decreased in numbers one week post application, but increased eight weeks post application in the tanks with higher chlorpyrifos concentrations (Figure 3.4B). A similar pattern was observed for nauplii and mature stages of copepods. Rotatoria taxa decreased (*Keratella tropica*) or increased (*Hexarthra mira*, *Filinia longiseta*, and *Asplanchna sp.*) in abundance (Table 1; Figure 3.4C).

The *Moina micrura* bioassay resulted in LC50 values of 0.7 $\mu\text{g/L}$ (24h) and 0.6 $\mu\text{g/L}$ (48h). A NOEC of 0.1 $\mu\text{g/L}$ was calculated for both 24 hours and 48 hours post application.

Table 3.1 NOECs (No Observed Effect Concentration) for invertebrates that showed a significant response in the Williams test calculations ($p \leq 0.05$). Concentrations ($\mu\text{g a.i./L}$) showed significant increases (+) or decreases (-); nm = not measured; > indicates a NOEC of $> 100 \mu\text{g/L}$.

	Effect	Sampling week												
		-3	-1	0	1	2	3	4	5	6	7	8	9	10
Arthropoda														
Crustacea														
Conchostraca	-	>	nm	nm	0	0.1	nm	10	nm	>	nm	>	nm	>
Ostracoda	-	>	nm	nm	10	10	nm	10	nm	10	nm	10	nm	10
Cladocera	-	>	>	>	1	1	1	10	10	10	>	>	>	>
<i>Ceriodaphnia cornuta</i>	- / +	>	>	>	1	>	>	>	>	>	>	0.1	>	>
<i>Moina micrura</i>	-	>	>	>	0.1	0	0.1	0.1	0.1	0.1	0.1	0.1	0.1	>
Copepoda (nauplii)	-	>	>	>	10	>	>	>	>	>	>	>	>	>
Copepoda (mature)	- / +	>	>	>	10	>	>	>	10	>	>	>	>	>
Calanoid	+	>	>	>	>	>	>	>	>	>	>	10	10	10
Cyclopoid	+	>	>	>	>	>	0	>	10	>	>	>	>	>
Insecta														
Corixidae I	-	>	nm	nm	>	>	nm	1	nm	>	nm	>	nm	>
Corixidae II	-	>	nm	nm	>	>	nm	0.1	nm	1	nm	>	nm	>
Rotifera														
<i>Keratella trópica</i>	-	>	>	>	10	>	>	>	>	>	>	>	>	>
<i>Hexarthra mira</i>	+	>	>	>	>	10	>	>	>	>	>	>	>	>
<i>Filinia longiseta</i>	+	>	>	>	>	>	>	0	1	10	10	>	10	>
<i>Asplanchna sp</i>	+	>	>	>	>	>	>	>	10	>	>	>	10	>
Platyhelminthes														
Turbellaria	+	>	nm	nm	1	1	nm	1	nm	>	nm	>	nm	>
Annelida														
Hirudinae	+ / -	>	nm	nm	>	0.1	nm	>	nm	>	nm	10	nm	>
Mollusca														
Physidae	+	nm	nm	>	nm	1	nm	>	nm	>	nm	>	nm	>
Planorbidae I	+	nm	nm	>	nm	10	nm	>	nm	>	nm	>	nm	>
Planorbidae II	+	nm	nm	>	nm	>	nm	>	nm	>	nm	>	nm	10

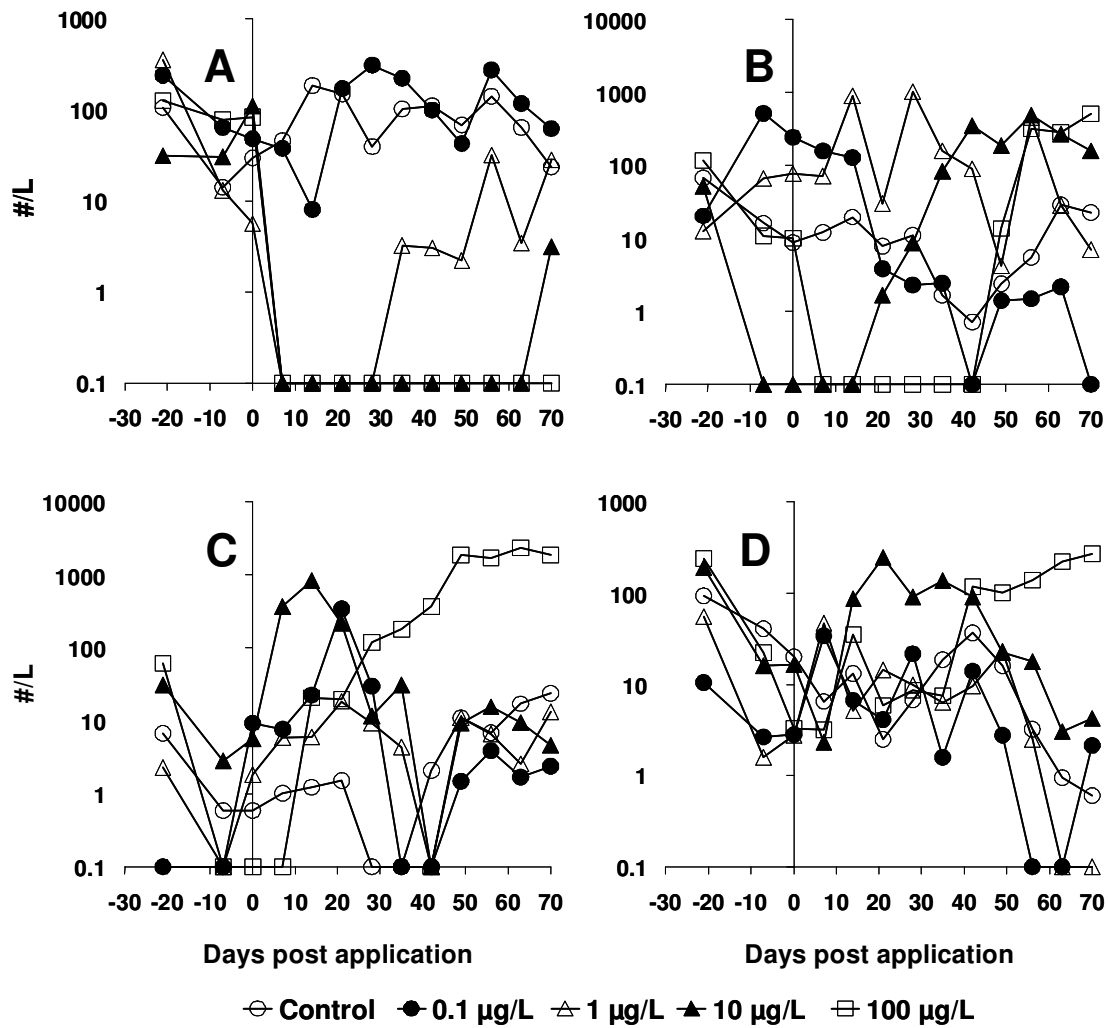


Figure 3.4 Dynamics in terms of numbers of the 4 zooplankton taxa that discriminated best in the PRC: *Moina micrura* (A), *Ceriodaphnia cornuta* (B), *Filinia longiseta* (C) and calanoid copepods (D). In the figures, a value of 0.1 denotes absence of the taxon.

Macroinvertebrate substrates

Over the experimental period, a total of 13 different taxonomic groups were identified from the pebble stone baskets. Besides insects, which were the most diverse group with 8 different families, also flatworms, clam shrimps, oligochaetes, leeches and ostracods were found. In the pre-treatment period, ostracods and clam shrimps (*Conchostraca*) were the most abundant organisms. The effects of the chlorpyrifos application on the macroinvertebrate community are visualised in the PRC diagram presented in Figure 3.5. The diagram shows large deviations from the controls for the two highest treatment levels and smaller ones for the 1 µg/L level.

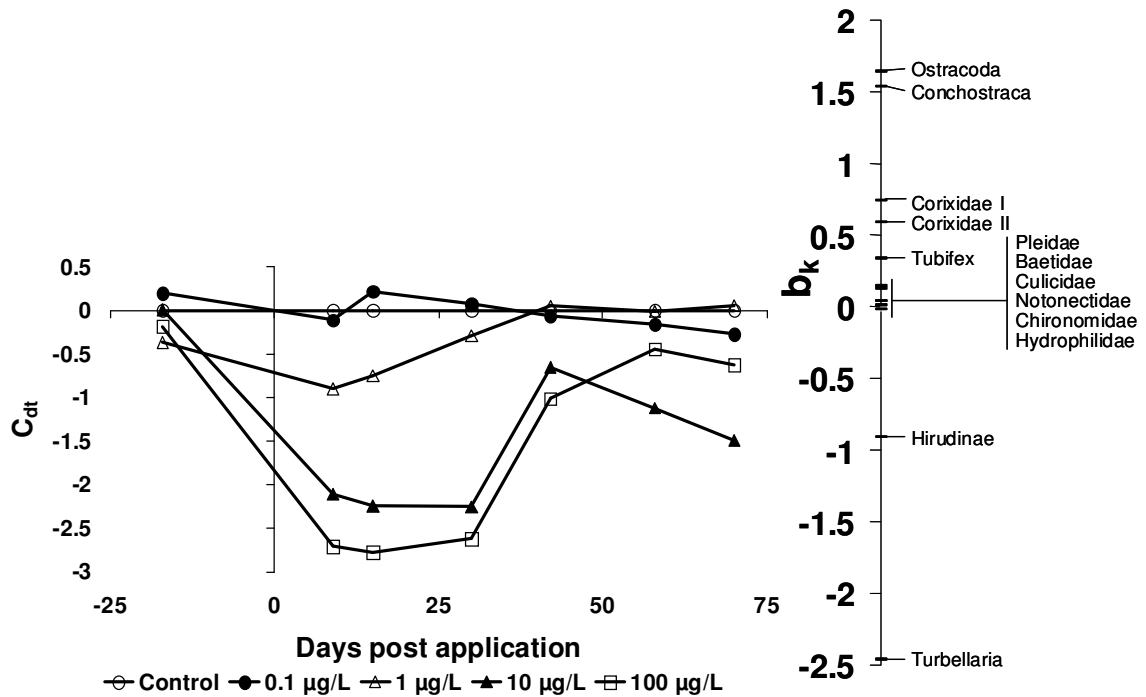


Figure 3.5 Principal response curves resulting from the analysis of the macroinvertebrate data set, indicating the treatment effects of chlorpyrifos on the macroinvertebrate community. Of all variance, 21% could be attributed to sampling date; this is displayed on the horizontal axis. Forty-nine percent of all variance could be attributed to treatment level. Of this variance, 50% 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. A Monte Carlo permutation test indicated that a significant part of the variance explained by treatment level is displayed in the diagram ($P = 0.003$).

All treated tanks more or less returned to a state resembling that of the controls within the experimental period. The diagram indicates that Ostracoda, Conchostraca and Corixidae decreased in numbers due to the treatment, while Turbellaria increased. The Monte Carlo permutation tests indicated significant treatment effects for the post-treatment sampling dates up to day 58. The Williams test indicated a significant treatment effect for the three highest chlorpyrifos levels only ($NOEC_{community} = 0.1 \mu\text{g/L}$).

All taxa identified from the macroinvertebrate substrates for which a NOEC was calculated are summarised in Table 3.1. The most prominent effect was a complete elimination of Conchostraca one week post application (Figure 3.6A; $NOEC = 0.1 \mu\text{g/L}$). Only in the $1 \mu\text{g/L}$ microcosms, individuals of Conchostraca were found again at the end of the experiment.

Ostracods disappeared from the highest dosage tanks (Figure 3.6B). At the two highest treatment levels, water boatmen (*Corixidae*) decreased in numbers relative to the controls, whereas flatworms (*Turbellaria*) increased. Even though *Corixidae* were completely eliminated at these concentrations, they returned to normal levels 8 weeks after application (Figure 3.6C). *Turbellaria* were absent or present in very low numbers in controls and at the lower chlorpyrifos dosages. Applications of 10 and 100 $\mu\text{g/L}$ led to a large increase in numbers of flatworms up to 4 weeks post application, after which numbers declined to the low numbers found in the controls (Figure 3.6D).

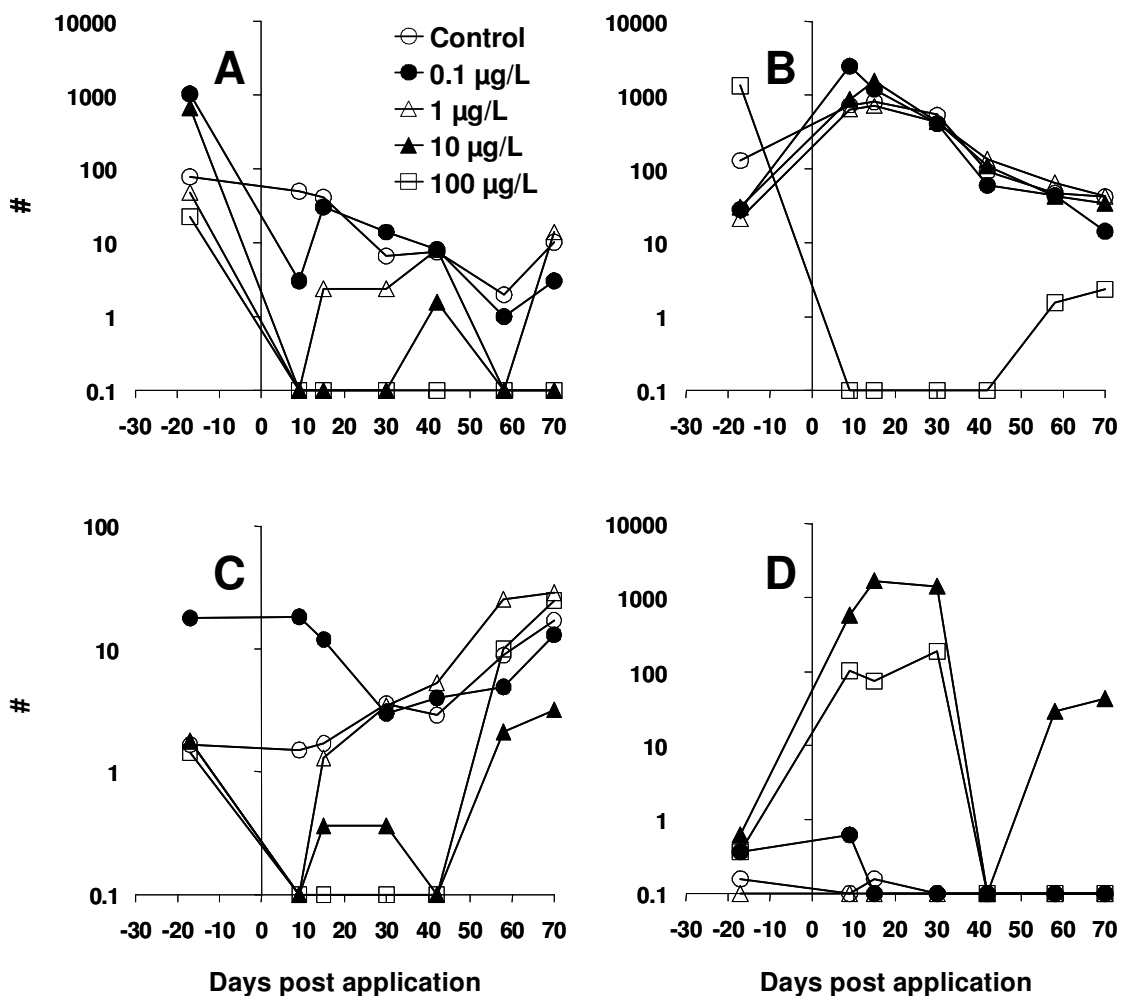


Figure 3.6 Dynamics in terms of numbers of the 4 groups of animals from the macro-invertebrate substrates that showed significant treatment effects of chlorpyrifos: Conchostraca (A), Ostracoda (B), Corixidae (C) and Turbellaria (D). A value of 0.1 denotes absence of the taxon.

Snails

Snails were represented by 3 families: Physidae, Planorbidae (2 species) and Ancyliidae. Increased abundances of snails were found at several moments in the experiment, though no NOECs for two consecutive sampling dates could be calculated (Table 3.1).

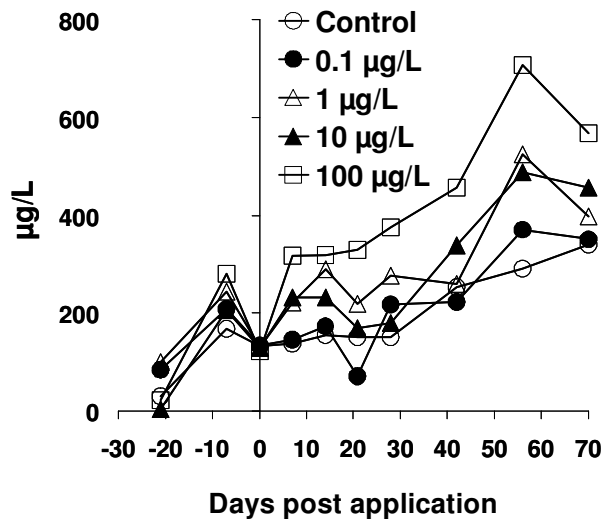


Figure 3.7 Dynamics of chlorophyll-a levels in the course of the experiment.

Chlorophyll-a

Chlorophyll-a values of the phytoplankton increased in all microcosms during the experimental period (Figure 3.7). The increase was substantially higher in the microcosms with the highest dosage than in controls, leading to significantly higher chlorophyll-a levels in these systems.

Chlorophyll-a content of the periphyton on glass slides varied substantially between sampling dates and treatments (from 1 to 500 µg/dm²), but no consistent treatment effects were found. Six weeks post application, periphyton chlorophyll-a levels at the highest treatment level were as much as 10 times higher than in controls (significant, data not shown).

Water quality parameters

No consistent treatment effects were recorded for dissolved oxygen, pH, conductivity or alkalinity. Dissolved oxygen (3 wks p.a.) and conductivity levels were significantly lower than those of the controls at the highest treatment level on two sampling dates (6 and 8 wks p.a.).

Discussion

Fate of chlorpyrifos

Chlorpyrifos disappeared rapidly from the water column, with disappearance rates similar to those found in studies in temperate regions (Figure 3.1, Crum and Brock, 1994; Leeuwangh, 1994). Initially, a faster decline of chlorpyrifos concentrations in the water compartment was expected under tropical conditions, since higher temperatures and light intensity have been reported to decrease the half-lives of organophosphorus insecticides (Schaefer and Dupras, 1970). The decrease in chlorpyrifos concentrations in water from approximately day 1 to day 10, however, is thought to be governed especially by partitioning processes (Leeuwangh, 1994). Macrophytes have been demonstrated to adsorb approximately 40% of the dose applied in macrophyte-dominated microcosm and mesocosm studies in temperate regions (Crum and Brock, 1994). Hence, the higher than expected chlorpyrifos concentrations in the water compartment are likely to be due to the absence of macrophytes. Instead, the high algal biomass in the plankton-dominated test systems of the present study (Figure 3.7) presumably bound a large part of the insecticide dose. Since unfiltered water samples were used for analysis, chlorpyrifos adsorbed to algae was included in the water compartment. Furthermore, the microcosms in the present study were approximately twice as deep as the test systems used in reference studies (Crum and Brock, 1994; Leeuwangh, 1994), meaning that the surface to volume ratio of the water was relatively low. This implies less evaporation of chlorpyrifos from the water, which has been demonstrated to play a significant role in the loss of chlorpyrifos from aquatic systems (Racke, 1993). The sediment surface to water volume was also low, so adsorption of chlorpyrifos from the water to the sediment could have played a smaller role than in the studies in temperate regions. This may explain why only a relatively small proportion of the insecticide became associated with the sediment (Figure 3.1). Relative contributions of deeper sediment layers gradually increased over time (Figure 3.2) due to processes like diffusion and bioturbation (Ruiz et al., 2001).

Effects of chlorpyrifos on invertebrates

The primary response to chlorpyrifos was a decline in arthropod invertebrate abundances. Clam shrimps (Conchostraca) and the cladoceran *Moina micrura* were the most susceptible to

chlorpyrifos (Table 3.1). Results of the in situ bioassay corresponded well with the effects on the *Moina micrura* population observed in the microcosms, as an NOEC of 0.1 µg/L was found for both.

The relatively small cladoceran *Ceriodaphnia cornuta* declined in numbers only at the highest chlorpyrifos concentration, and increased in numbers at the 1 and 10 µg/L dosages. In line with this, smaller cladoceran species have been found to be less sensitive to insecticides than larger species (Hanazato, 1998).

Ostracods and the rotifer *Keratella tropica* were moderately sensitive to chlorpyrifos. Their abundances were only significantly decreased relative to controls at the highest treatment level (Table 3.1). In the case of *Keratella tropica*, it is questionable if this was a direct effect of chlorpyrifos, because *Keratella* species are not known to be sensitive to insecticides (Van Wijngaarden et al., 2005b). The rotifers *Hexarthra mira*, *Filinia longiseta* and *Asplanchna sp.* increased in numbers in the higher dosage tanks. In line with this, non-sensitive Rotifera have frequently been reported to increase in numbers in insecticide-stressed aquatic systems, as a result of reduced competition and grazing pressure caused by the decline in cladocerans (Brock et al., 2000; Fleeger et al., 2003; Van Wijngaarden et al., 2005b).

Copepods as a group, i.e. cyclopoids and calanoids combined, as well as their immature stages (nauplii), decreased in numbers one week post application in the highest dosage tanks. Apparently, they recovered quickly, since increased abundances of calanoids and cyclopoids were found at several moments in the course of the experiment (Table 3.1).

Among the insects, numbers of Corixidae were significantly reduced relative to controls at the higher treatment levels, starting four weeks post application (Figure 3.6C). No significant treatment effects were observed on other insect species, mainly due to their low abundances in the control microcosms.

Overall, the NOECecosystem was set at 0.1 µg/L. This is based on the PRC diagram and the NOECcommunity calculated for the macroinvertebrates, and the PRC diagram for zooplankton. Although the NOEC calculations for the zooplankton community revealed a NOECcommunity of 1 µg/L, this is most likely due to the high level of variation in the controls, since deviations from the control were also found for the 1 µg/L treatment and to a lesser extent also for the 0.1 µg/L treatment level (Figure 3.3). These differences were, however, not confirmed by the univariate analysis (Table 3.1). Prolonged significant treatment effects on the dominant zooplankton (*Moina micrura*) and dominant species from the macroinvertebrate substrates (Conchostraca) were found at chlorpyrifos levels of 1 µg/L and higher.

Table 3.2 No observed effect concentration (NOEC), lowest observed effect concentration (LOEC) and time to recovery of the zooplankton and macroinvertebrate communities corresponding to the LOEC reported in microcosm and mesocosm studies after short-term exposure (single pulse in artificial streams and single application in lentic test systems) to chlorpyrifos. nm = not measured.

Study	Type of system	Reference	NOEC / LOEC ecosystem ($\mu\text{g/L}$)	Time to recovery zooplankton (wk)	Time to recovery Macroinvertebrates (wk)
Temperate					
The Netherlands	Lentic, outdoor, experimental ditches	Van den Brink et al. (1996)	0.1 / 0.9	4	4 - >55
USA (Kansas)	Lentic, outdoor, microcosms	Biever et al. (1994)	0.1 / 0.3 - 1	≤ 4	4-6
USA (Minnes.)	Lentic, outdoor, littoral enclosures	Siefert et al. (1989)	< 0.5 / 0.5	< 1	< 1
Australia	Lotic, outdoor, artificial streams	Pusey et al. (1994)	0.1 / 5	nm	< 6
The Netherlands	Lentic, indoor, laboratory microcosms				
	Mesotrophic; cool (16 - 18 °C)	Van Wijngaarden et al. (2005a)	0.1 / 1	5	nm
	Mesotrophic; warm (24 - 28 °C)	Van Wijngaarden et al. (2005a)	0.1 / 1	5	nm
	Eutrophic, warm (25 - 28 °C)	Van Wijngaarden et al. (2005a)	0.1 / 1	> 5	nm
Tropical					
Thailand	Lentic, outdoor, microcosms	This study	0.1 / 1	9	6-8

Ecological effect chain

The chlorpyrifos application led to a decline and elimination of several zooplankton and macroinvertebrate taxa. The resulting reduction in grazing pressure led to increased chlorophyll-a levels in phytoplankton. This increase in food availability resulted in an increase in snails and tolerant invertebrates. The higher algal densities also led to an occasional drop in conductivity and dissolved oxygen levels in the morning, due to increased respiration during the night. Similar ecological effect chains have been reported in various studies in temperate regions (e.g. Hanazato, 1998; Brock et al., 2000; Fleeger et al., 2003).

Comparison of thresholds in tropical and temperate zones

The sensitivity of the microcosm biocoenosis to chlorpyrifos was comparable to that found in studies in temperate regions (Table 3.2). Irrespective of the geographic position, a NOEC community of 0.1 µg/L is found. The same threshold level was found, irrespective of climatic test conditions, in laboratory microcosm studies by Van Wijngaarden et al. (2005a). The authors explained this from the fact that microcrustaceans are amongst the species that are the most sensitive to organophosphate exposure, and that these species are usually abundant in different types of test systems. The crustaceans in the present study appeared to be as sensitive as those in temperate zone studies, with Cladocera as the most sensitive group. The most abundant cladoceran in the present study, *Moina micrura*, exhibited a sensitivity in the bioassay (LC50 48h = 0.6 µg/L) comparable to *Daphnia magna*, a standard test species in temperate countries (LC50 48h = 1 µg/L; Kersting and Van Wijngaarden, 1992). In line with this, no major differences in species sensitivity distributions have been found between tropical and temperate freshwater arthropods (Maltby et al., 2005; Kwok et al., 2007).

The recovery of the zooplankton community was slower in our study under tropical conditions than has been observed under temperate conditions (Table 3.2). Even though one may expect a faster recovery of affected communities because of the high chlorophyll-a levels and high temperatures, the excessive increase in copepod and especially rotifer abundances probably hampered the recuperation of the cladoceran community. The recovery of the Corixidae took approximately 6 to 8 weeks, which is slightly longer than the 4 to 6 weeks reported for macroinvertebrate species in studies in temperate regions (Table 3.2). However, significant treatment effects and hence recovery were only found for one macroinvertebrate in

the present study. In a mesocosm study by Van den Brink et al. (1996), recovery of macroinvertebrates took between 4 and > 55 weeks, which made them conclude that the recovery of macroinvertebrates mostly depends on life strategies and infrastructural characteristics of the ecosystem.

The above findings support the use of temperate zone toxicity data for the risk assessment of pesticides for aquatic communities in tropical regions. However, more ecological data is required to improve our understanding of indirect effects and recovery of tropical freshwater communities.

Acknowledgement

This study was funded by the Portuguese government (scholarship SFRH/ BD/ 8213/ 2002). The authors are indebted to Mr. Manoj Yomjinda for nutrient analysis and to staff at the AIT hatchery for their assistance.

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CHAPTER 4

IMPACT OF SINGLE AND REPEATED APPLICATION OF THE INSECTICIDE CHLORPYRIFOS ON FRESHWATER PLANKTON COMMUNITIES UNDER TROPICAL CONDITIONS

(Submitted to *Ecotoxicology*)

Abstract

This paper describes the effects of a single and a repeated application of the organophosphorus insecticide chlorpyrifos on zooplankton and phytoplankton communities in outdoor microcosms in Thailand. Treatment levels of 1 µg/L were applied once or twice with a two-week interval. Both treatments led to a significant decrease in cladocerans followed by an increase in rotifers, although the extent by which species were affected was different. *Ceriodaphnia cornuta* was the most responding cladoceran after the first treatment, while most pronounced effects of the second treatment were found for *Moina micrura*. This is explained by differences in population dynamics at the time of application and the increase of *Microcystis* abundance over the course of the experiment. Several phytoplankton taxa either increased or decreased as a result of the chlorpyrifos-induced changes in zooplankton communities. Even though chlorpyrifos disappeared fast from the water column, effects on plankton communities persisted till the end of the experiment (42 days) when the insecticide concentrations had dropped below the detection limit.

Introduction

Before the late 1960s, the traditional agricultural practices in Thailand were in close interrelationship with the local environment. Occasional floods of rivers during the rainy season assured a continual fertility of the land and pesticides were hardly used (Heckman, 1979; Tonmanee and Kanchanakool, 1999). The Green Revolution led to an intensification of agricultural practises and the use of pesticides and fertilizers increased considerably throughout the years (Jungbluth, 2000). As a consequence, pesticide contamination of soil,

water and agricultural products have been reported throughout the country (Thapinta and Hudak, 2000).

Little research has been carried out, however, evaluating the environmental side-effects of agrochemicals in tropical countries like Thailand (Lacher and Goldstein, 1997; Gopal, 2005). The Thai ecotoxicological literature consists almost entirely of determinations of LC50 values for various freshwater species, invariably conducted using static tests, and basic freshwater community interactions are still largely unknown (Campbell and Parnrong, 2001).

In the present study, the fate and effects of a single and repeated application of the organophosphorous insecticide chlorpyrifos was evaluated in outdoor plankton-dominated microcosms in Thailand. Microcosms and mesocosms have frequently been used for the environmental risk assessment of several chemicals, like insecticides (see Maltby et al., 2005 and Van Wijngaarden et al., 2005 for reviews). These test systems provide more ecological realism as compared to laboratory bioassays since they include ecological processes like interactions between plankton populations while still allowing an experimental set-up.

In a previous microcosm experiment using larger test systems, the fate and effects of a single chlorpyrifos application was studied (Daam et al., *subm.*). However, farmers in Thailand administer pesticides with a high frequency on their land, whereby a biweekly interval is not uncommon (Van den Brink et al., 2003; Satapornvanit et al., 2004).

We, therefore, evaluated the effects of a single and repeated application of 1 µg chlorpyrifos/L on zooplankton and phytoplankton communities and its interactions in outdoor microcosms in Thailand. The interval between the two applications was set at two weeks to mimic local agricultural practices.

Materials and Methods

Experimental set-up

Ten circular experimental microcosms, each with a diameter of 0.76 m and a water depth of 0.56 m (water volume approximately 250 liters), were used for the experiment. The concrete tanks were coated with watertight non-toxic epoxy paint and set up outdoors at the hatchery of the Asian Institute of Technology (AIT), located approximately 42 km north of Bangkok (Thailand). The test systems were filled with water from the canal surrounding AIT after filtering through a net (mesh size 0.1 mm) to avoid fish and prawns entering the systems. No

sediment was added to keep the experimental set-up as simple as possible and, consequently, to facilitate interpretation of the (in)direct treatment effects. In the preparatory phase of the experiment (1 week prior to application), zooplankton was collected from the AIT canal and introduced into the microcosms. In this period, the water was circulated two times by exchanging 100 litres between the microcosms using a Perspex tube to achieve similarity between the communities in the systems. A nutrient addition of N (1.4 mg/L as urea) and P (0.35 mg/L as TSP) was applied twice a week during the entire experimental period.

Application and fate of the test substance

Chlorpyrifos was applied as an aqueous solution of Dursban to six microcosms at a concentration of 1 µg/L. This concentration was chosen because it corresponds with the LC50 of the cladoceran *Daphnia magna* (Kersting and Van Wijngaarden, 1992) and on the basis of an earlier experiment in similar systems, treatment effects were expected without completely eliminating the zooplankton community (Daam et al., *subm.*). Four other systems were untreated to serve as controls. Two weeks after the first application, three of the six applied tanks received a second application of 1 µg/L chlorpyrifos. After applications, the water in the microcosms was gently stirred in order to mix the insecticide over the water column. Subsamples of the treatment solutions were taken and subsequently analysed as described below for calculations of nominal concentrations.

Depth-integrated water samples of approximately 10-L were collected in a glass container using a Perspex tube one hour after application (initial concentration) and at various moments during the experiment. Of these samples, 750 mL were transferred to glass bottles and shaken with 50 ml n-hexane (HPLC grade) for one hour. A part of the upper liquid was collected and transferred to GC-vials for analysis by splitless injection of 3 µl on a HP 5890 Gas Chromatograph. GLC operating parameters: capillary column coated with HP-5, length 30 m, internal diameter 0.32 mm, film thickness 0.25 µm; initial oven temperature 70°C, increasing with 20°C/ min until a final temperature of 280°C which was kept constant for 10 min (total run time 20.5 min); detector: Electron Capture Detector; nitrogen flow 1.5 mL/min. The detection limit and recovery of chlorpyrifos were respectively 0.07 µg/L and 93.5 ± 6.7% (mean ± s.d., n = 6). Correction for the recovery was made when calculating chlorpyrifos residues in water.

Endpoints

Dissolved oxygen was measured in the morning at 1-week intervals with an YSI model 58 oxygen meter connected to an YSI 5739 probe approximately 10 cm under the water surface. Together with the oxygen, conductivity and pH were measured with a WTW conductivity meter and a CONSORT pH meter respectively.

At several moments during the course of the experiment, a bulk water sample of 10-L was collected in a bucket by taking several depth-integrated water samples using a Perspex tube. From this bulk sample, a subsample of 1-L was taken to study the phytoplankton community and another 1-L for determination of the phytoplanktonic chlorophyll-a concentration. Then, the bucket was emptied until a remainder of 5 litres was obtained which was transferred through a zooplankton net (mesh size 60 μm) to examine treatment effects on the zooplankton community.

The concentrated zooplankton sample was fixed with formol in a final concentration of 4%. The 1-L phytoplankton sample was stained with lugol and concentrated after sedimentation of 6 days. Additional lugol was added when needed to assure conservation of the samples. Subsamples of the zooplankton and phytoplankton samples were counted with an inverted microscope (magnification 100 - 400) and numbers were recalculated to numbers per litre microcosm water. Colony forming algae except *Microcystis aeruginosa* and *Microcystis incerta* were quantified by counting the number of colonies. *M. aeruginosa* and *M. incerta* form large 3-dimensional colonies that, especially when occurring in high abundances, are difficult to quantify with high precision. Therefore, these two species were quantified as single cells in subsamples of the phytoplankton samples after disintegration of the colonies by ultrasonication as described by Kurmayer et al. (2003).

Phytoplanktonic chlorophyll-a measurements were made using the 1-L water sample taken as described above. A known volume was concentrated over a Whatman GF/C glass fibre filter (mesh size 1.2 μm) until the filter was saturated. Filters were then air dried and extracted the same day using the method of Moed and Hallegraeff (1987).

Data analysis

Abundance data of zooplankton and phytoplankton were $\text{Ln}(Ax + 1)$ transformed prior to analysis, where x stands for the abundance value and Ax makes 2 by taking the lowest

abundance value higher than zero for x . This was done to down-weight high abundance values and approximate a normal distribution of the data (for rationale, see Van den Brink *et al.*, 2000).

Until the second chlorpyrifos application (14 days post first application), statistical significance of differences between the treatment and the control were calculated for all parameters using ANOVA. Analyses were performed with Community Analysis, version 4.3.05 (Hommen *et al.*, 1994). Statistical significance was accepted at $p < 0.05$. After the second application, statistical significance between the treatments and the control were calculated using the Dunnett's test and expressed as NOECs.

The zooplankton and phytoplankton data sets were analysed by PRC (Principal Response Curves) using the CANOCO software package version 4.5 (Ter Braak and Smilauer, 2002). PRC is based on the Redundancy Analysis ordination technique (RDA), the constrained form of Principal Component Analysis. The analysis results in a diagram showing the sampling day on the x-axis and the first Principal Component of the treatment effects on the community on the y-axis (see Figure 4.2 for an example). This yield a diagram showing the deviations *in time* of the treatments compared to the control. In this way PRC shows the most dominant community response to the treatment present in the data set. The species weights are shown in a separate diagram, and indicate the affinity the species have with this dominant response. The species with a high positive weight are indicated to show a response similar to the response indicated by PRC, those with a negative weight, one that is opposite to the response indicated by PRC. Species with a near zero weight are indicated to show a response very dissimilar to the response indicated by PRC or no response at all. 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 (Van den Brink and Ter Braak, 1999). After the first PRC component, more can be extracted from the remaining variation analogous to as described by Van den Brink and Ter Braak (1998). The second PRC shows the most important deviations from the first PRC, present in the data set.

Monte Carlo permutation tests were performed to assess the significance of the differences in community composition between the treatments and the controls. This was done by testing every treatment against the controls per sampling date.

Results

Fate of chlorpyrifos

The standard deviations within treatments for initial and nominal concentrations as well as the concentrations during the course of the experiment were mostly lower than 5% and always lower than 10% of the respective concentrations. The concentrations of chlorpyrifos decreased rapidly after both applications (Figure 4.1). Four and seven days after application, mean chlorpyrifos concentrations were respectively 28 - 21 % and 17 - 10 % (first - second application) of nominal concentrations.

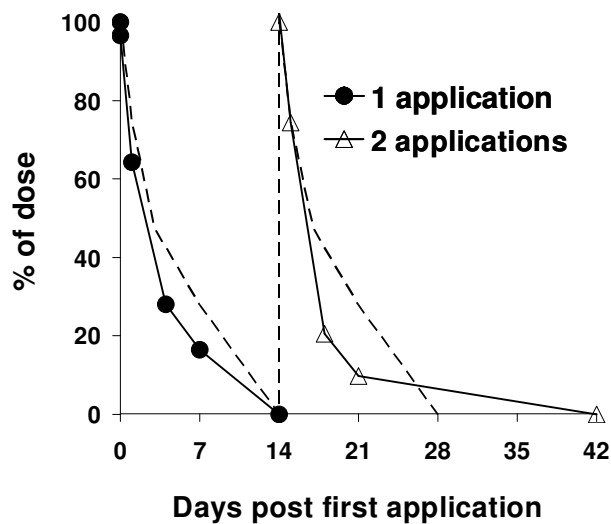


Figure 4.1 Dynamics of the chlorpyrifos concentrations in the water as a percentage of the dose applied. The dashed line indicates the chlorpyrifos concentrations following application of 1 $\mu\text{g}/\text{L}$ as measured in a microcosm study using larger test systems (Daam et al., *subm.*; for explanation: see text).

Zooplankton

Before application, the dominant species in the zooplankton samples belonged to the groups of Rotifera and Copepoda, while Cladocera and Ostracoda occurred in low numbers. During the course of the experiment, Cladocera and Ostracoda increased in numbers in the control systems while Copepoda showed the opposite trend. By the end of the experiment, Cladocera and Ostracoda, and to a lesser extent the Rotifera, dominated the control zooplankton community.

Analysis using the PRC method indicated that forty-two percent of all variance could be attributed to the treatments. Of this variance, 40% is displayed on the vertical axis of a first PRC (Figure 4.2A, $p < 0.01$) and another 18% on the vertical axis of a second PRC (Figure 4.2B, $p < 0.01$).

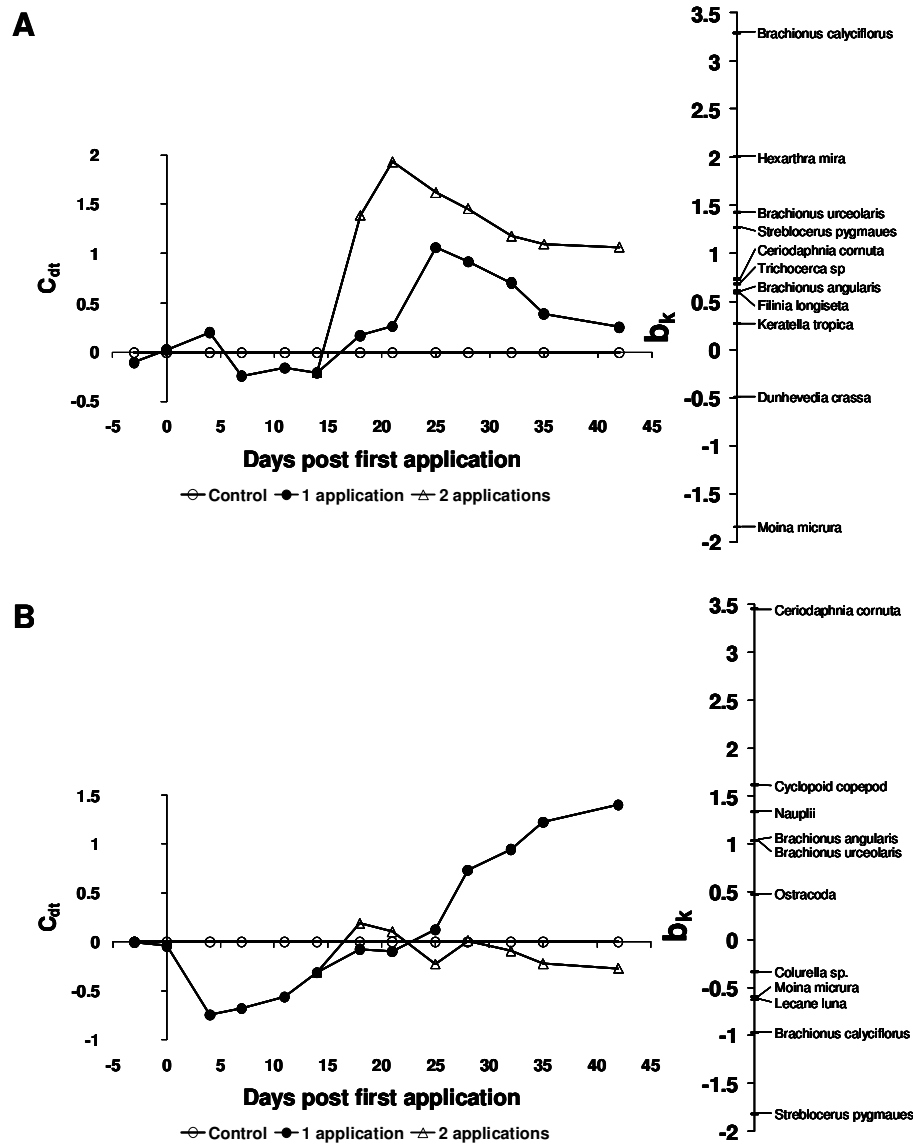


Figure 4.2 First (A) and second (B) Principal response curves resulting from the analysis of the zooplankton data set, indicating the effects of one or two applications of 1 $\mu\text{g/L}$ of the insecticide chlorpyrifos on the zooplankton community. Of all variance, 20% could be attributed to sampling date; this is displayed on the horizontal axis. Forty-two percent of all variance could be attributed to treatment level. Of this variance, 40% is displayed on the vertical axis of the first PRC (A) and 18% on the vertical axis of the second PRC (B). 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. A Monte Carlo permutation test indicated that the treatment regime had a significant influence on the community structure ($p = 0.011$) and that a significant part of the variance explained by treatment level is displayed in the first ($p = 0.024$) and second ($p = 0.001$) PRC.

The PRC diagrams of the zooplankton dataset show that both chlorpyrifos treatments led to deviations from the controls, which are confirmed by the results of the Monte Carlo permutation tests (Table 4.1).

Table 4.1 Results of Monte Carlo permutation performed per sampling date for the zooplankton data set. NP means calculation is not possible.

Day	1 application	2 applications
-3	0.308	NP
0	0.083	NP
4	0.016	NP
7	0.014	NP
11	0.041	NP
14	0.261	NP
18	0.309	0.030
21	0.030	0.030
25	0.030	0.030
28	0.030	0.030
32	0.075	0.058
35	0.051	0.105
42	0.051	0.124

After the first treatment, the first PRC diagram does not show a large deviation from control in the two weeks following application, whereas the curve of the second PRC clearly drops immediately after application. From two to three weeks onwards, both PRCs show deviations in zooplankton community from control for microcosms treated once with chlorpyrifos. The curve of the first PRC rises considerably after the second treatment, whereas the curve in the second PRC stays close to zero. The broad pattern that emerges from this is that the dominant short-term effects of the first chlorpyrifos application are best described by the second PRC and longer-term effects by a combination of the two PRCs, whereas effects of the second application are mainly shown by the first PRC.

The indicated response pattern for individual species is obtained by multiplying the respective species weights with the treatment (c_{dt}) scores in the corresponding PRCs and then summing the two products (Van den Brink and Ter Braak, 1998). This is thus especially relevant for the longer term effects of the first application. To facilitate the subtraction of the indicated response on species level, a plot of the weights of the different species on the first and second PRC is given in figure 4.3.

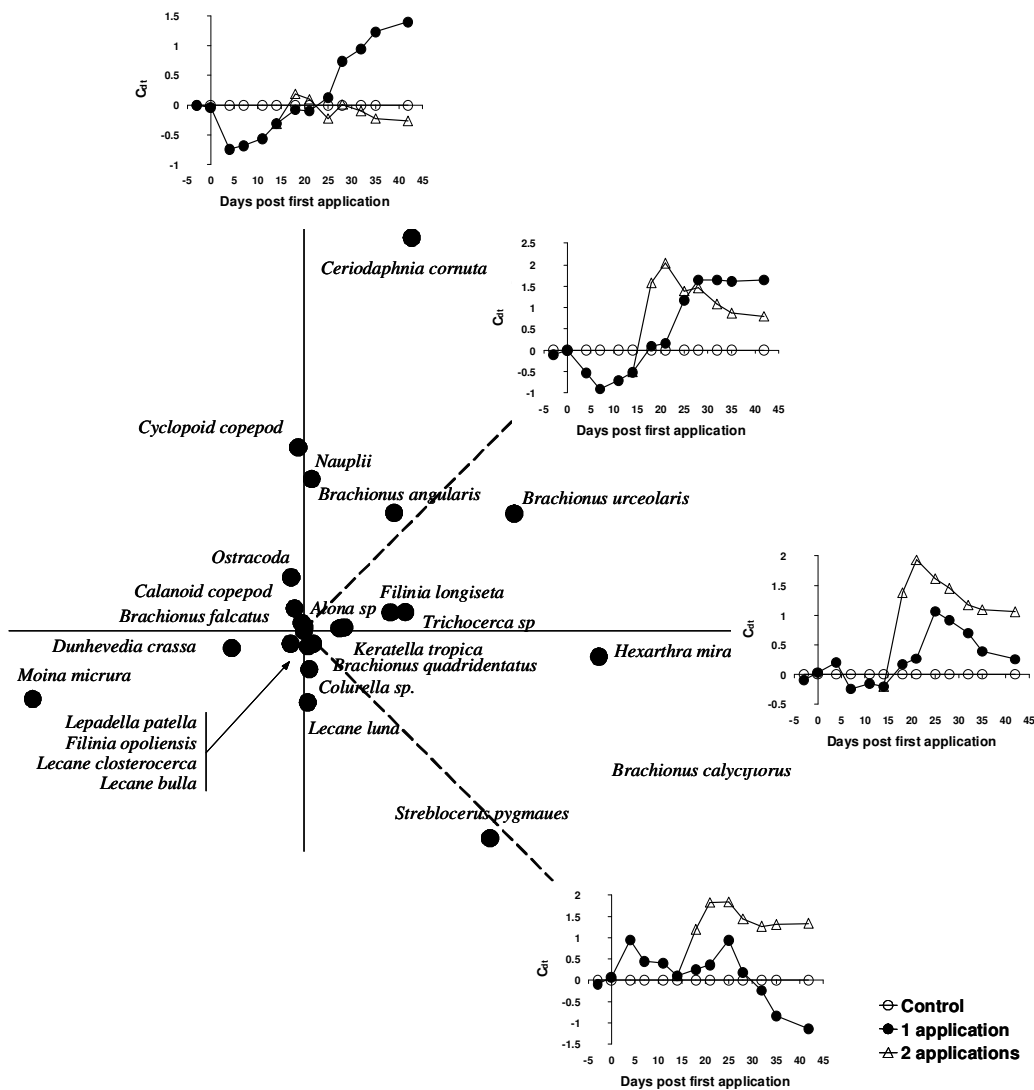


Figure 4.3 Two dimensional plot of the weights of the zooplankton taxa on the first (horizontal axis) and second (vertical axis) PRC, as given in Figure 4.2. The diagram in the corner applies to the taxa that have equal weights on the two PRC's.

Furthermore, calculated response curves with a ray of $+45^\circ$ and -45° from the horizontal axis are included in this diagram. Species coordinates that lay near the origin indicate that the corresponding species did not show a large response to the treatments. The species that are positioned along one of the axis have a response curve as indicated by the corresponding PRC. For instance, *Hexarthra mira* is located on the right side of the horizontal axis and thus has a response curve similar to the first PRC. *Streblocerus pygmaeus* has a positive weight in the first PRC and a negative weight in the second PRC, so its response curve is a combination of the first PRC and the inversed curve of the second PRC (Figure 4.3).

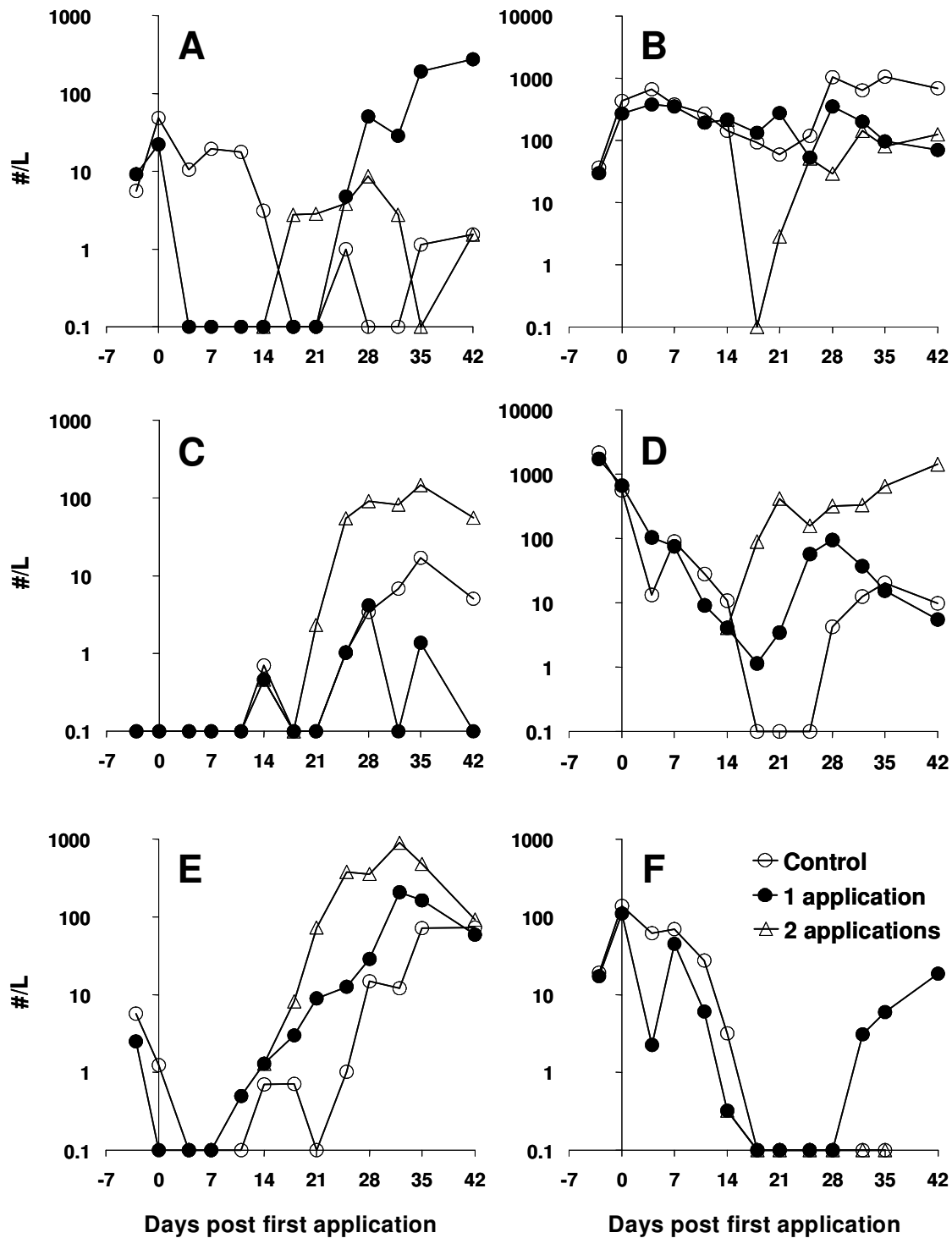


Figure 4.4 Dynamics in numbers of the zooplankton taxa with a species weight higher than 1.5 or lower than -1.5 in the two PRCs of the zooplankton dataset. Figures 4A through 4F show the geometric means of the abundances of the cladocerans *Ceriodaphnia cornuta* (A), *Moina micrura* (B), and *Streblocerus pygmaeus* (C); the rotifers *Brachionus calyciflorus* (D) and *Hexarthra mira* (E); and cyclopoid copepods (F). In the figures, a value of 0.1 denotes the absence of the taxon.

Table 4.2 NOECs (No Observed Effect Concentration) for zooplankton and phytoplankton populations that showed a significant response ($p \leq 0.05$). 0: NOEC = control; 1: NOEC = 1 application; >: NOEC ≥ 1 application (days -3 till 14) or 2 applications (days 18 till 42); (+): significant increase compared to controls; (-): significant decrease compared to controls; nm: not measured.

	Days post start first application												Fig	
	-3	0	4	7	11	14	18	21	25	28	32	35		42
ZOOPLANKTON														
Cladocera	>	>	0 (-)	>	>	>	1 (-)	>	>	>	>	>	>	
<i>Ceriodaphnia cornuta</i>	>	>	0 (-)	0 (-)	0 (-)	>	>	>	>	>	>	>	>	4A
<i>Moina micrura</i>	>	>	0 (-)	>	>	>	1 (-)	1 (-)	>	1 (-)	>	>	>	4B
<i>Streblocerus pygmaeus</i>	>	>	>	>	>	>	>	>	1 (+)	1 (+)	>	>	>	4C
Rotifers	0 (-)	>	0 (+)	>	0 (+)	>	1 (+)	0 (+)	0 (+)	0 (+)	1 (+)	>	>	
<i>Brachionus calyciflorus</i>	>	>	>	>	>	>	1 (+)	1 (+)	0 (+)	0 (+)	>	>	1 (+)	4D
<i>Brachionus urceolaris</i>	>	>	>	0 (-)	>	>	>	1 (+)	>	>	>	>	>	
<i>Colurella sp.</i>	>	>	>	>	0 (+)	>	>	>	>	>	>	>	>	
<i>Hexarthra mira</i>	>	>	>	>	>	>	>	1 (+)	1 (+)	>	0 (+)	>	>	4E
<i>Keratella tropica</i>	>	>	>	>	>	>	>	1 (+)	>	>	>	>	>	
<i>Trichocerca sp.</i>	>	>	>	>	>	>	>	1 (+)	>	>	>	>	>	
Copepoda	>	>	>	>	>	>	>	>	>	>	>	>	>	
Calanoid copepod	>	>	0 (-)	>	>	>	>	>	>	>	>	>	>	
Cyclopoid copepod	>	>	0 (-)	>	>	>	>	>	>	>	>	>	>	4F
Ostracoda	>	>	>	>	>	>	>	>	>	>	0 (+)	0 (+)	>	
PHYTOPLANKTON														
<i>Scenedesmus quadricauda</i> (A)	>	>	nm	0 (-)	nm	>	nm	1 (+)	nm	>	nm	>	nm	6B
<i>Coelastrum astroidesum</i>	>	>	nm	0 (-)	nm	>	nm	0 (+)	nm	>	nm	>	nm	6C
<i>Coelastrum microporum</i>	>	>	nm	0 (-)	nm	0 (-)	nm	>	nm	>	nm	>	nm	
<i>Oocystis borgei</i>	>	>	nm	0 (-)	nm	>	nm	>	nm	1 (+)	nm	>	nm	6D
<i>Microcystis aeruginosa/ incerta</i>	>	>	nm		nm	>	nm	1 (-)	nm	>	nm	>	nm	6A
<i>Nitzschia palea</i>	>	>	nm		nm	>	nm	1 (+)	nm	>	nm	>	nm	
Chlorophyll-a	>	0 (-)	nm	>	nm	>	nm	1 (-)	nm	1 (-)	nm	>	>	7A
WATER QUALITY														
Dissolved oxygen	>	>	nm	>	>	>	nm	>	nm	1 (+)	nm	>	>	7B

The dynamics of the taxa with a weight higher than 1.5 or lower than -1.5 with either one of the two PRCs are given in Figure 4.4A through 4.4F, whilst all taxa for which a statistical significance of difference was calculated are presented in Table 4.2. The most susceptible taxa belonged to the Cladocera, although they were affected differently by the two chlorpyrifos applications. The first application led to a complete elimination of *Ceriodaphnia cornuta*, and only a slight (though significant) decrease in numbers of *Moina micrura* (Figure 4.4A and 4.4B). However, *M. micrura* was completely eliminated by the second application, while numbers of *Streblocerus pygmaeus* increased in abundances compared to controls (Figure 4.4C). Except an isolated case of decreased abundance of *Brachionus urceolaris* 7 days after the first application,

rotifer species increased in abundances after the first and, more pronounced, after the second application (Table 4.2; Figure 4.4D and 4.4E). Calanoid and cyclopoid (Figure 4.4F) copepods decreased in numbers four days after the first application and ostracod abundances were higher in all treated microcosms compared to controls at the end of the experiment (Table 4.2).

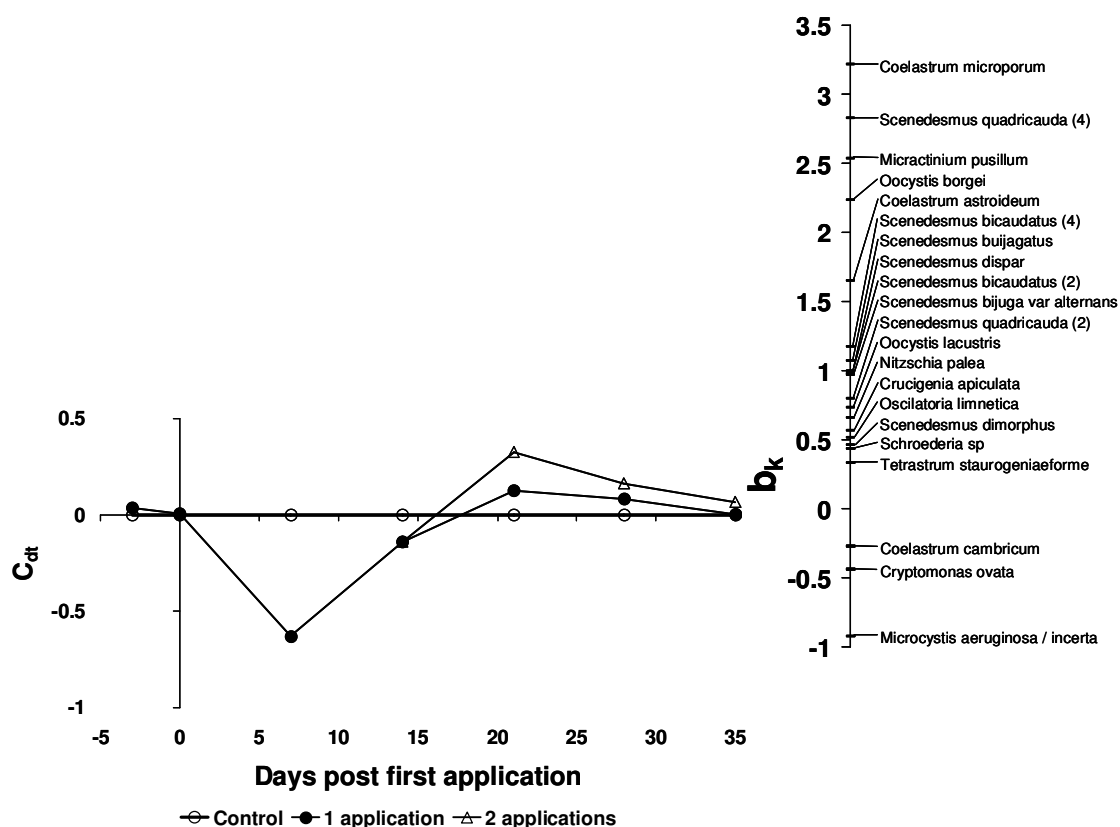


Figure 4.5 First Principal response curves resulting from the analysis of the phytoplankton data set, indicating the effects of one or two applications of 1 µg/L of the insecticide chlorpyrifos on the phytoplankton community. Of all variance, 58% could be attributed to sampling date; this is displayed on the horizontal axis. Ten percent of all variance could be attributed to treatment level. Of this variance, 42% is displayed on the vertical axis of the first PRC. 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. A Monte Carlo permutation test indicated that the treatment regime had a significant influence on the community structure ($p = 0.001$) and that a significant part of the variance explained by treatment level is displayed in the first ($p = 0.005$) PRC. (2): 2-cell colony; (4): 4-cell colony.

Phytoplankton

The PRC diagram resulting from the analysis of the phytoplankton data set is presented in Figure 4.5, whilst the results of the Monte Carlo permutation tests are given in Table 4.3. Most species have a positive weight in the diagram, indicating that most species decreased in abundances after the first application and increased slightly after the second application. Only *Microcystis aeruginosa/incerta* has a relatively high negative weight and is thus expected to show the opposite trend. These findings are confirmed by the univariate analysis, which calculated four negative treatment-related responses after the first application and four positive-related treatment effects after the second application (Table 4.2). In addition, a negative response on abundances of *Microcystis aeruginosa/incerta* was found after the second treatment (Figure 4.6A). Interestingly, *Scenedesmus quadricauda*, *Coelastrum astroideum* and *Oocystis borgei* were found to decrease after the first application and to increase the second application. Their dynamics are presented in figure 4.6B through 4.6D.

Table 4.3 Results of Monte Carlo permutation tests performed per sampling date for the phytoplankton data set. NP means calculation is not possible.

Day	1 application	2 applications
-3	0.962	NP
0	0.803	NP
7	0.005	NP
14	0.409	NP
21	0.265	0.030
28	0.574	0.183
35	0.669	0.888

Chlorophyll-a

Chlorophyll-a contents in control and microcosms that received only one application of 1 µg/L chlorpyrifos were high and rather constant during the experimental period (Figure 4.7A). The second insecticide application resulted in a decrease in chlorophyll-a content. Although levels remained lower than controls and once applied microcosms until the end of the experimental period, significant differences in chlorophyll-a levels between the different treatments were noted only up to three weeks after the second treatment (Table 4.2).

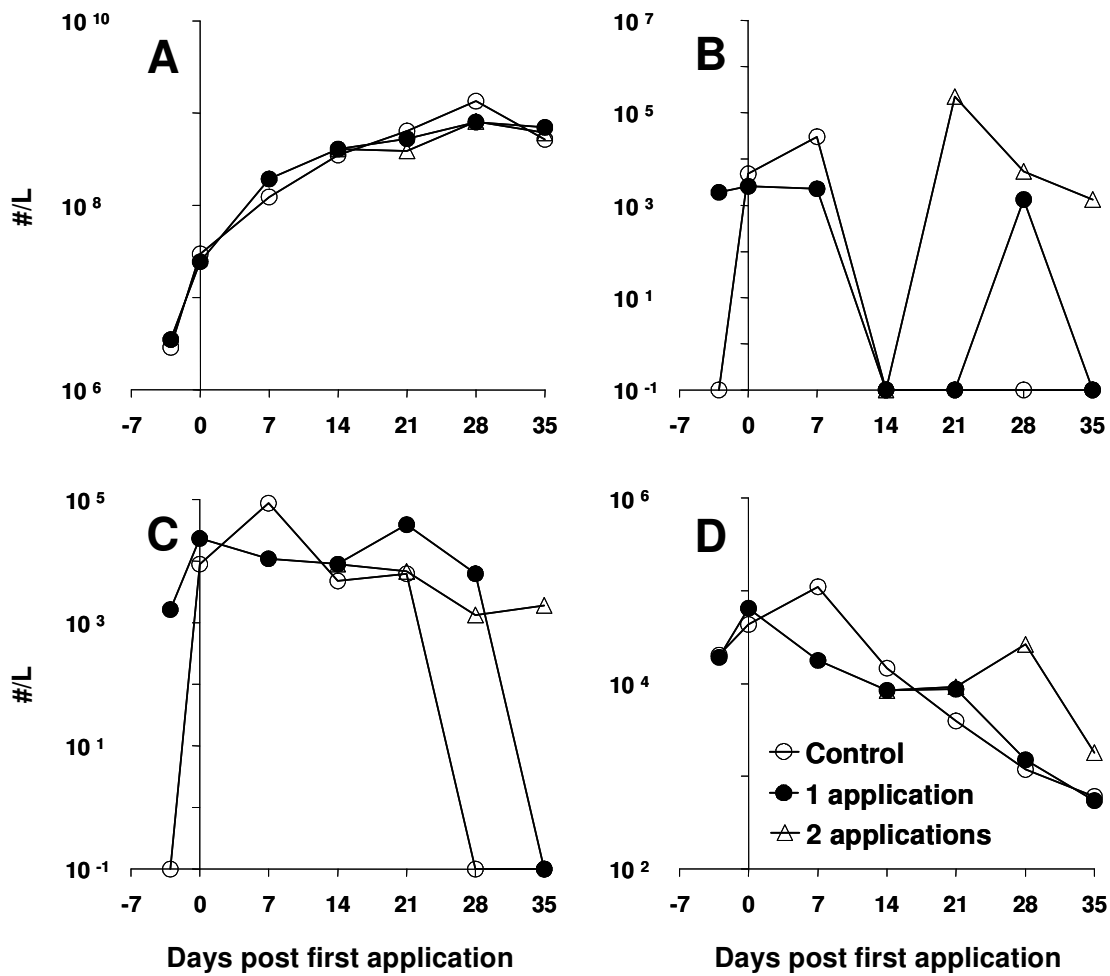


Figure 4.6 Dynamics in numbers of *Microcystis aeruginosa / incerta* (A), which dominated the phytoplankton community, as well as the dynamics in numbers of the three phytoplankton species that showed a decrease after the first chlorpyrifos application and an increase after the second treatment: *Scenedesmus quadricauda* 4-cell colonies (B), *Coelastrum astroideum* (C) and *Oocystis borgei* (D). A value of 10^{-1} denotes absence of the taxon.

Physicochemical Conditions

The overall trend in dissolved oxygen (DO) concentration during the experiment is visualized in figure 4.7B. By the end of the experiment, oxygen levels were lower compared to the initial phase of the experiment in controls and singly applied tanks. Microcosms that received two chlorpyrifos treatments, remained high levels of DO leading to a significant increase over controls until three weeks after the second treatment. No other significant treatment effects were found on physicochemical parameters.

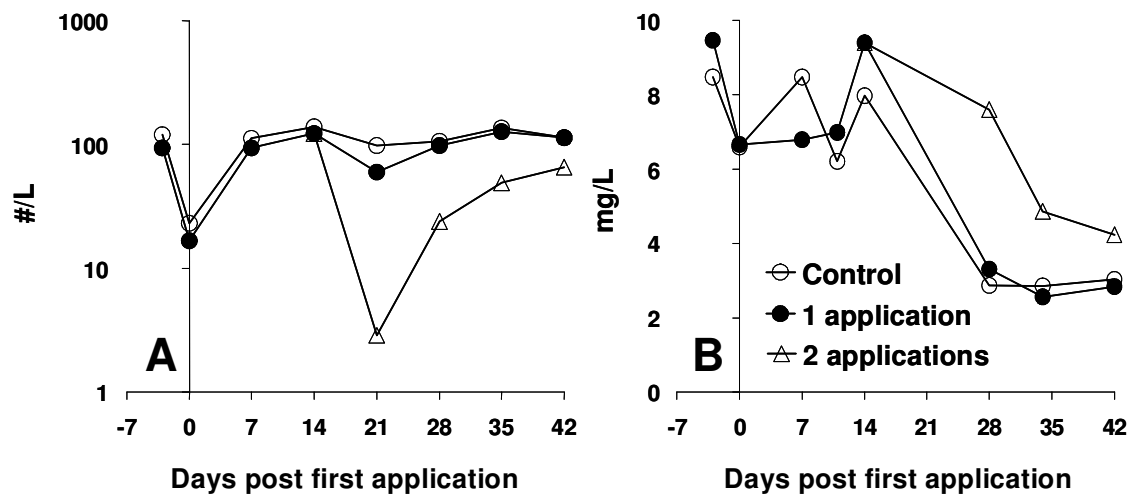


Figure 4.7 Dynamics of chlorophyll-a values (A) and morning dissolved oxygen levels (B).

Discussion

Fate of chlorpyrifos in the water

Chlorpyrifos disappeared fast from the water layer with disappearance rates slightly higher than those reported in a microcosm study carried out in Thailand evaluating single chlorpyrifos applications (Daam et al., subm. Figure 4.1). This may be explained by the fact that in the latter study, deeper test systems (length 1m, width 1m, water depth 1m) were used, meaning that the surface to volume ratio of the water in the present study was higher (2.3 times). This implies a relatively higher surface area for evaporation of chlorpyrifos from the water to the air, which has been demonstrated to play a significant role in the loss of chlorpyrifos from aquatic systems (Racke, 1993).

Representativeness of the zooplankton and phytoplankton communities

Rotifera was the most biodiverse group, among others represented by 5 *Brachionus* taxa and 3 *Lecana* taxa. Rotifera have indeed been reported to generally dominate tropical zooplankton communities (Segers, 2001). Furthermore, the warm water adapted Rotifera species *Filinia opoliensis*, *Hexarthra mira* and *Keratella tropica* (Kutikova, 2002) were found regularly in the zooplankton samples. Cladocera had a composition characteristic for tropical Asian freshwaters, i.e. *Daphnia* was absent and the smaller limnetic species *Moina micrura* and

Ceriodaphnia cornuta dominated the cladoceran community (Dumont, 1994). All zooplankton species identified in the present study were previously recorded in Thailand (Sanoamuang, 2001). Furthermore, from a study by Boonsom (1984), who associated zooplankton species with different habitats in Thailand, it can be concluded that the zooplankton communities were characteristic for Thai irrigation tanks and to a lesser extent for fish fields, rather than rivers and reservoirs.

In the phytoplankton samples, 27 of a total number of 41 taxa belonged to the phylum Chlorophyta. In line with this, chlorophyte biomass in the tropics has been reported to be high (Kalff and Watson, 1986) and Chlorophyta was the most diverse phytoplankton phylum in field studies carried out in different parts of Thailand (Pongswat et al., 2004; Ariyadej et al., 2004; Peerapornpisal, 1996). The cyanophyte *Microcystis aeruginosa* became the dominant species along the course of the experiment (Figure 4.6A). This dominance may be explained by the fact that lentic systems were used, since water bodies with a high degree of water column stability favour *Microcystis*. This is because *Microcystis* colonies can regulate their buoyancy, implying that during periods of water stability they have an advantage over other phytoplankton for nutrients and especially light (Dokulil and Teubner, 2000; Bonnet and Poulin, 2002). The experiment was carried out at the end of the rainy season, when direct sunlight is often blocked by cloud cover (Heckman, 1979), indicating that light may indeed be a limiting factor during this time of the year. In line with this, Vijanakorn et al. (2004) found *Microcystis* blooms in a reservoir in Thailand in the rainy season of 2002.

Direct treatment effects of chlorpyrifos on zooplankton

Both chlorpyrifos applications had pronounced but different effects on the zooplankton community. After the first application, the cladoceran *Ceriodaphnia cornuta* was eliminated and only a relatively small effect on *Moina micrura* was found (Figure 4.4A and 4.4B, Table 4.2). After the second application, however, *M. micrura* was the most responding zooplankton species and *C. cornuta* started to re-emerge even though this species was absent in control and singly applied microcosms at that time. This may be explained by differences in growth phase between these species at the time of application. Abundances of *C. cornuta* were relatively low at the time of the first application and showed a decreasing trend in controls, while *M. micrura* was relatively abundant and showed an increasing trend. In the period before the second application, *C. cornuta* was still absent and abundances of *M. micrura* were decreasing. In line

with this, Hanazato and Yasuno (1990) demonstrated that zooplankton populations were less susceptible for the insecticide carbaryl when applied in their growth phase than in their decreasing phase. A possible explanation for this is that increasing cladoceran populations contain more neonates, who have been demonstrated to be less sensitive to chlorpyrifos than older animals (Naddy et al., 2000). This may also elucidate why the first chlorpyrifos application significantly reduced the numbers of mature stages of copepods (cyclopoid, calanoid), while numbers of their immature stages (nauplii) were unaffected. No negative treatment effects were found after the second application on either mature or immature stages of copepods because they were absent in the controls.

Another reason for the larger impact on *M. micrura* after the second application compared to the first application may be the increased dominance over the experimental period by *Microcystis aeruginosa/incerta* (Figure 4.6A). This is because growth and reproduction of *M. micrura* has been reported to be severely reduced when reared with *Microcystis*, even when mixed with *Chlorella* (Hanazato and Yasuno, 1987). Other studies also concluded that cladocerans are affected by *Microcystis*, while rotifers and copepods are less vulnerable (Lampert, 1987; Ferrão-Filho, 2002). These studies report toxic effects of microcystins and mechanical interference of small colonies and filaments with the filtering process as possible underlying mechanisms.

Indirect effects of the insecticide

The decrease in zooplankton abundances led to increased abundances of several rotifers, followed by ostracods as a result of decreased competition and mechanical interference. The cladoceran population was more affected after the second chlorpyrifos application and therefore led to more pronounced effects on rotifers compared to the first application as well as an increase of the tolerant cladoceran *Streblocerus pygmaeus*.

Although cladoceran and copepod populations seemed recovered within three weeks after each application, rotifers and ostracods remained significantly increased in numbers up to five weeks post application (Table 4.2). This was presumably due to the increasing population trend of *M. aeruginosa/incerta* over the course of the experiment, which favoured rotifers over cladocerans as explained above. Ostracods have been reported to be indicative of stressed environments (Victor, 2002), implying that the plankton community was affected for a prolonged period even after the pesticide had completely disappeared from the microcosms.

Abundances of *Scenedesmus quadricauda* (4-cell colonies), *Coelastrum astroideum*, *C. microporum* and *Oocystis borgei* decreased in applied tanks one week after the first chlorpyrifos treatment. It is unlikely that this was the result of direct toxicity of chlorpyrifos since reported EC50 values of chlorpyrifos for algae are more than a thousand-fold higher than the concentration tested (Van Donk et al., 1992). The decrease of these phytoplankton taxa was probably the result of an increased grazing pressure by *Moina micrura* to maintain its population size. In line with this, three of these species (*S. quadricauda*, *C. astroideum* and *O. borgei*; Figure 4.6B through 4.6D), increased in abundances after *M. micrura* was completely eliminated by the second chlorpyrifos application. This further stimulated the growth of the tolerant cladoceran *Streblocerus pygmaeus*. Growth of the rotifers, however, is not likely to have increased further due to the increase in these phytoplankton taxa, which may be explained by differences in edible phytoplankton particles between rotifers and cladocerans. *M. micrura* and other small cladocerans have been considered to feed on particles smaller than 40 μm (Hanazato and Yasuno, 1987), while rotifers can handle particles up to 25 μm (Bergquist et al., 1985). *O. borgei* occurred in the phytoplankton samples as broad ellipsoidal colonies of mostly 4 cells with a length between 30 and 40 μm , indicating that this species could indeed be grazed by cladocerans, but not by rotifers. *S. quadricauda* 4-cell colonies had a maximum length of approximately 20 μm , which implies that these colonies could be grazed upon by cladocerans as well as rotifers. However, this species has two spines of 10 to 15 μm on each terminal cell, presumably hampering the grazing by rotifers. This is supported by Bergquist et al. (1985), who recorded an increase in *S. quadricauda* in the presence of small zooplankton and also ascribed this to the presence of its spines. *C. astroideum* and *C. microporum* formed compact colonies of mostly 16 and 32 cells and occasionally colonies with 8 cells and, only for *C. microporum*, 64 cells occurred. Although size varied considerably, colony size was around 30 μm for 16-cell colonies and 50 μm for 32-cell colonies, indicating that rotifers and cladocerans could filter colonies up to 16 and 8 cells, respectively. Interestingly, colony size of *C. microporum* increased from 17 ± 1 in controls to 30 ± 7 (means \pm SD; data not shown) in applied microcosms one week after the first application, which is in agreement with the hypothesis that *M. micrura* grazing increased on this species to recover its population size. The increase in *C. astroideum* after the second application, however, was calculated for all treated microcosms (NOEC = control) and no differences in colony size between treatments were found. This indicates that a factor other than *M. micrura* grazing was involved since abundances of the latter species in once applied tanks were comparable to controls. As a result of its elongated shape, the increase in numbers of *N. palea* (Length \pm 30-

60 μm ; Width \pm 3-5 μm) is also not likely to be the result of decreased *M. micrura* grazing alone.

The increased abundances of rotifers led to increased grazing on *Microcystis aeruginosa/incerta*, which subsequently decreased in abundance. Although the PRC indicates a decrease for both chlorpyrifos applications on day 21 and 28 (Figure 4.5), a statistical significant decrease could only be demonstrated for two applications on day 21 (Dunnett's test, $p < 0.05$). This corresponded to a decrease in abundance of approximately 40% compared to controls. Though not significant in the Dunnett's test, abundances of *Microcystis aeruginosa/incerta* in once applied microcosms were as much as 20% and 40% lower than control values on day 21 and 28, respectively.

Thus, as a result of decreased competition with *Microcystis aeruginosa/incerta*, numbers of *N. palea* and *C. astroideum*, as well as *S. quadricauda* and *O. borgei*, increased in numbers. In addition, as a consequence of the decreased *M. aeruginosa/incerta* biomass, chlorophyll-a levels decreased. This may have further stimulated *N. palea* growth since diatoms have been reported to be indicative of clean water environments (Brönmark and Hansson, 2005). Numbers of *N. palea* were indeed negatively correlated with chlorophyll-a levels over the experimental period (Pearson correlation test, $r = 0.47$; $p < 0.05$). The decreased phytoplankton biomass as indicated by the chlorophyll-a levels led to increased DO levels as measured in the morning due to reduced respiration in the night (Figure 4.7B).

Implications for risk assessment and recommendations for future research

The assessment of the risk of pesticides to the aquatic environment is currently based on dose-effect response studies using either single or continuous exposure regimes (e.g., EU, 1997). In normal agricultural practises, however, pesticides are generally applied repeatedly to ensure a sufficient protection of their crops. Hence, aquatic ecosystems surrounding agricultural fields are subject to repeated pesticide loads, which may influence toxic effect cascades on aquatic life. Indeed, Hanazato and Yasuno (1990) reported an increase in the magnitude of effects on the zooplankton community in experimental ponds after repeated applications compared to a single application of the insecticide carbaryl. After a single application of carbaryl, cladocerans were reduced but recovered soon and consequently suppressed rotifers through competition. Repeated applications suppressed cladocerans for a

prolonged period, which induced the occurrence of abundant rotifers (Hanazato and Yasuno, 1990).

Also in the present study, different effect patterns were observed after the first and second chlorpyrifos application. It appeared, however, that the larger impact of chlorpyrifos on the cladoceran *Moina micrura* after the second treatment was a result of its population dynamics at the time of application and the increase in *Microcystis* dominance, rather than an accumulation of toxicity. Due to the relatively larger reduction in total numbers of cladocerans, indirect effects on rotifers and the phytoplankton community composition were indeed more pronounced after the second treatment.

The apparent absence of increased toxicity on the cladoceran populations after the second application may be explained by a combination of the rapid degradation rate of chlorpyrifos and the time interval between the applications. In the experiment by Hanazato and Yasuno (1990), the repeated carbaryl application regime consisted of ten applications every other day. The interval of two weeks used in the present study to mimic realistic Thai agricultural practises appears to be sufficient to allow recovery of the cladoceran populations although effects on zooplankton community level lasted longer. In line with this, Naddy et al. (2000) demonstrated that daphnids could survive two 6-h 0.5 µg/L chlorpyrifos pulses if a minimum interval of 3 days was used between the treatments. These authors further stipulated that relationships among variables of pulsed exposures, including concentration, duration, interval, and frequency, need to be better evaluated and understood. This will not only allow investigating the response of organisms under more environmentally pragmatic exposure conditions, but may also provide additional information, such as the potential for recovery, resistance, or latent effects. This may be especially relevant for agricultural common practises in tropical countries like Thailand, where application frequency is high (Van den Brink et al., 2003; Satapornvanit et al., 2004). Thus, additional experimental research is required evaluating repeated applications of pesticides with different degradation rates and application intervals relevant for local agricultural practices to come to a better understanding of pesticide freshwater ecotoxicology in tropical countries like Thailand. In addition, since several pesticides are often applied together as a mix to specific crops (Jungbluth, 2000; Van den Brink et al., 2003; Satapornvanit et al., 2004), crop-based experiments mimicking specific pesticide treatment packages are needed to evaluate the actual ecological risk of pesticides for freshwater life.

Acknowledgements

This study was funded by the Portuguese government through FCT (scholarship SFRH/ BD/ 8213/ 2002). The authors are indebted to the staff at the AIT hatchery for technical assistance, and to Steven Crum for valuable contributions to the development of the chlorpyrifos analysis method.

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CHAPTER 5

EFFECTS OF THE FUNGICIDE CARBENDAZIM ON THE ECOLOGY OF OUTDOOR FRESHWATER MICROCOSMS IN THAILAND

(To be submitted to *Aquatic Toxicology*)

Abstract

The aim of this study was to analyse the effects of the benzimidazole fungicide carbendazim on the ecology of tropical freshwater model ecosystems and compare them with the effects observed in similar studies carried out in temperate regions. Plankton-dominated outdoor microcosms containing indigenous species were set up in Thailand and treated once with nominal concentrations of 3.3, 33, 100 and 1000 µg carbendazim/L. Carbendazim was less persistent in the water layer than in studies performed in the temperate zone, which is explained by higher pH, radiation levels and temperature in the present study. The macroinvertebrate community was most severely affected by the carbendazim application, with water boatmen (Corixidae) as the most sensitive group. Overall, the safety factors for toxicity values used by the European Union (Uniform Principles), obtained from standard toxicity tests with temperate species, also appear to ensure adequate protection for the freshwater community in tropical countries like Thailand. However, since macroinvertebrates are the most sensitive animals to carbendazim and these are not represented among the standard test species currently in use, laboratory toxicity tests using indigenous species should be included in the risk evaluation of fungicides like carbendazim.

Introduction

Environmental degradation of Asian tropical ecosystems has become a major focus for researchers and funding agencies alike. The region's wetlands are under particular pressure, and their status is of concern to many national and international bodies and environment agencies (e.g. UNEP, EU) as well as conventions (e.g. RAMSAR) (Satapornvanit et al., 2004; Van den Bosch et al., 2006; Berg et al., 2007). Although it is generally agreed that there is an urgent need for guidelines for good land-use practices that reduce or obviate the use of

agrochemicals, little is currently known about the fate and impact of these compounds in tropical environments (Bourdeau et al., 1988; Castillo et al., 1997; Racke, 2003).

In Thailand, 6,732 tons of fungicides were imported in 2003, approximately 13% of the total pesticide import (Chunyanuwat, 2005). Fungicides are mainly used for fruit and vegetables, and their use is expected to increase significantly in the coming years, due to falling prices for rice and a production restructuring programme by the Ministry of Agriculture to convert land currently used to cultivate rice, cassava, coffee and pepper into fruit orchards (Jungbluth, 1996).

In Thailand, each pesticide has to be tested by means of a risk-benefit assessment (to determine the recommended dose) and for its effects on humans and the environment. If a product has already been tested elsewhere, only missing toxicological data are requested (Jungbluth, 1996). Only paraquat is produced in Thailand itself; the USA and Germany are the two major countries from which pesticides are imported into Thailand (Jungbluth, 2000). This means that the ecotoxicological risk assessment is mostly based on toxicity values obtained from tests performed in temperate countries. Furthermore, water quality standards have not been established for many of the non-organochlorine compounds, which are generally present in relatively high concentrations in field samples (Thapinta and Hudak, 2000).

The aim of this study was to obtain a better understanding of the fate and effects of fungicides on tropical freshwater ecosystems, using carbendazim as a model substance. Carbendazim was chosen because it is commonly used in Thailand (Jungbluth, 1996) and because reference model ecosystem studies are available for temperate regions testing carbendazim (single-peak exposure: Slijkerman et al., 2004; chronic exposure: Cuppen et al. 2000, Van den Brink et al. 2000). Ultimately, this study aimed to validate whether toxicity threshold values derived from experiments with carbendazim in temperate regions can be applied to ensure adequate protection of freshwater populations in tropical regions like Thailand.

Materials and methods

Experimental design

On 28 February 2005, the fungicide Bavistin FL (active ingredient carbendazim) was applied once to 8 microcosms, in 4 duplicate doses (nominal levels: 3.3, 33, 100 and 1000 µg/L). Four other systems served as controls, and were therefore only treated with water. Treatments were

assigned randomly to the tanks, and applied by carefully pouring a solution of Bavistin FL into the tanks. Immediately after the application, the systems were gently stirred to mix the compound with the water column, while preventing an upflow of sediment particles.

Twelve plankton-dominated microcosms (length 1 m, width 1 m, height 1.15 m, water volume 1000 L) at the hatchery of the Asian Institute of Technology (AIT), 42 km north of Bangkok (Thailand), were allocated to the experiment. The tanks were freshly coated with a non-toxic epoxy paint to avoid any influence of previous experiments. The microcosms were filled with a 10-cm layer of sediment and a 1-m water column, taken from the canal surrounding AIT. The canal water was passed through a net (mesh size 0.1 mm) to avoid fish and prawns entering the systems.

In the preparatory phase of the experiment, zooplankton and macroinvertebrates were collected from the AIT canal and introduced into the microcosms. Over an acclimation period of 6 weeks, a biocoenosis was allowed to develop in the microcosms. In this period, the water was circulated twice a week by collecting 100 L from each microcosm into a container and gently pumping 100 L back to each microcosm, to achieve similarity between the communities in the systems. A nutrient addition of N (1.4 mg/L as urea) and P (0.18 mg/L as TSP) was added twice a week during the entire experimental period.

Fate of carbendazim

Nominal concentrations were calculated from an analysis of subsamples of the treatment solutions and the water volume of the microcosms. Concentrations in the tanks were determined 1 hour (initial concentration), 1 and 2 days as well as 1, 2, 4 and 8 weeks after application. This was done by collecting a 10-L depth-integrated water sample in a glass container, after which a subsample of approximately 300 mL was poured into a glass bottle and taken to the laboratory.

After filtering over Whatman GF/C filters, 250 mL water was extracted with octadecyl (C-18, supelco) solid phase extraction columns. The extraction columns were conditioned with 5 mL methanol and 5 mL distilled water. After extraction, the carbendazim was eluted from the column with 2 successive portions of 1.25 mL acetonitrile into glass test tubes. The samples were then diluted with water to a fixed volume of 5 mL and analysed with high performance liquid chromatography (HPLC). Subsamples of 100 μ L were injected with a Hitachi L-7200 autosampler. The mobile phase (water:acetonitrile = 40:60) was set at a flow rate of 0.7

mL/min. The analytical column used was a ZORBAX ODS (length 250 mm, width 4.6 mm) provided with a guard column of the same origin. The column was mounted in a Hitachi L-7300 oven, which was set at 40°C. Carbendazim was detected using a Hitachi L-7400 UV detector set at a wavelength of 220 nm. Under these conditions, the retention time for the carbendazim peak was 8 min and the detection limit in water was 2 µg/L. Carbendazim recovery from the water was $97 \pm 1.2\%$ (mean \pm sd, n = 6). Carbendazim concentrations were calculated using a calibration series based on external standards. Carbendazim concentrations presented here have been corrected for recovery.

Macroinvertebrate community

Two pebble baskets, incubated on top of the sediment in each microcosm, were used to study the effect of carbendazim on the macroinvertebrate community. Macroinvertebrates were sampled at approximately two-week intervals by gently retrieving the substrates using a net to prevent animals escaping. To collect the macroinvertebrates, the substrates were washed in a container, after which the content of the net was added. The animals were then identified, counted and subsequently released into their original microcosm. Since expertise and knowledge on macroinvertebrate identification in Thailand are still limited (Dudgeon, 2003), identification was only made to class level, to prevent misidentification. The data from the two baskets were pooled for statistical analysis.

Zooplankton and phytoplankton

At several moments during the experiment, a 10-L water sample was collected in a bucket by taking several depth-integrated subsamples using a Perspex tube. One litre was used for phytoplankton chlorophyll-a and alkalinity analysis. The bucket was then partially emptied into the microcosm from which it had been taken, leaving 5 L in the bucket. This remainder was passed through a zooplankton net (mesh size 60 µm) and preserved with formalin (final concentration: 4% V/V) to examine treatment effects on the zooplankton community.

Subsamples of the zooplankton sample were counted with an inverted microscope (magnification 100-400). Rotifers and cladocerans were identified to the lowest taxonomic level possible. Copepods were divided into nauplii (immature stages), calanoids and cyclopoids

(mature stages). Ostracoda were not further identified. Numbers were recalculated to numbers per litre of microcosm water.

Phytoplanktonic chlorophyll-a measurements were made using the 1-L water sample taken as described above. A known volume was concentrated over a Whatman GF/C glass fibre filter (mesh size 1.2 μm) until the filter was saturated. Filters were then air dried and extracted the same day using the method developed by Moed and Hallegraeff (1987).

Periphyton

Glass slides were used as an artificial substratum to study the treatment effects of carbendazim on chlorophyll-a content of periphyton. The slides were positioned in a glass frame that was suspended at approximately 10 cm below the water surface. Periphyton chlorophyll-a was sampled at two-week intervals by brushing five slides visually clean. Extraction of the pigments was performed using the ethanol method described by Moed and Hallegraeff (1987).

Community metabolism

Dissolved oxygen, pH, electrical conductivity (EC) and temperature were measured approximately 10 cm below the water surface two weeks before application and on a weekly basis after application. On sampling days, measurements were made in the morning (just after sunrise) as well as at the end of the afternoon (just before sunset). DO and pH measurements were made using a YSI model 58 oxygen meter connected to a YSI 5739 probe and a Consort C523 pH meter, respectively. EC and temperature were both measured with a Consort C532 conductivity meter. Alkalinity levels were determined at weekly intervals in 100-mL subsamples taken from a 1-L water sample obtained as described above, by titrating with 0.05 N HCl until pH 4.2.

The concentrations of ammonia, nitrate and ortho-phosphate were analysed at two-week intervals in a 1-L sample taken at approximately 10 cm below the water surface using the methods described in APHA (1992).

Decomposition

Effects of carbendazim treatment on the decomposition of particulate organic matter (POM) were studied using litter bags filled with *Musa* (banana) leaves. To this end, *Musa* leaves were collected from banana trees on the AIT campus, which are not treated with pesticides. Leaves were leached three times for two days to remove the more easily soluble humic compounds, and were dried in an oven at 60°C for three days. Subsequently, subsamples were dried at 105°C to establish the 60°C/105°C dry weight ratio.

A portion of 2 g dry weight (60°C) of *Musa* leaves was enclosed in a nylon bag with a mesh size of 200 µm (non-accessible to macroinvertebrates), closed with a stainless steel wire. Three litter bags were introduced into each microcosm at a depth of 20 cm on the day of fungicide application. A litter bag was retrieved from each microcosm 2, 4 and 8 weeks post application. The content was transferred to a white tray and gently washed to separate POM from other particles. Subsequently, the plant material was dried in aluminium foil to determine its dry weight (24 h; 105°C).

Data analysis

NOECs were calculated for all parameters using the Williams test, which assumes an increasing effect with increasing dose (Williams, 1972). Abundance data were $\ln(Ax + 1)$ transformed, where x stands for the abundance value and Ax makes 2 by taking the lowest abundance value higher than zero for x . This was done to down-weight high abundance values and approximate a normal distribution of the data (for rationale, see Van den Brink *et al.*, 2000). Analyses were performed with Community Analysis, version 4.3.05 (Hommen *et al.*, 1994) and statistical significance was accepted at $p < 0.05$. Effects were considered to be consistent when found on two consecutive sampling dates.

The zooplankton and macroinvertebrate data sets were analysed by PRC (Principal Response Curves) using the CANOCO software package, version 4.5 (Ter Braak and Smilauer, 2002). PRC is based on the Redundancy Analysis ordination technique (RDA), the constrained form of Principal Component Analysis. The analysis results in a diagram showing the sampling day on the x-axis and the first Principal Component of the treatment effects on the community on the y-axis (see Figure 5.2 for an example). This yields a diagram showing the deviations in time of the treatments compared to the control. In this way, PRC shows the most dominant

response to the treatment present in the data set. The species weights are shown in a separate diagram, and indicate the affinity the species have with this dominant response. The species with a high positive weight are indicated to show a response similar to that indicated by PRC, while those with a negative weight show one that is opposite to the response indicated by PRC. Species with a near zero weight are indicated to show a response very dissimilar to that indicated by PRC or no response at all. 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 (Van den Brink and Ter Braak, 1999).

Permutation tests were performed per sampling date using Ln-transformed treatment levels as explanatory variables to determine the significance of the treatment regime per sampling date. The NOEC values at community level for the zooplankton and macroinvertebrate communities were calculated for each individual sampling date by applying the Williams test to the sample scores of the first principal component of each sampling date (for rationale, see Van den Brink et al., 1996).

Results

Climatic conditions during the experiment

Figure 5.1 shows the meteorological conditions and water characteristics during the experimental period. The average air temperature during the time span of the experiment was 30°C (average min – max: 24 -36 °C), resulting in average water temperatures of 28°C in the morning and 32°C in the afternoon. These high temperatures, together with a high air humidity ($69 \pm 23\%$), are characteristic of the monsoon climate of the equatorial zone (McKay and Thomas, 1989). The experiment was performed between mid-January and the end of April 2005, i.e. partly in the cool season (November – February) and partly in the hot season (March - May).

Rain showers did not occur until mid-March, with an average daily evaporation of 5.9 mm, resulting in a negative net water balance (Figure 5.1). Based on these measurements by the meteorological station at AIT, the decrease in water level in the microcosms was approximately 6.6 cm over the entire experimental period.

To compensate for the preceding dry period, the cloud seeding technique described in European Patent Office (2004) was applied in the beginning of 2005 by the Thai government

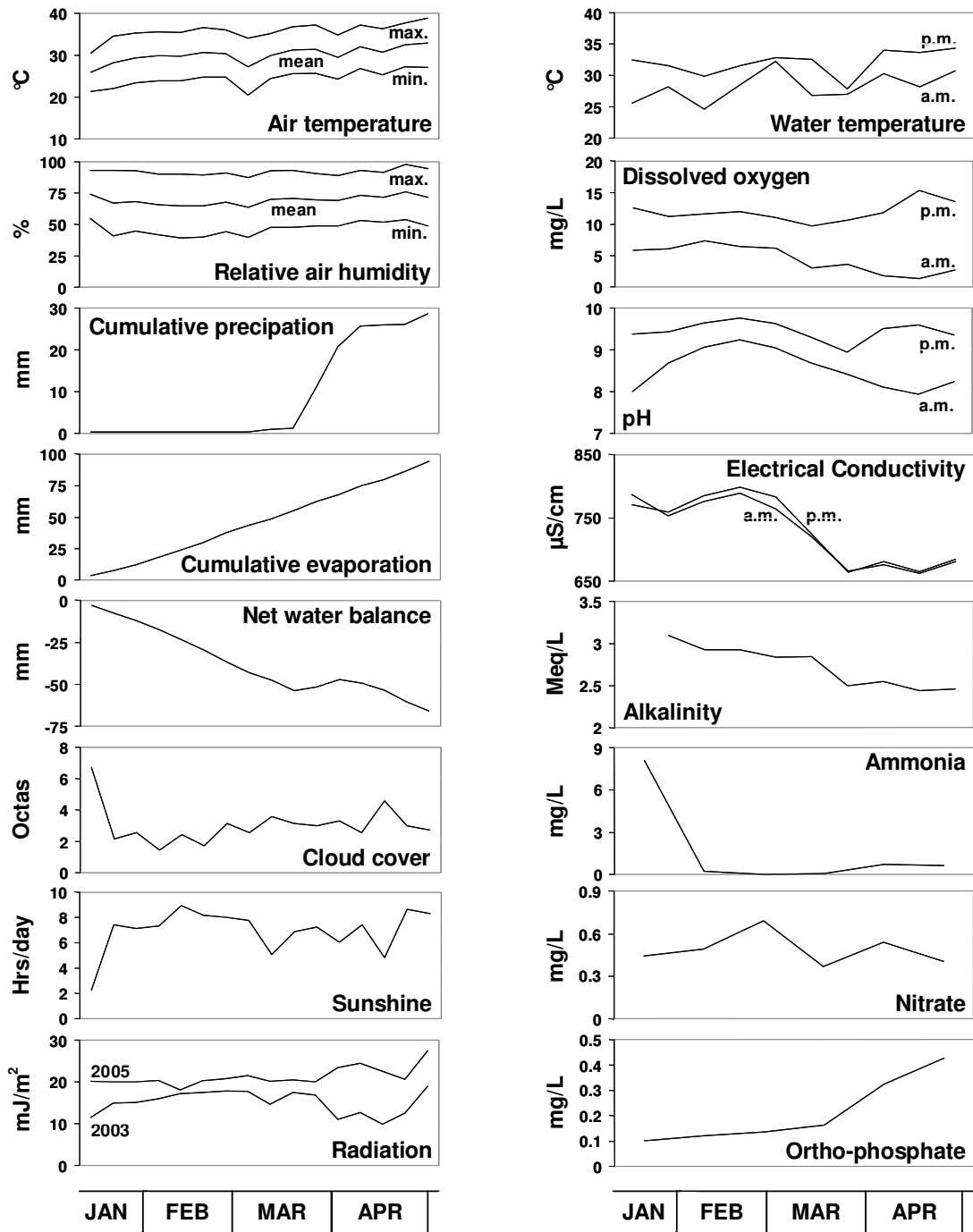


Figure 5.1 Meteorological conditions and physical/chemical water characteristics during the course of the experiment. Water parameter values are average values from the control microcosms. Data were obtained from the meteorological station at AIT.

to artificially produce rain. Compared to the same period in 2003, when microcosm studies with chlorpyrifos were carried out (Daam et al., *subm.*), cloud cover in 2005 was 6% higher, with approximately one hour of sunshine less per day. This resulted in relatively low radiation levels in the present study (Figure 5.1).

The water in the control microcosms was alkaline, with average pH values fluctuating between around 8.5 in the morning and 9.5 at the end of the afternoon. Dissolved oxygen (DO) levels in the morning were very low, with an average of 4 mg/L, which corresponds to a DO saturation of approximately 50%. DO measured in the afternoon was on average 7.5 ± 3.1 mg/L (mean \pm SD) higher than morning values. Electrical conductivity (EC) and alkalinity decreased slightly over the course of the experiment (Figure 5.1).

The microcosms were representative of hypereutrophic Thai plankton-dominated drainage ditches. As an indication of the nutrient status, mean ortho-phosphate levels increased during the experiment from 0.1 to 0.4 mg/L, levels that are characteristic of hypereutrophic water bodies (Brönmark and Hansson, 2005). Ammonia levels decreased rapidly in the pre-treatment period to < 0.8 mg/L and nitrate concentrations fluctuated slightly, between approximately 0.4 and 0.7 mg/L.

Carbendazim concentrations

Initial carbendazim concentrations measured 1 h after application were approximately 20% lower than the nominal carbendazim concentrations calculated from an analysis of the dose solutions and the water volumes in the microcosms (Table 5.1).

Table 5.1 Mean nominal and initial carbendazim concentrations (in $\mu\text{g/L} \pm \text{SD}$) and half-lives for the disappearance from the water phase per treatment level ($t_{1/2}$) as calculated over the experimental period.

Nominal concentration ($\mu\text{g/L}$)	Initial concentration ($\mu\text{g/L}$)	$t_{1/2}$ (days)
3.3 ± 0.1	2.5 ± 0.1	-
32.9 ± 0.1	25.2 ± 2.8	17 ± 1
99.0 ± 3.1	81.5 ± 4.5	15 ± 1
991.3 ± 18.5	832.5 ± 67.8	16 ± 0

Standard deviations within treatments were $8 \pm 5\%$ (mean \pm SD) over the course of the experiment. Irrespective of the dose applied, the half-life determined over the experimental period for the disappearance of carbendazim from the water phase ($t_{1/2}$) was 15 to 17 days.

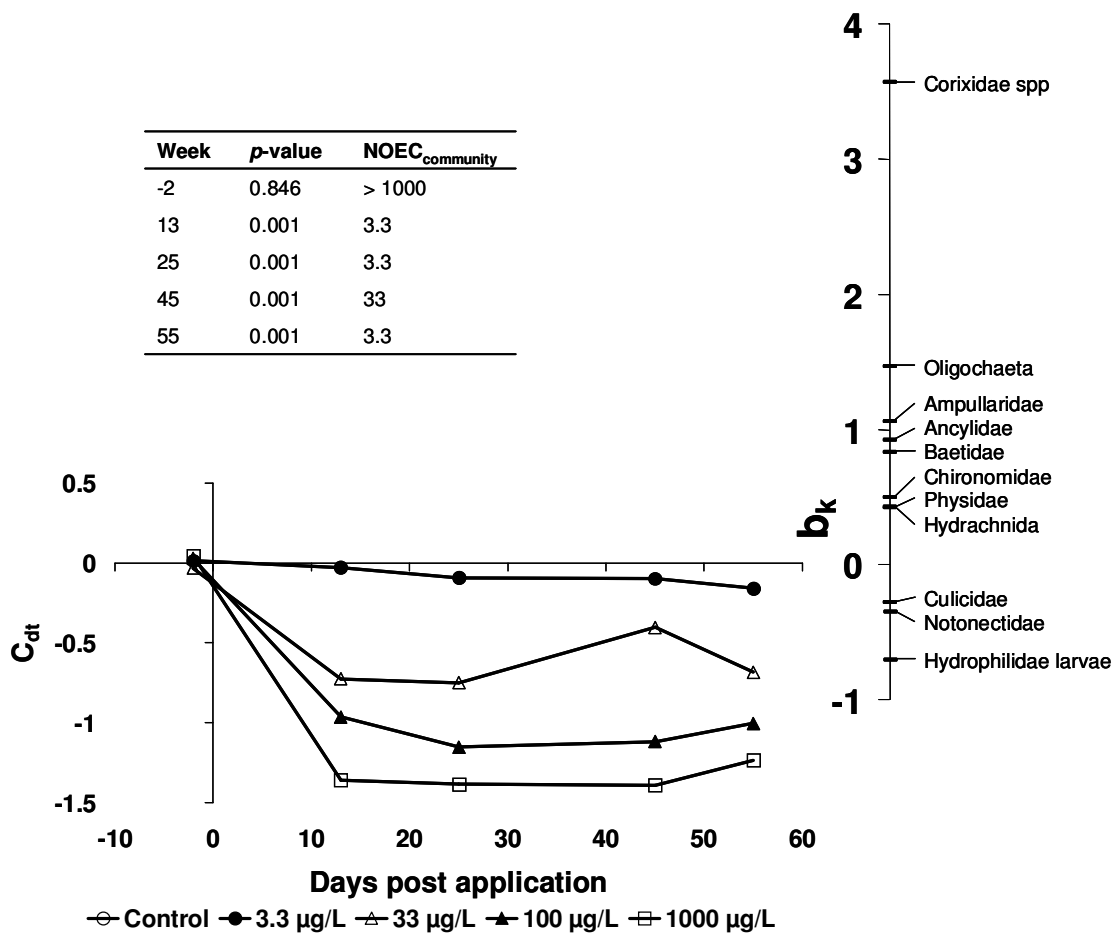


Figure 5.2 Principal response curves resulting from the analysis of the macroinvertebrate data set, indicating the effects of carbendazim treatment on the macroinvertebrate community. Of all variance, 35% could be attributed to sampling date; this is displayed on the horizontal axis. Forty percent of all variance could be attributed to treatment level; 45% of this 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. A Monte Carlo permutation test indicated that a significant part of the variance explained by treatment level is displayed in the diagram ($P = 0.002$). The results of the Monte Carlo permutation tests and Williams test on the PCA coordinates as performed for each individual sampling date for the macroinvertebrate data set are presented in the table accompanying the PRC diagram.

Macroinvertebrates

A total of 20 macroinvertebrate taxonomic groups were identified in the experiment. Insects were the most diverse macroinvertebrate class, with 10 different families. Most groups occurred in very low numbers, making it difficult to demonstrate statistically significant effects on many macroinvertebrate families and classes. Over the experimental period, 92% of the community in the control microcosms consisted of water boatmen (*Corixidae*), baetid mayflies (*Baetidae*), oligochaets, ostracods and apple snails (*Ampullariidae*).

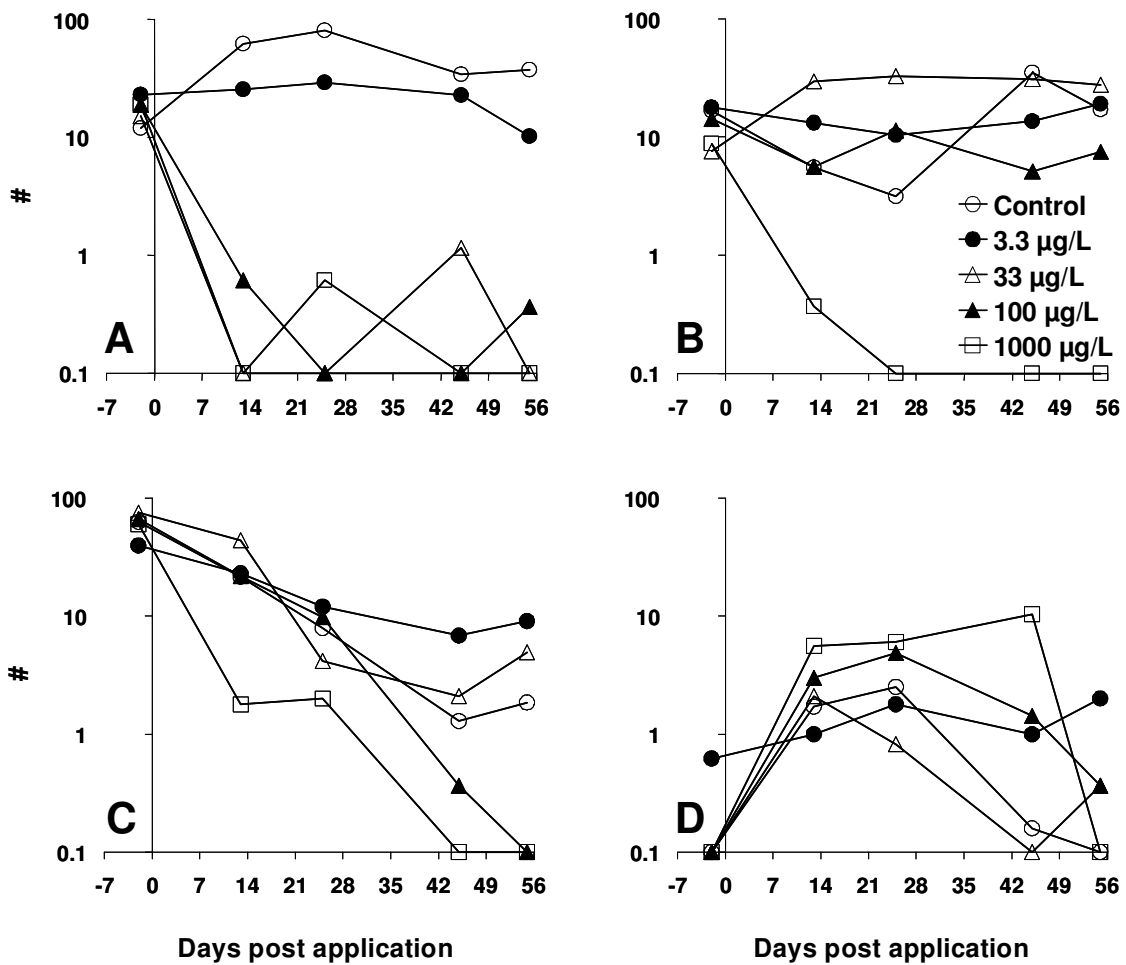


Figure 5.3 Dynamics in numbers of the four most important macroinvertebrates in the PRC analysis. Geometric means of *Corixidae* (A), *Oligochaeta* (B), *Ampullariidae* (C) and *Hydrophilidae* larvae (D) are shown in the figures, with a value of 0.1 denoting absence of the macroinvertebrate.

Statistical analysis of the PRC revealed that the community composition of the 33, 100 and 1000 µg/L microcosms differed significantly from that in the controls (Figure 5.2; Monte Carlo permutation test and Williams test, $p < 0.05$). The PRC also indicated a more pronounced effect with increasing doses of carbendazim. The water boatmen (Corixidae) had a relatively high positive weight in the diagram, indicating a major reduction in the abundance of this macroinvertebrate family in the treated microcosms compared to the controls. Indeed, Corixidae were found to have the lowest NOEC: 3.3 µg/L (Table 5.2, Fig. 5.3A). Other negatively affected species were Oligochaeta (Fig. 5.3B), Ampullariidae (Fig. 5.3C) and Baetidae, though NOECs could only be calculated for one or two sampling dates (NOECs 33 – 100 µg/L, Table 5.2). Water scavenger beetle larvae (Hydrophilidae) had a relatively high negative weight, indicating an increase in numbers relative to control values. This was confirmed with the Williams test, which calculated an increase at the two highest treatment levels (NOEC 33 µg/L; Fig. 5.3D).

Zooplankton

In terms of overall abundance, the control zooplankton community was dominated by rotifers, copepods and cladocerans, followed by ostracods. Rotifera were the most diverse group with 11 species, 4 of which belonged to the *Brachionus* family, while Cladocera were represented by 4 taxa. Immature stages of copepods (nauplii) had a high abundance throughout the experimental period in the controls, with an average of 380 per litre. In these microcosms, cyclopoid copepods increased over time, leading to slightly higher numbers compared to calanoid copepods at the end of the experiment.

The PRC of the zooplankton data shows a clear deviation from controls at the highest treatment level (Fig. 5.4). This visual difference was confirmed by the permutation tests and NOECcommunity calculations (NOEC = 100 µg/L). This NOEC was confirmed at the species level for several taxa (Williams test, Table 2). Cladocerans *Moina micrura* (Fig. 5.5A), *Ceriodaphnia cornuta* (Fig. 5.5B) and *Diaphanosoma* sp. occurred in significantly lower numbers at the highest treatment level. The rotifer *Keratella tropica* decreased in numbers (Fig. 5.5C), whilst the rotifers *Brachionus caudatus* (Fig. 5.5D) and, though less pronounced, *Lecane closterocerca* and *Euchlanis* sp., increased in numbers (Table 5.2). Ostracoda also had higher abundances and copepods were negatively affected mostly at a later stage in the experiment (Table 5.2).

Table 5.2 NOECs (No Observed Effect Concentration) per sampling week for invertebrates that showed a significant response in the Williams test calculations ($p \leq 0.05$). Concentrations ($\mu\text{g a.i./L}$) showed significant increases (\uparrow) or decreases (\downarrow); nm – not measured; > indicates a NOEC of $> 1000 \mu\text{g/L}$.

	Sampling week											Figure
	-1	0	0.1	1	2	3	4	5	6	7	8	
Arthropoda												
Crustacea												
Cladocera	>	>	>	100↓	100↓	100↓	100↓	>	>	>	100↓	
<i>Moina micrura</i>	>	>	>	100↓	>	100↓	>	>	33↑	>	100↓	5.5A
<i>Diaphanosoma</i> sp.	>	>	>	>	>	>	>	100↓	100↓	>	3.3↓	
<i>Ceriodaphnia cornuta</i>	>	>	>	>	100↓	>	100↓	100↓	100↓	>	100↓	5.5B
Ostracoda	>	>	>	>	>	>	>	>	33↑	33↑	>	
Copepoda	>	>	>	>	>	>	100↑	33↑	100↓	100↓	100↓	
Nauplii	>	>	>	>	>	>	>	>	100↓	100↓	100↓	
Calanoid	>	>	>	>	>	>	100↑	>	>	>	33↓	
Cyclopoid	>	>	100↓	>	>	>	>	>	100↓	100↓	33↓	
Insecta												
Corixidae	Nm	>	nm	nm	3.3↓	nm	3.3↓	nm	3.3↓	nm	3.3↓	5.3A
Baetidae	Nm	>	nm	nm	>	nm	33↓	nm	>	nm	>	
Hydrophilidae larvae	Nm	>	nm	nm	>	nm	33↑	nm	>	nm	>	5.3D
Rotifera												
<i>Brachionus caudatus</i>	>	>	>	>	>	100↑	100↑	100↑	100↑	>	>	5.5D
<i>Lecane closterocera</i>	>	>	>	>	100↑	>	>	>	>	>	>	
<i>Keratella trópica</i>	>	>	33↓	100↓	100↓	100↓	100↓	100↓	>	100↓	>	5.5C
<i>Lepadella patella</i>	100↑	>	>	>	>	>	>	>	>	>	>	
<i>Eucblanis</i> sp.	>	>	>	100↑	>	>	>	>	>	>	>	
Annelida												
Oligochaeta	Nm	>	nm	nm	>	nm	>	nm	33↓	nm	100↓	5.3B
Mollusca												
Ampullariidae	Nm	>	nm	nm	100↓	nm	>	nm	>	nm	>	5.3C
Ancyliidae	Nm	>	nm	nm	33↓	nm	100↓	nm	>	nm	>	

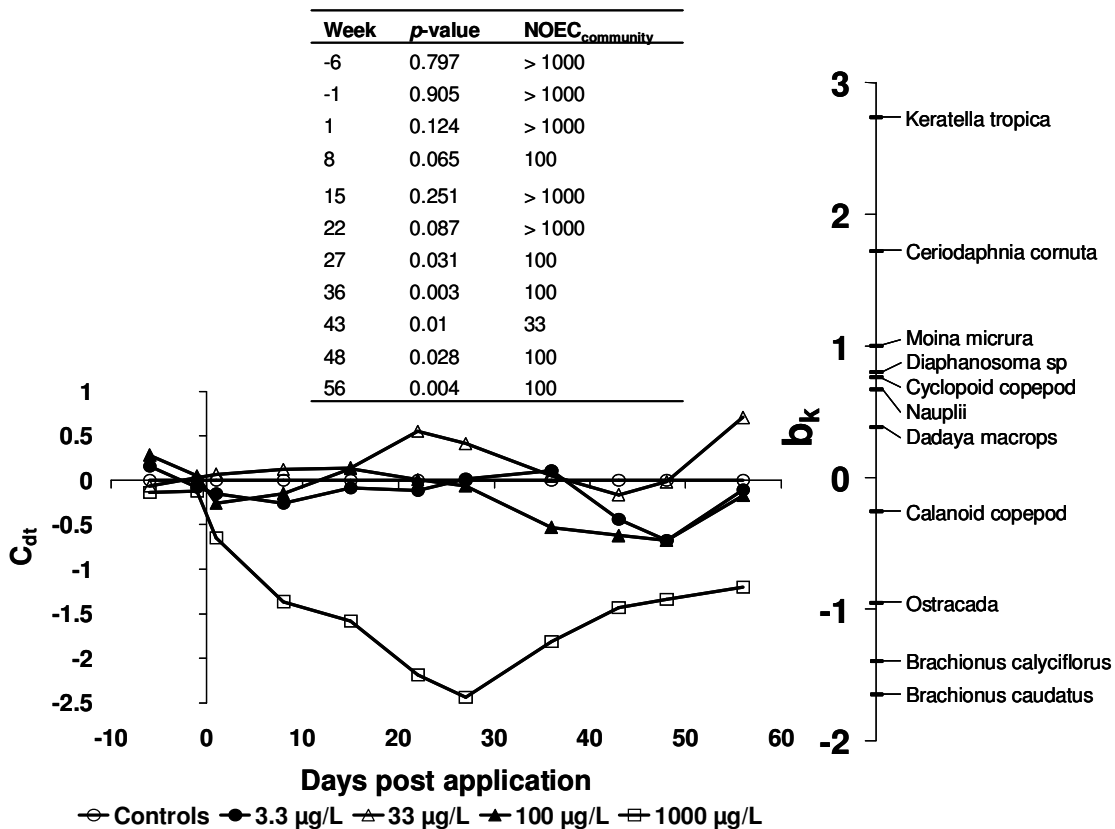


Figure 5.4 Principal response curves resulting from the analysis of the zooplankton data set, indicating the effects of carbendazim treatment on the zooplankton community. Of all variance, 21% could be attributed to sampling date; this is displayed on the horizontal axis. Forty-two percent of all variance could be attributed to treatment level; 40% of this is displayed on the vertical axis. The lines represent the course of the treatment levels over time. The species weight (bk) can be interpreted as the affinity of the taxon with the Principal Response Curves. A Monte Carlo permutation test indicated that a significant part of the variance explained by treatment level is displayed in the diagram ($P = 0.024$). The results of the Monte Carlo permutation tests and Williams test on the PCA coordinates as performed for each individual sampling date for the zooplankton data set are presented in the table accompanying the PRC diagram.

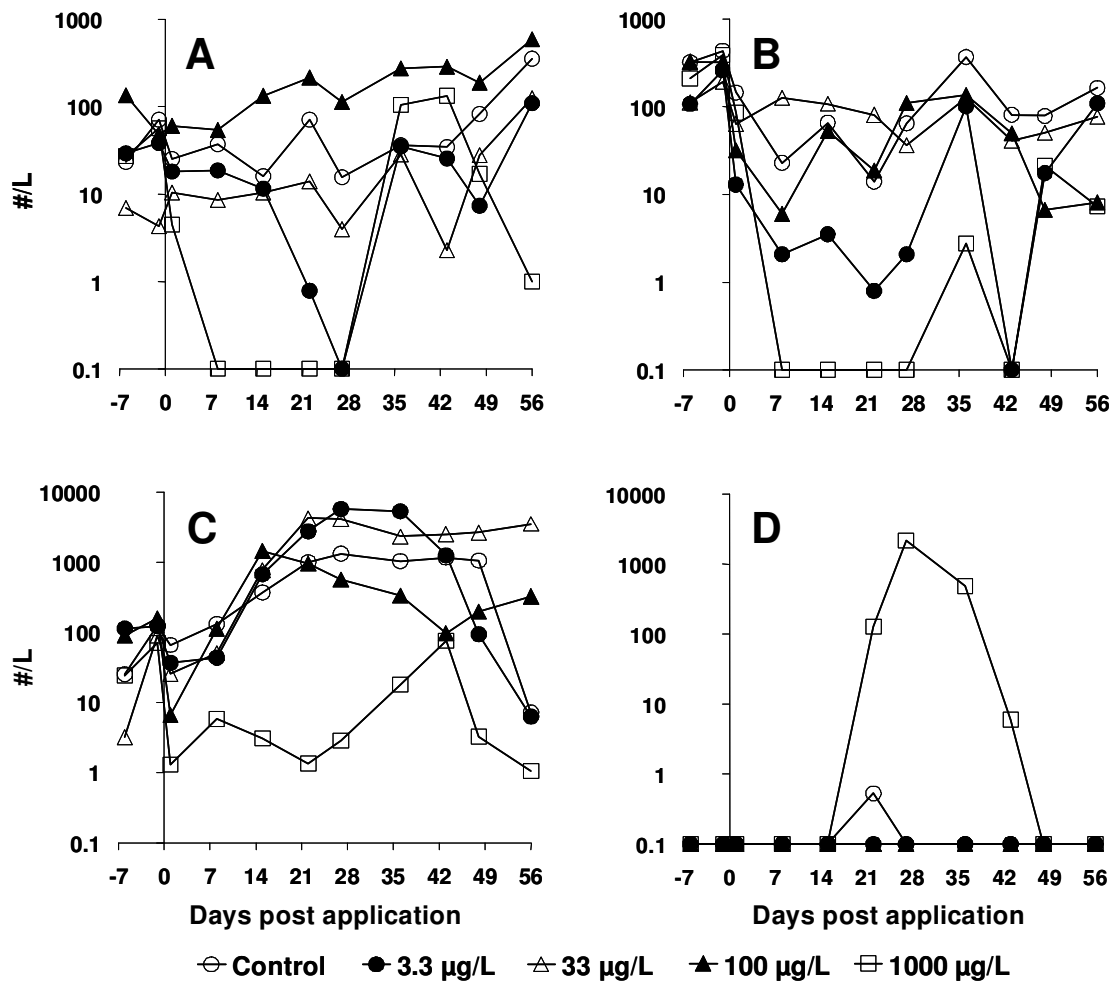


Figure 5.5 Dynamics of the four zooplankton taxa found to be the most discriminating ones in the PRC analysis. Figures 5.5A to 5.5D show geometric means for *Moina micrura* (A), *Ceriodaphnia cornuta* (B), *Keratella tropica* (C) and *Brachionus caudatus* (D). In the figures, a value of 0.1 denotes the absence of the taxon.

Chlorophyll-a phytoplankton and periphyton

Geometric mean phytoplanktonic and periphytonic chlorophyll-a levels during the experiment are presented in table 5.3. The chlorophyll-a content of the phytoplankton showed a significant increase 5 weeks post application in the highest treatment microcosms, while the same systems had decreased chlorophyll-a values 8 weeks post application. Periphytonic chlorophyll-a levels were significantly increased in these microcosms in the samples taken 4 weeks after application (Table 5.3).

Table 5.3 Geometric means of chlorophyll-a levels in phytoplankton ($\mu\text{g/L}$) and periphyton ($\mu\text{g}/\text{dm}^2$) during the experiment. Significant increases and decreases (Williams test, $p < 0.05$) relative to controls are indicated by a \uparrow and a \downarrow , respectively.

Phytoplanktonic chlorophyll-a ($\mu\text{g/L}$)						Periphytonic chlorophyll-a ($\mu\text{g}/\text{dm}^2$)					
Week	Control	3.3 $\mu\text{g/L}$	33 $\mu\text{g/L}$	100 $\mu\text{g/L}$	1000 $\mu\text{g/L}$	Week	Control	3.3 $\mu\text{g/L}$	33 $\mu\text{g/L}$	100 $\mu\text{g/L}$	1000 $\mu\text{g/L}$
-1	2.1	0.6	0.0	7.5	2.1	0	75.4	89.8	66.6	100.0	113.6
1	5.1	0.3	0.0	12.1	14.0	2	299.1	156.8	173.2	80.9	178.5
2	4.7	1.3	0.8	40.5	101.0	4	172.0	129.8	39.1	35.9	331.7 \uparrow
3	4.7	0.0	0.0	91.3	92.1	6	145.2	94.6	32.9	47.0	106.1
4	3.9	3.7	0.3	3.9	39.0	8	133.1	441.1	80.4	140.7	135.6
5	0.2	0.0	0.0	19.0	81.3 \uparrow						
6	2.1	1.2	4.1	27.0	46.8						
7	4.9	2.6	2.8	103.8	0.0						
8	55.0	25.9	60.8	113.2	0.0 \downarrow						

Water quality parameters

Several effects on water quality parameters were found, especially in the last 3 weeks of the experiment (Table 5.4). Dissolved oxygen and oxygen production, as well as pH, temperature and nitrate were significantly lower especially at the highest carbendazim concentration. At the highest treatment level, DO was even below 5 mg/L after day 43 (Figure 5.6). Alkalinity showed an increase at this treatment level (Table 5.4).

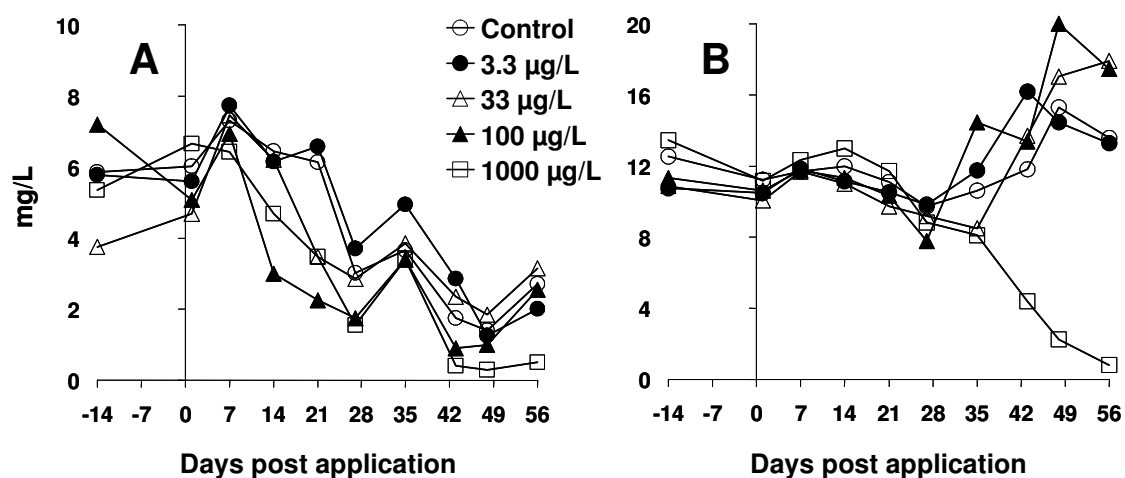


Figure 5.6 Dynamics of dissolved oxygen values as measured in the morning (A) and afternoon (B) at a depth of 10 cm.

Table 5.4 NOECs calculated for water quality endpoints in the microcosms. Significant treatment effects (Williams test, $p < 0.05$) resulted in either increased (\uparrow) or decreased (\downarrow) values in affected microcosms. In the table, $>$ indicates p values > 0.05 and NOECs > 1000 $\mu\text{g/L}$; nm = not measured. Dissolved oxygen production was calculated as the difference between morning and afternoon values.

Endpoint	Sampling week										
	-2	-1	0	1	2	3	4	5	6	7	8
Dissolved oxygen											
Morning	>	>	>	>	>	>	>	>	>	100 \downarrow	>
Afternoon	>	>	>	>	>	>	>	>	100 \downarrow	100 \downarrow	100 \downarrow
Production	>	>	>	>	>	>	>	>	100 \downarrow	100 \downarrow	100 \downarrow
pH											
Morning	>	>	>	>	>	3.3 \downarrow	3.3 \downarrow	>	>	>	100 \downarrow
Afternoon	>	>	>	>	>	>	>	>	100 \downarrow	100 \downarrow	100 \downarrow
Electrical conductivity											
Morning	>	>	>	>	>	>	>	>	>	>	>
Afternoon	>	>	>	>	>	100 \downarrow	>	100 \downarrow	100 \downarrow	>	>
Temperature											
Morning	>	>	>	>	>	>	>	33 \downarrow	>	>	>
Afternoon	>	>	>	>	>	>	33 \downarrow	>	100 \downarrow	100 \downarrow	100 \downarrow
Alkalinity	>	>	>	>	>	>	>	>	100 \uparrow	>	100 \uparrow
Ammonia	>	>	>	>	>	>	>	>	>	>	>
Nitrate	>	>	>	>	>	>	>	>	100 \downarrow	>	>
Ortho-phosphate	>	>	>	>	>	>	>	>	>	>	>

Table 5.5 Residual dry weights of *Musa* leaves per treatment level as % of initial biomass. The decay periods were 2, 4 and 8 weeks. The microcosms treated with the highest carbendazim concentration had significantly (Williams test, $p < 0.05$) lower decomposition levels after 8 weeks of incubation (indicated with an *).

Week	Control	3.3 $\mu\text{g/L}$	33 $\mu\text{g/L}$	100 $\mu\text{g/L}$	1000 $\mu\text{g/L}$
0 - 2	64.5	66.8	61.7	74.3	67.0
0 - 4	49.6	48.0	38.1	62.9	53.5
0 - 8	12.6	0.9	4.4	12.0	33.6 *

Decomposition

Table 5.5 shows the residual dry weights of the *Musa* (banana) leaves from the litter bags after decay periods of 2, 4 and 8 weeks. The residual dry weights of the leaves in the control test systems amounted to approximately 65, 50 and 13%, respectively. The microcosms treated with 1000 µg carbendazim/L had significantly higher residual dry weights after an 8-week incubation (Table 5.5).

Wolffia sp.

Five weeks after application, the floating plant *Wolffia* sp. started to emerge in the microcosms treated with the highest carbendazim concentration. Within a week, this resulted in the water surface becoming completely covered by this species. In the other microcosms, *Wolffia* sp. only covered a small part (<10%, data not shown) and only by the end of the experiment (i.e. 8 weeks p.a.).

Discussion

Influence of artificial rainmaking on ecosystem structure

As described above, radiation levels during the experimental period were relatively low for the time of the year as a result of artificial rainmaking (Figure 5.1). As a result, phytoplankton biomass was relatively low in the control and low-dose carbendazim microcosms, with rather large variation between the tanks (Table 5.3). As a consequence, abundances of several zooplankton taxa were low, and *Moina micrura* was even completely eliminated in the lowest treatment microcosm 4 weeks post application (Figure 5.5A). The abundance values of *Moina micrura* in the control and in all but the highest carbendazim dosage tanks correlated with the phytoplanktonic chlorophyll-a levels (Pearson correlation test, $r = 0.65$; $p < 0.05$). No significant correlation was found when the 1000 µg/L microcosms were included in this relation, confirming the toxic effect of this carbendazim concentration (see the results of the Williams tests and permutation tests on PRC).

Fate of carbendazim

Carbendazim was moderately persistent in the water layer, with a half-life for the disappearance of carbendazim from the water phase of 15 to 17 days (Table 5.1). This breakdown is relatively fast compared to a microcosm study performed by Slijkerman et al. (2004) in the Netherlands, which evaluated the fate and effects of a single carbendazim application. In the latter study, a carbendazim loss of 6 to 32%, was found four weeks after application, which is considerably lower than the $66 \pm 4\%$ (mean \pm SD) found in the present study.

This difference can be explained by the rather constant high pH values (a.m. 8.4 ± 0.6 , p.m. 9.3 ± 0.5 ; mean \pm SD) in our treated microcosms, since the degradation rate of carbendazim has been reported to be accelerated in alkaline solutions (especially at a pH of 9 and higher) with higher irradiation levels (Boudina et al., 2003). Although radiation levels were relatively low for the time of the year (see above), light intensity was presumably higher in the present study than in the outdoor microcosm study performed in the Netherlands by the end of September and October 2000. Furthermore, the higher temperatures in Thailand as compared to temperate regions may result in a higher microbial activity, which has been demonstrated to contribute greatly to the degradation of carbendazim (Tomlin, 2000).

Ecological effect chain

The hypothesised direct and indirect effect chains of carbendazim application on the structure and functioning of the ecosystem in the microcosms are visualised in Figure 5.7. The macroinvertebrate community was more sensitive to the fungicide than the zooplankton community (with NOEC_{community} values of 3.3 and 100 $\mu\text{g/L}$, respectively; Figures 5.2 through 5.5). Most effects emerged a week after application or later. Carbendazim has indeed been shown in the laboratory (Van Wijngaarden et al., 1998), as well as in the field (Cuppen et al., 2000; Van den Brink et al., 2000), to have a very slow mode of action. Of the several invertebrates affected (Table 5.2), Corixidae were the most susceptible, with complete elimination at 33 $\mu\text{g/L}$ (Figure 5.3A). The reduced grazing pressure as a result of the decline and elimination of several invertebrates led to increased levels of periphyton and phytoplankton (Table 5.3). Tolerant invertebrates increased in numbers, due to this increased food availability and reduced competition by sensitive invertebrates (Table 5.2).

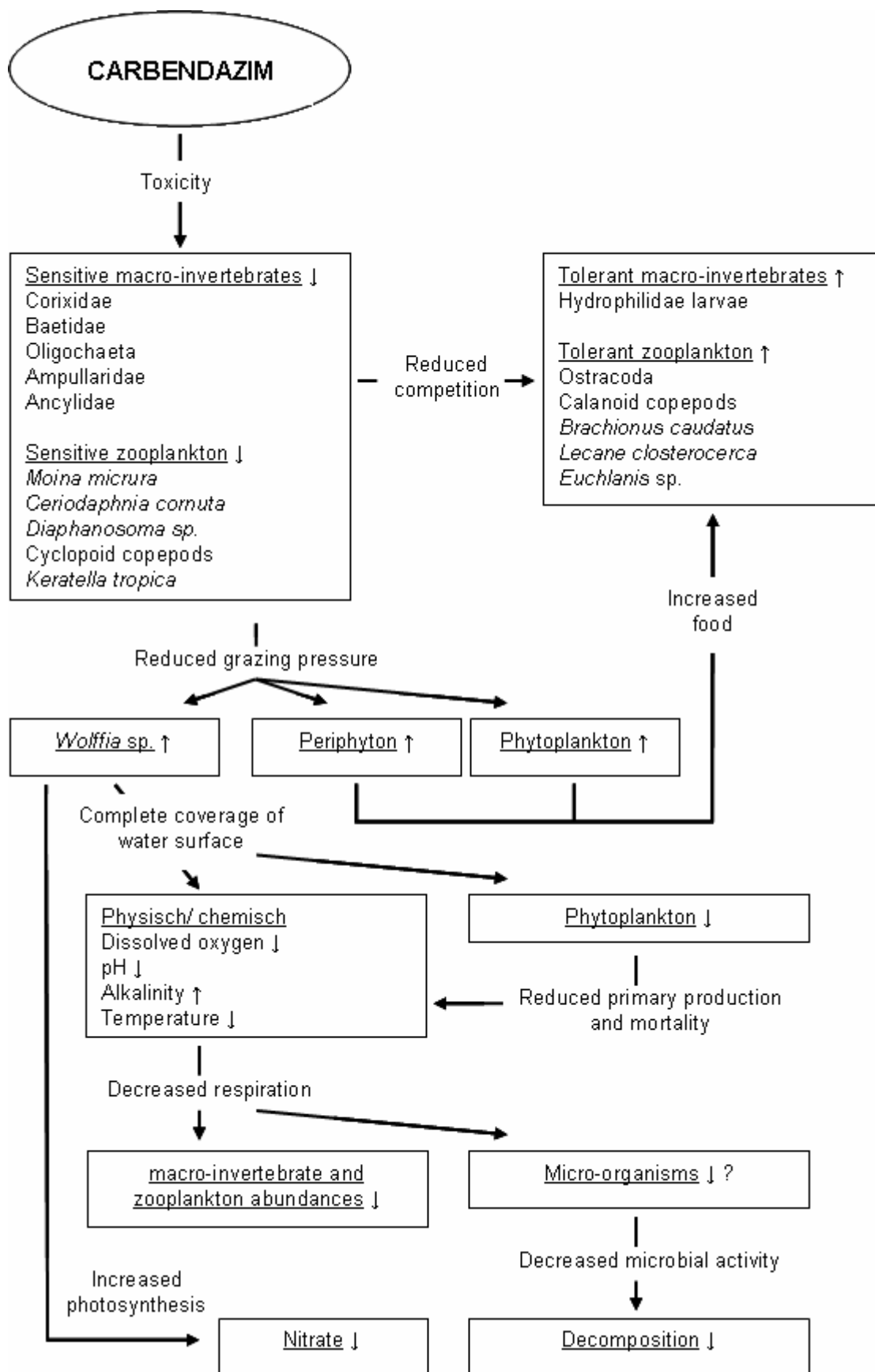


Figure 5.7 Schematic overview of the hypothesised direct and indirect effect chains of carbendazim application on ecosystem structure and functioning.

A bloom of the floating plant *Wolffia* sp. was observed in the microcosms treated with 1000 µg carbendazim/L at the end of the experiment. Van den Brink et al. (2000) also found an increase in macrophytes (*Elodea nuttallii*) after carbendazim applications of 330 and 1000 µg/L, and explained this by a reduced presence of pathogens of these macrophytes, directly or indirectly caused by carbendazim. In the present study, however, the *Wolffia* outbreak is more likely to have resulted from the complete elimination of apple snails (Ampullariidae). Apple snails are known to be efficient grazers of aquatic macrophytes and have even been reported to leave the water column to forage for plants (Carlsson et al., 2004; Dudgeon, 1999).

The complete covering of the water surface by *Wolffia* sp. led to a decrease in several water quality parameters (dissolved oxygen, pH, electrical conductivity and temperature) as well as phytoplankton biomass (Figure 5.6; Tables 5.3 and 5.4). This reduction in algal biomass further increased the effects on the physico-chemical parameters, as a result of a decrease in primary production (decreased DO and pH levels) and an increased decomposition of the phytoplankton biomass (increase in alkalinity). At the same time, primary production by *Wolffia* led to a decrease in nitrate levels but did not compensate for the decrease in dissolved oxygen levels. Presumably, a large part of the gas exchange by the dense *Wolffia* mat occurred with the ambient air, rather than with the water column. A study by Morris and Barker (1977) did indeed indicate that much of the oxygen produced by *Wolffia* mats is lost to the atmosphere.

The extremely low dissolved oxygen concentrations in the water column led to a decrease in total numbers of rotifers and copepods. Interestingly, the abundance of ostracods increased in zooplankton samples from the tanks treated with the highest doses (Table 5.2). Ostracods have previously been reported to be very resilient and indicative of stressed environments where most zooplankton is eliminated, such as anoxic water conditions (Green, 1959; Victor, 2002; Corbari et al., 2004).

Decomposition of the *Musa* leaves was reduced in the highest treatment tanks after a decay period of 8 weeks (Table 5.5). It is unlikely that this is the direct result of carbendazim, since no effect was observed after a 4-week decay period, when most of the fungicide had already been broken down. A more plausible explanation for this phenomenon is a decrease in microbial activity as a consequence of the very low oxygen concentrations. Since microorganisms were not studied in the present experiment, we can not confirm this hypothesis.

One of the aims of this study was to validate the use of the EU's ecological risk assessments for tropical countries like Thailand. This was done by determining the effects of carbendazim on macroinvertebrates, zooplankton, phytoplanktonic and periphytonic biomass and ecosystem functioning, using microcosms. The macroinvertebrate community was the most sensitive endpoint (NOEC_{community} 3.3 µg/L; Figure 5.2), with Corixidae as the most susceptible group (Figure 5.3; Table 5.2). Therefore, the overall NOEC_{ecosystem} is set at 3.3 µg/L. The same NOEC was calculated for the macroinvertebrate community and the ecosystem as a whole in a microcosm experiment performed by Cuppen et al. (2000) and Van den Brink et al. (2000) to evaluate the treatment effects of chronic (4 weeks) carbendazim exposure.

The zooplankton community in the present study appeared to be less sensitive than that in microcosm studies performed in the Netherlands (Table 5.6). This is probably the result of the limited number of cladoceran species and their relatively low abundances, since cladocerans have been reported to be the most sensitive zooplankton group (Van den Brink et al., 2000; Slijkerman et al., 2004).

Indirect effects of the carbendazim treatment on phytoplanktonic and periphytonic chlorophyll-a, as well as on functional parameters, differed from previously reported effects (Table 5.6). Indirect effects of pesticides, especially at higher concentrations, are known to vary considerably between different microcosm and mesocosm experiments (Van Wijngaarden et al., 2005; Fleeger et al., 2003).

Values of EC₅₀ (96 hours) and chronic NOEC (25 days) for *Daphnia magna*, which is the most susceptible standard laboratory test species for carbendazim used in temperate regions, are 87 and 26 µg/L, respectively (Van Wijngaarden et al., 1998). Therefore, applying safety factors of 0.01 and 0.1 to these toxicity values, as laid down in the Uniform Principles (EU, 1997), also appears to ensure adequate protection for the tropical microcosm community observed in this study. However, as discussed by Cuppen et al. (2000), the use of the standard test organisms from temperate countries (i.e. *Daphnia*, fish, algae) for compounds like carbendazim is questionable, since the most susceptible taxa are macroinvertebrates, which are not adequately represented. We therefore recommend including indigenous macroinvertebrate species in the local risk assessment of fungicides. Standard laboratory toxicity tests and in-situ field tests have already been developed for indigenous chironomids (Domingues et al., 2007) and

Macrobrachium species (Satapornvanit, 2006). Future research should focus on the taxonomy and ecology of Thai macroinvertebrate communities and the sensitivity of these taxa to pesticide (fungicide) stress.

Table 5.6 NOECs - LOECs (in µg/L) per endpoint found in the present study and those reported in other microcosm studies. For community metabolism, the table presents the lowest LOEC – NOEC combination from the following endpoints: dissolved oxygen (DO), electrical conductivity (EC), pH, temperature (T), alkalinity, ammonia, nitrate and ortho-phosphate. nm = not measured

Endpoint/ pesticide load	This study	Single peak	Constant
Macroinvertebrate community	3.3 – 33 ↓	nm	3.3 – 33 ↓
Zooplankton community	100 – 1000 ↓	2.2 – 21 ↓	33 – 100 ↓
Phytoplanktonic chlorophyll-a	100 – 1000 ↑ *	21 – 226 ↑	33 – 330 ↑
Periphytonic chlorophyll-a	100 – 1000 ↑	nm	> 1000
Community metabolism	3.3 – 33 ↓ *	21 – 226 ↑	> 1000
Based on:	pH a.m.	DO	
Decomposition	100 – 1000 ↓ *	nm	100 – 330 ↓
Overall NOECecosystem	3.3 – 33	2.2 – 21	3.3 – 33
Reference	–	Slijkerman <i>et al.</i> , 2004	Cuppen <i>et al.</i> , 2000; Van den Brink <i>et al.</i> , 2000
Type of model ecosystem	Lentic, outdoor Plankton-dominated	Lentic, outdoor ?	Lentic, indoor Macrophyte-dominated
Concentrations tested (µg/L)	3.3, 33, 100, 1000	2.1, 21, 226	3.3, 33, 100, 1000
Location	Thailand	The Netherlands	The Netherlands

* Effects on decomposition and main effects on community metabolism in the present study were most likely the result of the complete covering of the microcosms with the highest dosage by *Wolffia* sp., rather than of the carbendazim application. Furthermore, at the end of the experiment, a significant (Williams test, $p < 0.05$) decrease in phytoplanktonic chlorophyll-a was observed. See text for a detailed explanation.

Acknowledgements

This study was funded by the Portuguese government through FCT (scholarship SFRH/ BD/ 8213/ 2002). The authors are indebted to Phongnakhorn Yang-ngarm for nutrient analysis, to Apiyut Siyapan and the staff at the AIT hatchery for technical assistance, and to Steven Crum for valuable contributions to the development of the carbendazim analysis method.

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CHAPTER 6

ECOLOGICAL EFFECTS OF THE HERBICIDE LINURON IN TROPICAL FRESHWATER MODEL-ECOSYSTEMS: I. PRIMARY PRODUCERS

(Submitted to *Ecotoxicology and Environmental Safety*)

Abstract

Effects of a single application of the photosynthesis inhibiting herbicide linuron (0, 15, 50, 150 and 500 µg/L) on the ecology of outdoor plankton-dominated microcosms was studied in Thailand. This paper is the first in a series of two and discusses the effects on the primary producers up to 8 weeks after application. Herbicide concentrations in higher doses declined relatively slow in the initial phase following application due to a decrease in pH and herewith also in the hydrolysis of linuron. Degradation of linuron in the water column of lower dosed microcosms was slightly faster than reported in a microcosm study evaluating a single-peak treatments of linuron carried out in a temperate country, probably because of the high tropical temperatures in the present study.

The control phytoplankton community was dominated by Chlorophyta, which was also the most sensitive to the herbicide stress. Several chlorophytes belonging to the genera *Scenedesmus*, *Coelastrum* and *Pediastrum* were eliminated in higher linuron concentrations, whereas other chlorophytes increased in abundance. Diatom and cryptophyte taxa were tolerant and increased in numbers, while the cyanobacterium *Merismopedia tenuissima* initially decreased and in a later stage increased in abundance. *Chamaesiphon sp.* (Cyanophyta) dominated the periphyton community and was the most susceptible periphyton species. Succession of colonization in controls was from *Chamaesiphon sp.* to a community consisting of chlorophytes and diatoms. In higher linuron treatments, insensitive cyanobacteria and diatom taxa increased in abundances. As a consequence of functional redundancy, effects of the herbicide on the chlorophyll-a content of periphyton and especially phytoplankton did not always reflect the effects noted on community level. Thus, chlorophyll-a turned out not to be a sensitive indicator of herbicide stress.

Introduction

Numerous risk assessment studies have been performed over the past decades to evaluate potential risks of pesticides to aquatic organisms, ranging from toxicity tests with standard test species in the laboratory to field studies. These studies were almost exclusively carried out in temperate regions and hence, the fate and effects of agrochemicals on the aquatic ecosystem in the tropical zone are largely unknown (Castillo et al., 1997; Lacher and Goldstein, 1997; Racke, 2003). The chemical industry has grown rapidly in many developing tropical countries following the “Green Revolution” and the amount and variety of chemicals used has increased considerably (Bourdeau et al., 1989). There is thus an urgent need to validate whether ecotoxicological principles developed in the temperate zone are applicable to countries in the tropical zone.

Microcosm and mesocosms studies have often been used as test systems to determine the environmental fate of pesticides and their side-effects on aquatic ecosystems (see Van den Brink et al., 2006 for studies performed with herbicides). These test systems include more ecological realism than lower-tier laboratory single-species tests while still allowing an experimental set-up. For these reasons, microcosms and mesocosms have been proven a useful tool for the evaluation of pesticide stress including the experimental validation of the safety factors used to calculate no effect concentrations from laboratory toxicity threshold values (Van den Brink, 1999).

The present study aimed to evaluate the impact of the herbicide linuron on outdoor plankton-dominated in Thailand. This was done to validate the use of toxicity values for the herbicide linuron from studies performed in the temperate zone for countries in the tropical zone like Thailand. This paper is the first in a series of two and deals with the effects on the primary producers. The second paper summarizes the effects of the herbicide application on zooplankton and community functioning and focuses on the hazard assessment of linuron for tropical freshwater ecosystems (Chapter 7).

Materials and Methods

Experimental design and linuron application

The experiment was performed at the hatchery of the Asian Institute of Technology (AIT), located approximately 42 km north of Bangkok (Thailand). The twelve outdoor microcosms used for the experiment consisted of circular concrete tanks (diameter 0.75 m, height 0.65 m), newly coated with watertight non-toxic epoxy paint to avoid any influence from a previous experiment. The test systems contained a water layer of 0.55 m (water volume approximately 250 liters). Water was collected from the canal surrounding AIT after filtering through a net (mesh size 0.1 mm) to avoid fish and prawns entering the systems. No sediment was added to keep the experimental set-up as simple as possible and, consequently, to facilitate interpretation of the (in)direct treatment effects. In addition, a relatively low sorption of linuron was expected to the sediment because of the relatively low octanol-water partitioning coefficient of linuron ($\log K_{ow} = 3$). This is supported by Crum et al. (1998), who recorded a maximum of 6% of the linuron dose applied to experimental ditches to become associated with the sediment, despite its relatively high organic content (20 to 25%).

Additional zooplankton was collected from the AIT canal and introduced into the microcosms in the preparatory phase of the experiment. Over an acclimatization period of 5 weeks, a biocoenosis was allowed to develop in the microcosms. During this period, the water was circulated twice a week by collecting 100 L from each microcosm into a container and gently pumping 100 L back to each microcosm after mixing, to achieve similarity between the communities in the systems. A nutrient addition of N (1.4 mg/L as urea) and P (0.18 mg/L as TSP) was made twice a week during the entire experimental period.

Application and fate of the test substance

On the day of application, the treatment doses of linuron (nominal levels: 15, 50, 150, 500 $\mu\text{g/L}$), applied as Afalon Flow, were distributed evenly over the water surface of two microcosms for each concentration and mixed by stirring. Subsamples of the treatment solutions were taken to calculate nominal concentration levels. Four systems were untreated to serve as controls. The microcosms were randomly assigned to the different treatment levels.

Linuron concentrations in the microcosms were determined 1 hour (initial concentration), 1 and 2 days as well as 1, 2, 4 and 8 weeks after application. To this end, a 10-L depth-integrated water sample was collected in a glass container. After stirring, a subsample of approximately 300 mL was poured into a glass bottle and taken to the laboratory.

After filtering over Whatman GF/C filters, 250 mL water was extracted with octadecyl (C-18, supelco) solid phase extraction columns. The extraction columns were conditioned with 5 mL methanol and 5 mL distilled water. After extraction, the linuron was eluted from the column with 2 successive portions of 1.25 mL acetonitrile into glass test tubes. The samples were then diluted with water to a fixed volume of 5 mL and analysed with high performance liquid chromatography (HPLC). Subsamples of 100 μ L were injected with a Hitachi L-7200 autosampler. The mobile phase (water:acetonitrile = 60:40) was set at a flow rate of 1.0 mL/min. The analytical column used was a ZORBAX ODS (length 250 mm, width 4.6 mm) provided with a guard column of the same origin. The column was mounted in a Hitachi L-7300 oven, which was set at 40°C. Linuron was detected using a Hitachi L-7400 UV detector set at a wavelength of 254 nm. Under these conditions, the retention time for the linuron peak was 23 min with a detection limit in water of 0.2 μ g/L. Linuron recovery from the water was $102 \pm 2\%$ (mean \pm sd, n = 6).

Primary producers community structure

A depth-integrated water sample of 10-L was collected by means of a Perspex tube at several moments during the course of the experiment. A subsample of 1-L was used for the determination of the phytoplanktonic chlorophyll-a concentration. Another 1-L was stained with lugol and concentrated after sedimentation of 6 days. Additional lugol was added when needed, to assure conservation of the samples. Subsamples were counted with an inverted microscope (magnification 400 x) and densities were calculated as numbers per litre microcosm water. Colony forming algae except *Microcystis* species were quantified by counting the number of colonies. *M. aeruginosa* and *M. incerta* form large irregular colonies but appeared, although they were not frequently found, as fractions as well as individual cells. Transport, age, and/or fixation of the samples had apparently caused disaggregation of colonies. Therefore, to prevent overestimation of *Microcystis* species abundance in samples with many small clusters and underestimation in samples with a few larger colony fractions, *M. aeruginosa* and *M. incerta* were quantified by making cell number estimates.

Effects of linuron on the periphyton communities were studied with glass slides that served as artificial substratum. The slides were positioned in a glass frame that was suspended two weeks before application at approximately 10 cm below the water surface. At two-week intervals, the periphyton biomass of five slides was collected by brushing the slides visually clean. Preservation and identification of the samples was done as described for phytoplankton. For chlorophyll-a analysis, another five slides were brushed and collected periphyton biomass transferred to tap water.

Phytoplanktonic and periphytonic chlorophyll-a content

A known volume of phytoplankton and periphyton chlorophyll-a samples, prepared as described above, was concentrated over a Whatman GF/C glass fibre filter (mesh size 1.2 μm) until the filter was saturated. Filters were air dried and extracted the same day using the method described by Moed and Hallegraeff (1987).

Univariate and multivariate data analysis

NOECs (no observed effect concentrations) were calculated for all parameters using the Williams test, which assumes an increasing effect with increasing dose (Williams, 1972). Abundance data were $\text{Ln}(Ax + 1)$ transformed, where x stands for the abundance value and Ax makes 2 by taking the lowest abundance value higher than zero for x . This was done to down-weight high abundance values and approximate a normal distribution of the data (for rationale, see Van den Brink et al., 2000). Analyses were performed with Community Analysis, version 4.3.05 (Hommen et al., 1994) and statistical significance was accepted at $p < 0.05$.

The phytoplankton and periphyton data sets were analysed by PRC (Principal Response Curves) using the CANOCO software package, version 4.5 (Ter Braak and Smilauer, 2002). PRC is based on the Redundancy Analysis ordination technique (RDA), the constrained form of Principal Component Analysis. The analysis results in a diagram showing the sampling day on the x-axis and the first Principal Component of the treatment effects on the community on the y-axis (see Figure 6.2 as an example). This yields a diagram showing the deviations in time of the treatments compared to the control. In this way, PRC shows the most dominant response to the treatment present in the data set. The species weights are shown in a separate diagram, and indicate the affinity the species have with this dominant response. The species

with a high positive weight are indicated to show a response similar to that indicated by PRC, while those with a negative weight show one that is opposite to the response indicated by PRC. Species with a near zero weight are indicated to show a response very dissimilar to that indicated by PRC or no response at all. 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 (Van den Brink and Ter Braak, 1999).

Permutation tests were performed per sampling date using Ln-transformed treatment levels as explanatory variables to determine the significance of the treatment regime per sampling date. The NOEC values at community level for the phytoplankton and periphyton communities were calculated for each individual sampling date by applying the Williams test to the sample scores of the first principal component of each sampling date (for rationale, see Van den Brink et al., 1996).

Results

Linuron concentrations

Linuron concentrations in lower doses during the entire experimental period and until 4 weeks post application for the two highest doses had standard deviations within treatments that were always lower than 10% and mostly lower than 5%. Deviations in linuron concentrations between the two replica's of 150 µg/L and 500 µg/L dosed microcosms were 11% and 13% (4 weeks p.a.) and 18% and 32% (8 weeks p.a.), respectively.

Linuron disappeared moderately fast from the water (Figure 6.1). Concentrations decreased slower in the initial period after application in the two highest treatments. The half-life for the disappearance of linuron from the water-phase (DT50) as calculated over the experimental period was therefore slightly higher for the higher treatments (16 – 22 days) than for lower doses (8 – 10 days; Figure 6.1).

Phytoplankton

A total of 77 different phytoplankton taxa were identified from the phytoplankton samples. In terms of numbers of taxa as well as total abundances, control microcosms were dominated by

Chlorophyta, followed by Cyanophyta and Bacillariophyta. With a total of 16 taxa identified, *Scenedesmus* was the most diverse phytoplankton genus.

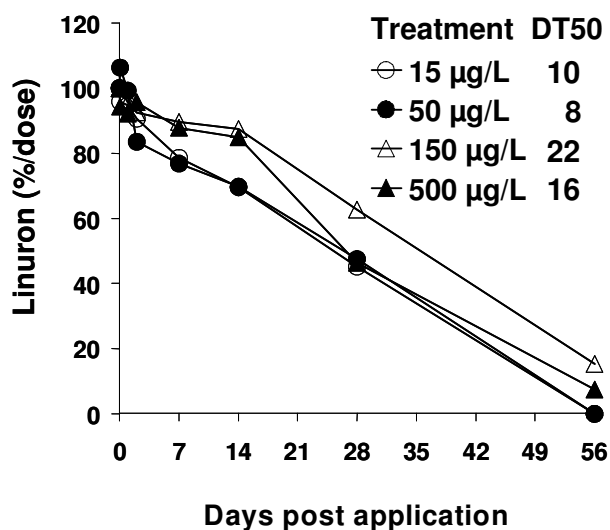


Figure 6.1 Dynamics of linuron concentrations as a percentage of the dose applied. Half lifetime for the disappearance of the herbicide from the water-phase per treatment ranged from 8 to 22 days, depending on the dose applied.

The PRC of the phytoplankton data set shows that the 500 µg/L, 150 µg/L and, to a lesser extent, the 50 µg/L treatments deviated from controls (Figure 6.2). These visual differences were confirmed by the permutation tests, which indicated significant treatment effect from two weeks post application onwards ($p < 0.01$; Table 6.1). The lowest NOECphytoplankton community was 15 µg/L and was calculated three weeks after the linuron treatment.

Table 6.1 Results of Monte Carlo permutation tests (P-value) and Williams test on the PCA set. coordinates (NOECcommunity) as performed for each sampling date for the phytoplankton data

Day	P-value	NOEC
-7	0.373	≥ 500
-2	0.658	≥ 500
7	0.049	≥ 500
14	0.005	50
21	0.001	15
26	0.001	50
35	0.002	50
42	0.001	50
47	0.005	50
55	0.003	150

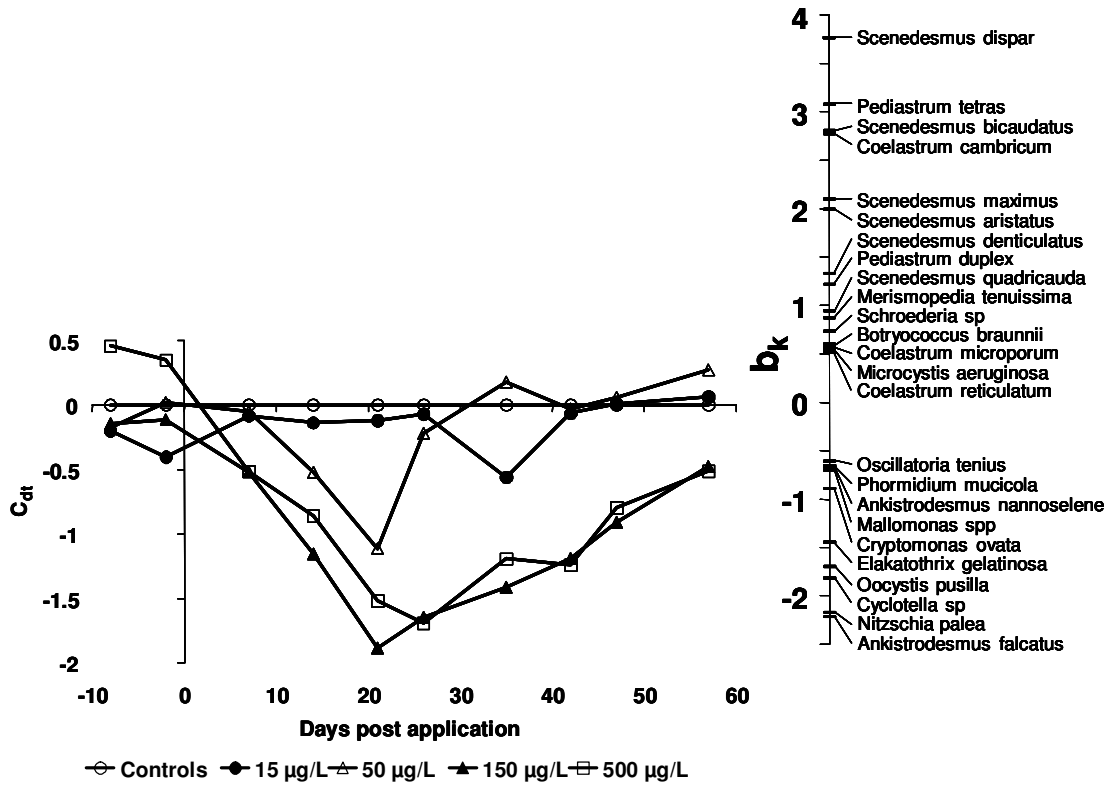


Figure 6.2 Principal response curves resulting from the analysis of the phytoplankton data set, indicating the treatment effects of linuron on the phytoplankton community. Of all variance, 20% could be attributed to sampling date; this is displayed on the horizontal axis. Thirty-six percent of all variance could be attributed to treatment level. Of this variance, 28% 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. A Monte Carlo permutation test indicated that a significant part of the variance explained by treatment level is displayed in the diagram ($P = 0.003$).

The dynamics of the 4 taxa with the highest species weight and the 4 taxa with the lowest species weight in the PRC are shown in Figure 6.3. The most seriously affected taxa were the chlorophytes *Scenedesmus dispar* (Figure 6.3A), *Pediastrum tetras* (Figure 6.3B), *Scenedesmus bicaudatus* (Figure 6.3C) and *Coelastrum cambricum* (Figure 6.3D). The taxa that were indicated to increase most in abundances were the chlorophytes *Ankistrodesmus falcatus* (Figure 6.3E) and *Oocystis pusilla* (Figure 6.3F), and the diatoms *Nitzschia palea* (Figure 6.3G) and *Cyclotella sp.* (Figure 6.3H).

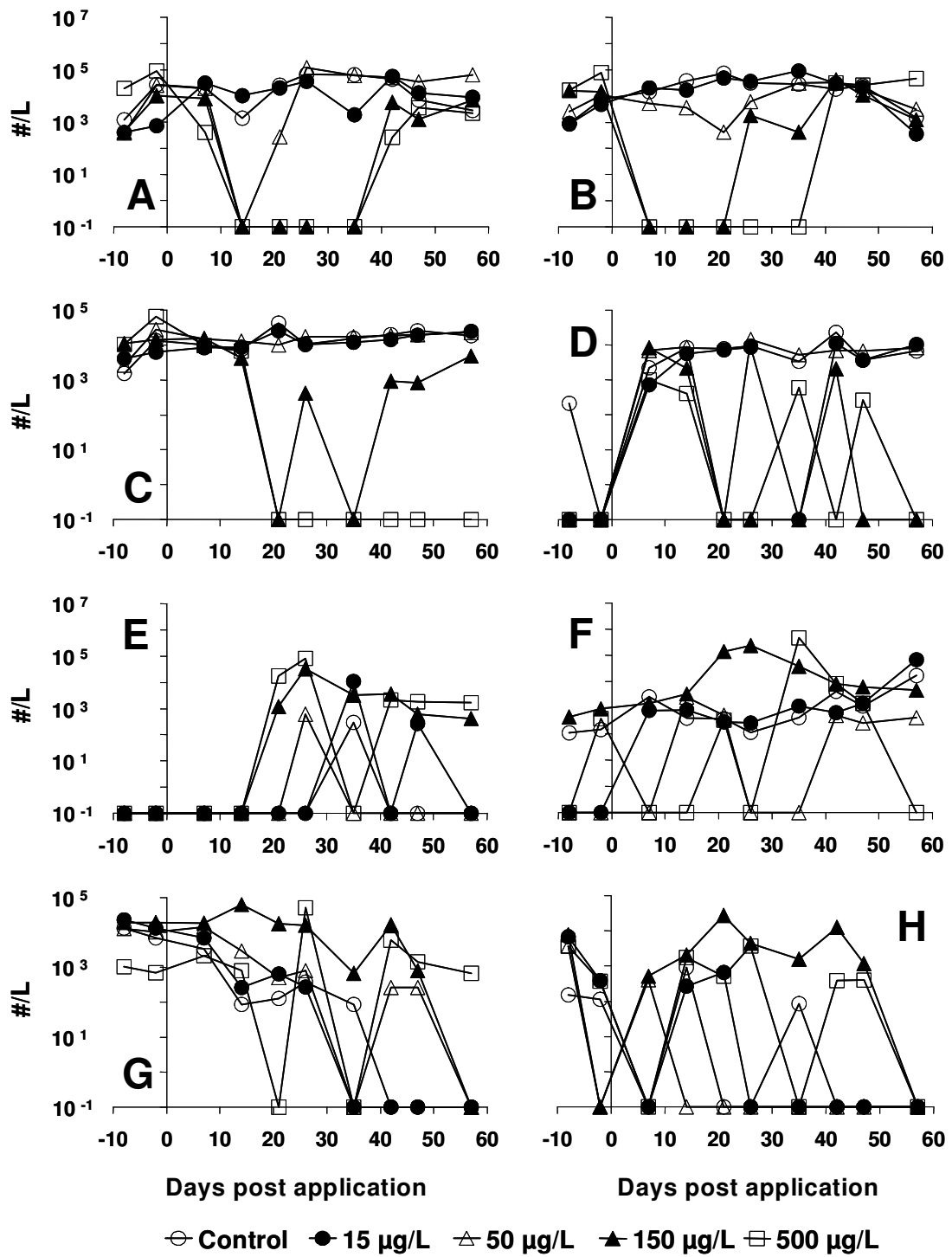


Figure 6.3 Dynamics in geometric means of the phytoplankton taxa with the highest positive (*Scenedesmus dispar* (A), *S. bicaudatus* (B), *Pediastrum tetras* (C) and *Coelastrum cambricum* (D)) and negative (*Ankistrodesmus falcatus* (E), *Oocystis pusilla* (F), *Nitzschia palea* (G) and *Cyclotella* sp. (H)) species weights in the PRC. In the figures, a value of 0.1 denotes the absence of the taxon.

Table 6.2 NOECs (No Observed Effect Concentration) calculated per sampling date for individual phytoplankton taxa in microcosms treated with linuron. Concentrations ($\mu\text{g a.i./L}$) showed significant increases (+) or decreases (-) compared to controls. In case of a complete elimination of a taxon, the table also indicates the lowest linuron concentrations and the sampling week this occurred as well as the sampling date of reappearance of the eliminated taxon for the different treatments. > indicates a NOEC of $> 500 \mu\text{g/L}$; NE = not eliminated.

	Days post application										Lowest concentration of elimination and sampling date
	-8	-2	7	14	21	26	35	42	47	57	
CHLOROPHYTA											
<i>Scenedesmus maximus</i>	>	>	>	15 (-)	15 (-)	15 (-)	50 (-)	50 (-)	50 (-)	>	50 (day 14)
<i>Scenedesmus aristatus</i>	>	>	150 (-)	15 (-)	15 (-)	50 (-)	50 (-)	50 (-)	>	>	50 (day 21)
<i>Scenedesmus quadricauda</i>	>	>	>	>	15 (-)	>	>	150 (+)	>	>	50 (day 14)
<i>Scenedesmus dispar</i>	>	>	150 (-)		15 (-)	50 (-)	50 (-)	50 (-)	>	>	50 (day 14)
<i>Scenedesmus tropicus</i>	>	>	>	50 (-)	>	>	>	>	>	>	150 (day 14)
<i>Scenedesmus bicaudatus</i>	>	>	50 (-)	15 (-)	15 (-)	150 (-)	50 (-)	>	>	>	150 (day 7)
<i>Scenedesmus denticulatus</i>	>	>	>	>	15 (-)	>	>	>	>	>	150 (day 21)
<i>Scenedesmus opoliensis</i>	>	150 (-)	>	>	>	>	>	>	>	>	NE
<i>Scenedesmus dimorphus</i>	50 (+)	>	>	>	>	>	>	>	>	>	NE
<i>Coelastrum reticulatum</i>	>	150 (+)	>	>	>	>	>	>	>	>	NE
<i>Coelastrum cambricum</i>	>	>	>	150 (-)	15 (-)	50 (-)	>	50 (-)	50 (-)	50 (-)	50 (day 21)
<i>Pediastrum tetras</i>	>	150 (+)	>	>	15 (-)	50 (-)	50 (-)	50 (-)	50 (-)	150 (-)	150 (day 21)
<i>Pediastrum duplex</i>	>	>	>	>	50 (-)	50 (-)	>	>	>	>	150 (day 21)
<i>Pediastrum simplex</i>	150 (+)	>	>	>	>	>	>	>	>	>	NE
<i>Oocystis pusilla</i>	>	>	>	>	>	>	50 (+)	>	>	>	NE
<i>Oocystis lacustris</i>	>	>	>	>	>	>	>	>	150 (+)	>	NE
<i>Tetraedron caudatum</i>	>	150 (+)	>	>	>	>	>	>	>	>	NE
<i>Botryococcus braunii</i>	>	>	150 (+)	>	>	>	>	>	>	>	NE
<i>Elakatothrix gelatinosa</i>	>	>	>	>	>	>	>	50 (+)	>	>	NE
<i>Ankistrodesmus falcatus</i>	>	>	>	>	>	50 (+)		50 (+)	>	>	NE
<i>Ankistrodesmus nanmoselene</i>	>	>	>	>	>	>	>	>	150 (+)	>	NE
<i>Monoraphidium sp</i>	>	>	>	>	>	>	>	>	>	15 (-)	NE

All taxa for which a NOEC was calculated are listed in Table 6.2. Many chlorophyte species, especially those belonging to the genera *Scenedesmus*, *Pediastrum* and *Coelastrum*, decreased significantly in numbers compared to control values. Several of these taxa were even eliminated at higher linuron doses.

Other chlorophytes, namely *Oocystis pusilla* (Figure 6.3F), *Botryococcus braunii*, *Elakatothrix gelatinosa*, *Ankistrodesmus falcatus* (Figure 6.3E) and *A. nanmoselene* had significantly higher numbers in the two highest linuron doses (Table 6.2). The cyanophyte *Merismopedia tenuissima* was eliminated in the two highest applied microcosms one week post application, while its numbers were increased over controls in a later stage of the experiment. Abundances of the diatoms *Nitzschia palea* (Figure 6.3G), *Cocconeis sp.*, *Surirella tenera* and *Cyclotella sp.* (Figure 6.3G) increased in higher linuron treatments. Remarkably, *N. palea* had significantly higher numbers in the 500 µg/L dose in weeks 2, 4, 6 and 8, while this species was eliminated at this dose in weeks 3 and 5 (Figure 6.3G). The cryptophytes *Chilomonas paramecium* and *Cryptomonas pyrenoidifera* were profited from the linuron stress in a later stage of the experiment; their abundances increased in the highest treatment (Table 6.2). Besides NOECs noted after the application of linuron, significant increased and decreased abundances of several species were randomly calculated in the pre-treatment period (Table 6.2).

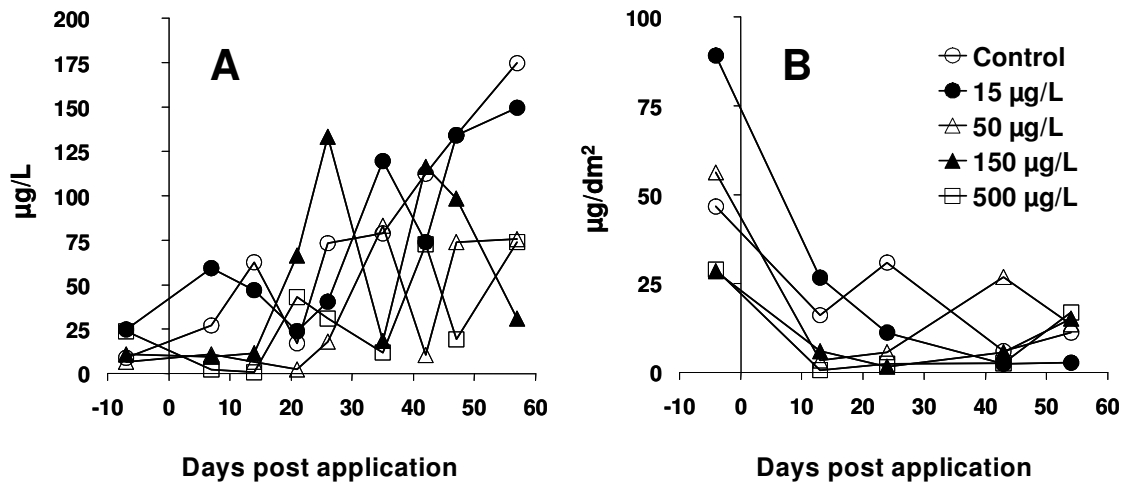


Figure 6.4 Dynamics of the chlorophyll-a content of phytoplankton (A) and periphyton (B) in the course of the experiment.

The chlorophyll-a content of the phytoplankton increased in the course of the experiment (Figure 6.4A). The dynamics of the 15 µg/L treatment resemble that of the control, while deviations were found for higher treatments. The Williams test revealed a significant decrease

in chlorophyll-a levels at the highest concentration in week 2 and 7, and for all but the lowest concentration in week 8.

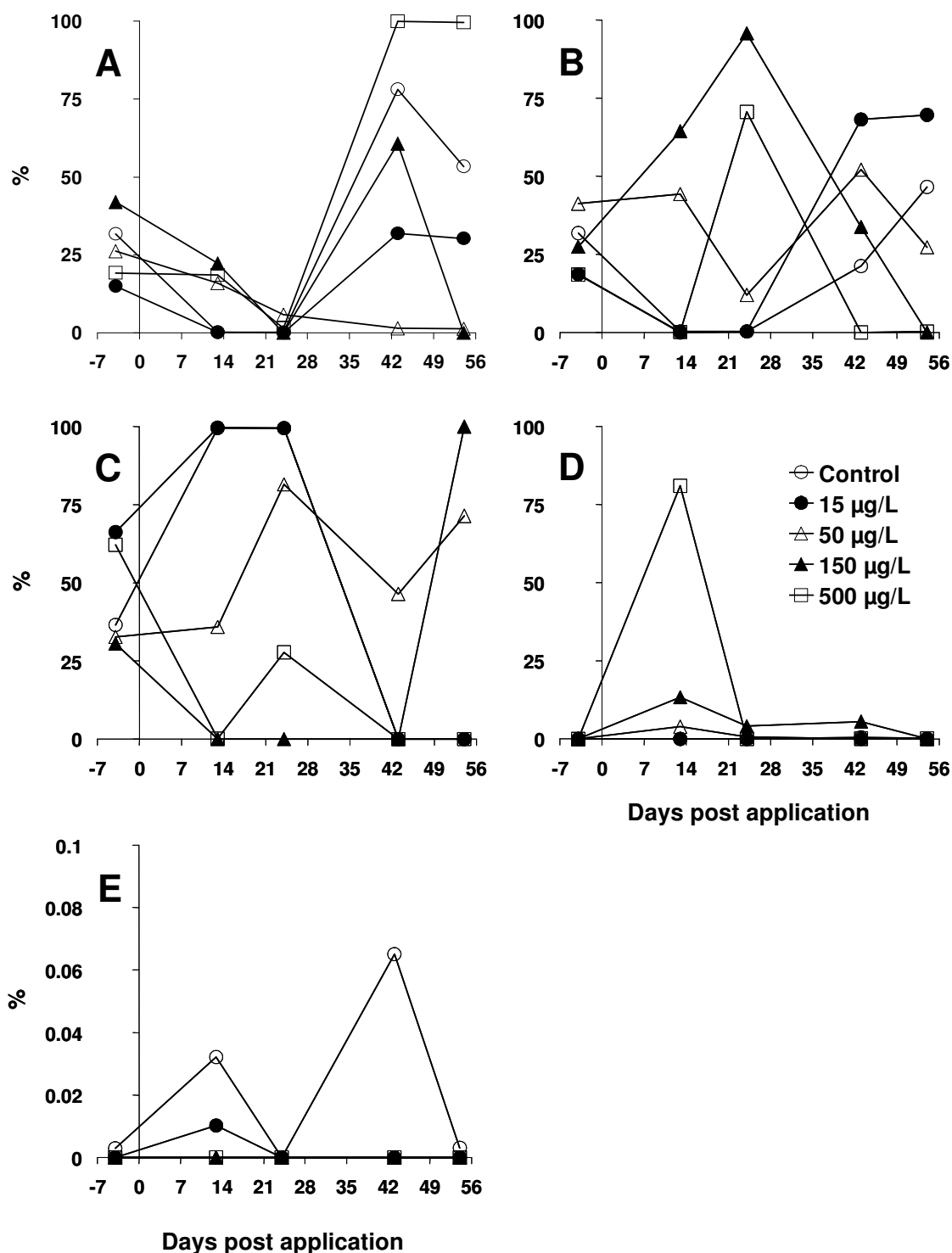


Figure 6.5 Relative contributions (% of total counts) of Chlorophyta (A), Bacillariophyta (B), the dominant cyanophyte *Chamaesiphon* sp. (C), other Cyanophyta (D), and Euglenophyta (E) for the different treatments in the periphyton community.

Periphyton

The succession of the periphyton community is visualized in Figure 6.5. After an incubation of 2 weeks, i.e. on day -3, the periphyton community in controls consisted of comparable numbers of Chlorophyta (Figure 6.5A), Bacillariophyta (Figure 6.5B) and Cyanophyta (Figures 6.5C and 6.5D). Euglenophyta, represented by *Euglena pisciformis* and *Phacus longispina*, were absent and were only found in very low numbers as compared to the other periphyton divisions during the entire experimental period (Figure 6.5E). Microscopic slides that were incubated for 4 to 6 weeks were completely (99.5%) dominated by the cyanobacterium *Chamaesiphon sp.* This species, however, completely disappeared after an incubation period of 8 and 10 weeks in control microcosms and replaced by chlorophytes and diatoms. Over the entire incubation period, a total number of 28 chlorophyte, 10 cyanophyte, 6 diatom and 2 euglenophyte taxa were found.

The diagram resulting from the PRC analysis of the periphyton dataset is given in figure 6.6. Of all variance, 28% and 36% could be attributed to sampling date and treatment level, respectively. The variance explained by sampling date is displayed on the x-axis, while 60% of the variance explained by treatment level is displayed on the y-axis. The diagram reveals that two and four weeks after application, the periphyton community of the three highest linuron concentrations deviated from controls (Figure 6.6). Indeed, Monte Carlo permutation tests and Williams test on the PCA coordinates calculated a NOEC of 15 µg/L for these sampling days (Table 6.3). *Chamaesiphon sp.* and *Nitzschia palea* have a species weight higher than 6 and lower than -1, respectively, while all other species have a species weight between -1 and 1 (Figure 6.6). This implies that *N. palea* is indicated to have increased most strongly in the higher treatment levels compared to the controls, *Chamaesiphon sp.* decreased even to a higher extent, and that the other species only showed a slight response to linuron or no response at all. Lowest NOECs were indeed calculated for *Chamaesiphon sp.* and *N. palea* (15 and 0 µg/L, respectively; Table 6.4), and their dynamics are presented in figure 6.7. During the first four weeks after application, numbers of *Chamaesiphon sp.* in controls and lowest dosed test systems increased, while numbers in the 50 µg/L dose remained constant and higher dosed systems even showed an elimination of this taxon (Figure 6.7A). For *N. palea*, intermediate concentrations had high numbers two weeks post application, while this species was absent in control, high- and low-dose treatments (Figure 6.7B). Two weeks later, *N. palea* was only

absent in controls with intermediate to high numbers in other treatments, leading to the calculation of a NOEC of 0 µg/L.

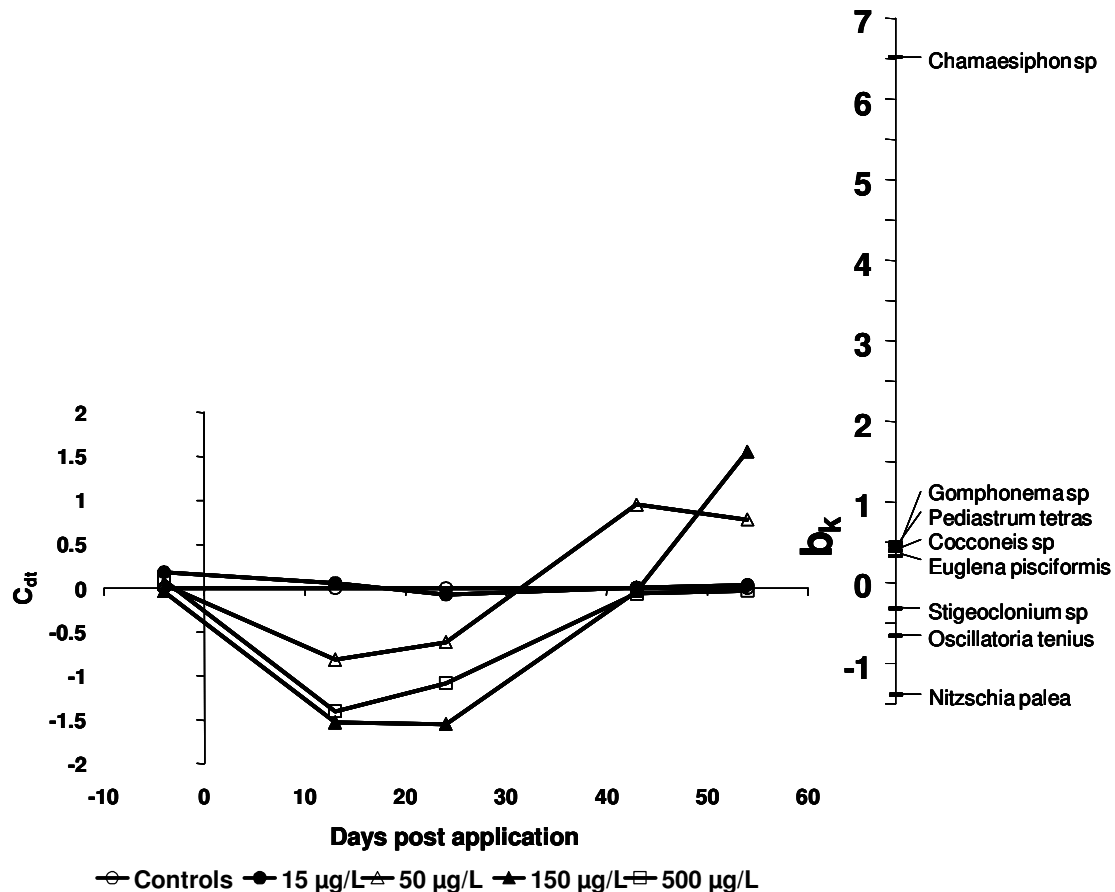


Figure 6.6 Principal response curves resulting from the analysis of the periphyton data set, indicating the treatment effects of linuron on the periphyton community. Of all variance, 28% could be attributed to sampling date; this is displayed on the horizontal axis. Thirty-six percent of all variance could be attributed to treatment level. Of this variance, 60% 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. A Monte Carlo permutation test indicated that a significant part of the variance explained by treatment level is displayed in the diagram ($P = 0.002$).

The only other diatom for which a NOEC could be calculated was *Gomphonema sp.*, which decreased in abundance two weeks post application at the highest dose. Chlorophytes decreased (*Pediastrum tetras* and *Cosmarium sp*) or increased (*Scenedesmus quadricauda* and *S. bicaudatus*) in numbers compared to controls. Besides the negative effects of linuron on

Chamaesiphon sp., effects calculated for other cyanobacteria taxa revealed increased abundances, namely for *Oscillatoria tenuis*, *Aphanocapsa sp.* and *Merismopedia tenuissima* (Table 6.4).

Table 6.3 p-values calculated with the Monte Carlo permutation tests and no observed effect concentrations (NOECcommunity) calculated by the Williams test on the PCA coordinates for the periphyton community data set.

Day	P-value	NOEC
-4	0.997	≥ 500
13	0.001	15
24	0.002	15
43	0.748	≥ 500
54	0.187	≥ 500

Table 6.4 NOECs (No Observed Effect Concentration) per sampling week for periphyton taxa that showed a significant response in the Williams test calculations ($p \leq 0.05$). Concentrations ($\mu\text{g a.i./L}$) showed significant increases (+) or decreases (-); > indicates a NOEC of > 500 $\mu\text{g/L}$.

	Days post application				
	-4	13	24	43	54
CHLOROPHYTA	>	>	>	>	>
<i>Scenedesmus quadricauda</i>	>	>	>	>	150 (+)
<i>Scenedesmus bicaudatus</i>	>	>	>	>	150 (+)
<i>Pediastrum tetras</i>	>	150 (-)	>	15 (-)	>
<i>Tetraedron minimum</i>	150 (+)	>	>	>	>
<i>Cosmarium sp</i>	>	150 (+)	>	>	150 (+)
CYANOPHYTA	>	>	>	>	>
<i>Oscillatoria tenuis</i>	>	50 (+)	>	>	>
<i>Chamaesiphon sp</i>	>	15 (-)	50 (-)	>	>
<i>Aphanocapsa sp</i>	>	150 (+)	>	>	150 (+)
<i>Merismopedia tenuissima</i>	>	>	>	>	150 (+)
BACILLARIOPHYTA	>	>	>	>	>
<i>Nitzschia palea</i>	>	>	0 (+)	>	>
<i>Gomphonema sp</i>	>	150 (-)	>	>	>

Effects of linuron on relative contributions of *Chamaesiphon sp* and periphyton groups are given in figure 6.5. The lowest concentration showed a succession similar to controls; initial dominance of *Chamaesiphon sp.*, followed by chlorophytes and diatoms. In the 50 $\mu\text{g/L}$ dose, the decrease in *Chamaesiphon sp.* abundances was accompanied with an increase in chlorophytes. At a linuron concentration of 150 $\mu\text{g/L}$, the complete elimination of *Chamaesiphon sp.* led to increases in subsequently diatoms and chlorophytes, after which *Chamaesiphon sp.* returned to dominate the periphyton community. In the highest dose,

Chamaesiphon sp. elimination caused a different succession pattern: dominance by other cyanophyta was followed by diatom domination and, from 6 weeks post application onwards, chlorophyta (Figure 6.5).

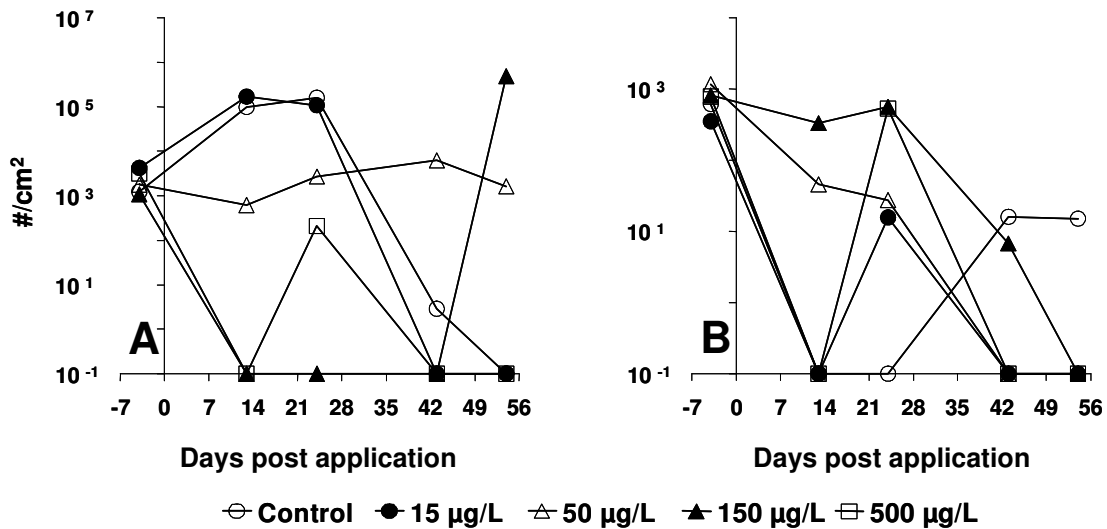


Figure 6.7 Dynamics in numbers (geometric means) of the taxon that dominated the phytoplankton community, *Chamaesiphon* sp. (A), and the taxon with the lowest species weight in the PRC of the periphyton dataset, *Nitzschia palea* (B). In the figures, a value of 0.1 denotes the absence of the taxon.

The dynamics in chlorophyll-a levels of the periphyton are presented in figure 6.4B. Linuron doses higher than 15 µg/L had decreased chlorophyll-a concentrations on day 13 (Williams test, $p < 0.05$).

Discussion

Fate of linuron in the water column

Linuron disappearance from the higher dosed microcosms followed a two-phase sequence (Figure 6.1). The initial loss of linuron was slower than that after two weeks post application. This may be explained by the drop in pH from values above 9 in control and lower doses to values near or just below 8 in the higher doses (Chapter 7) since hydrolysis of linuron has been reported to be slower at pH 6 and 8 than at pH 4 and 10 (Cserhati et al., 1976). In line with this, Van den Brink et al. (1997) also attributed the concentration-dependent rate of linuron

disappearance from the water phase they noted in laboratory microcosms to differences in pH regime.

A priori, the authors hypothesized a faster decline in linuron concentrations in the present study than observed in studies carried out in temperate countries since higher temperatures and nutrient levels have been demonstrated to accelerate the decomposition of herbicides (Cserhati et al., 1976; Lozano and Pratt, 1994; Pratt and Barreiro, 1998). Since the rate of herbicide loss appeared to be dose-dependent and pulsed or chronic treatment regimes may imply a loading of the herbicide, we can only compare the fate of linuron in the present study with that reported in a microcosm study by Slijkerman et al. (2005), evaluating single peak linuron concentrations comparable to those in the present study. In the study by Slijkerman et al. (2005), a decrease in concentration of 21%, 35% and 36% was noted 28 days after application of 20, 60 and 180 µg linuron/L. In our study, corresponding values (for 15, 50 and 150 µg/L) were 53%, 55% and 37%, which indeed suggests a slightly faster decomposition for the lower doses.

Representativeness of primary producer communities for tropical (Thai) freshwater ecosystems

Chlorophyta was the most abundant and diverse phytoplankton group in control communities. In line with this, Kalff and Watson (1986) reported that the fraction of chlorophyte biomass in the tropics is generally higher than in temperate lakes. Furthermore, approximately 40% (author's calculation) of the species recorded in a checklist of freshwater algae in Thailand belong to the division Chlorophyta (Wongrat, 1995). Several identified species belonging to the genera *Scenedesmus* were not recorded in the latter report nor in several field studies carried out in different parts of Thailand (Peerapornpisal et al., 2000a; Ariyadej et al., 2004; Pongswat et al., 2004). These include *S. bicaudatus* and *S. dispar*, which combined numbers made up approximately two-third of total phytoplankton numbers in controls throughout the present study. Based on a comparison between the number of phytoplankton species known in the world and in Thailand, Baimai (1995) concluded that the discovery of new species for Thailand may indeed be expected. This is supported by the fact that in recent studies of the Mae Sa stream in Chiang Mai (Northern Thailand), a total of 68 new species were recorded in field samples between 1997 and 1998 and another 51 between 1998 and 1999 (Peerapornpisal et al., 2000a; Pektong and Peerapornpisal, 2001). In addition, *S. bicaudatus* has

previously been recorded in Asia (Ling and Tyler, 2000) and several varieties of *S. dispar* were even first described for Vietnam and India (Hegewald and Silva, 1988).

Dominance of the periphyton community changed from *Chamaesiphon sp.* to chlorophytes and diatoms in the course of the experiment (Figure 6.5). In temperate freshwaters, the colonization of periphytic algae on a pristine surface is from small, flat cells with a large surface area attached to the substrate like diatoms to standing or stalked forms and eventually filamentous forms (Brönmark and Hansson, 2005). Based on this succession pattern, dominance of diatoms and *Chamaesiphon sp.* would be expected to be reversed. However, Brönmark and Hansson (2005) also noted that the succession stages as described above are reversed at high grazing pressure. Fish were absent in the microcosms so the consequently high abundances of zooplankton (see part II) presumably led to a first dominance of *Chamaesiphon*. In addition, *Chamaesiphon investiens* has been reported as a grazer-resistant species by Rosemond et al. (2000).

Chamaesiphon guilleri has been recorded in Thailand, and this species was found to be indicative of the oligo-mesotrophic zone of a stream in Thailand (Peerapornpisal et al., 2000b). Diatoms have been reported to be characteristic for high pH and may become dominant irrespective of the nutrients status of the water (Brönmark and Hansson, 2005). The average pH was approximately 0.5 unit lower during the dominance period of the *Chamaesiphon sp.* compared to the period diatoms and chlorophytes dominated the periphyton community. In addition, concentrations of several nutrients increased over the course of the experiments as a result of nutrient additions (Chapter 7). Thus, the increasing trend in pH and nutrient levels over the course of the experiment led to a competitive advantage of diatoms over *Chamaesiphon sp.* in time and consequently a shift in dominance by *Chamaesiphon sp.* to diatoms.

Pre-treatment NOECs for phytoplankton species

The NOECs in the pre-treatment period were always only calculated on individual sampling dates, and indicated increases in numbers compared to controls except *S. opoliensis* (Table 6.2). The latter species was indeed absent in both microcosms of the highest linuron dose. However, an absence was also noted in one of the control replicas. The NOECs indicating increased abundances for higher linuron doses were calculated for species that were mostly absent in controls and lower linuron doses on the corresponding sampling date. In addition, in two-third of the cases, the abundance of the species was also zero in one of the two replicates

and evidently sufficiently high in the other replicate to indicate significance. Based on the tropical weather conditions (e.g. high temperature: 30 ± 2 °C) and nutrient input, the microcosms exhibited a high productivity. This presumably caused opportunistic species to bloom occasionally, leading to relatively high single peak abundances in individual microcosms and consequently the calculation of random NOECs in the pre-treatment period.

Sensitivity of phytoplankton

Chlorophyta was the most affected phytoplankton division, followed by Cyanophyta (only *Merismopedia tenuissima*), whereas Bacillariophyta and Cryptophyta were tolerant to linuron stress and increased in abundances. Chlorophytes have indeed been reported to be more sensitive and diatoms and cryptophytes more tolerant to photosynthesis inhibitors (see Bérard et al., 1999 for several reference studies).

Of the Bacillariophyta, the pennate diatom *Nitzschia palea* and the centric diatom *Cyclotella sp.* were the most discriminate diatom taxa in the PRC (Figure 6.2) and the lowest NOECs were calculated for these diatoms (50 µg/L; Table 6.2). *N. palea* was found to be insensitive to the herbicides simetryn and pretilachlor and is assumed to be tolerant to various pollutants (Kasai, 1999). Centric diatoms remained predominantly longer in ponds treated with simetryn than in control ponds (Kasai and Hanazato, 1995). *Cryptomonas pyrenoidifera* was the cryptophyte with the most pronounced increase (Table 6.2, Figure 6.2). In line with this, predominance of *Cryptomonas* and *Mallomonas* species were associated with increased phytoplankton community tolerance after exposure to atrazine (deNoyelles et al., 1982). Although no NOECs on species level were calculated for *Mallomonas* species in the present study, the PRC did indeed indicate a (slight) increase for these Crysophyta species (Figure 6.2).

Toxic effects on chlorophytes increased with time, with a lowest NOECphytoplankton community of 15 µg/L calculated three weeks after application (Table 6.1). Effects of photosynthesis inhibiting herbicides are indeed known to increase with exposure duration (Gustavson et al., 2003), and Van den Brink et al. (1997) attributed this to survival of algae on their storage energy so effects only become apparent when this is exhausted.

Also on species level, a time-dependent treatment effect was found. *Scenedesmus bicaudatus* was the most severely affected species one week after application and a NOEC of 15 µg/L was calculated after two weeks. For most other *Scenedesmus* species, *Coelastrum cambricum* and *Pediastrum tetras*, this NOEC was only calculated on day 21 while the NOEC for *P. duplex* was

only 50 µg/L on that day (Table 6.2). Weiner et al. (2004) found that smaller phytoplankton taxa with greater surface area to volume ratios incorporated a larger part of the herbicide atrazine, and are consequently more sensitive to atrazine exposure. *S. bicaudatus* was indeed the smallest affected *Scenedesmus* taxon in the present study. Other *Scenedesmus* species were not always smaller than 4-cell colonies of *P. tetras* and young colonies of *C. cambricum*. However, the colony shape of *Scenedesmus* is rectangular whereas colonies of *P. tetras* and *C. cambricum* are spherical to circular, implying a larger surface area to volume ratio for *Scenedesmus* species and hence a larger herbicide sensitivity. Colonies of *P. duplex* measured up to 80 µm in diameter, which explains why a response was only found at the two highest linuron doses three and four weeks post application.

For the chlorophytes *Oocystis pusilla*, *O. lacustris*, *Ankistrodesmus falcatus*, *A. nannosele* *Botryococcus braunii*, *Elakatothrix gelatinosa* and the cyanobacterium *Merismopedia tenuissima*, NOECs of 50 µg/L or 150 µg/L indicating increased abundances were calculated at different sampling dates (Table 6.2). *B. braunii* was the most tolerant chlorophyte and increased in abundance one week after application in the highest applied tanks, which at that time contained 434 µg/L linuron. Kasai (1999) observed a high tolerance of *Dictyosphaerium* and ascribed this to a prevention of pretilachlor entering the cells because of the mucilaginous sheath of this species. The cells of *B. braunii* are embedded in a tough mucilage, and may thus be the reason of the high linuron tolerance of this species.

Four weeks post application, *A. falcatus* had increased numbers over controls in the 150 µg/L and 500 µg/L treatments, corresponding to linuron concentration levels of respectively 94 µg/L and 230 µg/L. LC₅₀ and NOEC values reported in laboratory tests for linuron are 4.9 and 2.5 µg/L, respectively (Crommentuijn et al., 1997), and *A. falcatus* has also been reported to be sensitive to other photoinhibitors, like atrazine (DeLorenzo et al., 2004). This thus implies that this species developed herbicide-tolerant populations in the present study.

Other increased numbers of chlorophytes at the higher linuron doses were subsequently found for *O. pusilla* (5 wks; NOEC 50 µg/L), *E. gelatinosa* (6 wks; NOEC 50 µg/L), *A. nannosele* and *O. lacustris* (7 wks; NOEC 150 µg/L). This indicates that the order of tolerance level (or competitive fitness at a similar tolerance level) was *O. pusilla* > *E. gelatinosa* > *A. nannosele* ≈ *O. lacustris*. Since linuron concentration levels were not analyzed on sampling dates corresponding to these NOECs and linuron laboratory toxicity studies are not known to the authors for these species, we can not evaluate whether these species are generally tolerant to linuron or if this tolerance was induced in the present study.

The PRC indicated a recovery of the phytoplankton community in the 50 µg/L treatment on day 26. From day 26 up to day 47, a NOECphytoplankton community of 50 µg/L was calculated, while this was 150 µg/L on day 55. This indicates recovery of the 150 µg/L treatment at the end of the experiment, while the phytoplankton community in the highest applied microcosms did not recover within the experimental period (Table 6.1).

Effects on periphyton community

The cyanophyte *Chamaesiphon sp.* was the most sensitive periphyton species, while the cyanophytes *Oscillatoria tenuis* and *Aphanocapsa sp.*, and, like in the phytoplankton community, the diatom *N. palea* increased in abundances (Figure 6.6 and 6.7; Table 6.4). This relatively high sensitivity of *Chamaesiphon sp.* may be explained by the fact that cells are (i) small and individually attached to a substrate (ii) enclosed only by a thin envelope and (iii) not embedded in a mucilaginous sheath (Ling and Tyler, 2000). In addition, the ultrastructure of *Chamaesiphon confervicola* has been reported to possess numerous pores in the mureic layer of the cell wall (Gromov and Mamkaeva, 1980), which may enhance the penetration of the cells by herbicides. In the 150 µg/L treatment, the dominance by *Chamaesiphon sp.* was replaced by diatoms followed by chlorophytes and a recovery of *Chamaesiphon sp.* (Figure 6.5). In the highest linuron concentration, however, the succession pattern of colonization was from other cyanobacteria to diatoms to chlorophytes. The chlorophyll-a of the phytoplankton was as low as 0.6 µg/L in the highest linuron dosed microcosms, while this was still 11 µg/L and not significantly reduced in the 150 µg/L on day 14. This implies that the grazing pressure of the zooplankton populations on the periphyton community was probably very high in the 500 µg/L applied microcosms. As discussed above for the succession of periphyton community, the enhanced grazing pressure in these microcosms presumably favored the large filamentous colonies of *Oscillatoria tenuis* and *Aphanocapsa sp.* rather than the unicellular diatoms as observed in the 150 µg/L.

Effects on periphyton at the community level were calculated for all but the lowest linuron concentrations on day 13 and 24, while on later sampling dates a NOECperiphyton community > 500 µg/L was calculated (Table 6.3). This implies a recovery of the periphyton community within 6 weeks in all affected microcosms. However, the response curves of the 50 µg/L (on day 43 and 54) and the 150 µg/L treatments (on day 54) lay higher than the control curve (i.e., the horizontal axis; Figure 6.6). This indicates periphyton communities

opposite to the affected community. This may be explained by the relatively high contribution of *Chamaesiphon* sp. to the periphyton community in these treatments by the end of the experiment (Figure 6.5C) and the high species weight of this species.

Chlorophyll-a

As a consequence of functional redundancy, i.e. the alteration of the phytoplankton community from sensitive to tolerant taxa, NOECs calculated for the chlorophyll-a content of the phytoplankton did not reflect the impact of linuron stress. For periphyton, the NOEC for effects on chlorophyll-a resembled the NOEC periphyton community on day 13 but did not indicate the change in periphyton community on day 24, presumably due to the increase in *N. palea* on that day (Tables 6.3 and 6.4). Thus, chlorophyll-a turned out not to be a sensitive indicator for herbicide stress, especially regarding the effects on phytoplankton.

Acknowledgement

This study was funded by the Portuguese government through FCT (scholarship SFRH/ BD/ 8213/ 2002). The authors are indebted to the staff at the AIT hatchery for technical assistance, and to Yuwadee Peerapornpisal for sending her doctoral thesis and providing valuable information regarding phytoplankton identification.

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CHAPTER 7

ECOLOGICAL EFFECTS OF THE HERBICIDE LINURON IN TROPICAL FRESHWATER MODEL-ECOSYSTEMS: II. ECOSYSTEM FUNCTIONING, ZOOPLANKTON AND HAZARD ASSESSMENT

(Submitted to *Ecotoxicology and Environmental Safety*)

Abstract

A microcosm study was carried out to evaluate the effects of linuron (nominal levels: 0, 15, 50, 150 and 500 µg/L) on the ecology of surrogates for tropical Thai freshwaters. Structural effects on primary producers and chlorophyll-a levels were discussed in a previous paper. The aim of the present paper was three-fold: Firstly, effects on ecosystem functioning and the zooplankton community were assessed and discussed in relation to the effects noted on algae. Secondly, threshold values for the different endpoints were compared with those reported in microcosm and mesocosm studies performed in the temperate zone. Thirdly, implications for the use of toxicity data derived from studies in temperate countries for tropical countries like Thailand are discussed in the discussion section. It is concluded that in terms of ecosystem *sensitivity*, the use of temperate toxicity data ensure sufficient protection of the freshwater ecosystem in tropical Thailand. However, since higher pesticide concentrations may be expected after application in flooded rice fields than in temperate ditch-dike systems, care should be taken when extrapolating *hazard* assessments of herbicides like linuron from temperate to tropical countries.

Introduction

Pesticides sprayed on agricultural fields to control pests may enter surrounding aquatic ecosystems and may therefore result in undesirable side-effects on aquatic organisms (Capri and Trevisan, 1998; Hill et al., 1994). To prevent adverse side effects on the aquatic environment, authorities in many countries require an assessment of the potential ecological risks before registration of a pesticide. The (first-tier) evaluation is usually based on laboratory toxicity tests with standard test species (algae, macrophyte, *Daphnia*, fish), eg EU (1997). Also in

Thailand, each pesticide has to be tested for effects on the environment prior to placing them on the market, as laid down in the Hazardous Substances act B.E. 2535 (1992). However, if a product has been tested elsewhere, only missing toxicological data are requested (Jungbluth, 1996). All pesticides except paraquat are imported to Thailand mainly from countries located in the temperate zone, where most of the ecotoxicity testing has been conducted (Bourdeau et al., 1989; Lacher and Goldstein, 1997; Abdullah et al., 1997; Jungbluth, 2000). Also in other tropical countries, water quality criteria (WQC) have been reported to rely on extrapolations from data obtained from studies carried out in countries from the temperate zone (Kwok et al., 2007). It thus becomes imperative to validate whether toxicity values obtained from these studies are applicable to tropical countries like Thailand.

This paper is the second in a series of two dealing with the effects of the herbicide linuron on outdoor plankton-dominated microcosms in Thailand. The first paper dealt with effects on phytoplankton and periphyton (Chapter 6). The purpose of the present paper was three-fold. Firstly, the effects on ecosystem functioning and the zooplankton community are discussed in detail the more since basic community interactions in Thai freshwaters are still largely unknown (Campbell and Parnrong, 2001). Secondly, the overall ecological effects noted in the present study are compared with those reported in microcosm and mesocosm studies performed in the temperate zone. Thirdly, implications for the use of toxicity data derived from studies in temperate countries for tropical countries like Thailand are discussed in the discussion section.

Materials and Methods

Experimental set-up

The outdoor microcosm study was conducted with twelve circular concrete tanks (diameter 0.75 m; total depth 0.65 m; water depth 0.55 m; water volume 250 L) at the hatchery of the Asian Institute of Technology (AIT). After a pre-treatment period of 5 weeks, linuron dose solution (nominal levels: 15, 50, 150, 500 $\mu\text{g/L}$; applied as Afalon Flow) were gently poured added in two microcosms for each concentration and mixed by stirring. Four other systems did not receive any treatment and served as controls. To stimulate phytoplankton growth and to compensate for losses, nutrient additions of N (1.4 mg/L as urea) and P (0.18 mg/L as TSP) were made twice a week during the entire experiment.

Details on the experimental design, application and fate analysis of the herbicide are described in part I (Chapter 6).

Water quality parameters

Two weeks before application and on a weekly basis after application, dissolved oxygen (DO), pH, electrical conductivity (EC) and temperature (T) were measured in the morning (just after sunrise) as well as at the end of the afternoon (just before sunset). On sampling days, measurements were made approximately 10 cm below the water surface using a YSI 58 oxygen meter connected to a YSI 5739 probe (DO), a Consort C523 pH meter (pH) and a Consort C532 conductivity meter (EC and T). Alkalinity levels were determined at weekly intervals in 100-mL subsamples taken from a 1-L water sample obtained as described below, by titrating with 0.05 N HCl until pH 4.2.

The concentrations of ammonia, nitrate, nitrite, total Kjeldahl nitrogen (TKN), soluble reactive phosphate (SRP) and total phosphorous were analysed at two-week intervals in a 1-L sample taken at approximately 10 cm below the water surface using the methods described in APHA (1992).

Zooplankton

From one week prior to application up to 8 weeks post application, depth-integrated water sample of 10-L were collected on a weekly basis using a Perspex tube. One liter was used for determination of treatment effects on the phytoplankton community structure, as described in part I. Another liter was transferred to plastic bottles for phytoplanktonic chlorophyll-a determination (see part I) and alkalinity (see above). The bucket was then partially emptied into the microcosm from which it had been taken, leaving 5 L in the bucket. This remainder was passed through a zooplankton net (mesh size 60 μm) and preserved with formalin (final concentration: 4% V/V) to examine treatment effects on the zooplankton community. Subsamples of the zooplankton sample were counted with an inverted microscope (magnification 100-400). Rotifers and cladocerans were identified to the lowest taxonomic level possible. Copepods were divided into nauplii (immature stages), calanoids and cyclopoids (mature stages). Ostracoda were not further identified. Numbers were recalculated to numbers per litre of microcosm water.

Data analysis

A detailed description and rationale for the data analysis of the abundance values of phytoplankton and periphyton data sets are given in part I (Chapter 6). Since the same methods were used for the zooplankton dataset, only a brief description is given in this section.

NOECs were calculated for all parameters using the Williams test, which assumes an increasing effect with increasing dose (Williams, 1972). Abundance data were $\text{Ln}(Ax + 1)$ transformed, where x stands for the abundance value and Ax makes 2 by taking the lowest abundance value higher than zero for x . Analyses were performed with Community Analysis, version 4.3.05 (Hommen *et al.*, 1994) and statistical significance was accepted at $p < 0.05$.

The response of the zooplankton community to the linuron application was analysed by the Principal Response Curves method (PRC) using the CANOCO software package, version 4.5 (Ter Braak and Smilauer, 2002). 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 (Van den Brink and Ter Braak, 1999). In addition, permutation tests were performed per sampling date using Ln -transformed treatment levels as explanatory variables to determine the significance of the treatment regime per sampling date. The NOEC values at community level for the zooplankton community were calculated for each individual sampling date by applying the Williams test to the sample scores of the first principal component for each sampling date separately (for rationale, see Van den Brink *et al.*, 1996).

Results

DO-pH-alkalinity-conductivity syndrome

In control microcosms, DO ranged between 5.1 mg/L in the morning and 12.4 mg/L at the end of the afternoon, leading to an average DO production of 7.3 mg/L. pH was alkaline with morning values (8.9 ± 0.3 ; mean \pm SD) lower than those measured in the afternoon (9.6 ± 0.3 ; mean \pm SD). Late afternoon levels of electrical conductivity (EC) were on average 11 $\mu\text{S}/\text{cm}$ higher than in the morning. EC values dropped on day 21, with average EC values 165 - 177 $\mu\text{S}/\text{cm}$ (afternoon – morning) lower in the period before day 21 than the period

thereafter. Alkalinity decreased over the experimental period in controls from 2.9 meq/L in the pre-treatment period to 1.7 meq/L on the last day of the experiment.

Table 7.1 No observed effect concentrations (NOECs; Williams test, $p < 0.05$) per sampling date (weeks p.a.) for water chemistry parameters. Concentrations ($\mu\text{g a.i./L}$) showed significant increases (+) or decreases (-); > indicates a NOEC of $> 500 \mu\text{g/L}$; DO = dissolved oxygen; EC = electrical conductivity; TKN = Total Kjehldahl Nitrogen; TP = Total Phosphorus; SRP = soluble reactive phosphorus.

	-4	-2	-1	0	1	2	3	4	5	6	7	8
DO am	NM	>	NM	NM	15 (-)	>	>	15 (+)	150 (+)	150 (+)	>	>
DO pm	NM	>	NM	NM	15 (-)	15 (-)	>	>	>	>	>	>
DO prod	NM	>	NM	NM	150 (-)	15 (-)	15 (-)	15 (-)	>	150 (-)	150 (-)	>
pH am	NM	>	NM	NM	15 (-)	150 (-)	>	>	>	>	>	>
pH pm	NM	>	NM	NM	15 (-)	15 (-)	>	150 (-)	>	>	>	>
EC am	NM	>	NM	NM	50 (+)	15 (+)	50 (+)	>	>	>	>	>
EC pm	NM	>	NM	NM	150 (+)	15 (+)	>	>	>	>	>	>
Alkalinity	NM	NM	150 (+)	NM	15 (+)	15 (+)	50 (+)	150 (+)	150 (+)	15 (+)	15 (+)	>
Ammonia	>	>	>	NM	NM	15 (+)	NM	>	NM	>	NM	>
Nitrate	>	>	>	NM	NM	>	NM	150 (+)	NM	150 (+)	NM	>
Nitrite	>	>	150 (+)	NM	NM	150 (+)	NM	50 (+)	NM	150 (+)	NM	150 (+)
TKN	>	>	>	NM	NM	150 (+)	NM	>	NM	>	NM	>
TP	>	>	>	NM	NM	15 (+)	NM	150 (+)	NM	150 (+)	NM	>
SRP	>	>	>	NM	NM	15 (+)	NM	150 (+)	NM	50 (+)	NM	150 (+)

The NOECs calculated with the Williams test per sampling date on physicochemical parameters are presented in Table 7.1. Morning and afternoon DO levels (Figure 7.1A and 7.1B), as well as DO production (Figure 7.1C), were decreased at all but the lowest linuron concentration. Interestingly, DO measured in the morning increased over control values 4 weeks post application at these doses, while 5 and 6 weeks after application this was only noted for the $500 \mu\text{g/L}$ (Table 7.1). For pH, the lowest NOEC calculated was also $15 \mu\text{g/L}$, although decreased levels returned faster to control levels (Figure 7.1D). The decrease in DO and pH was accompanied with an increase in EC (Figure 7.1E) and, more prolonged, alkalinity (Figure 7.1F). No NOEC $< 500 \mu\text{g/L}$ was calculated on the last sampling day, indicating that DO, pH, EC and alkalinity levels regained control levels within the experimental period.

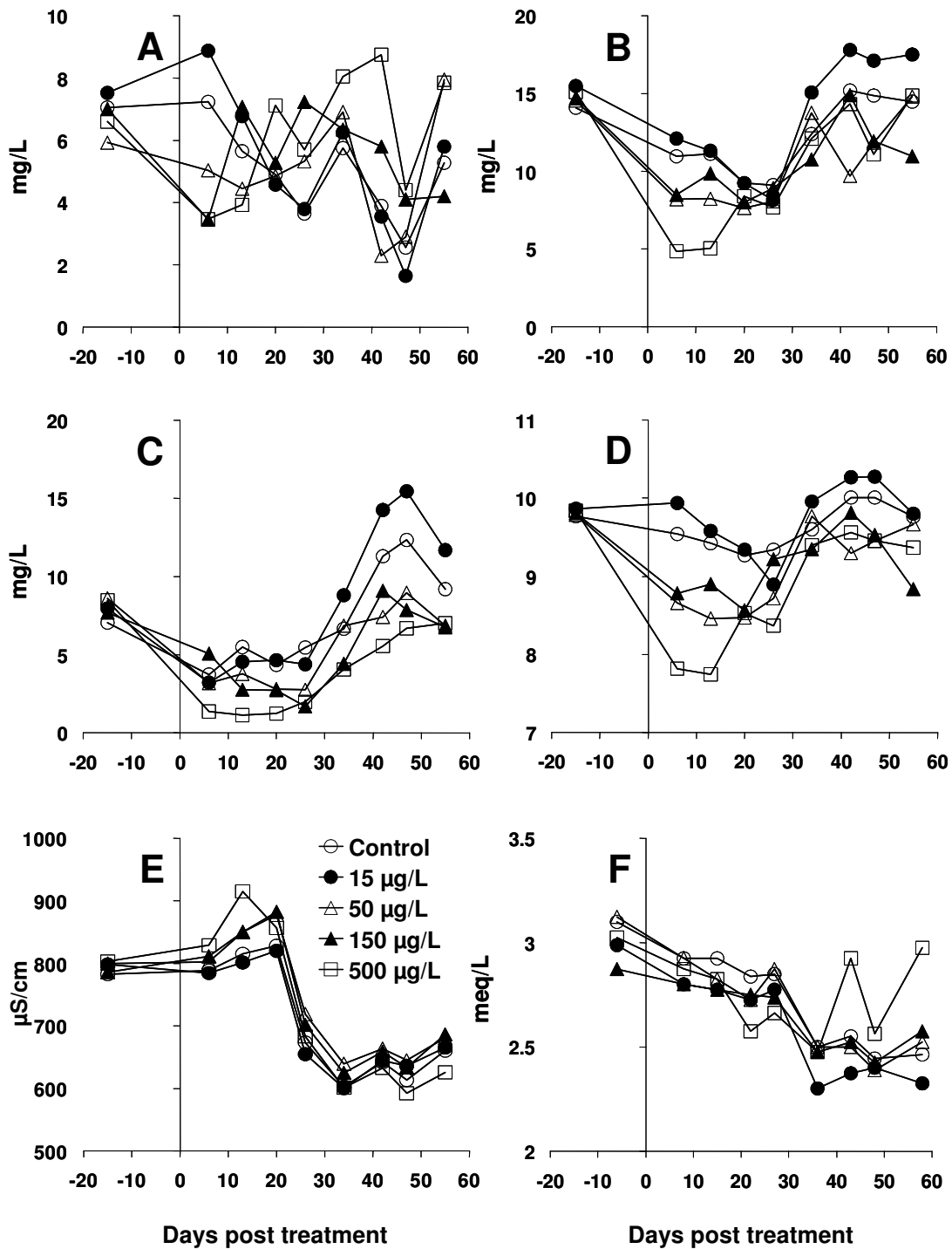


Figure 7.1 Dynamics of the measurements on the DO-pH-alkalinity-conductivity syndrome. Figures 7.1A through 7.1C show means of measurements per treatment level of dissolved oxygen (DO) concentrations measured in the morning (A), by the end of the afternoon (B) and the difference between these two levels; DO production (C). Dynamics in late afternoon values of pH (D) and electrical conductivity (E), as well as alkalinity measured at a depth of 10 cm (F) are shown in Figures 7.1D through 7.1F.

The drop in alkalinity in the control microcosms as described above was accompanied with a drop in total Kjehldahl nitrogen (TKN). Inorganic nitrogen levels over the experimental period were made up of mostly ammonia (mean: 0.12 mg/L), followed by nitrate (mean: 0.05 mg/L), while nitrite concentrations were always lower than 0.01 mg/L and averaged 0.005 mg/L. Total phosphorus (TP) and soluble reactive phosphate (SRP) concentrations increased during the experimental period from 0.42 mg/L to 0.85 mg/L and from 0.24 mg/L to 0.38 mg/L, respectively. SRP made up approximately half of the TP concentration.

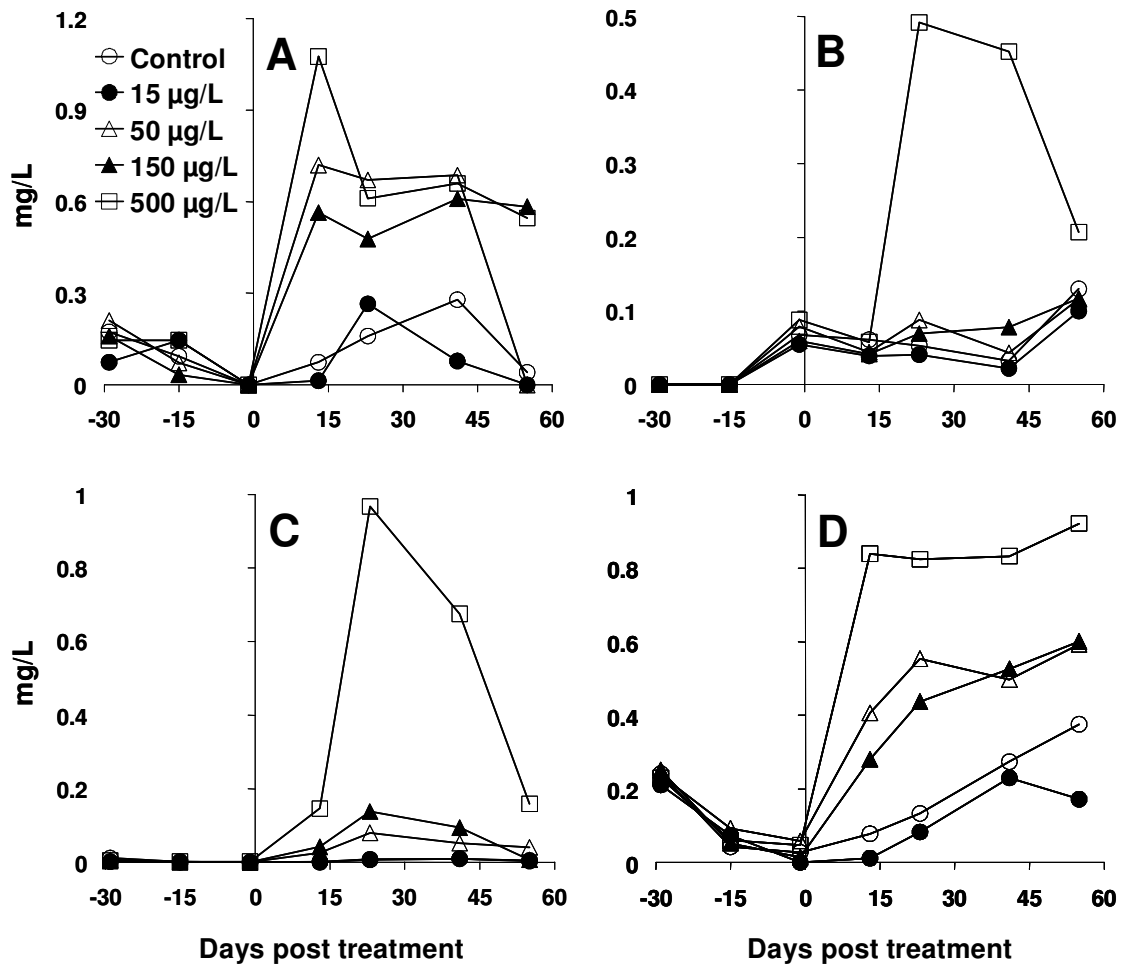


Figure 7.2 Dynamics of ammonia (A), nitrate (B), nitrite (C) and soluble reactive phosphate (SRP; D) concentrations per treatment level.

Two weeks post application, ammonia concentrations were increased in all but the lowest treatment (Figure 7.2A), while nitrite (Figure 7.2C) and TKN were increased on that day in the highest treatment only (Table 7.1). Although nitrite remained increased in the higher linuron applied microcosms, ammonia and TKN returned to control values within 4 weeks after application. Nitrate increased in concentration only at the highest dose 4 and 6 weeks after application. Linuron also had a prolonged effect on TP and SRP concentrations (Figure 7.2D). Lowest NOECs for both parameters were calculated two weeks post application (i.e., 15 µg/L). Effects on TP were calculated up to 6 weeks for the highest linuron concentration, while effects on SRP levels remained at this treatment level till the end of the experiment (Table 7.1).

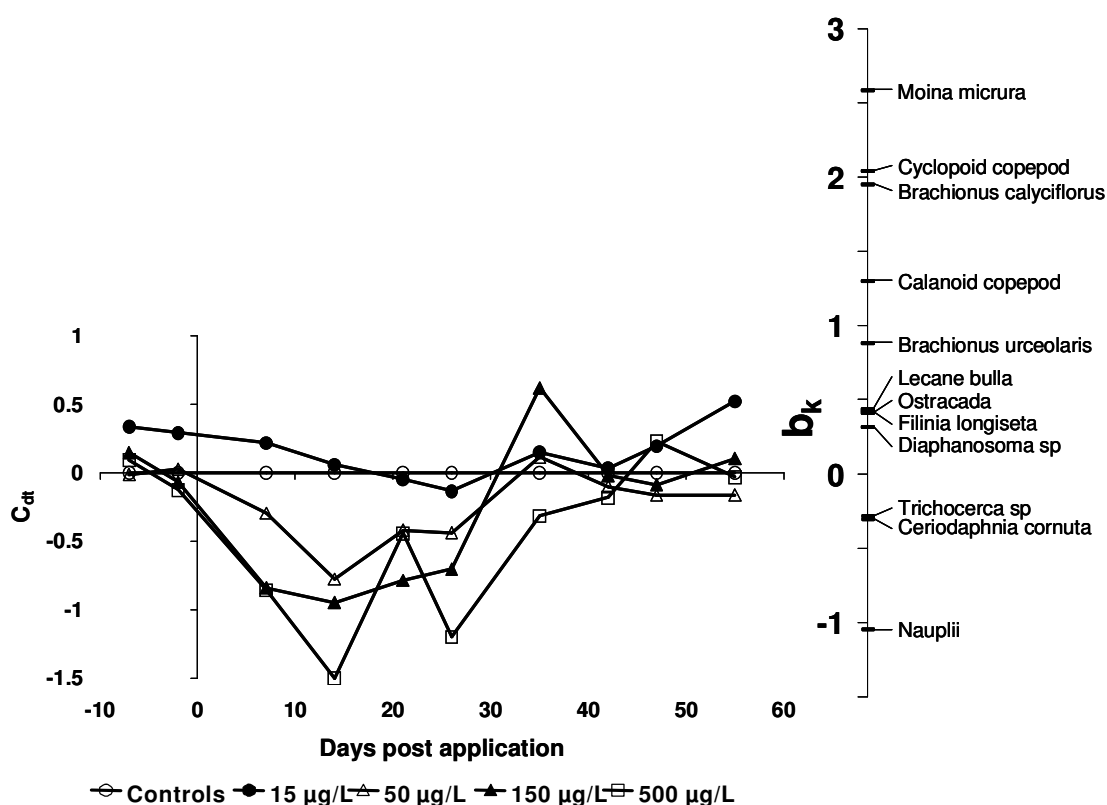


Figure 7.3 Principal response curves resulting from the analysis of the zooplankton data set, indicating the treatment effects of linuron on the zooplankton community. Of all variance, 32% could be attributed to sampling date; this is displayed on the horizontal axis. Twenty-four percent of all variance could be attributed to treatment level. Of this variance, 24% is displayed on the vertical axis. The lines represent the course of the treatment levels in time. The species weight (bk) can be interpreted as the affinity of the taxon with the Principal Response Curves. A Monte Carlo permutation test indicated that a non-significant part of the variance explained by treatment level is displayed in the diagram (P = 0.804).

Zooplankton

A total number of 11 rotifer taxa were identified from the zooplankton samples, with *Brachionus* (4 taxa) and *Lecane* (3 taxa) as most diverse genera. Cladocera was represented by *Moina micrura*, *Diaphanosoma sp.*, *Ceriodaphnia cornuta* and *Streblocerus pygmaeus*, while Copepoda (Cyclopoida, Calanoida, nauplii) and Ostracoda were not identified to the species level. In terms of total abundances, the control microcosms changed from rotifer-dominated to cladoceran-dominated in time. Total numbers of copepods were high in the pre-treatment period and decreased over the experimental period. Abundances of ostracods slightly increased throughout the experiment although total numbers remained low.

Table 7.2 Results of Monte Carlo permutation tests (P-value) and Williams test on the PCA coordinates (NOECcommunity) as performed for each sampling date for the zooplankton data set.

Day	P-value	NOEC
-7	0.946	≥ 150
-2	0.939	≥ 150
7	0.005	15
14	0.002	5
21	0.286	≥ 150
26	0.038	50
35	0.148	≥ 150
42	0.431	≥ 150
47	0.219	≥ 150
55	0.322	≥ 150

The PRC of the zooplankton data set is presented in figure 7.3, whilst the results of the Monte Carlo permutation tests are given in Table 7.2. Thirty-two percent of all variance could be attributed to sampling date and is thus displayed on the horizontal axis. Of the total variance, 24% could be attributed to treatment level. Although a Monte Carlo permutation test indicated that the part displayed on the vertical axis was non-significant (24%; $p = 0.804$), the PRC reveals a clear concentration-dependent effect with an increasing effect in the order $500 \mu\text{g/L} > 150 \mu\text{g/L} > 50 \mu\text{g/L} > 15 \mu\text{g/L} \approx \text{control}$.

The species weight (b_k), which is shown on the right side of the PRC diagram, can be interpreted as the affinity of the taxon with the Principal Response Curves. Species with a high positive weight, thus *Moina micrura*, *Brachionus calyciflorus*, and cyclopoid and calanoid copepods

(combined the mature copepods), are indicated to have decreased most strongly in the higher treatment levels (Figure 7.3).

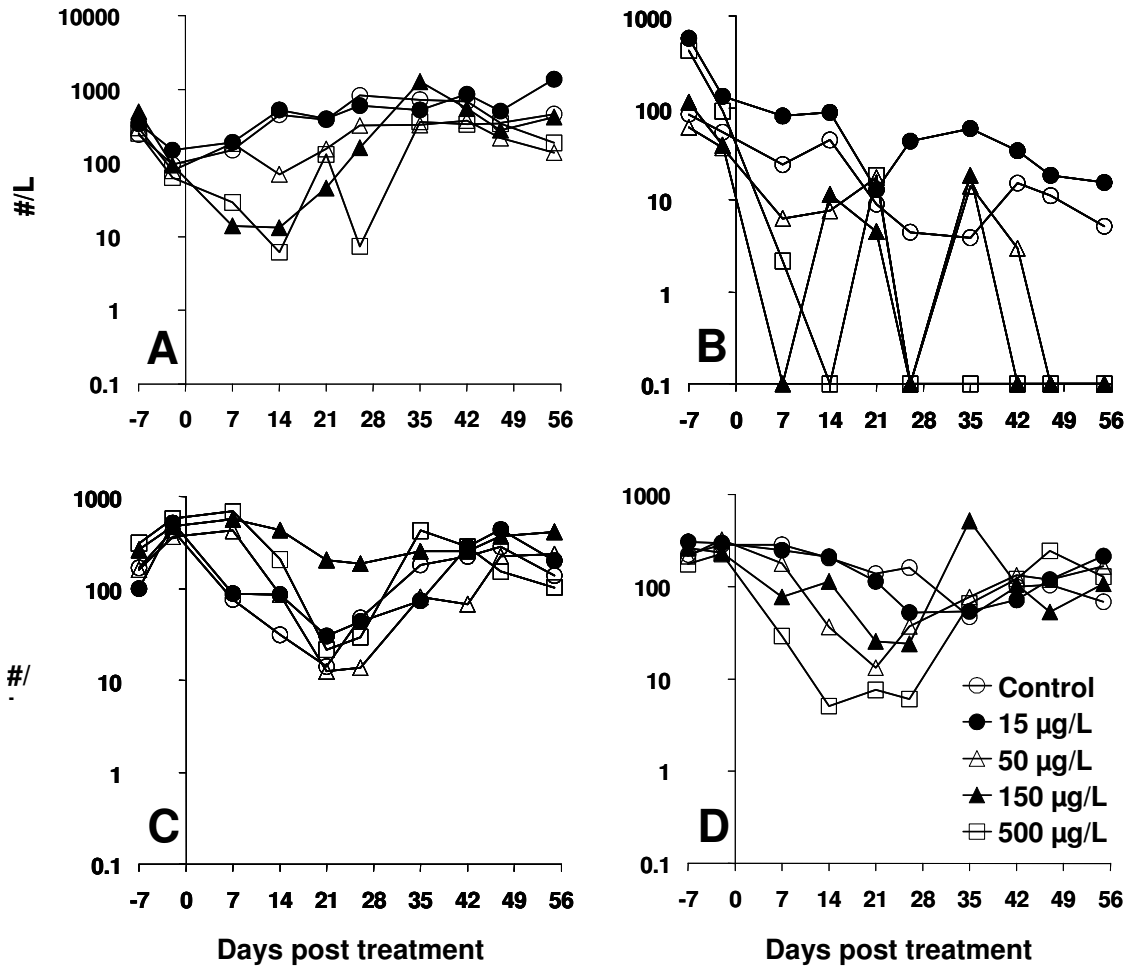


Figure 7.4 Dynamics in numbers of the most discriminate zooplankton taxa in the PRC analysis. Figures 7.4A through 7.4D show the geometric means of the numbers per liter counted per treatment level of *Moina micrura* (A), *Brachionus calyciflorus* (B), immature copepod stages; Nauplii (C) and mature copepods, i.e. the sum of abundances of cyclopoid and calanoid copepods (D). In the figures, an abundance of 0.1 denotes absence of the taxon.

Nauplii (immature copepod stages) has a relative high negative weight and thus increased most strongly as a result of linuron. These effects were confirmed by the univariate NOEC calculations using the Williams test (Table 7.3) and their dynamics presented in Figure 7.4. Abundances of *M. micrura* (Figure 7.4A) were decreased compared to controls in the two highest concentrations. Besides the negative treatment effect on *B. calyciflorus* (Figure 7.4B), the rotifers *Lecane closterocerca* and *Trichocerca* sp. increased in abundances at higher linuron doses

(Table 7.3). The decrease in mature copepods (calanoids and cyclopoids) was accompanied with an increase in immature copepod stages (nauplii) in all but the lowest treatment (Figures 7.4C and 7.4D).

Table 7.3 No observed effect concentration (NOEC) of zooplankton taxa per sampling date calculated by the Williams test ($p < 0.05$). Concentrations ($\mu\text{g a.i./L}$) showed significant increases (+) or decreases (-); > indicates a NOEC of $> 500 \mu\text{g/L}$.

	Days post application									
	-7	-2	7	14	21	26	35	42	47	55
CLADOCERA	>	>	>	15 (-)	>	150 (-)	>	>	>	>
<i>Moina micrura</i>	>	>	50 (-)	50 (-)	>	150 (-)	>	>	>	>
ROTIFERA	>	>	>	>	>	>	>	>	>	>
<i>Brachionus calyciflorus</i>	>	>	50 (-)	>	>	>	>	>	>	>
<i>Lecane closterocerca</i>	>	>	150(+)	>	>	>	>	>	>	>
<i>Trichocerca sp</i>	>	>	>	>	>	>	50 (+)	>	>	>
COPEPODA	>	>	150(+)	>	>	>	>	>	>	>
Nauplii	>	>	15 (+)	50 (+)	>	>	>	>	>	>
Cyclopoid copepod	>	>	50 (-)	150 (-)	50 (-)	>	>	>	>	>
Calanoid copepod	>	>	>	15 (-)	>	>	>	>	>	>
Copepoda mature stages	>	>	50 (-)	150 (-)	150 (-)	>	>	>	>	>

Discussion

Effects of linuron on ecosystem functioning

The primary effect of linuron is an inhibition of the photosystem II electron flow (Snel et al., 1998). Consequently, dissolved oxygen (DO) production was suppressed as a result of the herbicide application (Figure 7.1C), leading to the decreased DO concentrations in the higher linuron doses (Figures 7.1A and 7.1B). The increased DO morning values in a later stage of the experiment may be explained by a decreased respiration during the night resulting from a decrease in algal and zooplankton abundances.

Uptake of CO_2 and biologically available forms of nitrogen and phosphorus from the water decreased as a result of the reduction in primary production and may thus explain the decrease in pH and the increase in ammonia, nitrate and soluble reactive phosphate (Figure 7.2A, 7.2B and 7.2D). The increase in nitrite most likely resulted from the decrease in DO levels (Figure 7.2C). The low DO concentrations presumably hampered a full nitrification to nitrate, while levels were apparently still high enough to prevent denitrification to N_2 and consequently a loss of excess nitrogen to the atmosphere. The release of dissolved substances from the death

of primary producers (see part I) further increased nutrient concentrations and led to increased levels of alkalinity and electrical conductivity (EC).

The above does not explain the increase in TKN (total Kjeldahl nitrogen) and TP (total phosphorus) since in these parameters inorganic forms as well as organically bound phosphorus and nitrogen are included. Thus, although inorganic forms increased in higher doses, this would be expected to be compensated by organic forms, i.e. embedded in primary producers, in controls and lower doses. A possible explanation for this may be that higher nitrogen and phosphorus losses occurred in control and lower dosed microcosms due to ecological processes such as algal biomass renewal and energy transfer between primary producers and zooplankton.

Responses of the zooplankton community to linuron stress

The decline in abundances of the cladoceran *Moina micrura* in the 150 µg/L and 500 µg/L treatments were relatively fast (Table 7.3), indicating a possible role of direct toxicity. The EC₅₀ (24h) and EC₅₀ (96h) values of linuron reported for the standard cladoceran species *Daphnia magna* are respectively 310-590 µg/L (Stephenson and Kane, 1984) and 7000 µg/L (Hernando et al., 2003), implying that if direct toxicity played a role this was slight and only for the highest linuron dose.

A more likely explanation for the decrease in abundances of *M. micrura*, however, is the decrease in planktonic and epiphytic algal communities as discussed in chapter 6. Although several phytoplankton and periphyton species indeed decreased in abundances, other (insensitive) taxa increased in abundance. Algal biomass, measured as chlorophyll-a content, was therefore only slightly affected, especially for phytoplankton (see part I). Apparently, the tolerant primary producers were less edible species than the species that had disappeared. To test this hypothesis, the phytoplankton species were divided in edible and inedible algae and their dynamics compared between the controls and treatments that showed effects on *M. micrura* (Figure 7.5). *M. micrura* has been considered to feed on particles smaller than 40 µm in size (Hanazato and Yasuno, 1987), so algal species > 40 µm were regarded inedible. In addition, heavily spined *Scenedemus* taxa smaller than 40 µm in colony size were excluded as edible algae since spines have been reported to hamper the filtration process by mechanical interference, like for *S. quadricauda* (Bergquist et al., 1985). For the 500 µg/L, the decrease in edible algae reflects the decrease in abundances of *M. micrura*.

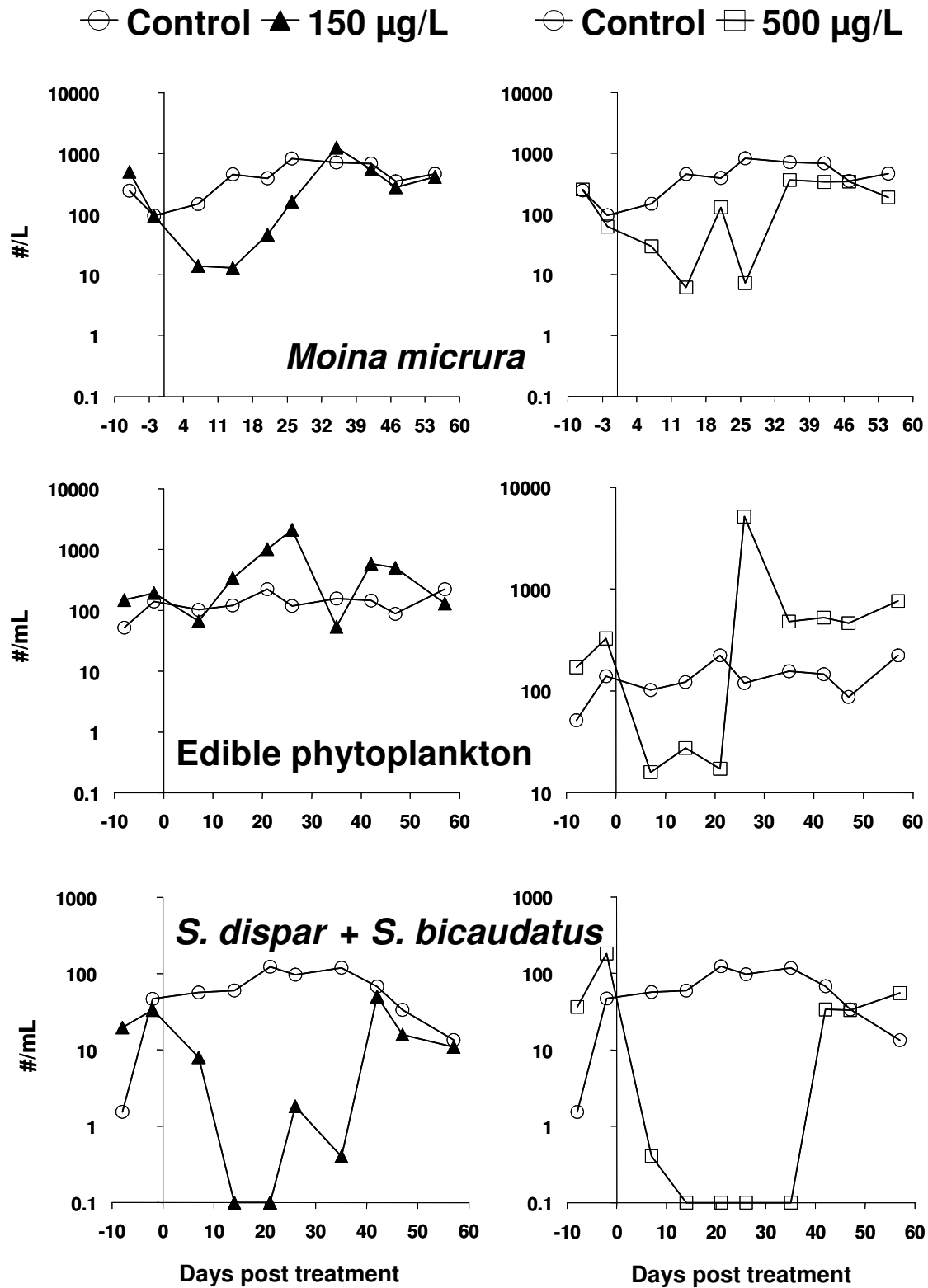


Figure 7.5 Dynamics in numbers of *Moina micrura* (upper figures), total numbers of edible phytoplankton (central figures) and combined numbers of *Scenedesmus dispar* and *Scenedesmus bicaudatus* (lower figures) in the 150 µg/L (left) treatment and 500 µg/L (right) compared to control values. An abundance of 0.1 denotes absence of the taxon.

Furthermore, the abundance peaks in edible species on day 14 and, more pronounced, on day 26 are followed by increased numbers of *M. micrura* on day 21 and a recovery to control levels on day 35, respectively (Figure 7.5). The dynamics of edible species in the 150 µg/L treatment, however, does not show decreased numbers following application. In addition, the recovery of *M. micrura* goes along with an increasing trend of edible species abundances to levels higher than in controls. The decrease in *M. micrura* at this treatment level appears to be more related with the decrease in *S. dispar* and *S. bicaudatus*, the small chlorophyte species that dominated the control phytoplankton community (Figure 7.5). Apparently, the species that replaced *S. dispar* and *S. bicaudatus* in the first two weeks after linuron application may be considered edible for a mechanical point of view, but had a lower nutritious value. Although no significant increases were noted on day 7 and 14 for phytoplankton species, several taxa had peak abundances in one of the replicas in the 150 µg/L treatment. The chlorophytes *Oocystis borgei*, *O. pusilla*, and *Tetraedron minimum*, and the diatoms *Gomphonema parvulum* and *Cyclotella* sp. combined made up almost 65% of the phytoplankton community on these sampling days, while this was only 13% in controls. Diatoms possess a tough frustule, and high grazer pressure has been reported to induce silicification of this frustule (Pondaven et al., 2007). Also the small *T. minimum* is surrounded with a thick cell wall. *Oocystis* colonies were found to be grazing resistant in a study by Vanni and Temte (1990), which may be explained by the protective sheet around the cells of this colonial species. Indeed, thick cell walls and protective sheets have been reported as mechanisms allowing cells or colonies to pass the gut of the zooplankton unaffected (Brönmark and Hansson, 2005).

Interestingly, *S. dispar* and *S. bicaudatus* only seemed to recover fully *after* complete recovery of *M. micrura*. Grazing pressure stabilized after recovery of *M. micrura*, so species with energy-costly specialized defense adaptations presumably lost their competitively advantage over small fast growing algae without any protection, like *S. bicaudatus* and *S. dispar*.

The decrease in numbers of *S. bicaudatus* and *S. dispar* may have caused a decrease in abundances of the euplanktonic rotifer *Brachionus calyciflorus* (Figure 7.4B). The periphytic rotifer species *Lecane closteroerca* and *Trichocerca* sp., however, were found to increase at higher linuron concentrations 1 week and 5 weeks post application, respectively (Table 7.3). At least for *L. closteroerca*, this was not due to an increase in food, since periphyton biomass was largely reduced at the highest linuron dose on day 14, as indicated with a chlorophyll-a content was 0.7 µg/dm². A possible explanation for the increased rotifer abundances may be an increase in detritus as a result of death of primary producers or the edible micro-organisms induced by

this, since rotifers have been reported to consume organic detritus and bacteria (Kutikova, 2002).

Although exceptions are common, most Calanoida are planktonic fine particle filter feeders, while Cyclopoidae belong to the micro-predators and feed on small invertebrates but also eat algae (Alekseev, 2002). Thus, the decline in mature copepod abundances (Figure 7.4D) resulted from the reduced availability of algae and/or decreased zooplankton abundances. Interestingly, immature stages of copepods (nauplii) increased in abundances (Figure 7.4C). Feeding habits of nauplii have not been studied intensively, but in tropical marine water a similar effect pattern was found as in the present study. Naupliar growth rates appeared to be uncoupled from chlorophyll concentration, while adults became food-limited, indicating a difference in diet between nauplii and later copepod stages (Hopcroft and Roff, 1998). Although Roff et al (1995) indeed concluded that nauplii can thrive on a diet of bacteria and picoplankton, Finlay and Roff (2004) concluded that copepod nauplii have a similar diet as later copepod stages. In case micro-organisms were an edible food source for the nauplii in the present study, their increased abundance may be explained by a proliferation of micro-organisms as discussed for rotifers. In case of the contrary, the development of nauplii to mature stages may have been delayed as a result of decreased food availability while hatching of resting eggs was unaffected, consequently leading to increased numbers of nauplii.

Ecological effect chain

A schematic overview of the direct and indirect effects of the linuron application is presented in Figure 7.6. The direct effect of linuron was the blockage of the electron transport in the Hill reaction of photosystem II. As a result of the decreased photosynthesis, DO and pH decreased while EC, alkalinity and nutrient concentrations increased. Furthermore, since primary producers could no longer support their energy need, several algal taxa decreased in abundances or were even eliminated. The consequently reduced respiration overnight led to increased DO morning values.

As a result of decreased competition and increase in nutrients, tolerant primary producers increased in abundances. The decrease in food caused by the death of primary producers and because the altered algal community contained less digestible species for zooplankton taxa, which consequently decreased in numbers. Copepod nauplii, however, had higher numbers at higher linuron concentrations, which may be due to i) increased detritus and/or edible micro-

organisms resulting from primary producer death or ii) a delay in copepod development as a result of decreased food availability in combination with unaffected hatching of resting eggs.

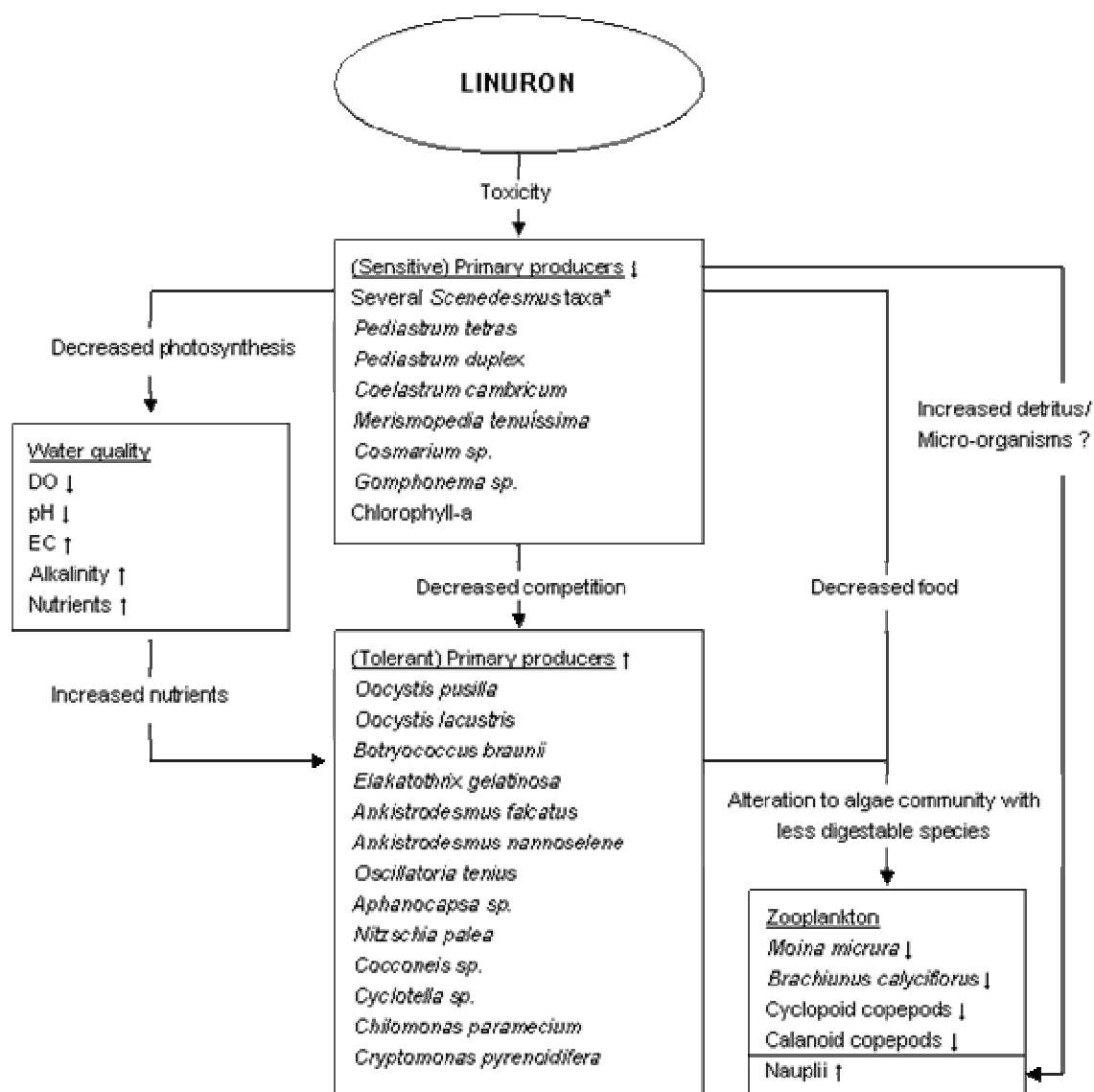


Figure 7.6 Schematic overview of the hypothesised direct and indirect effect chains of carbendazim application on ecosystem structure and functioning.

Comparison of results with other microcosm and mesocosm studies

In Table 7.4, the no observed effect concentrations (NOECs) and lowest observed effect concentrations (LOECs) for the different endpoints are compared with those noted in microcosm and mesocosm studies evaluating linuron performed in temperate regions. Toxicity

values reported in a study by Stephenson and Kane (1984) were not included since only one high linuron concentration (1000 µg/L) was tested so no LOEC – NOEC could be estimated. Effects of herbicides in microcosm and mesocosm studies evaluating herbicides depend on system structure and exposure regime, which may impede the comparison of threshold values (Brock et al., 2000; Van den Brink et al., 1997; Gustavson et al., 2003). For example, submerged macrophytes are reported to be more sensitive than algae to the auxin-simulater 2,4-D but equally vulnerable to photosynthesis-inhibiting herbicides, which may be explained by their difference in toxicological mode of action (Van den Brink et al., 2006). Compared with systems dominated by macrophytes, plankton communities are usually characterized by a higher proportion of short-lived species (phytoplankton, zooplankton), lower biomass but higher turnover rates, and a less diverse macroinvertebrate community (Brock and Budde, 1994). Thus, although sensitivity may be similar for plankton and macrophytes, recovery and adaptation (recovery of functionality) in plankton-dominated systems is more rapid and indirect effects on higher trophic levels (zooplankton) are only observed at higher concentrations (Brock et al., 2000). Although the threshold values for the zooplankton community in the present study were in line with those from the reference studies (Table 7.4), effects were indeed clearly different from those in the macrophyte-dominated systems carried out in The Netherlands. In the latter studies, linuron application led to an increase in cladocerans (Slijkerman et al., 1999) and a decrease in rotatoria (Cuppen et al., 1997). An increase in the size of the dominant zooplankton species has also been reported for other photosynthesis inhibitors, e.g. atrazine (Hamilton et al., 1989) and simazine (Jenkins and Buikema, 1990). Cuppen et al. (1997) explained the increase in cladocerans after linuron stress with the increased numbers of the small phytoplankter *Chlamydomonas*, a readily edible chlorophyte. In the present study, an opposite effect was found: abundances of the cladoceran *M. micrura* decreased in the higher linuron applied microcosms. In the laboratory microcosm study by Daam and Van den Brink (2007), a slight decrease in total numbers of cladocerans was noted, but only after chlorophyll-a levels had been 0-1.7 µg/L for over two weeks. In the present study, however, effects on the zooplankton community were instantaneous, more pronounced and occurred at chlorophyll-a concentrations of approximately 1.5 µg/L (in 500 µg/L dose) as well as 10 µg/L (in 150 µg/L dose). This may be explained by the fact that the tolerant algal species were less edible and/ or had a lower nutritious value, and the order of a magnitude higher threshold food concentration for tropical cladocera than for their temperate counterparts (Sarma et al., 2005).

Table 7.4 No observed effect concentrations (NOEC; in $\mu\text{g/L}$) and lowest observed effect concentrations (LOEC; in $\mu\text{g/L}$) for different endpoints calculated in the present study and those reported in other microcosm studies. NM = not measured; NR = not recorded; NP = not present.

Endpoint/ pesticide load	Single peak	Single peak	Single peak	Pulsed (3x at 4-wk intervals)	Constant (4 wk)
Phytoplankton					
Community	15 - 50	No clear dose-effect ¹	50 - 150	> 50	15 - 50 ⁵
Chlorophyll-a	15 - 50	>180	5 - 50	> 50	50 - 150
Periphyton					
Community	15 - 50	NM	NM	> 50	15-50 ⁵
Chlorophyll-a	15 - 50	NM	NM	> 50	15 - 50
Macrophytes	NP	NR ²	NP	> 50	0.5 - 5 ⁶
Community metabolism	15 - 50	<20 - 20	0.5 - 5	0.5 - 5	0.5 - 5
Based on:	DO, pH, EC, nutrients	DO, pH	DO, pH	DO, pH	DO, pH
Zooplankton community	15 - 50	20 - 60 ³	5 - 50 ⁴	> 50	5 - 15 ⁷
Overall NOEC - LOECecosystem	15 - 50	<20 - 20	0.5 - 5	0.5 - 5	0.5 - 5
Reference	This paper; Part I	Slijkerman et al., 2005	Daam and Van den Brink. (2007)	Kersting and Van Wijngaarden, 1999; Van Geest et al., 1999	Van den Brink et al., 1997; Cuppen et al., 1997
Type of model ecosystem	Lentic, outdoor Plankton-dominated Microcosms (250 L)	Lentic, outdoor Macrophyte-dominated Microcosms (3000 L)	Lentic, indoor Plankton-dominated Microcosms (8.5 L)	Lentic (0-1 wk p.a.)/ lotic (1-4 wk p.a.), outdoor, macrophyte-dominated experimental ditches (mesocosms; 60000 L)	Lentic, indoor Macrophyte-dominated Microcosms (600 L)
Duration (weeks)	8	4	3	22	10
Concentrations tested ($\mu\text{g/L}$)	15, 50, 150, 500	20, 60, 180	0.5, 5, 50, 150	0.5, 5, 15, 50	0.5, 5, 15, 50, 150
Location	Thailand	The Netherlands	The Netherlands	The Netherlands	The Netherlands

¹ Besides increase in cyanobacteria after three weeks in all treated tanks, effects did not increase with increasing dose

² Growth of the macrophyte *Elodea* recorded as “reduced” but statistical significance of different treatments unclear

³ Only slight effect; increase in rotifers and cladocerans on day 6 and 13, respectively

⁴ Based on decrease in total numbers of cladocerans.

⁵ Based on multivariate analysis. The authors also reported a decrease in *Chroomonas* sp. in phytoplankton and *Cocconeis* sp. in periphyton with lowest NOEC = 0.5 $\mu\text{g/L}$.

⁶ Based on in-situ bioassay. Standing stock of *Elodea nuttallii* in microcosms: NOECbiomass reduction = 15 $\mu\text{g/L}$.

⁷ Based on decrease in Rotatoria

In the laboratory plankton-dominated microcosm study by Daam and Van den Brink (2007), linuron was more persistent than in the present study. Nevertheless, threshold values for the phytoplankton communities in the present study were lower compared to that study and comparable with those of the study evaluating chronic linuron exposure (Table 7.4). Periphyton was not studied in the single-peak reference studies, and was also equally vulnerable on community level in the present study after a single-peak application as in the chronic linuron study. It thus appears that the algal communities in the present study were relatively sensitive compared to the studies carried out in the temperate zone. Differences in community structure between the studies may be a possible explanation for this. The phytoplankton and periphyton communities in the present study at the time of application were dominated by sensitive *Scenedesmus* taxa and *Chamaesiphon* sp., respectively. In the chronic linuron study by Van den Brink et al. (1997), the algal communities were dominated by diatoms, which have been reported to be relatively tolerant to herbicide stress (Bérard et al., 1999). In addition, the phytoplankton community in the laboratory study by Daam and Van den Brink (2007) was only studied three weeks after application, by which time adaptation of phytoplankton taxa may have occurred. The chlorophyte *Monoraphidium* sp. dominated the phytoplankton community in the latter study and this genus has been reported to obtain tolerance to the photosynthesis inhibitor simetryn (Kasai, 1999).

Effects on community metabolism were less pronounced in the present study compared to the macrophyte-dominated as well as the plankton-dominated reference studies (Table 7.4). This may be related with the high tropical temperatures (average water temperature $\pm 30^{\circ}\text{C}$), since a temperature-dependent detoxification of atrazine has been demonstrated in laboratory experiments (Bérard et al., 1999). The authors attributed this to increased turnover rates of the DI protein, which is the specific target of photosynthetic inhibitors, with increasing temperature. Thus, high turnover rates in the present study may have resulted in a quick detoxification of the PSII activity at the lowest linuron concentration, and consequently an absence of effects on community metabolism at this treatment level.

The overall NOEC in the present study was set at 15 $\mu\text{g/L}$, since this threshold was calculated for all endpoints tested (Table 7.4). In other microcosm and mesocosm experiments, the NOEC_{community} was mostly 0.5 $\mu\text{g/L}$. In those studies, this NOEC was calculated for community metabolism, which was less sensitive in the present study as described above.

Recovery

Table 7.5 summarizes the time in weeks to recovery for the different endpoints. Only the phytoplankton community and the community metabolism of the highest linuron dose did not return to controls within the experimental period, i.e. 8 weeks.

Table 7.5 Time to recovery (in weeks) per endpoint as noted in the present study.

Treatment/endpoint	Phytoplankton	Periphyton	Zooplankton	Community metabolism
50 µg/L	4	>8	3	5
150 µg/L	8	>8	3	5
500 µg/L	>8	>8	5	>8

A comparison of recovery observed in the present study with the reference studies evaluating linuron is hampered because i) endpoints were only measured at the end of the experiment (i.e., 3 wks p.a.; Daam and Van den Brink, 2007), ii) no effects were noted at concentrations tested (Kersting and Van Wijngaarden, 1999; Van Geest et al., 1999), iii) the experimental period was too short to demonstrate recovery (i.e., 3 wks; Slijkerman et al., 2005), iv) a chronic exposure of 4 weeks was evaluated so recovery of most endpoints did not occur within the 5 weeks following the end of the treatment (Van den Brink et al., 1997; Cuppen et al., 1997).

In Brock et al. (2000), the effects and recovery of herbicides on freshwater microcosms and mesocosms are reviewed and compared. To enable a good comparison, the reported test concentrations were transformed to “toxic units” (TU) by dividing these concentrations by the (geometric mean of) EC_{50} value(s) of the most sensitive standard algal species recommended by the OECD (1984): *Scenedesmus subspicatus*, *Selenastrum capricornutum* or *Chlorella vulgaris*. For linuron, the lowest EC_{50} of the latter species is recorded for *S. subspicatus*; 16 µg/L (Crommentuijn et al., 1997). This implies that the treatment levels in the present study, i.e. 15 µg/L, 50 µg/L, 150 µg/L and 500 µg/L, correspond to respectively 0.9, 3.1, 9.4 and 31.3 TU's.

The effects and recovery of community metabolism, phytoplankton, periphyton and zooplankton in the present study are compared in Table 7.6 with other photosynthesis inhibiting herbicide studies reviewed in Brock et al. (2000) using the toxic unit approach explained above. The relatively high NOEC for community metabolism (0.9 TU compared to a range of 0.01 – 1 TU's) is confirmed, while recovery appears to occur at comparable

concentrations. Periphyton did not recover within the experimental period, which also would not be expected based on the reference studies at the TU's tested. Phytoplankton recovery within 8 weeks as found for the 150 µg/L dose (9.4 TU's) lies at the end of the range (1.7 – 8.9 TU's) of photosynthesis inhibiting herbicides, indicating that recovery at this treatment level was relatively fast. For the zooplankton communities this is more prominent: recovery within 8 weeks in the present study occurred at TU's much higher than reported in reference studies (Table 7.6).

Table 7.6 Range of classified effects (In toxic units. For explanation: see text) following single application of photosynthetic inhibitors in model ecosystem studies and those observed in the present study. Reference ranges were derived from Brock et al. (2000).

Endpoint	No effect	Clear short-term effect (< 8 wks)	Clear long-term effect (> 8 wks)
Community metabolism			
Reference studies	0.007 – 1.2	0.28 – 37**	3 – 75
This study	0.9	3.1 & 9.4	31.3
Phytoplankton			
Reference studies	0.28 – 4.3***	1.7 – 8.9	0.75 – 217
This study	0.9	3.1 & 9.4	31.3
Periphyton			
Reference studies	0.075 (0.03 – 1.5)*	0.28 (0.28 – 15)*	1 – 5.7
This study	0.9	-	3.1 & 9.4 & 31.3
Zooplankton			
Reference studies	0.15 - 15	2 (1.1 – 5.4)*	217 (not available)*
This study	0.9	3.1 & 9.4 & 31.3	-

* only one reference study with single application available. Range in brackets for single, multiple or continuous exposure.

** an outlier of 370 TU was also noted.

*** an outlier of 63 TU was also noted.

Brock et al. (2000) discussed that recovery of systems strongly depending on macrophytes may take longer than in plankton-dominated test systems. This may thus explain why the recovery of the phytoplankton community was found to be at the end of the range observed for the reviewed studies since these studies used either plankton-dominated or macrophyte-dominated test systems. In addition, higher rates of population increase have been reported for tropical cladocerans, like *M. micrura*, than for temperate cladocerans (Sarma et al., 2005). This implies that tropical cladoceran populations may regain control abundances quicker after environmental conditions are favorable again and may thus explain the recovery of the zooplankton community at relatively high TU's in the present study.

Implications for risk assessment of linuron in Thailand

Currently, uncertainty factors applied to ecosystem generated threshold values for setting regulatory acceptable concentrations range from 1 (US EPA) to a variable factor, which is based on a case-by-case evaluation of studies (EU) (Van Wijngaarden, 2006). For atrazine, a well studied photosynthesis inhibitor (see Brock et al., 2000 for a review), an uncertainty factor of 2.5 was determined (Brock et al., in press). We assumed that the protection goal was to protect ecosystems from any significant effect observed in the microcosms. Significance was accepted at $p < 0.05$, indicating that an effect smaller than 5% was determined not to harm the aquatic environment. The Regulatory Acceptable Concentration (RAC, Brock et al., 2006) was calculated by applying an uncertainty factor of 2.5 to the lowest NOEC in the present study (15 µg/L), making 6 µg/L.

The EC50 for the most susceptible temperate standard test species (according to OECD, 1984) is 16 µg/L, which was calculated for *Scenedesmus subspicatus* (Crommentuijn et al., 1997). By multiplying the EC50 with a safety factor of 0.1, as indicated by the Uniform Principles (EU, 1997), the no effect concentration (NEC) is 0.16 µg/L. Thus, the threshold values calculated from temperate laboratory toxicity values appear to ensure adequate protection for the aquatic community in tropical countries like Thailand in the case of a single-peak exposure to the photosynthesis inhibitor linuron.

However, it should be noted that even though the *effect assessment* of linuron on the tropical aquatic ecosystem in Thailand may be sufficiently covered by the temperate NEC, this does not automatically imply that the hazard of this herbicide is the same between temperate and tropical conditions. Herbicides in Thailand are frequently used on rice plantations (Jungbluth, 1996). In case pesticides are applied to flooded rice fields, this implies that pesticides are sprayed directly in the water. Consequently, spray drift will be much higher (100%) than in ditch-dike systems in for instance the Netherlands, where only a relatively small part of the pesticide will enter the ditches surrounding the agricultural fields. Thus, for an *exposure assessment* point of view, the predicted environmental concentration (PEC) is very likely to be higher in (tropical) flooded rice fields and consequently the hazard, even though the sensitivity of the ecosystem may be the same.

Acknowledgement

This study was funded by the Portuguese government through FCT (scholarship SFRH/ BD/ 8213/ 2002). The authors are indebted to the staff at the AIT hatchery for technical assistance.

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CHAPTER 8

GENERAL DISCUSSION

Influence of experimental set-up of microcosms on the effect assessment of pesticides

In the present thesis, the influence of the experimental set-up on the fate and effects of pesticides with different modes of action were studied by performing microcosm studies ranging from small indoor laboratory test systems in The Netherlands (Chapter 2) to larger-scale outdoor test systems under tropical conditions in Thailand (Chapters 3 through 7). The organophosphorous insecticide chlorpyrifos was chosen as a model substance for acetylcholinesterase inhibiting insecticides, the benzimidazole fungicide carbendazim for beta-tubulin synthesis inhibitors and the phenylureum herbicide linuron for photosynthesis inhibiting herbicides. These pesticides were chosen since reference model ecosystem studies were available, enabling an evaluation of the degree in similarity of the observed fate and effects.

In tables 8.1 to 8.3, the threshold values on ecosystem level noted in the experiments with respectively chlorpyrifos, carbendazim and linuron are compared with those reported in other microcosm and mesocosm studies carried out with these compounds. To facilitate comparisons, the effects are classified into five “effect classes” as used by Brock et al. (2000a, b): class 1 = no effect; class 2 = slight effect; class 3 = clear short-term effect (recovery < 8 wks); class 4 = clear effect but experimental period too short to demonstrate recovery; class 5 = clear long-term effect (recovery > 8 wks).

The sensitivity of the tropical freshwater ecosystem to chlorpyrifos appeared to be comparable to temperate freshwater ecosystems (Table 8.1). This may be explained with the fact that cladocerans are the most susceptible species to organophosphates, and sensitivity to chlorpyrifos of the dominant cladoceran in the tropical microcosms, *Moina micrura* ($LC_{50} = 0.6 \mu\text{g/L}$. Chapter 3), was found to be in the range of response variability of 3 or more reported for standard laboratory test species (Sprague, 1985; Baird et al., 1989) with the temperate standard test species *Daphnia magna* ($LC_{50} = 1 \mu\text{g/L}$. Kersting and Van Wijngaarden, 1992).

Table 8.1 Comparison of threshold values noted in the studies carried out with the *insecticide chlorpyrifos* in this thesis with those reported in other microcosm and mesocosm studies. Class 1: no effect; Class 2: slight effect; Class 3: clear effect, recovery < 8 wks p.a.; Class 4: clear effect but experimental period too short to demonstrate complete recovery within 8 wks p.a.; Class 5: clear effect, recovery > 8 wks.

Pesticide treatment	Test system	Location Reference	Effect class 1	Effect class 2	Effect class 3	Effect class 4	Effect class 5
Single	Outdoor, lentic, microcosms	USA (Kansas) Biever et al. (1994)	0.1	0.3	1.0	-	3.0
Single	Outdoor, lentic, littoral enclosures	USA (Minnes.) Brazner et al. (1989)	-	-	0.5	6.3	-
Single	Outdoor, lentic, experimental ditches	The Netherlands Van den Brink et al. (1996)	0.1	-	0.9	-	6.0
Pulsed (6 hrs)	Outdoor, lotic, artificial streams	Pusey et al. (1994)	0.1	-	5.0*	-	-
Single	Outdoor, lentic, microcosms	Thailand Chapter 3	0.1	-	1.0**	-	1.0**
Single/ Repeated	Outdoor, lentic, microcosms	Thailand Chapter 4	-	-	1.0	-	-
Single	Indoor, lentic, laboratory microcosms	USA (Minnes.) Stay et al. (1989)	-	0.5	-	5.0	-
Single	Indoor, lentic, laboratory microcosms	The Netherlands Van Wijngaarden et al. (2005)	0.1	-	1.0 ^A	1.0 ^B	-
Single ^C	Indoor, lentic, laboratory microcosms	The Netherlands Chaper 2	0.005	-	-	0.05	-
Continuous (7 wks)	Indoor, lentic, microcosms	The Netherlands Van den Brink et al. (1995)	-	-	-	0.1	-
Continuous (3 wks)	Outdoor, lotic, experimental streams	Australia (Queensland) Ward et al. (1995)	-	-	0.1	-	5.0

* Relatively fast recovery due to continuous input of propagulus.

** Recovery of communities in multivariate analyses < 8 wks, but recovery of the dominant cladoceran *Moina micrura* > 8 wks.

^A Under “temperate” (16°C; relatively low light intensity 14h; productive) conditions.

^B Under “mediterranean” (26°C; relatively high light intensity 12h; highly productive) conditions.

^C Study without sediment, resulting in a relatively slow disappearance of chlorpyrifos from the water phase

Table 8.2 Comparison of threshold values noted in the studies carried out with the *fungicide carbendazim* in this thesis with those reported in other microcosm and mesocosm studies. Class 1: no effect; Class 2: slight effect; Class 3: clear effect, recovery < 8 wks p.a.; Class 4: clear effect, but experimental period too short to demonstrate complete recovery within 8 wks p.a.; Class 5: clear effect, recovery > 8 wks.

Pesticide treatment	Test system	Location Reference	Effect class 1	Effect class 2	Effect class 3	Effect class 4	Effect class 5
Single	Outdoor, lentic, microcosms	Thailand This thesis (Chapter 5)	3.3	-	-	-	33
Single*	Outdoor, lentic, microcosms	The Netherlands Slijkerman et al. (2004)	2.2	21	-	226	-
Single*	Indoor, lentic, microcosms	The Netherlands This thesis (Chapter 2)	33	-	-	100	-
Continuous (4 wks)	Indoor, lentic, microcosms	The Netherlands Cuppen et al. (2000); Van den Brink et al. (2000)	3.3	-	-	-	33

* Effects based on zooplankton community; macroinvertebrate community not studied.

Table 8.3 Comparison of threshold values noted in the studies carried out with the *herbicide linuron* in this thesis with those reported in other microcosm and mesocosm studies. Class 1: no effect; Class 2: slight effect; Class 3: clear effect, recovery < 8 wks p.a.; Class 4: clear effect, but experimental period too short to demonstrate complete recovery within 8 wks p.a.; Class 5: clear effect, recovery > 8 wks.

Pesticide treatment	Test system	Location Reference	Effect class 1	Effect class 2	Effect class 3	Effect class 4	Effect class 5
Single	Outdoor, lentic, microcosms	Thailand This thesis (Chapter 6 & 7)	15	-	-	-	50
Single	Outdoor, lentic, pond enclosures	UK Stephenson and Kane (1984)	-	-	-	1000	-
Single	Outdoor, lentic, microcosms	The Netherlands Slijkerman et al. (2005)	-	-	-	20	-
Single	Indoor, lentic, microcosms	The Netherlands This thesis (Chapter 2)	0.5	-	-	5	-
Pulsed (3X at 4-wk intervals)	Outdoor, lentic (0-1 wk p.a.) - lotic (1-4 wk p.a.), experimental ditches	The Netherlands Kersting and Van Wijngaarden (1999); Van Geest et al. (1999)	0.5	5	15	-	-
Continuous (4 wks)	Indoor, lentic, microcosms	The Netherlands Van den Brink et al. (1997); Cuppen et al. (1997)	0.5	-	5	-	15

The extent by which a freshwater community responds to pesticide stress depends on a combination of ecosystem sensitivity and the (biologically available) concentration of a compound. Because closed systems without macrophytes and sediment were used in the laboratory experiment described in chapter 2, chlorpyrifos was found to be more persistent compared to larger-scale studies also evaluating single-peak chlorpyrifos treatments. Thus, although the zooplankton (cladoceran) community in the laboratory microcosms was similar in the latter studies, effects were found to be more comparable with those observed in studies with a chronic chlorpyrifos exposure regime (Table 8.1). This is supported by Van Wijngaarden et al. (2005), who noted the same threshold values for chlorpyrifos as those reported in larger-scale outdoor microcosms and mesocosms using “open” small laboratory test systems containing sediment.

Non-arthropod macroinvertebrates are the most sensitive taxa to carbendazim. Therefore, lower threshold values were noted in the study by Slijkerman et al. (2004) and the laboratory microcosm experiment described in chapter 2 since these studies did not evaluate effects on this animal group (Table 8.2). The macroinvertebrate community in Thailand appeared to be as sensitive as reported in indoor macrophyte-dominated microcosms with chronic carbendazim exposure in The Netherlands (Cuppen et al., 2000), although most susceptible taxonomic groups were not the same. In the latter study, as well as in laboratory bioassays carried out with temperate macroinvertebrate taxa (Van Wijngaarden et al, 1998), “worm”-like taxonomic groups such as Oligochaeta, Turbellaria and Hirudinae were the most sensitive macroinvertebrates (NOEC 3.3-3.4 µg/L). In Thailand, however, oligochaetes were found to be moderately sensitive (NOEC 33 µg/L), possibly because a single-peak exposure regime was applied, whereas the water boatmen Corixidae was the most susceptible macroinvertebrate group (NOEC 3.3 µg/L).

Evidently, the primary producers (algae, macrophytes) and ecosystem metabolism (e.g., dissolved oxygen, pH) are the most severely affected endpoints after treatment with the photosynthesis inhibiting herbicide linuron. The overall sensitivity observed in the laboratory study was in agreement with that reported in reference studies, whereas the Thai microcosms appeared less sensitive (Table 8.3). This may be explained by the fact that community metabolism was less affected by linuron due to higher turnover rates of the DI protein, the specific target of photosynthesis inhibiting herbicides, at higher temperatures (Chapter 5).

Recommended experimental set-up of microcosm studies with different pesticides

As described in the general introduction, larger test systems are closer to the real-world, whereas smaller test systems are less complex and results are therefore easier to interpret. Thus, the choice of the experimental set-up of a test system should depend on the research question (Chapter 2; Leewangh et al., 1994). For studies that are initiated with the purpose of estimating an ecologically or regulatory acceptable concentration, lowest NOEC(s) and consequently direct effects on most sensitive endpoints are of utmost importance. Thus, based on the data generated in this thesis and reported in the literature as discussed in the previous section, minimum requirements for the experimental set-up of model ecosystems assuring that most sensitive endpoints are adequately taken into account may be elucidated (Table 8.4).

Table 8.4 Recommended model ecosystem for the three pesticides tested, based on the most sensitive endpoints(s) and disappearance rates of the compounds.

Pesticide	Most sensitive endpoint	Disappearance rate	Recommended microcosm
Chlorpyrifos	Zooplankton (Cladocera)	Fast	Small-scale indoor microcosm
Carbendazim	Macroinvertebrate community	Slow	Larger-scale outdoor microcosm/mesocosms
Linuron	Primary producers/community metabolism	Slow	Small- (algae) to intermediate-scale (macrophyte) indoor/outdoor microcosms

For organophosphorous insecticides like chlorpyrifos, small indoor test systems may be considered suitable because i) most sensitive taxa belong to the Cladocera, which can be easily reared in smaller laboratory test systems ii) the robustness of the NOECecosystem for reported microcosm and mesocosm studies (Table 8.1). Closed microcosms without sediment may lead to an overestimation of the sensitivity of the ecosystem, and should therefore only be used if sediment is not that important for exposure and/or if lakes are the ecosystems of concern.

Small test-systems will not allow the development of a very rich community of macroinvertebrates, which were discussed to contain the most susceptible taxa to fungicides like carbendazim (Chapter 3). Consequently, larger microcosms or mesocosms are required for an appropriate risk assessment of fungicides. Since reproduction and oviposition of insects are hampered in laboratory rooms (Cuppen et al., 2000), outdoor systems are preferable over indoor systems. Plankton-dominated model ecosystems have been reported to have a less

diverse macroinvertebrate community than test systems where macrophytes dominate the primary producer community (Brock and Budde, 1994). Also in the AIT canal, which was the source of water, sediment and additional zooplankters and macroinvertebrates for the microcosms, macroinvertebrates were noted to be concentrated in the roots of the floating plant *Pistia stratiotes*, an originally South American species that has become pan-tropical (Mitchell and Gopal, 1991). Other macrophytes in this canal were confined to another floating plant, *Wolffia sp.*, and the rooted *Nelumbo nucifera* and *Typha angustifolia*, plants that use their expansigenous aerenchyme to supply their roots with atmospheric oxygen (Große, 1996; Seago Jr. et al., 2005). Free floating plants have indeed been reported to be abundant in tropical freshwaters, as a result of extensive asexual vegetative reproduction, potentially exponential population increase and horizontal mobility (Talling and Lemoalle, 1998). As we saw in chapter 5, the bloom of *Wolffia sp.* in higher treated carbendazim led to a complete coverage of the water surface and consequently an extensive reduction in ecosystem structure and functioning. The inclusion of floating plants in microcosms is therefore not recommendable. Furthermore, macrophytes in general were noted to be very scarce in Thai farm canals. Macrophytes have been discussed to be susceptible to tropical hydrological regimes that produce marked changes in water level (Talling and Lemoalle, 1998). Thus, the extensive drainage regimes and the use of little boats passing through the irrigation canals, sometimes pulled forward by walking through the canal and subsequently disturbing the sediment (personal observations), may explain this scarcity of macrophytes. For risk assessment purposes, the irrigation canals are the most direct contamination sites and are consequently intended to be simulated in model-ecosystems. Thus, the inclusion of macrophytes in Thai model ecosystems may be questionable and hence the use of plankton-dominated microcosms is recommended.

Most sensitive endpoints for photosynthesis inhibiting herbicides like linuron are community metabolism and primary producers (algae, macrophytes). Effects on these endpoints in the small laboratory microcosms were comparable with those observed in more complex test systems (Table 8.3). In the same way as discussed for chlorpyrifos, the fact that the systems were closed led to a more prolonged exposure. Since effects of herbicides have been reported to increase with exposure duration (Gustavson et al., 2003; Van den Brink et al., 1997), this should be taken into account when interpreting results from closed test systems. The phytoplankton community in the microcosm study evaluating repeated chlorpyrifos applications was highly dominated by the cyanobacteria *Microcystis*. This was explained by the

fact that the experiment was carried out at the end of the rainy season, when *Microcystis* has a competitive advantage over other algae due to limited light conditions (Chapter 4). Indeed, the microcosms of the linuron microcosm study in Thailand, which was carried out at the end of the cold season and the beginning of the hot season, exhibited a diverse phytoplankton community dominated by chlorophytes (Chapters 6). Thus, to ensure an adequate representation of the most sensitive endpoint, future outdoor model ecosystems studies evaluating herbicides in Thailand should be carried out in the end of the cold season or the beginning of the hot season. For the same reason, outdoor microcosm and mesocosm studies in temperate countries are recommended to be carried out in spring to mid-summer (Giddings et al., 2002). For indoor herbicide effect assessments, this limitation may be overcome by seeding test systems from mixed algae cultures.

For evaluation of herbicide stress using macrophyte-dominated test systems, the microcosms as described in chapter 1 are evidently too small to allow a diverse macrophyte community. Medium-sized indoor and outdoor microcosms, however, have been proven to sustain a suitable macrophyte community for the evaluation of effects on this endpoint following herbicide stress (e.g., Van den Brink et al., 1997 and Slijkerman et al., 2005).

The use of temperate toxicity data for tropical risk assessment of pesticides

One of the aims of this thesis was to validate whether toxicity data generated in the temperate zone can be used for the tropical risk assessment of pesticides. This was done by carrying out microcosm experiments in Thailand and comparing (semi-field) ecosystem sensitivities with those reported in similar studies performed in temperate countries (chapters 3 to 7).

Previous studies aiming at a validation of the protective value of temperate toxicity threshold values for tropical freshwaters have focused mainly on a species (assemblage)-level based approach by comparing sensitivities of species between temperate and tropical freshwaters using Species Sensitivity Distributions (SSDs). For example, Dyer et al (1997) compared the SSDs of temperate, coldwater and tropical fish for 6 compounds (carbaryl, DDT, lindane, malathion, PCP and phenol). Temperate fish appeared to be more sensitive for DDT than tropical fish, while for the other compounds no significant difference in sensitivity was found. Maltby et al. (2005) compared sensitivities of temperate and tropical arthropods to the insecticides chlorpyrifos, fenitrothion and carbofuran. Although they reported that HC5 values of these pesticides were generally lower for tropical arthropods, these differences were

not statistically significant. Based on the comparison of Australian and non-Australian organisms exposed to the organochlorine insecticide endosulfan, Hose and Van den Brink (2004) indicated that the sensitivity of organisms to toxicants appears to be independent of their geographic origin. The most extensive comparison of temperate and tropical species sensitivities was made in a recent study by Kwok et al. (2007). In this study, SSDs of temperate and tropical species assemblages without separation into taxonomic groups were constructed for 18 chemical substances (ammonia, 9 metals, 2 narcotics and 6 pesticides: carbaryl, chlordane, chlorpyrifos, DDT, lindane and malathion). The authors noted confounding influences on results because i) the best fit parametric SSD model was only valid for 10 chemicals with a satisfactory goodness of fit; ii) tropical tests were conducted at a significant higher temperature than temperate tests for 13 of the tested chemicals; iii) the quantity and quality of the tropical data was lower (Kwok et al., 2007). These factors may be the reasons why overall differences in sensitivities appeared not to be consistent, although trends in species sensitivities to different chemicals between tropical and temperate aquatic organisms could be demonstrated. For 6 chemicals (among which the insecticides chlordane and chlorpyrifos), tropical organisms tended to be more sensitive than their temperate counterparts. However, for several other chemicals, especially metals, the opposite trend was noted. Based on their findings, Kwok et al. (2007) recommended the use of an extrapolation factor of 10 for coverage of 95% of the chemicals with a 90% protection level if the water quality standard is primarily based on temperate species and a priori knowledge of the sensitivity of tropical species is very limited or not available.

The consistency of threshold values from model ecosystem studies performed in different parts of the world has recently been studied by Van den Brink et al. (2006) for herbicides and Van Wijngaarden (2006) for insecticides. Van den Brink et al. (2006) concluded that there is a surprising degree in threshold values from studies evaluating herbicides performed in different parts of the world (i.e. USA, Canada, Europe) and different application regimes (i.e. single, repeated, constant). A similar conclusion was made by Van Wijngaarden (2006) for the insecticides chlorpyrifos and lambda-cyhalothrin, who reported that concentrations leading to 'no' to 'slight and transient' effects (Effect classes 1 and 2) were remarkably consistent among reviewed model ecosystem studies performed in Europe, the USA, and a study by Pusey et al. (1994) carried out in southeastern Queensland, Australia, just south of the tropic of Capricorn. Brock et al. (accepted) calculated the spread (i.e. the ratio of upper and lower limits of the 95% confidence interval) in effect classes 1 and 2 threshold values from the same studies reported

in Van Wijngaarden (2006) as well as studies performed in Europe and the USA with the PSII inhibitor atrazine as a measure of geographical variability. In this way, calculated uncertainty factors for the geographical extrapolation of threshold values for chlorpyrifos, lambda-cyhalothrin and atrazine were respectively 2.9, 2.6 and 2.5 (Brock et al., accepted). That these values are relatively low may be illustrated with the fact that, as mentioned earlier, variability in responses in standard single species tests with similar compounds may be a factor 3 or more (Sprague, 1985; Baird et al., 1989).

From the data generated in this thesis, it appears that the consistency in threshold values across the temperate zone, i.e. mostly Europe and USA, is also valid for the tropical zone. Effect classes 1 and 2 threshold values from the microcosm studies with chlorpyrifos and carbendazim in Thailand are well in the range of those reported in studies carried out in the temperate zone (Tables 8.1 and 8.2). For linuron, the class 1 threshold value is even relatively high compared to reference values due to the discussed lower threshold values for the community metabolism (Table 8.3). These findings thus support the use of temperate toxicity data from model ecosystem studies carried out in the temperate zone for the ERA in tropical countries. Evidently, additional tropical model ecosystem studies are required to evaluate whether this is also valid for a wider array of compounds and on a larger tropical geographical scale. Special attention should be paid to herbicides due to the influence of experimental design and fate on the impact of these chemicals.

Implications for tropical pesticide hazard assessment

The hazard assessment of pesticides is based on a comparison of a predicted no effect concentration and the predicted concentrations of the pesticides in aquatic ecosystems surrounding agricultural areas following pesticide application. Thus, although from an *effect assessment* point of view the use of temperate toxicity data may be validated, this does not automatically mean that the *hazard assessment* of pesticides may be directly extrapolated from temperate to tropical regions. In other words, due to eventual discrepancies in estimated *exposure assessments* between temperate and tropical situations, the determined hazard of a pesticide may be lower or higher.

The Predicted Environmental Concentration (PEC) in the EU is usually calculated with the help of a computer model (e.g. TOXSWA) for a standardized freshwater system on the basis of the recommended dose used for pest control and the expected drift percentage and runoff

or drainage fractions (FOCUS, 2001). The application of pesticides to flooded rice fields implies 100% spray drift, whereas in the European scenarios (e.g. Dutch ditches) only a relatively small part of the pesticide is expected to enter the ditches surrounding the agricultural fields (chapter 7). Also in Thai mixed fruit and vegetable farms spray drift may be expected to be relatively high, since pesticides are sprayed on small strips of land closely surrounded by water using boat application methods or knapsack spraying (Van den Brink et al., 2003). In line with this, spray drift in TOXSWA scenario's for mixed fruit and rice fields in Thailand and Sri-Lanka were set at 30 to 100% (Satapornvanit et al., 2004; Van den Brink et al., 2003), whereas this was set at only 3% for ditches in The Netherlands (Adriaanse, 1996). More research is thus required to quantify pesticide concentrations in aquatic ecosystems surrounding Thai farms following pesticide application.

Final considerations

The microcosm experiments described in this thesis support the extrapolation of temperate toxicity data for the risk evaluation of pesticides in Thailand. However, the use of temperate toxicity data is often disputed as a sustainable way to assess chemical hazards. For example, Do Hong et al. (2004) noted that the common standard organisms used for aquatic ecotoxicology tests, *Daphnia magna* and *Ceriodaphnia dubia*, do not exist in Vietnam. *Daphnia* has indeed been reported to be largely absent in the tropics (e.g., Dumont, 1994), and *D. magna* and *C. dubia* are also not listed in reviews of zooplankton species existing in Thailand (Boonsom, 1984; Sanoamuang, 2001). Do Hong et al. (2004) developed a toxicity test with the autochthonous organism *Ceriodaphnia cornuta* and noted higher sensitivities for this species compared to *D. magna* to potassium dichromate, diazinon, methyl parathion and mercury. Lahr (2000) assessed the ecological risks of insecticides to temporary pond ecosystems in the Sahel and concluded that the invertebrate communities in general did not have a higher vulnerability or more resilience compared to their temperate counterparts. However, the fate and effects varied according to the type of insecticide, although there was no general trend. The author therefore discussed that the effect assessment for pesticides should be based on toxicity tests with representative, preferably indigenous test species and not (solely) on the type of standard data which is currently used in industrialized countries (Lahr, 2000).

Potential differences in sensitivity of aquatic organisms are acknowledged and incorporated in the ERA of some jurisdictions. For instance, the Canadian water quality guidelines require

toxicity data of at least one coldwater species, e.g. trout, and one warm water species, e.g. fathead minnow (Canadian Council of ministers of the Environment, 1991). Australia and New Zealand exhibit a wide range of ecosystem types, including tropical, temperate, arid, alpine and lowland ecosystems. Therefore, the use of site-specific ERA has been promoted and applied by the authorities in Australia and New Zealand (ANZECC and ARMCANZ, 2000; Van Dam et al., 2004).

Also in the microcosm studies described in this thesis, differences in fate and effects on individual species were noted. For instance, the most susceptible taxonomic group to carbendazim in Thailand was Corixidae, whereas worm-like species are reported as most sensitive in temperate regions. The dissipation rates of lower linuron concentrations were indicated to be relatively fast and the ecosystem appeared less sensitive than reported in temperate studies. Therefore, although the use of temperate toxicity data may be validated as an immediate measure, the replacement by data generated with local fate and toxicity data may be necessary for a sustainable tropical risk assessment especially when indirect effects and recovery are of concern.

Surrogates for *Daphnia magna* may be the above mentioned *C. cornuta*, to which *Moina micrura* may be added the more as this species appeared to be more sensitive than *C. cornuta* to the pesticides tested. Both species were abundant in the microcosms and are frequently recorded for Thailand (Boonsom, 1984; Sanoamuang, 2001) and other tropical Asian freshwater bodies (Dussart et al., 1984).

Using local test species may be especially relevant when assessing the effects of fungicides. As discussed in chapter 5, it is questionable whether *D. magna* may fully represent the macro-invertebrate community and Corixidae was found to be the more susceptible to carbendazim than the present cladoceran species. Dudgeon (1999) reported that in several Asian countries, an unusual rich assemblage of Corixidae is found. Thus, the development of a laboratory toxicity test with a local representative of this macroinvertebrate family is highly recommended when evaluating the risk of fungicides in Thailand. Standard laboratory toxicity tests and in-situ field tests were already developed for indigenous chironomids (Domingues et al., 2007) and *Macrobrachium* species (Satapornvanit, 2006).

Regarding algal test species, there is a paucity of exclusively tropical phytoplankton species in the tropics (Kalff and Watson, 1986). Furthermore, the *Scenedesmus* species assemblage in the present study appeared to be adequately protected based on the laboratory toxicity data from the standard temperate algal species *S. subspicatus*. Higher water temperatures and

(consequently) different exposure durations were shown to influence sensitivity of primary producers to the herbicide linuron (Cserháti et al., 1976; Gustavson et al., 2003; Van den Brink et al., 1997). Thus, laboratory tests under tropical test conditions using for instance *S. dispar*, which was the dominant *Scenedesmus* taxon in the linuron microcosm study, may be expected to lead to higher threshold values compared to those generated under temperate test conditions. The influence of temperature on threshold values in bioassays has been reported to be both species and chemical specific (Kwok et al., 2007). Other herbicides, and pesticides in general, may thus lead to lower or higher threshold values when tested under tropical conditions compared to those generated in the temperate zone.

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